APPLICATION OF MASS SPECTROMETRY AND PROTEOMICS TO STUDY KIDNEY FUNCTION; THE CONCEPT OF RENAL INTRACRINE REGULATION

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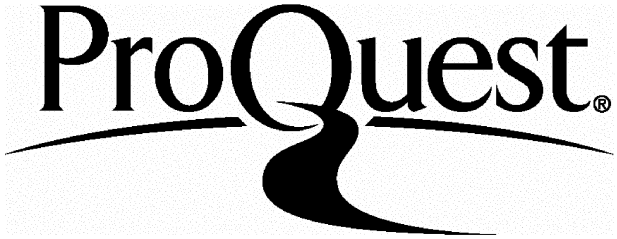

ABSTRACT

The accepted view on hormonal regulation of renal function is that hormones operating on the basolateral side of renal tubular cells control the expression, localisation and activity of proteins involved in key renal functions.

The studies presented in this thesis aimed at the exploration of the idea that bioactive peptides present in the tubular fluid (or 'pre-urine') may also have a role in controlling renal tubular cell function. To this end, urine from renal Fanconi syndrome (FS) patients was analysed and compared with that of normal individuals. In addition, the protein composition of apical and basolateral membranes from rat renal tubular cells was also investigated.

Methods for the extraction of polypeptides from urine were investigated. Implementation of these methods for the analysis of FS urine (with emphasis on Dent's disease) gave insights into the nature of low molecular weight proteinuria and suggested that the reuptake of proteins from the glomerular filtrate shows some kind of specificity and it is not as promiscuous as previously thought. In addition to plasma proteins, numerous peptides with previously reported bioactive actions were detected in both normal and FS urine, although the relative abundance of these peptides was altered in FS patients.

In a separate set of experiments, several proteins with potential roles in signal transduction were found in apical membrane segments of renal tubular cells. The presence of bioactive peptides in Dent's and normal urine and the finding that proteins with signalling roles are located on apical membranes support the notion of an intracrine system operating in the lumen of the healthy tubules. It is concluded that an alteration on the hormonal composition of the tubular fluid, as in diseases that lead to proteinuria, may contribute to the progression of these diseases with the end result of renal dysfunction and ultimately kidney failure.

ABBREVIATIONS

An alphabetically indexed list of abbreviations used in this thesis is presented below. Hyphenation of acronyms is prevalent in mass spectrometry and analytical chemistry in general; the individual terms and common concatenations are listed below.

β-2-GP I	β-2-glycoprotein I
1DE	One-dimensional gel electrophoresis
2DE	Two-dimensional gel electrophoresis
2D-LC	Two-dimensional liquid chromatography
ACN	Acetonitrile (Methyl cyanide)
ADH	Antidiuretic hormone (vasopresin)
ADIF	Autosomal dominant idiopathic Fanconi syndrome
AmBic	Amonium bicarbonate ($NH_2H_2CO_3$)
Ang	Angiotensin
APS	Ammonium persulphate
AQ	Aquaporin
ATP	Adenosine triphosphate
BBM	Brush border membrane (apical membrane)
BK	Bradykinin
BLM	Basolateral membrane
BME	β-mercaptoethanol
BMP	Bone morphogenic protein
BPB	Bromophenol blue
C18	Octadecyl silane packing material
cAMP	Cyclic AMP
CBB	Colloidal coomassie brilliant blue
CID	Collision-induced dissociation
Da	Dalton
DHB	Dihydroxybenzoic acid
DTT	Dithiothreitol
EGF	Epithermal growth factor
ESI	Electrospray ionisation
FS	Renal Fanconi syndrome
HCCA	α-cyano-4-hydroxy cinammic acid
HMWP	High molecular weight proteinuria
HPLC	High-performance liquid chromatography
HPX	Hemopexin
IAM	Iodoacetamide
ICAT	Isotope-coded affinity tag
IEF	Isoelectric focusing
IGF	Insulin-like growth factor
IGFBP	IGF binding protein
IPG	Immobilised pH gradient
IR	Infra-red
LC	Liquid chromatography
LC-MS	On-line liquid chromatography with mass spectrometric detection
LIFT	Proprietry technology used to focus fragment ions in a single

	MALDI-PSD experiment
LMWP	Low molecular weight proteinuria
m/z	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption/ionisation
Mr	Molecular weight
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
Na/K ATPase	Sodium/potassium ATPase pump
NaPi	Sodium phosphate cotransporter
PAGE	Polyacrylamide gel electrophoresis
PDGF	Platelet-derived growth factor
PEDF	Pigment epithelium-derived factor
pI	Isoelectric point
Pi	Inorganic phosphate
PKA	Protein kinase A (cAMP-regulated protein kinase)
PKC	Protein kinase C
ppm	Parts per million
PSD	Post-source decay
PT	Proximal tubular cell
PTH	Parathyroid hormone
Q-TOF	Quadrupole time-of-flight
RBP	Retinal binding protein
RP	Reversed-phase
SCX	Strong cation exchange
SDS	Sodium dodecyl sulphate
SPE	Solid phase extraction
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TIC	Total ion chromatogram
TOF	Time-of-flight
TTR	Transthyretin
UV	Ultraviolet
VDBP	Vitamin D binding protein

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1. Introduction

1.1. Biological Background

The kidney has several roles in maintaining mammalian body homeostasis. Of importance, these roles include allowing the excretion of toxic substances such as end-products of metabolism, while retaining others that are essential or of value, such as water, energy sources (e.g., sugars and amino acids), salts, vitamins, etc. Reabsorption of important molecules such as water and salts is under tight hormonal and neuronal control, such that organisms can quickly adapt to changes in their environment. Without being comprehensive or exhaustive, this section aims to summarize general aspects of hormonal regulation of kidney function as well as the features of renal physiology and pathophysiology that are relevant to the studies presented in this thesis.

1.1.1. Hormonal Regulation of Renal Function

General aspects

The functional unit of the kidney is the nephron; it has been estimated that there are 0.8 to 1.2 million nephrons per human kidney [1]. Anatomically and functionally nephrons are divided into two main structures: the Malpighian capsule and the tubules. The Malpighian capsule consists of the glomerulus and the Bowman capsule, while the tubules can be subdivided into the proximal and distal tubules, the loop of Henle, and the connecting segment. Afferent and efferent arteries are separated by a capillary plexus that is the site of filtration of blood. The glomerulus produces an ultrafiltrate of plasma. The physical basis of this ultrafiltration is provided by a filtration barrier, which is formed by fenestrated capillary endothelium, a basement membrane, and slit pores between the visceral epithelial cells that are on the other side of the basement membrane (to the endothelium and known as podocytes).

The hydraulic pressure of blood in the capillaries and the permeability of the filtration barrier determine ultrafiltration. About 180 liters of glomerular filtrate - that is, tubular fluid - are produced per day. The precise volume is determined by the glomerular filtration rate, which can be, in turn, regulated by peptide hormones such as angiotensin (Ang) II, endothelins, epithermal growth factor (EGF), among others (reviewed in [2]). Neuronal innervations also seems to play a role in the regulation of glomerular filtration rate [2].

Size and charge of the filtration barrier pores determine which molecules are filtered; it is generally accepted that substances with a molecular radius of ~500 nm are normally excluded from the filtration, whereas those with a radius of 200 nm are freely filtered [2]. This means that large proteins are normally not present in the glomerular filtrate and that the molecular weight cut-off for filtration is between 60 to 70 kDa for globular proteins. In contrast, other physiologically important molecules, such as vitamins, minerals, water, and sugars, are freely filtered.

Certain small molecules present in the tubular fluid are selectively reabsorbed along the tubules so that they only appear in urine in significant amounts when their concentrations in plasma are above the normal range levels. The bulk of this reabsorption occurs in the proximal tubule and it is mediated by proximal tubular (PT) cells, which are a specialized type of renal epithelial (tubule) cell. As with all renal epithelial cells, PT cells are polarized so that their cell membrane is divided into three anatomically and functionally distinctive domains: the basal, the lateral, and the apical membranes. The basal membrane faces the interstitial space (and peritubular capillary blood); the lateral side mediates cell-cell contacts and it is rich in adhesion molecules; finally, the apical membrane, also called brush border membrane (in the proximal tubule) due to its numerous invaginations, faces the lumen of the tubules and it is rich in ion channels and transporters that mediate the reabsorption of solutes from the tubular fluid.

The passage of solutes at the apical membrane is by facilitated diffusion under their concentration gradient, which is generated by active transport on the basal membrane. For example, a Na^+/K^+-ATPase operating at the basolateral membrane actively pumps Na^+ ions outside the cell. This creates a concentration gradient that drives the energy free transport of Na^+ ions from the tubular fluid into the cell through apical Na^+ channels and transporters. In many cases, entry of Na^+ ions is coupled with the co-transport of other molecules (symport), i.e., with the entry of e.g., inorganic phosphate (Pi), glucose or amino acids.

Regulation of this absorptive process in the proximal tubule is thought to be mediated by hormones present in the interstitial fluid and operating on the basolateral membrane; these hormones act from blood and modulate the activity and expression of ATP driven transporters operating basolaterally. In addition, the signal induced by certain regulatory factors diffuses from the basal side of tubular cells to the apical side such that

regulation occurs at the level of transcriptional expression and localization of co-transporters and ion channels at the brush border membrane.

Additionally, it has been recently found that peptide hormone receptors are also located on the apical side of tubular cells so that regulation may also takes place by the action of bioactive peptides present in the lumen of tubules [3]. For example, receptors for Ang II [4], insulin-like growth factor (IGF)-I and II [5], transforming growth factor (TGF)-β [6], Guanylin [7], and parathyroid hormone (PTH) [8] have been found to be located on apical membranes of tubular cells and the intraluminal concentrations of Ang II and IGF-I were reported to be ~4 nM and 1 nM, respectively [9-11]. In this respect, it was found that Ang II stimulates ion transport across tubular cells when administrated into the lumen of the tubules to a greater degree than when it acted at the basolateral membrane [12]. The presence of peptide hormones in the tubular fluid could arise from their filtration at the glomerulus as well as from the expression and secretion of these peptides by renal cells. For example, it has been reported that renal cells express and secrete bioactive peptides into the tubular fluid; these include angiotensin [4;9;12-14], EGF [15], platelet derived growth factor (PDGF) [16], kininogen (bradykinin precursor) [17;18], and osteopontin [19;20].

Regulation of phosphate absorption

An example of a reabsorption process that is regulated by the apical expression of the transporter is that of inorganic phosphate (Pi), which is mediated by members of the Na^+/Pi co-tranporters (NaPi) gene family [21]. The main kidney NaPi isoform is the type IIa Na^+/Pi (NaPi-II) and it is expressed in PT cells and located at the brush border membrane. It has been estimated that ~80% of filtered phosphate is reabsorbed in the first segment of the proximal tubule [21]. The mechanisms by which phosphate reabsorption is regulated are not completely known, but it is believed that this regulation is mainly mediated by exocytic insertion and endocytic retrieval of NaPi molecules at the brush border membrane from and into subapical vesicles. It is also suspected that phosphorylation plays a part in the modulation of NaPi-II activity because there is some evidence that this co-transporter is a phosphoprotein [21], but no experimental evidence exists regarding how phosphorylation may modulate NaPi-II activity. Certain peptide hormones (chiefly PTH) and dietary phosphate decrease the rate of Pi to be reabsorbed by PT cells by promoting the retrieval of NaPi-II molecules into early subapical endosomes

and, conversely, those factors that increase the rate of Pi reabsorption promote the insertion of transporters on the brush border [21].

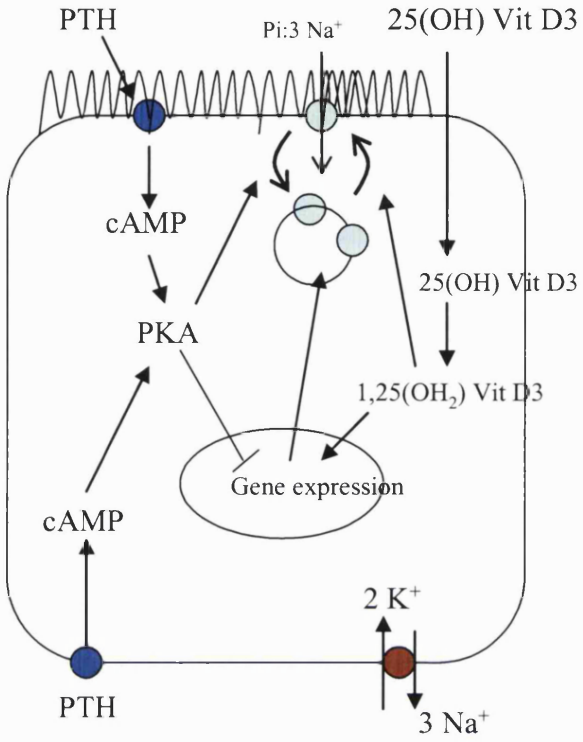


Figure 1.1.1. Mechanism and regulation of inorganic phosphate (Pi) reabsorption in PT cells.
Pi reabsorption is mediated mainly by NaPi-II, which is inserted into the plasma membrane from subapical endosomes. Parathyroid hormone (PTH) inhibits Pi reabsorption by retrieval of the NaPi-II molecules into endosomes, which are then taken by lysosomes and degraded. Down regulation of NaPi-II gene expression is also mediated by PTH, which signals via the cAMP/PKA signalling pathway. Inactive 25(OH) vitamin D3 enters PT cells bound to its binding protein. In the kidney 25(OH) vitamin D3 is converted to the active 1,25(OH) vitamin D3 by kidney hydroxylases. Pi reabsorption is enhanced by 1,25(OH) vitamin D3 by upregulation of NaPi-II gene expression and perhaps also by promoting the insertion of NaPi-II from endosomes into the apical membrane. Other factors may influence Pi reabsorption (not shown on in the figure for simplicity).

PTH is the peptide hormone that contributes the most to the regulation Pi reabsorption and its role is to decrease reabsorption so that infusion of PTH results in an elevated excretion of Pi in urine (i.e., phosphaturia). PTH receptors exists on the basolateral as well as the apical membranes [8], and although the molecular events that take place between receptor activation and retrieval of the transporter from the membrane are not completely understood, it is believed that the cAMP/protein kinase A and the DAG/protein kinase C signalling pathways are turned on as a result of PTH receptor activation [21]. NaPi-II interacts with PDZ containing protein 1 (reviewed in [22]); but the precise biochemical and molecular events that take place between the activation signal

transduction cascades and the retrieval of NaPi-II into endosomes to its final degradation in lysosomes are unknown. Figure 1.1.1 summarises the molecular mechanism of Pi reabsorption.

In contrast to PTH and dietary phosphate, which decrease reabsorption of Pi from the tubular fluid, vitamin D is believed to play a role in increasing Pi reabsorption. The mechanism seems to involve an upregulation of NaPi-II gene expression or changes in the lipid composition of membranes.

Other factors have been found to have an effect on Pi reabsorption, including insulin, growth factors (such as IGF, TGF-beta and EGF), atrial natriuretic peptide, thyroid hormone, glucocorticoids, and glucagon, among others [21]. Some of these factors stimulate Na-Pi cotransport (e.g., IGF-I, and insulin), while others decrease it (e.g., EGF and TGF-beta). Since studies from which the above discussion is derived were conducted to test the effect of specific factors, it is not known the precise contributions of each of the factors to the regulation of Pi reabsorption. Therefore, knowledge of the composition of the interstitial fluid around PT cells would be required in order to know the contributions of each factor to Pi homeostasis. Furthermore, as mentioned above, receptors for some of these bioactive peptides are thought to be located on the apical side of tubular cells. Thus, the composition of the tubular fluid should also be considered when making a model of Pi regulation together with the knowledge of receptor availability and their response to a mixture of factors (rather than just considering a factor at a time).

Regulation of water reabsorption

The regulation of Pi reabsorption, briefly discussed above, is an example of a regulatory process that is understood to occur at the proximal tubule. However, other processes are regulated in more distal parts of the tubule. One example is that of water reabsorption. As with Pi, the bulk of filtered water is reabsorbed by PT cells, but the fine-tuning of water homeostasis and its regulation occurs in the distal tubule and collecting duct. Similarly, as with the regulation of Pi, modulation of water reabsorption is controlled by exocytic insertion and endocytic retrieval of vesicles containing water channels into the apical membrane.

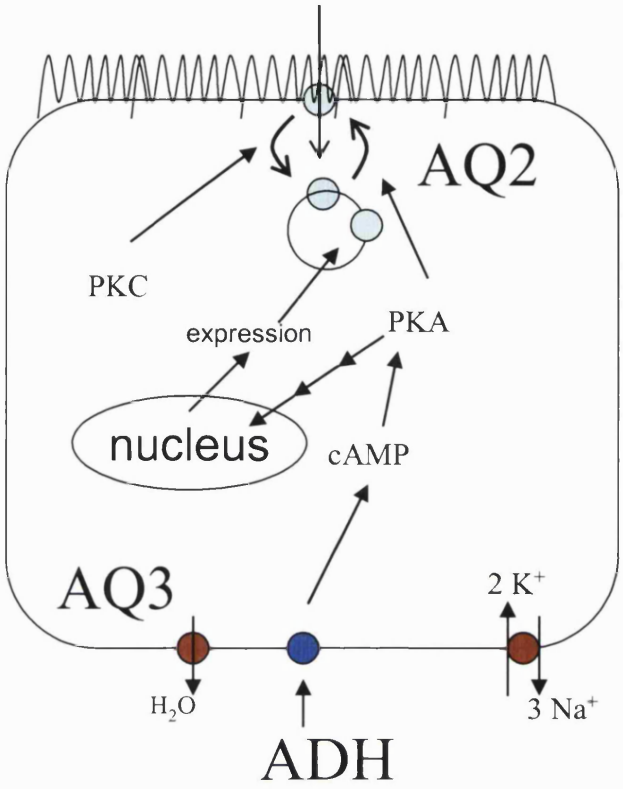


Figure 1.1.2. Mechanism and regulation of water reabsorption from the distal tubules.
Water is reaborbed from the glomerular filtrate in distal tubule and collecting duct by water channels (aquaporin 2, AQ2, in the apical membrane and AQ3 at the basolateral). Factors that increase the activity of PKA, such as antidiuretic hormone (ADH), promotes the insertion of AQ2 from subapical endosomes into the apical membrane. Conversely, diuretic factors promote the retrieval of AQ2 from the membrane into endosomes and signal through PKC. PKA also influences AQ2 gene expression.

This process is understood in some detail and it has been the subjects of several recent reviews [23-25] (Figure 1.1.2). Vasopressin, acting on basolateral receptors, promotes the insertion of vesicles containing aquaporin 2 into the apical membrane [26]. Aquaporin 2 is one of the members of the aquaporin gene family that was found shortly after cloning the first water channel [27]. Upon vasopressin activating its receptor, the intracellular cAMP levels are increased. This is a consequence of vasopressin receptor activating tetrameric G proteins, which in turn, activate adenylate cyclase; the latter catalyses the conversion of ATP into cAMP, which then activates protein kinase A (PKA). It was found that PKA phosphorylates aquaporin 2 at a conserved serine residue (Ser-256) and, as a result, this water channel is translocated from subapical vesicles to the plasma membrane, where it mediates the reabsorption of water from the tubular fluid (reviewed in [23]) along the concentration gradient generated by the basolateral water channel isoforms aquaporins 3 and 4 [23]. The retrieval of aquaporin 2 from the apical membrane seems to

be promoted by factors that increase the activity of protein kinase C (PKC) such as phorbol esters and prostaglandin E2, although it has been found that PKC does not directly phosphorylate aquaporin 2 [28] so that it was proposed that retrieval may be caused by rearrangements on the cytoskeleton.

Other factors may play a role in water homeostasis; among these, bradykinin (BK) seems to augment water (and salt) lost by the kidney [29]. BK is formed from its precursor kininogen, which is synthesized, among other tissues, by proximal and distal renal tubular cells [17;30]. Proteolytic action of tissue kallikrein converts kininogen into kallidin II, which is then further hydrolysed by peptidases into BK. Kininases exist which cleave and thus inactivate BK. Since BK acts by activating PKC, the inhibitory properties of this peptide in water reabsorption may be explained by the actions of PKC on the retrieval of aquaporin 2 from the apical membrane of distal tubular cells. BK receptors seem to be expressed in distal tubular cells and located in both apical and basolateral membranes [30-33]; therefore, it seems possible that intraluminal BK may have a role in regulating water homeostasis.

Other factors have been found to influence water permeability of the collecting duct including endothelins [34], prostaglandin [35], and perhaps also other factors that increase the PKC or PKA signalling pathways such as EGF [36] also have an effect on water reabsorption. In addition, nucleotides may also have an effect in water regulation [37]. The relative contributions of these factors in regulating water absorption are not known because of the difficulties in testing the responses of biological systems to a combination of hormones and because of the lack of knowledge on the precise composition of the interstitial and tubular fluids.

1.1.2. Reabsorption of polypeptides from the glomerular filtrate

In contrast to the reabsorption of solutes from the tubular fluid discussed in the preceding section, which occurs down their concentration gradients, the reuptake of polypeptides is thought to occur by receptor-mediated endocytosis, a process that requires the direct input of metabolic energy. This process also occurs in the first segment of the proximal tubular cells and at least two of the receptors that mediate this process have been identified and named gp330/megalin and the cobalamin receptor, also known as cubilin [38]. These two proteins seem to mediate the reabsorption of most, if not all, peptides and proteins in the tubular fluid. Furthermore, megalin has been implicated in the reabsorption of certain drugs [39;40]. Megalin is a member of the very low density lipoprotein receptor

(VLDL-R) gene family and shares homology with other VLDL-R [41]. The size of megalin is about 460 kDa and seems to contain a single transmembrane domain. Its cytoplasmic tail has several EGF like repeats [41]. In contrast, cubilin does not contain any predicted transmembrane region and it is thought that this protein is anchored to the brush border membrane by its interaction with megalin [42].

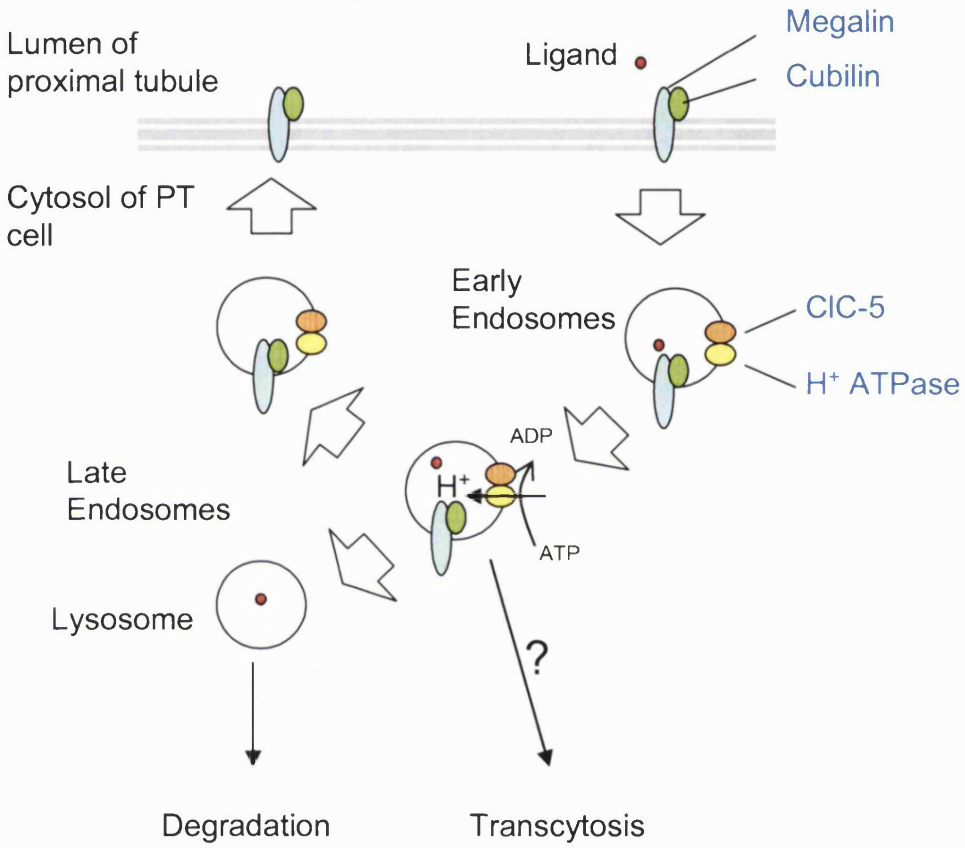


Figure 1.1.3. The megalin-cubilin endocytic pathway in renal PT cells.
In this diagram, ligands are defined as any of the molecules known to bind megalin and/or cubilin. Upon binding, the ligand-megalin-cubilin complex is endocytosed in early endosomes, which mature into late endosomes and then lysosomes, where the ligand is taken and degraded. As an alternative intracellular route for the ligand, certain peptide hormones have been found to be transcytosed so that they escape degradation. The role of the vacuolar ATPase and ClC-5 channel is to allow for the acidification of late endosomes so that ligands dissociate from the receptor complex, which then recycles back to the plasma membrane. Disruption of any of these processes could lead to the manifestations of the renal Fanconi syndrome (see later).

The megalin/cubilin-mediated endocytic pathway operates like classical receptor-mediated endocytosis of proteins [43;44] as exemplified by the transferrin retrieval pathway (Figure 1.1.3). In this pathway, ligands such as proteins and peptides bind either megalin or cubilin (or both). This prompts the internalization of the receptor-ligand complex into early endosomes, which are clathrin coated. How this binding triggers the internalization process is not yet known in detail but adaptor proteins such as Dab2 [45] may have a role

in mediating endocytosis. Certain GTPases such as Rab5 have been known for some time to play a role in receptor mediated endocytosis and they may also prove to have a role in megalin-cubilin internalization [46]. Once inside the cells, these early subapical endosomes are matured into late endosomes and their lumen acidified by a vacuolar H^+-ATPase to ~ pH 5.5; this allows for the dissociation of the ligand from the receptor [47]. Late endosomes transfer their cargo to lysosomes where they are degraded, while the receptor is recycled back to the plasma membrane so that another round of receptor mediated endocytosis can take place.

Other members of this pathway have been identified. For example, a chloride channel, termed ClC-5, seems to be needed for allowing the passage of chloride ions alongside protons into endosomes so that their acidification is not electrogenic. Loss of function of the ClC-5 channel in knocked-out mice resulted in low molecular weight proteinuria and Dent's disease patients, who also have low molecular weight proteinuria, have been found to have mutations in the CLCN5 gene, which codes for the ClC-5 protein. Endosomes in PT cells of CLCN5 knocked-out (KO) mice showed a slow trafficking rate from subapical endosomes to the apical membrane [48]. Similarly, Dent's disease patients had a low concentration of megalin in urine when comparing them to normal individuals, which suggest that less megalin is shed from tubular cells into urine because there is less of it present at the apical membrane [49]. Megalin also interacts with the Na^+/H^+ exchanger isoform 3 (NHE3) and although the significance of this is not fully clear, NHE3 may also have a role in counteracting the electrogenic acidification of endosomes. And since the activity of NHE3 is regulated [50], this link may also provide a way of regulating protein reuptake and could explain the decrease of proteinuria by drugs that inhibit the formation of peptide hormones, e.g., Ang II by angiotensin converting enzyme (ACE) inhibitors or receptor blockers [51]. Another chloride channel isoform, namely ClC-4, has recently also been implicated in megalin-cubilin mediated endocytosis [52].

The megalin-cubilin endocytic pathway is probably important for the salvage of amino acids, which otherwise would be lost in urine in the form of protein. It is estimated that about 0.1% of albumin is filtered from plasma and present in the glomerular filtrate [53]. This corresponds to 8 g of albumin filtered every day. Furthermore, plasma contains other proteins with lower molecular weight than albumin and therefore they are filtered more readily than albumin. As discussed above, megalin and cubilin mediate the reuptake

of many proteins including albumin [54;55]. Thus, large amounts of amino acids would be lost in urine if this pathway was defective and this is indeed the case as exemplified by mutations that cause low molecular weight proteinuria (see later).

In addition, transcytosis of certain vitamins seems to be dependent on the megalin-cubilin endocytic pathway and thus this pathway may be also essential for the salvage of vitamins such as retinol [56;57] and thyroglobulin [58], for which transcytosis from the tubular fluid into the interstitial fluid is dependant on their binding to megalin and/or cubilin. Other vitamins are endocytosed by this pathway bound to their carrier proteins including vitamin D3 and vitamin B12 [59-61], which is also important for the reabsorption of peptide hormones such as insulin and PTH [62;63]. The fact that the megalin-cubilin pathway is needed for the activation of vitamin D3 precursor into its active form [64] highlights the importance of this pathway for normal vitamin physiology [65]. Lipoproteins [66] and advanced glycation end products [67] also seem to be ligands of megalin and/or cubilin.

In addition to its expression in kidney, megalin is expressed in brain and KO mice showed abnormalities in brain development [43]. Later, megalin was implicated in the biology of sonic hedgehog, a growth factor involved in central nervous system development [68]. These findings could be explained by direct signalling functions of megalin or by a function of this protein in endocytosing and thus terminating the signal induced by this growth factor [69]. Moreover, megalin could also be involved in signalling indirectly by mediating the internalisation of hormones, such as retinol, that have their receptors located intracellularly [69]. Recently, the cytoplasmic tail of megalin has been shown to interact with proteins with signalling roles and a role in signal transduction has been suggested for megalin [70-72], although no conclusive evidence is yet available in this respect.

1.1.3. The renal Fanconi syndrome

The renal Fanconi syndrome (FS), previously known as Lignac-de Toni-Debre-Fanconi, is attributed to have been described by Abderhalden in 1903 (reviewed in [73]). Later, Lignac (1921), Fanconi (1931), Toni (1933), and Debre (1934) independently described a condition characterized by renal disease, dwarfism, rickets, and albuminuria. In 1936, Fanconi suggested that these studies were describing the same condition, and in 1943 McCune proposed to collectively call this disorder FS [73].

There are several forms of the FS and all of them present different degrees of the same manifestations including defective reabsorption of several solutes and bone disease (rickets in adults and osteomalacia in children) [73]. Solutes that fail to be reabsorbed and appear in urine at abnormally high concentrations include glucose, urate, phosphate, bicarbonate, water, potassium, amino acids, peptide hormones, and proteins [73]. Clinical features include polyuria (large water excretion in urine), dehydration, hypokalaemia (low potassium concentration in blood), rickets, and impaired growth. A consistent clinical finding is low molecular weight proteinuria (LMWP).

It is important to distinguish between high molecular weight proteinuria (HMWP) and LMWP. In the former, proteins appear in urine as a consequence of an overload of proteins in the tubular fluid secondary to disruption of the glomerular filter. The reabosorption mechanisms are overwhelmed and thus proteins leak into urine. In contrast, in LMWP the glomerular filter is not compromised and the defect is in the reabsorption of polypeptides from the glomerular filtrate. Therefore, LMWP is also known as tubular proteinuria. The fact that FS patients have LMWP suggested that the defect is on the transport mechanism that mediates the uptake of proteins and other solutes from the glomerular filtrate. Indeed, it has become apparent that a dysfunction of any of the steps involved in megalin-cubilin endocytic pathway can or could result in FS [73].

There are not known human diseases caused by mutations in the megalin gene, possibly because loss of function of megalin would be lethal, as it is in megalin KO mice [74;75]. These mice show developmental abnormalities including brain malformations and most of them die at birth. Only one in fifty megalin KO mice reach adulthood and they show the manifestations of the FS. Recently, another megalin KO mice, with a conditional targeted mutation of the kidney gene, has been reported [76], which allowed to study the effects of megalin loss of function in the kidney in more detail. It was shown that megalin KO mice have poor bone mineralisation, probably due to loss of vitamin D in urine, and confirmed previous studies that pointed to the importance of the megalin-cubilin endocytic pathway in the intake of vitamin D precursor bound to its carrier protein into the kidney PT cells. This step is important for the subsequent conversion of the inactive 25-hydroxy vitamin D_3 into the active 1,25-dehydroxy vitamin D_3 by kidney specific hydroxylases [74;75].

Mutations in the cubilin gene that lead to dysfunction of cubilin are associated with a form of anaemia due to defective absorption of vitamin B_{12} from the intestine [77].

Patients with Imerslund-Grasbecks disease have mutations in the cubilin gene [78] and dogs with defective processing of cubilin have also been reported [79]. In addition to anaemia, due to defective absorption of vitamins from the intestine, Imerslund-Grasbecks patients and dogs with mutations in the cubilin gene present the manifestations of the FS including LMWP.

As briefly outlined in the preceding section of this thesis, Dent's disease, a congenital form of the FS linked to the Xp11.22 chromosome, is caused by loss of function of the CLCN5 gene, which codes for the ClC-5 protein [80-82]. In addition to FS, Dent's patients often develop kidney stones (nephrolithiasis). It was shown that other X-linked conditions, in addition to Dent's disease, such as X-linked recessive nephrolithiasis, and X-linked recessive hypophosphataemic rickets are caused by *CLCN5* mutations and it was proposed to collectively call all these conditions Dent's disease [80]. As discussed in Chapter 1.1.2, the role of the ClC-5 channel seems to be in the endosomal acidification needed for the dissociation of the receptor-ligand complex [83] as suggested by the co-localisation of ClC-5 with the vacuolar ATPase proton pump in PT cells [84]. There also exist two murine models of Dent's disease, which show the manifestations of the FS, including LMWP [85;86]. It has been demonstrated that recycling of the megalin-cublin receptor complex is disrupted in CLC-5 KO mice [48].

It is not known how a defect in the reabsorption of proteins can cause the manifestations of FS and kidney stones. Piwon *et al.* proposed that the hypercalcuria and phosphaturia is secondary to the LMWP [85]. As discussed in Chapter 1.1.1, the reuptake of filtered phosphate is mediated by NaPi-II in PT cells and regulated by PTH and vitamin D_3. There are basolateral and apical receptors for PTH and an important route for the intake of vitamin D into PT cells is through the endocytosis of vitamin D binding protein (VDBP). Since the effect of PTH is to decrease the reuptake of phosphate from the tubular fluid, an increased intraluminal PTH concentration would produce phosphaturia. And indeed, this is a common manifestation of Dent's disease and other forms of the FS.

Lowe's syndrome, another X-linked congenital form of the FS, is characterized by cataracts and mental retardation, in addition to having LMWP and the other manifestations of the FS [87]. The gene mutated is the oculocerebrorenal syndrome of Lowe (OCRL). This gene codes for a phosphatidylinositol (PtdIns)-(4,5)-bisphosphate (P_2) 5-phosphatase, which catalyses the conversion PtdIns(4,5)P_2 into PtdIns(4)P. The OCRL phosphatase is located in the lysosomal membrane and in the trans-Golgi network [88;89].

It has been suggested that loss of function of this protein results in an accumulation of inositol phospholipids and that this leads to a defective trafficking of endosomes [89]. Another hypothesis to account for the LMWP observed in Lowe patients is that abnormal concentrations of inositol phospholipids lead to a defective regulation of the cytoskeleton, which in turn leads to defective trafficking of endosomes [90]. This hypothesis is based on the observation that PtdIns(4,5)P_2, the substrate of OCRL, can dissociate profilin-actin complexes and it also binds certain cytoskeletal binding proteins such as Ezrin, Radixin, and Moesin [90]. Thus, it is possible that Lowe patients have a more severe phenotype than patients with other forms of the FS because a defect on endosome trafficking would lead to malfunction of many cell types in several organs since the OCLR enzyme is expressed in several organs, whereas ClC-5 expression is restricted to the kidney. Alternatively, or in addition, it has been suggested that the pathophysiology of Lowe syndrome is attributable to leakage of lysosome enzymes into the circulation, which subsequently mediate cell injury [91].

Other congenital diseases that give rise to the manifestations of the FS include: cystinosis, Wilson disease, glycogen storage disease type I, fructose intolerance and autosomal dominant idiopathic FS (ADIF). The molecular bases of ADIF have not been identified, although the gene have been mapped to a large region of chromosome 15 [92]. Diseases of carbohydrate metabolism also lead to FS. At least in part, this may be because a defective production of metabolic energy impairs ATP generation and therefore function of ATP-dependent processes, such as the vacuolar proton ATPase required for endosomal acidification [73].

In addition to the genetic causes introduced above, environmental factors can also produce the symptoms of the FS, among these certain chemotherapeutic drugs and heavy metals have been described to give some of the manifestations of the FS [73]. The molecular bases by which these compounds produce FS are not known; but it is believed that cadmium inhibits the vacuolar ATPase pump that co-localises with ClC-5 and is responsible for acidifying endosomes in PT cells [93]. FS phenotype is thus probably produced by inhibition of receptor-mediated endocytosis by a mechanism similar to that described above for Dent's disease.

1.2. Analytical Methods

The aim of this section is to briefly introduce and discuss the physicochemical basis of the analytical methods used during the course of the studies presented in this thesis. Two main methods were used to separate proteins, namely gel electrophoresis and liquid chromatography (LC). The separated proteins were then analysed by mass spectrometry (MS). A brief overview of these methods will be presented below. In the next section it will be discussed how the combination of analytical approaches is currently used in the field of proteomics to characterize qualitatively whole proteomes and to make relative quantitative analyses of changes that occur in these proteomes as a result of a stimulus, genetic defect, disease, etc.

1.2.1. Gel Electrophoresis of Proteins

The word electrophoresis describes a process in which charged molecules migrate in an electric field [94]. If the charges of these molecules differ they will migrate from one of the poles to the other at different velocities and this provides the basis for separation. Electrophoresis of proteins for analytical purposes is commonly carried out in acrylamide gels [94]. These gels are formed by the polymerisation of acrylamide molecules with occasional crosslinks provided by N,N,N',N'-methylenebisacrylamide (bis-acrylamide).

Acrylamide gels contain pores, the sizes of which are determined by the concentration of acrylamide and bis-acrylamide present in the gel. A common form of gel electrophoresis of proteins is that of sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). In this form of electrophoresis, proteins are reduced and denatured in the presence of the anionic detergent SDS, which binds strongly to the amino acids of the reduced protein (on average one SDS molecule binds to every other amino acid). As a result, the intrinsic charge of the protein is overwhelmed by the negative charges of SDS, and therefore, proteins of similar molecular weight have the same apparent charge. Under these conditions, the separation by PAGE is believed to be mainly determined by the sieving effects of the pores present in the acrylamide gels. Therefore, protein migration in SDS-PAGE can be related to the size or molecular weight of the protein [94].

Another form of electrophoresis of proteins is that of isoelectric focusing (IEF) [94]. Proteins are amphoteric molecules, with different proteins having different isoelectric points (pI), and therefore they can be separated on this basis. The method of IEF relies on

introducing ampholytes in the acrylamide gel that form a pH gradient upon the application of an electric field. Since gels with very low porosity are used (4%) sieving effects are negligible. Ampholytes are mixtures of synthetic polyamino-polycarboxylic acids that cover a specific pH range (e.g. pH 3 to 10). When the protein mixture is loaded and an electrical field applied, proteins migrate to the position in the gel until they reach the region of the gel in which the pH matches their isoelectric point, i.e. the point in the gel in which their charge is zero [94]. Because charge is zero, the protein accumulates or 'focusses' in that gel region and does not further migrate in the electric field.

Two-dimensional gel electrophoresis (2DE) combines the two electrophoretic methods described above; in the first dimension proteins are separated on the bases of pI by IEF, while in the second they are separated by SDS-PAGE according to their molecular weight. This mode of electrophoresis was simultaneously described for proteins in 1975 by O'Farrell [95] and Klose [96] and is currently widely used for the separation and visualisation of whole proteomes.

With the introduction of gels with immobilized pH gradients (IPG) for the IEF step, the reproducibility of 2DE-based separations improved. Other technological advances included the inclusion of chaotrophes (e.g., urea and thiourea) and neutral or zwitterionic detergents (e.g., CHAPS) in the buffer used for the IEF separation [97]. The inclusion of these solubilising reagents is thought of being of particular importance because otherwise proteins tend to precipitate when they reach their pI.

Current research trends in the field of 2DE are directed at improving the solubilisation of as many protein classes as possible during the IEF (e.g., [97-101]) and to the development of detection methods with more dynamic range and sensitivity than the classic staining of proteins that use silver nitrate and Commassie blue dyes (e.g., [102-104].

1.2.2. Liquid Chromatography

Much or the practical work carried out during the studies described in this thesis was performed using liquid chromatography (LC), either by itself as the main separation method, or in combination with gel electrophoresis to increase the separation capacity of the analytical approach.

Molecules can be separated on the basis of their different partition or distribution between two immiscible phases [105]. In column LC these two phases are provided by a stationary phase, which consists of chromatographic beads with distinct functionality, and

a liquid mobile phase. Molecules can be separated when their distribution coefficients (Kd) are different. Kd is defined as the concentration of a molecule in the stationary phase divided by its concentration in the mobile phase.

Several forms of liquid chromatography are available, including reversed-phase (RP), ion exchange, size exclusion chromatography, affinity chromatography, etc. The practical studies described in this thesis were carried out using high performance LC (HPLC) and the types of chromatography used were RP and ion exchange. The principles of these types of LC are briefly described in the following.

Reversed-phase liquid chromatography

In RP-LC, proteins and peptides are separated according to their hydrophobicity [105]. The functional groups on RP stationary phase are provided by aliphatic carbon chains; these are hydrophobic (non-polar) and mediate the retention of peptides and proteins by hydrophobic and van der Waals interactions. The mobile phase is relatively polar. Due to the fact that proteins and peptides have similar Kd values, they cannot be well separated by isocratic elution. Instead, gradient elution is normally carried out in order to separate these molecular species [106;107].

The choice of stationary phase depends on the hydrophobicity of the analytes under study. Thus, more hydrophobic species, such as proteins can be separated by relatively hydrophilic alkylsilane stationary phases such as butyl (C4) or octyl (C8) (i.e. chromatographic silica beads derivatised with aliphatic chains containing 4 or 8 carbon atoms) containing relatively large pore sizes. Derivatised silica beads with pore sizes of 30 nm are normally used for the separation of proteins, although the use of gigaporous beads, with pore sizes of 100 to 300 nm, have been reported for fast chromatography of proteins [108] and these were used for some of the experiments described in this thesis (see later). In contrast, small peptides are better separated with columns packed with beads derivatised with octadecyl groups (i.e. C18) containing relatively small pore sizes (10 nm are normally used in commercially available columns for peptide analysis).

Typically, loading and equilibration is carried out using an aqueous mobile phase containing trace organic solvent and acid. Polypeptides are eluted with increasing concentration of organic solvent (organic modifier) in the mobile phase [106;107]. RP-LC is currently widely used for the analysis of proteins and peptides due to its high resolution. Moreover, RP-LC is easily hyphenated (i.e., coupled) to mass spectrometry and

consequently this form of LC is now extensively used in proteomic related applications for high throughput analysis (e.g., [109-113])

The solvophobic theory has been used to explain the mechanism of retention in RP [114]. An intuitive and non-mathematical description of this theory is that there is a gain of entropy of the system as a result of hydrophobic residues in the analyte escaping from water molecules, which would otherwise form structured lattices around the molecule, thus decreasing entropy. As a result, the interaction of non-polar groups with the bonded stationary phase is thermodynamically favoured. Whether the retention in RP occurs by partition or absorption is not known, but the solvophobic theory seems to explain both models [114].

Ion exchange chromatography

In ion exchange chromatography, the stationary phase consists of charged functional groups and separation of molecular species occurs on the bases of their differences in charge [105]. The mobile phase is composed of a solution containing the counter ion of that present in the stationary phase. There are two main types of ion exchangers, namely anion exchangers and cation exchangers. The form of ion exchange used here was strong cation exchange (SCX), in which sulfonic acid groups are linked to the stationary phase; these groups are negatively charged and ionised at any pH. Polypeptide samples dissolved in a solvent of low pH and ionic strength are loaded in the SCX column so that all the polypeptides have at least one positive charge, which interacts with the negative charge of the sulfonic acid groups. Elution is carried out by increasing the ionic strength of the mobile phase or by increasing its pH. This mode of chromatography has been used for the analysis of peptides and proteins [115] and currently it is used for 2D-LC based separations in proteomics (e.g., [116]).

Microcapillary and Nano-flow Liquid Chromatography

Traditionally, analysis by packed HPLC is carried out in columns with an internal diameter (ID) of 4.6 mm [105]. These so called analytical columns have typical flow rates of 1 ml/min. Columns with IDs of 1 and 2 mm are termed narrow bore columns and those packed in capillaries with IDs of 800 μm, 500 μm, 300 μm and 150 μm are known as microcapillary (μ) columns. Columns with narrower IDs are available; these are termed 'nanoflow' columns and have internal diameteters of 100 μm, 75 μm, and 50 μm.

The advantages of performing HPLC separations in columns with narrow IDs have been reviewed [117-119]. The ID of the column influences none of the parameters used to measure the efficiency of a LC separation, although length does have an effect. For example, the efficiency of an HPLC column is often measured in terms of plate numbers (N), which is defined as follows [105]

$$N = 16\left(\frac{t_R}{w}\right)^2$$

where t_R is the retention time and w is the width of the peak at its base. N is an important parameter because other parameters such as resolution and peak capacity of the column are related to it.

It has been found that N does not change as a function of the ID of the column [119]. If an analyte eluted from a chromatographic column producing a peak with a width at half height of say 0.5 minutes, in conventional HPLC, in which the flow rate is 1 ml/min, the volume of the peak would be ~ 500 µl, whereas if the separation was carried out using a 75 µm ID column, in which the flow rate is 200 nl/min, the volume would be ~ 100 nl. It follows that 75 µm ID columns can concentrate the analyte 5000 times more than 4.6 mm ID columns. In the preceding discussion it is assumed that both columns have the same efficiency (i.e., that the peak width produced with these two systems are the same). Analyte concentration is important for its detection by UV absorption, which is the most common detection method in LC, as indicated by the definition of absorbance (Abs) derived from the Lambert-Beer Law [120]:

$$Abs = \varepsilon\, c\, l$$

where Abs is proportional to analyte concentration (c), the length of the path through the solution (l) and the extinction coefficient (ε) of the analyte. Thus, by using a 75 µm column instead of a 4.6 mm column, and consequently, increasing the concentration of analyte by a factor of 5000 the sensitivity of a chromatographic system with UV detection increases by the same factor. The sensitivity by other detection methods, such as electrospray ionisation (ESI) mass spectrometry, whose response is also concentration dependent, is also improved by column miniaturization. In the case of ESI, the observed increase of analytical sensitivity is also attributable to a more efficient ionisation at low flow rates (this will be discussed in more detail below).

There are technical problems associated with the reduced flow rates used in microcapillary LC (μLC) and nanoflow LC (nanoLC). One of them is that relatively small dead volumes (i.e. the volume between the end of the column and the detector) can have detrimental effects in the resolution because of diffusion of the analyte after separation and concentration. Reduction of the ID of the connecting tubes minimises this dead volume but some extra-column effects are commonly observed. Injectors with low dead volumes are also commercially available. Another problem is that at low flow rates diffusion would occur in the UV detector. The Lambert-Beer Law predicts that, in addition to concentration, absorbance is dependent on the optical path length in the UV detector. However, if this path length was too large diffusion of the analyte would occur such that this would lead to dispersal and peak broadening. To solve this problem, flow cells for UV detectors have been developed with U- and Z-shaped configurations that minimize dead volumes while maintaining a relatively long optical path [121].

1.2.3. Biological mass spectrometry

The development of the first mass spectrometer is attributed to J.J. Thomson, who at the beginning of the 20th century measured the charge-to-mass ratios (e/m) of several atoms and small molecules (reviewed in [122]). In the first halve of the 20th century developments in ionisation methods and analysers occurred with the concomitant application of mass spectrometry in the fields of organic chemistry for the elucidation of chemical structures and for the analysis of environmental and industrial samples. It was not until the beginning of the 1990s, however, that the field of biological mass spectrometry became significant. This was due to the introduction of electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI) methods that allowed for the ionisation of macromolecules such as proteins and peptides.

In this section I shall briefly discuss the aspects of mass spectrometry that are relevant to the studies presented here and will not consider applications of biological mass spectrometry such as analysis of nucleotides, carbohydrates, lipids and small molecules of biological origin. These uses of biological mass spectrometry for analysing these molecules were not explored during the course of the experimental work presented here.

A mass spectrometer can be defined as an instrument capable to measure the mass of molecules. Mass spectrometers consist of three main parts; namely, an ion source, a mass analyser, and a detector [123]. In addition, sample inlets and recorders are needed, but they are not part of the mass spectrometer as such. In order to be analysed in the

mass analyser molecules have to be ionised and transferred to the gas phase. Early ionisation methods such as electron impact (EI) and chemical ionisation (CI) introduce a large internal energy in the analyte molecule, which as a result fragments. The fragmentation pattern can be used in structure determination or as a fingerprint of the molecule. Libraries exist where fragmentation patterns of unknowns can be compared with those in the library [123]. Unfortunately, these ionisation modes are too energetic and consequently inefficient for polypeptide analysis. Fast atom bombardment (FAB) ionisation was introduced at the beginning of the 1980's and this method proved to be more effective in ionising peptides [124;125], although its low sensitivity made it unsuitable for the analysis of biological material, from where only small sample amounts are routinely available. Introduction of less energetic ('softer') ionisation methods, namely ESI and MALDI, at the end of the 1980's made it possible analysing peptides and proteins with the sensitivity required for most biological applications. The bases of ESI and MALDI will be briefly described below.

With regard to the mass analysers, many different instruments exist, but they all have in common that they separate the ions produced in the ion source according to their mass-to-charge ratio (m/z). Examples of mass analysers include magnetic sectors, electric sector, quadrupole ion filters, ion traps, time-of-flight (TOF), and Fourier transform ion cyclotron resonance MS. In the studies described in this thesis quadrupole and TOF mass spectrometers were used; the physical principles that govern the separation of ions in these types of mass spectrometers will be briefly outlined below.

Once the ions produced in the ion source have been separated in the mass analyser they are detected in one of the several types of detectors that have been developed; the most commonly used detector in modern mass spectrometers is the electron multiplier. The signal detected is sent to a computer which records the m/z value of the detected ions and displays them graphically in a mass spectrum.

Matrix-assisted laser desorption/ionisation

MALDI was first introduced by Karas and Hillenkamp [126] and Tanaka [127] in 1988 as a soft ionisation method with which relatively large macromolecules can be analysed. In this ionisation method the sample to be analysed is mixed with an excess of matrix molecules and allowed to crystallise. The matrices used in MALDI are typically acidic compounds (carboxylic acids are normally used) with an absorption in the region of the laser wavelength. The analyte/matrix crystals are usually placed in high vacuum and

irradiated with a laser beam (Figure 1.2.1). Routinely, UV nitrogen lasers are used at a wavelength of 337 nm, although the use of IR lasers has been reported [128;129].

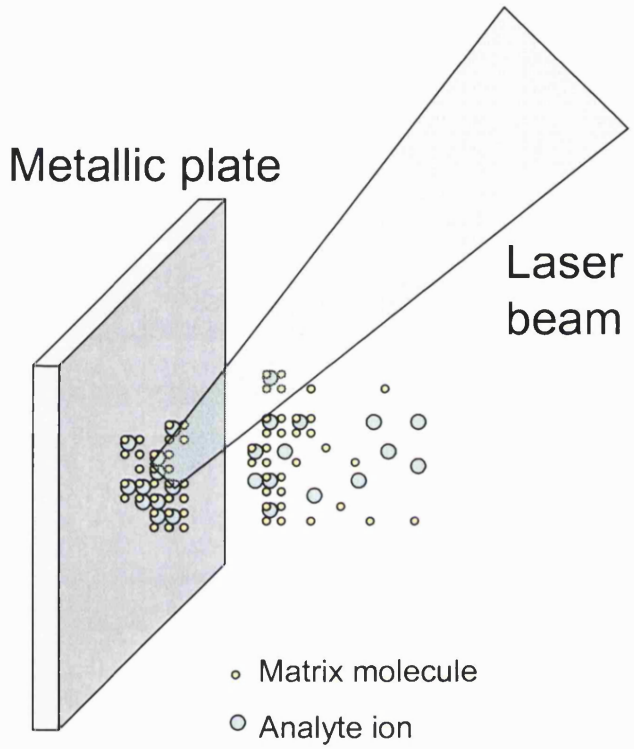


Figure 1.2.1. Ion formation by MALDI.

Analyte molecules are co-crystallised with an excess of matrix molecules. A hypothesis to account for ion formation by MALDI is that irradiation of these crystals with a laser beam desorbs matrix-analyte ion clusters, which then undergo gas phase reactions. As a result such clusters dissociate to leave free analyte and matrix ions.

The process of ion formation in MALDI is not very well understood. It has been proposed that after laser irradiation of the matrix-analyte crystals, clusters of matrix-analyte are formed by a complex combination of thermal desorption, sublimation/evaporation, and ablation by phase explosion induced by the laser beam (reviewed in [130]). The relative contributions of these processes to the desorption mechanism and whether other processes occur after laser irradiation are not yet clear. After desorption (or at the same time) ions are formed, most probably by the cluster ionisation mechanism (review by [131]). This theory assumes that analyte ions are preformed in the crystal, which explains why most matrices used for UV MALDI are acidic molecules. Recently, it has been shown that particles of a wide size distributions are also formed, some of them being as large as 1

μm [132]. Upon desorption, matrix-analyte ions clusters are formed and they undergo proton transfer reactions, which for peptides can be schematised as follows [131]:

$$\{(M + nH)^{n+} + (n-1)A^- + x\ ma\} \rightarrow MH^+ + (n-1)AH + x\ ma$$

where M: analyte; ma: matrix; A: anion; {...}: cluster.

Protonated analyte ions (MH^+) are formed, whose m/z values can then be measured by MS analysers; TOF analyser is the most commonly used, although instruments exist that hyphenate MALDI to ion traps, FT-ICR, and Q-TOF analysers.

Electrospray ionisation

ESI is another soft ionisation method that was introduced by Fenn [133] at approximately the same time as MALDI, although the principles of the formation of ions from macromolecules in atmospheric pressure had been described by Dole in the late 1960s [134]. The process of ion formation by ESI is depicted in Figure 1.2.2. In ESI, ions are formed from solution by the application of high voltage to a capillary from where the sample is sprayed.

The mechanism by which ions are formed in ESI is understood in some detail. Application of a high voltage creates a fine spray which consists of droplets of solvent containing analyte ions [135;136]. These droplets protrude from the capillary tip, solvent evaporates and the droplets divide into smaller droplets as a result of fission that takes place when the repulsion is greater than the cohesive forces that hold the drop together. When the droplets are small, ions are formed by one of the two mechanisms that have been proposed, namely the field evaporation mechanism proposed by Thomson and Iribarne [137] or the solvent evaporation model proposed by Dole *et al.* [134]. In the former, desolvated ions are formed by repulsion between the opposite charges in molecule and those on the droplet surface, which eject the molecule out of the droplet. The solvent model postulates that solvent evaporates from the nanometre size droplets formed as a result of repetitive fission until analyte molecules are left as ions.

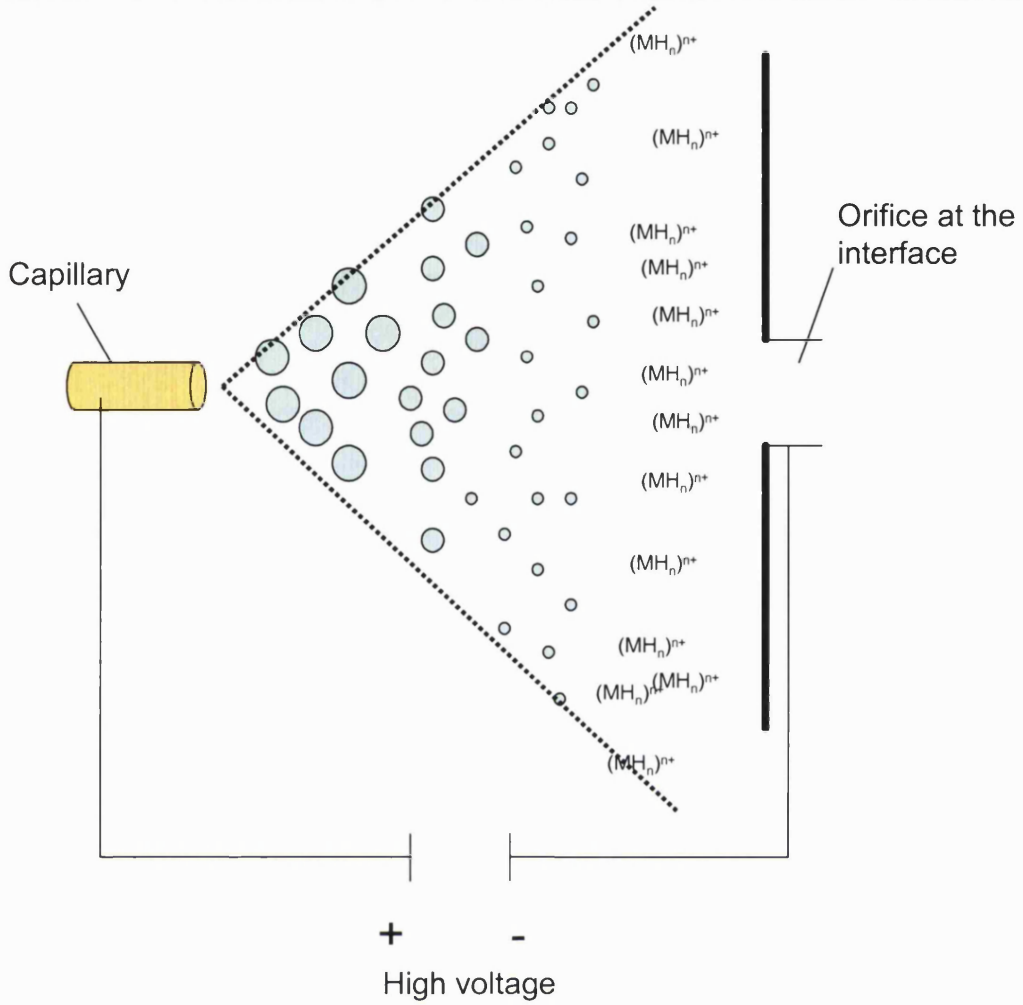


Figure 1.2.2. Ion formation by ESI.
Infusion of analyte molecules (dissolved in an aqueous-organic solvent) through a capillary at high voltage produces a spray of highly charged droplets that decrease in size as they undergo fission and desolvation. An electrospray plume is formed (represented by dashed lines in this diagram). Final analyte ions may be formed when the solvent molecules evaporate to leave free ions; alternatively, ions may be ejected from the small droplets as a result of the repulsion between the opposite charges on the droplet surface and those on the ions. Note that due to the relatively small orifice at the interface between the ion source and the mass analyser, only a small proportion of the formed ions can enter the analyser. At low flow rates the size of the initial droplets and the electrospray plume diameter decrease so that a larger proportion of the formed ions enter the analyser. This partially accounts for the observed increase in sensitivity in nanoESI.

A feature of ESI is the formation of multiply charged ions. This extends the mass range of mass analysers. In ESI only a small proportion of ions are analysed because the orifice at the interface between the mass spectrometer and the ion source has to be small in order to prevent dissipation of the vacuum in the analyser. This is a problem for all ionisation methods that occur at atmospheric pressure, and could be one of the causes why atmospheric pressure MALDI mass spectrometry is not as sensitive as low pressure MALDI [138].

Several authors have described an increased sensitivity of ESI when low flow rates in the inlet capillary are used (reviewed in [135]) and Wilm and Mann proposed the term nanoelectrospray or nanospray (nanoESI) for this variation of ESI [139;140]. As illustrated in Figure 1.2.2, ions generated in the ESI plume are accelerated towards a negative charge in the orifice at the interface. The radius of the electrospray plume is proportional to the flow [139]. Therefore, by decreasing the flow rate a larger proportion of the formed ions can be analysed, thus increasing the sensitivity of the system.

Another reason why low flow rates in ESI produce higher ion yields is because the size of the original droplets generated in the ESI plume are smaller than in ESI operating at larger flow rates. Wilm and Mann [139] predicted that at a flow rate of 1-10 μl/min the initial droplets are about 1 μm in diameter and contain more than 150,000 molecules. Conversely, at 25 nl/min the droplet diameter may be ~ 180 nm and contain, on average only one molecule per droplet. Therefore, less fission events need to take place before the ion is desolvated (or ejected from small droplets) so that the ionisation process may be much more efficient at low flow rates. Sub-femtomole sample consumption was reported when using nanoESI emitters in combination with quadrupole MS [140].

Multipole ion filters

Having dealt with some of the basic aspects of ion formation in the two ionisation methods employed in the work presented here, I shall now briefly describe the basics of the mass analysers used.

Quadrupole ion filters were introduced by Wolfang Paul in the 1950's (reviewed in [122;123]). Mass spectra in quadrupoles ion filters are obtaining by a scanning mechanism. Quadrupoles are the most common of all multipoles, although modern instruments may be constructed with hexapoles. The theoretical background to these devices is considerably complicated. In essence, a quadrupole mass filter consists of two pairs of cylindrical rods placed in parallel to which direct current (DC) and radio frequency (RF) voltages are applied. One pair of opposite rods has negative DC voltage, while the other pair has positive charge. The voltages of these parallel rods are changed and the DC/RF kept constant. At specific voltage settings the trajectory of ions with a defined m/z value will be stable and only these will reach the detector, while the trajectory of ions with other m/z values became unstable and they are diverted to the rods. A mass spectrum is obtained by a scanning mechanism by changing the voltages such that ions with only one m/z value are transmitted at a time. In tandem mass spectrometers, in where quadrupoles are used as

ion guides to allow the passage of ions rather that to scan the whole mass range, DC voltages are switched off and the quadrupole operates in the so-called RF mode only.

The sensitivity of quadrupole mass spectrometry is low when used to scan wide mass ranges because of the limited duty cycle of these analysers; i.e., when an ion is being transmitted all the other ions are diverted away from the detector (the larger the m/z range the larger duty cycle). Nevertheless, its sensitivity is increased when used to monitor only one particular analyte in applications such as quantitation of drugs by LC-MS.

Time-of-flight mass spectrometry

Ions are separated by TOF on the basis of the time that ions take from the ion source to reach the detector through a field-free region [141]. This time can be correlated to m/z using a simple function. In a simple TOF mass analyser, ions are accelerated into a field-free region and allowed to separate according to their m/z. However, ions formed by MALDI, although suited to analysis by TOF because they are formed in a pulse, have energy distributions arising from their differences in initial kinetic energies, and because of their spatial and temporal distributions. For these reasons, devices have been introduced in modern TOF apparatus that correct for these energy distributions [141]. In this, reflectrons (also called ion mirrors) correct for initial kinetic energy distributions, while delayed extraction and configurations in which ions are accelerated orthogonally correct for the temporal and spatial distributions of ion formation in MALDI. Ion packets can also be collected from ESI after being focused by a quadrupole and accelerated orthogonally in push-pull regions towards the field-free region of the TOF, and consequently, ESI has also been hyphenated to TOF analysers (e.g. the Mariner™ mass spectrometer commercialised by Applied Biosystems).

In addition to their use in obtaining accurate mass measurements, MALDI-TOF MS can be used for the primary structure of peptides through a process called post source decay (PSD), in which increased internal energy is deposited in the analyte, which as a result fragments in the field-free region of the TOF analyser [123]. The fragmentation pattern can be used for the primary structure determination of the peptide as for tandem mass spectrometry described below.

Tandem mass spectrometry (MS/MS)

The purpose of tandem mass spectrometry (MS/MS) experiments is to fragment ions such that structural information can be obtained from the fragment ions. There are two main types of MS/MS, MS/MS in space and MS/MS in time [123;142]. The latter is carried out in ion traps and will not be discussed here in any detail. MS/MS in space is performed in tandem mass spectrometers containing two mass analysers on-line (Figure 1.2.3). The first analyser is capable to isolate the ion to be analysed, which is then fragmented in a collision cell; a second mass analyser determines the m/z values of the fragment ions produced. Several types of tandem mass spectrometers exist, including triple quadrupoles, quadrupole-time-of-flight (Q-TOF), and TOF/TOF mass spectrometers. The work described below was carried out using two different Q-TOF instruments and a TOF/TOF.

Peptides fragment in their backbone and this information can be used to determine their amino acid sequence *de novo*. For convention (proposed in 1984 by Roepstorff and Fohlman [143]) carboxyl terminal fragment ions (i.e. fragment ions that retain the charge at the C terminus) are termed x, y, and z ions, while the amino terminal peptides are named a, b, c fragment ions (Figure 1.2.4). The differences in masses between fragment ions of the same type give the sequence amino acid residues in the peptide chain. Often, sequencing of a few amino acids is enough for the identification of the protein from which the peptide was derived.

The Q-TOF instruments commercialised by MDS-Sciex and Micromass allow for Data Dependent Acquisition experiments. In this, eluents from HPLC runs are fed directly into the ESI-MS and the m/z values of the eluting peptides are recorded in MS mode only. When a multiply charged ion is detected (peptide ions are normally multiply charged by ESI) the mass spectrometer switches to MS/MS mode; the peptide ion is selected for fragmentation and a MS/MS spectrum is produced. After a predetermined time the mass spectrometer switches back to MS mode so that other eluting peptides can be detected and sequenced. In this way several peptides can be automatically sequenced in a relatively short time.

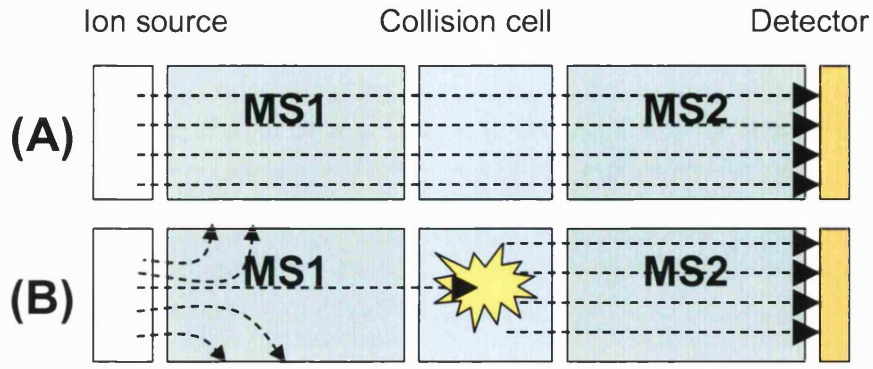


Figure 1.2.3. The principle of tandem mass spectrometry in space.
Tandem mass spectrometers have two mass analysers (MS1 and MS2) connected in line via a collision cell. MS1 is commonly a quadrupole (although instruments with ion traps and TOF in MS1 are commercially available). MS2 may be another quadrupole (as in triple quadrupoles), a TOF (e.g., the Q-Tof™ and the Qstar™, developed by Micromass and MDS-Sciex, respectively), an ion trap, or an ion cyclotron resonance analyser. Tandem mass spectrometers can produce mass spectra and tandem mass spectra.

(A) MS experiment: ions with different m/z values produced at the source follow the ion optics path in the MS/MS instrument (represented here by arrowed broken lines) and are separated according to their m/z values so that an MS spectrum is recorded.

(B) MS/MS experiment: MS1 isolates a previously determined ion which is then fragmented in the collision cell. The fragment ions thus produced are subsequently separated according to their m/z in MS2 and recorded by a detector. This process, termed collision induced dissociation (CID), creates a MS/MS spectrum that can be used for determining the structure of the molecule. In the case of peptides, their primary structure can be determined *de novo*.

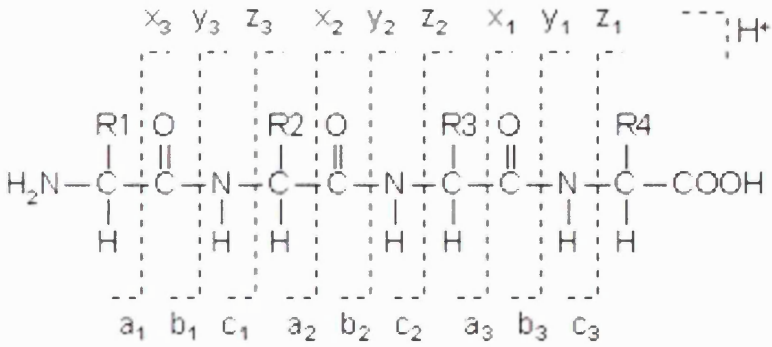


Figure 1.2.4. Nomenclature of peptide fragment ions generated in MS/MS or PSD experiments.
Peptides fragment more or less randomly at their backbone due to the relatively large internal energy deposited in CID or PSD experiments; a series of fragments are generated that differ in one amino acid residue. When the charge is retained by the C-terminus these ions are termed x, y or z; when the charge is at the N-terminus they are termed a, b or c ions. (Figure reproduced from Matrix Science website at www.matrixscience.com).

Liquid chromatography mass spectrometry (LC-MS)

LC has been hyphenated to MS both on-line and off-line. In off-line LC-MS fractions from the LC run are collected and then the molecules present in these fractions analysed by any of the MS methods described above (or indeed by any other method). In on-line LC-MS the eluent from the LC is directly fed into the mass spectrometer. This mode of LC-MS is commonly carried out using ESI-MS. As discussed above, a great improvement in sensitivity can be achieved when the ESI process is performed at low flow rates. In addition, the concentration capacity of nanoLC is greater than that of conventional LC and nanoLC requires passage of the mobile phase at low flow rate. Moreover, the solvents used in RP-LC are compatible with ESI. Consequently, the hyphenation of nanoLC and nanoESI-MS/MS has been very successful for the analysis of proteins and peptides with great sensitivity (e.g [144]). When LC is combined with ESI and MS/MS instruments that allow for DDA a large amount of peptides can be sequenced in a relatively short time (e.g., [116]).

1.3. The proteomics workflow.

The analytical methods used during the studies presented in this thesis have been introduced in the preceding discussion. In the following, a brief account is presented on how these methods are combined for the analysis of gene expression in the field that is known as proteomics.

1.3.1. Targeted approaches for the detection and quantitation of proteins

In conventional biochemistry, a common method to detect and quantify proteins is that of immunochemistry. This approach is in principle very powerful provided antibodies for the proteins under study are available. Due to the specificity of antibodies virtually any protein or peptide can be detected in a background of other proteins without the need for sample pre-treatment. In addition to their use in Westerns blots and immunoassays such as ELISA, antibodies can be used for localising proteins in cells and tissues, thus making possible to detect the intracellular localization of the protein under study.

Several investigators have used immunochemical methods for identification of several proteins or peptides in kidney tissues and in urine in a proteomic scale. For example, the group of Knepper and colleagues has developed antibodies for each of the transporters involved in sodium reuptake from the glomerular filtrate [145]. These antibodies have been used to follow the changes of their target in animal models of disease after perturbation of defined physiological parameters [146-149]. Norden el al. have also used immunochemical methods for the quantitation of several proteins in both Fanconi syndrome patients and control urine, and these studies provided information on the nature of LMWP [150;151].

Although this strategy is powerful to follow the change in expression levels of specific proteins, it is not suitable for the identification of new candidates involved in physiological processes because there is a limit on the number of antibodies that can be used at a time, and the promises of comprehensive protein arrays is yet to be fulfilled. Furthermore, immunochemical methods are somehow biased in that it is the investigator who chooses the set of antibodies to be used. Problems of specificity, expense, and availability of antibodies should also be considered. Finally, for obvious reasons, antibodies cannot be used to follow the expression of genes whose products have not yet been characterized.

1.3.2. Two-dimensional gel electrophoresis and mass spectrometry-based methods

The bases of protein separation by 2DE have been introduced in section 1.2.2 of this chapter. Changes in gene expression as a result of disease or different cell states have been analysed with this method, which is the most widely used approach in proteomics for protein profiling [152-155]. Typically, the protein samples to be compared are separated in parallel gels. After staining and scanning, gels spots that show an altered level of expression are excised, and in-gel digested with a suitable protease (trypsin is almost always used). The peptides produced are then analysed by MS, most commonly by MALDI-TOF MS, or nanoLC-ESI-MS/MS. The technical advances in biological MS, outlined in Chapter 1.2.3, and bioinformatics during the 1990s meant that mass spectrometers have now enough sensitivity for the identification of proteins showing a faint signal in silver stained gel spots.

An approach for the identification of proteins using in-gel digestion and MS data is that of peptide mass fingerprinting (PMF) [156] in which the m/z values of the peptide ions observed in the MALDI-TOF mass spectrum are used to search a protein database. Search algorithms have been developed that compare those m/z values to those derived from the theoretical digestion of all proteins in the database (e.g., [157;158]). The protein present in the gel spot is identified when the theoretical and the observed m/z values are the same within a defined mass error. Algorithms that assess the statistical probability that the hit is correct have also been developed [157;158], although operator intervention is needed to identify false positives.

An alternative to MALDI-TOF MS and PMF for the identification of gel separated proteins is LC-ESI-MS/MS followed by sequence-tag searches [156], in which the peptides generated by in-gel digestion of the protein spot are separated by LC and detected and sequenced by MS/MS. Sequencing by MS is carried out using tandem mass spectrometers, as outlined in Chapter 1.2.3.

Proteomics studies using the approach of 2DE in combination with MS have demonstrated the usefulness of these methods to compare gene expression profiles of related proteomes. For example, 2DE has been used in the context of nephrology to identify proteins differentially expressed in kidney medulla and cortex [159;160], to analyse proteins patterns of workers exposed to toxic levels of cadmium [161], to identify changes in gene expression in renal cells as a result of hypoxia [162], and to comparatively analyse rat urinary proteomes before and after sodium overload [163].

1.3.3. Liquid chromatography-tandem mass spectrometry based methods

As an alternative to 2DE-based methods, in which proteins are separated and then analysed one at a time by MS, strategies based on LC-MS/MS are being used for the 'shotgun' identification of all the proteins in a sample (reviewed in [164]). In this method, proteins are first digested with a suitable protease and the peptides generated (there could be several hundred thousand if a whole cell lysate is to be analysed) are separated by two-dimensional (2D)-LC; the first dimension usually being SCX-LC and the second RP-LC. Peptides are detected and sequenced on-line by MS/MS. Peptides are thus separated by charge in the first dimension and by hydrophobicity in the second. The mass spectrometer separates eluting peptides according to their m/z value, and therefore, MS provides a third dimension of separation. For this reason this approach has been termed multidimensional protein identification technology (MudPiT) by the group of Yates, which was one of the first groups that implemented this approach for large scale proteomics [113]. A 90 minute reversed phase LC run can, in combination with ESI-MS/MS detection, sequence about 900 peptides/run. Therefore, a 2D-LC-MS/MS experiment in which 20 SCX fractions are analysed could generate 18,000 MS/MS spectra, which in an ideal scenario, could generate the same number of peptide sequences.

The drawback of this method, when compared with 2DE based approaches, is that quantitation is not straightforward such that labelling with stable isotopes is needed for relative quantitation. Methods for labelling peptides prior to 2D-LC-MS/MS include isotope coded affinity tags (ICAT) first introduced by the Aebersold group [165;166] and later commercialised by Applied Biosystems. The ICAT reagents are alkylating compounds that label cysteine amino acid residues. One of the samples to be analyzed is labelled with an ICAT reagent containing nine ^{13}C, while the other is labelled with the same compound but containing the common ^{12}C. After labelling, the samples are mixed and analysed by 2D-LC-MS/MS as described above for MudPiT.

A problem associated with the ICAT strategy is that these reagents label cysteine residues and therefore proteins that do not contain this amino acid in their coding region cannot be quantified with this method. Recently, other investigators have reported the development of compounds based on the ICAT principle [167]. Alternative strategies have also been described that involve metabolic labelling by growing cell cultures in media containing amino acids that incorporate heavy stable isotopes [168], or in media in which ^{15}N replaces ^{14}N as an nitrogen source [169].

1.4. Aims and Scope

The primary aim of the studies that constitute the subject of this thesis was to use mass spectrometry based analytical approaches to investigate the notion that factors present in the tubular fluid have a role in controlling renal physiology. As discussed in preceding sections of this chapter, there are several published studies suggesting that luminal factors contribute to the hormonal regulation of solute transport in renal tubules. For instance, several receptors for bioactive peptides are known to be located on luminal membranes, and in addition, renal cells express bioactive peptides; since many of them appear in urine, they probably are also present in tubular fluid. These observations point to a model of renal physiology in which intracrine regulation (i.e., regulation from the luminal side of tubular cells) plays an important homeostatic function in the kidney, in addition to the roles performed by classical autocrine and paracrine hormonal systems.

In spite of the evidence already present, the relative amounts of peptide hormones in the tubular fluid remain unknown. Therefore, it is not possible to predict which peptide(s) may have greater contributions to the regulation of tubular cell function. Moreover, it has been shown that actions of growth factors on cultured cells may vary depending on whether cells are exposed to individual factors in isolation or as part of a mixture. Thus, it would be of great interest to know the hormonal composition of interstitial and tubular fluids. In this regard, one of the aims of the studies presented in this thesis was to investigate the peptide hormone composition of renal tubular fluid.

Renal FS patients have a defect on the reabsorption of proteins and peptides from the glomerular filtrate, but it is generally accepted that the physical integrity of the glomerular filter is not affected in these patients. Consequently, the urinary composition of FS patients is believed to be close to that of normal tubular fluid. Urine samples from these patients were analysed for peptides and proteins using some of the approaches introduced in previous sections of this chapter. The results obtained during the course of these experiments are the subject of Chapters 3 and 4.

Proteomic methods were also used to analyse the proteomes of cortical apical and basolateral membranes in order to detect the presence of proteins that may be involved in signal transduction cascades. For a signalling system to operate there must be extracellular components as well as intracellular ones. In this context, experiments presented in Chapter 5 of this thesis suggest that proteins with previously reported signaling functions are

present at the apical membrane of tubular cells and this further supports the existence of an intracrine control of renal function.

2. Experimental

2.1. Mass spectrometry

Mass spectrometry-based experiments were carried out using instruments located and maintained at the Bioanalytical Chemistry laboratory (Dr R. Cramer, head) of the Ludwig Institute for Cancer Research (UCL branch, Prof M.D. Waterfield, director) or in the Mass Spectrometry Facility (University of California in San Francisco, Prof.. A.L. Burlingame, director).

2.1.1. MALDI-TOF MS and MALDI-TOF/TOF MS/MS

Instrumentation

MALDI-TOF MS experiments were carried out using one of the following instruments:

Voyager Elite XL ™ (PE Biosystems, Framingham, MA, USA)

This is a low pressure MALDI-TOF instrument equipped with a UV nitrogen laser (337 nm wavelength) that operates at pulse rate of 3 ns and also an IR Q-switched Erbium-Yttrium-Aluminium-Garnet (Er-YAG) laser (2.94 μm wavelength) and a pulse rate of about 100 ns.

Ultraflex TOF/TOF ™ (Bruker Daltonics, Bremen, Germany)

This is also a low pressure MALDI-TOF instrument equipped with LIFT technology so that in PSD experiments the fragments ions are recorded at once obviating the need for spectra stitching. Since there is only one analyser, it may be argued that this instrument is capable to perform MS/MS experiments in time.

4700 Proteomics Analyzer MALDI-TOF/TOF ™ (PE Biosystems, Foster City, CA, USA)

This is also a low pressure MALDI-TOF/TOF capable of high collision energy fragmentation in a collision cell located in between two TOF analysers. This instrument can perform MS/MS experiments in space.

Sample preparation and analysis

Samples to be analysed by MALDI-TOF MS were prepared using the dried droplet protocol. Standard laboratory procedures in the Bioanalytical Chemistry laboratory of the LICR were followed. In short, the sample analyte was mixed with an excess of

matrix. Typically, 0.5 µl of sample solution was mixed with 1 µl of saturated solutions of 2,5-dihydroxybenzoic acid (2,5-DHB, Bruker Daltonics or Sigma) or α-cyano-4-hydroxycinammic acid (HCCA, Hewlett-Packard, Böblingen, Germany). Saturated solutions of 2,5-DHB were freshly prepared just before analysis by dissolving an excess of solid matrix in HPLC grade water. HCCA was purchased as already made solutions in methanol. After mixing sample and matrix solutions on the MALDI target, they were dried with a stream of warm air.

Analysis was performed by averaging the spectra produced by 50 to 300 laser shots depending on the individual signal-to-noise ratio (S/N). Laser intensity was varied depending on the observed signal intensity so that peaks were not saturated. Spectra showing saturated peaks were discarded. Instruments were externally calibrated using a standard mixture of peptides for the analysis of low molecular weight peptides such as those produced from protein digestions. Internal calibration was performed when the spectra contained autolytic peptides of known mass derived from the protease. When larger polypeptides were analysed the calibration was performed externally using a mixture of proteins.

2.1.2. ESI-QTOF MS

Instruments

Q-Tof™ instrument (Micromass, Manchester, UK)

This is a hybrid instrument consisting of a quadrupole (Q1) and a TOF connected through another quadruple (Q2) that is used as a collision cell. When scanning a large mass range, Q1 and Q2 operate in RF mode only so that ions with a wide m/z range are transmitted to a push-pull region where ions are accelerated orthogonally towards the field-free region of the TOF. In MS/MS experiments Q1 isolates the desired ion and transmits it to Q2 where it fragments due to collision induced dissociation (CID).

QSTAR™ (Sciex / PE Biosystems, Foster City, CA, USA)

This is another hybrid Q–TOF mass spectrometer with ion optics similar to those described above for the Q-Tof™ instrument.

Mariner™ (PE Biosystems, Foster City, CA, USA)

This instrument consists of an ESI ion source, a quadrupole operating in RF mode, and a TOF region. The quadrupole focuses the ions towards a push-pull region,

which accelerates ion packets towards the field free region of the TOF mass analyser. As for the Qstar and Q-Tof, in the Mariner the acceleration towards the TOF is orthogonal. The quadrupole operates in RF mode only; therefore this instrument cannot perform MS/MS experiments, although structural information can be obtained by in-source fragmentation.

Operation of ESI-Q-Tof

The mass spectrometers described above were equipped with nanoflow ion sources and fused silica ESI emitters with 50 μm I.D. tapered to 15 μm at the tip (PicoTip™, New Objective, Woburn, MA, USA). These tips are not coated with conductive material and the high voltage was applied at a liquid metal junction about 3 cm from the tip of the emitter. Sample was introduced from a syringe pump or from the eluent of HPLC runs at flow rates of 0.2 to 2 μl/min depending on the application. Optimal operation parameters were determined empirically by infusing a standard peptide. Typical spray tip potential was 1800-3500 V depending on the tip of the emitter and the flow rates. Voltages at the orifice of the interface ranged from 40 to 150 V. The Q-Tof™ and QSTAR™ instruments were calibrated using the fragment ions generated from a MS/MS fragmentation of [Glu^1]-Fibrinopeptide B. The Mariner™ was calibrated using the doubly and triply charged neurotensin.

2.1.3. LC-ESI-QTOF MS

NanoflowLC was performed in an Ultimate™ HPLC system (LC Packings, Amsterdam, Netherlands) or in an ABI system (Applied Biosystems) converted to low flow rates by means of a precolumn split constructed in-house.

The Ultimate system consisted of an autosampler (Famos™) that loaded 5 μl of sample solution at a flow rate of 40μl/min onto a peptide trap column (0.3 x 1 mm, PepMap, LC Packings) placed at a switching valve (Switchos™). Peptides loaded in the peptide trap column were washed for 2 minutes at 40 μl/min with 0.1% formic acid. After this time, peptides were eluted using gradient elution at 200 μl/min onto a main analytical column (C18 PepMap, 75 μm x 15 cm). Solvent A was 0.1% formic acid and solvent B was 80% acetonitrile (ACN) / 0.08% formic acid. Elution was carried out from 5%B to 45%B in 30, 60, 90, or 120 minutes depending on the application.

The ABI system consisted of a 140C pump, a 750B UV detector in which the flow cell had been replaced by a low volume flow cell (35 nl) with Z configuration (LC

Packings), and a rheodyne injector (Rohnert Park, CA, USA) model 8125 in which the sample loop was replaced by a peptide trap column as above. Sample solutions were loaded in a Hamilton air-tight syringe (10, 25, and 100 µl syringes were used) and injected manually directly in the peptide trap column, washed with 10 to 250 µl of 0.1% formic acid and eluted with the same gradient elution conditions as for the Ultimate system described above with the exception that in addition to 75 µm columns, columns with other IDs were also used. For 150 µm x 150 mm columns (C18 PepMap) the flow rate was 1 µl/min. Flow rates were obtained by means of a precolumn split that consisted of a T-junction and a 50 µm I.D. capillary tube of 0.5 m in lenght. The flow rate of solvent eluting from the column was measured using a calibrated glass micropipette and the flow rates in the pump were modified so as to achieve the desired flow rate at the column.

The eluents of the HPLC runs were analysed on-line by one of the ESI mass spectrometers described above. When the Q-Tof or the QSTAR were used they were operated in Data Dependent Acquisition, which allows for the automatic switching from MS to MS/MS experiments whenever an ion of a predetermined nature is detected.

2.2. Liquid chromatography

2.2.1. Column packing

Several methods for packing capillary HPLC columns were tried and tested.

Frits were constructed as previously published using porous filters [117;170;171], porous ceramic plugs [172], or unions containing stainless steel screens [173].

Briefly, capillaries were fritted with PVDF porous filters (Millipore) by placing the filter on a plastic surface and using the end of a capillary (320 µm ID) to cut a section of the filter, which was then pushed inside the capillary using a narrower capillary (280 µm OD). The narrower capillary was then taken out, applied epoxy glue, and inserted again into the wider capillary. After allowing drying, the column was ready to be packed.

Capillaries were also fritted using potassium silicate solutions that formed a porous plug at the end of the capillary. In this, 300 µl potassium silicate solution (SiO_2:K_2O, 21:9, Merk) was mixed with 100 µl formamide, vortexed for 1-2 minutes, centrifuged, and the solution positioned inside the capillary by capillary action; the end of the capillary was inserted in the solution for 3-4 s. Polymerisation was performed by placing the capillary in heat block at 37°C for 1 h.

A final method for column fritting was tested in which beads are retained by placing stainless steal unions containing integrated frits at the end of the capillary to be packed. These unions are commercially available from Valco Instruments (Schenkon, Switzerland) and columns packed using these frits were very robust.

Once the column was fritted, it was packed as described in the literature by introducing chromatographic beads suspended in a slurry into the capillary at high pressure [117;170;171;173]. Briefly, the non-fritted end of the capillary was placed at the end of a slurry reservoir, which consisted of an empty stainless steal column (4.6 mm I.D. x 5 cm long) whose ends had been drilled with a 1 mm drill. The slurry reservoir was filled with slurry (packing material suspended in organic solvent). The end of the slurry reservoir that is not connected to the column to be packed was connected to a HPLC pump. The flow rate of the HPLC pump was set at 100 µl/min, and thereafter adjusted so that the backpressure was ~2000 p.s.i. until the column was packed. Columns were left conditioning overnight at 2000 p.s.i., after which time the flow was switched off from the pump and the column left depressurising from the frit until the backpressure reading at the pump was zero (typically 24 h.).

2.2.2. Reversed phase HPLC

Off line LC-MS experiments were performed in microcapillary HPLC (µLC) columns (POROS 10 R2, 320 µm I.D. x 250 mm long), which were packed in house as described above in fused silica tubes using stainless steel unions with integrated frits. Gradient elution was carried out from 15% B to 60% B in 30 minutes after an initial isocratic step of 8 minutes at 5% B. Mobile phase A was 0.1% TFA and mobile phase B was 80% ACN/0.1% TFA. The ABI HPLC system described above was used for these experiments. The sample loop was 5 µl and the flow rate was 20 µl/min.

Off-line LC-MALDI-TOF/TOF experiments were also conducted. This system consisted of an Ultimate HPLC (LC Packings) equipped with a 75 µm x 150 mm column (PepMap, LC Packings) and operated as described above for the on-line LC-ESI-MS/MS analyses with the exception that the eluents of these LC runs were directly spotted on MALDI plates by a robot (Probot, LC Packings). The flow rate from the column was 330 nl/min and that of the matrix solution (saturated CHCA in 70% methanol / 0.4% TFA) was 800 nl/min. The LC eluent and the matrix were mixed at a T-junction and spotted on the MALDI target every 30 seconds. Solvent A and B were as above and the gradient was

from 2% to 12% B in 5 minutes, from 12% to 30% B in 50 minutes, and from 30% to 90% B in 3 minutes. One hundred spots were analysed per run in a MALDI-TOF/TOF instrument (Proteomic analyser 4700, Applied Biosystems).

2.2.3. Strong cation exchange HPLC

Separation of proteins and peptides by SCX was carried out in columns packed in-house using one of the methods described above. PEEK tubes with 508 and 760 μm I.D. were used and they were cut to 50, 100 or 200 mm depending on the application. Column packing was as described above using the following packing material: POROS S 10 (Applied Biosystems, USA), ceramic SCX material (Sigma), or Polysulphoethyl aspartamide (PSE) (polyLC, USA). Commercially available columns packed with PSE (2.1 x 10 mm) were also used.

In a typical SCX-based HPLC separation, the column was equilibrated with at least 10 column volumes of 0.1% formic acid / 20% ACN. After loading the sample, the column was washed with the same solvent until the UV absorbance reached the base-line line. Peptides and proteins were eluted by increasing concentrations of ammonium acetate dissolved in 0.1% formic acid / ACN. The ABI HPLC system described above was used for these experiments. The sample loop was 100 μl and the flow rate was varied depending on the column used. For 320 μm I.D. columns packed with ceramic beads the flow rate was 20 μl/min. For 760 μm I.D. columns packed with POROS S10 beads the flow rate was 100 μl/min and when columns of the same dimensions were packed with PSE material the flow rate was 50 μl/min.

For some applications potassium chloride or ammonium chloride substituted ammonium acetate. These two salts are transparent at 214 nm so that separations can be monitored by UV absorption. Typical parameters for polypeptide separation by SCX using gradient elution were as follows. Solvent A was 20% ACN / 0.1% formic acid and solvent B was 500 mM KCl dissolved in A. Equilibration was at 100% A for at least 10 column volumes until a stable baseline in the UV chromatogram was observed. After loading the sample it was waited until the base- line returned to the starting position before applying a linear gradient of 0% to 50% B in 15 minutes, followed by 50% to 100 %B in 2 minutes.

2.3. Gel electrophoresis

Gel electrophoresis was carried out using the standard laboratory procedures in the Cancer Proteomics Laboratory of the Ludwig Institute for Cancer Research (University College London branch, Prof. M. Waterfield, director). These are briefly outlined below.

2.3.1. One-dimensional SDS-PAGE

1D-SDS-PAGE was performed in either commercially available precasted 12% Tris-glycine gels, (ReadyGels, BioRad, Hemel Hempstead, Herts) or by casting them in-house using the solutions and buffers listed on Table 2.1.

Protein samples were mixed with sample buffer and boiled for 5 minutes at 100 °C. Small gels (10 cm) were run at a constant current of 50 mA, for 1-2 h, whilst large (1.5 mm x 15 cm x 20 cm) format gels were run with a constant current of 8-10 mA per gel for 16-20 hours until the BPB front had run out the gel. Following electrophoretic separation of proteins on gels, separated protein molecules were visualised using either of two mass spectrometry compatible staining procedures (see below).

Table 2.1. Composition of SDS-PAGE solutions

Resolving gel	
Reagent	**Final concentration**
Tris-HCl pH 8.8	280 mM
Acrylamide/bisacrylamide (37.5:1 stock solution	10-20%,
SDS	0.1%
N,N,N',N'-Tetramethylethylenediamine (TEMED)	0.15%
Ammonium persulphate (APS)	0.05%
Stacking gel	
Reagent	**Final concentration**
Tris-HCl pH 6.8	125 mM
Acrylamide/bisacrylamide	4%
SDS	0.1%
APS	0.05%
TEMED	1%
Sample Buffer	
Reagent	**Final concentration**
Tris pH 6.8	134 mM

SDS	(4% w/v)
Bromophenol blue (BPB)	0.06% (w/v)
β-mercaptoethanol (BME)	6% (w/v)
Glycerol	20% (w/v)

2.3.2. Two-dimensional PAGE

Samples for 2DE were dissolved in 8M urea / 2M thiourea / 4% CHAPS / 10 mM Tris pH 7.2, mixed with ampholites and DTT (85 mM final concentration), and applied to immobilised pH gradient (IPG) gel strips pH 3-10 (Amersham Biosciences, UK). The strips were allowed to rehydrate overnight at room temperature. Isoelectrophocusing was performed in a Multiphor II apparatus (Amersham Biosciences, UK) using the program in table 2.2.

Table 2.2. Program for the isolectricfocusing of proteins in IPG strips

Step	Time (hours)	Conditions
1	1/60	300 V, 5 mA, 10 W
2	0.5	300 V, 5 mA, 10 W
3	3	3500 V, 5 mA, 10 W
4	21	3500 V, 5 mA, 10 W
5	25	500 V, 5 mA, 10 W

After focusing, strips were equilibrated in 6M urea / 0.1 M Tris pH 6.8 / 30% glycerol / 1% SDS containing 5 mg/ml (65 mM) dithiothreitol DTT for 10 minutes followed by incubation in 45 mg/ml (240 mM) iodoacetamide (IAA) in the same solutions.

The second dimension was carried out using a BioRad gel electrophoresis system (BioRad, Laboratory Inc, USA) that allowed for running six gels in parallel with dimensions 18 cm x 25 cm x 1.5 mm. Gels for the second dimension were prepared and run as for SDS-PAGE.

2.3.3. Gel staining and analysis

Gel separated proteins were visualized by either colloidal coomassie blue using Coomassie G-250 dye as described in Neuhoff *et al.* [174;175] or by silver nitrate staining using the procedure of Shevchenko *et al* [176]. Both staining methods are compatible with subsequent mass spectrometry analysis and have comparable sensitivities. The advantage

of colloidal Coomassie blue staining over silver staining is that the intensity of bands or spots is more linear with relation to protein concentration than the silver staining method. Conversely, silver staining is faster than Coomassie staining when high sensitivities are required.

Protein staining using Coomassie blue G-250 has been described [174;175]. Briefly, gels were fixed from 3 h to overnight in 50% (v/v) ethanol / 2% (v/v) phosphoric acid. After washing the gels three times with water (1/2 hours per wash), gels were incubated with staining solution (34% (v/v) MeOH / 17% (w/v)$(NH_4)_2SO_4$ / 3% (v/v) phosphoric acid) for 1hour, after which time 0.7 g/L (solid) Coomassie Blue G-250 was added to the solution. Gels were then left shaken until bands of the required intensity were visible. The end point of labelling is reached after 3-4 days of incubation. Sensitivity was about 10-50 ng of protein (BSA).

Mass spectrometric compatible silver staining was carried out as described with minor modifications [176]. Briefly, gels were fixed using 40% ethanol / 10% acetic acid overnight and then washed with 50% ethanol for 10 minutes followed by 3 washes in water (10 min/wash). Gels were then sensitised in 0.02% sodium thiosulphate for 1min, washed in water twice (1 min/wash) and stained with pre-cooled 0.1% silver nitrate for 30 minutes at 4 °C. After washing twice with water (1 min/wash), the signal was developed by incubating in 0.04% formalin, 2% sodium carbonate. The reaction was stopped with 1% acetic acid.

Gels were scanned using a densitometer (BioRad model GS-800 calibrated densitometer) and analysed (curated) using the software package Melanie™. For 2D gels, spot intensity was expressed as optical density (OD) normalised to total OD.

2.4. Enzymatic digestion of proteins

2.4.1. In-solution digestion

Proteins present in HPLC fractions were digested with trypsin dissolved in 25 mM ammonium bicarbonate. The amount of trypsin used was adjusted so that the ratio substrate:trypsin was between 50:1 and 20:1 (w/w). The reaction was performed overnight at 37 °C and DTT to a final concentration of 1 mM was added after digestion to reduce disulphide bridges.

When reduction and alkylation was performed before proteolytic digestion, protein solutions were solubilised in 6M urea / 0.05% SDS/ 50 mM ammonium bicarbonate / 5 mM Tris(2-carboxyethyl)phosphine (TCEP), heated at 50 °C for 1 hour and subsequently labelled with the alkylating agent (either iodoacetamide or ICAT reagent) for 2h at 37°C in the dark. Before adding trypsin, samples were diluted to final concentrations of 1.2M urea and 0.01% SDS.

2.4.2. In-gel digestion

In-gel digestion was performed using published procedures [177;178]. Briefly, gel pieces from silver stained gels were de-stained using H_2O_2 (1%v/v); this step was omitted when pieces from CBB stained gels were subjected to in-gel digestion. The following was performed for both silver and CBB stained gel pieces. After washing the gel pieces 3 times with 50% ACN, they were dried in a SpeedVac until all the solvent had been evaporated. Sufficient 10mM DTT (in 25mM ammonium bicarbonate, pH8) was added to cover gel pieces, which were then incubated for 45min at 50°C, after which time the DTT solution was removed and sufficient 50mM IAM (in 25mM AmBic pH8) added to cover gel pieces. Incubation was for 1hr at room temperature in the dark. After washing the gel pieces with 50% ACN and drying in the SpeedVac as above, 50 ng of trypsin dissolved in 25 mM AmBic was added and incubated overnight at 37 °C. Tryptic peptides were extracted from the gel pieces by the addition of 5% TFA / 50 % ACN (this was done a total of 3 times). Extracted peptides were concentrated to dryness in the SpeedVac and then dissolved with a suitable volume 0.1% formic acid.

2.5. Data Analysis

Proteins were identified by either peptide mass fingerprinting (PMF) or sequence tag searches (STS) using MS or MS/MS data, respectively. For PMF, m/z values from MALDI-TOF MS spectra were fed into MS-Fit (Protein Prospector). For internally calibrated spectra, the tolerated mass error was set to 50 ppm and for externally calibrated spectra to 200 ppm. Routinely, m/z values were searched against the NCBI protein database restricted to the entries of the organism under study (human for the urinary polypeptide analysis and mammalian or rodent for the membrane proteome experiment).

For STS, data from the nanoLC-MSMS runs were converted to peak list files, which were then fed into MASCOT to search the NCBI protein database. Mass error tolerance was set up to 100 ppm for the parent ions and 150 ppm for the fragment ions. As for PMF, searches were restricted to the taxonomy of the organism under study. Considered modifications for both STS and PMF were oxidation of methionines, pyro-glutamation of N-terminus, and carbamidomethylation of cysteines. When searching with data from ICAT experiments, ICAT modified cysteines were considered instead of carbamidomethyl cysteine. In-solution digestion in the presence of IAM can modify N-termini of peptides. Therefore, in these cases carbamidomethylation of the N-terminus was also considered as a possible modification.

For PMF, a protein was identified when more than 5 peptides matched an entry and those covered more than 35% of the protein. For STS, a protein was identified when 2 peptides matched a protein and the Mowse score given by MASCOT was well above the statistical probability that the hit was correct. In cases where only one peptide matched a protein, the correctness of the hit was confirmed my manual assignation of all the peaks in the MS/MS spectrum to theoretical fragment ions. In some cases, when it was observed that the MS/MS spectra were of good quality but not statistically significant hits were returned by MASCOT, these were interpreted *de novo*.

2.6. Extraction of polypeptides from urine

2.6.1. Precipitation

Three different methods for the precipitation of urinary proteins were investigated. Dye precipitation was carried out as described by Marshall [161] using Coomassie blue dye R-250. Acetone precipitation was carried out in 50% acetone at –20°C. Finally, methanol/chloroform precipitation was carried out as described [179].

2.6.2. Solvent extraction

Urine samples were treated with 3 x 900 µl ether:ethyl acetate (1:1). The organic layer was discarded and peptides in the aqueous layer precipitated with methanol:chloroform (3:1). The pellet was redissolved in 0.1 % formic acid.

2.6.3. Liquid chromatography

Two types of RP column were used for the extraction of polypeptides from urine: solid phase extraction (SPE) cartridges (OASIS, reversed phase cartridges, 80 Å pore size,

Waters, MA, USA) and POROS R20 (20 μm bead size, 2000 Å pore size, Applied Biosystems) packed in house in 760 μm x 50 mm PEEK tubes.

Extraction by SPE was carried out by gravity flow as follows. A volume of urine containing 10 μmol of creatinine was added to an equal volume of 1% TFA. Cartridges were conditioned with 1 ml of 80% methanol / 0.1% formic acid and equilibrated with 1 ml 4% methanol / 0.1% formic acid. The sample was applied twice thought the cartridge, which was then washed with 1 ml 4% methanol/0.1% formic acid. Peptides were eluted using 1 ml of 80% methanol / 0.1% formic acid. Eluted peptides were dried in a SpeedVac and reconstituted in 5% acetonitrile/0.5% formic acid.

Extraction by HPLC was carried out as follows. Urine samples were injected in an HPLC system equipped with an RP column connected on-line to a SCX column. Samples were injected in the RP column (packed in-house with POROS R2 20 in a PEEK tube with dimensions 50 x 0.76 mm) and washed with 20 column volumes of 4% ACN/0.1% TFA at a flow rate of 100 μl/min. A pre-equilibrated SCX column (packed in housed with POROS HS in a PEEK tube with dimensions 50 x 0.76 mm) was then connected to the end of the RP column and peptides eluted directly from the RP column to this SCX column using 150 μl of 80% ACN/0.1% TFA. Peptides bound to the SCX column were washed with 20 column volumes of 4% ACN/0.1% TFA, after which time polypeptides were eluted with 150 μl of 2 M ammonium acetate dissolved in 25% ACN/0.1% TFA. Eluted polypeptides were dried in a Speed Vac to remove the volatile ammonium acetate, re-issolved in 5% ACN/ 0.1% TFA, and the protein quantified using the Bradford assay [180].

2.7. Labelling of polypeptides with isotope coded affinity tags

In some experiments proteins were labelled with ICAT reagents prior to their separation by SCX followed by RP LC and their analysis by MS. In this, proteins were resuspended in 6M urea / 50 mM ammonium bicarbonate / 5 mM Tris(2-carboxyethyl)phosphine and subsequently labelled with the cleavable ICAT reagents for 2h at 37°C in the dark. Equal amounts of each of the samples to be compared were mixed, adjusted to about ~1M urea, and sequence grade trypsin to a final ratio of 1:20 (w/w) was added to the reaction mixture, which was then incubated overnight at 37° C.

ICAT labelled peptides were separated from free label by SCX HPLC as described in section 2.2.3. This step was also used to separate the peptide mixture into fractions.

Labeled peptides present in SCX fractions were separated from unlabelled ones by avidin affinity chromatography using manufacturer's recommendations. The biotin moiety of the ICAT label was removed by acid cleavage using manufacturer's instructions. The solution used to cleave the ICAT reagent was removed by Speed-Vac evaporation and ICAT-labelled peptides redissolved in 0.1% formic acid. Identification and quantitation of ICAT pairs was carried out by LC-MS/MS as described in section 2.1.

2.8. Operation of the column switching liquid chromatography tandem mass spectrometry set up

2.8.1. Description of the system

The column switching consisted of three columns connected through two HPLC injectors. The flow rates were provided by independent HPLC pumps and fuse silica tubing (50 μm ID) was used for delivery of the mobile phase.

Column 1 was a SCX column packed in-house to 1 cm in 320 μm I.D. fused silica capillary using Ceramic beads (Sigma) with 50 μm mean bead size. These beads were held in place in the column by a restrictor (a 50 μm I.D. / 280 μm O.D. fused silica capillary) at the end of the 320 μm capillary. Thus, this column did not have a frit (i.e., it was not plugged) which minimized sample losses due to absorption. Column 2 was a PepMap column (LC Packings) with dimensions 300 μm x 1 mm. Column 3 was also a PepMap column (LC Packings) with dimensions 75 μm x 150 mm.

HPLC pumps were two ABI 140B delivery systems. Pump 1 operated isocratically at a flow rate of 40 μl/min and the mobile phase was 0.1% formic acid / 5 % ACN. Pump two was programmed for gradient elution and operated as described above for RP nanoLC-MS/MS.

Injector 1 was a Rheodyne injector model 7010 fitted with a 40 μl sample loop. Injector 2 was a Rheodyne 8125 in which the loop was replaced by column 2. A pre-column flow rate split was made so that the flow from pump 2 was decreased to 200 nl/min.

2.8.2 Operation of the system

The sample was injected through injector 1 into column 1 at 40 μl/min and the column washed for 2 minutes. Molecules that did not bind column 1 were diverted to

waste. After washing, column 1 and 2 were connected in-line. Polypeptides were eluted from column 1 directly to column 2 by injecting 40 µl of 500 mM ammonium acetate from injector 1. Eluting peptides were trapped in column 2, which was then washed to remove salts in the mobile phase coming from pump 1. Injector 2 was then switched so that column 2 and 3 were now in-line. Pump 2 then delivered a gradient of ACN that eluted peptides into a ESI-MSMS instrument (QStar, MDS-Sciex/ABI).

2.9. Proteomic analysis of membrane preparations

Rat renal cortical membrane preparations were generous gifts from Dr. Joanne Marks (Royal Free Hospital, London, UK) and Dr. Juerg Biber (University of Zurich, Switzerland).

Brush border membranes (BBM) were prepared by Dr. Marks using the method of Biber [181] in which BBM are separated from total membrane by magnesium precipitation. BBM as well as basolateral membranes (BLM) were also prepared by Dr. Biber using a method based on free-flow electrophoresis as described by Kaufmann [8].

The protein contents of BBM and BLM preparations were quantified using the Bradford assay and proteins separated by 12% SDS-PAGE. Proteins were visualized by colloidal CBB G-250 staining and the whole lane cut into 20 gel pieces irrespective of stain intensity. The identities of the proteins present in these gel pieces were determined by in-gel digestion, nanoLC-ESI-MS/MS, and STS searches as described above.

2.10. Patients

All patients participating in the study had clinical and laboratory features of the renal Fanconi syndrome and had been described before in detail [80-82, 150, 151]. The molecular genetic features of the Dent' disease patients studied had been elucidated [80-82]. Patient 1 had total CLCN5 deletion, patient 2 has mutation W279X, patient 3 has a splice-site mutation that leads to deletion of codons 132-241, and patient 4 has mutation R34X. Each of these mutations is associated with loss of function of the ClC-5 chloride channel. Two Lowe syndrome patients were also studied [150-151]. These are brothers and, in addition to renal Fanconi syndrome, these patients have mental and growth retardation and visual impairment. A patient with ADIF, described previously [150-151], was also studied. Patients presented creatinine clearances of between 60 and 101 ml/min. Control urine was collected from three male subjects with no history of renal disease.

Urine samples were obtained from Dr. Antony Norden (Department of Clinical Biochemistry, Addenbrookes Hospital, Cambridge). The study was approved by the local research ethics committee, and all the subjects gave their informed consent. Samples were frozen in liquid nitrogen, transported in dry ice and stored at -80 °C until the day of analysis.

3. Analysis of urinary polypeptides by liquid chromatography and mass spectrometry

This chapter is divided into two sections. The first one describes the studies that were carried out in order to find and optimise a method suitable for the extraction of polypeptides from urine. It will be shown that although several methods were tested for the extraction of peptides and proteins from urine, only liquid chromatography based on SCX could separate peptides of a wide molecular weight range from other urinary compounds.

The second section presents an account on the analysis of renal Fanconi patient's urinary polypeptides using the methods tested and reported in the first part of the chapter. Possible implications for the pathophysiology of the FS will be discussed.

3.1. Methods for the extraction of polypeptides from urine

This section describes results of experiments aimed at investigating the suitability of methods for the extraction of polypeptides from urine such that they are in a form suitable for mass spectrometry (MS) analysis. The main criteria used here to define an efficient extraction procedure were specificity (that is, the extraction method ideally should only isolate polypeptides), comprehensiveness (i.e. all polypeptides should be extracted by this method), and high recovery. The latter was defined as fraction of polypeptide recovered as a function of amount of polypeptide present in the original samples. Recovery was an important parameter because although urine is available in large volumes, specimens from patients are sometimes unavailable or scarce. Moreover, it was anticipated that the method, once optimised, could be used for analysing other biological fluids, such as tubular fluid obtained during micropuncture experiments, for which the volumes available are very small.

Urine is a complex mixture in which peptides are only present at trace levels. Reversed phase (RP) liquid chromatography (LC) was first used to investigate whether polypeptides could be separated from other urinary compounds on the basis of hydrophobicity. In gradient RP-LC compounds elute from the column according to their hydrophobic character, more hydrophobic compounds eluting later. However, experimental evidence demonstrated that RP, by it self, was not suitable for the extraction of peptides from urine. Consequently, it was concluded that it is necessary to extract urinary components prior to LC-MS analysis. Several methods have been described for the extraction of polypeptides from biological fluids [182], including solvent extraction [183;184], solid phase extraction (SPE) [185;186], ultrafiltration [187;188], precipitation [161;189], dialysis [190;191], or a combination of these [192;193]. Two of these approaches, namely precipitation and solvent extraction, were investigated during the course of the studies described in this thesis, but they proved to be unsuitable to the extraction of small peptides and proteins; precipitation did not separate peptides from other urinary compounds, while solvent extraction showed low recovery. The use of methods that rely on separation of molecular species by size was not investigated here because the aim was to be able to analyse small peptides as well as proteins.

Finally, ion exchange chromatography, specifically strong cation exchange (SCX), proved to be effective, in combination with RP-LC and MS, to extract, detect and characterise urinary peptides and small proteins. The approach was first used for the

analysis of peptides in normal urine, and was later implemented for the characterization of peptides and proteins in Normal and renal Fanconi patient's urines. The results of these experiments are described below in some detail.

3.1.1. Extraction of urinary peptides by reversed phase liquid chromatography

Experiments aimed to assess the possibility that peptides could be separated from matrix compounds by reversed phase chromatography showed that many compounds coeluted with standard peptides spiked in urine (Figure 3.1.1). In a representative experiment, control urine was spiked with angiotensin (Ang) I to a concentration of 10 nM. One microliter (containing 10 fmol Ang I) of this solution was injected in the chromatographic system described in the legend to Figure 3.1.1. The guard column was washed with 150 µl 0.1 % formic acid so that salts and other hydrophilic compounds were directed to waste before switching the valve to the 'inject' position. The length and internal diameter of the guard column were 1 mm and 300 µm, respectively, and thus its volume was about 0.07 µl. This means that the guard column was washed with a volume of acidified water equivalent to more than 2000 times its chromatographic bed volume. The solvent used for washing was diverted to waste. After switching the injector so that guard column and analytical column were on-line, a 0.75 % B/min gradient was applied, which eluted compounds according to their hydrophobicity into the ESI-MS. Figure 3.1.1 shows the results of one of the runs. Ang I had a t_R of 45.5 minutes approximately and many compounds co-eluted with Ang I; this complicated its detection.

It was evident from these experiments that although most peptides of interest are above 1 kDa, the fact that peptides are detected in ESI-MS as multiply charged species means that their m/z falls within the range of singly charged matrix compounds, a fact that complicates peptide analysis.

The nature of the matrix compounds detected in the experiment described in Figure 3.1.1 is unknown. The presence of organic acids in urine has been reported [194;195]. These are small compounds, however, and therefore they may not be the species that seem to interfere with peptide analysis (Figure 3.1.1). The largest organic acid that could be found in the studies cited is arachidonic acid with a Mr of 304 Da [194;195].

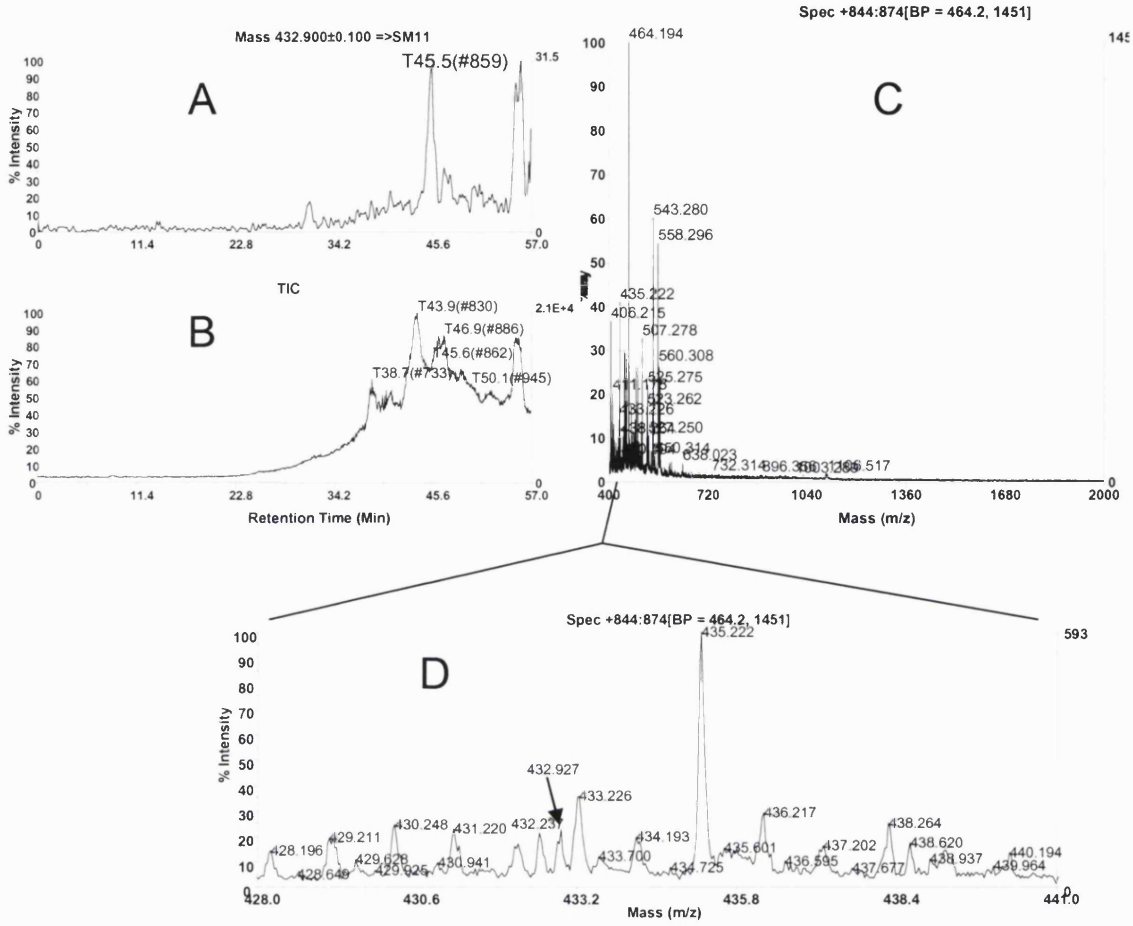


Figure 3.1.1. LC-MS analysis of peptides in untreated urine.

Control urine was spiked with angiotensin (Ang) I to a concentration of 10 nM and centrifuged to sediment particulates. An aliquot of 1 µl of this spiked urine was separated by LC and the eluent detected by an ESI-TOF mass spectrometer. Total ion chromatogram and extracted ion chromatogram for Ang I are shown in **B** and **A**, respectively.**(C)** Spectrum centered at t_R 45.5 min in were Ang I eluted (20 seconds acquisition). (**D**) Closer view of the spectrum at (C) centered at peak corresponding to Ang I. Separation was carried out in a RP C18 PepMap (150 x 0.150 mm) column at a flow rate of 1 µL/min. After loading the sample in a guard column and washing, gradient elution was carried out from 5% to 45% B in 30 minutes. Solvent A was 0.1% formic acid and B was 80% acetonitrile in A. Although Ang I was detected in the spectrum shown in (D) at m/z 432.9 $^{+3}$, the signal to noise ratio was small due to co-eluting of endogenous singly charged urinary compounds.

Nevertheless, the technique employed to analyse organic acids in the cited work was gas chromatography with mass spectrometric detection, so that failure to detect larger organic anions could be caused by poor transfer of larger species to the gas phase as normally observed in electron impact and chemical ionisation MS. In independent investigations, other compounds found in urine included sugars [196], organic cations (e.g creatinine and steroid hormones), and other organic anions (e.g., nucleotides and long chain fatty acids) [197]. The presence of fatty acids and other lipids, such as steroid hormones and bile acids, in urine was of particular concern because these compounds

have been described as "column killers" [105]. Consequently, it was decided that an extraction procedure prior to RP-LC/MS was necessary in order to separate peptides from urinary matrix compounds.

3.1.2. Extraction of urinary peptides by organic solvent precipitation

Precipitation of proteins in organic solvents, such as ethanol, chloroform and acetone, salts, or in acids, e.g., trichloroacetic acid, is a common procedure employed in protein chemistry for the separation of proteins from other compounds such as salts and detergents. This separation method is based in the fact that proteins denature in these solvents such that their hydrophobic amino acid residues, which in aqueous environments face the interior of the protein, became exposed. Interaction of the hydrophobic chains by van der Waals forces between these residues is thought to occur with the end result of aggregation, and consequently, precipitation [198;199]. After centrifugation, un-precipitated material remains in the supernatant, which is aspirated and discarded.

Experiments aimed at assessing the usefulness of this method for the separation of small peptides revealed that other compounds co-precipitate with peptides such that although precipitation with organic solvents or dyes has been used for the extraction of proteins from urine prior to proteomic studies [161;189;200], this method may not be suitable for the isolation of small peptides before LC-MS analyses.

3.1.3. Extraction of urinary peptides by solvent-solvent extraction

Solvent-solvent extraction has been used to extract organic anions from urine [183;184] and it has been used for the extraction of peptides from tissues [184]. In this procedure organic acids were extracted with a 1:1 ether:ethyl acetate solution. By acidifying urine prior to extraction the ionisable groups are protonated and thus solubility of organic anions in the organic layer increases.

A variation of this procedure was used in the experiments described in this thesis to extract organic anions into the organic layer, which was then discarded. Peptides in the aqueous layer were subsequently precipitated. This step was introduced because it was observed that the aqueous layer contained pigments, which interfered with peptide analysis.

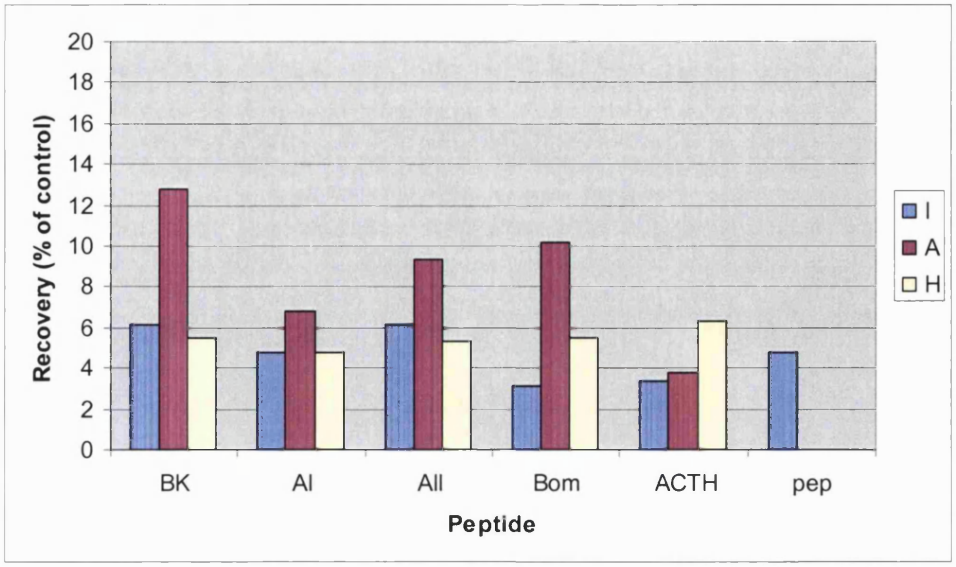


Figure 3.1.2. Recovery of peptides from solvent-solvent extraction experiments. The result of a representative experiment is shown, in which 300 μL urine spiked to 100 nM with several peptides was treated with 3 x 900 μl ether:ethyl acetate (1:1). The organic layer was discarded and peptides in the aqueous layer precipitated with methanol:chloroform (3:1). The pellet was redissolved in 50 μl 0.1 % formic acid and 2 μl (about 1.2 pmol if recovery was 100%) analysed by LC/MS. Percentages are shown relative to control (1.2 pmol of untreated peptides dissolved in water). BK: bradykinin; AI: angiotensin I; AII angiotensin II; Bom: bombesin. ACTH: adrenocorticrotopic hormone; pep: a synthetic peptide sequence VHLTPVEK. Legend: I, intensities; A, areas; H, heights.

In experiments aimed to test the performance of this method, control urine was spiked with a mixture of standard peptides to a concentration of 100 nM and 300 μl were used for these experiments. To assess percentage recovery of peptides, the height and area under the peak of the ion chromatogram, as well as the intensity of the peaks in the spectrum were measured and compared with those of the untreated standards dissolved in water (this was the control). Although the standard peptides were detected by the mass spectrometer, the recovery was poor (Figure 3.1.2). The percentage recovery was similar irrespective of the response measured (intensities, areas, or heights) and it was never greater than 15% (Figure 3.1.2). The reasons for this poor recovery are not known but could be caused by inefficient precipitation of small peptides.

3.1.4. Extraction of polypeptides from urine by reversed phase and strong cation exchange solid phase extraction

Solid phase extraction (SPE) is a term frequently used to describe a purification process using chromatographic material (e.g. C18 beads) packed in disposable cartridges [105]. In this work, SPE cartridges as well as unpacked chromatographic beads were used to extract peptides from urine.

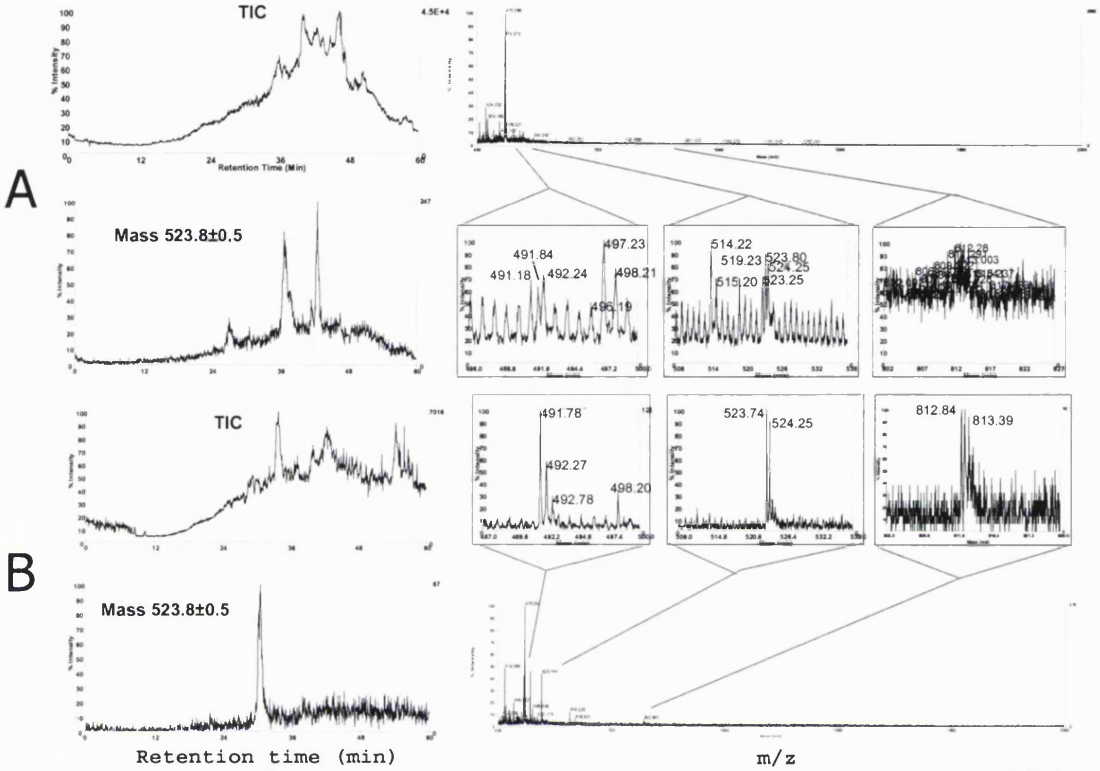


Figure 3.1.3. Solid phase extraction of urinary peptides using RP and SCX chromatography.

(A) RP LC-MS of 100 fmol angiotensin II (Ang II, m/z 523.74) spiked in 10 µl urine and treated with RP SPE before LC-MS. Total ion chromatogram and extracted ion chromatogram of Ang II are shown on the left. Mass spectra at the time of Ang II elution are shown on the right with insets showing the spectrum of Ang II (middle), and two endogenous peptides (left and right) that co-eluted with Ang II. (B) As in (A) but sample was purified off-line by SCX chromatography prior to RP LC-MS analysis. Note that before purification the extracted ion chromatogram indicated that there were three ions with an m/z value of 523.8 in urine (see A); after extraction only one component had this m/z value, which corresponded to Ang II.

The results of one of these experiments are shown in Figure 3.1.3.A, which illustrates that urine treated with reversed phase (RP) beads still contained many non-proteinaceous organic compounds similar to those found in untreated urine (compare with Figure 3.1.1). However, when peptides were extracted with RP beads followed by strong cation exchange (SCX) beads the signal-to-noise ratio of a standard peptide (previously spiked in control urine) increased dramatically (Figure 3.1.3.B). Inspection of the spectrum shown in Figure 3.1.3.B revealed that in addition to the standard peptide Ang II, there were also other multiply charged peaks that could correspond to endogenous peptides. Doubly charged ions at 491.8 and 811.4 m/z units are visible in the spectrum of the sample treated with SCX; these peptides, however, would had been overlooked in the

sample treated with RP beads only. Later experiments demonstrated that the ion at 491.8 m/z units is an uromodulin fragment.

These studies demonstrated that SCX chromatography provides a means to separate peptides from neutral and acidic urinary compounds. Peptides are zwitterionic molecular species but by lowering the pH to 3 most, if not all, peptides are positively charged. Therefore, they can be retained by the SCX beads with sulfonic acid functionality (which is negatively charged at any pH). After washing these beads with a low ionic strength buffer to remove acidic and neutral compounds, peptides are eluted with a high ionic strength buffer, which disrupts the ionic interactions between the opposite charges on the SCX beads and peptides. This procedure thus separates polypeptides from neutral and acidic urinary compounds.

3.1.5. Manufacture of microcapillary liquid chromatographic columns

The efficiency of the extraction procedure should increase if carried out in packed columns. For this reason it was decided to pack chromatographic columns in silica and PEEK capillaries using commercially available packing materials. The concept of packing microcapillary columns is straightforward; a capillary tube is fritted (i.e. plugged) at one end and the packing material with the desired functionality is inserted from the other end at high pressure [119]. However, there are several technical difficulties associated with packing columns.

One of them is fritting. Several reports exist describing fritting of capillaries by sintering methods [201], restrictors and tapers [202;203], porous filters [117;170;171], porous ceramic plugs [172], and unions containing stainless steel screens [173]. McCormack *et al.* reported the use of Teflon filters to frit capillary columns [171]. However, Davis et al claimed that hydrophilic PVDF filters have less affinity for peptides than Teflon so that columns constructed with these frits are less likely to cause losses of analyte by absorption to the filter [117]. The manufacturer also reports that hydrophilic PVDF binds less protein than Teflon [204] (this company sells both Teflon and PVDF filters). Therefore, a hydrophilic PVDF filter (0.65 μm pore diameter) was initially used for the work described in this report.

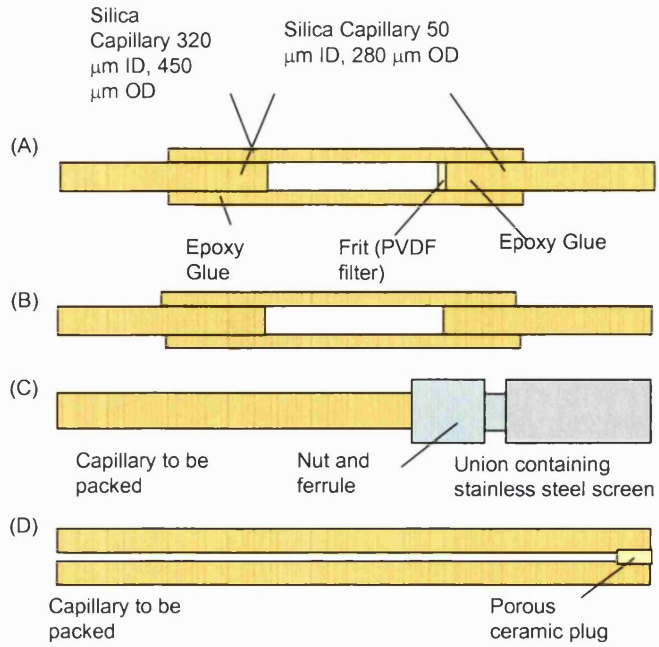


Figure 3.1.4. Methods for the construction of microcapillary liquid chromatographic columns.
The figure shows schematically the different methods that were used to manufacture the chromatographic columns used during the work described in this thesis. (A) Silica capillaries were fritted by inserting a PVDF filter in a 320 µm I.D. silica tube by forcing it using a 280 µm O.D. / 50 µm I.D. silica tube. Epoxy glue was used to glue the two capillaries. (B) Fritless columns were constructed as in (A) but the filter was not placed. This type of frits is suitable for packing beads with large diameters. (C) Packing of columns using stainless steel frits. (D) Construction of porous ceramic frits. An empty column was used as slurry reservoir and filled with chromatographic media. Water at high pressure from a HPLC pump was used to drive these beads into the fritted capillary.

In this procedure, the PDVF porous filter is inserted at one end of the capillary by forcing it inside with a slightly narrower capillary, which is then glued to the main capillary with epoxy glue (Figure 3.1.4). The manufacture of columns containing this type of frit was relatively easy; however, they proved to be weak because the epoxy dissolved after a few hours or days of operation, which was probably caused by the organic and acidic solvents employed in HPLC and the high pressures used.

Thus other, more robust, methods to construct frits were investigated (Figure 3.1.4). Silica and PEEK capillaries were readily fritted using commercially available stainless steel unions containing integrated frits. This procedure is simple and gave good results in this work and columns packed using these frits have been reported to withstand very high pressures [173]. Finally, silica capillaries were fritted using porous ceramic plugs, the manufacture of which was difficult to optimise. Nonetheless, columns packed with these frits were robust and showed good performance (Figure 3.1.5).

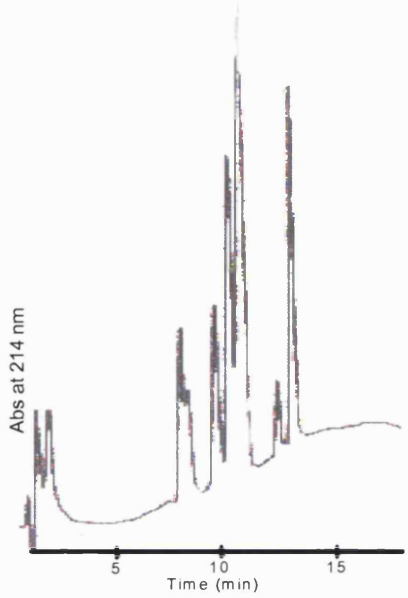


Figure 3.1.5. Performance of columns packed in house using porous ceramic frits.
The performance of a column (dimensions 70 µm x 15 cm) packed in house with RP perfusion chromatography material was tested by injecting a mixture of peptides (5 pmol each of insulin, Ang I, Ang II, BK, ACTH 17-38, neurotensin, and fibrinopeptide). Gradient elution from 5%B to 50%B in 10 minutes was carried out at a flow rate of 2 µl/min (50 µl/min before pre-column split). Separation was monitored by UV absorption at 214 nm.

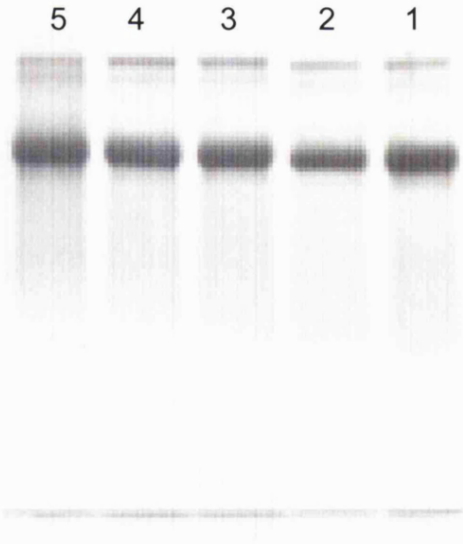


Figure 3.1.6. Recovery of proteins from in-house packed columns.
A PEEK capillary tube, dimensions 0.508 x 50 mm, was packed with POROS R2 20 RP beads. The media was retained using a stainless steel frit. The sample consisted in a mixture of proteins (BSA, casein and myoglobin), which was loaded in the column at 50 µL/min. The column had been previously equilibrated with 4% ACN/0.1% formic acid. After the baseline in the UV trace returned to the starting position, proteins were eluted using 50% ACN/0.1% formic acid and subsequently loaded in a 12 % SDS-PAGE. Lanes: 1 and 2, control (20 pmol of protein); 3, 20 pmol of protien after extraction; 4 and 5, 20 pmol of protein after extraction and previously dissolved in 8M urea/1mM DTT / 10 mM IAM.

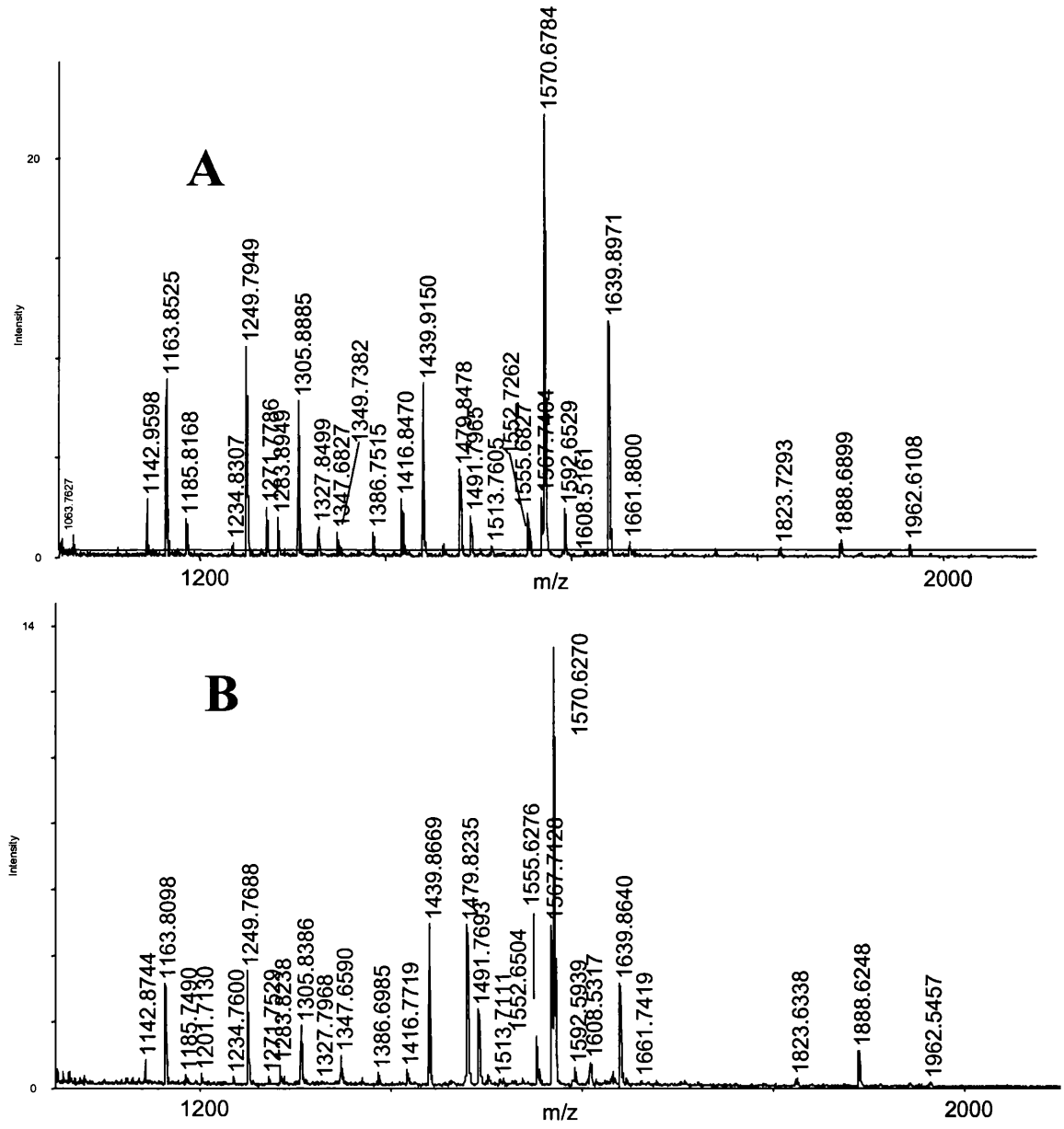


Figure 3.1.7 Recovery of peptides from RP and SCX columns.
The figure shows MALDI-TOF-MS spectra of a BSA tryptic digest before (A) and after (B) extraction by both RP and SCX chromatography with in-house packed columns. Just before analysis samples were mixed with fibrinopeptide (m/z 1570.6), which served as an internal standard to normalize ion intensities. Ion intensities of peptides in B were 75 % ± 73 (mean ± SD) of those in A (n = 23 peptides). Experiments were conducted by injecting sixty micrograms of a BSA tryptic digest in a RP column (packed in housed with POROS R2 20 in a PEEK tube with dimensions 50 mm x 0.8 mm). After washing the column, a pre-equilibrated SCX column (packed in housed with POROS HS in a PEEK tube with dimensions 50 mm x 0.8 mm) was then connected to the end of the RP column and peptides eluted directly from the RP column to this SCX column. The SCX column was then washed with a low ion strength buffer, after which step polypeptides were eluted with a high ionic strength solvent.

The recovery of proteins and peptides from columns constructed using stainless steel frits and packed with perfusion chromatography media was investigated. Figure 3.1.6 shows that after extraction the recovery of proteins was virtually quantitative and that addition of solubilizing, reducing and alkylating agents did not interfere with the recovery of proteins. In a different set of experiments, peptides produced by digesting BSA with trypsin were extracted by RP followed by SCX chromatography in in-house packed columns. Eluted peptides were analysed by MALDI-TOF MS and their intensities compared with the intensities of an equal amount of untreated peptides (assuming 100% recovery). The ion intensities were compared with a standard peptide (GFP) spiked in the sample just before MS analysis. The results, presented in Figure 3.1.7, indicated that all BSA tryptic peptides were recovered from the RP and the SCX columns, and that their intensities after extraction with respect to GFP were about 70% of those before extraction.

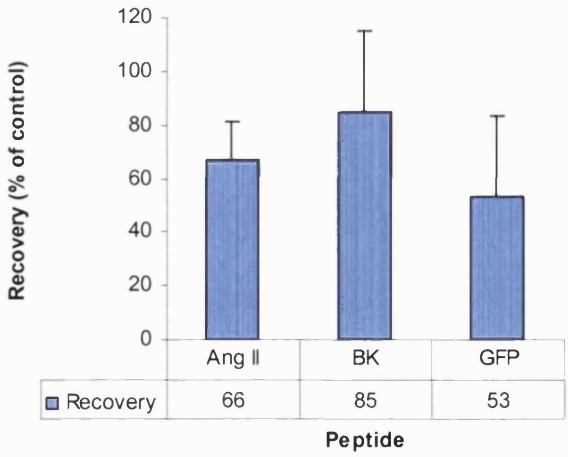


Figure 3.1.8. Recovery of urinary peptides from in-house packed chromatographic columns.
Control urine was spiked with standard peptides to a concentration of 1 nM. One hundred microliters of this sample were desalted by RP SPE and subsequently extracted with a 10 mm x 0.32 mm SCX column. Solvent A was 5% ACN/ 0.5 % formic acid and solvent B was 500 mM ammonium acetate dissolved in solvent A. After loading the sample in the pre-equilibrated column, this was washed with solvent A until a stable baseline was obtained in the UV chromatogram. Peptides were then eluted with 100 % solvent B. After drying the samples in a SpeedVac, they were reconstituted in 0.1% formic and analysed by LC-MS. The graph reports percentage of ion counts for each peptide after extraction with respect to the same parameter before extraction. Figure shows mean ± SD (n = 3).

In separate experiments aimed at investigating whether matrix compounds in urine had a detrimental effect in the performance of these columns, standard peptides were spiked in urine, desalted using SPE cartridges and further separated from other urinary compounds by SCX in packed capillaries. After extraction, LC-MS was used to compare

ion intensities of the peptides before and after extraction. Figure 3.1.8 shows that after extraction, extracted peptides produced between 50 and 80 % of the control peptide intensities.

3.1.6. Column switching liquid chromatography for the extraction and analysis of urinary peptides

Having found in the experiments thus far described that SCX chromatography is an effective method for the separation of peptides from other compounds in urine, the peptide content of urine from renal Fanconi syndrome patients and control subjects was investigated. A suitable workflow for the extraction and analysis of urinary peptides would be to extract peptides from urine by SCX, after which the eluted peptides would be in a form amenable to RP LC-MS analysis.

The aim of the experiments described in this section was to assemble a system that would allow for high recoveries of peptides while maintaining specificity. Sample losses commonly occur, among other reasons, as a result of transfer of liquid between tubes and in the walls of pipette tips because it is virtually impossible to collect and dispense the entire liquid sample from vessel to vessel. In order to overcome this problem, column switching techniques have been developed [105], in which the eluent from one column is transferred directly to another column using a system of valves such that sample losses on the walls of vessels are obviated. Once the sample is injected in the system no further operator handling of the analyte is required. Several reports exist on the use of column switching methods for the extraction of peptides [205-207] and other molecules [208] from biological fluids before their mass spectrometric analysis. Recently, column switching methods have also been used for the two-dimensional separation of peptides in proteomic studies [209].

In a study aimed at assessing the feasibility of column-switching LC-MS for the analysis of urinary peptides, a system was assembled that consisted of three columns (a short SCX, a short RP peptide trap column, and a "nano-flow" analytical column) connected via two HPLC injectors. Two HPLC pumps served the mobile phase to the SCX and to the RP analytical column independently. Figure 3.1.9 shows a scheme for the operation of this system and experiments aimed at identifying the sensitivity that could be achieved by it demonstrated that low femtomole amounts of standard peptides spiked in urine could be detected using this system Figure 3.1.10.

In order to assess the suitability of this system for the analysis of peptides in urine, peptides in urine samples from three forms of the renal Fanconi syndrome and control subjects were analysed. Plasma samples were also analysed for comparison. It was found that numerous small peptides were present in these specimens when only 5 μl of sample was used. Representative elution profiles are displayed in Figure 3.1.11 and the identities of the peptides identified are shown in Table 3.1.1.

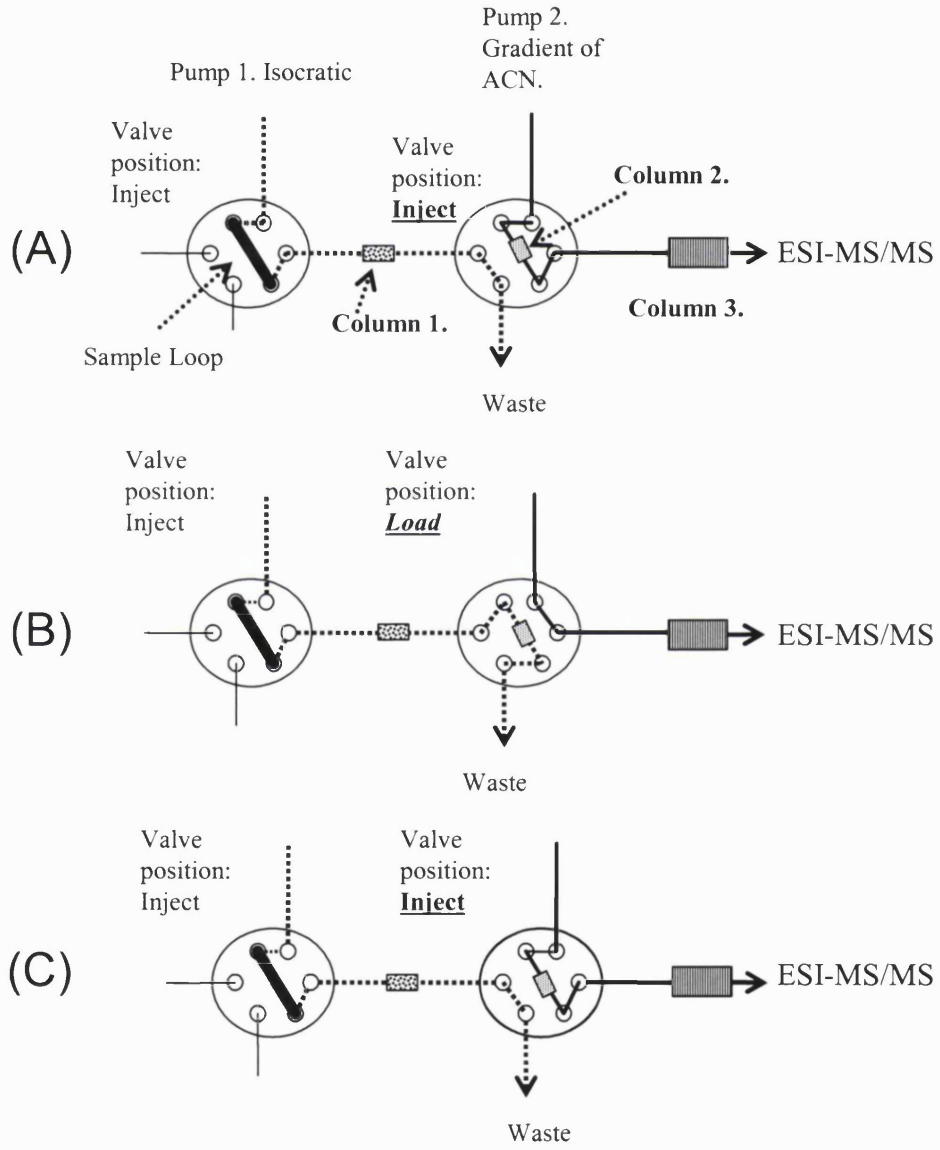


Figure 3.1.9. Operation of the Column-switching.
(A) The sample is loaded on column 1 (SCX), which is then washed with low ionic strength buffer from pump 1 (broken line). Neutral and acidic compounds are diverted to waste. **(B)** A high ionic strength solvent from pump 1 is used to elute peptides bound to column 1 directly to a reversed phase peptide trap column (column 2) placed at the sample loop of a second injector. Peptides are trapped to this second column, which is then washed to remove salts. **(C)** A gradient of acetonitrile (solid line) elutes peptides from column 2 to the analytical column 3 (C18 RP), which then separates peptides according to their hydrophobicity. Detection and characterization of eluting peptides was carried out on-line by ESI-Q-Tof MS using a QSTAR instrument.

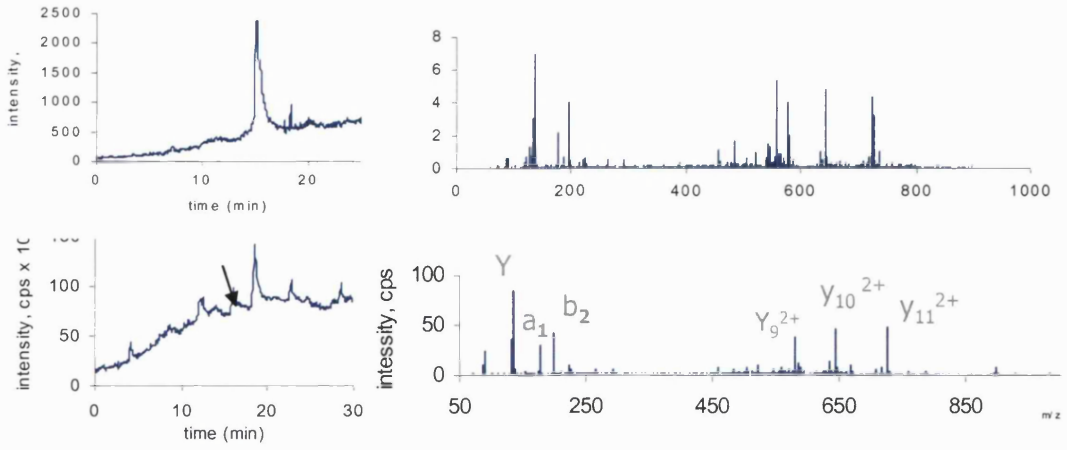


Figure 3.1.10. Sensitivity of the column switching LC-ESI-MS/MS method.
(Top Panel) Selected ion monitoring of m/z 558.3 at 1 fmol level. Urine was spiked with neurotensin (Mr 1671.9) to a concentration of 1 nM. One microliter of this solution was mixed with a low ionic strength solution and analysed with the column-switching LC-MS/MS system. Q1 was set up to transmit m/z 558.3 only and the ion current at this m/z value is shown on the left.
(Bottom Panel) Data dependent acquisition (DDA) Experiment at 10 fmol level. Urine was spiked with several peptides to a concentration of 10 nM each. One microliter of this solution was analysed using full mass range (300 to 2000 m/z) in the first quadrupole and DDA. In this mode of mass spectrometry, ions are selected for MS/MS only when the ion has a previously determined intensity in the MS scan. All peptides were observed at 10 fmol level and the figure shows the spectrum of neurotensin as an example. The arrow points to the time where neurotensin eluted.

All of the peptides identified are protein fragments probably derived from degradation by proteases. Most protein fragments identified in urine were also identified in plasma, which suggest that these fragments originated in plasma and were subsequently filtered in the kidney, although the contribution of kidney peptidases and proteases to this degradation cannot be excluded.

An exception is uromodulin, which was found present in all urine samples but was not detected in plasma because this is a protein of renal origin. It was also found that certain peptide hormones, namely IGF-II, angiotensinogen, and kallidin II, were detected in some FS patients but not detected in normal urine or plasma. Another potentially bioactive peptide, pigment epithelium-derived factor, was found in plasma and a FS patient but was not found in normal urine. The potential significance of these results will be discussed in the next section of this chapter.

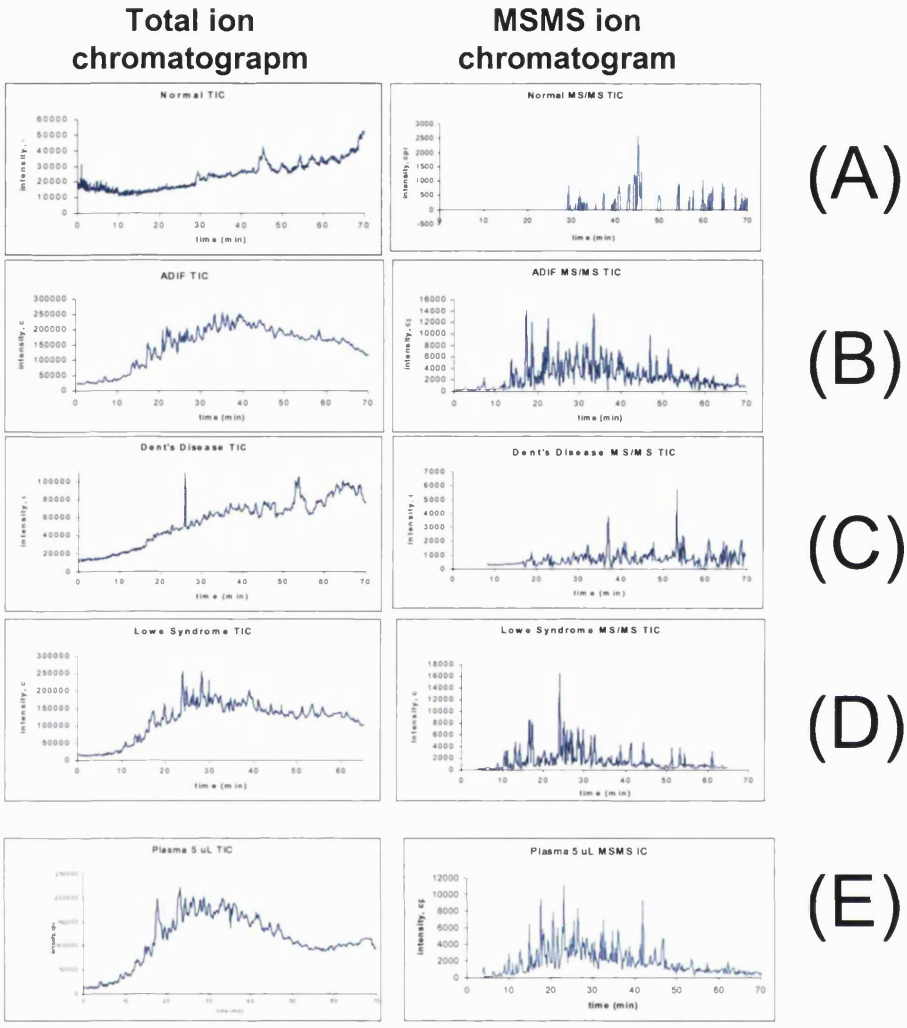


Figure 3.1.11. Elution profile of small urinary and plasma peptides.

Urinary peptides (5 µL) were analysed by the column-switching LC-MS/MS system. Many peptides were detected and sequenced in normal urine (A) and three inherited forms of the Fanconi syndrome including ADIF (B), Dent's disease (C), and Lowe syndrome (D). Plasma peptides were also analysed (E).

Table 3.1.1. Peptides identified by column switching LC-ESI-MS/MS.
The table lists all the gene products identified in each sample category together with the number of protein fragments or peptides that matched the named gene product. Percentage coverage and mascot scores are also tabulated.

ADIF

Accession No.	Protein Name	# peptides	Coverage (%)	Mascot Score
gi|223373	alpha-1-microglobulin	18	27	417
gi|28762	apolipoprotein A-IV	18	24	400
gi|4699583	Zinc-Alpha-2-Glycoprotein, Chain B	16	25	398
gi|178775	apolipoprotein AI	10	36	178
gi|33357205	Retinol Binding Protein	7	21	141

gi|23243412	Uromodulin, Tamm-Horsfall glycoprotein	4	2	141
gi|1195503	beta 2-microglobulin	13	39	140
gi|7770227	alpha2-HS glycoprotein	5	8	135
gi|224917	lipoprotein CIII	4	29	118
gi|230651	Transthyretin	7	31	97
gi|32171249	prostaglandin D2 synthase 21kDa (brain)	4	13	94
gi|7427517	heparan sulfate proteoglycan 2	8	2	93
gi|13937967	Similar to semenogelin I	6	12	85
gi|181387	cystatin C	3	19	74
gi|27692693	Albumin	5	8	72
gi|532198	Angiotensinogen	3	10	55
gi|17028367	Gelsolin	2	8	54
gi|7245524	Transferrin, Human Serum Chain A,	2	7	38
gi|422816	Pigment epithelium-derived factor	4	13	34
gi|482007	Ig kappa chain - human	2	10	26

Dent's disease

Accession No.	Protein Name	# peptides	Coverage (%)	Mascot Score
gi|2521983	alpha2-HS glycoprotein	6	5	113
gi|32171249	prostaglandin D2 synthase 21kDa (brain	3	13	108
gi|2914175	Apolipoprotein A-I	4	7	87
gi|23243412	Uromodulin,Tamm-Horsfall glycoprotein	3	2	78
gi|223130	fibrinogen betaB 1-118	1	11	71
gi|231241	Alpha1-Antitrypsin	4	61	64
gi|563320	apolipoprotein A-IV	2	11	61
gi|4699583	Zinc-Alpha-2-Glycoprotein	4	10	46
gi|230651	Transthyretin (Chain A, Prealbumin)	2	30	41
gi|7427517	heparan sulfate proteoglycan 2	3	1	37
gi|30023	pre-pro-alpha-2 type I collagen	2	5	27
gi|224917	lipoprotein CIII	2	29	16

Lowe syndrome

Accession No.	Protein Name	# peptides	Coverage (%)	Mascot Score
gi|178775	Apo-AI (proapolipoprotein)	12	31	221
gi|181387	cystatin C	7	32	199
gi|223373	apha-1-microglobulin	9	23	182
gi|17028367	gelsolin	6	20	163
gi|1942682	beta-globin (Human Hemoglobin)	5	26	148

gi|223057	fibrin alpha C term fragment	8	42	97
gi|177836	alpha-1-antitrypsin precursor	3	7	88
gi|7770227	alpha2-HS glycoprotein (PRO2743)	2	7	82
gi|28762	apolipoprotein A-IV precursor	6	14	80
gi|23241675	albumin	11	10	79
gi|219978	transthyretin	5	24	66
gi|224917	lipoprotein CIII	4	22	66
gi|229138	kallidin II	1	100	65
gi|1827805	Factor D, Chain A, Complement Activating Enzyme	5	28	63
gi|319896	hemoglobin alpha chain	2	12	56
gi|32171249	prostaglandin D2 synthase 21kDa	2	13	55
gi|30157167	similar to cytokeratin 8 [Homo sapiens]	3	6	47
gi|4504707	inositol polyphosphate-4-phosphatase, type II, 105kD; inositol polyphosphate 4-phosphatase II; 4-phosphatase II [Homo sapiens]	2	3	44
gi|137116	Uromodulin, Tamm-Horsfall glycoprotein	1	2	41
gi|34616	beta-2 microglobulin [Homo sapiens]	3	9	39
gi|33004	put. IGF-II [Homo sapiens]	1	5	32
gi|38026	Zn-alpha2-glycoprotein [Homo sapiens]	4	15	31

Normal

Accession No.	Protein Name	# peptides	Coverage (%)	Mascot Score
gi|23243412	Uromodulin, Tamm-Horsfall glycoprotein	4	3	119
gi|23241675	albumin precursor	4	10	49
gi|219978	transthyretin	1	9	34
gi|13787109	Alpha-1- Antitrypsin	4	10	28
gi|224917	lipoprotein CIII	1	22	26
gi|38026	Zn-alpha2-glycoprotein	1	8	23
gi|296672	Retinol Binding Protein	1	10	20

Plasma

Accession No.	Protein Name	# peptides	Coverage (%)	Mascot Score
gi|28762	apolipoprotein A-IV precursor [Homo sapiens]	38	58	748
gi|4699583	Chain B, Human Zinc-Alpha-2-Glycoprotein	10	29	176
gi|178775	apolipoprotein AI	8	26	163

gi|2521983	alpha2-HS glycoprotein	6	8	128
gi|224917	lipoprotein CIII	5	29	128
gi|230014	beta-2-microglobulin	10	57	116
gi|32171249	prostaglandin D2 synthase 21kDa	3	11	114
gi|21361198	alpha-1 antiproteinase, antitrypsin, member 1;	9	10	112
gi|12653501	Pigment epithelium derived factor	6	12	106
gi|763431	albumin	6	12	77
gi|339685	transthyretin	5	52	72
gi|223373	alpha-1-microglobulin	4	16	64
gi|296672	RBP [Homo sapiens]	2	26	53
gi|1827805	Chain A, Human Factor D, Complement Activating Enzyme	5	32	41

3.2. Analysis of polypeptides in the urine of renal Fanconi patients

The preceding section demonstrated that peptides can be extracted from urine by SCX. However, although the column switching set-up described above was useful for characterizing small peptides, larger polypeptides could not be detected with that method because the fragmentation of large polypeptides needed for the generation of MS/MS spectra, and hence sequence information, is inefficient for large peptides. For this reason, virtually all methods reported in the literature for the analysis of polypeptides by LC-MS/MS involve digesting the polypeptide mixture with a protease (normally trypsin), after which the peptide mixture is separated by LC (or 2D-LC) and the peptides detected and sequenced by MS/MS [116]. Another limitation was that only small sample volumes could be loaded in the column switching set-up.

This section describes the results obtained from the analysis of renal FS and control urinary peptides using a strategy that involved extraction and separation of peptides and small proteins by RP SPE followed by SCX LC. Fractions collected from these LC runs were digested with trypsin, and the peptides thus generated were further separated by RP-LC and analysed on-line by ESI-MS/MS. Three Dent's disease, one Lowe syndrome, one ADIF, and three Normal samples were studied. These analyses were standardized to creatinine concentration to compensate for the difference in water excretion between individual samples.

3.2.1. The peptide composition of renal Fanconi and Normal urines

To generate a profile of small and medium size polypeptides isolated from urine of renal FS patients, peptides were de-salted by RP SPE and separated from acidic and neutral compounds by capillary SCX. It was evident from the experiments presented in the previous section of this chapter that the complexity of the urinary peptide content of these samples was high. Therefore, SCX was also used to separate the polypeptide mixture obtained by SPE. Sample pre-treatment in this fashion showed an acceptable recovery of small and medium size peptides (about 60 – 80 %, see previous section of this chapter). This allowed for the selective enrichment of small polypeptides and proteins.

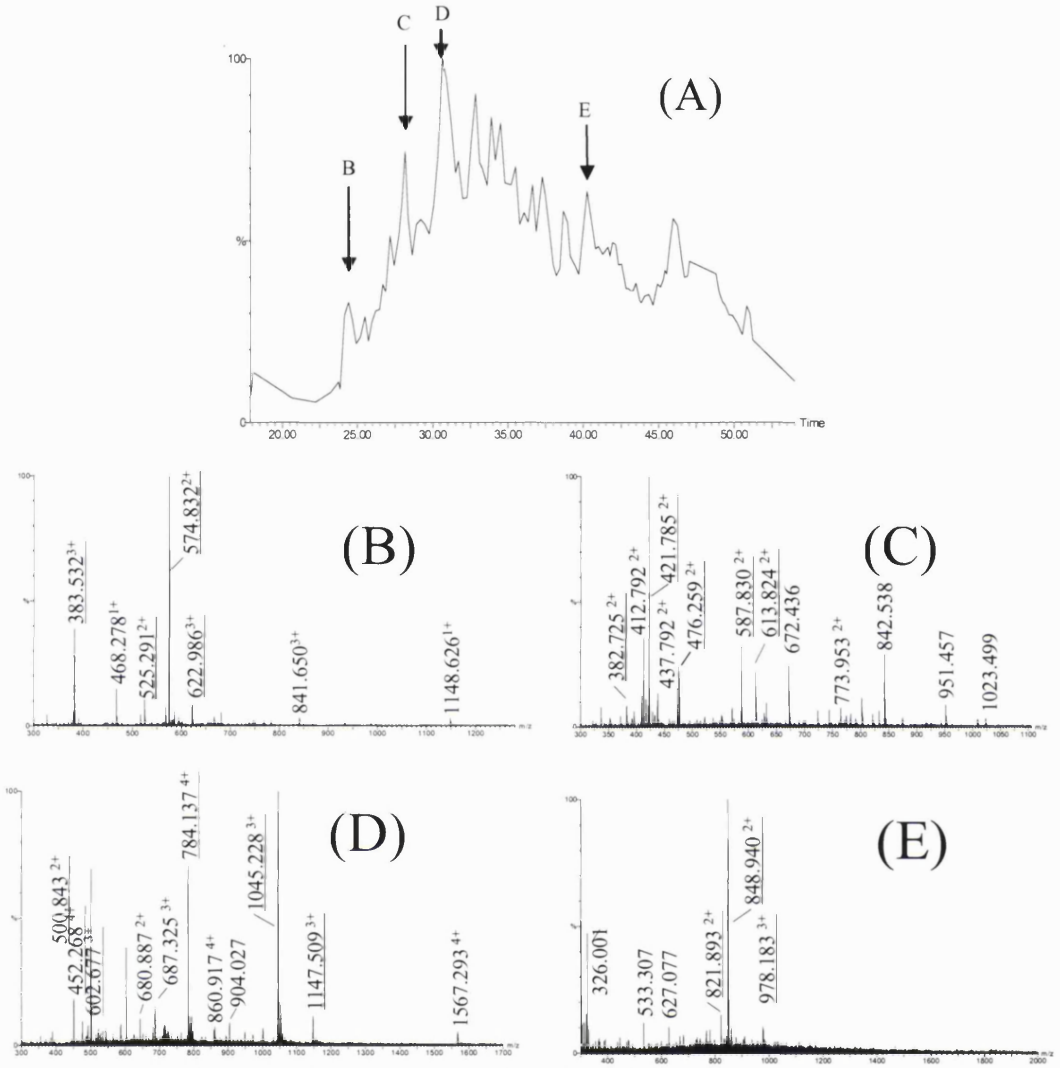


Figure 3.2.1. LC-MS/MS Survey Scan of One FS Urinary SCX Peptide Fraction. Peptides were extracted from urine by SPE and SCX chromatography. SCX LC was also used to separate peptides, which were further separated by RP LC and analysed by MS/MS. (A) Total ion chromatogram (TIC) of one of the SCX fractions as analysed by LC-ESI-MS/MS. (B), (C), (D), and (E) show single MS survey scans (2 seconds acquisition) at times marked by arrows B, C, D, and E in the TIC shown in (A). Underlined m/z values were selected for MS/MS. Note that not all multiply charged ions were selected for MS/MS and therefore no sequence information could be derived from these ions.

In order to detect and characterize as many peptides as possible, extensive fractionation by SCX was necessary (see Figure 3.2.1). In contrast to most multidimensional LC-MS/MS approaches described in the literature (e.g., [113;165;210]), which involve proteolytic digestion of a protein mixture prior to fractionation by SCX, in the present study, tryptic digestion was performed after fractionation in order to keep all the resultant peptides in the same fraction, thus facilitating identification by automated database searching.

Figure 3.2.1 illustrates the complexity of the peptide mixture following trypsin digestion. Inspection of TOF-MS spectra collected at any point during the chromatographic run showed that several peptides co-eluted at any one time. The Data Dependent Acquisition capability of MassLynx™ allowed for the switching from MS to MS/MS mode when peptides of a predetermined intensity are detected. Hence, more abundant ions are selected more readily than low abundant ones, thus abundant species are more likely to be selected. Since ESI normally produces multiply charged peptide ions, parameters were chosen so that only multiply charged ions were selected for MS/MS. Figure 3.2.1 shows four MS scans at four different time points of the LC-ESI-MS/MS run (at 2 seconds/scan). Twenty-six putative peptides were recorded, of which eighteen were selected for MS/MS. Hence, as a rough estimate, and according to the data presented in Figure 3.2.1, about 70% of eluting multiply charged ions were selected for fragmentation.

The majority of polypeptides identified were serum polypeptides (Figure 3.2.2), which correlated well with published lists of plasma polypeptides [211;212]. A large number of peptides having potential biological activity were also identified in FS patients (Table 3.2.1), which were absent from the Normal urine, when the analysis was standardized to creatinine concentration. Since samples underwent tryptic digestion in order to create peptides more amenable to tandem mass spectrometry, it is not possible to assert whether the full-length species were present in the native specimens. However, in many instances a large percentage of the polypeptide was sequenced. As an illustration, Figures 3.2.3 shows that sequence coverage of 88% was achieved for the IGF-II polypeptide chain, which virtually represents full sequence coverage, considering that tryptic peptides with less than four amino acids are usually not recorded. Examples of MS/MS spectra that led to the identification of selected bioactive peptides is presented in Appendix I, while Appendix II lists all the gene products identified during the course of these experiments.

Bradykinin and kallidin II were also detected as full-length species. These peptides can be generated by trypsin cleavage of their common precursor (kinin). However, nanoLC-MS/MS experiments of the undigested polypeptide fraction of FS urine demonstrated that both bradykinin and kallidin II are present in these samples before tryptic digestion. In addition to serum proteins, cellular, extracellular and membrane associated proteins were also present (see Figure 3.2.2 and Appendix II).

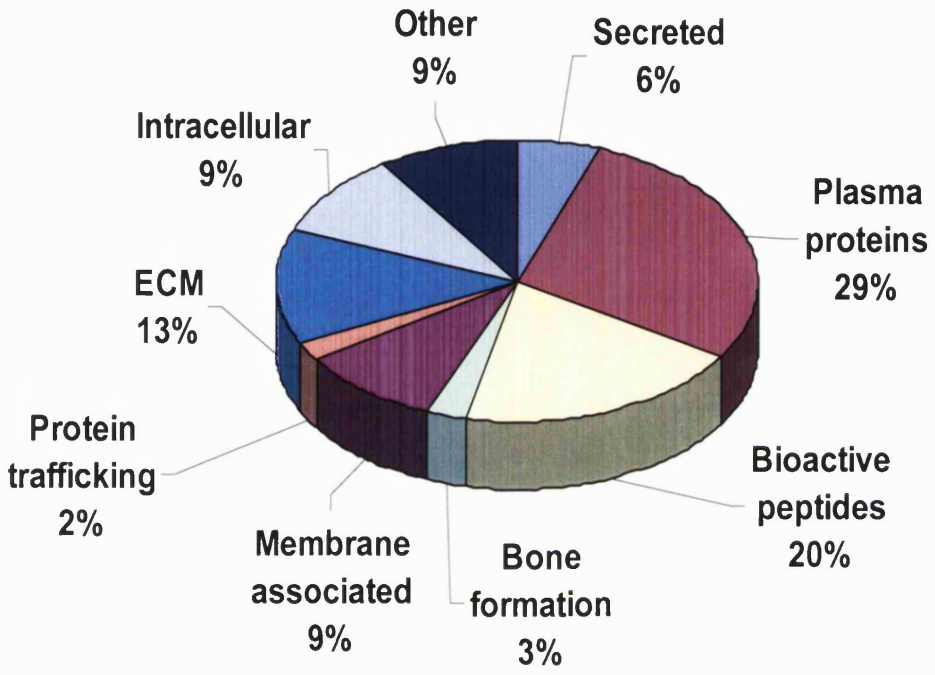


Figure 3.2.2. Classification of identified polypeptides from urine of Fanconi syndrome patients.
The majority of the identified polypeptides are abundant plasma components. Furthermore, a large number of the detected peptides have potential biological activity.

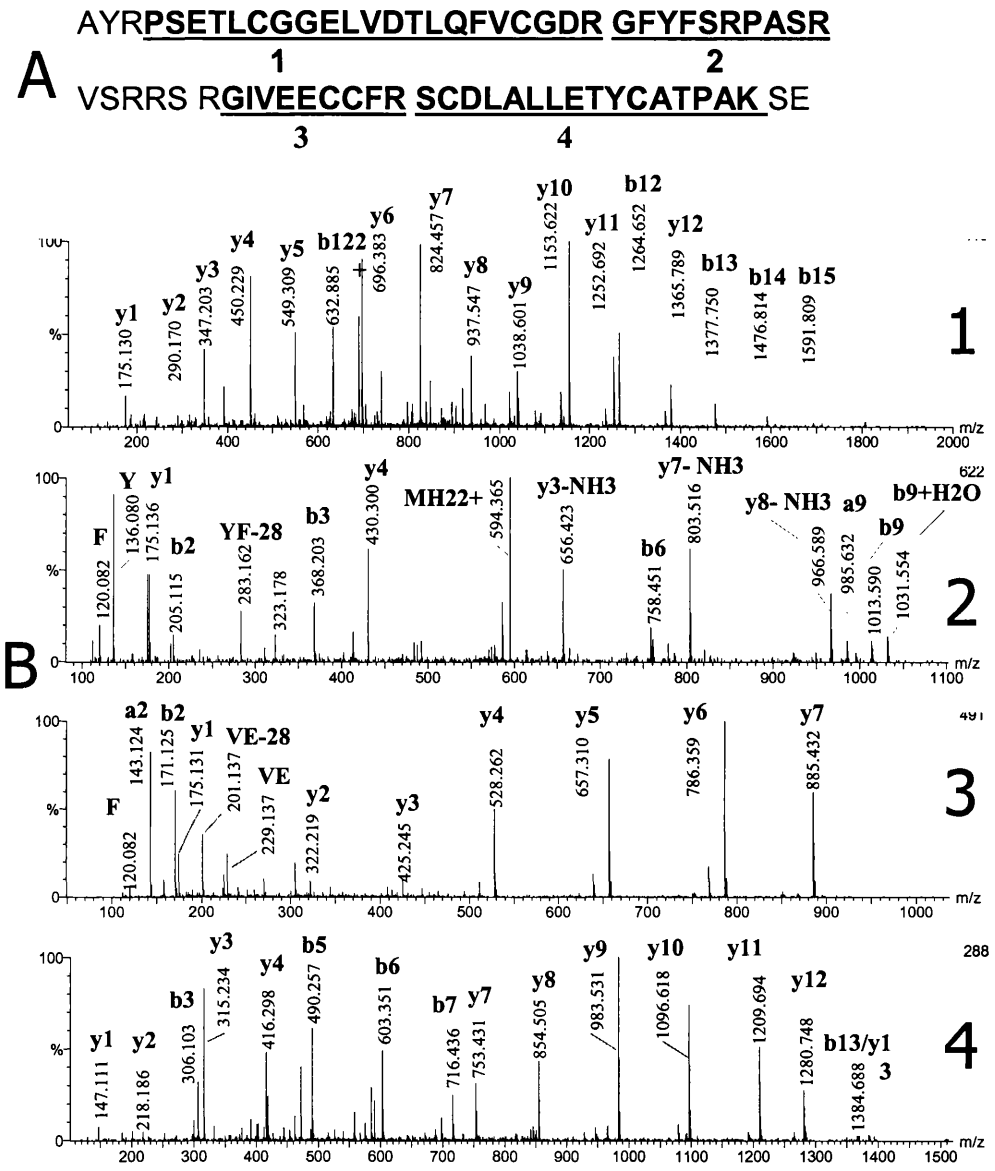


Figure 3.2.3. Mass spectra of IGF-II.
Mass spectra and percentage coverage of IGF-II tryptic peptides is shown. **(A)** Sequence of IGF-II (NCBI g.i. number 1000058). Underlined peptides labelled 1, 2, and 3 were sequenced by MS/MS **(B)** Mass spectra of tryptic peptides that derived data for sequence determination (depicted bold underlined in A). Sequence coverage of 88% was achieved.

Table 3.2.1. Examples of bioactive peptides found in Dent's disease urine by LC-MS/MS.

Name#	NCBI GI	Patient 1		Patient 2		Patient 3	
		N° of peptides*	Coverage (%)**	N° of peptides*	Coverage (%)**	N° of peptides*	Coverage (%)**
Chemokin; member 15 (leukotactin-1)	4759072	1	10	4	61	3	17
Chemokine CC-3,	4759070	3	50	4	48	2	30
IGF-I	755741	0	0	1	36	1	21
IGF-II	1000058	2	49	4	88	1	10
Dermcidin	16751921	1	12	1	10	1	10
Diazepam binding inhibitor	10140853	2	23	0	0	3	32
Gastric inhibitory polypeptide	4758436	1	11	0	0	2	11
Hepcidin antimicrobial peptide	10863973	1	19	0	0	1	19
Platelet basic protein	4505981	5	27	4	19	2	14
Stromal cell derived factor	13399638	2	36	0	0	1	15
Pigment epithelium-derived factor	1144299	1	3	3	11	1	3

These peptides were detected in Dent's but not in Normal urine when the analyses were standardized to 2.5 μmol creatinine.

* Peptides that generated sequence information for the identification of the named peptide.

** Percentage of the peptide sequenced by MS/MS.

3.2.2. Comparison with published reports on the composition of renal Fanconi urinary peptides

Norden *et al* detected and quantified insulin and PTH in the urine of FS patients using immunochemical assays [151], but these two peptides were not detected in this study. A possible reason for this discrepancy could be the limited dynamic range of reversed phase LC-MS. In data-dependent acquisition experiments, ions detected by the mass spectrometer are selected for CID, so that sequence information can be generated. When several peptides co-elute the more intense ions are selected instead of less intense ones, inducing a bias towards more prevalent peptides. Furthermore, in the electrospray plume generated in ESI, peptides compete for available protons for ionisation [135]. When several peptides co-elute from the reversed phase column, peptides with greater proton affinity ionise more efficiently. Consequently, although insulin and its fragments ionise well by ESI, it is possible that the relative abundance of insulin or PTH tryptic fragments with relation to other co-eluting peptides was such that these species did not produce ions of enough intensity for mass spectrometric detection.

Nevertheless, an advantage of mass spectrometry is that it is operator unbiased and, since MS is not an affinity-based method, detection of molecular species is not restricted to the availability of antibodies. Furthermore, the problem of antibody cross-reactivity and specificity is avoided by using mass spectrometric methods.

In this respect, it should be noted that Norden *et al.* [151] standardised their immunoassays to creatinine concentration. Since the ratio of protein to creatinine is 50 to 100 times higher in disease than in control, any degree of antibody cross-reactivity would have amplified the signal by the same factor in their assays. It has been reported that immunoassays overestimated insulin concentrations when compared with LC-MS using internal standards labelled with stable isotopes even when the same protein amounts were used for the analysis [213]. Thus, other, more systematic, studies would be required to answer the question of whether the discrepancy of the results presented in this thesis and those reported by Norden *et et al.* is due to over estimation of concentration in the cited paper, or a limitation of the approach reported in this thesis and based on LC-ESI-MS/MS.

In this respect, it should be possible to use the antibodies used for the immunochemical detection of insulin and PTH in Norden et al [151] to affinity purify

these molecules, which could then be analysed by MS. Any degree of cross-reactivity could then be detected by MS.

3.2.3. Possible implications for the pathophysiology of the renal Fanconi syndrome

The results presented above indicated that there are several polypeptides that are detectable in FS patients, but not in normal urine when the analyses were standardized to creatinine concentration (to correct for differences in water excretion). Possible implications of these findings are discussed below.

Although the three inherited forms of the renal FS studied have different genotypes, all of them show LMWP, which suggest that the reabsorption of polypeptides from the glomerular filtrate is compromised in these patients [73]. Lowe's syndrome patients have a more severe and systemic phenotype than other FS patients [214]. In addition to FS, these patients suffer from cataracts, blindness, and mental and growth retardation. It has been suggested that in these patients a leak of lysosomal enzymes may result in generalized cell injury [91]. However, it is not clear how a defect on the re-uptake of metabolites by PT cell results in the progressive loss of kidney function, which is also a feature of Dent's disease and ADIF.

It has been proposed that the toxicity of proteinuria *per se* may be responsible for this damage (reviewed in [215]). Albumin overload, resulting from glomerular disease has been reported to induce apoptosis [216], and an enhanced rate of endocytosis leading to activation of as yet unidentified signal transduction pathways [217], which result in the upregulation of pro-inflamatory cytokines [218]. However, in contrast to glomerular proteinuria, in tubular proteinuria there is not usually an associated overload of albumin, since the integrity of the glomerular membrane is not compromised until FS is advanced; thus, other factors may contribute to the progressive kidney failure observed in FS patients. Thus an alternative explanation to account for the progressive loss of kidney function in FS patients is that, as in some glomerular diseases [219], biologically active peptides contribute to the pathophysiology of FS.

Results presented in this thesis and elsewhere [220], and data reported in other studies [151], showed that there is a large number of potentially bioactive polypeptides in the urine of these patients, and therefore, they must also be at high concentrations in tubular fluid. Many of these polypeptides are known to be present in serum (e.g., chemokine 14 and 15, IGF I and II, BMP-1, EDGF and PDGF) [221] and are filtered. It follows that failure of re-uptake by PT cells of these peptides will result in the

concentration of these cytokines and chemokines in more distal portions of the renal tubular lumen, potentially exposing the lining of epithelial cells to direct stimulation.

Luminal membrane receptors have already been demonstrated for a number of circulating and locally produced bioactive peptides [3], suggesting the proposed intrarenal paracrine or autocrine system may be overwhelmed in FS. For example, TGF-β has been linked to progressive glomerular and tubular injury [222]. Although, this particular cytokine was not detected in the urine of FS patients, BMP-1, a member of the TGF-β family was found in FS but not in normal urine. As described for glomerular diseases, such cytokines and chemokines can upregulate the production of extracellular matrix proteins and recruit immune cells, which in turn have a role in producing more peptides with biological activity. The consequence is an accumulation of extracellular matrix proteins and interstitial fibrosis, which contribute to progressive renal failure [219].

4. Comparative proteomic analysis of Dent's and Normal urine

Experiments described in Chapter 3 revealed the presence of several polypeptides in the urine of renal Fanconi syndrome patients, which were not detectable in normal urine when the analysis was standardized to creatinine concentration. However, some of these molecular species could be normal components of urine, and failure to detect them could be due to limited dynamic range of the analytical methodology. Protein concentration in the urine of renal Fanconi syndrome patients is fifty to a hundred times higher than in the urine from control subjects. Methods used for the identification of proteins are dependent on the amount of analyte present in the sample such that proteins present at high levels are detected more readily than those present at low levels. Therefore, since the analyses described in the previous chapter were standardized to volume (corrected for water excretion by standardizing to creatinine) the analytical method detected more proteins in FS urine than in control samples.

Therefore, to investigate whether the proteins found in renal Fanconi syndrome urine are normal components of urine, although present at lower concentrations, a comparative proteomic strategy was set up, which focused on the qualitative profile of a form of FS (Dent's disease) urine versus normal urine. To this end, the analyses were standardized to protein concentration. Two main approaches were used for the separation of polypeptides, namely gel electrophoresis and liquid chromatography. MS and MS/MS were used for the identification and/or quantitation of the separated proteins. The results obtained by these two approaches, and the conclusions and new knowledge acquired by analysing the data, are the subject of this chapter and will the described in turn.

4.1. Analysis of urinary polypeptides by gel electrophoresis

Although 1D SDS-PAGE is relatively tolerant to the presence of low concentration of salts, the isoelectric focusing of proteins in the first dimension of 2DE requires the sample to be free of charged compounds. Furthermore, proteins in normal urine are present at low concentrations, and therefore, they have to be concentrated in order to load a relatively large amount of protein on the IPG strips. Although several methods for the extraction of proteins from urine prior to gel electrophoresis have been described in the literature (reviewed in [161]), experiments described in Chapter 3 demonstrated that RP LC was an efficient method for desalting urinary proteins. Therefore, it was decided to use RP LC to desalt urinary proteins. These desalted proteins were in a solvent (concentrated acetonitrile in trace acid) amenable to dry in vacuum. The dried protein extracts were then reconstituted in Laemli buffer for 1D SDS-PAGE or in loading buffer for 2DE prior to their separation by gel electrophoresis.

4.1.1. One dimensional SDS-PAGE of Dent's and normal urinary proteins

The patterns of urinary proteins derived from four Dent's disease patients and four normal individuals were compared by 1D SDS-PAGE. Samples were desalted by perfusion RP LC and equal amounts of protein loaded on each gel lane. As Figure 4.1.1 shows, the patterns were very similar within sample groups and strikingly different between Dent's and normal individuals. Specifically, bands labelled 1 to 4 in Figure 4.1.1, among others, appeared in all normal samples with high intensity, whereas they were absent from the Dent's samples. In contrast, bands 7 and 8 in the same gel produced higher intensities in Dent's samples than in normal samples. These patterns were also seen when another purification methods, i.e., precipitation, was used to concentrate proteins (data not shown).

Gel bands from the gel displayed in Figure 4.1.1 were excised and subjected to in-gel digestion. The resultant tryptic peptides were analysed by LC-ESI-MS/MS. The identity of proteins in 10 of the bands could be determined and Table 4.1.1 lists all the proteins identified in these experiments. In eight of these bands there was more than one protein and some proteins were identified in more than one band, which is probably a consequence of post-translational modifications, most probably proteolytic digestion. A total of 24 different gene products were identified with confidence; Mascot scores were high (well above the limit of statistical confidence, see Table 4.1.1) and at least 2 peptides derived sequence information that matched the listed protein (Table 4.1.1). The exceptions

were α-1 antichymotrypsin, kininogen, T4-binding globulin, complex forming glycoprotein HC, transthyretin, and cystatin C, for which only one peptide was sequenced or had low Mascot scores. Nevertheless, further inspection of the MS/MS spectra indicated that the matches were probably correct. As an example, Figure 4.1.2 shows the MS/MS spectra of transthyretin and kininogen tryptic peptides. All the fragment ions could be assigned to theoretical fragmentations of the peptides.

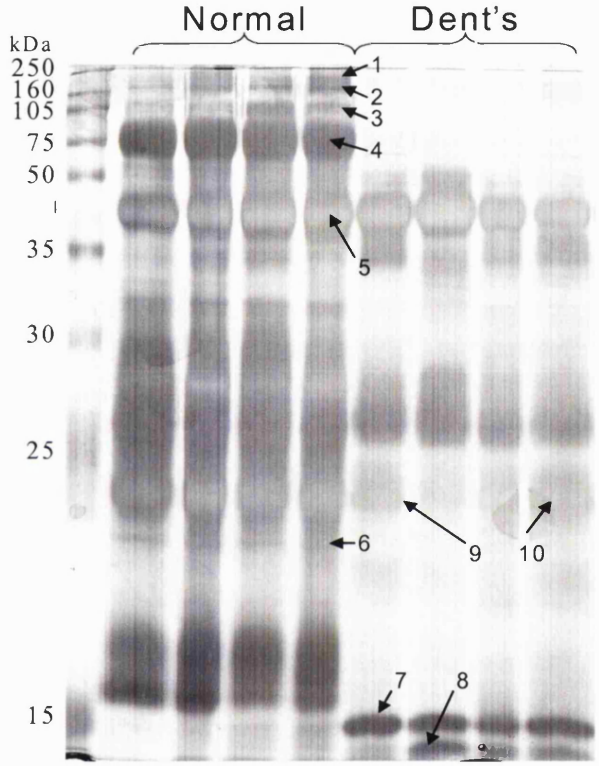


Figure 4.1.1. 1D SDS-PAGE of urinary proteins.
Proteins were desalted by perfusion RP LC, quantified and same amounts of protein loaded in each lane of a 12% acrylamide gel. After silver nitrate staining, numbered bands were excised, in-gel digested and analysed by LC-ESI-MS/MS. Results of these analyses are displayed in Table 4.1.1.

Table 4.1.1. Proteins identified by 1D SDS-PAGE and LC-ESI-MS/MS

Band	NCBI Acc No.	Protein Name	Mr	pI	PEP	Mascot Score	COV (%)
1	4557503	cubilin; intrinsic factor-cobalamin receptor;	407465	5.15	1	78	0
2	4557503	cubilin; intrinsic factor-cobalamin receptor;	407465	5.15	12	578	5
	6806919	low density lipoprotein-related protein 2; megalin	540349	4.89	9	507	2
	4758712	maltase-glucoamylase; brush border hydrolase;	210939	5.26	2	84	1
3	4503491	epidermal growth factor (beta-urogastrone);	137565	5.56	7	362	5
	30030	alpha-1 collagen VI (AA 574-1009)	48497	5.52	4	235	11
	4502095	membrane alanine aminopeptidase	109842	5.27	3	94	4
4	137116	uromodulin (Tamm-Horsfall urinary glycoprotein)	72451	5.05	14	292	13
	5031863	galectin 3 binding protein; L3 antigen; Mac-2-binding protein;	66202	5.13	3	157	7
	15489339	Similar to RIKEN cDNA 2610528G05	64937	7.68	2	145	5
	514366	poly-Ig receptor	76443	5.38	2	141	4
5	3212456	Human Serum Albumin	68425	5.67	21	723	30
	177827	alpha-1-antitrypsin	46787	5.42	4	168	13
	113584	Ig alpha-1 chain C region	38486	6.08	3	129	7
	28332	alpha 1 antichymotrypsin	25217	5.64	1	82	5
	17511435	roundabout homolog 4, magic roundabout	86335	9	1	73	2
	137116	uromodulin (Tamm-Horsfall urinary glycoprotein)	72451	5.05	2	71	3
	125507	Kininogen precursor (Alpha-2-thiol proteinase inhibitor)	72984	6.34	1	49	1
	1351236	Thyroxine-binding globulin precursor (T4-binding globulin)	46637	5.87	1	40	2
6	539611	perlecan precursor - human	479245	6.05	3	143	0
7	219978	prealbumin (transthyretin)	16023	5.52	2	25	19
	137116	uromodulin (Tamm-Horsfall urinary glycoprotein)	72451	5.05	1	10	2
8	181387	cystatin C	16047	9	1	28	7
	219978	prealbumin (transthyretin)	16023	5.52	1	14	19
9	178775	proapolipoprotein (apo AI)	28944	5.45	11	505	31
	125145	Ig kappa chain C region	11773	5.58	3	159	45
	33700	immunoglobulin lambda light chain	25519	6.81	2	70	14
	32171249	prostaglandin D2 synthase 21kDa (brain)	21243	7.66	1	28	8
10	306958	immunoglobulin kappa chain	12017	8.07	2	188	30
	178775	proapolipoprotein(apo AI)	28944	5.45	1	49	4
	223373	complex-forming glycoprotein HC	20592	5.84	1	44	12
	21669179	immunoglobulin lambda light chain	11601	7.96	1	17	15

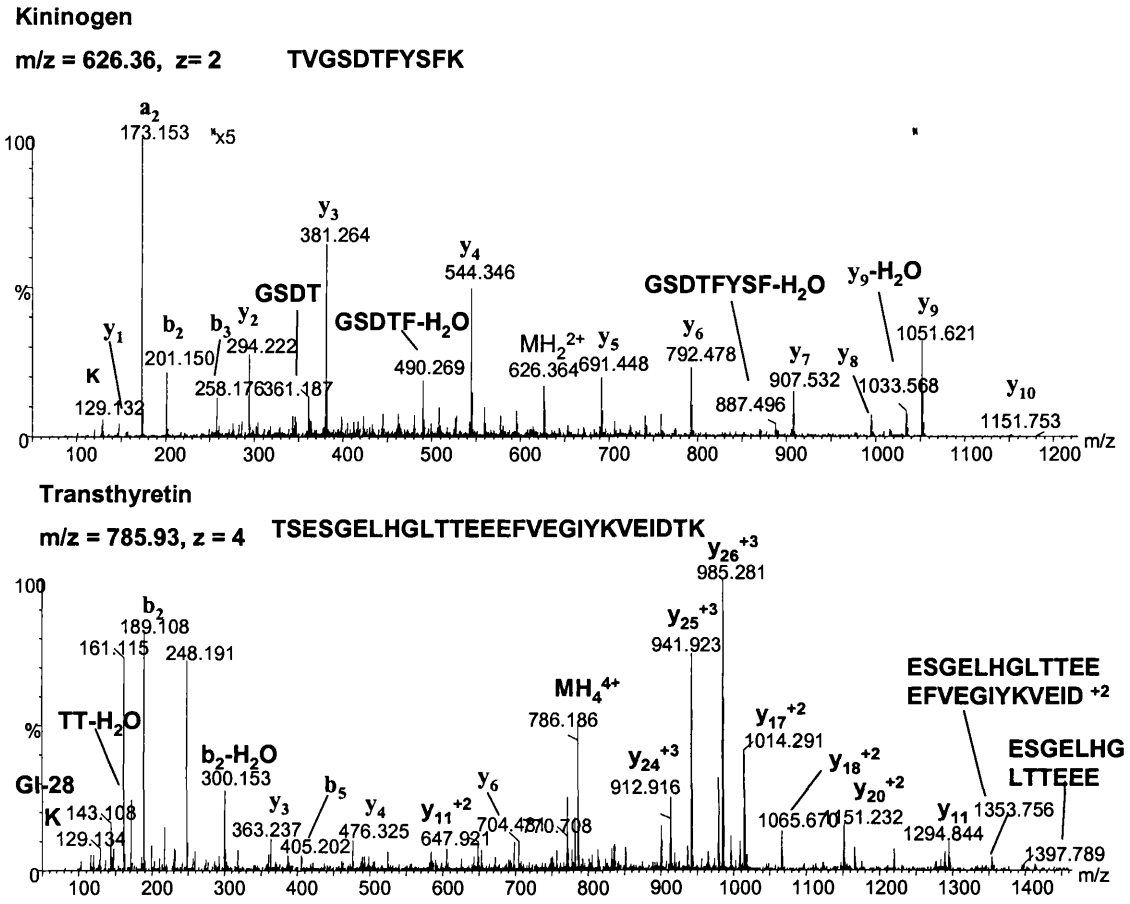


Figure 4.1.2 Tandem Mass Spectra of peptides matching kininogen and transthyretin.
Kininogen and transthyretin were identified in bands 5 and 7, respectively. The identities were obtained by MASCOT searches and confirmed by manual assignment of all major peaks in the spectra.

From the a methodological standpoint, the significance of these results is that the method used to extract polypeptides from urine, namely perfusion RP LC using gygaporous C18 beads, is effective for the isolation of both large and small proteins. The largest protein identified was megalin, which has a Mr in excess of 500 kDa, while the smallest was transthyretin (10 kDa). In addition, experiments using HPLC and MALDI-TOF MS revealed that small peptides were also isolated with this method (see below).

Regarding their biological relevance, these data show that several large proteins are present in the urine of normal subjects at higher levels than that of Dent's disease individuals when the analysis is standardized to protein concentration. These proteins are of kidney origin. For example, megalin and cubilin are expressed on the apical side of proximal tubular cells [77] and have been found previously in normal urine at relatively

high concentrations [49]. Uromodulin, a protein expressed on the apical side of distal renal tubular cells [223-226], has been reported to be the most abundant protein in normal urine [226] and the results presented here agree with this statement. The EGF precursor and kininogen are the precursors of bioactive peptides and they are also expressed on the apical side of tubular cells [227]. Therefore, these proteins are probably shed (or actively secreted) into the tubular fluid, which would explain their appearance in normal urine. Since plasma proteins are almost quantitatively reabsorbed by normal PT cells but not by Dent's PT cells [38;73;77], proteins of kidney origin represent a larger proportion of the normal urinary proteome when compared with that of Dent's patients.

4.1.2. Two dimensional gel electrophoresis of Dent's and normal urinary proteins

Since most of the bands from 1D SDS-PAGE contained more than one protein, it is not possible to draw definite conclusions regarding the relative amounts of protein in the samples. For this reason, the patterns of desalted proteins from normal and Dent's subjects were also compared by 2DE, which is a separation method with more resolution power than 1D SDS-PAGE. Equal amounts of urinary proteins from three normal and three Dent's patients were run in separate gels and, after electrophoretic separation, the proteins were visualized by silver staining. Representative gels are shown in Figure 4.1.3.

The spot patterns obtained by 2DE were analyzed with Melanie™ for quantitative determination of differences. The optical density (OD) of each spot was expressed as a function of total OD. Although it has been shown that intensity of silver staining proteins does not produce a linear response as a function of concentration [228], this staining method has been extensively used to detect qualitative differences in protein abundance between related proteomes (e.g., [159;177;178;229]). The results obtained here indicated that there are qualitative differences between normal and Dent's urinary proteomes (Figure 4.1.3 and 4.1.4).

Since the aim was to identify proteins present at different levels as well as those that were common in both samples, virtually all the spots that could be visualized with the naked eye were cut and analysed by in-gel digestion prior to MS. In total, 192 spots (2 x 96 well plates) were excised from each of the two gels displayed in Figure 4.1.3 (384 in total) and in-gel digested.

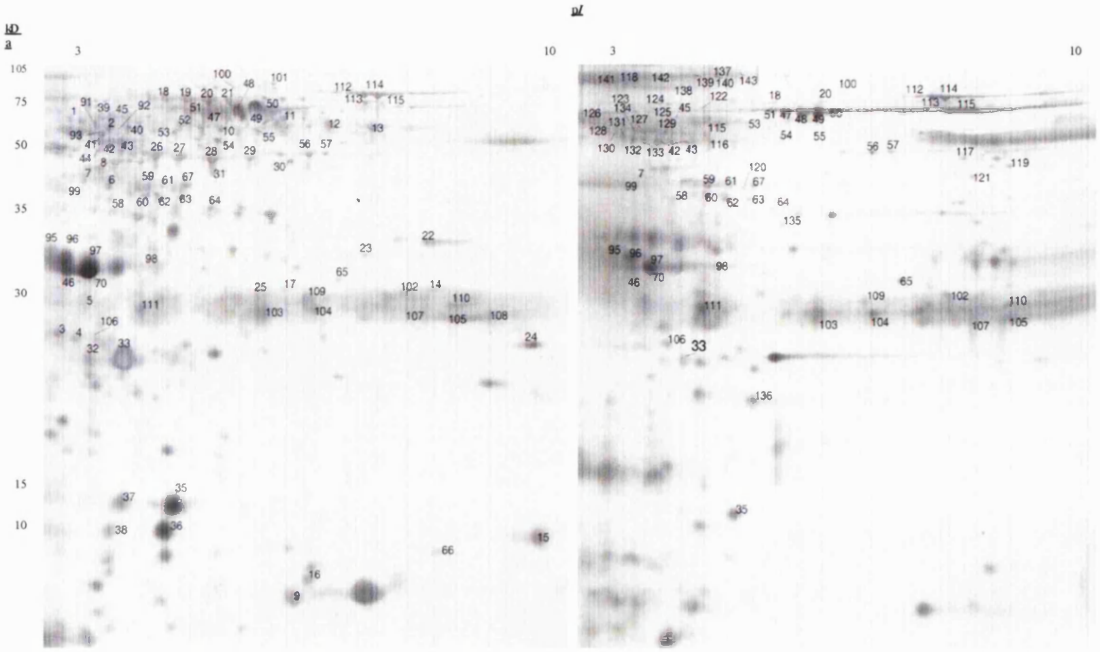


Figure. 4.1.3. Two-dimensional gel electrophoresis analysis of Normal and Dent's urinary proteomes.
Equal amounts (150 μg) of Dent's (left) and Normal (right) urinary proteins were loaded in IPG strips pH 3-10 and focused for 24 hours. The second dimension was in 15% acrylamide gels, which were subsequently stained with silver nitrate. Gels were curated using Melanie™. All the spots that could be visualized were excised from the gel, in-gel digested, and analysed by MALDI-TOF MS and/or LC-ESI-MS/MS. Numbered spots returned significant hits and the protein(s) present in these are listed in Table 4.1.2.

The resultant tryptic peptides were analysed by MALDI-TOF-MS and/or LC-ESI-MS/MS followed by peptide mass mapping or sequence tag searches, respectively. The identity of the proteins found in 174 of these spots were determined, which lead to fifty different gene products (Table 4.1.2)

An example of the data obtained by MALDI-TOF MS and their analysis by peptide mass fingerprinting is given in Figure 4.1.5, which shows the MALDI-TOF spectrum of spot 33 in Figure 4.1.3. The m/z values were fed into MS-Fit and used to search the NCBI protein database (restricted to *Homo sapiens* entries). The search returned a statistically significant hit (Mowse score 3.81e+005, Figure 4.1.5D); the protein was retinol binding protein, and the peptides identified covered 76% of the protein. Since all the spectra were internally calibrated with known autolytic tryptic peptides, the mass accuracy was within 50 ppm, with the exception of a peak at 3140.76 m/z units, which was not assigned to a RBP peptide because of the relatively large mass error. Close inspection of the spectrum revealed that this mass error was due to poor peak shape (Figure 4.1.5C insert), which tilts the centroid mass of the peak to the right. This is probably due to low ion abundance, which in turn, leads to poor ion statistics. Increasing the mass error

tolerance would lead to the inclusion of the peak at 3140.76 m/z units in the list of matched peptides. The results are convincing in that the most intense peaks were assigned to RBP or to trypsin. Furthermore, three peptides containing methionine (M) were detected as pairs 16 m/z units apart. This is due to partial oxidation of M which has been shown to add confidence to protein identification [230].

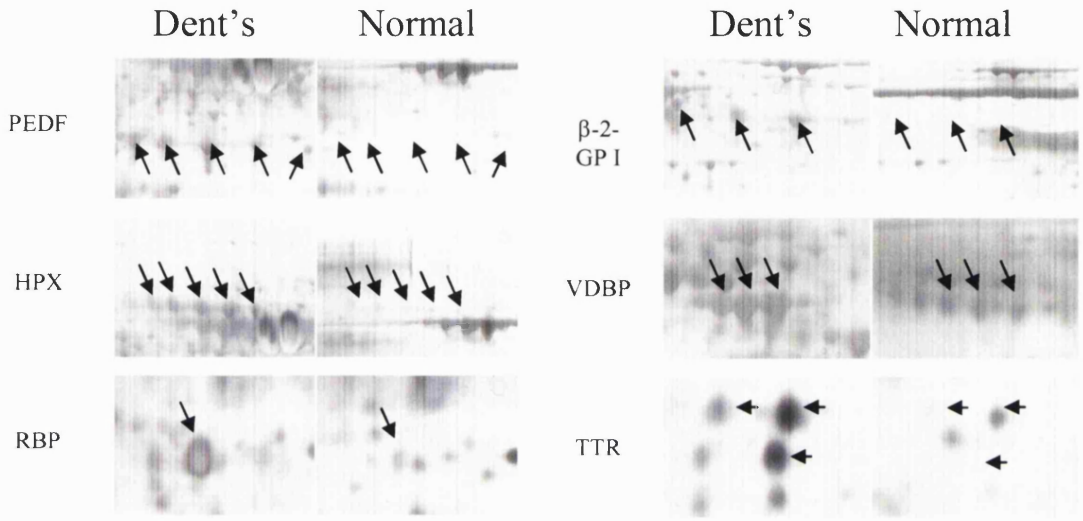


Figure 4.1.4. Two-dimensional gel electrophoretic profiles of selected urinary proteins.
Selected areas of the gels in Figure 4.1.3 showing differential spot patterns are enlarged. Dent's disease proteins are shown on the left and normal proteins on the right. Arrows point to the electrophoretic migration position of the named gene product. Abbreviations: PEDF, pigment epithelium-derived factor; HPX, hemopexin; RBP, retinol binding protein; β-2-GP I, β-2-glycoportein I; VDBP, vitamin binding protein; TTR, transthyretin.

Several proteins were present in more than one spot, which probably reflect the presence of isoforms due to post-translational modification or partial digestion of the protein. These modifications are known to alter the pI and/or Mr of the protein. On the other hand, several spots were also found to contain more than one protein.

Table 4.1.2.A. Proteins found at higher levels in Dent's than in normal by 2-DE

Protein	NCBI Acc. No.	MS Method	Spot No.*	Peptides/coverage (%)	
				Normal	*DENT'S*
Angiotensinogen	BC011519	MALDI-TOF	1	ND#	7/16
			2	ND	13/33

Apo-AI	A15879	MALDI-TOF	3	ND	26/81
			4	ND	9/42
			5	ND	6/28
Apo-AIV	M14566	MALDI-TOF	6	ND	29/65
			7	ND	28/65
			8	ND	10/28
β-2-microglobullin	V00567	MALDI-TOF	9	ND	6/50
β-2-glycoprotein I	P02749	MALDI-TOF	10	ND	12/49
			11	ND	12/40
			12	ND	12/40
			13	ND	9/37
Carbonic anhydrase	P00915	MALDI-TOF	14	ND	8/42
Cystatin C	M27891	MALDI-TOF LC-ESI-MSMS	15	ND	10/41 3/19
Cystatin M	Q15828	MALDI-TOF	16	ND	6/43
Complement factor H-related	M84526	MALDI-TOF	17	ND	8/57
Hemopexin	X02537	MALDI-TOF	18	8/25	10/32
			19	ND	10/34
			20	9/29	10/34
			21	ND	10/34
IGF binding protein 2	P18065	MALDI-TOF	22	ND	8/35
			23	ND	7/28
Lysozyme	gi|126628	LC-ESI-MSMS	15	ND	1/9

Neutrophil lipocalin	S75256	MALDI-TOF	24	ND	18/90
Phosphatidylethanolamine binding protein	AY037148	MALDI-TOF	25	ND	7/48
Pigment epithelium derived factor	U29953	MALDI-TOF	26	ND	14/40
			27	ND	16/41
			28	ND	18/43
			29	ND	13/33
			30	ND	13/39
			31	ND	8/24
RBP	X00129	MALDI-TOF	32	ND	13/76
		LC-ESI-MSMS	33	ND	4/32
Profilin 1	NM_005022	LC-ESI-MSMS	15	ND	2/21
Transthyretin		MALDI-TOF	35	ND	7/86
		LC-ESI-MSMS	36	ND	3/18
		LC-ESI-MSMS	37	ND	3/37
		LC-ESI-MSMS	38	ND	2/24
Vitamin D Binding protein	S67527	MALDI-TOF	39	ND	8/29
			40	ND	15/43
			41	ND	14/36
			42	16/43	ND
			43	8/23	5/8
			44	ND	7/26

Table 4.2.B. Proteins found at similar levels in Dent's and Normal by 2-DE

Protein	Acc. No.	MS Method	Spot No.*	# Peptides/Coverage	
				Dent's	Normal
Antithrombin III	P01008	MALDI-TOF	45	9/24	ND[#]
Alcohol dehydrogenase	P14550	MALDI-TOF	46	15/47	ND
Albumin	AF119890	MALDI-TOF	47	28/52	42/76
			48	48/69	32/57
			49	19/32	30/50
			50	26/43	25/44
			51	17/36	32/54
			52	15/27	ND
			53	7/14	ND
			54	26/42	ND
			55	11/24	ND
			56	22/41	ND
			57	21/38	ND
			58	7/15	14/33
			59	22/33	ND
			60	15/28	34/53
			61	13/26	21/42
			62	22/39	ND
			63	13/25	16/27
			64	14/24	20/30
			65	20/24	ND
			66	10/15	ND

			67	ND	21/43
			68	ND	21/39
			69	ND	18/23
Complex forming glycoprot. HC	223373	MALDI-TOF	70	ND	12/54
α-1-antitrypsin	X17122	MALDI-TOF	127	8/29	9/31
			128	6/36	11/27
			93	12/39	8/25
			129	ND	12/25
α-1-microglobulin	P02760	MALDI-TOF	95	7/20	14/32
			96	ND	11/31
			97	17/33	5/18
			98	9/26	ND
Gelsolin	P06396	MALDI-TOF	99	5/10	12/22
			7	17/26	8/16
			100	9/18	ND
			101	21/35	ND
Ig kappa light chain	AB004304	MALDI-TOF	102	5/32	ND
			103	8/35	6/42
			104	9/40	9/40
			105	5/32	ND
			106	ND	7/42
			107	ND	5/40
			108	5/41	7/51
Ig lamda light chain	U07991	MALDI-TOF	109	5/41	5/28
			110	5/38	ND

Prostaglandin D synthase	P41222	MALDI-TOF	111	11/55	4/24
Transferrin	P02787	MALDI-TOF	112	23/40	10/18
			113	32/49	36/43
			114	11/19	21/42
			115	ND	15/27
Zn-α-2-glycoprotein	P25311	MALDI-TOF	144	9/38	9/34
			145	ND	9/35

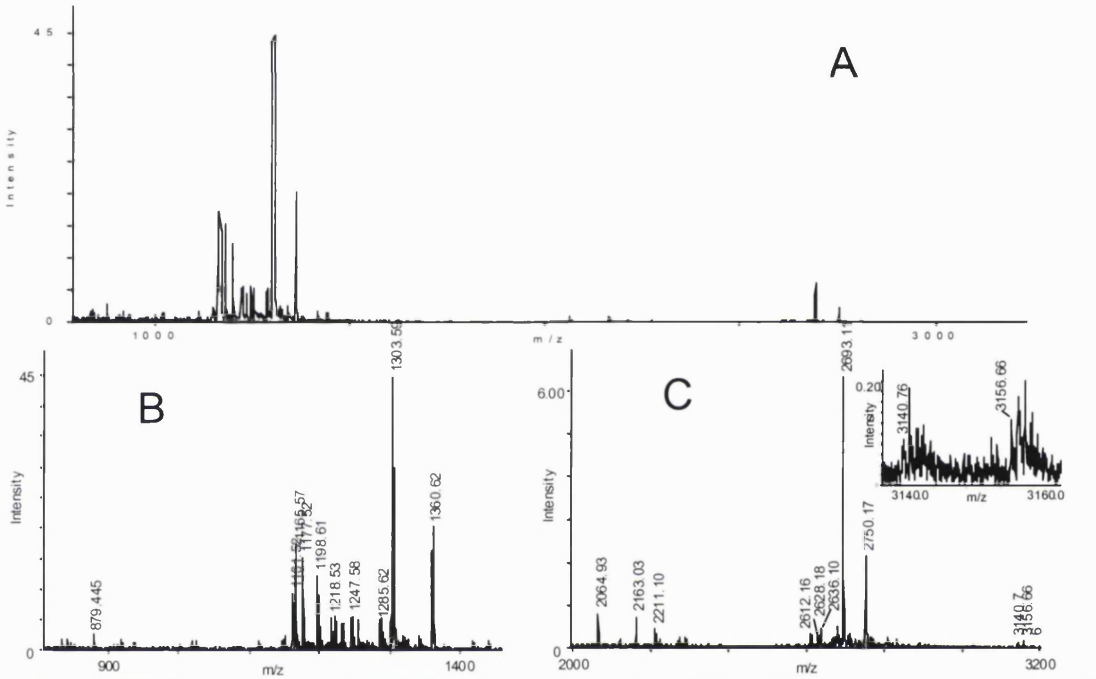
Table 4.2.C. Proteins found at higher levels in Normal than in Dent's by 2-DE

Protein	Acc. No.	MS Method	Spot No.*	# Peptides/ Coverage	
				Dent's	Normal
Acid phosphatase	BC016344	MALDI-TOF	93	ND[#]	11/27
			115	ND	8/25
			116	ND	12/25
Alpha-enolase	P06733	MALDI-TOF	117	ND	9/32
ACE	P22966	MALDI-TOF	118	ND	8/11
Betaine--homocysteine S-methyltransferase	Q93088	MALDI-TOF	119	ND	8/29
Glutaminyl-peptide cyclotransferase precursor	Q16769	MALDI-TOF	120	ND	8/26
Guanylate cyclase soluble, beta-1 chain	Q02153	MALDI-TOF	121	ND	7/14
Heat shock cognate 71 kDa protein	P11142	MALDI-TOF	122	ND	14/28
Kininogen	P01042	MALDI-TOF	42	ND	18/26
			93	ND	10/17

			115	ND	9/16
			123	ND	8/16
			124	ND	9/19
			125	ND	5/15
			126	ND	20/44
			127	ND	15/22
			128	ND	13/20
			129	ND	9/16
			130	ND	15/19
			131	ND	13/17
			132	ND	21/31
			133	ND	11/16
			134	ND	8/18
L-lactate dehydrogenase B chain	P07195	MALDI-TOF	135	ND	10/35
MBL-associated serine protease (MASP)-2		MALDI-TOF	136	ND	10/53
Myeloperoxidase	A90533	MALDI-TOF	121	ND	12/17
Mdm-1 protein	M20823	MALDI-TOF	137	ND	14/27
Polymeric-immunoglobulin receptor	P01833	MALDI-TOF	138	ND	11/17
Similar to orosomucoid		MALDI-TOF	139	ND	5/22
Uromodulin	M17778	MALDI-TOF	118	ND	12/20
			139	ND	7/14
			140	ND	13/18
			141	ND	19/25

		142	ND	16/19
		143	ND	8/17

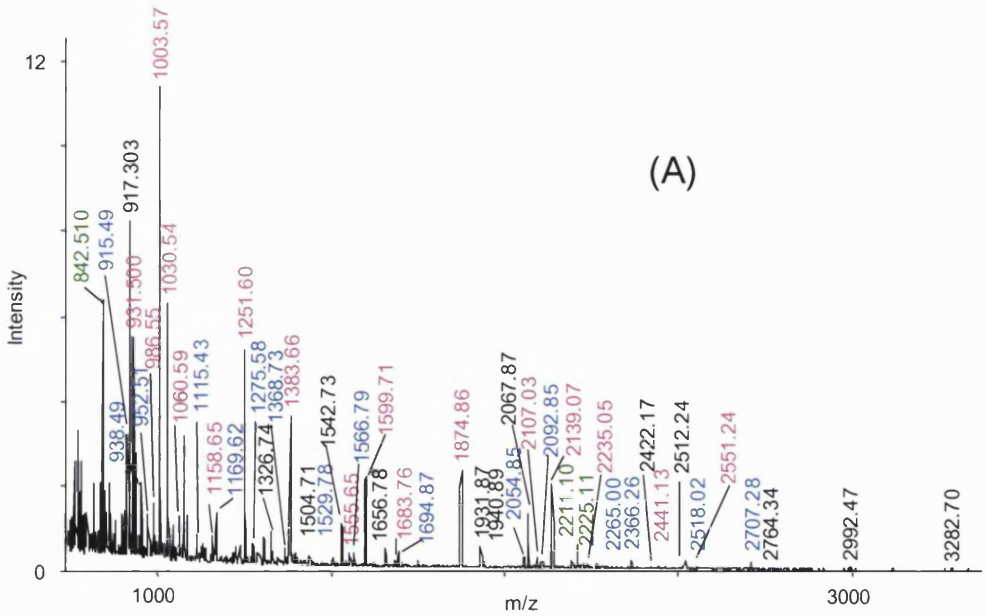
* Numbers correspond to the spot label in Figure 4.2.1
\# ND, undetermined/undetected



13/41 matches (31%). 22868.1 Da, pI = 5.48. Acc. # X00129. HOMO SAPIENS. precursor RBP .

m/z submitted	MH+ matched	Delta ppm	start	end	Peptide Sequence	PTM
879.4441	879.4576	-15.3585	27	33	(R) VKENFDK(A)	
879.4441	879.4325	13.2415	29	35	K) ENFDKAR(F)	
1161.5222	1161.5403	-15.5749	36	45	(R) FSGTWYAMAK(K)	
1165.5688	1165.5853	-14.179	156	166	(R) DPNGLPPEAQK(I)	
1177.5195	1177.5352	-13.3376	36	45	(R)FSGTWYAMAK(K)	1Met-ox
1198.6143	1198.6261	-9.8191	106	115	(R) DPNGLPPEAQK(I)	
1285.6205	1285.6323	-9.1736	170	179	(R)QRQEELCLAR(Q)	pyroGlu
1302.6229	1302.6588	-27.592	170	179	(R) QRQEELCLAR(Q)	
1303.594	1303.6217	-21.2708	183	193	(R) LIVHNGYCDGR(S)	
2064.9266	2064.9701	-21.0492	138	155	(R) LLNLDGTCADSYSFVFSR(D)	
2612.1564	2612.1649	-3.2459	79	101	(R) LLNNWDVCADMVGTFTDTEDPAK(F)	
2628.1766	2628.1598	6.3948	79	101	(R)LLNNWDVCADMVGTFTDTEDPAK(F)	1Met-ox
2693.1139	2693.1214	-2.7976	116	137	(K) GNDDHWIVDTDYDTYAVQYSCR(L)	
3156.66	3156.5047	49.2	46	74	(K)KDPEGLFLQDNIVAEFSVDETGQMSATAK(G)	1Met-ox

Figure 4.1.5. MALDI-TOF mass spectrum and peptide mass map of spot 33.
The protein in spot 33 from the Dent's gel in Figure 4.1.3 was in-gel digested and the resultant peptides analysed by MALDI-TOF MS. The m/z values in the MALDI-TOF mass spectrum shown in (A) with zoom views in (B) and (C) were used to search the NCBI protein database using the search engine MS-Fit. Result of the search is displayed on the panel bottom paned, which shows that 13 of the 41 submitted peptides matched RBP, and these peptides covered 76% of the protein. A peak at 3140.76 m/z units (C insert) did not match to RBP due to poor mass accuracy. However, this peptide probably is the non-oxidised version of 3156.66 m/z units.



(B)

1. 16/68 matches (23%). 52951.1 Da, pI = 5.34. Acc. # S67527. HOMO SAPIENS. vitamin D-binding protein/group specific component; DBP/GC .		
m/z submitted	**MH⁺ matched**	**Delta ppm**
915.4861	915.4576	31.1242
938.4877	938.4835	4.4886
952.5144	952.5104	4.2299
1115.4306	1115.4138	15.0888
1169.6237	1169.6418	-15.457
1275.5799	1275.568	9.3588
1368.7298	1368.7739	-32.1864
1529.785	1529.7851	-0.0973
1566.7895	1566.8168	-17.4132
1694.8715	1694.9117	-23.7454
2054.8526	2054.8653	-6.1931
2092.789	2092.8262	-17.7655
2264.9961	2264.9375	25.8895
2366.263	2366.2706	-3.232
2518.0235	2518.0172	2.4907
2707.2768	2707.2099	24.7283

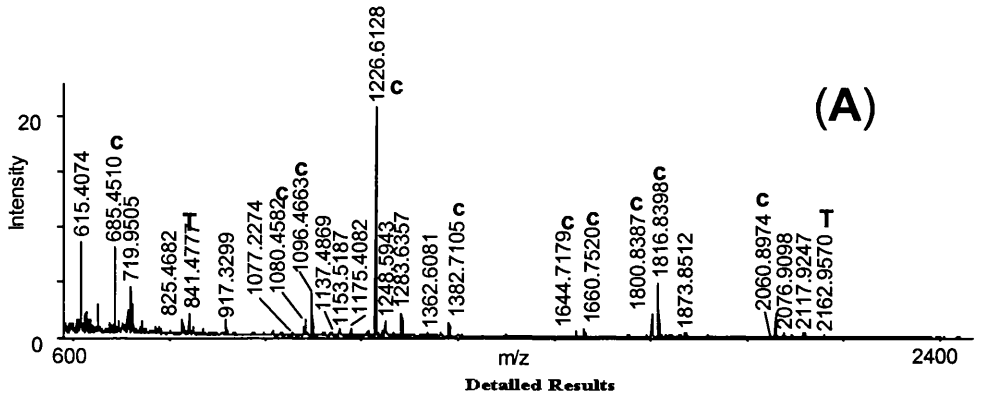
(C)

3. 17/68 matches (25%). 47883.6 Da, pI = 6.29. Acc. # P01042-01. HOMO SAPIENS. SPLICE ISOFORM LMW OF P01042 (kininogen)		
m/z submitted	**MH⁺ matched**	**Delta ppm**
931.5004	931.4889	12.338
986.5465	986.5423	4.2169
1003.5734	1003.5689	4.495
1030.5446	1030.5362	8.159
1060.5946	1060.5692	23.9274
1158.6531	1158.6312	18.9406
1251.5959	1251.5897	4.9189
1383.6585	1383.6619	-2.4302
1555.6549	1555.6434	7.4107
1599.7067	1599.7477	-25.64
1683.7579	1683.7383	11.6202
1874.8561	1874.8785	-11.9734
2107.0304	2107.017	6.3521
2139.0698	2139.0821	-5.7589
2235.0501	2235.112	-27.685
2235.0501	2235.112	-27.685
2441.1331	2441.1043	11.7867
2512.2376	2512.2182	7.7083

Figure 4.1.6. Mass spectrometric analysis of spot 42.
(A) MALDI-TOF mass spectrum of spot 42 from the gel corresponding to normal proteins. The MS-Fit search returned two significant 'hits' corresponding to VDBP (B) (Coverage: 43%, Mowse Score: 7.75e+007) and kininogen (C) (Coverage: 37%, Mowse Score: 2.97e+007). Peaks labelled in green and black were from trypsin or unassigned, respectively. Peaks labelled in red matched kininogen peptides, while those labelled in blue matched VDBP peptides.

For example, mass mapping using data from MALDI-TOF MS of the proteins present in spot 42 (Figure 4.1.3.A; Normal gel) returned two hits with significant MOWSE scores (Figure 4.1.6). The proteins were kininogen and VDBP. In one other instance, that of the analysis of spot 15 (Figure 4.1.3.A; Dent's gel) MALDI-TOF MS and peptide mass fingerprinting identified only one protein in the spot (Figure 4.1.7), whereas LC-ESI-

MS/MS of the same spot returned three hits (Figure 4.1.8 and Table 4.1.3). The observed pI and Mr of Spot 15 are about 9 and 15 kDa, respectively. Similarly, the theoretical pI and Mr of the identified proteins, namely cystatin C, profilin 1, and lysozyme, are close to those values (Table 4.1.3). Thus, it can be concluded from the analysis of this data that identification of one protein from single gel spots does not necessarily mean that there is only one protein in that particular gel region.



Detailed Results

1. 10/26 matches (38%). 15799.4 Da, pI = 9.00. Acc. # P01034. HOMO SAPIENS. (CST3)Cystatin C precursor (Neuroendocrine basic polypeptide) (Gamma-trace) (Post-gamma-globulin)..

(B)

m/z submitted	MH^+ matched	Delta ppm	start	end	Peptide Sequence (Click for Fragment Ions)	Modifications
685.4510	685.4361	21.7615	72	77	(R)ALQVVR(A)	
1080.4582	1080.4533	4.5658	63	71	(K)ASNDMYHSR(A)	
1096.4663	1096.4482	16.5248	63	71	(K)ASNDMYHSR(A)	1Met-ox
1226.6128	1226.6057	5.7655	52	62	(R)ALDFAVGEYNK(A)	
1382.7105	1382.7068	2.6477	51	62	(R)RALDFAVGEYNK(A)	
1644.7179	1644.7903	-44.0242	35	50	(R)LVGGPMDASVEEEGVR(R)	
1660.7520	1660.7852	-20.0057	35	50	(R)LVGGPMDASVEEEGVR(R)	1Met-ox
1800.8387	1800.8914	-29.2753	35	51	(R)LVGGPMDASVEEEGVRR(A)	
1816.8398	1816.8863	-25.6132	35	51	(R)LVGGPMDASVEEEGVRR(A)	1Met-ox
2060.8974	2060.9612	-30.9722	102	118	(K)TQPNLDNCPFHDQPHLK(R)	

Click link below to search for another component.
16 unmatched masses:
Click individual mass to do a non-specific cleavage search.
615.4074 719.9505 825.4682 841.4777 917.3299 1077.2274 1137.4869 1153.5187 1175.4082 1248.5943 1283.6357 1362.6081 1873.8512 2076.9098 2117.9247 2162.9570

The matched peptides cover **41%** (60/146AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 79493

Figure 4.1.7. Analysis of spot 15 by MALDI-TOF.
(A) MALDI-TOF-MS spectrum of the in-gel digested spot 15 from the gel displayed in Figure 4.1.3. Peaks corresponding to cystatin C and trypsin are labelled with 'C' and 'T', respectively. (B) Detailed report from MS-Fit showing the ions that matched cystatin C tryptic peptides. No other match could be obtained with the unassigned ions

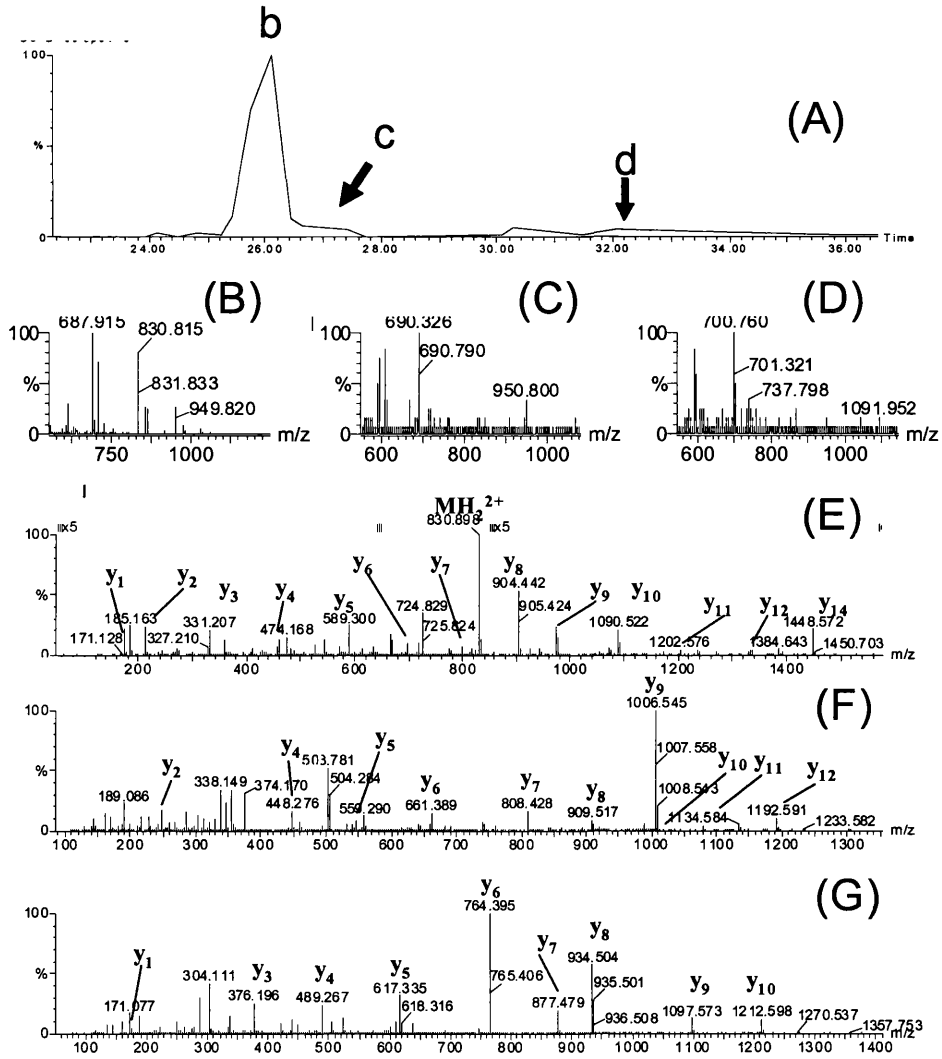


Figure 4.1.8. Analysis of spot 15 by LC-ESI-MS/MS.
Examples of three peptides that generated MS/MS spectra for the identification of three different proteins are given. (A) Extracted ion chromatogram of ions at m/z 830.9, 690.3, and 700.8. (B), (C), and (D) show MS survey spectra at points b, c, and d, respectively, on the ion chromatogram in (A). (E) MS/MS spectrum of ion at m/z 830.82 whose sequence matched cystatin C. (F) MS/MS spectrum of ion at m/z 690.33, that matched profilin 1. (G) MS/MS spectrum of ion at m/z 700.76 that corresponded to a lysozyme peptide. Other details of this analysis are given on Table 4.1.3.

Table 4.1.3. Proteins identified from the analysis spot 15 by LC-ESI-MS/MS

NCBI gi	Protein name	# Peptides /coverage (%)	Theoretical pI/Mr (Da)
4503107	cystatin C	3/19	9.00/16085
4826898	profilin 1	2/21	8.44/15299
4930016	Lysozyme	1/9	9.28/15157

Examination of the 2DE data revealed that certain proteins are found to be at higher levels in the urine of Dent's disease patients when compared with normal urine (Table 4.1.2). Among these, there were several lipoproteins, growth factors, and vitamin/metal binding proteins (Table 4.1.2 and Figures. 4.1.3 and 4.1.4). In particular, the spots corresponding to apolipoprotein AI and apolipoprotein AIV were detected in three spots each and occupied 1.0 ± 0.2 % (2 subjects) and 1.1 ± 0.1 % (2 subjects) of the total spot volume, respectively. For hemopexin, which was detected in 6 spots (probably is present in urine in at least 6 different isoforms), the % volume was 1.8 ± 0.4 (2 subjects,). Pigment epithelium derived factor (PEDF) also produced 6 isoforms in these gels and the %OD was 2.1 ± 0.1 (2 subjects, 12 spots). Finally, the % volume of retinol binding protein (RBP), transthyretin and vitamin D binding protein (VDBP) were 4.2 ± 0.2 (2 subjects, 2 isoforms spots), 8.5 ± 2.3 (2 subjects, 5 isoforms), and 1.1 ± 0.2 (2 subjects, 3 isoforms), respectively. From all proteins detected at higher levels in Dent's urine only three proteins were detected in normal urine; these were VDBP (% volume of 0.5 ± 0.2; 3 subjects), hemopexin (% volume of 0.4 ± 0.2; 3 subjects) and transthyretin (% volume = 0.9 ± 0.1; 3 subjects).

Some other proteins found at higher levels in normal urine than in Dent's urine are listed in Table 4.1.2. Among these, kininogen was observed at considerable levels in the urine of normal individuals (% volume of 2.6 ± 0.6; 3 subjects, 43 spots). However, kininogen was not detected in the urine of Dent's patients by 2DE. Uromodulin also occupied a relatively large percentage of the normal urinary proteome (% volume of 2.6 ± 0.9; 3 subjects, 18 spots) but was not found in Dent's urine. Finally, there were proteins present at similar level in normal urine and the urine of Dent's patients (Table 4.1.2). Most of these proteins are abundant plasma proteins such as albumin, microglobulins, proteins involved in coagulation, and immunoglobulins.

As with the 1D SDS-PAGE analysis, the 2DE data suggested that proteins of kidney origin occupy a large proportion of the normal urinary proteome, whereas plasma proteins dominate the Dent's proteome. Nevertheless, several proteins of plasma origin seem to be present at similar levels in both samples, including albumin and several microglobulins, whereas others, e.g., vitamin binding proteins, are found at higher levels in Dent's urine when the analysis is standardized to protein concentration. The significance of these results will be discussed in detail later. These findings suggested that there is some degree of specificity in the reabsorption of polypeptides from the glomerular filtrate by PT

cells. A detailed discussion of the possible significance of these results will be presented in section 4.5.

4.2. Analysis of Dent's and Normal urinary proteomes by liquid chromatography and mass spectrometry

The experiments described in the first part of this chapter revealed the presence of qualitative differences in the protein composition between Dent's disease patients and normal individuals. These differences could be physiologically relevant or they could have been introduced at the time of analysis or during sample preparation. Although repetition of the experiments adds statistical significance to the results, when the source of error is intrinsic to the method, repetition of the experiments would not detect the presence of artifacts, if present. Therefore, two completely different analytical strategies based on liquid chromatography and MS/MS were employed as an orthogonal means to validate and complement the data obtained by gel electrophoresis. Separation of polypeptides by liquid chromatography has been described by several laboratories as an alternative to 2DE for the separation and analysis of full length (e.g., [109;110;220;231] or digested proteins (e.g.e.g., [113;210]) . After separation, protein characterization is carried out by MS/MS, either on-line by ESI coupled to ion traps [113;210] or Q-Tof instruments [220;232], or off-line using MALDI coupled to TOF/TOF mass spectrometers [232].

Quantitation by LC-MS is more complicated than by 2DE, and although the signal intensity in LC-ESI-MS is related to analyte concentration [144;233-238]], signal suppression due to co-elution of matrix compounds or other analytes could make the accurate quantitative analysis by LC-MS unreliable [239-241]. This is specially the case when small differences in analyte concentration or amounts are present. Thus, addition of internal standards can improve quantitation in LC-MS and is widely employed in pharmacological and environmental analyses (e.g.*e*.g., [242;243]). Recently, isotope labelled internal standards have also been used for the absolute quantitation of proteins [234;244] and peptides by LC-MS. The ideal internal standard is an isopically labelled version of the analyte of interest. When the aim is to compare the amounts of protein present in two samples, one of the samples is labelled with a compound containing a tag with heavy isotopes (e.g., ^{13}C, ^{15}N, or D) while the other sample is labelled with the same compound but containing normal, light, isotopes. Since the chemical and physical characteristics of these compounds are identical (except for Mr), labelled peptides co-elute during RP and SCX LC and have the same behaviour during ionisation in the ion source of the mass spectrometer. This is the basis of the method named isotope coded affinity tags (ICAT), which was introduced by Gygi and Aebersold [165;166]. Recently, other investigators have

reported the development of compounds based on the ICAT principle [167;245]. Alternative strategies have been described that involve metabolic labelling by growing cell cultures in media containing amino acids [168;246] with heavy isotopes or ^{15}N as an nitrogen source [169]. These are elegant approaches, albeit they are restricted to the analysis of proteins extracted from cell cultures and cannot be applied to the analysis of proteins obtained from *in-vivo* sources.

In the experiments described below, proteins were first extracted from urine using a combination of RP and SCX chromatography in order to separate polypeptides from inorganic and organic salts respectively. By doing this, compounds that are relatively hydrophobic and positively charged at acidic pH are extracted, whereas organic and inorganic salts, organic acids, bile acids, and lipids are diverted to waste. Polypeptides are relatively hydrophobic and therefore RP columns retain them, whereas salts are not retained and appear in the solvent front. Proteins and peptides are also zwitterionic species, but at pH 2-3 most, if not all, peptides are positively charged so that they are retained by beads with SCX functionality; this provides a physicochemical basis for the separation of peptides from neutral and acidic compounds. Thus, after extraction, peaks in UV chromatograms are likely to correspond to the absorbance of peptides and proteins and are probably free from most matrix interferences. Proteins and peptides were then analysed by MALDI-TOF MS before and LC-ESI-MS/MS after, digestion of the samples with trypsin. Approximate relative quantitation was obtained by using the intrinsic information in the mass spectra. As stated above, ion intensity in ESI is dependant on analyte concentration, and although the issues of ion suppression are a concern, ion intensities of several peptide ions corresponding to the same protein added confidence to the quantitation. The results of these experiments were later validated by independent experiments using multidimensional chromatography of ICAT labelled peptides. This section describes the results and the conclusions of these two studies.

4.2.1. Microcapillary reversed phase liquid chromatography of urinary proteins

As stated above RP LC is a powerful method for the separation of molecular species on the basis of their hydrophobicity, more hydrophobic compounds eluting later than less hydrophobic (more hydrophilic) ones. Miniaturization of conventional LC has several advantages including an increase in sensitivity. In the experiments described here microcapillary LC (μLC) was used for the separation of polypeptides after extraction by RP and SCX. A μLC RP column packed in house in 320 μm x 25 cm fused silica capillaries

was used. ; the packing material was gygaporous beads (2000 Å pore size) with C18 RP functionality, which allows for the separation of relatively large polypeptides at a relatively high flow rate (and thus short analysis time).

After the extraction step, the protein content was quantified and 5 µg injected in the µLC system in which the separation was monitored by UV absorption at a wavelength of 214 nm. Representative chromatograms are depicted in Figure 4.2.1 showing significant differences in the elution profiles between Dent's and normal urinary proteins. Fractions, collected every 3 minutes (60 µl volume) from these runs, were lyophilised and analysed by MALDI-TOF MS for intact Mr determination. Figure 4.2.2 summarises all the ions with m/z values identified during MALDI-TOF MS analysis. Several ions were detected in one set of samples but not in the other, indicating qualitative differences between the two samples. It should be noted that many ions with low m/z values were detected by MALDI-TOF MS, whereas 2DE analyses did not show many spots at this molecular weight (Figure 4.2.2). In contrast, MALDI-TOF MS failed to detect the large proteins that were readily detectable by 2DE. These results highlight the fact that large proteins do not ionise efficiently by MALDI so that small and medium size polypeptides are detected preferentially. It can be concluded from this experiment that the 2D separation afforded by LC-MALDI-TOF complements the data obtained by 2DE.

In order to investigate the identity of the observed peptide ions, fractions were digested with trypsin. The resultant tryptic peptides were further separated by nano-flow LC and sequenced by ESI-MS/MS. Figure 4.2.3 summarises all gene products identified during these experiments. Although the number of tryptic peptides detected per gene product has been shown to correlate with their abundance in the original sample [247], it is arguable whether this parameter can be taken as an accurate indication of protein concentration. Nevertheless, a higher number of tryptic peptides detected per protein correlates with a higher probability that the identification is correct [157]. For proteins generating only one tryptic peptide, the identity was confirmed by manual interpretation of the MS/MS spectrum.

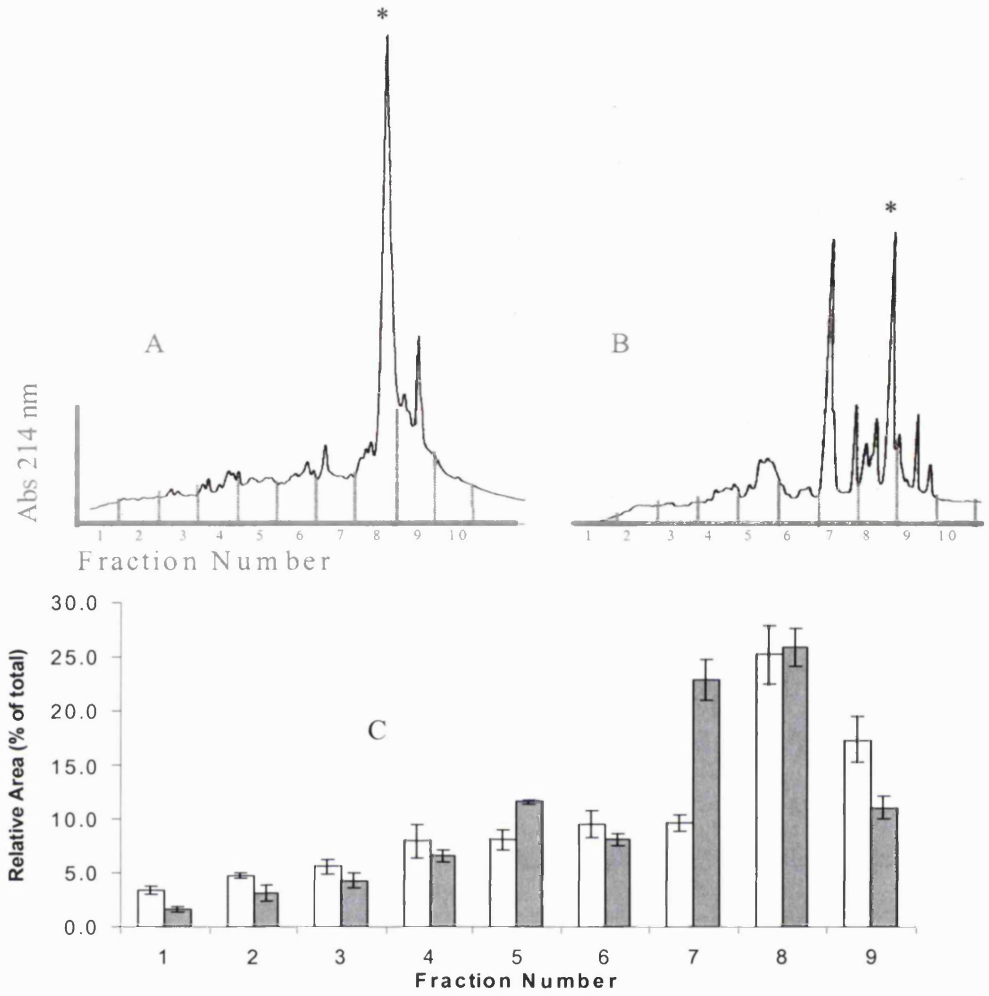


Figure 4.2.1. Chromatographic elution profile of urinary proteins.
Polypeptides (5 μg) purified from urine by RP followed by SCX chromatography were loaded on a capillary LC system consisting of a RP column (dimensions 320 μm x 25 cm) packed in house with POROS™ 10 R2 beads. The flow rate was 20 μl/min, the sample was injected at 5%B and gradient elution was from 15%B to 60%B in 30 minutes after an isocratic step of 8 minutes at 5%B. Solvent A was 0.1% TFA and solvent B was 80% ACN in A. Representative UV chromatograms for normal (A) and Dent's (B) samples are displayed. HPLC runs were carried out in triplicate for 4 patients and 4 healthy subjects and the elution profiles were highly consistent within sample groups. Ten fractions were collected every 3 minutes, lyophilised, and the proteins were either analysed by MALDI-TOF MS for intact Mr determination or they were digested with trypsin. Nano-flow LC-ESI-MS/MS was then used to determine the identity of the gene products in each fraction. Peaks marked with an asterisk have the same retention time as BSA. In (C), the area under each fraction was calculated and plotted normalized to total area (clear bars, normal; grey bars, Dent's). Differences in fractions 1, 2, 5, 7, and 9 are statistically significant (t test, $p > 0.05$, $n = 3$).

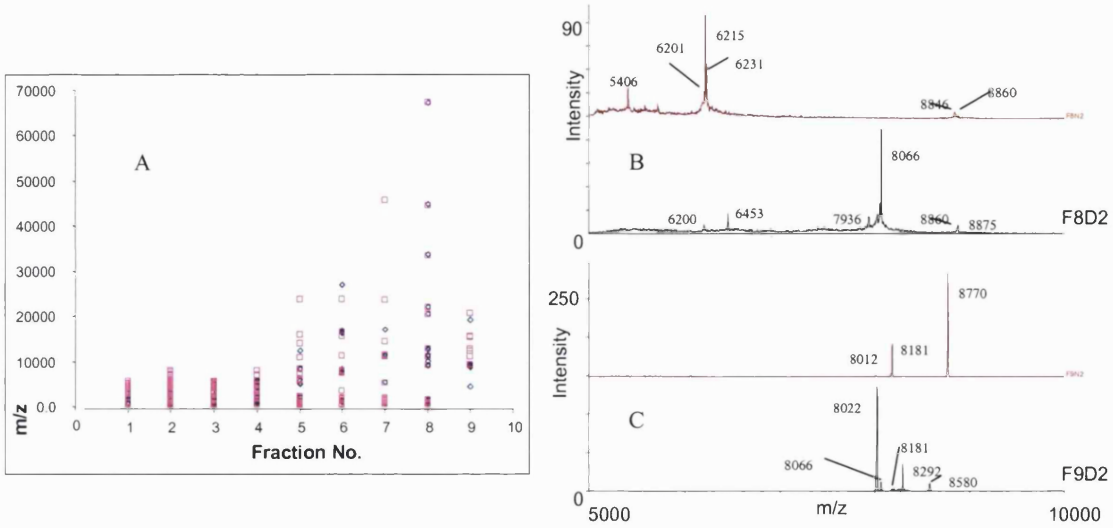


Figure 4.2.2. Plot of the m/z values found in each of the capillary LC fractions. Fractions from the μLC runs were dried and reconstituted in 0.1 % formic acid. An aliquot (1μL) was mixed with DHB and crystalised under a stream of cold air. Monoisotopic values are shown up to 3000 m/z units and average values from 3000 to 70000 m/z units. (Dent's, squares; Normal m/z, diamonds). (A) Plot of the m/z values found in each μLC fraction. (B) Spectra from fraction 8 (normal top, Dent's bottom). (C) Spectra from fraction 9 (normal top, Dent's bottom).

The data obtained by μLC and nano-flow LC-ESI-MS/MS suggests that apolipoprotein (apo) AIV occurs at higher levels in the urine of Dent's patients when compared compared with urine of normal individuals (Figure 4.2.4). Although several peptides corresponding to apo-AIV were detected in Dent's and normal urines, apo-AIV peptides in Dent's urine showed consistently higher ion intensities than the same peptides from Normal urines. Furthermore, when more than one peptide ion had the same m/z value as the apo-AIV peptide, the relative intensity of the apo-AIV peptide with respect to this other ion was greater in Dent's than in Normal (Figure 4.2.4).

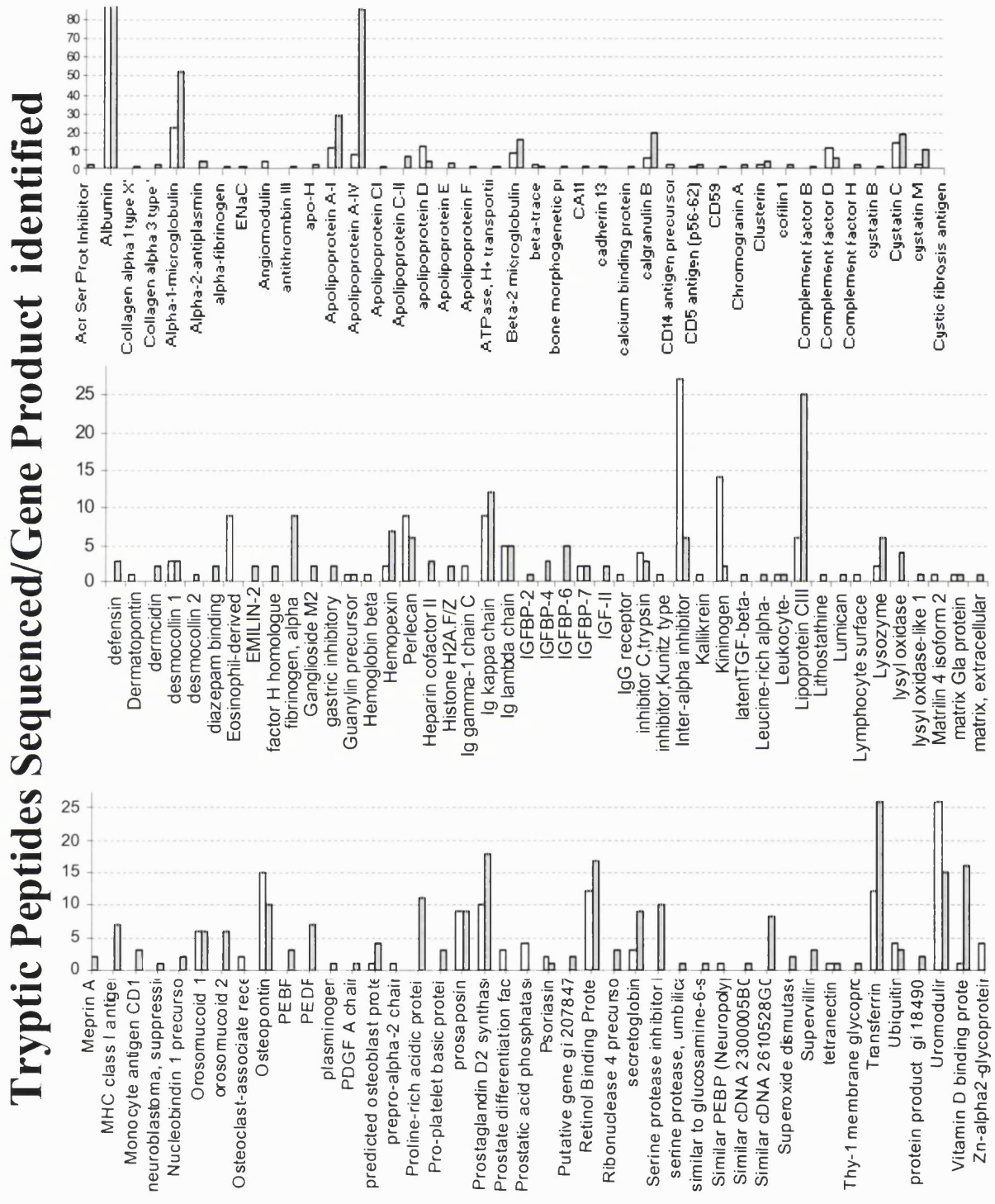


Figure 4.2.3. Polypeptides identified by μLC.
Proteins in fractions collected from the microcapillary LC runs were digested with trypsin and the peptides thus generated were sequenced by LC-MS/MS. Uninterpreted mass spectra were used to interrogate the NCBI protein database using the Mascot search engine. Clear bars, control; grey bars, Dent's.

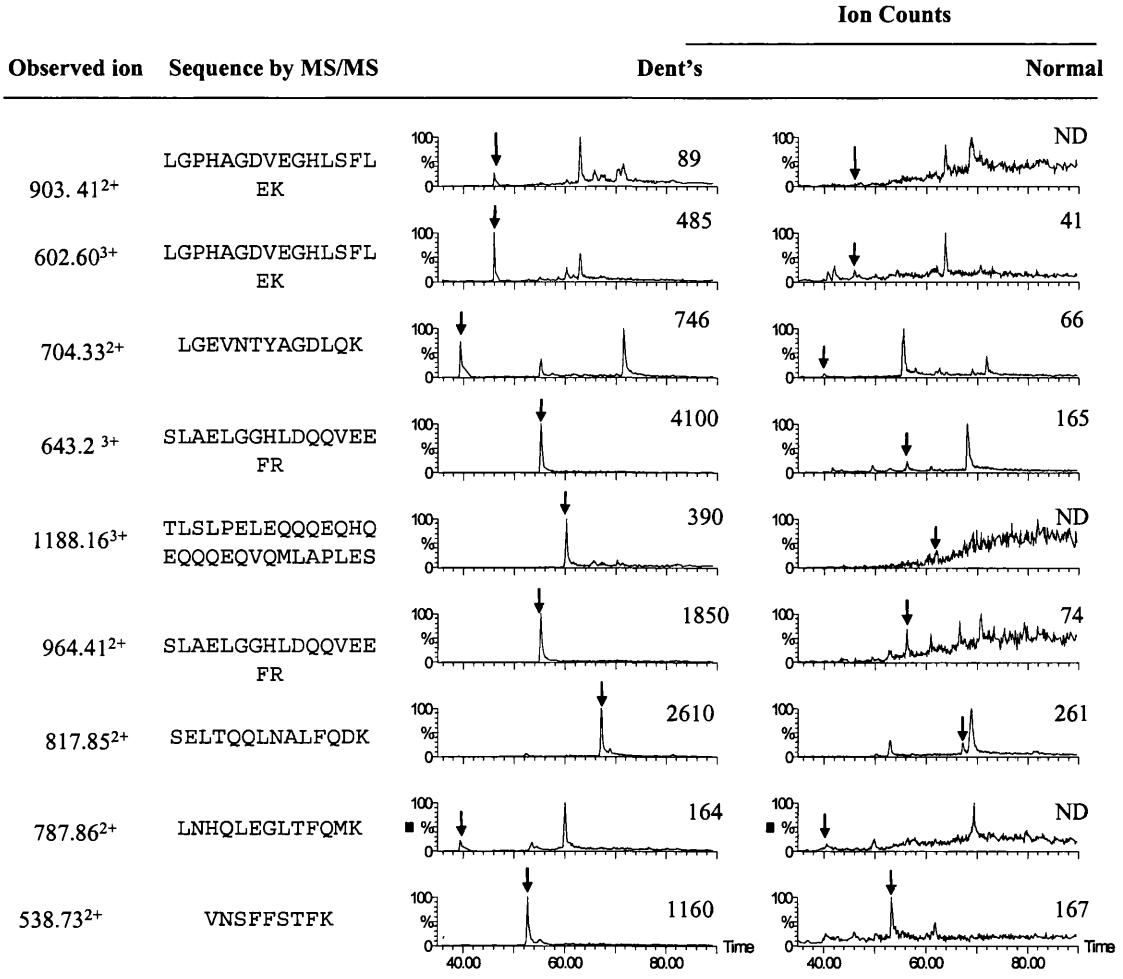


Figure 4.2.4. Chromatographic elution profiles of apo-AIV tryptic peptides.
The figure shows extracted ion chromatograms and ion intensities of some of the ions that produced sequence information matching apo-AIV peptides. The experiments are further described in the text and in Figures 4.2.1 and 4.2.3. Arrows point to the chromatographic retention time of the named peptide ion. In some cases, more than one ion had the same m/z value. For example, extracted ion chromatogram at m/z 817.85 produced two peaks, only one corresponding to a apo-AIV peptide. In this case the apo-AIV peptide ion at 817.85 m/z units produced more intense signal in Dent's than in Normal with respect to this other ion with the same m/z value.

Other lipoproteins were detected in these analyses; with the exception of apo-D, all apolipoproteins seem to be present at higher levels in the urine of Dent's disease patients when compared compared with that of Normal individuals when the analysis is standardised to protein amounts (Figure 4.2.5).

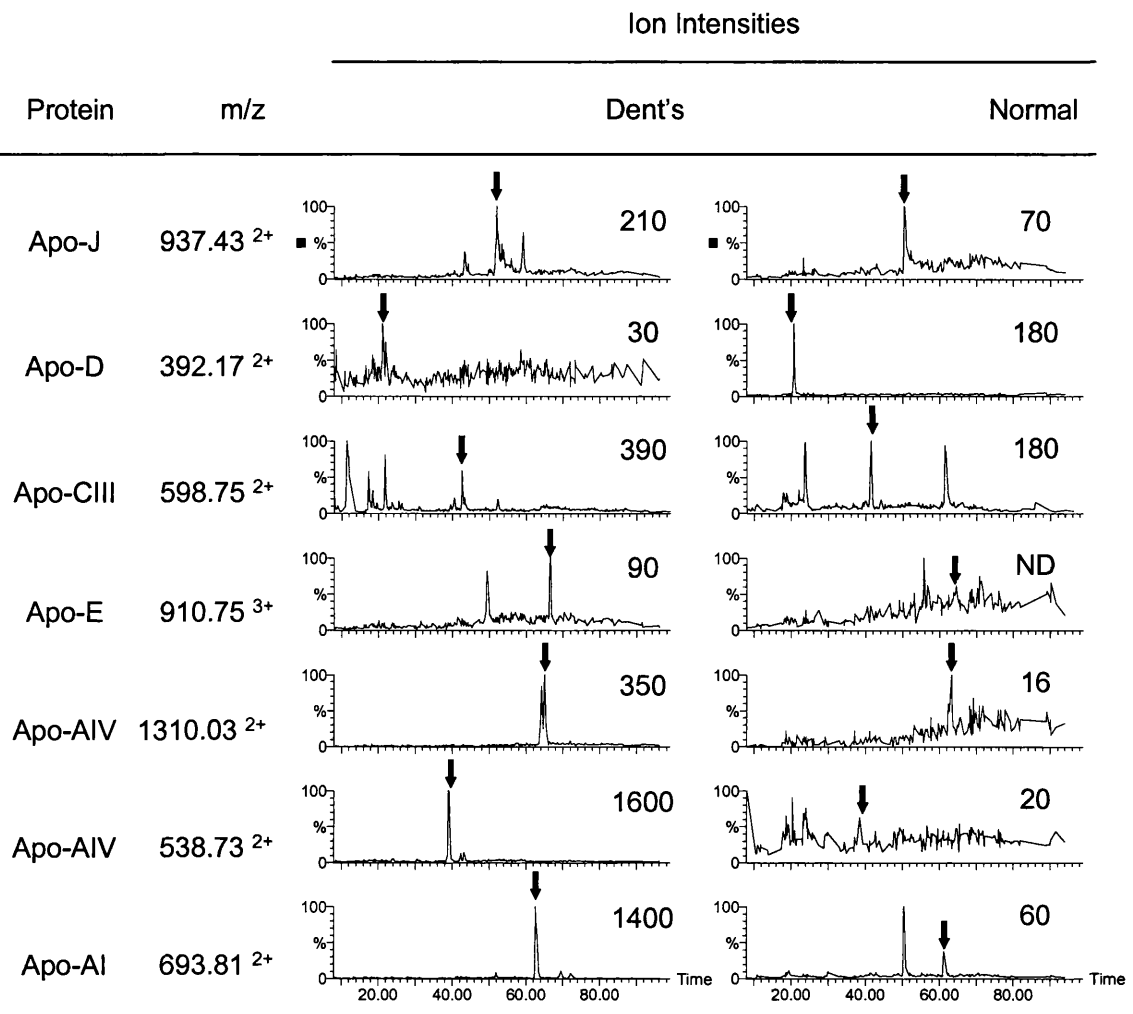


Figure 4.2.5. Chromatographic elution profile of selected apolipoproteins.
The figure shows the extracted ion chromatograms and retention times of selected apolipoproteins together with their ion intensities. Arrows point to the chromatographic retention time of the named peptide ion. These are results obtained from the experiments described in the captions of Figure 4.2.1 and 4.2.3.

Similarly, Figure 4.2.6 shows the extracted ion chromatograms of some of the tryptic peptides that provided sufficient sequence information for the identification of carrier proteins. As in the 2DE analysis, IGFBP-2, hemopexin, β-2-glycoprotein I, VDBP, and RBP seemed to be present at higher levels in the urine of Dent's patients, while tryptic peptides of uromodulin and IGFBP-7 produced stronger signals in normal urine (Figure 4.2.6). These data agree with and thus validate the data obtained by the 2DE analyses.

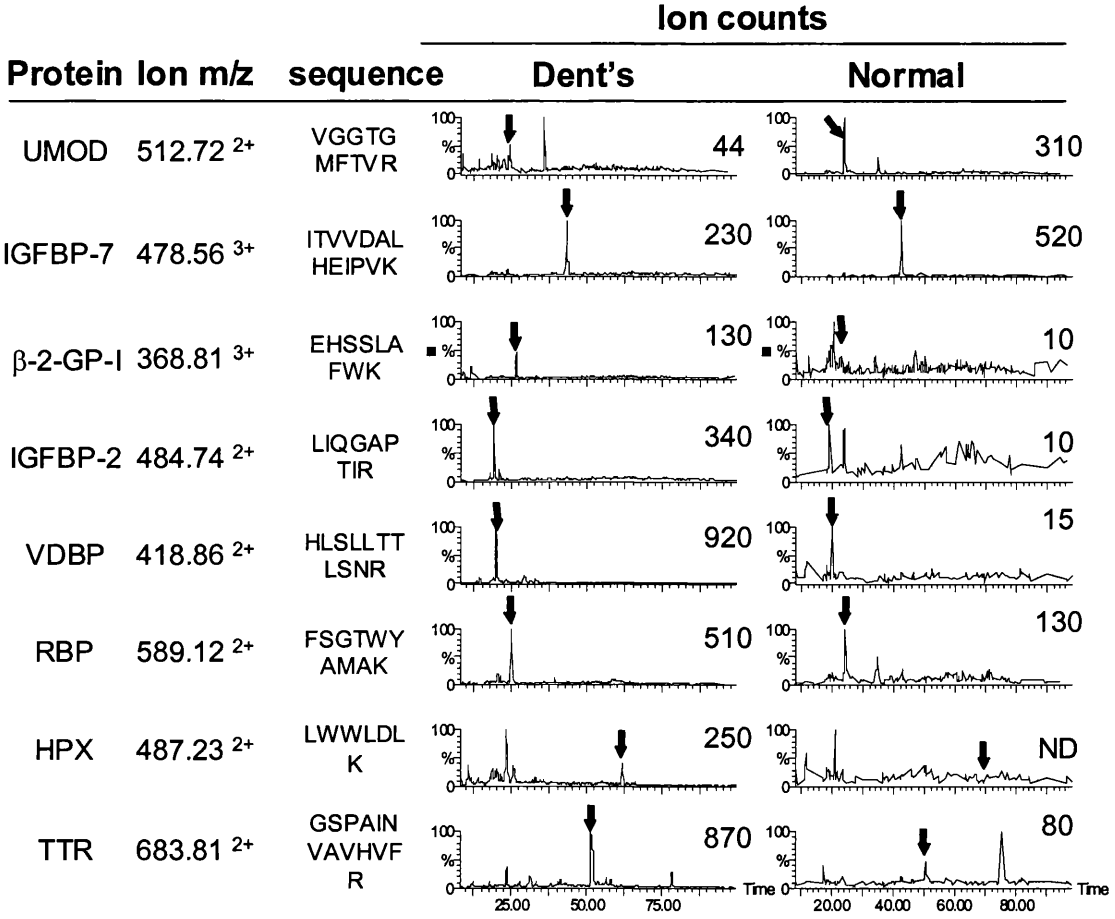


Figure 4.2.6. Chromatographic elution profile of selected carrier proteins.
The extracted ion chromatograms and retention times of tryptic peptides corresponding to the named proteins are shown together with their ion intensities. Arrows point to the chromatographic retention time of the named peptide ion.

Finally, polypeptides with potential signalling function were also detected. The elution profiles of some of their tryptic peptide products are displayed in Figure 4.2.7. In agreement with the 2DE data (see Chapter 4.1) kininogen peptides produced stronger ion intensities in the normal samples, whereas PEDF tryptic peptides were found in Dent's but not in normal specimens. Some other potentially bioactive peptides not seen in the MS analysis of the excised 2DE gel spots were detected in Dent's but not in normal urine by this μLC approach, including pro-platelet basic peptide (PBP), insulin-like growth factor II (IGF-II), diazepam binding inhibitor (DBI), platelet derived growth factor (PDGF), and bone morphogenic protein-1 (BMP-1) (Figure 4.2.7).

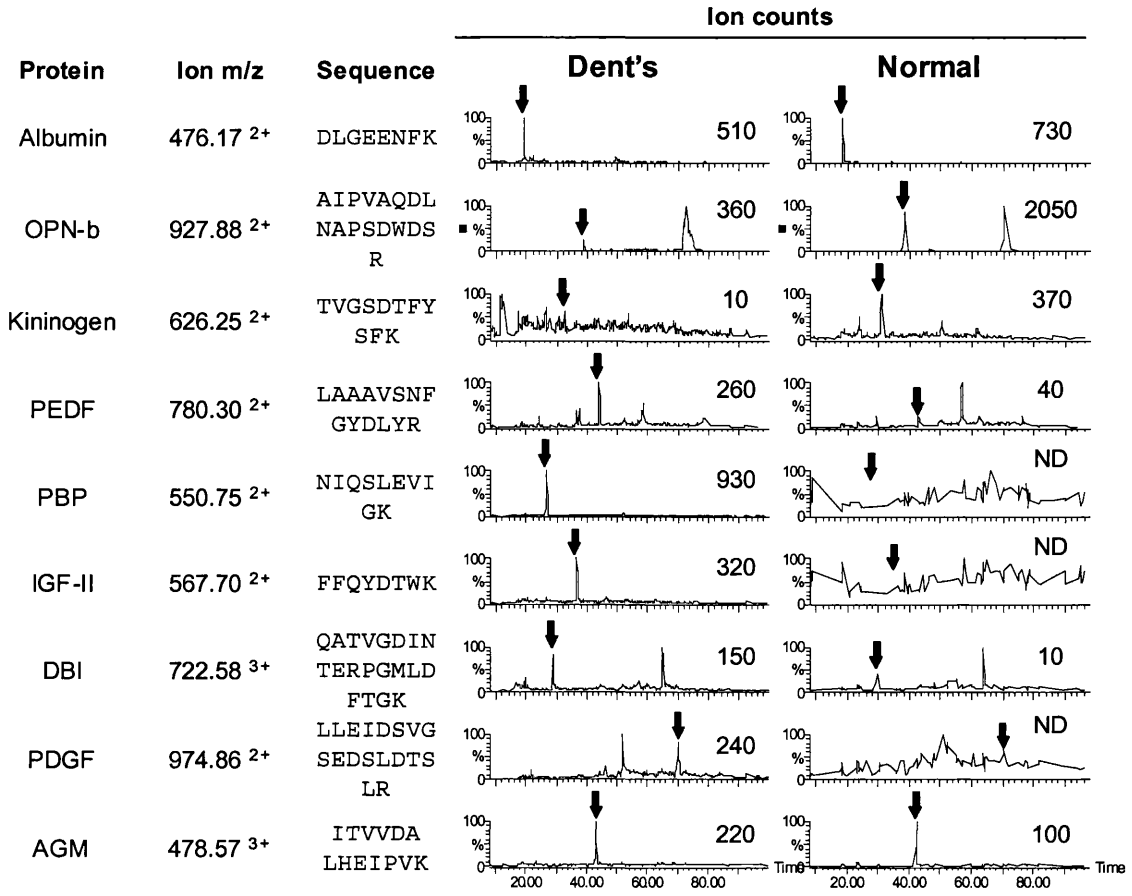


Figure 4.2.7 Chromatographic elution profile of selected bioactive peptides. The extracted ion chromatograms and retention times of tryptic peptides corresponding to the named proteins are shown together with their ion intensities. Arrows point to the chromatographic retention time of the named peptide ion. The elution of an albumin peptide is shown for comparison.

4.2.2. Multidimensional liquid chromatography of proteins labelled with stable isotopes

The number of ions produced from their neutral precursors by ESI has been repeatedly shown to be directly proportional to the original analyte concentration [144;233-238]. However, this may only be the case when the ionization occurs free from interfering compounds, which may suppress the ionisation of the analyte under study. Extensive separation, by e.g. LC, so that individual components of a complex mixture elute at different time points, can increase the ionisation efficiency and narrows the chances of ion suppression due to interferences from matrix or other analytes [239;240]. Nevertheless, the accuracy of quantitative data obtained by LC-ESI-MS is limited and conclusions obtained using this information may only be drawn when large and consistent differences between the case and control samples are observed. In any case, validation with a different method should be used to support the data obtained by LC-ESI-MS and in this thesis a novel

method, termed isotope coded affinity tags (ICAT) [165], was used to further investigate the differences between the urinary proteomes of Dent's disease patients and Normal subjects.

The method of ICAT was first introduced by Gygi *et al.* [165] and the reagents were later commercialised by Applied Biosystems. The first commercially available reagents consisted of three parts; a thiol reactive group (so that these reagents alkylate cysteine residues), a linker, and a biotin moiety. In one of the reagents, deuterium atoms (D) substitute for eight of the protons in the linker, so that the difference in mass between the light and the heavy ICAT reagents is 8 Da. Several problems with these reagents were observed. First, for doubly labelled compounds the difference in mass is 16 Da, which is the same as an oxidised methionine, a common modification in proteins and peptides. Thus, due to this ambiguity a doubly labelled peptide could be confounded with an oxidized peptide and this in turn could also lead to erroneous assignments of ICAT pairs. Second, analogues incorporating deuterium and protium elute at slightly different times during RP-LC such that compounds labelled with D elute with a few seconds difference to those containing normal H isotope. This makes quantitation time consuming and difficult to automate. Finally, the label was found to be too bulky and made the ionisation and fragmentation of labelled peptides inefficient. Therefore, later developmental efforts were directed to overcome these problems and a new version of these reagents was commercialised, which contains nine ^{13}C atoms instead of eight D as the isotope used for relative quantitation, the difference between the two reagents is 9 Da instead of 8 Da, and they contain an acid cleavable group so that the bulky biotin moiety is removed before LC-MS/MS analysis.

These new cleavable ICAT reagents (cICAT) were used to label Dent's and Normal urinary proteins; Dent's proteins were labelled with the heavy reagent, while Normal proteins were labelled with the light reagent. Prior to labeling, proteins were precipitated using 50% acetone and further extracted using SCX LC. Tryptic digestion was carried out after labelling and mixing the two samples, and separation of the peptides thus generated was carried out by SCX LC, using a gradient of ammonium chloride in trace formic acid. Four fractions per urine sample were collected. Avidin affinity chromatography was used to purify labelled peptides in each of the SCX fractions as recommended by the manufacturer. Cleavage of the biotin moiety was also performed using manufacturer's recommendations.

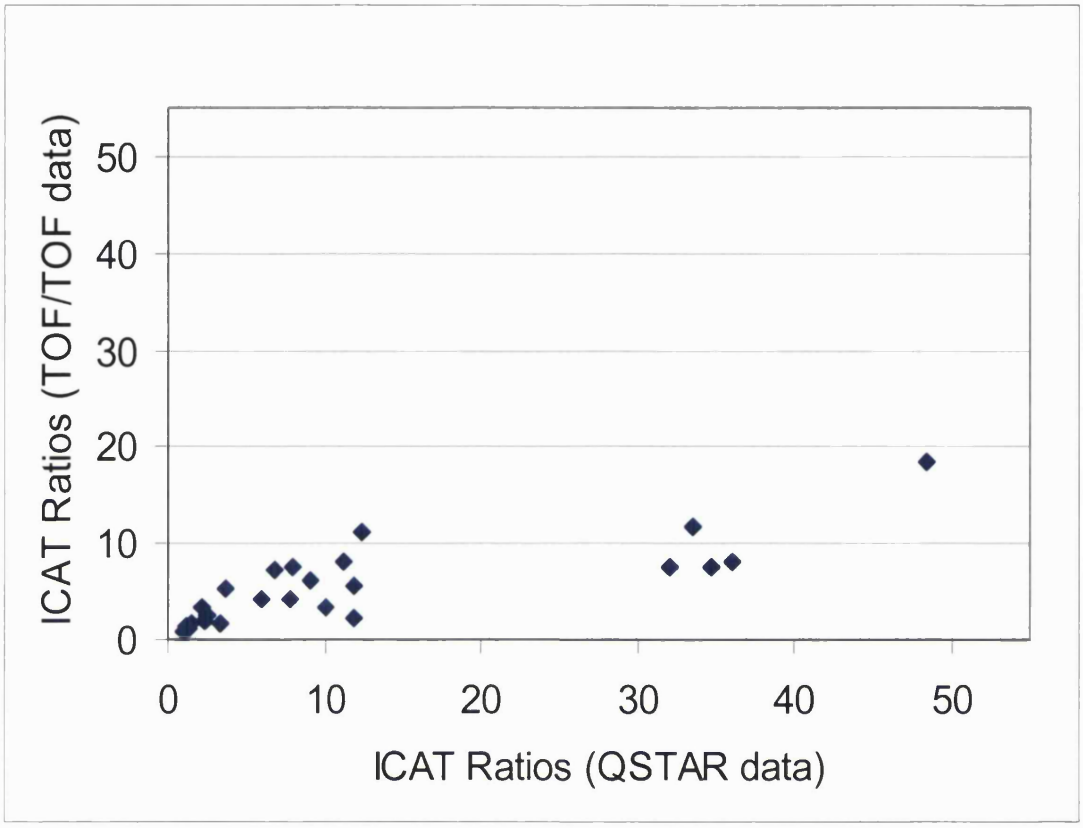


Figure 4.2.8. Comparison of the quantitative data obtained by LC-ESI-Q-TOF and LC-MALDI-TOF/TOF analytical platforms.
ICAT ratios as obtained by LC-ESI-Q-TOF were ploted aginst the same values obtained by LC-MALDI-TOFTOF. The values correlated more when the ratios were small.

Identification and quantitation of the labelled peptides was then carried out by LC-MS/MS. Two different analytical platforms were used; namely, on-line nano-flow LC-ESI-Q-Tof, and off-line nano-flow LC-MALDI-TOF/TOF MS. The quantitative results obtained by these two different analyses are compared in Figure 4.2.8, which shows that results from the two platforms largely agreed when the ratios were small; however, for large ICAT ratios the LC-ESI-MS based method gave larger ratios than the LC-MALDI-MS method. These results may reflect a larger dynamic range of the Q-Tof instrument (QSTAR) when compared with the TOF/TOF one.

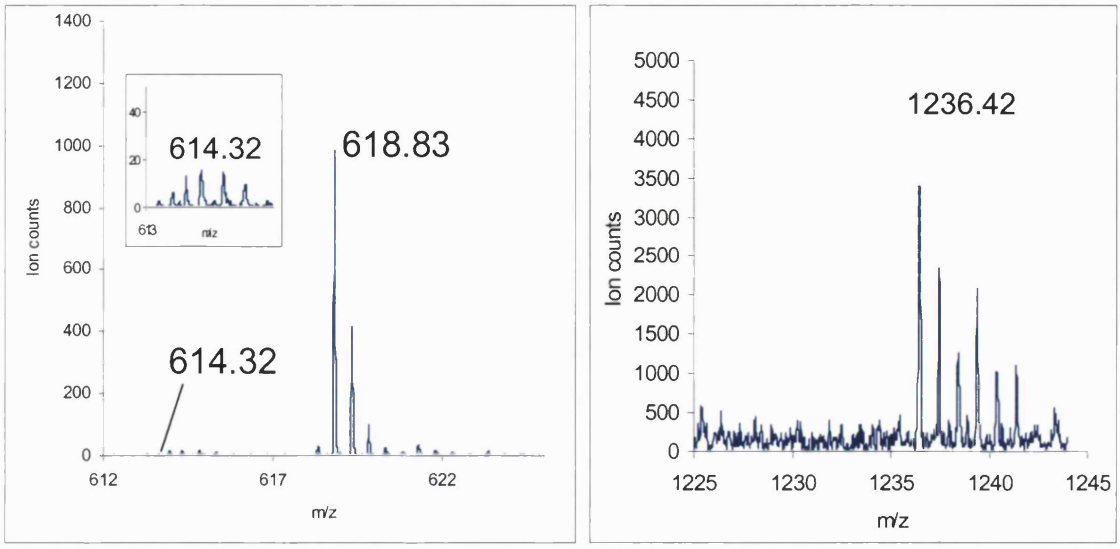


Figure 4.2.9. Relative quantitation of an angiogenin tryptic peptide demonstrating the dynamic range of LC-MALDI-TOF/TOF and LC-ESI-Q-TOF. Angiogenin appeared as a singlet by MALDI analysis (right panel) and only the peptide labelled with the heavy ICAT reagent could be identified. In contrast, the ESI analysis (left panel) detected both the light and the heavy labelled peptides; the ratio heavy:light for this ion pair was 39.7:1.

Another example to illustrate the larger dynamic range of the LC-ESI method is given in Figure 4.2.9. Angiogenin was found to be 40 times more abundant in Dent's than in Normal urine by LC-ESI-MS when the analysis was standardised to protein concentration. However, angiogenin was detected as a singlet by LC-MALDI-MS so that quantitation was not possible with this method in this occasion. This greater dynamic range of the ESI method may be due to higher noise levels and signal saturation in the TOF/TOF mass spectrometer, and by adjusting the laser intensity for each spot to prevent saturation the dynamic range of the TOF/TOF would have improved. Being an online technique, the recording of ions is continuous in LC-ESI-MS such that spectra are collected with virtually no time lapse with respect to their elution from the LC column. Therefore, the detector is less likely to be saturated than in off-line LC-MALDI-MS in where fractions from the LC run are collected every 30 seconds so that all the components eluting in this time frame are analysed in a single MS experiment. Nevertheless, some components were identified by LC-MALDI-MS only, and therefore, both methods provided complementary information.

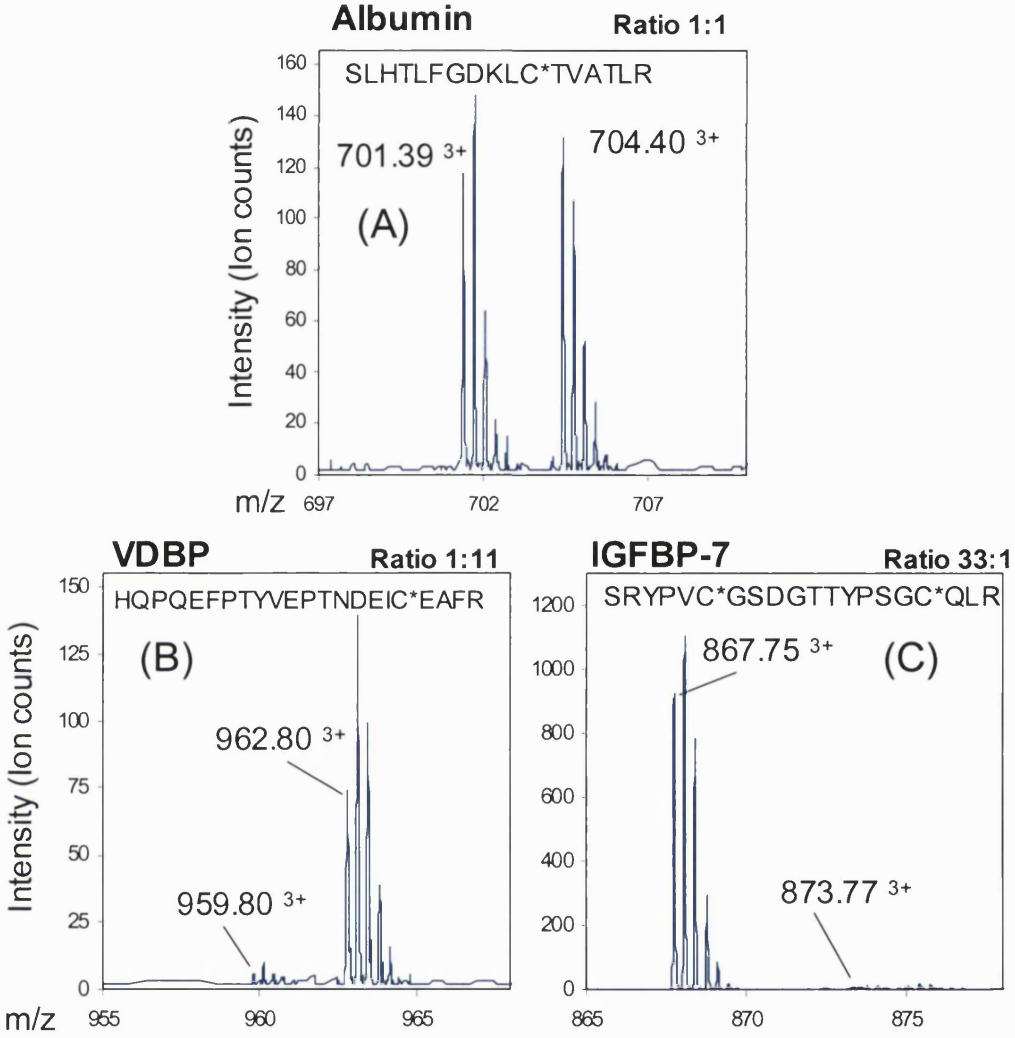


Figure 4.2.10. Representative ion pairs used for the relative quantitation of urinary proteins by multidimensional LC-ESI-MS of ICAT labeled peptides.
Dent's urinary proteins were labeled with the heavy ICAT reagent, whereas normal urinary proteins were labeled with the light reagent. Fifty μg of each labelled sample were then mixed, digested with trypsin and the resultant peptides analyzed by 2D-LC-ESI-MS/MS. The ratio of the heavy (Dent's; right peaks):light (Normal; left peaks) pairs were calculated by dividing the ion intensities of the first isotopes. Three examples of peptide ion pairs are shown (A, B and C). The difference between the heavy and the light ICAT reagents is 9 Da. Since the peptides shown ionized to produce triply charged ions, the difference between the ion pairs is 3 m/z units for albumin and vitamin D binding protein (VDBP) (these peptides contain only one cysteine residue) and 6 m/z units for insulin-like growth factor binding protein -7 (IGFBP-7) (this peptide contains two cysteine residues). The Figure shows that albumin was present at similar levels in both samples (A); conversely, VDBP was 11 fold more abundant in Dent's urine (B), whereas IGFBP-7 was 33 times more abundant in Normal urine (C).

Table 4.2.1 shows the quantitative results obtained with this method and Figure 4.2.10 illustrates representative ICAT ion pairs obtained by LC-ESI-MS. These data confirmed the results obtained by the 2DE and μLC analyses and showed that several

proteins of plasma origin were present at similar levels in Dent's and normal urine when the analysis was standardized to total protein content (Table 4.2.1). In contrast, and in agreement with the 2DE and μLC data, carrier proteins such as HPX, RBP, VDBP, β-2-GP-I, and several IGFBPs were present at higher levels in Dent's urine samples. Several cytokines were also present at higher levels in Dent's samples, again in agreement with the results obtained by μLC. However, results disagreed for transferrin, TTR, OPN, and PEDF. TTR and OPN do not have a cysteine residue in their coding sequence and therefore they cannot be labelled by ICAT reagents, while PEDF only has one cysteine-containing tryptic peptide; however, this peptide was not detected among the complex peptide mixture generated upon trypsin digestion of the whole sample. Transferrin appeared to be present at higher levels in Dent's urine by ICAT, which also disagreed with the 2DE data. This probably reflects a saturation of signal in the silver-stained gels. On the other hand, the detection of uromodulin, kininogen, and EGF precursor, and others, present at higher levels in normal urine by ICAT agreed with the gel electrophoresis and/or μLC data (Table 4.2.1 and Figure 4.2.10).

Table 4.2.1. ICAT ratios.
The ratios of Dent's over Normal urinary proteins as determined by the ICAT strategy are shown as average ± standard deviation of 4 Dent's samples over a pool of 5 normal urine samples.

Protein	Ratio D/N
Proteins found at higher levels in Normal	
latent TGF-β BP	> 0.02
CD59	0.021 ± 0.01
uromodulin	0.028 ± 0.007
IGFBP7	0.027 ± 0.018
poly-Ig receptor	0.031 ± 0.008
EGF (urogastrone)	0.039 ± 0.01
TNF receptor superfamily	0.080 ± 0.047
kininogen	0.084 ± 0.008
CD27 (TNF receptor)	0.086 ± 0.009
perlecan	0.099 ± 0.05
lithostathine	0.13 ± 0.036
urokinase	0.14 ± 0.005
urinary protein 1	0.24 ± 0.1
TNF	0.28 ± 0.1
Proteins found at similar levels	
Ig γ chain	0.46 ± 0.2
Ig κ	0.60 ± 0.11
cystatin M	0.93 ± 0.4
albumin	1.0 ± 0.3
pancreatic ribonuclease	1.0 ± 0.9
α-2-HS-glycoprotein	1.3 ± 0.3
α-1-microglobulin	1.4 ± 0.4

orosomucoid	1.6 ± 1.2
cystatin C	1.7 ± 1.0
zinc-α-2-glycoprotein	1.9 ± 0.7
Proteins found at higher levels in Dent's	
Carriers	
IGFBP2	2.1 ± 0.8
transferrin	5.9 ± 1.3
RBP	7.9 ± 2.7
hemopexin	9.1 ± 4.6
VDBP	11 ± 2.7
β-2-glycoprotein I	13 ± 8.2
IGFBP5	16 ± 1.3
IGFBP-4	25 ± 12
IGFBP6	38 ± 21
Complement components	
complement factor B	2.5 ± 0.88
complement factor C2	9.1 ± 0.7
complement factor H	24 ± 0.33
complement factor D	34 ± 10
Bioactive peptides	
IGF-II	7.4 ± 2.2
fibronectin FN70	9.2 ± 1.1
chemokine 14	11 ± 5.2
angiogenin	34 ± 11
neutrophil activating peptide (PBP)	>40

4.3. Comparison of the results presented in this chapter with published studies on the proteome of urine

Urine is the most readily available biological fluid and therefore its analysis provides the least intrusive method for the diagnosis of specific conditions. As a result, considerable interest has been placed in the characterization of the urinary proteome with the emphasis in its comparison with the proteome in different disease states [161]. Examples of applications in which urinary proteomics have been used to identify markers of diseases include several types of cancer [248-251], rheumatoid arthritis [252], glomerular disease [190;229;253], unspecific inflammatory abscess [247] and cadmium nephrotoxicity [161]. Recently, analysis of proteins in urine has also been used to study kidney function after perturbation of a defined physiological parameter [163].

When comparing the protein patterns of urine as determined by 2DE it is found that these patterns vary between studies. Figure 4.3.1 shows the gel images from five studies in where normal proteins were separated by 2DE. The gel image from the Danish Centre for Human Genome Research (Figure 4.3.1.A) shows the urinary patterns after separation by IEF in the first dimension and SDS PAGE in the second dimension. No details on the staining or extraction procedure are given in their database (at proteomics.cancer.dk). The intensity of the spot corresponding to uromodulin is weaker in this image than in the image obtained from a representative gel obtained during this study (Figure 4.3.1.F), whereas orosomucoid displays a strong intensity in gel A but not in gel F. Figure 4.3.1.B displays the gel patterns as determined by IPG on the first dimension and SDS PAGE on the second after silver nitrate staining and extraction by dye precipitation [161]. This pattern is similar to the one in F with the exception that orosomucoid (also known as α-1-acid glycoportein) was not detected in F, probably due to its high acidity.

The gel image in Pang et *et* al. [247] in Figure 4.3.1.C was obtained after SPE extraction using RP disks and Sypro Ruby staining [247]. The lack of albumin and transferrin is quite surprising in this gel since these are the two of the most abundant proteins in urine. This may be due to artefacts introduced during the staining procedure or to degradation or low recovery of proteins during SPE extraction. In a parallel investigation the authors detected these two proteins by LC-MS/MS after proteolytic digestion of the whole sample [247].

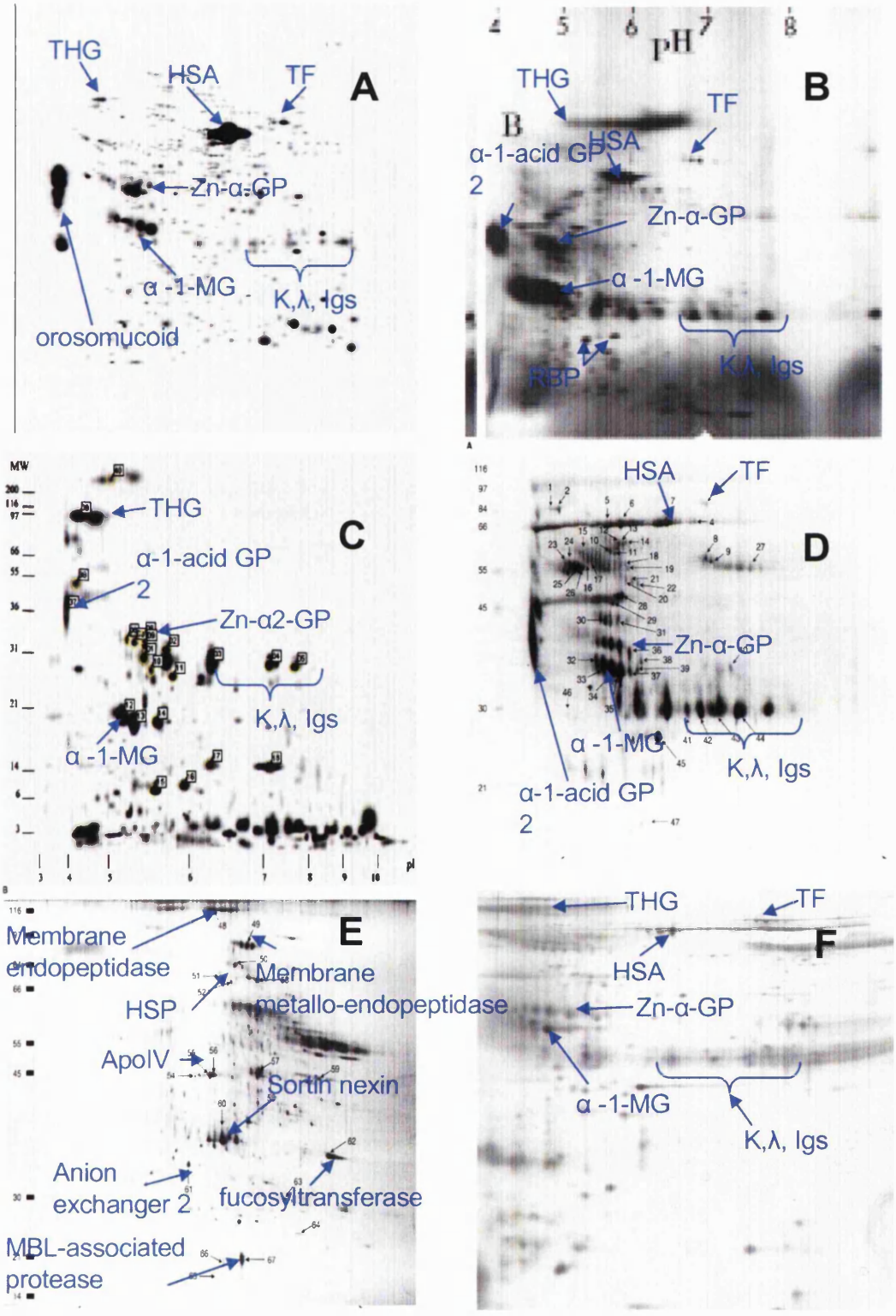


Figure 4.3.1. Comparison of published studies on urinary proteomes by 2DE. Patterns of human urinary proteomes from normal individuals as previously published are shown (A-E) and compared compared with one of the gels obtained from this work (F). Gel images are reproduced from: A, the Danish Centre for Human Genome Research (at proteomics.cancer.dk); B, Marshall *et al.* [161]; C, Pang el al [247]; D and E, Thongboonkerd et *et* al. [189]; F, this study (normal subjects). The identities of the protein present in some of the major spots are shown.

Thongboonkerd *et al.* reported two methods for the extraction of proteins from urine, namely precipitation using organic solvent, and ultracentrifugation [189]. The protein patterns obtained by these two procedures were strikingly different (see Figure 4.3.1.D and E). Precipitation gave a pattern that is consistent with the patterns obtained in this study and that of Marshall *et al.*, with the exception that uromodulin, the most abundant protein in urine, was not detected by Thongboonkerd *et al.* In contrast, ultracentrifugation enriched for membrane proteins [189]. It has been suggested that urine contains a low density 'membrane fraction' that arises from exocytosis of proteins in defined vesicles [254].

In view of this comparison, it can be argued that variation exists in the 2DE patterns of urinary proteins as reported by different groups, probably due to differences in sample preparation and/or staining method.

Relative quantitation of proteins as determined by 2DE is somewhat misleading since uromodulin, the most abundant protein in urine [223;226], produced low spot intensity with respect to other protein spots in most of the studies reported in the literature. The low representation of hydrophobic proteins in 2D gels has been documented [98;100;101;255-258] so that alternatives for the analysis of hydrophobic proteins based on 1D SDS-PAGE (e.g., [259]) or LC-MS (e.g. [260]) have been described. Furthermore, uromodulin has a quaternary structure consisting of four subunits and it also forms complexes with other proteins [280]. Perhaps because of its tendency to form aggregates, uromodulin is prone to precipitation, and is found in kidney stones [261]. This is exploited in its purification [262] and may be a cause of its low representation in 2D gels. In contrast to the 2DE data, the 1D SDS-PAGE data presented in this thesis (see Figure 4.1.1) agreed with the assertion that uromodulin is the most abundant protein in normal urine, and therefore results obtained with by the latter method may be more indicative of the true nature of the sample under study. However, the low resolution of 1D SDS-PAGE makes it unsuitable for most proteomic applications dealing with complex protein mixtures.

Marshall *et al.* investigated the protein patterns in the urine of subjects occupationally exposed to cadmium [161]. Since cadmium exposure causes the manifestations of renal Fanconi syndrome [73] it would be expected to find a correlation between their data and the data presented here. The cited study indicated that several low

molecular weight proteins such as RBP, apo-AI, β2-microglobulin, α-1 microglobulin, and Igs are present at higher levels in case versus control subjects [161]. These findings agreed with the data obtained here for all the proteins except for α-1 microglobulin and the Igs, for which no significant differences were observed in this study. The study was based on 2DE and gels were stained with silver nitrate. The low dynamic range of silver nitrate signal intensity as a function of protein amount has been documented [102;263] and the results presented by Marshall *et al.* [161] were not followed by other analytical methods. Therefore, the discrepancy in α-1 microglobulin and Igs amounts between their data and this data could be explained by saturation of signal in the silver stained 2D gels.

The implementation of LC-MS/MS methods for the characterization of urinary proteins has also been reported [112;247]. In the studies cited, Pang *et al.* [247] identified 36 proteins by 1D-LC-MS/MS and 90 by 2D-LC-MS/MS in the urine of a subject before and after suffering from an inflammatory condition, whilst Spahr *et al.* [112] identified 124 protein in commercial lyophilised urinary proteins by 1D-LC-MS/MS. The data presented by these authors is consistent with the data presented here and most of the reported proteins in these papers were also found during the course of this work. It should be noted, however, that certain bioactive peptides present in renal Fanconi patients were not detected in these two studies, including IGF-I, IGF-II, BMP-1, PDGF, and PBP, which reinforces the data presented in this thesis, and may indicate that the presence of these peptides in urine is specific for renal Fanconi syndrome patients.

More targeted proteomic studies, based on immunochemistry, aimed at the identification of proteins and peptides in the urine of renal Fanconi patients have also been described. Norden *et al.* reported quantitative analyses of several proteins in the urines from the same subjects used during this work [151;264]. Nevertheless, direct comparison of their data with this data is difficult because these authors standardised their analyses to creatinine concentration, whereas in the studies reported here the standardisation were to protein concentration. Thus, in order to compare these two datasets the concentration values for each of the proteins reported in Norden *et al.* was divided by the concentration of albumin. For this comparison, albumin was chosen to normalise all the other proteins because albumin has the same concentration ratio with respect to other protein in Dent's and in Normal urine (see Figure 4.2.10).

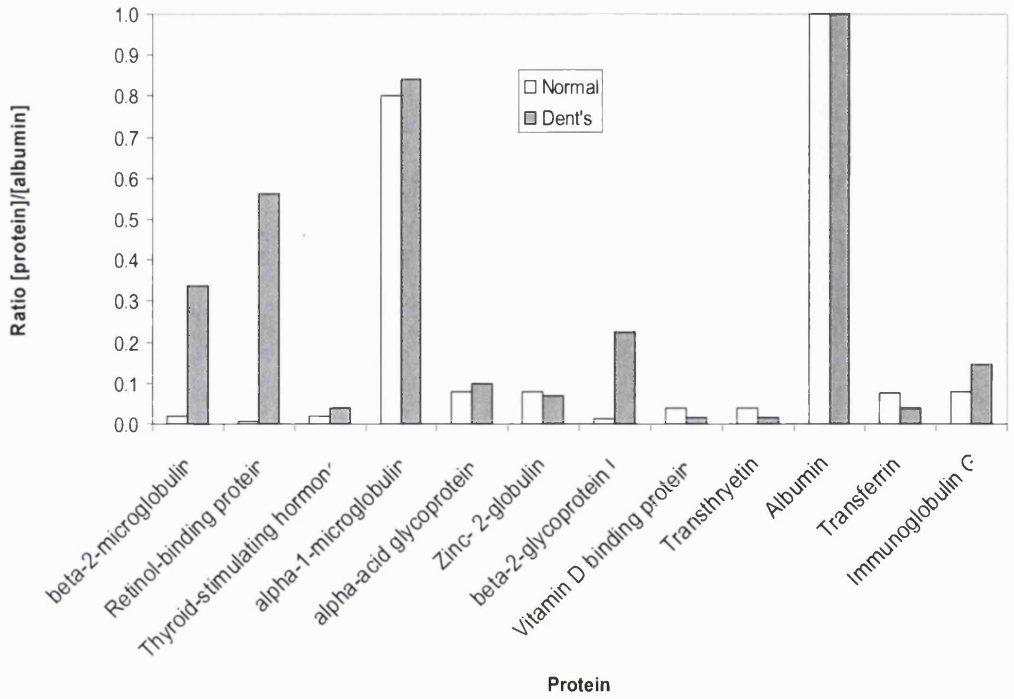


Figure 4.3.2. Ratio of urinary proteins to albumin as determined by immunochemistry.
Concentration values reported in Norden *et al.* [151] were divided by the concentration of albumin (also reported in the cited paper). The figure shows that the ratios of RBP, beta-2-microglobulin and beta-2-glycoprotein I normalised to albumin concentration are higher in Dent's than in Normal.

Results of this reinterpretation of the data in Norden *et* al. are displayed in Figure 4.3.2, which shows that the ratios of RBP, β-2-GP I, and β-2 microglobulin to albumin are higher in Dent's than in Normal. Conversely, the ratios of other proteins to albumin are approximately the same in Dent's and Normal. Therefore, the results agree with the data presented in this thesis for RBP, β-2-GP I, and β-2 microglobulin, which were found at higher levels with respect to total protein amounts in these studies, and also for α-1 microglobulin, IgG, α-acid glycoprotein, and Zn-2-glycoprotein, which were found at similar levels with respect to total protein in this study and also at similar levels with respect to albumin by Norden *et al.* The results disagreed for VDBP, transthyretin, and transferrin, which were found at higher levels in the urine of Dent's patients with respect to total protein in the studies presented in this thesis, but at similar levels with respect to albumin in Norden *et al.* The concentration values given by Norden *et al.* for proteins in control urine are only approximate (the value is reported as < 0.1 mg/mmol creatinine) [151]; thus, the actual concentrations could be much smaller than the reported values. Therefore, more accurate measurements of the concentration of these proteins in control

and Dent's urines would be needed in order to make a more appropriate comparison of the data obtained by immunoassays with the data presented in this thesis.

4.4. Lessons learned from the direct comparison of three analytical approaches to urinary proteomics

The results presented in Sections 1 and 2 of this chapter indicated that there are several differences in the qualitative composition of Dent's disease urinary proteome when compared compared with that of normal subjects. These differences were found by independent analytical approaches aimed at the identification and relative quantitation of proteins. Although the results obtained by these different approaches gave consistent results for most proteins, there also were some inconsistencies, the possible source of which is discussed below.

2DE is the most frequently used separation method to compare the polypeptide patterns of related samples because of its resolution power, which allows for the display of several hundreds to thousands of protein spots. However, there are several problems associated with this technique [265]. For example, 2DE is not suitable for the separation of very hydrophobic, large, small, and very basic or acidic proteins;, and low abundant proteins are masked by high abundant ones in most 2D gels. In addition, not all proteins can be visualised by silver or other staining methods [266]. For example, it has been shown that certain acidic proteins, such as OPN, are poorly stained by silver nitrate [266]. This may explain why this protein was readily detected by the μLC method used this study but was not identified by 2DE. An additional caveat of 2DE based proteomic studies is that, in some occasions, gel spots contain more than one protein, making quantitation unreliable. Results presented in section 1 of this chapter showed that spots from 2D gels sometimes consist of a mixture of proteins, and often MALDI-TOF MS and peptide mass mapping analysis does not allow, by itself, for the identification of several proteins in a mixture.

Nevertheless, 2DE is the most widely used separation method in proteomics because of its robustness, its advanced state of development, and because it provides a visual platform for the qualitative and quantitative comparison of related proteomes [156;267]. Furthermore, the data obtained by 2DE is easy to visualize and interpret and it is the only method employed in this laboratory that allows for the identification of proteins isoforms based on pI heterogeneity. Indeed, in the studies that are the subject of this thesis 176 proteins were identified by 2DE, which corresponded to 50 different genes. This means that, on average, each gene was identified as 3.5 protein isoforms. These isoforms may arise by post-translational modifications such as proteolysis or covalent modifications

with phosphates, lipids, or sugars. The nature and extent of these modifications were not investigated here.

Liquid chromatography has also been used prior to mass spectrometry for the separation of proteins [109;110;231;268] or their proteolytic products [112;113;165;166;210;247;269]. The advantage of this approach is that it is more sensitive and less time consuming, as well as less biased, than 2DE such that results from these strategies only partially overlap with those obtained by 2DE analysis. In the experiments described here, over 120 proteins were detected by the μLC approach, whereas only 50 were detected by the 2DE method. This is particularly significant when we consider the fact that 10 times more protein was used for the 2DE analysis than for the μLC runs. However, some large proteins may not elute in a defined peak or they may precipitate during the chromatographic run so that they may not be detectable by this method. Moreover, quantitation by non-gel based proteomic methods, albeit feasible, is more challenging than by 2DE because isotopic labeling is needed for accurate quantitation.

One of these isotope labelling strategies, namely multidimensional LC of ICAT labelled proteins [165], was employed in the experiments described in this thesis. The data obtained with this method validated the data obtained with the other two methods in most of the cases. An important discrepancy between the data obtained by ICAT and that obtained by 2DE was observed for transferrin, for which the ICAT strategy showed that this protein is about 5 times more abundant in Dent's urine when the analysis is standardized to protein concentration, whereas 2DE analysis did not show differences for this protein. One of the problems associated with silver nitrate staining of gel separated proteins is that the staining intensity saturates at high protein concentration and therefore this staining procedure is only suitable for the detection of qualitative differences between two samples. For more quantitative work, other staining procedures, such as those based on fluorescence emission, may give larger dynamic range. Another problem associated with the ICAT strategy is that not all proteins contain cysteine residues in their coding sequence. For example, TTR, PDGF, and several apolipoproteins appeared to be present at higher levels in Dent's when compared with the same amounts of normal urinary protein by μLC and/or 2DE. However, these proteins could not be quantified by the ICAT strategy because they do not contain cysteine residues.

Polypeptides are heterogeneous from a physicochemical point of view; properties of proteins and peptides, such as hydrophobicity, pI, and charge, range enormously and

some of them are very difficult to solubilise in aqueous solvents (e.g., membrane proteins), whereas others are readily soluble in water. Conversely, hydrophobic proteins are better solubilised in organic solvents, whereas hydrophilic ones precipitate in these solvents. These different physical and chemical properties reflect the fact that polypeptides perform very different physiological functions in the cell and extracellularly, protein functions ranging from structural to catalytic. This makes certain proteins very difficult to purify and there do not exist 'off-the-self' methods for the isolation of proteins, as for e.g. DNA, because purification methods normally exploit some kind of physical property of the molecule of interest. Consequently, due to the same physicochemical heterogeneity of proteins that enables them to carry out diverse biological functions, no single separation technique may be completely adequate for comprehensively analysing all the proteins in a sample. Thus, an important lesson learned during the course of the studies described in this thesis is that proteomic studies may benefit by adopting several separation methods before mass spectrometric analysis if a comprehensive picture of the proteome under study is to be obtained.

4.5. Rationalization of the differences observed between the proteomes of Dent's and Normal urinary proteomes

The aim of this chapter section is to summarise and interpret the data presented in the previous sections of this chapter. Table 4.5.1 shows a selection of the proteins identified during the course of the experiments described in Sections 1 and 2 of this chapter.

Table 4.5.1. Summary of proteins identified in the urine of Dent's and Normal individuals.

Selected proteins identified in the urine of Dent's and Normal subjects are tabulated. A detailed description of the results is presented in sections 4.1 and 4.2 of this chapter. In all cases the term 'levels' refers to amount of protein in relation to total protein and it does not mean to imply actual concentration with respect to volume or to creatinine.

A. Proteins found at similar levels in the urine of Dent's and Normal subjects

Protein	Method*	Function**
Albumin	μLC, 2DE, ICAT	Colloid osmotic pressure of blood
α-1-antitrypsin	μLC, 2DE, ICAT	Coagulation
α-1-microglobulin	μLC, 2DE, ICAT	Immune system
Ig κ	μLC, 2DE, ICAT	Immune system
Ig λ	μLC, 2DE, ICAT	Immune system
Gelsolin	μLC, 2DE,	Regulates cytoskeleton in cells. The function of the plasma isoform is unknown
Zn-α-2-glycoprotein	μLC, 2DE, ICAT	Plasma protein. Involved in the turnover of lipids.
Orosomucoid	μLC, ICAT	Immune system
Ribonuclease	μLC, ICAT	Secreted. Role in digestion.

* Method used for the identification of the named protein
** Function of the named protein as reported in [270]

B. Proteins found at higher levels in the urine of Dent's patients

Protein	Method*	Function**
	Apolipoproteins	
Apo-AI	2DE	Carrier of lipids
Apo-AIV	2DE, μLC	Carrier of lipids
Apo-CIII	μLC	Carrier of lipids
Apo-E	μLC	Carrier of lipids
	Bioactive Peptides	
IGF-II	μLC, ICAT	Cytokine
PEDF	μLC, 2DE	Cytokine
PDGF	μLC	Cytokine
Angiogenin	μLC, ICAT	Angiogenesis
PBP	μLC, ICAT	Chemokine
Chemokine 14	ICAT	Chemokine
Fibronectin FN70	μLC, ICAT	Cytokine
	Complement Components	
Factor H related	2DE, μLC	Complement complex formation
Factor B	ICAT, μLC	Complement complex formation
Factor C2	ICAT	Complement complex formation
Factor D	ICAT, μLC	Complement complex formation
	Carrier Proteins	
β-2-glycoprotein I	2DE, ICAT, μLC	Carrier of phospholipids and other negatively charged compounds
Hemopexin	2DE, ICAT, μLC	Carrier of heme
IGFBP-2	2DE, ICAT, μLC	Carrier of IGF
Transthyretin	2DE, μLC	Carrier of thyroid hormone and retinol
VDBP	2DE, ICAT, μLC	Carrier of vitamin D
RBP	2DE, ICAT, μLC	Carrier of retinol
Neutrophil lipocalin	2DE	Carrier of lipophilic substances
	Other	
β-2-microglobulin	2DE	Immune system (MHC class I molecule)

* Method used for the identification of the named protein

** Function of the named protein as reported in [270]

C. Proteins found at higher levels in the urine of Normal subjects

Protein	Method*	Function**
Latent TGF-β BP	ICAT, μLC	Binds and modulates TGF-β actions
CD59	ICAT, μLC	Inhibits complement formation
Uromodulin	ICAT, μLC, 1DE, 2DE	Binds and may modulate cytokine activity
IGFBP7	ICAT, μLC	Binds and may modulate IGF actions
Urokinase	ICAT, μLC	Activates plasmin into an active form
TNF receptor	ICAT	Receptor for TNF
Kininogen	ICAT, μLC, 2DE	Precursor of Bradykinin and other bioactive peptides
EGF precursor	ICAT, 1DE	Precursor of EGF
TNF	ICAT	Cytokine
Urinary protein 1	ICAT, μLC	Unknown
Lithostathine	ICAT, μLC	Inhibit crystal formation
Megalin	1DE	Endocytosis of proteins
Cubilin	1DE, μLC	Endocytosis of proteins
Poly-Ig receptor	2DE, μLC, ICAT	Transcytosis
Acid phosphatase	2DE, μLC	Enzyme. Physiological role unknown
Osteopontin	μLC	Bioactive peptide

* Method used for the identification of the named protein
** Function of the named protein as reported in [270]

Dent's disease is the result of a defective chloride channel termed ClC-5 [80]. In non-affected individuals this channel has a role in the megalin-mediated endocytic pathway of proteins in proximal tubular epithelial cells. A defective ClC-5 results in inefficient delivery of megalin from endosomal compartments to the apical brush border plasma membrane such that proteins present in the glomerular filtrate, normally reabsorbed in this tubular segment, leak into urine producing the symptom of low molecular weight (or tubular) proteinuria. Indeed, the quantitatively elevated excretion of proteins in the urine of Dent's disease patients and animal models of this disease is well documented [85;86;151;264;271-273]. However, no studies were available at the date of writing this thesis describing the qualitative composition of the proteome of Dent's disease urine with comparison to that of normal urine. Since the ClC-5 channel is involved in the megalin-mediated endocytosis, results described herein are probably also relevant to the function of this pathway.

It was found that several plasma proteins are present in normal and Dent's urine at similar levels (Table 4.4.1.A). Conversely, several transport proteins seemed to be present at higher levels in the urine of Dent's patients than in that of control subjects when

the analysis was standardised to total protein amount (Table 4.4.1.B). In a healthy nephron most proteins present in the glomerular filtrate are reabsorbed in the first segment of the proximal tubule by megalin-mediated endocytosis [38;77]. However, some proteins fail to be reabsorbed so that trace amount of protein is always present in normal urine.

The results presented herein may be interpreted by assuming that the endocytic apparatus in proximal tubular cells has a higher affinity for transport proteins than for other protein classes. Thus, certain transport proteins such as hemopexin, VDBP, RBP, transthyretin and β-2-glycoprotein I, which transport valuable molecules in plasma and are readily filtered given their size, may be almost completely reabsorbed by cells expressing functional ClC-5 channels and therefore appear in normal urine to a lesser degree than other filtered proteins with less affinity for this endocytic machinery. Conversely, in Dent's patients they appear in urine at the same proportion than other filtered proteins because this pathway is defective. See also Figure 4.5.1 for further explanation of this interpretation.

As shown in Figure 4.3.2, previously reported results also showed that the ratio of RBP to albumin concentration is on average about 40 times higher in the urine of Dent's disease patients than in normal urine [151;264]. Thus, the reinterpretation of these results support the notion that RBP is reabsorbed to a greater extent than albumin from the glomerular filtrate, assuming that the integrity of the glomerular membrane is not compromised.

Another protein class that appears to have a great affinity for megalin is the apolipoprotein family (Table 4.4.1.B). Megalin is a member of the low-density lipoprotein receptor family [41], and therefore, although megalin has affinity for several different proteins and peptides [44], it may have greater affinity for the lipoprotein family of proteins than for other proteins. Table 4.4.1.B also shows that several complement components were detected at higher levels in the urine of Dent's patients when compared compared with that of normal subjects. Interestingly, complement factors and apolipoproteins have been found to be nephrotoxic either by themselves or by the action of their lipid content on epithelial tubular cells [274-279]; thus these findings may be clinically relevant.

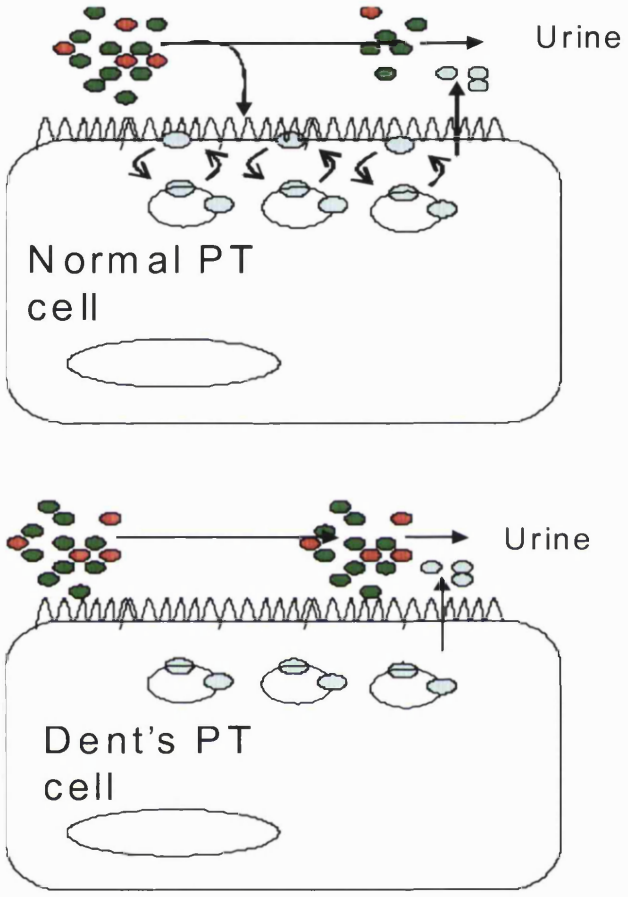


Figure 4.5.1.Interpretation of results.
The hypothetical case of two proteins, one with high affinity (4 molecules, red circles) and the other with low affinity (10 molecules, green circles) for the megalin-cubilin complex, is considered. In proximal tubular (PT) cells expressing a functional receptor mediated endocytic pathway (top panel), proteins with high and low affinity are almost completely reabsorbed from the glomerular filtrate and only trace protein amounts appear in urine. When this pathway is defective as in FS patients (bottom panel) most, if not all, filtered protein appear in urine. In the hypothetical case presented here 3 out of 4 molecules (75 %) of the high affinity protein is reabsorbed by normal PT cells whereas only 5 out of 10 molecules (50 %) of the low affinity protein is reabsorbed. If only these two proteins were in urine, the high affinity protein would represent 16 % of total protein in normal urine, whereas in Dent's urine its proportion would be 29%. It follows that high affinity proteins represent a larger percentage of the Dent's urinary proteome when compared with that of normal individuals. Therefore, the results presented here can be interpreted by assuming that the proteins found at proportionally higher levels in Dent's urine represent proteins reabsorbed by normal PT cells from the glomerular filtrate in preference to other proteins. Proteins of kidney origin (blue circles) represent a larger proportion of the normal urinary proteome and consequently these results can also be used to infer which of the proteins present in normal urine are of kidney origin.

Other proteins found in Dent's urine but not in normal urine in this study and previous studies [151;264] include cytokines and chemokines such as angiotensinogen, IGF-II, PEDF, BMP-1, and platelet basic protein among others. In contrast, kininogen was found in normal urine at higher levels than in Dent's urine when the analysis was standardised to total protein amount, which suggests that the source of this protein is renal.

Angiotensin converting enzyme (ACE) was also found in normal urine suggesting that molecular machinery involved in the angiotensin system is present in tubular fluid. Since receptors for some of these and other peptides have been located on the luminal side of tubular epithelial cells [3-5;8], the altered relative concentrations of bioactive peptides in the tubular fluid may contribute to a dysregulation in cell signalling. In this respect, a greater angiotensin concentration in the tubular fluid of Dent's patients may contribute to an alteration in fluid and electrolyte homeostasis.

In addition, several proteins with putative regulatory function are present at lower levels in the urine of Dent's patients than in that of normal subjects when the analysis is standardized to total protein. For example, uromodulin, a protein whose putative function is to bind and modulate cytokine activity [280], is present at lower levels in Dent's urine with respect to total protein than in normal urine. Other proteins involved in regulation and found at lower levels in Dent's than in normal are CD59 (which is involved in inhibiting complement complex formation [281]), IGFBP7 and TGF-β BP (which, as their name indicate, are involved in binding and perhaps regulating, IGF and TGF-β respectively). Another protein found in Normal urine at higher levels than in Dent's urine is lithostathine, a protein involved in inhibiting calcite crystal formation in pancreas [282]. Interestingly, a common manifestation in Dent's disease is accumulation of crystals, which end up in the formation of kidney stones. Thus, the proposed regulatory role of these proteins may be overwhelmed as a result of the defective reabsorption of polypeptides in the first segment of proximal tubular cells in Dent's patients. Taken together, these alterations may contribute to the progressive kidney failure observed in these patients by the aberrant stimulation of apical receptors on tubular cells with the concomitant dysregulation of gene expression and cell function.

Therefore, an important conclusion derived from the studies presented in this chapter is that, in addition to quantitative differences, there exist several qualitative differences between the urinary proteomes of Dent's and Normal individuals. The elevated

amounts of transport proteins in samples form Dent's disease patients suggest that the endocytic apparatus that is disrupted in these patients may have evolved to have more affinity for proteins that transport valuable molecules in plasma such as vitamin binding proteins than for other protein classes (e.g., albumin). This endocytic apparatus may also have more affinity for bioactive peptides so that small plasma molecules do not disrupt the proposed 'intracrine' homeostatic control of peptides secreted by renal tubular cells and present in the tubular fluid. Finally, a further role of the proximal tubular endocytic machinery may be to protect distal parts of the nephron from the cytotoxic effects of certain plasma proteins such as the lipoproteins and complement components. It is conceptually possible that the cubilin-megalin receptor system has different affinities for different proteins. Alternatively, the observed differential affinity might be explained by assuming that there exist other, as yet unidentified, receptors specific for different proteins in the proximal tubule.

5. Proteomic analysis of basolateral and apical membranes from kidney cortex

Results presented in the previous two chapters indicated that there are several peptides with potential biological activity in the urine of both normal individuals and FS patients. Interestingly, some of these peptides (e.g., EGF precursor and kininogen) represent a larger proportion of the normal urinary proteome when compared with that of Dent's disease urine. Conversely, other bioactive peptides (e.g., IGF-I and II) are present at higher levels in Dent's urine than in normal urine when the analysis was normalized to protein amounts. Thus, it is open to consideration whether an alteration of the bioactivity of the tubular fluid in these patients contributes to the progression of the disease and to its manifestations.

It may be proposed that there is a signalling network that operates from the apical side of renal tubular cells such that peptides present in the tubular fluid have a role in controlling the physiology of these cells. Furthermore, results presented in Chapter 4 suggest that there may be specificity in the reabsorption of polypeptides from the glomerular filtrate, and this finding strongly suggests that either megalin has different affinities for different ligands, or there are other, as yet unidentified, receptors expressed on proximal tubular cell apical membranes.

To investigate the possibility of intracellular signaling occurring from the apical side of renal tubular cells, proteins present in renal proximal tubular (PT) cells brush border membranes (BBM) and basolateral membranes (BLM) were analysed using proteomic approaches and the results of these experiments constitute the topic of this chapter. It was anticipated that these experiments may also be able to detect the presence of other putative receptors for proteins on the apical side of PT cells, if present.

PT cells are the main type of cells in the renal cortex so that homogenates from this renal segment are mainly composed of PT cells. Glomeruli are also located in the cortex but treatment with collagenase separates glomeruli from tubular cells. There is only a limited knowledge regarding the protein composition of the BBM and the BLM from renal tubular and other epithelial cells. The main function of PT cells is to reabsorb solutes from the glomerular filtrate. But, in addition to ion channels and transporters involved in the reabsorption of solutes and their release into blood, there also are protein components

that regulate the activity and localisation of these proteins, and there also exist several cytoskeletal proteins that provide a scaffold for the other proteins to interact.

As discussed in previous chapters, most studies aimed at characterizing complex proteomes use 2DE for the separation of proteins, which are then identified one at a time using MS. This approach is very powerful and has given insights into changes in gene expression secondary to specific perturbations of biological systems [267]. However, it is now accepted that not all proteins may be amenable to 2DE analysis [265]. Among these, hydrophobic proteins such as integral membrane proteins may not enter the first dimension in 2DE, and therefore membrane proteins may be underrepresented in 2DE gels [257;258]. Although advances in 2DE technology may overcome this problem in the future [97;98;255], some reports exist that use 1D SDS-PAGE or 2D-LC followed by MS/MS for the analysis of membrane proteins. For example, the proteomes of mitochondria [283], Golgi membranes [284], peroxisomes [285], lipid rafts [286], chloroplasts membranes [287], and plasma membranes of cancer cell lines [288], have been analysed using 1D SDS-PAGE as the separation method followed by identification by MALDI-TOF and/or LC-ESI-MS/MS. An advantage of 1D over 2DE is that membrane proteins are readily solubilised by the detergent SDS; unfortunately, the anionic character of SDS makes it incompatible with IEF such that intrinsic membrane proteins are not efficiently analysed by 2DE.

As an alternative to gel electrophoresis, protein analysis by 2D-LC-MS/MS has also been reported for the analysis of membrane proteins in a high throughput manner. The groups of Yates (e.g., [258]) and Aebersold [166] have used approaches based on 2D-LC-MS/MS for the analysis of brain and microsomal membrane proteins, respectively. Since this approach involves digesting the protein mixture with a protease prior to two rounds of LC, problems concerning the solubility of proteins are avoided. Peptides are more soluble than proteins in aqueous solvents and since each protein produces several peptides upon enzymatic digestion, the probability that some of them are going to be soluble in aqueous solvents, and thus detected, increases.

Although 2D-LC-MS and 1DE-LC-MS based approaches for 'shotgun' proteomics are increasingly used for the analysis of proteins, there are not studies assessing and comparing directly their performance. In the studies presented in this thesis, these two analytical strategies were investigated for membrane protein analysis. In the first of the approaches to be compared, proteins from cortical brush border membranes were

separated by SDS-PAGE, and subsequently, the complete gel lane was cut to obtain 15-20 gel pieces containing the complete membrane proteome. Proteins were extracted from the gel pieces by in-gel digestion and the identity of these proteins determined by LC-ESI-MS/MS. In the second approach, the membrane protein mixture was digested with a protease, and the resultant peptide mixture separated by two rounds of chromatography; namely, SCX followed by RP-LC. As in the SDS-PAGE based method, the detection and characterization of eluting peptides was carried out by on-line ESI-MS/MS. This mode of mass spectrometry provides sequence information, and therefore, identification of the gene products is, in most cases, unambiguous.

In preliminary experiments using enriched brush border membrane preparations obtained by magnesium precipitation and differential gradient centrifugation more than two hundred proteins were detected; most of them have been previously reported to be located at the apical membrane. In addition to transporters and ion channels known to be involved in the reabsorption of solutes from the glomerular filtrate, many membrane proteins known to be involved in signal transduction were also identified, which suggests that signalling may also be occurring at the apical side of tubular cells. However, there also were proteins that are markers of the basolateral membrane, which indicates that the membrane preparation was not of enough purity; in addition to apical membrane proteins, proteins from other membrane compartments were also present in these preparations. Therefore, in order to obtain a more accurate description of the apical membrane proteome of proximal tubular cells, other methods for separating apical and basolateral membranes were investigated. A free flow electrophoresis method was expected to provide a purer source of apical membrane and also basolateral membrane preparations so that a quantitative comparison could be made, which, in turn, would reveal how much each protein was enriched in the respective membrane preparation.

5.1. Analysis of proteins present in brush border membranes obtained by magnesium precipitation

Enrichment of brush border membranes from cortical kidney segments by magnesium precipitation was described in 1981 by Biber *et al.* [181] as an improvement of a method previously described by Booth and Kenny [289]. This is now a method widely used to enrich for the components of renal apical membranes (e.g., [290]) and it is based on the fact that the brush border and the basolateral membranes have different lipid, carbohydrate and protein ratios (a fact that reflects their different functional roles) such that they have different charges and densities. Thus, these two membrane segments can be separated by density gradient centrifugation using divalent cations; the most commonly used ones being magnesium and calcium [291]. In addition to brush border membranes, it is also known that the method isolates components of endosomes and it has been acknowledged that these preparations may also isolate other components in addition to apical membrane proteins [291], although the precise nature of the components isolated by this method was not known at the time of writing this thesis.

Cortical brush border membrane preparations obtained by the method of Biber were a gift from Dr. J. Marks at the Royal Free Hospital, London. Seventy micrograms of protein were separated by either SDS-PAGE (undigested) or by SCX after proteolysis of all the protein components. In both cases the final identification of components was by LC-ESI-MS/MS using long gradient runs in order to increase the peak capacity of the system. About 300 to 500 MS/MS spectra could be generated per each SCX or gel piece fraction when 90-minute gradients were employed.

The gel lane shown in Figure 5.1.1 containing the entire BBM proteome was cut into 19 gel pieces of equal size and the proteins present in each gel piece were in-gel digested with trypsin. The tryptic peptides thus generated were separated by RP nanoLC and detected and sequenced as they eluted from the run by on-line ESI-MS/MS. A total of about 7000 MS/MS spectra were collected, which led to the identification of 251 proteins. These proteins are listed in Appendix III.

Proteins present in BBM preparations were also analysed by 2D-LC-MS/MS. To this end, proteins were digested with trypsin and the resultant peptide mixture was separated by SCX HPLC. Fractions collected from these runs were further separated and analysed by LC-ESI-MS/MS as above for the in-gel digested proteins. A total of 20

fractions were collected and peptides were present in 16 of these fractions. About 3000 MSMS spectra were collected and this led to the identification of 146 gene products.

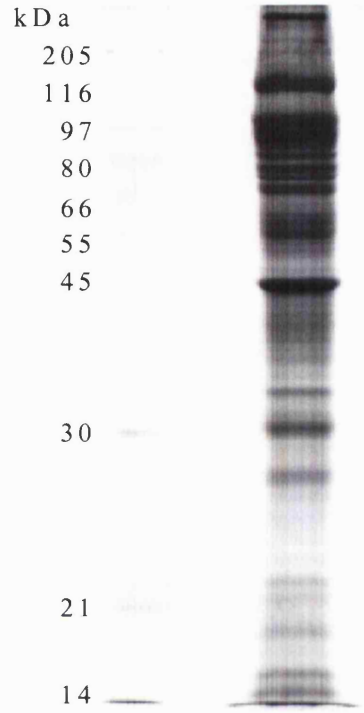


Figure 5.1.1. SDS-PAGE of BBM preparations.
Proteins (70 μg) present in BBM vesicles obtained by differential centrifugation were separated on a 12% acrylamide gel. After staining with colloidal Coomassie blue, the whole lane was cut regardless of stain intensity to make 19 fractions of approximately equal size. Proteins were extracted by in-gel digestion and the resultant tryptic peptides analysed by LC-MS/MS

Table 5.1.1. Comparison of statistical values from 2D-LC and SDS-PAGE approaches

	Method	
Parameter	**SCX**	**SDS-PAGE**
Average pI	7.0	6.9
Average Mr (Da)	71075.6	61348.0
Average coverage (%)	10.7	11.6
Average peptides/protein ID	10.9	7.2
Average Mascot Score	154.6	232.4
Total number of Identifications	146	251

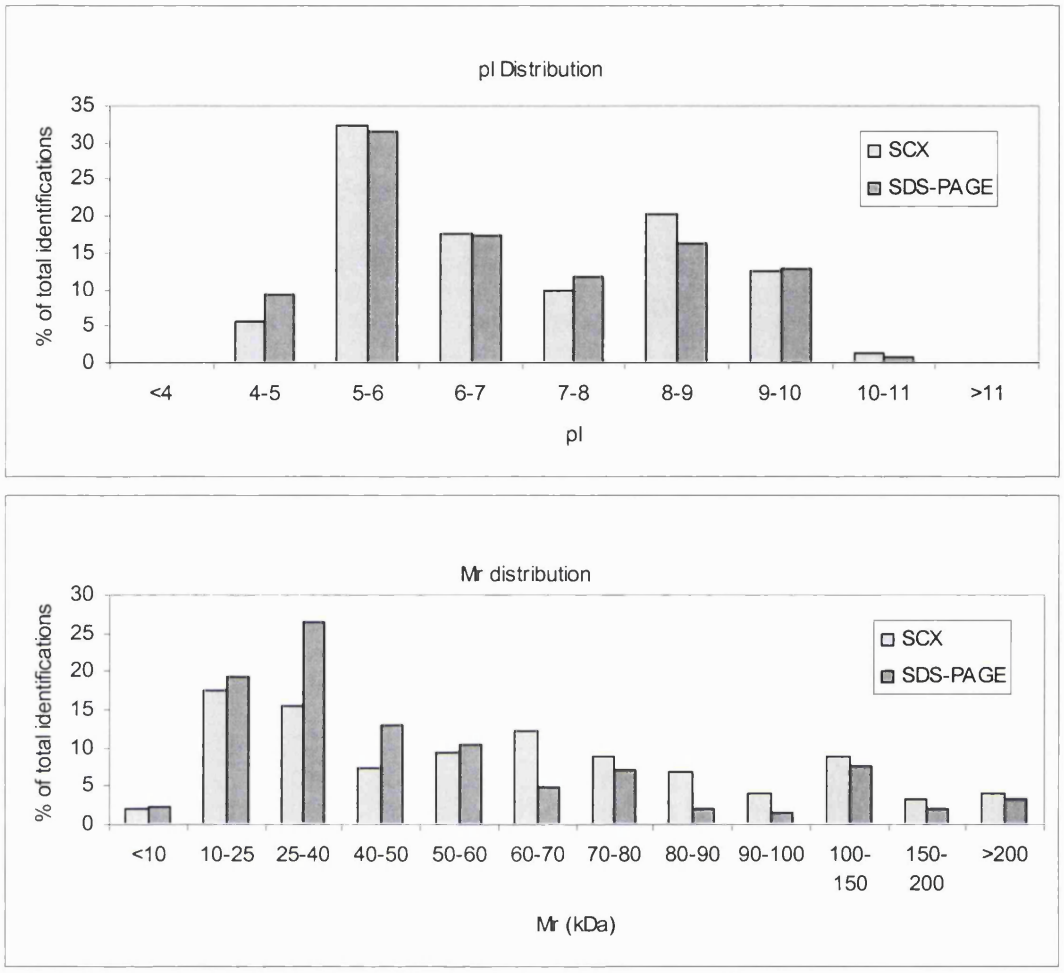


Figure 5.1.2. Distribution of pI and Mr of the proteins identified in BBM preparations by two different proteomic approaches.
The theoretical pI and Mr of the proteins identified in BBM were plotted against the percentage of total number of proteins identified. No obvious differences were observed in the pI distributions (top panel), whereas the SCX method seemed to detect larger proteins than the SDS-PAGE method (bottom panel).

In order to investigate whether these methods are biased towards certain protein classes, the Mr and pI values of the proteins identified were compared. Table 5.1.1 shows that there is no difference in the average pI value of the proteins identified by these methods. However, proteins identified by the SDS-PAGE method were on average smaller in size than those identified by the 2D-LC approach. Similarly, Figure 5.1.2 indicates that there were not apparent differences in the pI distributions of the proteins identified by these methods, whereas proteins identified by the 2D-LC method were on average larger than those identified by the gel approach (Figure 5.1.2). In 2D-LC experiments proteins were digested prior to separation by HPLC. Since larger proteins produce more tryptic peptides than smaller proteins the chances that they are going to be

identified in automated data acquisition experiments is greater, and this may explain why larger proteins were more represented in 2D-LC experiments. Another explanation to account for the observed differences in the average and distribution of Mr obtained by the two methods under comparison is that the migration of proteins in SDS-PAGE along the gel is not linear with respect to their Mr. Instead, migration of proteins in PAGE is proportional to the log(10) of their Mr. Thus, if the gel lane is cut into sections of equal size, those pieces at the lower end of the gel contain a narrower Mr range than those cut at the top of the gel. Consequently, in SDS-PAGE experiments low molecular weight proteins are more likely to be identified, when the whole lane is cut into pieces of equal size.

The quality of the identifications obtained by these two methods was also compared. Parameters used to assess the probabilities that the identifications are correct include number of peptides that generated sequence information matching the gene product, the sequence coverage achieved, and other statistical factors such as the Mascot or Mowse scores included in bioinformatics packages. Table 5.1.1 shows that proteins identified by the SDS-PAGE method had, on average, a greater coverage than those identified by the 2D-LC method. In contrast, more peptides matched the identified protein in the 2DE-LC analysis than in the SDS-PAGE approach. This may seem counterintuitive since more peptides matching a given protein should cover a greater percentage of its sequence. But, in fact, this observation agrees with the fact that larger proteins were identified by the 2D-LC method (see Table 5.1.1 and Figure 5.1.2). A larger protein generates more peptides and therefore more of these are detected by LC-MS/MS. However, since the proteins identified by the 2D-LC method were on average larger than those found by the SDS-PAGE method, these peptides covered a smaller sequence of the protein.

More proteins were detected by the SDS-PAGE than by the 2D-LC approach (Table 5.1.1). Moreover, running of gels and their subsequent handling was, in the hands of the author, more robust than SCX HPLC. Protocols for running gels are better established than those for HPLC in most biochemistry laboratories and less instrumentation is required for SDS-PAGE than for HPLC. For these reasons, further experiments were carried out using the SDS-PAGE approach whenever possible.

Proteins with diverse functions were identified (Figure 5.1.3 and Table 5.1.2). As expected, many proteins involved in the reabsorption of solutes from the tubular fluid

were detected including ion channels and transporters. Several endopeptidases, already known to be located on apical membranes, were also detected. Moreover, proteins involved in the trafficking of endosome were identified, which suggest that these membrane preparations also enrich for endosome components. Several membrane-associated proteins that have been reported to have roles in signal transduction were also identified. If the membrane preparations were pure, this would indicate that signalling is also accruing at the apical membrane. Finally, the fact that proteins, whose functions have not been determined yet, were also found in these fractions highlights the potential of this approach for the discovery of novel functions for the apical side of tubular cells and for the annotation of the genome (i.e. for sorting each gene product to its intracellular location).

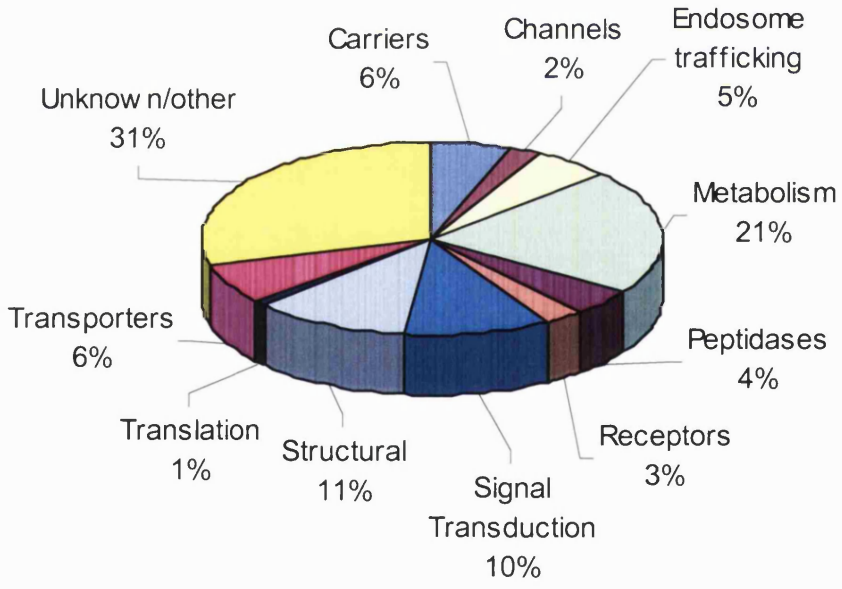


Figure 5.1.3. Function of the identified gene products present in BBM vessicles.
A total of 251 proteins were identified and classified into arbitrarily selected functional categories

Proteins, which are believed to be located exclusively on the basolateral membrane, such as the Na^+/K^+-ATPase and the glucose transporter 2, were also detected. This was not totally unexpected because although the method used to isolate BBM is

known to enrich for the components of the BBM, it is also known that this method does not allow for the complete isolation of this membrane segment [181;291]. However, the precise composition of membrane vesicles obtained by this method had not been reported before and the results reported here are novel in that respect.

Table 5.1.2. Examples of the proteins found in BBM membrane preparations.

Channels
intracellular chloride ion channel protein p64H1
chloride intracellular channel 5
barttin
voltage-dependent anion channel 1 [Mus musculus]
Endosome trafficking
lysosomal membrane glycoprotein-type B precursor
similar to Vacuolar ATP synthase subunit d (V-ATPase d subunit) (Vacuolar proton pump d subunit) (V
syntaxin 3
syntaxin binding protein Munc18-2
Metabolism
ATP synthase alpha chain, mitochondrial precursor
aldehyde dehydrogenase family 9, subfamily A1; 4-trimethylaminobutyraldehyde dehydrogenase [Rattus
Cytochrome P450 4A2 precursor (CYPIVA2) (Lauric acid omega-hydroxylase) (P450-LA-omega 2) (P450 K-5
FRUCTOSE-1,6-BISPHOSPHATASE (FBPASE)
Peptidases
dipeptidyl-peptidase IV (EC 3.4.14.5), membrane-bound form precursor – rat
kidney aminopeptidase M; Leucine arylaminopeptidase 1
kidney-derived aspartic protease-like protein
X-prolyl aminopeptidase (aminopeptidase P) 2, membrane-bound
Glutamyl aminopeptidase (EAP) (Aminopeptidase A) (APA)
Receptors
beta-1-adrenergic receptor – rat
low density lipoprotein receptor-related protein 2; glycoprotein 330; low density lipoprotein-relat
cubilin; cubilin (intrinsic factor-cobalamin receptor)
folate receptor 1 (adult); folate binding protein 1; folate receptor 1
similar to G-protein coupled receptor 112 [Homo sapiens]
Signal transduction
guanine nucleotide-binding protein alpha 11 subunit
guanine nucleotide binding protein (G protein) alpha 12
GTP-binding protein (G-alpha-i2)
guanine nucleotide-binding protein, beta-2 subunit [Mus musculus]
annexin VI
Annexin V
Ser-Thr protein kinase related to the myotonic dystrophy protein kinase
Annexin A4 (Annexin IV) (Lipocortin IV) (36 kDa zymogen granule membrane associated protein) (ZAP36
phospholipid scramblase 1
Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1 (Transducin beta chain 1)
Transporters
FXYD domain-containing ion transport regulator 2 isoform b; ATPase, Na+/K+ transporting, gamma 1 po
Sodium/potassium-transporting ATPase beta-1 chain (Sodium/potassium-dependent ATPase beta-1 subunit
Multidrug resistance protein 1 (P-glycoprotein 1)
low affinity Na-dependent glucose transporter (SGLT2)
solute carrier family 34 (sodium phosphate), member 2

Unknown
PDZ domain containing 1
similar to RIKEN cDNA 0610006H10 gene [Mus musculus]
similar to CG10869-PA [Drosophila melanogaster]
similar to hypothetical protein AF506821 [Mus musculus]
hypothetical protein XP_243379
hypothetical protein XP_228339
similar to hypothetical protein MGC32871 [Homo sapiens]
similar to hypothetical protein DKFZp434I1117.1 - human (fragment)
similar to Hypothetical protein KIAA0173
similar to KIAA0802 protein [Homo sapiens]

5.2. Analysis of proteins present in brush border and basolateral membrane preparations

The method of magnesium precipitation combined with differential density centrifugation is useful for the enrichment of BBM from kidney cortexes. However, as shown in the experiments described in the previous section, this method does not purify BBM components to homogeneity so that proteins from other subcellular fractions are also present in these preparations. Consequently, it is not possible to assert with confidence which of the identified proteins is a BBM protein and which one is a contaminant. Therefore, a different strategy was used to investigate the protein composition of BBM and also BLM vesicles. Since it may not be possible to purify to homogeneity a given subcellular compartment, it may be necessary to experimentally determine which components are enriched in a given preparation in order to characterise the proteome of the organelle under study.

The group of Murer has used a method based on free flow electrophoresis to separate BBM from BLM extracted from kidney cortex [8] and samples of these preparations were used as a source to investigate and to compare the proteomes of these two subcellular compartments.

Same amounts of BBM and BLM proteins were loaded and separated in SDS-PAGE gels, which were subsequently stained with colloidal Coomassie blue (Figure 5.2.1). As with the experiments described in the previous section, the whole gel lanes were cut into pieces. However, because of the lessons learned from the experiments described in the preceding section, this time the pieces were cut into sections of approximately the same Mr range instead of pieces of equal size.

A total of 344 and 454 proteins were detected in the BBM and BLM fractions, respectively. After deletion of proteins that appeared in more than one gel section, 268 proteins were identified in the BBM and 332 in the BLM fractions. The proteins identified are listed in Appendix IV. As with BBM preparations obtained by differential density centrifugation, these membrane vesicles contained a mixture of proteins known to be located to the respective membrane sections; but there also were contaminating components of the other membrane section, the cytosol and from mitochondria.

To illustrate the data obtained during the course of these experiments, the analysis of gel pieces 1 and 17 will be described in some detail, as follows. The gel piece in fraction

1 comprised proteins with an apparent Mr greater than 250 kDa as determined by co-migration of Mr standards. Analysis of the peptides generated by in-gel digestion of Fraction 1 corresponding to BLM and BBM by LC-ESI-MS/MS produced a total of 461 and 511 MS/MS spectra, respectively, leading to the identification of 30 proteins in the BLM and 32 in the BBM. Tables 5.2.1 and 5.2.2 list all the BLM and BBM proteins identified in these gel fractions.

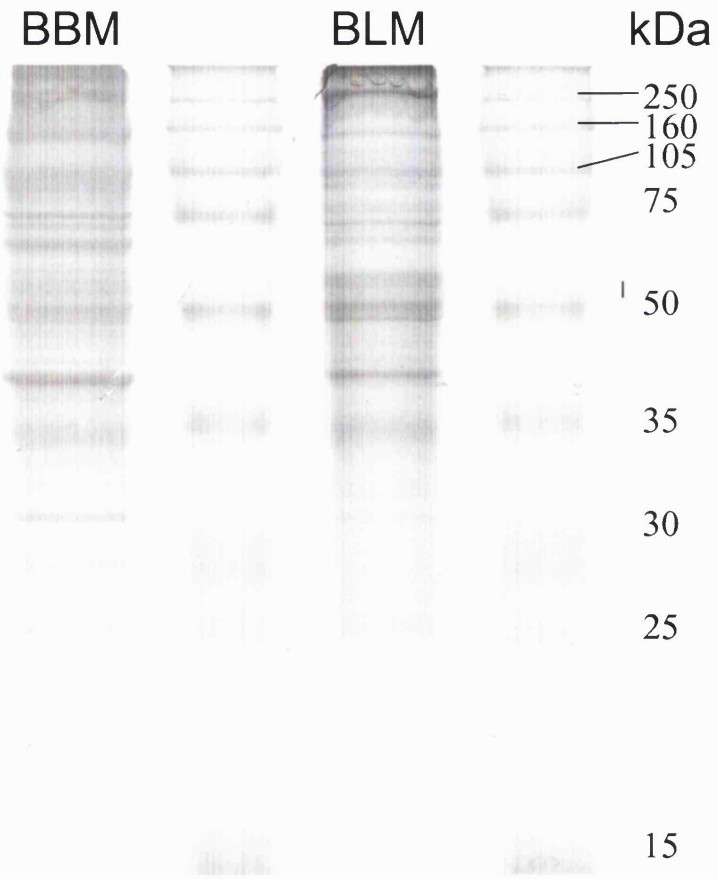


Figure 5.2.1. SDS-PAGE of BBM and BLM preparations.
Proteins (100 μg) present in BBM and BLM vesicles obtained by free flow electrophoresis (Dr. Biber, Zurich) were separated in a 12% acrylamide gel. After staining with colloidal Coomassie blue, both lanes were cut regardless of stain intensity to make 20 fractions of approximately equal Mr ranges. Proteins were extracted by in-gel digestion and the resultant tryptic peptides analysed by LC-MS/MS

Table 5.2.1. Proteins identified in gel fraction 1 of the brush border membrane analysis.

NCBI Acc. No.	Protein Name	# peptides	Mascot Score	Coverage (%)
13562118	Megalin (low density lipoprotein receptor-related protein 2)	90	3152	16
358959	ATPase alpha1,Na/K	26	991	28
27710072	similar to Maltase-glucoamylase, intestinal	34	967	5
17380501	Spectrin alpha chain,	21	788	9
7106421	spectrin beta 2 isoform 2; beta-spectrin 2,	17	620	8
16758040	cubilin; (intrinsic factor-cobalamin receptor)	18	554	6
12018248	low affinity Na-dependent glucose transporter (SGLT2)	6	270	10
6981236	myosin, heavy polypeptide 9; Myosin, heavy polypeptide 9, non-	8	221	5
1083802	sodium-chloride transporter, Thiazide-sensitive - rat	2	161	2
13990959	ATPase, H+ transporting, lysosomal V0 subunit A isoform 4	6	156	7
267413	Aquaporin-CHIP (Aquaporin 1)	3	144	14
20908689	RIKEN cDNA 4632401C08	2	128	3
8393886	solute carrier family 22 member 6; organic cationic transporter-like 1	6	113	8
22074648	retinoic acid inducible protein 3	1	106	2
27733113	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	4	105	2
72475	multidrug resistance protein 1a -	2	105	1
19924057	mucin and cadherin-like; mu-protocadherin	2	102	2
13786160	organic anion transporter; organic anion transporter 3	4	86	5
28487424	similar to apical iodide transporter , putative	2	83	3
6981544	solute carrier family 34, member 1; Solute carrier family 34 (sodium phosphate), member 1	3	81	5
11560059	aminopeptidase A	3	80	3
27733107	similar to multidrug resistance-associated protein	3	78	10
1053142	LX1 (organic cation transporter OCT1A	2	72	4
54130	sodium/potassium ATPase beta subunit	2	70	9
30023556	ATP-binding cassette transporter ABCG2	3	60	4
28570190	Na+ dependent glucose transporter 1	2	58	3
400621	Sodium- and chloride-dependent creatine transporter 1 (CT1)	1	58	2
7513988	high-affinity carntine transporter, CT1 -	2	56	3
27661930	solute carrier family 22 (organic cation transporter)-like 2	2	56	3
2696709	RST [Mus musculus] (solute carrier family 22 (organic cation transporter)-like 2	3	51	3

6644384	sodium bicarbonate cotransporter	4	49	3
28488992	hypothetical protein XP_289590	1	48	3

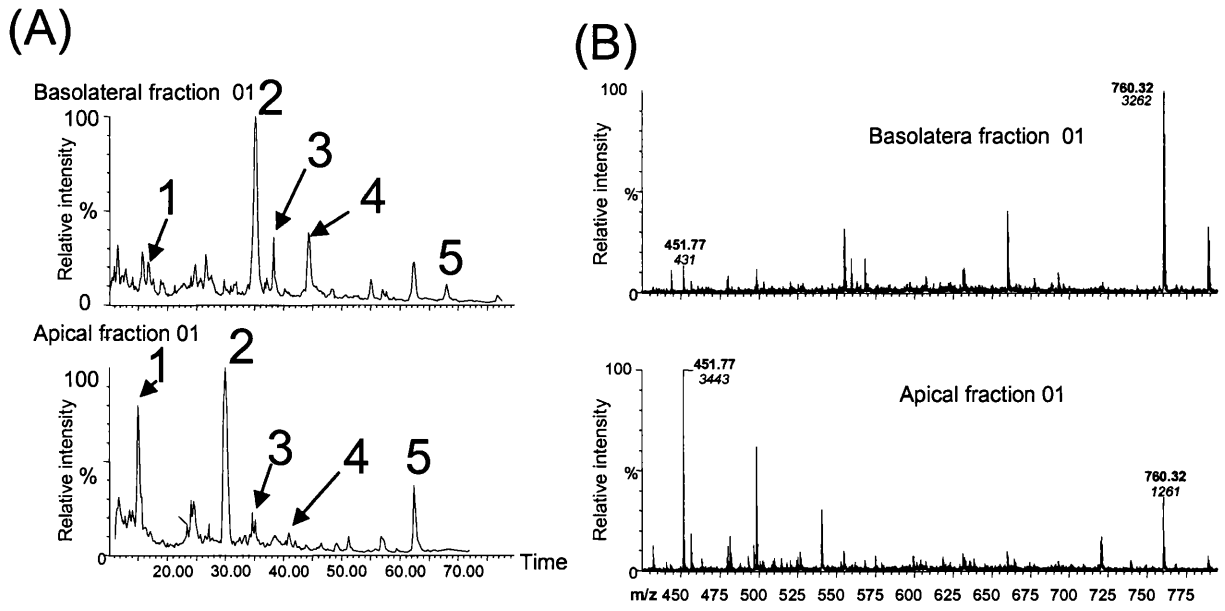
Table 5.2.2. Proteins identified in gei fraction 1 of the basolateral membrane analysis.

NCBI Acc. No.	Protein Name	# peptides	Mascot Score	Coverage (%)
17380501	Spectrin alpha chain,	75	2877	31
358959	ATPase alpha1,Na/K	62	1937	42
30348966	spectrin beta 2 isoform 1;	46	1677	20
13562118	Megalin (low density lipoprotein receptor-related protein 2)	21	787	5
7514087	sodium bicarbonate cotransport protein NBC	11	221	10
1083802	sodium-chloride transporter, Thiazide-sensitive	3	183	3
6753138	ATPase, Na+/K+ transporting, beta 1	7	159	22
8393886	solute carrier family 22 member 6; organic cationic transporter-like 1	6	141	7
27718935	similar to solute carrier family 25	5	137	17
625305	myosin heavy chain non muscle form A	3	119	1
6981542	solute carrier family 16, member 1; Solute carrier 16	2	110	4
1709296	Solute carrier family 12 member 1 (Bumetanide-sensitive sodium-(potassium)-chloride cotransporter 2	3	106	2
13786160	organic anion transporter; organic anion transporter 3	4	96	5
1053142	LX1 (organic cation transporter OCT1A	2	90	4
346261	H+/K+-exchanging ATPase (EC 3.6.3.10) ATP1AL1	3	84	28
267413	Aquaporin-CHIP (Aquaporin 1)	2	82	10
28570190	Na+ dependent glucose transporter 1	2	78	3
12408328	sodium-dependent high-affinity dicarboxylate transporter 3	2	73	4
627754	phosphate carrier protein precursor, mitochodrial, splice form B	1	65	3
13272554	cytokeratin KRT2-6HF [Mus musculus]	2	63	8
70637	ubiquitin - Mediterranean fruit fly	2	59	33
1783347	organic cation transporter	1	58	2
4038352	breast cancer resistance protein (ATP-binding cassette, sub-family G, member 2;	2	54	2
89939	Ca2+-transporting ATPase (EC 3.6.3.8)	2	53	1
127827	low affinity Na-dependent glucose transporter (SGLT2)	2	44	2

The main function of the cells located in the renal cortex, namely PT cells, is to reabsorb molecules filtered at the glomerulus. It was thus expected to identify a large number of ion channels and transporters in these analyses and, as the list of proteins presented in Table 5.2.1 shows, this was indeed the case. It can be appreciated from these analyses that most of the identified proteins were present in both membrane fractions, which could mean that their protein composition is similar. However, it is known that PT cells (the predominant type of cell in kidney cortex) are polarized so that they express certain proteins exclusively at the BLM (the Na^+/K^+ ATPase being the classical example), whereas other proteins are located at the BBM only (e.g., megalin). Therefore, the identification of proteins in both membrane preparations may indicate that the method employed to obtain these preparations does not isolate to homogeneity the components of a single subcellular compartment.

Figure 5.2.2 shows the elution profile of some of the peptides from Fraction 1 that produced MS/MS spectra matching to megalin and Na^+/K^+-ATPase sequences. As expected, megalin peptides produced more intense ion signals in the BBM, whereas Na^+/K^+-ATPase peptides showed more intense signals in the BLM vesicles analysis. These observations agree with the established notion that megalin and the Na^+/K^+-ATPase are markers of the BBM and BLM, respectively. Thus, these results confirm previously reported results and indicate that markers for the BBM and BLM are enriched in the respective membrane preparation.

The elution profile of multidrug resistance protein (MRP) and MRP associated protein peptides, shown in Figure 5.2.3, illustrates that these two proteins are located at the BBM. Although the presence of MRP on the BBM has been reported before [292], the presence of MRP at the PT BBM was not described previously.



Peak Number	Peptide m/z (and identity)	Ion counts	
		Basolateral Band 01	Apical Band 01
1	418.2 (Megalin)	570	2400
2	451.8 (Megalin)	430	3400
	760.3 (Na/K ATPase)	3300	1300
3	431.7 (Na/K ATPase)	700	200
4	843.9 (Na/K ATPase)	740	70
5	780.4 (Megalin)	120	320

Figure 5.2.2. Analysis BBM and BLM protein markers in fraction 1.
(A) Extracted ion chromatograms of some of the peptides that matched megalin and Na/K ATPase alpha 1 subunit in fraction 1 of BBM and BLM. Numbered peaks correspond to the elution profile of the respective numbers on the table. Ions with m/z values at 451.8 (a megalin peptide) and 760.3 (a Na/K ATPase peptide) coeluted in peak 2. Note that although the retention times were different in the two runs due to different dead volumes the relative retention times were consistent. (B) Mass spectrum at peak 2 showing the intensities of ions at 451.8 (megalin) and 760.3 (Na^+/K^+ ATPase). Peaks are labelled with the m/z value and intensity (ion counts, in italics).

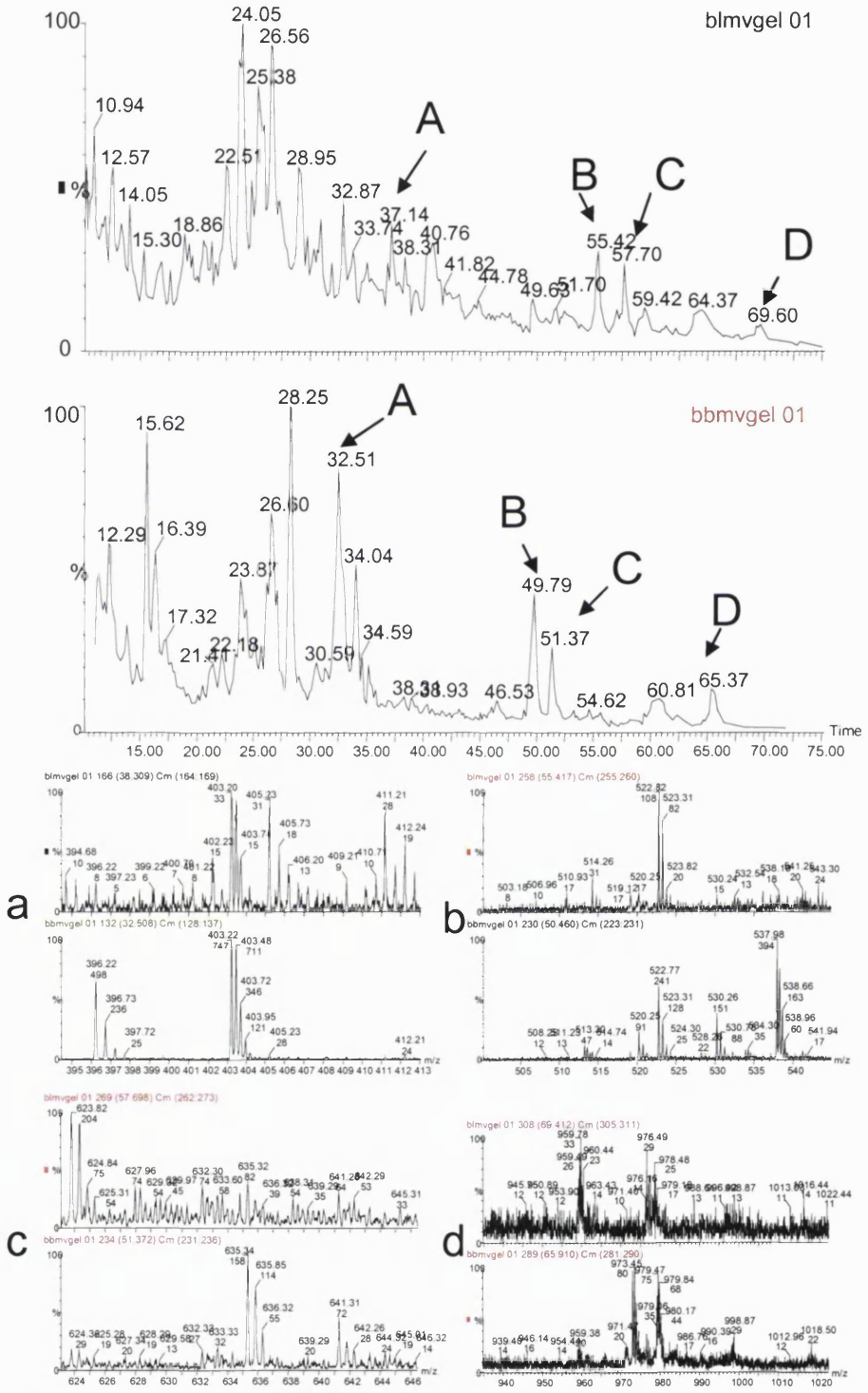


Figure 5.2.3. Elution of multidrug resistance protein and multidrug resistance protein-associated protein peptides in fraction 1.
Top panel shows the extracted ion chromatograms of peptides that derived sequence information for the identification of multidrug resistance protein (MRP) and MRP-associated protein. Peptides eluting at peaks marked with capital letters are shown centered in the spectra with the respective letter. Peaks corresponding to MRP peptides are shown in spectra *b* and *c*; those corresponding to MRP-associated protein are shown in *a* and *d*. Peaks in the spectra are labelled with their m/z value and with their intensity (ion counts).

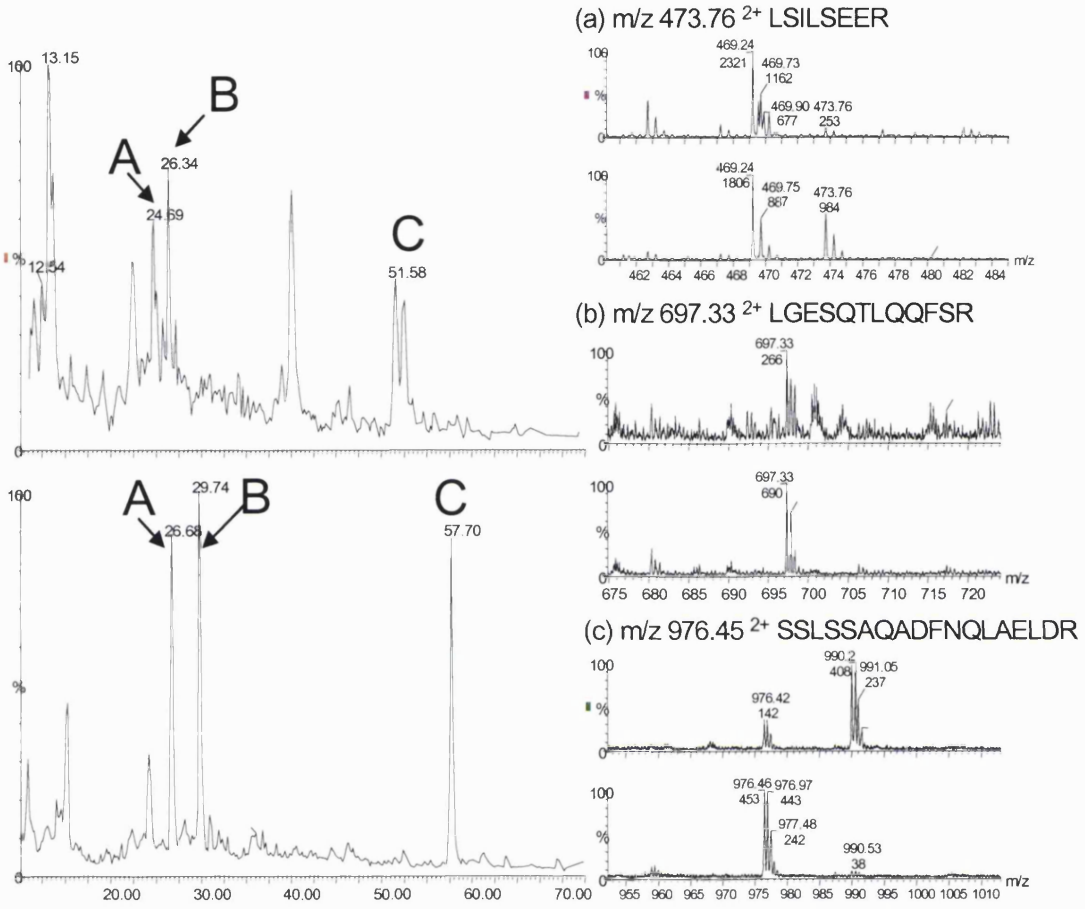


Figure 5.2.4. Detailed analysis of spectrin in fraction 1.

The elution profile (extracted ion chromatograms) of three of the spectrin peptides BBM (top) and BLM (bottom) are shown on the left panel. The mass spectra centered at the labelled peaks are shown on the right. Peaks on the spectra are labelled with their m/z value and with the ion intensity (underneath). Note that all spectrin peptides produced stronger ion intensities in BLM than in BBM fractions. The spectrin peptide at m/z 473.76 coeluted with a aquaporin 1 peptide (469.24 m/z units); the ratio of the spectrin peptide to the AQ1 peptide was larger in the BLM fraction. Similarly, the spectrin peptide at 976.4 coeluted with a megalin peptide at 990.2 m/z units. The megalin peptide ion was more intense in the BBM fraction, whereas the spectrin peptide was more intense in the BLM fraction.

Conversely, the data presented in Figure 5.2.4 indicate that spectrin may be more abundant at the BLM, a finding which is consistent with previously reported studies [293]. Spectrin is a cytoskeletal protein and its presence in BLM, but not in BBM may be important, to anchor BLM specific proteins to the BLM. Other protein, PDZK-1, produced more intense signals in the BBM analyses (data nor shown). PDZK-1 has been implicated in organizing the cytoskeleton of BBM [294;295]. Thus, the presence of different cytoskeletal proteins in BBM and BLM probably contribute to maintaining cell polarity.

Table 5.2.3. Proteins identified in gel fraction 17 of the brush border membrane analysis

NCBI Acc. No.	Protein Name	# peptides	Mascot Score	Coverage (%)
6754976	peroxiredoxin 1; proliferation-associated gene A; osteoblast specific factor 3;	5	85	16
267413	Aquaporin-CHIP (Aquaporin 1)	2	76	10
1633081	Catechol O-Methyltransferase	1	68	7
92339	GTP-binding protein rab1B - rat	3	68	21
1927215	ERS-24 [Cricetulus griseus] (SEC22 vesicle trafficking protein-like 1	1	60	6
420272	GTP-binding protein rab14 - rat	2	58	20
9790225	calcium binding protein P22	1	57	8
4758988	RAB1A, member RAS oncogene family; RAB1, member RAS oncogene family	3	57	20
1185280	glutathione S-transferase [Sus scrofa]	3	50	10
1710027	Ras-related protein Rab-5C	2	50	11
2465729	TFAR15	1	49	4
131794	Ras-related protein Rab-5A	2	49	11
234746	RAS-related protein MEL	1	48	6
27664498	similar to ribosomal protein L15, cytosolic	1	47	6
66313	glutathione peroxidase (EC 1.11.1.9) I -	1	42	6
19527236	RIKEN cDNA 1110014L17	1	40	4
28336	mutant beta-actin (beta'-actin)	1	39	4
996057	gp25l2	1	39	5
576133	Chain A, Glutathione S-Transferase Yfyf (Class Pi) (E.C.2.5.1.18	1	37	7
6647578	Membrane associated progesterone receptor component 1	2	37	11

The analysis of Fraction 17 led to the identification of 25 and 20 proteins in the BLM and BBM, respectively, from a total of 241 and 351 MS/MS spectra. As with the analysis of Fraction 1 described above, most of the identified proteins were present in both membrane sections. Many G proteins were present in this fraction (Table 5.2.3 and Table 5.2.4) and inspection of the elution profiles of these G proteins indicated that they may be present in both the BLM and the BBM. Some of these G proteins, e.g., rab5, are related to Ras and have been involved in endosomal trafficking and they may also play a role in signal transduction [46].

Table 5.2.4. Proteins identified in gel fraction 17 of the basolateral membrane analysis

NCBI Acc. No.	Protein Name	# peptides	Mascot Score	Coverage (%)
gi|92022	GTP-binding protein, 23K	5	229	27
gi|20071222	Ndufs3 protein	3	172	14
gi|121712	Glutathione S-transferase Ya chain	7	144	24
gi|6754976	peroxiredoxin 1; proliferation-associated gene A; osteoblast specific factor 3;	5	111	23
gi|4758988	RAB1A, member RAS oncogene family; RAB1, member RAS oncogene family	4	110	18
gi|267413	Aquaporin-CHIP (Aquaporin 1)	2	86	7
gi|4758984	RAB11A, member RAS oncogene family; RAB 11A, member oncogene family	3	84	12
gi|128867	NADH-ubiquinone oxidoreductase 24 kDa subunit, mitochondrial precursor	2	83	9
gi|57806	unnamed protein product	2	83	4
gi|18606182	Rab5c protein	2	81	10
gi|19705465	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1	3	74	9
gi|6678049	synaptosomal-associated protein 23;	2	73	10
gi|19354269	RIKEN cDNA 0610006F02 gene	3	72	7
gi|86755	ADP,ATP carrier protein T2	2	71	7
gi|6755973	lin 7 homolog c; vertebrate homolog of C. elegans Lin-7 type 3	3	66	16
gi|27716317	similar to succinate dehydrogenase complex, subunit B, iron sulfur (Ip); iron-sulfur	2	61	6
gi|14144	COII	3	60	10
gi|307331	differentially expressed protein	1	60	4
gi|7514011	membrane protein	1	58	4
gi|203237	calbindin-d28k	2	54	7
gi|53450	manganese superoxide dismutase [	1	51	6
gi|131796	Ras-related protein Rab-6A (Rab-6)	2	48	10
gi|4506609	ribosomal protein L19; 60S ribosomal protein L19	1	46	4
gi|19424238	camello-like 1	3	41	9

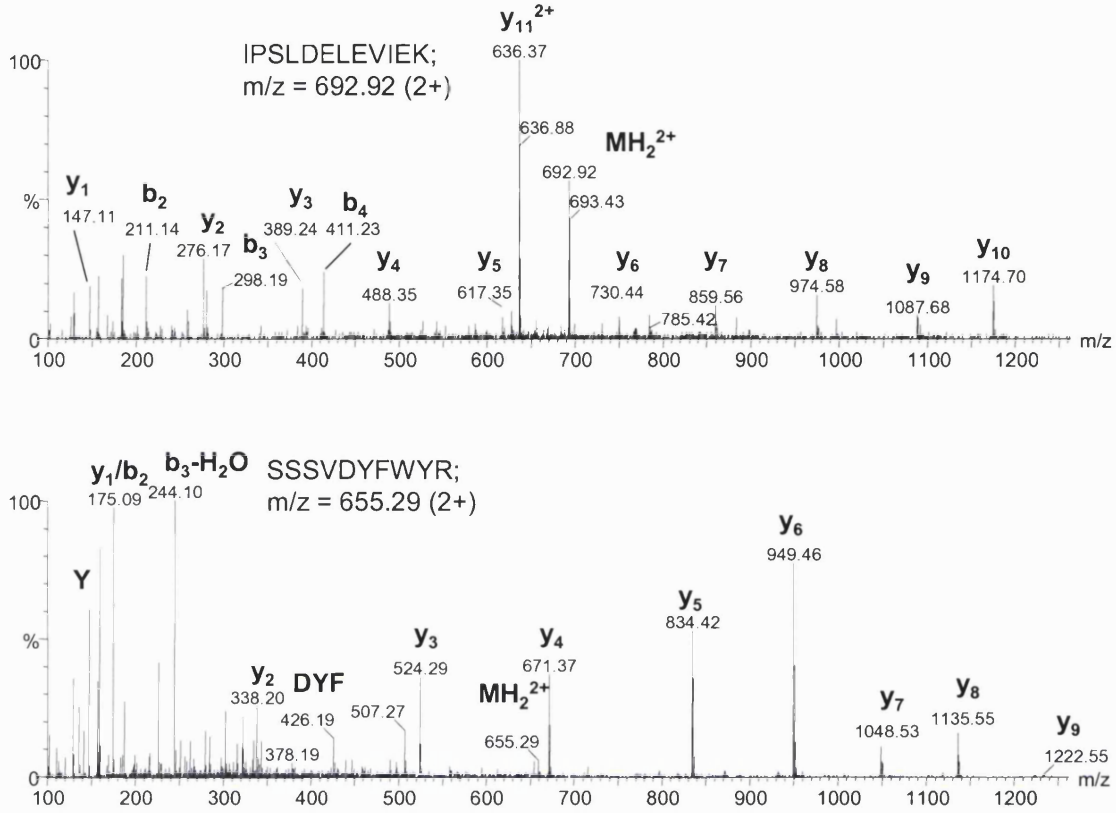
Some proteins with unknown function were detected in these analyses. For example, two peptides in the analysis of the gel fraction 3 matched to an entry in the NCBI protein database with gene identifier number gi|20908689 (Figure 5.2.5). These peptides appeared to be more abundant in the apical membrane preparations and they may be derived from an apical protein. This protein entry, named RIKEN cDNA 4632401C08,

originated from the translated version of a cDNA clone reported by the RIKEN Mouse Gene Encyclopaedia Project (FANTOM consortium) [296]. This cDNA clone was derived from mouse skin tissue and to the knowledge of the author their expression in kidney has not been reported before, although homologous proteins are known to be expressed in the kidney (Table 5.2.5).

Table 5.2.5. Selection of Proteins with Homology to RIKEN cDNA 4632401C08.
Some of the protein sequences returned by homology using Blast searches against the sequence of RIKEN cDNA 4632401C08 are shown

IDE	SIM	LALI	BEXPE	Organism	PROTEIN	Tissue expression	Function	Ref
100	100	634	0.0	Mus musculus	4632401C08Rik protein	Kidney	ND	[296]
53	70	596	e-171	Mus musculus	Na^+ and Cl^--dependent transporter XTRP2.	Kidney	Na:neurotransmitter symport	[297]
40	58	606	e-169	Bos Taurus	Na^+ and Cl -dependent neurotransmitter transporter NTT73	Retina	Na:neurotransmitter symport	[298]
52	69	596	e-168	Rattus norvegicus	Renal osmotic stress-induced Na-Cl organic solute cotransporter	Kidney cortex	Na:neurotransmitter symport	[299]
53	72	575	e-166	Homo sapiens	Hypothetical protein FLJ31236	Kidney	Na:neurotransmitter symport	[300]
45	63	608	e-161	Homo sapiens	Neurotransmitter transporter RB21A	Unknown	Na:neurotransmitter symport	[301]
46	64	581	e-161	Mus musculus	X transporter protein 3 similar 1	Unknown	Na:neurotransmitter symport	[302]

Definitions:
IDE : percentage of pairwise sequence identity
SIM: percentage of similarity
LALI: number of residues aligned
BEXPE: blast expectation value
PROTEIN: one-line description of aligned protein

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1   MVRLVLPNPG LEERIPSLDE LEVIEKEEAG SRPKWDNKAQ YMLTCVGFCV
51  GLGNVWRFPY LCQSHGGGAF MIPFLILLVF EGIPLLYLEF AIGQRLRKGS
101 MGVWSSIHPA LKGIGIASMF VSFMVGLYYN TIIAWVMWYF FNSFQEPLPW
151 SECPLNQNQT GYVEECAKSS SVDYFWYRET LNISTSISDS GSIQWWILLC
201 LTCAWSVLYV CIIRGIETTG KAVYITSTLP YVVLTIFLIR GLTLKGATNG
251 IVFLFTPNIT ELSNPNTWLD AGAQVFYSFS LAFGGLISFS SYNSVHNNCE
301 MDSVIVSVIN GFTSVYAATV VYSIIGFRAT ERFDDCVNTN ILTLINGFDL
351 PEGNVTSENF EAYQQWCNAT NPQAYAQLKF QTCDINSFLS EGVEGTGLAF
401 IVFTEAITKM PVSPLWSVLF FIMLFCLGLS SMFGNMEGVV VPLQDLNITP
451 KKWPKELLTG LICLGTYLIA FIFTLNSGQY WLSLLDSFAG SIPLLIIAFC
501 EMFAVVYVYG VDRFNKDIEF MIGHKPNIFW QVTWRVVSPL IMLVIFLFFF
551 VIEVNKTLMY SIWDPNYEEF PKSQKIPYPN WVYAVVVTVA GVPCLSIPCF
601 AIYKFIRNCC QKSDDHHGLV NTLSTASVNG DLKN
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Figure 5.2.5. Sequence and analysis of a novel renal membrane protein.
Data base searches using uninterpreted MS/MS spectra from the BBM gel fraction 3 returned several hits. One of them was a protein named RIKEN cDNA 4632401C08, whose sequence is shown here (bottom panel) with the peptides sequenced in red. MS/MS spectra of the ions that lead to sequence information are shown in the top panel. Prediction of membrane topologies using the SOSUI script revealed that RIKEN cDNA 4632401C08 codes for a transmembrane protein with 12 putative transmembrene domains (shown here in blue).

The translated version of RIKEN cDNA 4632401C08 codes for a putative protein of 634 amino acid residues. Hydrophobicity analysis of RIKEN cDNA 4632401C08 was carried out using the SOSUI script (available at www.expasy.org). Results revealed that this protein has 12 predicted transmembrane domains (Figure 5.2.5). Transmembrane

prediction using other publically available software also predicted RIKEN cDNA 4632401C08 is a putative transmembrane protein with a topology consisting of 12 transmembrane domains (not shown).

Blast searches revealed that this protein has 53 % similarity and 70 % homology to a mouse Na^+:neurotransmitter transporter termed XTRP2 and it is also similar to other sodium coupled neurotransmitter symporters (Table 5.2.5). The mouse XTRP2 protein is mainly expressed in kidney and it is predicted to be a transmembrane protein. Thus RIKEN cDNA 4632401C08 may be a novel transmembrane protein expressed in renal cortex and located on the apical membrane of PT cells. Its homology to Na^+:neurotransmitter symporters suggest that this protein may also have a role in the reuptake of this class of molecule.

To summarize, although the results presented in this section comparing the proteomes of BBM and BLM vesicles indicated that the methods to isolate these vesicles isolate qualitatively similar proteomes, semi-quantitative analysis reveals that certain proteins are enriched in specific membrane preparations. As expected, the Na^+/K^+-ATPase is enriched in BLM, whereas megalin is enriched in the BBM. The fold enrichment as determined by ion counts produced by ESI-MS is about 4 fold for these components in their respective membrane compartment. This contrasts with published results that suggest a greater enrichment fold (a 10 to 40 fold enrichment of Na^+/K^+-ATPase activity in BLM has been reported [8]). This discrepancy may be caused by the limited quantitative accuracy of ESI-MS. Thus, as discussed in the previous chapter, although quantitative data generated by ESI-MS can give an approximate indication of analyte abundance, it was decided to employ isotopic labelling to obtain a more accurate picture of BBM and BLM proteomes. Preliminary experiments using this approach are described in the next section of this chapter. Finally, the identification of proteins of at present unknown functions highlights the potential of this method for the identification of novel proteins and for the annotation of the genome.

5.3. Analysis of proteins in BBM and BLM vesicles by isotope coded affinity tags, liquid chromatography and mass spectrometry

Results presented in the previous section of this chapter demonstrated that although the markers of BBM and BLM are enriched in their respective preparation, the proteomes of BBM and BLM vesicles isolated by free flow electrophoresis are qualitatively similar, and the fold enrichment of the proteins identified is not sufficiently high to unambiguously assign a subcellular location to proteins of otherwise unknown localization. For this reason it was decided to use the isotope-coded affinity tags (ICAT) strategy in order to determine with a greater degree of accuracy the fold enrichment of each protein in these membrane vesicles. It was envisaged that this would lead to greater certainty as to the intracellular localization of the identified gene products.

To this end, proteins in BBM were labelled with the light ICAT reagent, while BLM proteins were labelled with the heavy ICAT reagent. Equal amounts of labelled protein (100 μg) from each membrane fraction were mixed, digested with trypsin, and the resultant tryptic peptide mixture separated from the free ICAT reagent and into fractions by SCX LC. The labelled peptides were separated from non-labelled ones by avidin affinity chromatography and then analysed by LC-ESI-MS/MS. Preliminary experiments are presented in this chapter section to illustrate the approach.

Figure 5.3.1 shows that, as expected, markers for the BBM and BLM were enriched in their respective membrane preparation. The fold enrichment of megalin and the Na^+/K^+-ATPase in the BBM and BLM, respectively, was about 6 fold. Several cytosolic proteins were also isolated in these membrane preparations. As an example, Figure 5.3.1 shows that, aldolase B, an abundant cytosolic protein was present in both membrane preparations at similar levels. The interpretation of this finding is not easy; it may be that, as proposed before [283], components of a defined cytosolic metabolic pathway associate at the interface of membranes so that transfer of reactants and products is more efficient. Since aldosase B protein appears at similar levels in both membranes (and also in mitochondrial preparations [283]), its association with biological membranes may be unspecific. Alternatively, this protein may not associate with biological membranes, and it may just be a contaminant whose appearance in subcellular preparations may be due to the belief that it may not be possible to completely separate all the components present in an organelle from cytosolic components.

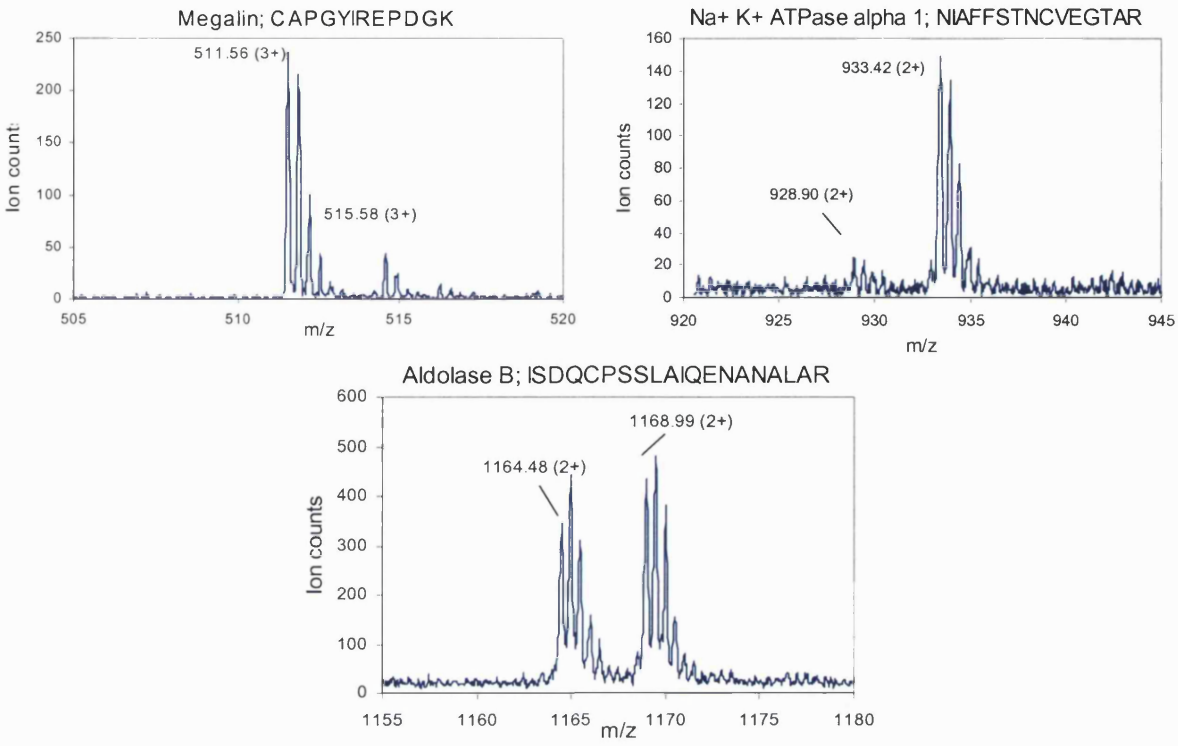


Figure 5.3.1. Ratio of megalin and Na+/K+-APTase in BBM and BLM as determined by LC-MS/MS of ICAT-labelled protiens.
BBM and BLM were labelled with the light and the heavy ICAT reagents, respectively. Therefore, peaks corresponding to BBM peptide-derived proteins appear to the left of those from BLM. After mixing equal amounts of BBM and BLM proteins (100 μg), these were digested with trypsin. The resultant tryptic peptides were separated by SCX followed by RP LC. Detection and identification were by on-line ESI-MS/MS. Spectra show approximately five fold enrichment of megalin and the Na+/K+-APTase in the BBM and BLM vesicles, respectively. The spectrum of a putative contaminant protein, aldolase B, is shown for comparison.

Other cytosolic proteins were found in these membrane preparations in this experiment and also in experiments described in the previous two sections of this chapter (see Appendix III and IV). Another reason why these cytosolic proteins are found in vesicles is that when vesicles form after membrane disruption these abundant cytosolic proteins are trapped inside the vesicles. Treatment with basic buffers has been reported to deplete membrane separations of cytosolic components [303]. Alternatively, or in addition, enrichment of membrane components may be accomplished by purification using affinity methods [304].

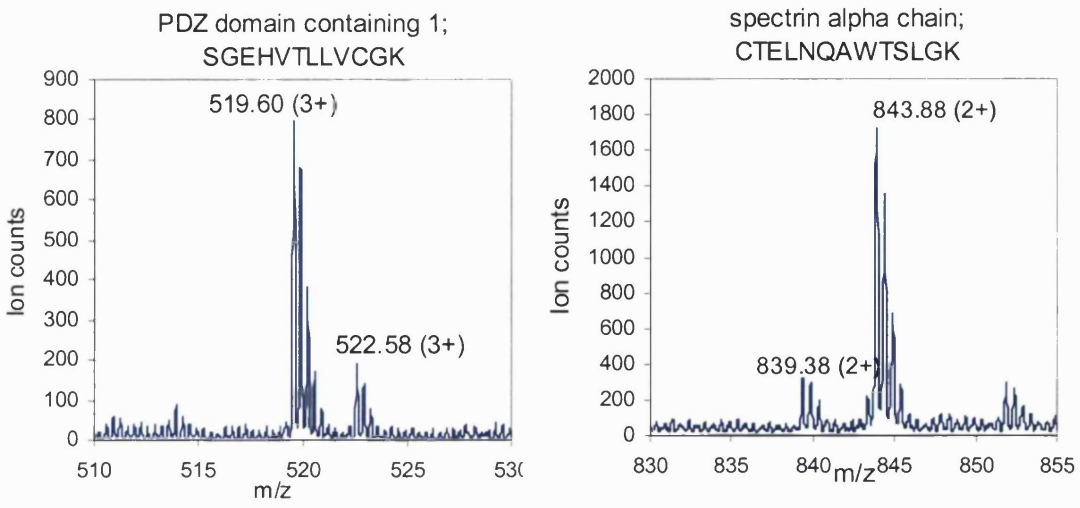


Figure 5.3.2. ICAT ratios of spectrin and PDZK-1 in BBM and BLM vesicles.
The experiment is described in the legend of Figure 5.3.1. This figure shows that a peptide derived from PDZK-1 was about five-fold enriched in the BBM, while an spectrin peptides was five-fold enriched in the BLM.

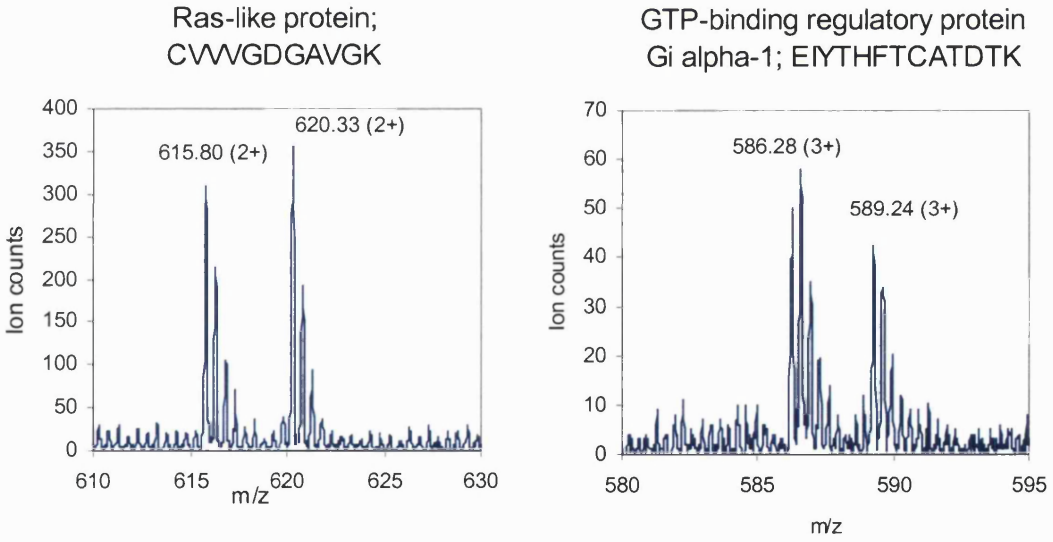


Figure 5.3.3. ICAT ratios of two G proteins in BBM and BLM vesicles.
The experiment is described in the legend of Figure 5.3.1. This figure shows that peptides derived from two different G proteins are found at similar levels in both membrane sections.

Components of the cytoskeleton were identified in both membrane preparations. As with the results described in the previous section of this chapter, spectrin appeared to be a component of BLM, whereas PDZK-1 appeared to be exclusively apical (Figure 5.3.2).

Other proteins that are known to be membrane associated were found to be located in both membrane separations. In this group are included G proteins with Ras homology that have been previously involved in signal transduction and in the regulation of membrane trafficking [46] (Figure 5.3.3). Other proteins involved in membrane trafficking, such as adaptor protein 1 and 2, and clathrin were also detected and found to be located in the BBM. This is consistent with the role of PT cells in receptor receptor-mediated endocytosis of comopunds present in the glomerular filtrate.

5.4. Summary

Results presented in this chapter have demonstrated the application of mass spectrometric-based approaches for the identification of membrane proteins in a manner suitable for proteome wide studies. It is predicted that these methods, in combination with cell fractionation techniques, will lead to the identification of not only the proteomes of each cell type but also the subcellular location of each gene product. The characterization of the mitochondrial proteome has already been reported [283] and attempts to analyse the plasma membrane proteomes of certain cell types [258] and Golgi membranes [259] are also published.

Currently, there is a great interest in the elucidation of the proteomes of specific organelles [154;305-308] in the view that this information can lead to a better understanding of the function of each subcellular structure. Ultimately, this knowledge may lead to a better understanding of cell biology and physiology. Nevertheless, it is recognized that the cell fractionation methods currently available do not isolate subcellular organelles or fractions to homogeneity, although protein components specific for the respective organelle may be enriched with respect to other cellular components. For example, Taylor *et al.* analysed the proteome of heart mitochondria by SDS-PAGE separation of proteins followed by excision of gel bands, the proteins present in which were identified by MALDI-TOF MS and LC-ESI-MS/MS [283]. Therefore, this approach was very similar to the one of the methods used during the course of the experimental work described here and presented in the first section of this chapter. The difference is that Taylor *et al.* did not explore 2D-LC-MS/MS as an alternative to SDS-PAGE followed by LC-MS/MS. More than 400 protein identification were reported in the cited paper [283], many of them were known mitochondrial proteins, and there also were proteins that had not been previously reported in mitochondria, including proteins with potential signalling roles. Surprisingly, proteins involved in carbohydrate metabolism, and thought to be exclusively cytosolic, were also found in these mitochondrial preparations. The authors speculate that the association of enzymes involved in a defined metabolic pathway with the outer mitochondrial membrane may facilitate the transfer of products and substrates thus making the pathway more effective.

Nevertheless, the fact that introduction of 'contaminant' proteins in the isolation of cellular organelles may be unavoidable cast doubt on the assignation of the intracellular location of a given protein. Results describing the proteome of organelles would be less

ambiguous if it was possible to determine the extent of enrichment of each component in its respective subcellular preparation and then compare it with the enrichment of known and *bona fide* markers of the organelle under study. In this respect, the experimental work presented in this chapter has demonstrated that contaminant proteins are present in the proteomes of the BBM and BLM vesicles. An approach for quantitative proteomics based on isotopic labelling was then used (Section 5.3), which provided information on how enriched proteins from BBM are with respect to those in BLM vesicles. This was done in a multiplex and high throughput fashion compatible with proteome wide studies. Further experiments using the approach described above could be carried out in the future to make a more comprehensive description of the proteomes present in BBM and BLM vesicles.

Another lesson learned from the results obtained during these experiments is that the set of proteins identified may be different depending on the method used. It has been reported that the average molecular weight of mitochondrial proteins is about 50 kDa [283]. Here, I have shown that the calculated average molecular weight of a given proteome may be dependent on the method used for their analysis. Indeed, the proteome of BBM isolated by magnesium precipitation and differential centrifugation had an average molecular weight of ~ 60 or ~ 70 kDa, depending upon whether the analysis was carried out by SDS-PAGE or SCX, respectively. Although this conclusion may not be surprising and a careful theoretical consideration may have predicted it, to the best of the author's knowledge, this phenomenum has not been reported before.

Several hundred proteins were identified in both membrane preparations (Sections 5.1 and 5.2). Many channels and transporters were identified, consistent with the function of the cells present in the renal cortex, which is to mediate the reuptake of solutes freely filtered at the glomeruli and present in the tubular fluid. The endocytic receptors megalin and cubilin were also identified together with proteins involved in receptor-mediated endocytosis, including the adaptor proteins AP-1 and AP-2, clathrin and small GTPases that regulate endocytosis (e.g., Rab5). The latter seemed to be present in both BBM and BLM, whereas adaptor proteins were more abundant in BBM vesicles.

Some proteins with signalling functions were present in both membranes. For example, Ras and Ras related proteins were identified in BBM and BLM, which suggests that at least part of the molecular machinery of the Ras signal transduction pathway is located on the apical side of PT cells. Failure to identify receptors for hormones in these

analyses may be explained by the low stochiometric concentrations of these proteins on membranes. Since intracellular cascades amplify the signal generated at the receptor, only low stochiometric amounts of receptors are needed to generate an intracellular signal cascade, and consequently their concentration on membranes is low when compared with cytoskeletal, structural and other 'house-keeping' proteins. Therefore, although hormone receptors for the peptides identified in chapters 3 and 4 could not be detected, their presence on the apical side of tubular cells cannot be excluded. Also, renal cortexes contain PT cells and the site of regulation may be on more distal parts of the tubules.

Nevertheless, the presence of proteins with potential signalling roles in the BBM preparations suggests that factors present in the tubular fluid may be capable to stimulate tubular cells.

Several structural proteins, such as spectrin and PDZK-1, which are located at the BLM and BBM, respectively, were also identified. Spectrin is known to be located at the BLM and this protein binds other proteins located at this membrane segment such as the Na^+/K^+-ATPase [309]. On the other hand, PDZK-1 interacts with NaPi-II [303;309] and the experiments presented in this thesis suggest that this protein is exclusively located at the BBM. These proteins form part of the cytoskeleton and their association with membranes may serve to dock proteins specific for their respective membrane compartment. This may provide a structural basis for membrane architecture and organization, which ultimately determine cell polarity.

To summarize, approaches to determine the proteomes of BBM and BLM from renal cortical cells were assessed and implemented. Several hundred membrane components were identified during these experiments. Ultimately, unambiguous assignation of proteins to their respective sub-membranous localization will need quantitative methodologies. In this respect, although immunochemical methods can be used for the relative quantitation of proteins, proteome wide quantitative methods, such as those described in this chapter, may be used instead in a faster and less biased fashion to unambiguously characterize the proteomes of subcellular compartments.

6. Concluding Remarks and Outlook

The purpose of the studies presented in this thesis was to develop and implement mass spectrometry-based analytical methods to test the idea that polypeptide hormones operating from the tubular lumen of nephrons may have a role in controlling renal physiology. Chapter 3 has briefly described the development of methods, and their preliminary applications to extract polypeptides from urine in a form amenable to analysis by MS. Chapter 4 presented an account on the proteome of normal and Dent's disease urine. Finally, studies aimed at the identification of the proteomes of BBM and BLM were described in Chapter 5.

Experimental work performed during the studies presented here indicates that a combination of chromatographic techniques can extract polypeptides from urine efficiently. These studies, presented in Chapter 3, demonstrate that by combining RP with SCX chromatography peptides with theoretical molecular weights ranging from 1 kDa to 600 kDa can be extracted. Therefore, the advantage of this method when compared with methods based on size exclusion (e.g., dialysis, ultrafiltration, or gel filtration) is that polypeptides of a wide molecular weight range can be isolated. This is illustrated in experiments presented in Chapter 3, where data is presented showing that numerous peptides were detected and sequenced by MS/MS using this approach. The results of these experiments have been published [220;272].

Chapter 3 also illustrates a method for the analysis of peptides present in small volumes of biological fluids. These experiments were presented in the 50th conference of the American Society for Mass Spectrometry [310] and demonstrated that peptides can be analysed with high sensitivity by a combination of capillary chromatographic columns with mass spectrometric detection. This system could be used for the analysis of peptides present in tubular fluid. However, the complexity of the system means that two HPLC pumps and a mass spectrometer have to be exclusively dedicated to these analyses. Therefore, other, less instrumentally demanding, methods will have to be investigated for the analysis of tubular fluid. In this respect, MALDI-TOF may be more tolerant to the presence of low molecular weight compounds that may interfere with peptide analysis, and preliminary experiments (not shown in this thesis) suggest that a single extraction by capillary RP-LC can, in combination with MALDI-TOF, be used for the analysis of urinary peptides with high sensitivity.

Chapter 4 is an account of the experimental work carried out during the studies aimed at the analysis of Dent's disease and normal urinary proteomes. Unlike previous studies on the peptide composition of the urine of Dent's disease and other forms of the FS, the analysis was standardised to protein concentration rather than to volume of urine or creatinine concentration. Therefore, the results present a qualitative picture of these proteomes, albeit the differences in qualitative composition were quantified using the intrinsic information obtained by LC-ESI-MS, 2DE, and also the ICAT strategy.

It was found that the parameter of ion intensity obtained in LC-ESI-MS experiments correlates well with the quantitative results obtained by 2DE and LC-MS of ICAT labelled peptides. Recently, it has been shown that ion intensities produced in LC-ESI-MS experiments can be correlated with protein abundance [233]. The intensity generated in ESI-MS is proportional to analyte concentration, and although ion suppression due to interferences with other analytes may be of concern, when these differences are large, as in biological fluids, quantitative information obtained by LC-ESI-MS can be used as a first indication of differences in the proteomes of case versus control samples.

An important conclusion regarding the methodology used during these studies is that a single proteomic approach may fail to identify all the proteins present in a biological sample. This is probably due to the heterogeneous physicochemical nature of proteins; since analytical approaches exploit of the analytes' properties for their identification, the fact that the physical and chemical properties of proteins differ also means that more than one approach will be needed for comprehensive proteomic analyses.

Regarding their biological significance, perhaps the most important conclusion of the results presented in this thesis is that the megalin-cubilin receptor complex may have different affinities for different proteins. Thus, vitamin-binding proteins seem to be reabsorbed more efficiently from the glomerular filtrate than other plasma proteins such as albumin. This finding strongly suggests that the salvage of vitamins (bound to their carriers) is physiologically more important than the salvage of amino acids (in the form of protein) such that this endocytic pathway may have evolved to have more affinity to this type of molecule.

Other protein classes for which this pathway seems to have more affinity are the lipoproteins, bioactive peptides, and complement components. These proteins have been reported to be toxic to cultured cells and they may also be toxic to renal tubular cells.

Consequently, another role of this pathway may be to protect tubular cells from the bioactivity of plasma components and it may explain why endocytosis occurs in the first segment of the tubules. Indeed, an important physiological role of the megalin-cubilin endocytic pathway may be to protect distal tubular cells from the bioactivity of plasma proteins and to allow the intracrine regulation of renal function - that may operate at the lumen of the tubules - to work unperturbed.

These results may be explained by assuming that the megalin-cubilin receptor complex has different affinities to different ligands. Alternatively, there may be other receptors that mediate the reuptake of specific proteins from the glomerular filtrate. This possibility was explored and the results presented in Chapter 5, where studies aimed at the characterisation of the BBM and BLM proteomes have been described. Megalin and cubilin were the only proteins with known roles in endocytosis that could be identified in these analyses. Therefore, these results do not support the notion of other endocytic receptors for proteins present in the proximal tubule. However, this possibility cannot be completely excluded because the MS/MS data was used to interrogate a protein database. Since not all the gene products have been added to this database there may be still unidentified genes with roles in endocytosis and expressed in the BBM of PT cells. Once the sequence of the rat genome is completed and all the open reading frames identified, the MS data generated in the experiments described in Chapter 5 could be used to interrogate the annotated rat genome. It may be predicted that other proteins may be identified with this exercise.

Results presented in Chapter 5 also confirmed that there are proteins present in BBM with potential roles in signal transduction and consequently these results reinforce the notion that luminal factors could contribute to the regulation renal function.

The studies presented in this thesis have demonstrated that there is an alteration in the relative amounts of peptides and proteins in Dent's disease urine when compared compared with normal urine. One of the manifestations of this and other forms of the FS is progressive renal failure. It can be proposed that progression of renal disease in FS patients is secondary to the alteration in urine composition, which, at least in part, reflects the composition of tubular fluid. Bioactive peptides in normal tubular fluid may have functions in maintaining cell polarity and in providing autocrine and paracrine loops with roles in keeping an extracellular composition compatible with cell survival. The results in Chapter 4 also identified proteins of renal origin that may have a role in regulating this

process. In diseases that lead to proteinuria, such as FS and diabetes, these regulatory systems may be overwhelmed and an abnormal stimulation of luminal receptors may lead to an altered stimulation of signal transduction pathways that would result in an abnormal regulation of gene expression.

If this hypothesis were true, it would be expected to find differences in renal gene expression between normal and animal models of the disease such as the CLCN-5 and the megalin knock-out mice. Consequently, experiments aimed at the characterisation of gene expression in CLCN-5 KO and normal mice kidneys could be carried out to investigate the possibility of an altered renal gene expression in these mice.

It has also been reported that in renal cells from CLCN5 KO mice and Dent's disease patients there is an altered delivery of apical proteins to the BBM [48;311]. Therefore, the ClC-5 protein may be involved in sorting proteins from the ER and Golgi to the BBM. Proteomic strategies as those used in Chapter 5 could be used to investigate this hypothesis further and to determine which apical proteins, if any, fail to be delivered to the BBM in PT cells of CLCN5 KO mice.

I have also speculated that there may be an altered bioactivity in the tubular fluid of patients with tubular proteinuria, which is based on the observation that the peptide hormone composition of FS urine is different from that of normal subjects. Functional experiments could be carried out in order to investigate the extent of this alteration; cultured renal tubular cells could be exposed to FS and normal urine and/or urinary proteins. The response of these cells could then be investigated by, for example, measuring the phosphorylation of known components of signal transduction pathways.

In conclusion, the experimental work carried out during the studies presented in this thesis identified an approach for the extraction of polypeptides from urine in a form compatible with MS analysis. Implementation of these methods for the analysis of FS urine (with emphasis on Dent's disease) gave insights into the nature of low molecular weight proteinuria and suggested that the reuptake of proteins from the glomerular filtrate shows some kind of specificity and it is not as promiscuous as previously thought. The presence of bioactive peptides in Dent's and normal urine and the finding that proteins with signalling roles are located on apical membranes support the notion of an intracrine system operating in the lumen of the healthy tubules. An alteration on the hormonal composition of the tubular fluid, as in diseases that lead to proteinuria, may contribute to

the progression of these diseases with the end result of renal dysfunction and ultimately kidney failure.

7. Apendices

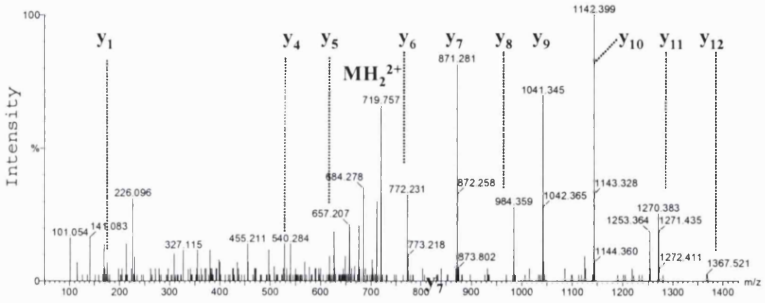
I. Examples of tandem mass spectra

Spectra of putative bioactive peptides are shown. These MS/MS spectra were obtained by analyzing the peptide fraction of renal Fanconi syndrome urine [272]. The experiments are described in some detail in Chapter 3.2 of this thesis. Only the y-ion series are labelled for clarity.

IGF-I

APQTGIVDECCFR

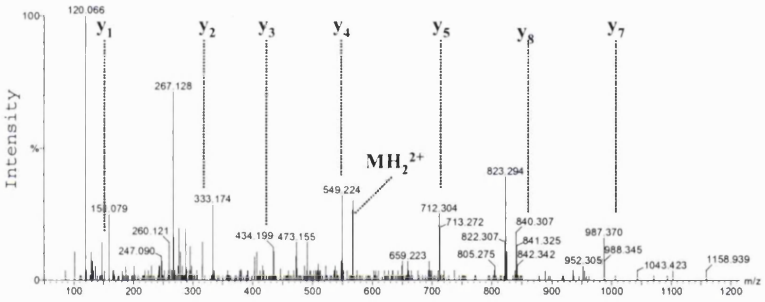
[M+2H] $^{2+}$: m/z 719.72



IGF-II

FFQYDTWK

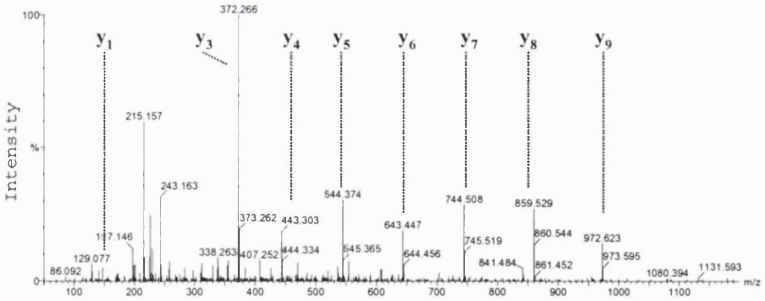
[M+2H] $^{2+}$: m/z 567.77



PEDF

ELLDTVTAPQK

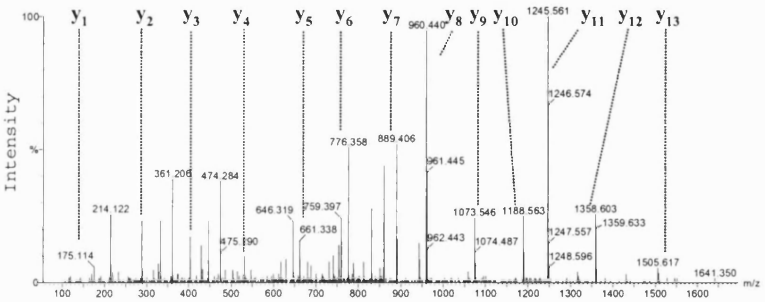
[M+2H] $^{2+}$: m/z 607.84



BMP-1

AAAFLGDIALDEED LR

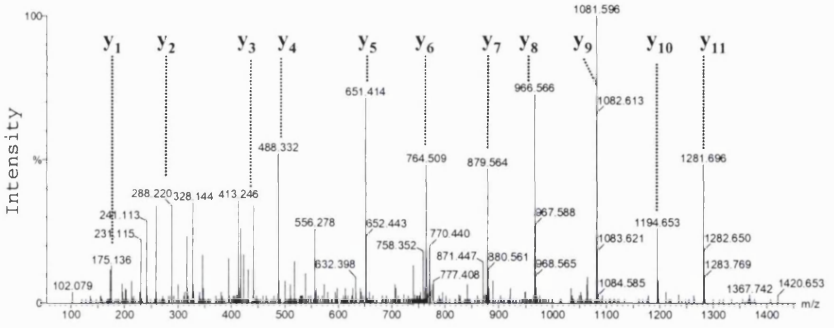
[M+2H] $^{2+}$: m/z 859.94



PBP

EESLDSDLYAELR

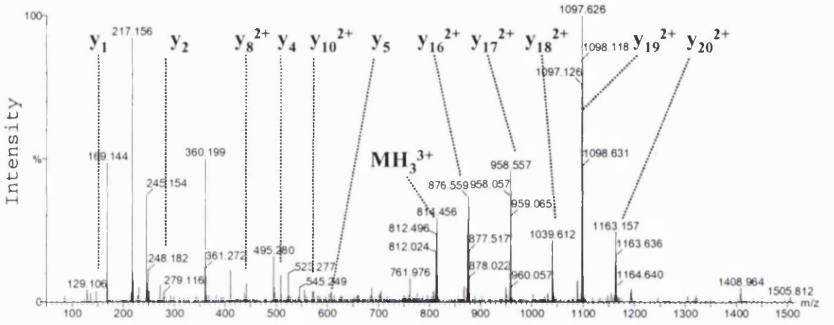
[M+2H] $^{2+}$: m/z 770.40



CC-3

IMDYYETNSQCSKP GIVFITK

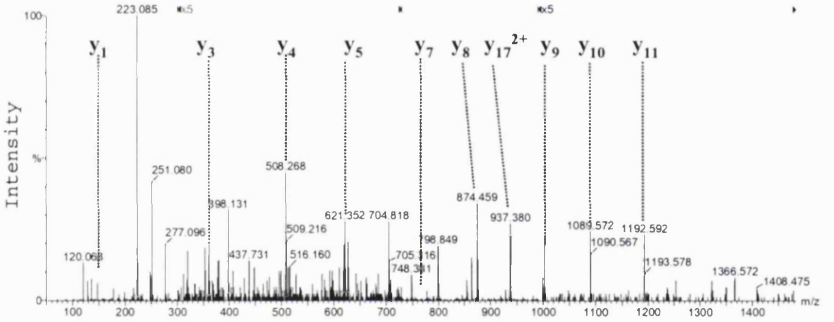
[M+3H] $^{3+}$: m/z 813.10



LEUKOTACTIN-1

SYFETSSECSKPGVI FLTK

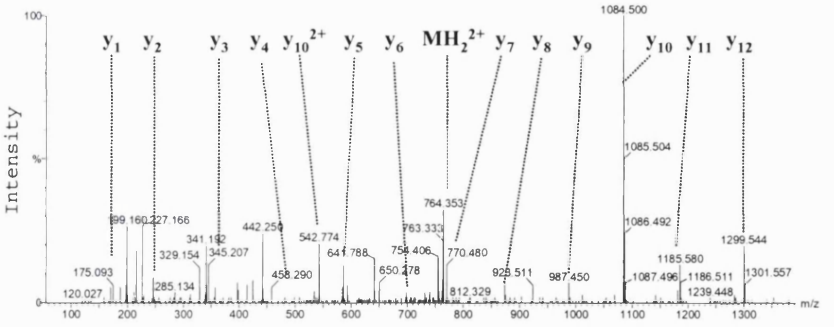
[M+3H] $^{3+}$: m/z 708.25



SDF-1

ILNTPNCALQIVAR

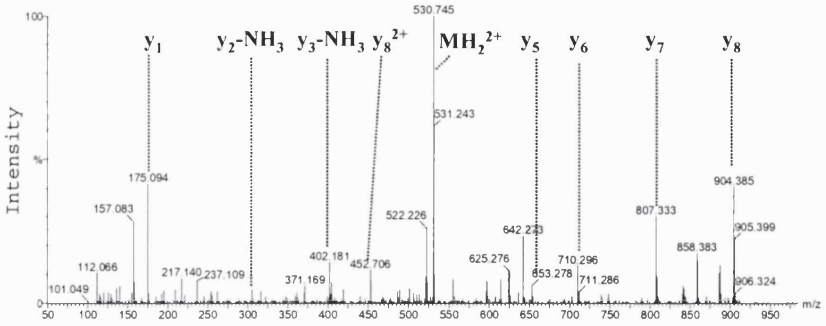
[M+2H] $^{2+}$: m/z 763.35



BK

RPPGFSPFR

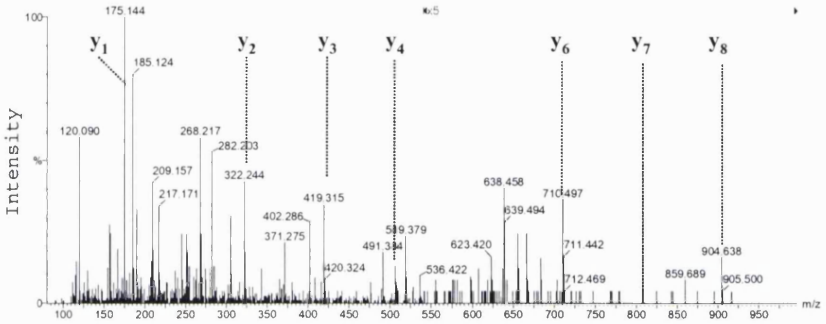
[M+2H] $^{2+}$: m/z 530.72



KALLIDIN II

KRPPGFSPFR

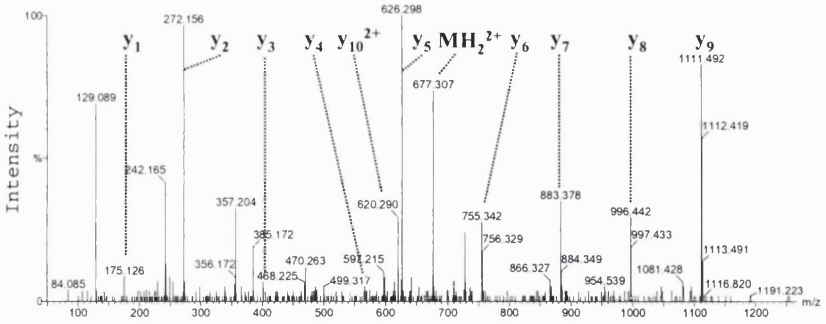
[M+3H] $^{3+}$: m/z 396.93



GUANYLIN

LKDLQEPQEPR

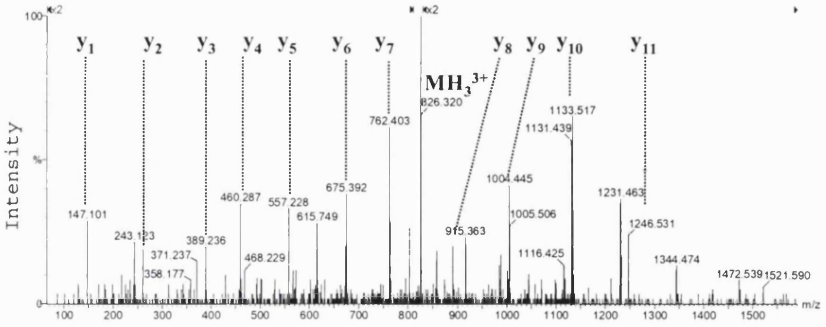
[M+2H] $^{2+}$: m/z 676.79



CHROMOGRANIN A

HSGFEDELSEVLEN QSSQAELK

[M+3H] $^{3+}$: m/z 826.00



II. Proteins Detected in FS Urine and Control

Proteins identified during the experiments described in Chapter 3.2 are tabulated. Analyses were standardized to 2.5 μmol of createnine. The results are expressed as number of times the named gene product was identified over the number of samples analysed.

Protein Name	gi # (NCBI)	Lowe*	ADIF*	Dent's*	Normal*
Secreted Proteins					
Beta-trace	410564	0/1	0/1	2/3	0/3
Latent transforming growth factor binding Protein 2	4557733	1/1	0/1	3/3	0/3
Pancreatic ribonuclease 1	35281	1/1	0/1	2/3	0/3
Pancreatic secretory trypsin inhibitor	225872	1/1	0/1	2/3	0/3
Pepsinogen A activation peptide	223220	0/1	0/1	2/3	0/3
Prolactin-inducible protein	4505821	0/1	1/1	0/3	0/3
Proline rich acidic protein	16265875	1/1	0/1	2/3	0/3
Secreted protein, acidic, cysteine rich	14722770	1/1	0/1	0/3	0/3
Secretory leukocyte protease inhibitor; Mucus proteinase inhibitor	4507065	0/1	0/1	1/3	0/3
Uveal autoantigen	12240161	1/1	0/1	0/3	0/3
Plasma Proteins					
Albumin	28590	1/1	1/1	3/3	2/3
Alpha-1-antitrypsin	1703025	1/1	1/1	1/3	0/3
Alpha-1-glycoprotein	112892	1/1	1/1	1/3	0/3
Alpha-1-microglobulin	4502067	1/1	1/1	3/3	1/3
Alpha-2-antiplasmin precursor	178751	1/1	0/1	1/3	0/3
Alpha-2-glycoprotein, zinc	4502337	0/1	0/1	3/3	0/3
Alpha-2-HS-glycoprotein	112909	1/1	1/1	1/3	2/3
Apolipoprotein E	178849	0/1	0/1	1/3	0/3
Apolipoprotein CI	178834	1/1	0/1	0/3	0/3
Apolipoprotein F	4502165	1/1	0/1	0/3	0/3
Apolipoprotein A-I	4557321	1/1	1/1	2/3	0/3
Apolipoprotein A-Ii	4502149	1/1	1/1	3/3	0/3
Apolipoprotein A-IV	178779	1/1	1/1	2/3	0/3
Apolipoprotein C-Ii	4502159	1/1	1/1	1/3	0/3
Apolipoprotein CIII	224917	0/1	1/1	3/3	0/3
Beta-2-microglobulin	1195503	1/1	1/1	3/3	1/3
Beta-fibrinogen	532595	1/1	1/1	3/3	0/3
Beta-fibrinogen	399492	0/1	1/1	1/3	0/3
B-factor, properdin	13278732	0/1	0/1	2/3	0/3
C1 inhibitor	15310473	0/1	1/1	1/3	0/3
Clusterin	4502905	1/1	0/1	2/3	0/3
Coagulation factor VII	6753806	0/1	1/1	0/3	0/3
Complement 9	4502511	0/1	0/1	2/3	0/3
Complement component 3,	13649325	0/1	1/1	3/3	0/3
Complement component 4	4502501	1/1	1/1	3/3	0/3
Complement component D	4503309	1/1	1/1	3/3	0/3
Cystatin A	4885165	0/1	0/1	0/3	1/3
Cystatin C	181387	1/1	1/1	3/3	1/3

Cystatin M	4503113	1/1	1/1	3/3	0/3
Fibrinogen, alpha chain	4503689	0/1	1/1	2/3	0/3
Gelsolin	4504165	0/1	0/1	2/3	0/3
Haptomyoglobin	3337390	0/1	0/1	2/3	0/3
Hemoglobin alpha chain	122412	0/1	0/1	0/3	1/3
Hemoglobin beta chain	122669	0/1	0/1	0/3	1/3
Hemopexin	14773705	0/1	1/1	1/3	0/3
Ig kappa chain	2623585	1/1	1/1	3/3	0/3
Ig lambda chain	87886	1/1	1/1	3/3	0/3
IGF binding protein 2	306926	1/1	0/1	3/3	0/3
IGF binding protein 4	10835021	1/1	0/1	3/3	0/3
IGF binding protein 6	11321593	1/1	1/1	3/3	0/3
Inter-alpha (globulin inhibitor), H2 polypeptide	4504783	0/1	0/1	1/3	0/3
Kazal type 5 serine proteinase inhibitor	5803219	1/1	1/1	2/3	0/3
Leuserpin (thrombin inhibitor)	187236	0/1	1/1	1/3	0/3
Lysozyme	4557894	0/1	0/1	3/3	1/3
Mannan-binding lectin serine protease 2 (bactericidal)	5729915	0/1	1/1	1/3	0/3
MHC class I antigen	4521334	0/1	1/1	3/3	0/3
Plasminogen	387031	0/1	0/1	1/3	0/3
Retinol-binding Protein	230284	1/1	1/1	2/3	0/3
Serum Amyloid 4A	825716	1/1	1/1	3/3	0/3
Transferrin	7245524	0/1	0/1	2/3	0/3
Transthyretin	339685	1/1	1/1	3/3	0/3
Bone formation					
Biglycan	4502403	0/1	1/1	0/3	0/3
Matrix Gla protein (inhibitor of bone formation)	4505179	1/1	0/1	3/3	0/3
Osteoblast protein	7661714	0/1	0/1	1/3	0/3
Osteopontin	129260	1/1	1/1	3/3	3/3
Sclerosin (inhibitor of bone formation)	13376846	0/1	0/1	1/3	0/3
Membrane Associated Proteins					
ADP-ribosyltransferase	14727509	0/1	1/1	0/3	0/3
CD59 (AA 2-103)	1340180	1/1	0/1	1/3	1/3
E48 antigen	1519481	1/1	0/1	2/3	0/3
Ems1	14767683	0/1	1/1	0/3	0/3
Fn14 for type 1 transmembrane protein	7706186	1/1	0/1	0/3	0/3
Guanylate cyclase activator 2A	14742571	1/1	1/1	2/3	0/3
Integrin alpha-7 precursor	4504753	0/1	1/1	2/3	0/3
K12 protein precursor	4506869	0/1	1/1	1/3	0/3
Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	5174521	1/1	1/1	1/3	0/3
Meprin (PABA peptide hydrolase) type I membrane protein; (a peptidase)	13643919	0/1	1/1	1/3	0/3
Mesothelin	14777095	0/1	1/1	0/3	0/3
Pancreatic secretory granule membrane major glycoprotein GP2	6539530	1/1	0/1	1/3	0/3
Prion protein	4506113	1/1	0/1	1/3	0/3
Prostaglandin D2 synthase (found in normal urine)	4506251	0/1	0/1	3/3	0/3

Retinoic acid receptor responder 2 (tazarotene induced)	4506427	0/1	0/1	2/3	0/3
Sodium/potassium-transporting ATPase gamma chain	2506207	0/1	0/1	0/3	1/3
Uromodulin	137116	0/1	1/1	2/3	3/3
Proteins Involved in Protein Trafficking					
Asialoglycoprotein receptor 2	4502253	0/1	1/1	0/3	0/3
Polymeric immunoglobulin receptor	14739061	0/1	0/1	1/3	0/3
Sortilin-related receptor	13639866	1/1	1/1	1/3	0/3
Vacuolar ATPase	14768401	0/1	1/1	0/3	0/3
Growth factors/hormones					
Adrenomedulin	4501945	0/1	0/1	1/3	0/3
Angiogenin	4557313	0/1	0/1	1/3	0/3
Bone morphogenic protein 1	4502421	1/1	1/1	0/3	0/3
Bradykinin (in kininogen)	4504893	1/1	1/1	2/3	1/3
Calprotectin	806925	1/1	0/1	0/3	0/3
Chemokin; member 15	4759072	0/1	0/1	2/3	0/3
Chemokine 1	4506831	0/1	0/1	1/3	0/3
Chemokine CC-3, member 14	4759070	0/1	0/1	3/3	0/3
Chromogranin A	1070547	1/1	1/1	1/3	0/3
Defensin alpha	4758146	1/1	1/1	3/3	2/3
Defensin beta	4885146	1/1	0/1	0/3	0/3
Dermcidin (survival-promoting peptide)	16751921	1/1	0/1	3/3	0/3
Diazepan bingding inhibitor (possible neuropeptide)	10140853	1/1	1/1	2/3	0/3
Gastric inhibitory polypeptide (glucagon like)	4758436	0/1	0/1	2/3	0/3
Granin-like neuroendocrine precursor	7019519	0/1	1/1	0/3	0/3
Hepcidin antimicrobial peptide	10863973	0/1	0/1	2/3	0/3
IGF-I	6832905	0/1	0/1	3/3	0/3
IGF-II	2135482	0/1	1/1	2/3	0/3
INL 3; relasin-like factor	3851207	0/1	0/1	1/3	0/3
Kallidin II	229138	0/1	1/1	3/3	0/3
Kininogen	4504893	0/1	1/1	3/3	0/3
Megakaryocyte stimulating factor	12720074	0/1	0/1	1/3	0/3
Motilin	4557034	0/1	1/1	0/3	0/3
Neuropeptide Y	4505449	0/1	1/1	0/3	0/3
Neutrophil activating peptide-2 (Dsdly)	1942554	1/1	0/1	0/3	0/3
Pigment epithelium-derived factor	1144299	0/1	1/1	3/3	0/3
Platelet basic protein	4505981	0/1	0/1	3/3	0/3
Platelet-derived growth factor	6119621	1/1	0/1	1/3	0/3
Proenkephalin	5453876	0/1	1/1	0/3	0/3
S100 calcium binding protein (psoriasin)	4389153	0/1	1/1	3/3	0/3
Secretogranin V	112850	1/1	0/1	0/3	0/3
Stromal cell derived factor (cytokine homology)	13399638	0/1	0/1	2/3	0/3
Survival/evasion peptide	15375076	0/1	1/1	0/3	0/3
VGF nerve growth factor inducible	14747294	0/1	0/1	0/3	2/3
Vitronectin	72146	0/1	1/1	0/3	0/3
ECM proteins					

Biglycan	4502403	0/1	1/1	0/3	0/3
Procadherin alpha 6	9256592	1/1	0/1	0/3	0/3
Cadherin 13	9789905	0/1	1/1	2/3	0/3
Collagen alpha 1 (I)	8134354	1/1	1/1	2/3	1/3
Collagen alpha 2 (I)	1705534	1/1	0/1	3/3	0/3
Collagen type IV, alpha 2	17475174	0/1	0/1	1/3	0/3
Collagen type VI, alpha 3		1/1	0/1	1/3	0/3
Desmocollin 1	457464	1/1	1/1	2/3	0/3
Desmocollin 2	13435366	0/1	0/1	1/3	0/3
Desmocollin 4	13435369	0/1	0/1	2/3	0/3
Fibrillin 2	4503667	0/1	0/1	1/3	0/3
Fibronectin	2497972	1/1	0/1	0/3	0/3
Lumican	1708878	0/1	0/1	1/3	0/3
Lysyl oxidase	187278	0/1	1/1	2/3	0/3
lysyl oxidase-like 1	15929303	0/1	0/1	3/3	0/3
MT-MMP	2135759	1/1	0/1	1/3	0/3
Dystroglycan 1	4758116	0/1	1/1	1/3	0/3
Spondin 1	14762106	1/1	0/1	1/3	0/3
Spondin 2	6912682	0/1	1/1	1/3	0/3
Neurexin	1083729	0/1	1/1	0/3	1/3
Perlecan	11602963	0/1	1/1	2/3	0/3
Proteinase 3 (PRTN3)	17455614	0/1	0/1	1/3	0/3
Proteoglycan 1, secretory granule; serglycin	450600445	0/1	0/1	1/3	0/3
Tubulointerstitial nephritis antigen	6449324	0/1	0/1	1/3	0/3
Cellular proteins					
Beta-actin	481515	1/1	0/1	1/3	0/3
Cyclophilin A	3659980	0/1	1/1	3/3	0/3
Diazepan bingding inhibitor	10140853	0/1	0/1	2/3	0/3
Fatty acid binding protein 1	4557577	0/1	0/1	1/3	0/3
Filamin 2	8885790	0/1	0/1	1/3	0/3
Glutaredoxin	4504025	0/1	1/1	0/3	0/3
IsoAsp protein carboxyl methyltransferase	1096024	0/1	0/1	1/3	0/3
Myoglobin	386872	0/1	1/1	1/3	1/3
Phosphatidylethanolamine-binding protein (Raf kinase inhibitor protein; RKIP)	14585855	0/1	0/1	2/3	0/3
Profilin	4826898	0/1	1/1	1/3	0/3
Ribonuclease 2 (lysosomal)	1350811	0/1	0/1	2/3	0/3
Similar to glucosamine-6-sulfatase (lysosomal)	14786242	0/1	1/1	2/3	2/3
Small proline-rich protein 3	4885607	0/1	0/1	0/3	1/3
Superoxide dismutase	515251	1/1	1/1	1/3	0/3
Thioredoxin	4507745	1/1	0/1	0/3	0/3
Thymosin beta-4	136580	0/1	0/1	1/3	0/3
Ubiquitin B	11024714	1/1	1/1	2/3	2/3
Other					
ADAM-TS precursors (metalloproteinase)	12644483	0/1	1/1	0/3	0/3

Beta-microseminoprotein	17451678	1/1	0/1	2/3	0/3
DJ14N1.2 calcium binding protein	12314268	0/1	1/1	0/3	0/3
DWF-1 protein; Ellis van Creveld syndrome protein	7657073	0/1	0/1	1/3	0/3
Eosinophil-derived neurotoxin; ribounuclease 2	4506549	0/1	0/1	0/3	1/3
Gastric-associated differentially-expressed protein YA61P	4758436	0/1	0/1	2/3	0/3
Prostate stem cell antigen	5031995	0/1	0/1	0/3	1/3
Putative gene	20784470	1/1	0/1	1/3	0/3
Semenogelin I	4506883	1/1	1/1	0/3	0/3
Semenogelin II	4506885	1/1	1/1	0/3	2/3
Seminal peptide 20	225667	0/1	1/1	0/3	0/3
SPR1	7662665	0/1	0/1	0/3	1/3
Sulfated glycoprotein-2	338057	1/1	1/1	0/3	0/3
Unnamed protein product	16551700	0/1	0/1	2/3	0/3
Unnamed protein product	16554039	0/1	0/1	1/3	0/3
Unnamed protein product	7460042; 10432882	0/1	0/1	0/3	1/3
Unnamed protein product	12383068, 10438196, 17028332	0/1	0/1	1/3	0/3

* Number of protein hits per number of samples analyzed

III. Proteins detected in brush border membranes isolated by magnesium precipitation and differencial centrifugation

Brush border membranes were isolated from rat renal cortex following the procedure of Biber [181] by Dr. J. Marks (Royal Free Hospital, London). The table lists all the proteins that were identified in these preparations. Experiments are described in Chapter 5.1. Proteins are grouped in arbitrarily chosen functional categories.

Accesion No.	Protein Name	Mr	pI	PEP	SCR	COV
Carriers						
13540689	hemoglobin alpha-chain	6927	6.62	1	70	18
27704910	diazepam binding inhibitor; Diazepam binding inhibitor (GABA receptor modulator, acyl-Coenxyme A bi	10021	8.78	1	38	20
27710758	hemoglobin, beta	15969	7.88	12	519	69
27709532	similar to Hemoglobin beta chain, minor-form	15972	8.91	9	371	53
27720219	hemoglobin beta chain, minor - rat	15982	9.04	10	444	61
27714209	major beta-hemoglobin	15983	7.88	5	220	36
7441446	similar to solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), membe	32817	9.55	3	127	11
111734	albumin	68674	6.09	5	297	8
8394307	solute carrier family 12, member 1; Solute carrier family 12, member 1	120519	8.05	2	40	2
Channels						
91940	chloride intracellular channel 5	28281	5.64	6	93	10
6981170	intracellular chloride ion channel protein p64H1	28615	5.92	7	168	20
1170902	Aquaporin-CHIP (Aquaporin 1)	28812	7.7	3	129	10
27677076	voltage-dependent anion channel 1 [Mus musculus]	30737	8.62	2	77	8
13937379	barttin	33873	4.36	1	36	4
Membrane trafficking						
6681095	ATPase, vacuolar, 14 kD	13362	5.52	1	50	10
17902245	similar to ATPase, H+ transporting, lysosomal 13kD, V1 subunit G isoform 1; ATPase, H+ transporting	13702	6.75	2	79	27
1351954	similar to Vacuolar ATP synthase subunit d (V-ATPase d subunit)	16865	5.82	6	265	45
18426816	similar to ATPase, H+ transporting, lysosomal 31kD, V1 subunit E isoform 1	26127	8.44	4	102	15
7159085	similar to Vacuolar ATP synthase subunit H (V-ATPase H subunit) (Vacuolar proton pump H subunit)	26765	5.95	1	74	8
728819	similar to ATPase, H+ transporting, lysosomal 34kD, V1 subunit D; ATPase, H+ transporting lysosomal	28291	9.44	5	184	13
1620451	syntaxin 3	33236	5.32	3	154	12
27673084	lysosomal membrane glycoprotein-type B precursor	43100	7.15	1	43	2
6981146	ATPase, H+ transporting, lysosomal (vacuolar proton pump), beta 56/58 kDa, isoform 2	56515	5.57	1	83	2
11262122	similar to ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B, isoform 1; ATPase,	62766	5.2	7	282	12
27709748	syntaxin binding protein Munc18-2	66654	6.31	2	96	6

27708570	similar to ATPase, H+ transporting, lysosomal (vacuolar proton pump), alpha 70 kDa, isoform 1	71924	7.53	2	61	6
11560095	GTP-binding regulatory protein Gs alpha-XL chain - rat	91566	5.23	1	69	1
Metabolism						
25282441	cytochrome-c oxidase (EC 1.9.3.1) chain IV, cardiac - rat (fragment)	2623	5.44	1	33	52
517440	multidrug resistance protein 4	7541	8.01	1	81	20
8248819	similar to ATP synthase, H+ transporting, mitochondrial F0 complex, subunit f, isoform 2; hypotheti	10457	9.69	1	47	12
7514079	cytochrome c, somatic [Mus musculus]	11598	9.61	2	114	20
13929166	thioredoxin	11666	4.8	1	43	12
27717207	peroxiredoxin 6	22165	8.94	1	41	6
27709856	similar to actin gamma, cytoskeletal - rat	22196	5.5	2	72	11
27705116	glutathione S-transferase, alpha 1; Glutathione-S-transferase, alpha type (Ya)	25303	8.78	3	99	14
119740	similar to palmitoyl protein thioesterase-like protein [Mus musculus]	25590	5.23	2	41	3
1050930	similar to Esterase D	28065	5.95	1	60	7
13592043	hydroxyacyl glutathione hydrolase; glyoxalase II; round spermatid protein RSP29	28878	6.46	1	63	4
27708936	ATP synthase gamma chain, mitochondrial	30172	8.87	1	57	4
27668132	2-4-dienoyl-Coenzyme A reductase 2, peroxisomal; putative peroxisomal 2,4-dienoyl-CoA reductase	31272	8.51	4	132	10
27697107	ketohexokinase	32729	6.24	1	67	5
13994119	N-ethylmaleimide sensitive fusion protein attachment protein alpha	33171	5.3	3	142	15
27659444	monoglyceride lipase	33478	6.92	1	60	4
27665018	Sulfotransferase K2 (rSULT1C2A)	34817	7	5	203	18
27664758	hydroxyarylamine sulfotransferase (EC 2.8.2.-) 2A - rat	34817	7	9	219	21
27720565	similar to glyceraldehyde-3-phosphate dehydrogenase [Mus musculus]	35526	6.45	1	120	5
4501887	retinol dehydrogenase type I	35639	9.14	1	31	3
27700285	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (38 kDa BFA-dependent ADP-ribosylation substrate)	35813	8.43	1	33	4
20302075	lactate dehydrogenase A	36427	8.45	1	68	4
13937391	malate dehydrogenase-like enzyme	36460	6.16	7	176	14
223556	aldo-keto reductase family 1, member A1; aldo-keto reductase family 1, member A1 (aldehyde reductas	36483	6.84	7	270	27
12018296	lactate dehydrogenase B; Lactate dehydrogenease B	36589	5.7	4	269	15
6980964	aldo-keto reductase family 7, member A2 (aflatoxin aldehyde reductase)	37574	6.27	1	57	4
27717949	long chain alpha-hydroxy acid oxidase =FMN-dependent alpha-hydroxy acid-oxidizing enzyme EC 1.1.3.1	39045	7.9	3	118	7
13929182	fructose-bisphosphate aldolase (EC 4.1.2.13) A - rat	39235	8.31	3	102	5
27687605	similar to aldolase B	39500	8.66	7	211	25
27685199	aldolase B	39579	8.66	7	124	20
6978809	FRUCTOSE-1,6-BISPHOSPHATASE (D-	39584	5.54	8	240	20

	FRUCTOSE-1,6-BISPHOSPHATE 1-PHOSPHOHYDROLASE) (FBPASE)					
19924057	Sorbitol dehydrogenase (L-iditol 2-dehydrogenase)	42807	6.83	1	42	4
2143593	gamma-butyrobetaine hydroxylase	44517	6.23	1	30	1
2792500	Phosphoglycerate kinase 1	44526	7.53	3	119	8
1709863	arginosuccinate synthetase 1	46467	7.63	1	46	3
11024674	isocitrate dehydrogenase 1; Isocitrate dehydrogenase 1, soluble	46705	6.53	1	35	2
13994159	enolase 1, alpha	47086	6.16	4	229	16
13928928	Alpha enolase (2-phospho-D-glycerate hydro-lyase) (Enolase 1)	47171	5.84	10	624	37
27681923	H^+ -transporting two-sector ATPase (EC 3.6.3.14) beta chain, mitochondrial - rat (fragment)	50738	4.9	3	100	9
27662142	glutathione synthetase; Glutathione synthetase gene	52312	5.48	2	58	6
13123941	aldehyde dehydrogenase family 9, subfamily A1; 4-trimethylaminobutyraldehyde dehydrogenase [Rattus	53618	6.57	2	89	6
5729999	alkaline phosphatase (EC 3.1.3.1), hepatic precursor - rat	57774	6.26	2	79	3
4502211	Cytochrome P450 4A2 precursor (CYPIVA2) (Lauric acid omega-hydroxylase) (P450-LA-omega 2) (P450 K-5	57932	9.18	1	54	2
4504301	Cytochrome P450 4A3 (CYPIVA3) (Lauric acid omega-hydroxylase) (P450-LA-omega 3)	58195	9.18	4	236	9
461475	ATP synthase alpha chain, mitochondrial precursor	58790	9.22	2	81	5
8393428	Liver carboxylesterase 4 precursor (Carboxyesterase ES-4) (Microsomal palmitoyl-CoA hydrolase) (Kid	62235	6.29	2	56	5
27692078	TRANSKETOLASE (TK)	67601	7.23	1	49	3
27705852	similar to aldehyde dehydrogenase 8A1 isoform 1; aldehyde dehydrogenase 12; aldehyde dehydrogenase	72498	8.79	1	85	3
22096350	Glutamylcysteine gamma synthetase light chain; glutamate-cysteine ligase, catalytic subunit [Rattus	72573	5.41	6	266	10
27721405	similar to Arylsulfatase B	75010	9.1	1	45	2
27667796	multifunctional protein 2	79141	8.49	1	43	1
18034791	ferroxidase (EC 1.16.3.1) precursor - rat	120588	5.39	1	31	<1
92930	similar to Putative adenosylhomocysteinase 3 (S-adenosyl-L-homocysteine hydrolase) (AdoHcyase) [Rat	131257	7.59	3	41	1
117164	similar to Maltase-glucoamylase, intestinal	773446	5.23	4	183	<1
Peptidases						
27665236	Glutamyl aminopeptidase (EAP) (Aminopeptidase A) (APA)	15090	7.03	1	73	13
5803225	lysozyme	16718	9.32	4	99	22
92350	Microsomal dipeptidase precursor (MDP) (Dehydropeptidase-I) (Renal dipeptidase) (RDP)	45477	5.68	3	116	10
27721231	kidney-derived aspartic protease-like protein	45601	9.07	1	44	4
27660601	similar to Xaa-Pro dipeptidase (X-Pro dipeptidase) (Proline dipeptidase) (Prolidase) (Imidodipeptid	52373	6.96	1	58	4
27702708	X-prolyl aminopeptidase (aminopeptidase P) 2,	76032	5.49	6	274	10

	membrane-bound					
130751	membrane metallo endopeptidase; Membrane metallo-endopeptidase (neutral endopeptidase/enkephalinase	85741	5.75	5	263	8
27731185	dipeptidyl-peptidase IV (EC 3.4.14.5), membrane-bound form precursor - rat	90869	5.88	2	42	1
12229956	aminopeptidase A; glutamyl aminopeptidase; APA; angiotensinase; BP-1/6C3	107927	5.24	29	994	27
16757986	aminopeptidase M	109170	5.34	7	386	10
14165170	kidney aminopeptidase M; Leucine arylaminopeptidase 1	109380	5.3	14	805	16
Receptors						
6678467	folate receptor 1 (adult)	29438	6.49	2	31	7
6671746	arginine-vasopressin V1b receptor	46668	9.36	1	29	1
4433351	beta-1-adrenergic receptor - rat	50172	9.12	1	32	1
4894188	similar to laminin receptor	53435	9.22	2	34	3
111948	similar to G-protein coupled receptor 112 [Homo sapiens]	319696	5.83	3	35	<1
19923092	cubilin; cubilin (intrinsic factor-cobalamin receptor)	398732	5.4	1	51	<1
27712982	low density lipoprotein receptor-related protein 2; glycoprotein 330; low density lipoprotein-relat	518937	5.03	62	2378	13
Signal transduction						
9910340	NHE3 kinase A regulatory protein E3KARP					
17985949	GTP-binding regulatory protein G42 alpha chain - rat (fragments)	8742	4.43	3	175	51
27712230	similar to Annexin A13 (Annexin XIII) (Annexin, intestine-specific) (ISA)	10964	4.97	1	61	15
400240	protein kinase, cAMP-dependent, regulatory, type 2, alpha	28250	5.9	2	112	9
12018248	similar to calmodulin	35066	7.72	1	36	4
27672726	Annexin V	35401	4.97	5	175	11
15100179	Annexin A4 (Annexin IV) (Lipocortin IV) (36 kDa zymogen granule membrane associated protein) (ZAP36	35852	5.31	1	92	5
119146	Ras-related GTP-binding protein RAGA [Homo sapiens]	36543	7.62	1	37	3
6755963	phospholipid scramblase 1	36687	4.82	1	72	5
728931	guanine nucleotide-binding protein, beta-2 subunit [Mus musculus]	37307	5.6	5	229	12
27661203	Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1 (Transducin beta chain 1)	37369	5.47	4	163	8
8394381	annexin II - rat	38951	8.07	7	433	25
13928688	GTP-binding protein (G-alpha-i2)	40473	5.28	2	39	7
27710072	guanine nucleotide binding, protein, alpha inhibiting polypeptide 3	40496	5.5	2	36	7
601865	guanine nucleotide-binding protein alpha 11 subunit	42000	5.91	1	42	4
8393446	guanine nucleotide binding protein (G protein) alpha 12	44037	9.84	1	61	2
27709414	GNAS complex locus; Guanine nucleotide-binding protein G-s, alpha subunit; guanine nucleotide bindi	45635	5.69	3	152	8
92233	guanosine diphosphate dissociation inhibitor 3; rab GDI beta; guanosine diphosphate (GDP) dissocia	50653	5.66	3	99	6

15375320	Diphor-1	52218	5.31	13	271	21
16758644	guanylate kinase associated protein	74187	5.35	1	34	1
19705537	annexin VI	75706	5.39	3	101	6
130223	similar to lymphocyte alpha-kinase [Homo sapiens]	109722	6.09	2	49	<1
27661169	similar to phosphatidylinositol polyphosphate 5-phosphotase isoform b [Homo sapiens]	115611	8.39	2	32	1
1703279	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta 1 (PLC-beta-1) (Phospholipase C-beta	138257	5.86	1	60	<1
9506737	Ser-Thr protein kinase related to the myotonic dystrophy protein kinase	196940	6.3	1	40	<1
8393706	renin-binding protein	48360	5.61	1	29	1
27713828	rab GDI alpha	50488	5.05	1	49	4
Structural						
16758404	keratin K5	9390	5.34	1	55	15
27667246	profilin	14978	8.46	2	113	20
19705431	Myosin light chain alkali, smooth-muscle isoform (MLC3SM)	16964	4.46	1	61	8
27706040	similar to coactosin-like 1; coactosin-like protein [Mus musculus]	31298	7.6	5	198	13
2493465	similar to actin related protein 2/3 complex subunit 2; ARP2/3 protein complex subunit 34 [Homo sap	34469	6.84	2	43	3
1304381	ERM-binding phosphoprotein	38807	5.7	3	41	7
27678772	similar to tubulin alpha-2 chain - mouse (fragments)	39075	4.69	2	92	8
13928690	actin beta - rat	41724	5.29	21	531	48
27715349	similar to F-actin capping protein alpha-1 subunit (CapZ alpha-1)	48160	6.05	1	91	2
205169	tubulin, alpha 4; tubulin alpha 4 [Mus musculus]	49892	4.95	4	231	13
267413	tubulin beta chain 15 - rat	49905	4.79	1	74	3
16758754	tubulin alpha	50210	4.94	1	51	4
17105370	similar to Tubulin alpha-1 chain (Alpha-tubulin 1)	50248	4.94	4	226	14
16758474	EZRIN	54140	6.16	7	175	16
27720175	clathrin assembly protein short form	64616	9.02	2	138	4
1421099	moesin	67697	6.16	2	83	3
238482	T-plastin	70260	5.42	5	160	8
16758040	similar to villin [Mus musculus]	70547	6	2	99	3
204582	mucin and cadherin-like; mu-protocadherin	90920	4.69	3	168	3
1352184	Alpha-actinin 4 (Non-muscle alpha-actinin 4) (F-actin cross linking protein)	104720	5.24	3	163	4
841197	similar to Keratin, type II cytoskeletal 2 oral (Cytokeratin 2P) (K2P) (CK 2P)	107656	5.61	4	76	2
67548	similar to myosin VIIb [Mus musculus]	167030	9.38	1	49	<1
68186	similar to keratin protein K6irs [Homo sapiens]	181694	5.53	1	76	<1
27714457	clathrin, heavy polypeptide (Hc)	191477	5.5	17	878	14
19424178	similar to keratin complex 2, basic, gene 20 [Mus musculus]	238777	6.04	3	61	1
27659024	similar to protocadherin 16 precursor; fibroblast cadherin FIB1; cadherin 19; fibroblast cadherin 1	320951	4.95	2	31	<1
6981210	similar to 1 beta dynein heavy chain	530771	5.92	2	31	<1

12018320	PDZ domain containing 1	56853	5.23	5	54	16
Protein synthesis						
8394009	histone H2B	13766	10.39	1	33	7
20806141	14.5 kDa translational inhibitor protein (Perchrolic acid soluble protein)	14295	7.79	5	234	37
27733133	Elongation factor 1-alpha 1 (EF-1-alpha-1) (Elongation factor 1 A-1) (eEF1A-1) (Elongation factor T	50082	9.1	2	91	4
Transporters						
27708940	FXYD domain-containing ion transport regulator 2 isoform b; ATPase, Na+/K+ transporting, gamma 1	7234	8.89	1	56	21
13592101	neutral and basic amino acid transporter protein	15958	5.16	1	47	7
13591953	similar to apical iodide transporter , putative [Homo sapiens]	21106	6.13	1	78	5
27708308	Sodium/potassium-transporting ATPase beta-1 chain	35179	8.83	4	131	15
111409	NaPi-2 alpha	36045	5.98	1	42	2
694108	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 3;	39419	9.41	2	45	3
8393101	similar to amino acid transporter XAT2 [Mus musculus]	43357	9.75	1	50	3
27719091	similar to 597 aa protein related to Na/glucose cotransporters	45331	8.99	1	46	2
27687753	similar to ABC transporter ABCG2 [Homo sapiens]	59678	8.68	2	81	3
9506497	high-affinity carntine transporter, CT1 - rat	62398	7.59	1	57	3
13591951	sodium-coupled ascorbic acid transporter 1	65221	6.09	4	155	8
7513988	low affinity Na-dependent glucose transporter (SGLT2)	72914	8.04	7	334	10
22538395	solute carrier family 34 (sodium phosphate), member 2	75942	7.85	2	46	2
27662850	solute carrier family 3, member 1	78457	5.48	4	44	7
13591894	Na,K-ATPase alpha-1 subunit	113192	5.27	4	215	4
27680713	Multidrug resistance protein 1 (P-glycoprotein 1)	141298	8.62	1	47	1
Other/unknown						
27715409	vesicle-associated membrane protein 8 (endobrevin)	11313	8.93	1	47	10
16758390	membrane-associated protein 17	12235	4.8	1	74	10
20376826	similar to RIKEN cDNA 0610006H10 gene [Mus musculus]	17077	4.81	2	62	10
13925523	similar to crystallin, lamda 1 [Mus musculus]	17451	6.08	2	49	9
1857139	kidney-specific membrane protein	25210	6.22	3	73	18
13591955	prominin - rat	96569	6.14	2	100	2
847653	H4 histone family, member A [Homo sapiens]	11360	11.36	2	86	19
3868786	similar to RIKEN cDNA 5730403E06 [Mus musculus]	11483	7.9	1	74	13
16758110	macrophage migration inhibitory factor	12538	7.74	1	48	7
8048915	similar to envelope protein	13627	9.64	2	37	6
25453414	vesicle associated membrane protein 2B	14500	5.44	1	63	12
27683583	similar to RIKEN cDNA 4930524N10 [Mus musculus]	15662	8.63	1	30	3
27672073	beta 2 globin [rats, Sprague-Dawley, Peptide, 146 aa]	15851	8.57	12	529	69

25742757	RS43 protein - rat (fragment)	16111	5.46	2	48	12
27716023	3-hydroxyanthranilate 3,4-dioxygenase	17372	5.5	1	55	8
25354513	peptidylprolyl isomerase A (cyclophilin A)	17863	8.34	4	217	30
92362	similar to RIKEN cDNA 1200011D11 [Mus musculus]	18098	4.72	3	75	30
285323	similar to pantophysin; Pan I; DNA segment, Chr 12, ERATO Doi 446, expressed [Mus musculus] [Rattus	18164	9.1	1	42	6
205632	destrin - rat	18375	7.78	1	43	6
13928998	cofilin 1; cofilin 1, non-muscle	18521	8.22	2	106	15
223096	cofilin 2, muscle [Mus musculus]	18698	7.66	2	56	13
396431	similar to mlrq-like protein [Mus musculus]	19689	9.11	1	56	6
27720877	ADP-ribosylation factor 6 [Homo sapiens]	20069	9.04	1	59	6
27696500	similar to RIKEN cDNA 1700025B18 gene [Mus musculus]	20787	8.73	1	75	11
27704574	hypothetical protein XP_243379	22922	8.75	1	32	4
6756041	hypothetical protein XP_228339	24006	9.38	1	30	2
27718935	trypsin (EC 3.4.21.4) II precursor - rat	26226	4.69	2	44	8
9507103	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; DNA segme	27754	4.73	2	147	10
27665254	phosphoglycerate mutase type B subunit	28828	7.07	3	60	13
204570	tyrosine 3/tryptophan 5 -monooxygenase activation protein, epsilon polypeptide; 14-3-3 epsilon; mit	29155	4.63	1	68	4
27670103	similar to EH-domain containing 1 [Mus musculus]	30308	8.52	1	32	3
1729977	glutamate-cysteine ligase , modifier subunit; glutamate cysteine ligase (gamma-glutamylcysteine syn	30529	5.36	3	65	10
1346320	similar to glycoprotein M6A [Homo sapiens]	31333	4.86	2	74	6
4557469	hypothetical protein XP_222453	32348	9.2	2	52	3
8393215	ADP,ATP carrier protein, heart/skeletal muscle isoform T1 (ADP/ATP translocase 1) (Adenine nucleoti	32968	9.81	2	57	8
17105346	similar to RIKEN cDNA 1110014J22 gene [Mus musculus]	33005	6.16	1	32	3
13591914	hypothetical protein XP_242425	35246	5.83	1	39	3
57806	similar to hypothetical protein MGC32871 [Homo sapiens]	35741	9.31	2	32	3
205055	Aflatoxin B1 aldehyde reductase (AFB1-AR)	36718	6.83	1	58	4
71620	calpactin I heavy chain	38654	7.55	1	41	3
1619606	actin, gamma 1 propeptide; cytoskeletal gamma-actin; actin, cytoplasmic 2 [Homo sapiens]	41766	5.31	30	753	55
114395	107 kDa sialoglycoprotein	41819	7.68	1	47	2
6651165	Centaurin-alpha2 protein	43496	9.35	1	30	1
10198602	CTL target antigen	43577	8.2	3	120	9
27683737	similar to radixin [Homo sapiens]	43708	7.15	6	214	15
1730213	acid phosphatase 2; Acid phosphatase 2, lysozymal	48288	6.16	1	32	2
8392842	similar to RIKEN cDNA 1110020M19 [Mus musculus]	48447	9.72	2	45	2
6981196	similar to hypothetical protein AF506821 [Mus musculus]	48652	9.41	2	33	2
915345	similar to MHC class Ib antigen	49386	6.05	3	54	6

1086315	syndapin IIab	51506	5.19	1	47	2
13592097	similar to RE15159p [Drosophila melanogaster]	52276	5.06	2	128	5
2842665	cysteine sulfinic acid decarboxylase	53692	7.55	1	71	2
25742748	similar to Myosin VI	59320	5.46	5	233	12
27710174	EH-domain containing 3	60769	6.04	2	112	4
27684953	gamma-glutamyltranspeptidase (AA 1-568)	61571	7.21	9	210	14
1703320	similar to hypothetical protein MGC37700 [Mus musculus]	61932	6.16	1	46	2
2506157	dnaK-type molecular chaperone hsp72-ps1 - rat	70884	5.43	9	383	13
120707	meprin A (EC 3.4.24.18) beta chain - rat	75003	5.23	1	52	4
347019	hypothetical protein XP_240301	75582	9.1	5	31	3
27721515	similar to hypothetical protein MGC33971 [Homo sapiens]	77273	6.25	1	55	2
27679356	similar to plastin 1 (I isoform) [Homo sapiens]	79204	5.48	3	114	6
7432967	meprin 1 alpha	85085	5.6	1	49	1
27711270	similar to KIAA1919 protein [Homo sapiens]	88809	8.91	1	42	<1
114523	adaptor-related protein complex 2, beta 1 subunit; adaptin, beta 2 (beta); clathrin-associated/asse	104486	5.22	1	115	1
13562118	similar to hypothetical protein DKFZp434I1117.1 - human (fragment)	106342	5.98	2	37	<1
27670717	similar to connexin39 [Mus musculus]	112204	8.55	1	50	1
2331224	similar to CG10869-PA [Drosophila melanogaster]	120819	6.23	3	59	1
56578	similar to Hypothetical protein KIAA0173	139462	9.28	2	38	<1
11968088	similar to Vigilin (High density lipoprotein-binding protein) (HDL-binding protein)	142109	6.53	3	46	2
16924020	similar to KIAA0802 protein [Homo sapiens]	197740	5.85	2	31	<1
27672179	similar to Protein KIAA1404	212474	8.2	2	32	1
2494386	polyubiquitin	11234	5.43	1	38	16
27666180	similar to heat shock protein 84 - mouse	83229	4.97	6	158	4
27714615	Heat shock protein HSP 90-beta (HSP 84)	83264	5.06	2	49	1
27661268	hemoglobin alpha chain	15275	8.45	5	200	41
27707688	CDC10 (cell division cycle 10, S.cerevisiae, homolog)	50476	8.82	1	50	2
27718949	Alkaline phosphatase, tissue-nonspecific isozyme precursor (AP-TNAP) (Liver/bone/kidney isozyme) (T	57623	6.42	3	130	5
9845234	Beta-2-glycoprotein I precursor (Apolipoprotein H) (Apo-H) (B2GPI) (Beta(2)GPI)			1		

Definitions:

Accesion No: gene identifier number as reported in the NCBI protein database

Mr and pI: the theoretical molecular weight and isoelectric point based on the reported amino acid sequence.

PEP: number of peptides that derived MS/MS spectra for sequence determination of the nemed protein.

SCR: Mascot score. In general, scores above 40 are significant. When scores bellow this threshold were found, the MSMS spectra was reinterpreted manually.

COV: sequence coverage of the identified protein in percentage

IV. Proteins identified in renal cortical membrane fractions isolated by free-flow electrophoresis

Proteins identified in BBM and BLM isolated using the procedure of free-flow electrophoresis [8]. Experiments are described in Chapter 5.2.

A. Proteins identified in basolateral membrane fractions

Accesion No.	Protein Name	Mr	pI	PEP	SCR	COV
17380501	Spectrin alpha chain, brain (Spectrin, non-erythroid alpha chain) (Alpha-II spectrin)	285261	5.2	75	2877	31
358959	ATPase alpha1,Na/K	114132	5.3	62	1937	42
30348966	spectrin beta 2 isoform 1; beta-spectrin 2, non-erythrocytic; beta fodrin; spectrin G; brain spectr	275164	5.66	46	1677	20
2494386	Liver carboxylesterase 4 precursor (Carboxyesterase ES-4) (Microsomal palmitoyl-CoA hydrolase)	62634	6.29	45	1159	50
7106421	spectrin beta 2 isoform 2; beta-spectrin 2, non-erythrocytic; beta fodrin; spectrin G	251686	5.36	33	1169	16
1352384	Protein disulfide isomerase A3 precursor (Disulfide isomerase ER-60) (ERp60) (58 kDa microsomal	57044	5.88	27	750	54
6755863	tumor rejection antigen gp96; tumor rejection antigen (gp96) 1 [Mus musculus]	92703	4.74	25	768	29
92350	H+H+-transporting two-sector ATPase (EC 3.6.3.14) beta chain, mitochondrial - rat (fragment)	50738	4.9	24	932	59
31543464	pyruvate carboxylase	130436	6.34	23	760	22
6978607	catalase	60062	7.07	22	688	39
117164	Cytochrome P450 4A2 precursor (CYPIVA2) (P450-LA-omega 2)	58274	9.18	22	731	41
13591914	kidney aminopeptidase M; Leucine arylaminopeptidase 1	109779	5.3	22	874	23
16758808	erythrocyte protein band 4.1-like 3	123118	5.03	21	590	18
13562118	low density lipoprotein receptor-related protein 2; glycoprotein 330; megalin	537697	5.03	21	787	5
1352384	Protein disulfide isomerase A3 precursor (Disulfide isomerase ER-60) (ERp60)	57044	5.88	21	688	41
71620	actin beta - rat	42066	5.29	20	560	44
72957	chaperonin groEL precursor - mouse	61074	5.91	20	791	39
6981210	membrane metallo endopeptidase;	86425	5.75	20	618	26
6981450	ATP-binding cassette, sub-family D (ALD), member 3; Peroxisomal membrane protein 1 [Rattus norvegic	75780	9.32	19	575	30
6166042	Cytochrome P450 2C23 (CYPIIC23) (Arachidonic acid epoxygenase)	57024	8.68	19	412	29

111948	dipeptidyl-peptidase IV (EC 3.4.14.5), membrane-bound form precursor - rat	91610	5.88	17	487	17
27658450	similar to cadherin 16 [Mus musculus]	94401	4.59	17	514	20
8394307	solute carrier family 3, member 1	78913	5.48	17	450	21
6678571	villin 2 [Mus musculus]	69417	5.83	17	462	22
6679891	alpha glucosidase 2, alpha neutral subunit [Mus musculus]	109791	5.75	16	258	16
11693172	calreticulin	48137	4.33	16	257	36
25742739	fatty acid Coenzyme A ligase, long chain 2; Acyl CoA synthetase, long chain	79155	6.6	16	507	24
13994159	annexin VI	76106	5.39	15	374	22
448251	beta spectrin (beta fodrin)	272627	5.63	15	455	6
404105	'human alpha-catenin' [Homo sapiens]	100702	6.06	14	475	16
238482	long chain alpha-hydroxy acid oxidase =FMN-dependent alpha-hydroxy acid-oxidizing enzyme EC 1.1.3.1	39502	7.9	14	425	43
11560059	aminopeptidase A	108499	5.19	13	295	12
114523	ATP synthase alpha chain, mitochondrial precursor	58904	9.22	13	448	26
4503131	catenin (cadherin-associated protein), beta 1, 88kDa; catenin	86069	5.53	13	395	16
4758012	clathrin heavy chain; clathrin, heavy polypeptide-like 2 [Homo sapiens]	193260	5.48	12	397	8
19424318	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	79179	9.28	12	363	17
6978847	flavin-containing monooxygenase 1	60427	8.66	12	389	29
13928998	PDZ domain containing 1	57252	5.23	12	355	27
27683443	similar to hypothetical protein MGC13047 [Homo sapiens]	116513	7.78	12	517	13
18203577	Sodium/potassium-transporting ATPase alpha-4 chain (Sodium pump 4) (Na+/K+ ATPase 4)	115940	5.52	12	320	9
13929034	solute carrier family 27 (fatty acid transporter), member 32; solute carrier family 27	71447	8.82	12	387	18
6981486	ribophorin I	68376	6.05	11	275	23
7514087	sodium bicarbonate cotransport protein NBC - rat	116822	6.44	11	221	10
8745552	voltage-dependent anion channel 1 [Sus scrofa]	30822	8.62	11	512	44
17902245	EZRIN	54254	6.16	10	327	20
13385454	glycine amidinotransferase (L-arginine:glycine amidinotransferase) [Mus musculus]	48779	8	10	220	25
27370516	isocitrate dehydrogenase 2 (NADP+), mitochondrial [Mus musculus]	51358	8.88	10	259	22
28336	mutant beta-actin (beta'-actin) [Homo sapiens]	42128	5.22	10	251	28
129731	Protein disulfide isomerase precursor (PDI) (Prolyl 4-hydroxylase beta subunit)	57315	4.82	10	232	18

7657583	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 13;	74819	8.77	10	334	16
4502099	solute carrier family 25, member 5; 2F1; adenine nucleotide translocator 2 (fibroblast)	33102	9.76	10	298	28
207603	truncated UDP-glucuronosyltransferase (EC 2.4.1.17)	47347	8.5	10	289	27
6680027	glutamate dehydrogenase [Mus musculus]	61640	8.05	9	309	18
126897	Malate dehydrogenase, mitochondrial precursor	36030	8.83	9	384	34
205660	NADPH-cytochrome P-450 oxidoreductase	77285	5.3	9	298	15
27684065	similar to NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial precursor (Complex I-75Kd)	45829	5.12	9	449	27
418146	Ubiquinol-cytochrome C reductase complex core protein 2, mitochondrial precursor	48400	9.16	9	401	22
33086580	Ab2-305	43767	6.09	8	232	14
19705431	albumin	70670	6.09	8	340	17
1619606	aldolase B	40035	8.66	8	285	27
19705543	Mg87 protein	38851	8.95	8	99	17
7514017	multifunctional beta-oxidation protein 2, peroxisomal - rat	79781	8.49	8	264	13
205632	Na,K-ATPase alpha-1 subunit	114504	5.27	8	219	7
32189340	solute carrier family 25 member 4; adenine nucleotide translocator 1 (skeletal muscle); ADP/ATP tra	33174	9.84	8	229	23
6753138	ATPase, Na+/K+ transporting, beta 1 polypeptide; sodium/potassium ATPase beta subunit [Mus musculus	35571	8.83	7	159	22
66063	acyl-CoA oxidase (EC 1.3.3.6) chain A, peroxisomal splice form II - rat	75042	8.24	7	217	19
3121992	Aldehyde dehydrogenase, mitochondrial (ALDH class 2) (ALDH1) (ALDH-E2)	54813	5.83	7	321	16
22096350	Alpha enolase (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase) (NNE) (Enolase 1)	47513	5.84	7	196	20
728931	ATP synthase gamma chain, mitochondrial	30229	8.87	7	203	23
2119726	dnaK-type molecular chaperone grp75 precursor - rat	73984	5.87	7	309	10
6980972	glutamate oxaloacetate transaminase 2; Glutamate oxaloacetate transaminase 2, mitochondrial (aspart	47683	9.13	7	238	17
121712	Glutathione S-transferase Ya chain (GST class-alpha)	25706	9.04	7	144	24
72222	heat shock protein 90-beta [validated] - human	83584	4.97	7	224	10
1586819	myosin VI:SUBUNIT=heavy chain	147614	9.01	7	281	5
21704020	NADH dehydrogenase (ubiquinone) Fe-S protein 1 [Mus musculus]	80724	5.51	7	362	12
7549225	PALS2-alpha splice variant [Mus musculus]	61172	5.82	7	153	15

191521	protein 4.1	79257	5.98	7	151	11
2501206	Protein disulfide isomerase A6 precursor (Protein disulfide isomerase P5) (Calcium-binding protein)	47590	4.95	7	356	19
20885919	RIKEN cDNA 2310039D24 [Mus musculus]	34393	6.03	7	135	10
27658990	similar to carboxylesterase precursor	63210	6.02	7	195	12
27717159	similar to dihydrolipoamide dehydrogenase [Mus musculus]	54574	7.96	7	172	17
8393886	solute carrier family 22 member 6; organic cationic transporter-like 1	61468	9.21	7	143	10
6978966	type II brain 4.1 minor isoform	107463	5.14	7	115	9
18373323	UDP glycosyltransferase 1 family, polypeptide A6	60837	8.91	7	218	14
6678573	villin [Mus musculus]	93256	5.72	7	340	9
6093769	Voltage-dependent anion-selective channel protein 2 (VDAC-2) (Outer mitochondrial membrane protein	32327	7.44	7	259	21
8393886	solute carrier family 22 member 6; organic cationic transporter-like 1	61468	9.21	6	141	7
8392833	acetyl-coenzyme A dehydrogenase, medium chain; Acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight-	46925	8.63	6	131	14
6978435	acyl-Coenzyme A dehydrogenase, very long chain; Very long chain Acyl-Coa dehydrogenase; Acyl-Coa de	71047	9.01	6	100	9
2804273	alpha actinin 4 [Homo sapiens]	102661	5.27	6	182	7
33086632	Ba1-651	165451	5.49	6	269	6
6435547	Chain A, Crystal Structure Of A Mammalian 2-Cys Peroxiredoxin, Hbp23.	22250	8.34	6	88	22
30316364	Corticosteroid 11-beta-dehydrogenase, isozyme 2 (11-DH2) (11-beta-hydroxysteroid dehydrogenase type	42616	9.38	6	154	11
2117706	dihydrolipoamide S-acetyltransferase (EC 2.3.1.12), liver - rat (fragment)	48117	5.97	6	146	12
627424	dolichyl-diphosphooligosaccharide-protein glycotransferase (EC 2.4.1.119) 50K chain - human	46145	5.42	6	173	11
7106303	EH-domain containing 1 [Mus musculus]	60622	6.35	6	171	11
92263	gamma-glutamyltransferase (EC 2.3.2.2) precursor - rat	61913	7.21	6	225	10
6981198	meprin 1 beta	80169	5.16	6	280	9
628012	myosin I myr 4 - rat	116858	9.48	6	135	6
13436359	oxoglutarate dehydrogenase (lipoamide) [Homo sapiens]	117059	6.4	6	134	4
27731305	similar to RIKEN cDNA 0610009I16 [Mus musculus]	27898	7.6	6	219	29
9506891	solute carrier family 3, member 2; antigen identified by monoclonal antibodies 4F2; solute	58150	5.19	6	273	15

	carrier					
27718935	similar to solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator)	33159	9.55	5	137	17
4557817	3-oxoacid CoA transferase precursor; Succinyl CoA:3-oxoacid CoA transferase; succinyl-CoA:3-ketoaci	56578	7.14	5	87	10
18426866	acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase) [Rattus norvegicu	42244	8.09	5	233	16
13591894	aldo-keto reductase family 1, member A1; aldo-keto reductase family 1, member A1	36711	6.84	5	82	16
601865	aminopeptidase M	109569	5.34	5	219	6
2098349	Apocytochrome B5, Ph 6.2, 298 K, Nmr, 10 Structures	11204	5.03	5	160	56
20302061	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit (oligomycin sensitivity conferri	23440	10.03	5	184	24
19527064	ATPase, H+ transporting, V1 subunit B, isoform 1; ATPase, H+ transporting, lysosomal	57154	5.19	5	207	13
6753138	ATPase, Na+/K+ transporting, beta 1 polypeptide; sodium/potassium ATPase beta subunit [Mus musculus	35571	8.83	5	125	14
13929196	beta-alanine-pyruvate aminotransferase	57905	8.33	5	148	10
25282419	calnexin	67612	4.49	5	114	9
5295992	chaperonin containing TCP-1 theta subunit	60088	5.44	5	208	10
483111	cytochrome c - guinea pig (tentative sequence)	11581	9.61	5	122	29
8393180	cytochrome c oxidase, subunit 4a; cytochrome c oxidase, subunit IV; cytochrome c oxidase, subunit I	19559	9.45	5	111	31
92022	GTP-binding protein, 23K - rat	23075	5.21	5	229	27
13591884	integrin alpha 1; integrin, alpha 1	132151	5.65	5	119	4
631730	matricin - mouse	61021	6.23	5	164	11
4826846	myosin VI [Homo sapiens]	149989	8.75	5	147	4
806754	Na,K-ATPase beta subunit	35270	8.84	5	89	14
11245971	nicotinamide nucleotide transhydrogenase [Mus musculus]	114449	7.53	5	149	4
13786160	organic anion transporter; organic anion transporter 3	59718	9.1	5	114	7
6754976	peroxiredoxin 1; proliferation-associated gene A; osteoblast specific factor 3; macrophage 23 Kd	22390	8.26	5	111	23
6679465	protein kinase C substrate 80K-H; alpha glucosidase II, beta-subunit [Mus musculus]	59725	4.41	5	156	7
13928974	ribophorin 2	69132	5.72	5	236	9
27659946	similar to mKIAA0417 protein [Mus musculus]	94296	6.29	5	214	6
27711856	similar to solute carrier family 26, member 9, isoform a; anion transporter/exchanger-9	171003	7.96	5	202	4

	[Homo sapie					
27721521	similar to ubiquinol-cytochrome c reductase core protein I [Homo sapiens]	28220	5.09	5	161	21
420115	spectrin beta chain - dog (fragment)	81297	5.47	5	115	6
57806	unnamed protein product	61913	7.21	5	190	8
383059	vacuolar H ATPase:SUBUNIT=70kD	68911	5.46	5	95	10
119717	Ezrin (p81) (Cytovillin) (Villin 2)	69470	5.94	4	63	5
13786160	organic anion transporter; organic anion transporter 3	59718	9.1	4	96	5
18034773	acetyl-CoA synthetase 2-like; acetyl-Coenzyme A synthetase 2 [Mus musculus]	75317	6.51	4	72	5
6978431	acetyl-Coenzyme A dehydrogenase, long-chain; Acyl Coenzyme A dehydrogenase, long chain [Rattus norv	48242	7.63	4	87	8
5031595	actin related protein 2/3 complex subunit 4; Arp2/3 protein complex subunit p20 [Homo sapiens]	19768	8.53	4	82	22
6981184	alpha-methylacyl-CoA racemase; Methylacyl-CoA racemase alpha	40035	6.22	4	94	10
117097	Cytochrome c oxidase polypeptide VA	12485	5.01	4	85	34
266684	Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondri	47668	8.17	4	82	7
6753762	epoxide hydrolase 1, microsomal [Mus musculus]	52714	8.43	4	91	7
2492741	Estradiol 17 beta-dehydrogenase 4 (17-beta-HSD 4) (17-beta-hydroxysteroid dehydrogenase 4) (HSD IV)	79891	8.77	4	102	6
119717	Ezrin (p81) (Cytovillin) (Villin 2)	69470	5.94	4	89	5
120707	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (38 kDa BFA-dependent ADP-ribosylation substrate)	36098	8.43	4	171	15
65987	glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) (EC 1.2.1.12) - pig	35914	6.9	4	88	9
90626	histone H4 (clone 53) - mouse	11317	10.87	4	98	38
18483253	hypothetical protein MMT-7 [Mus musculus]	67876	9.12	4	134	6
8393636	integrin, beta 1	91687	5.77	4	103	7
188696	methylmalonate semialdehyde dehydrogenase	46975	7.21	4	138	9
6754810	N-myc downstream regulated 1 [Mus musculus]	43437	5.69	4	229	15
18959236	perosisomal 2-enoyl-CoA reductase	32698	8.89	4	136	18
6679237	pyruvate carboxylase; pyruvate decarboxylase [Mus musculus]	130344	6.25	4	90	3
4758988	RAB1A, member RAS oncogene family; RAB1, member RAS oncogene family [Homo sapiens]	22891	5.93	4	110	18
3511277	reduced in osteosclerosis transporter [Mus	59806	8.69	4	107	6

	musculus]					
21313668	RIKEN cDNA 2310001A20 [Mus musculus]	46576	5.97	4	109	9
16758640	SAC1 (supressor of actin mutations 1, homolog)-like; suppressor of actin 1	67509	6.84	4	140	6
18426858	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	72596	6.75	4	103	6
135826	Thiosulfate sulfurtransferase (Rhodanese)	33384	7.84	4	158	17
313014	vacuolar proton ATPase [Homo sapiens]	26271	8.45	4	43	15
346261	H^+ / +/K +-exchanging ATPase (EC 3.6.3.10) ATP1AL1 - human (fragment)	10542	4.24	3	84	28
625305	myosin heavy chain nonmuscle form A - human	227799	5.53	3	119	1
1709296	Solute carrier family 12 member 1 (Bumetanide-sensitive sodium-(potassium)-chloride cotransporter 2	122648	7.88	3	106	2
1709863	14.5 kDa translational inhibitor protein (Perchloric acid soluble protein)	14352	7.79	3	112	26
25282441	2-4-dienoyl-Coenzyme A reductase 2, peroxisomal; putative peroxisomal 2,4-dienoyl-CoA reductase [Ra	31614	8.51	3	109	14
6753074	adaptor protein complex AP-2, mu1; adaptor-related protein complex AP-2, mu1; clathrin-associated A	49965	9.57	3	45	5
1703286	Aminopeptidase N (pAPN) (Alanyl aminopeptidase) (Microsomal aminopeptidase) (Aminopeptidase M)	109139	5.15	3	135	3
1167996	ankyrin G119	120266	8.54	3	69	2
4557343	antiquitin; antiquitin 1; aldehyde dehydrogenase 7A1 [Homo sapiens]	55845	6.44	3	174	7
19705465	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1	28965	9.39	3	74	9
1673514	B-cell receptor associated protein [Homo sapiens]	23621	9.57	3	54	10
9845234	calpactin I heavy chain	38939	7.55	3	96	8
19424238	camello-like 1	24964	8.73	3	41	9
18543177	citrate synthase; citrate synthase precursor	52176	8.53	3	65	6
14144	COII [Oryctolagus cuniculus]	26159	4.66	3	60	10
16758434	D-amino acid oxidase	39138	6.7	3	123	8
28380037	Dimethylaniline monooxygenase [N-oxide forming] 1 (Hepatic flavin-containing monooxygenase 1) (FMO	60818	8.94	3	142	6
8393243	dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenyme A; dodecenoyl-Coenzyme A delta isome	32348	9.55	3	133	15
21759113	Electron transfer flavoprotein alpha-subunit, mitochondrial precursor (Alpha-ETF)	35360	8.62	3	60	11
21313290	electron transferring flavoprotein, dehydrogenase [Mus musculus]	68903	7.34	3	86	4

11024674	ERM-binding phosphoprotein	39149	5.7	3	58	9
19527026	expressed sequence AA959742 [Mus musculus]	35312	9.57	3	73	11
16554572	FXYD domain-containing ion transport regulator 2 isoform b; ATPase, Na+/K+ transporting, gamma 1 po	7261	8.89	3	86	34
6677905	golgi apparatus protein 1; selectin, endothelial cell, ligand [Mus musculus]	137524	6.45	3	55	2
346707	GTP-binding regulatory protein beta chain - mouse (fragment)	13867	5.5	3	95	22
89580	GTP-binding regulatory protein Gi alpha-2 chain - bovine (fragments)	9111	4.4	3	108	42
348274	GTP-binding regulatory protein Gi alpha-3 chain - guinea pig	41210	5.5	3	106	9
2494884	Guanine nucleotide-binding protein G(T), alpha-1 subunit (Transducin alpha-1 chain)	40411	5.4	3	46	8
70716	histone H2B - bovine	13767	10.32	3	74	21
19424338	hydroxyacyl dehydrogenase, subunit B	51667	9.5	3	106	5
8393573	hydroxysteroid 11-beta dehydrogenase 2; Hydroxysteroid dehydrogenase, 11 beta type 2 [Rattus norvegicus}	44098	9.39	3	69	6
21426769	kidney-specific protein (KS)	64603	8.54	3	76	6
8393641	kynurenine aminotransferase 2; kynurenine aminotransferase II	48096	8.08	3	90	7
6755973	lin 7 homolog c; vertebrate homolog of C. elegans Lin-7 type 3 [Mus musculus]	21935	8.52	3	66	16
14010637	membrane-bound aminopeptidase P [Mus musculus]	76855	5.56	3	103	4
285323	meprin A (EC 3.4.24.18) beta chain - rat	75687	5.23	3	132	5
20071222	Ndufs3 protein [Mus musculus]	30358	6.4	3	172	14
2143900	peptidylprolyl isomerase (EC 5.2.1.8) B, 20.3K - rat	20352	9.35	3	92	16
22202631	programmed cell death 8 isoform 3; apoptosis-inducing factor [Homo sapiens]	35787	7.07	3	82	8
4505773	prohibitin [Homo sapiens]	29843	5.57	3	130	11
119531	Protein disulfide isomerase A4 precursor (Protein ERp-72) (ERp72)	72271	5.13	3	43	3
3929114	putative lung tumor suppressor [Homo sapiens]	58111	5.96	3	43	5
4758984	RAB11A, member RAS oncogene family; RAB 11A, member oncogene family [Homo sapiens]	24492	6.12	3	84	12
1710630	Retinol dehydrogenase type II (RODH II)	35973	8.89	3	121	9
6755354	ribosomal protein L6 [Mus musculus]	32648	10.77	3	58	9
19354269	RIKEN cDNA 0610006F02 gene [Mus musculus]	22578	8.53	3	72	7
27661336	similar to alpha glucosidase II alpha subunit; likely ortholog of mouse G2an alpha glucosidase 2	24692	5.96	3	81	19

27679634	similar to antiquitin - rat (fragment)	17605	6.43	3	81	22
20910231	similar to hypothetical protein FLJ32569 [Homo sapiens] [Mus musculus]	55742	5.99	3	158	6
27667200	similar to hypothetical protein MGC30702 [Mus musculus]	67088	8.75	3	122	6
27677028	similar to Spectrin beta chain, brain 1 (Spectrin, non-erythroid beta chain 1) (Beta-II spectrin) (	56057	6.89	3	134	8
6644384	sodium bicarbonate cotransporter	122228	6.4	3	59	2
114395	Sodium/potassium-transporting ATPase beta-1 chain (Sodium/potassium-dependent ATPase beta-1 subunit	35578	8.83	3	96	10
1083802	sodium-chloride transporter, Thiazide-sensitive - rat	111445	7.98	3	183	3
13928930	solute carrier family 15, member 2; solute carrier family 15 (H+/peptide transporter), member 2 [Ra	82009	8.25	3	72	3
8393886	solute carrier family 22 member 6; organic cationic transporter-like 1	61468	9.21	3	56	4
4758162	synapse-associated protein 97; discs large homolog 1; presynaptic protein SAP97 [Homo sapiens]	103443	5.63	3	52	2
135536	T-COMPLEX PROTEIN 1, ALPHA SUBUNIT (TCP-1-ALPHA) (CCT-ALPHA) (65 KD ANTIGEN)	60814	5.71	3	96	5
1729977	Transketolase (TK)	68342	7.23	3	74	4
72870	translation elongation factor eEF-1 alpha chain - mouse	50437	9.1	3	94	6
14010881	UDP-glucuronosyltransferase	61455	7.95	3	68	7
12831209	unconventional myosin Myr2 I heavy chain	118815	9.41	3	69	2
4558732	voltage dependent anion channel	30062	9.11	3	118	16
267413	Aquaporin-CHIP (Water channel protein for red blood cells and kidney proximal tubule) (Aquaporin 1)	29040	7.7	2	82	10
4038352	breast cancer resistance protein [Homo sapiens] (ATP-binding cassette, sub-family G, member 2; breast cancer resistance protein; mitoxantrone resist)	72897	8.85	2	54	2
89939	Ca2+-transporting ATPase (EC 3.6.3.8) - rabbit	135535	5.42	2	53	1
13272554	cytokeratin KRT2-6HF [Mus musculus]	42471	5.11	2	63	8
1053142	LX1 (organic cation transporter OCT1A)	62452	6.22	2	90	4
28570190	Na+ dependent glucose transporter 1	52141	8.86	2	78	3
12408328	sodium-dependent high-affinity dicarboxylate transporter 3	66455	8.09	2	73	4
6981542	solute carrier family 16, member 1; Solute carrier 16 (monocarboxylic acid transporter), member 1 [	53831	8.62	2	110	4
70637	ubiquitin - Mediterranean fruit fly	8446	6.56	2	59	33

1079478	2-oxoglutarate carrier protein - human	34229	9.92	2	89	7
20149758	3-mercaptopyruvate sulfurtransferase; e [Mus musculus]	33231	6.11	2	49	7
520469	597 aa protein related to Na/glucose cotransporters	65292	8.54	2	47	3
113159	Aconitate hydratase, mitochondrial precursor (Citrate hydro-lyase) (Aconitase)	86449	8.24	2	57	3
86755	ADP,ATP carrier protein T2 - human	33188	9.71	2	71	7
2138326	ankyrin-3 [Mus musculus]	103226	6.12	2	64	2
113969	Annexin A11 (Annexin XI) (Calcyclin-associated annexin 50) (CAP-50)	54326	7.53	2	69	3
114562	ATP synthase beta chain, mitochondrial precursor	56318	5.8	2	60	3
20806153	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	17619	5.03	2	106	13
20897610	brain abundant, membrane attached signal protein 1 [Mus musculus]	22074	4.5	2	52	15
203237	calbindin-d28k	30225	4.8	2	54	7
223872	calmodulin	16696	4.14	2	52	22
400542	chaperonin 10, cpn10 [Rattus norvegicus=rats, liver, Peptide Mitochondrial, 101 aa]	10764	8.91	2	77	23
116850	Cofilin, non-muscle isoform	18792	8.16	2	58	15
203658	Cu-Zn superoxide dismutase (EC 1.15.1.1)	15871	5.88	2	88	15
13928828	cytochrome P450, 4a10	58766	9.24	2	70	3
1070444	cytochrome-b5 reductase (EC 1.6.2.2), microsomal form - rat	34347	8.56	2	75	9
7706495	DnaJ (Hsp40) homolog, subfamily B, member 11; ER-associated DNAJ; ER-associated Hsp40 co-chaperone;	40774	5.81	2	74	7
16758848	endoplasmic retuclum protein 29	28614	6.23	2	47	8
16758174	flavin-containing monooxygenase 3	60606	8.44	2	67	3
68186	fructose-bisphosphate aldolase (EC 4.1.2.13) A - rat	39830	7.06	2	45	6
111734	GTP-binding regulatory protein G42 alpha chain - rat (fragments)	8742	4.43	2	74	25
1051238	guanine nucleotide-binding protein	42268	5.58	2	53	5
121012	Guanine nucleotide-binding protein beta subunit 4 (Transducin beta chain 4)	38071	5.6	2	124	6
2494886	Guanine nucleotide-binding protein, alpha-13 subunit (G alpha 13)	44378	8.11	2	50	5
16758446	isocitrate dehydrogenase 3 (NAD+) alpha	40044	6.47	2	68	6
6981112	isovaleryl Coenzyme A dehydrogenase	46862	8.03	2	65	4
16758758	lectin, mannose-binding, 1; endoplasmic reticulum-golgi intermediate compartment protein 53 [Rattus	58206	5.92	2	47	4
127827	low affinity Na-dependent glucose transporter	74083	7.14	2	44	2

	(SGLT2)					
6981170	lysozyme	17174	9.32	2	72	17
126889	Malate dehydrogenase, cytoplasmic	36625	6.16	2	69	6
400240	Microsomal dipeptidase precursor (MDP) (Dehydropeptidase-I) (Renal dipeptidase) (RDP)	45933	5.68	2	70	4
1401252	mlrq-like protein [Mus musculus]	8509	9.3	2	70	24
625305	myosin heavy chain nonmuscle form A - human	227799	5.53	2	65	1
128867	NADH-ubiquinone oxidoreductase 24 kDa subunit, mitochondrial precursor	26854	6	2	83	9
27819651	nicastrin	79035	5.51	2	71	2
4505237	palmitoylated membrane protein 1; membrane protein, palmitoylated 1 (55kD); erythrocyte membrane pr	52492	6.91	2	74	2
18677765	paralemmin	42072	4.86	2	76	4
627754	phosphate carrier protein precursor, mitochodrial, splice form B - bovine	40434	9.37	2	63	5
4505897	plastin 1; I-plastin; Plastin-1; I isoform [Homo sapiens]	70707	5.33	2	46	3
984249	protein kinase [Sus scrofa]	93089	4.76	2	64	3
18606182	Rab5c protein [Mus musculus]	23626	8.64	2	81	10
131796	Ras-related protein Rab-6A (Rab-6)	23692	5.42	2	48	10
16758640	SAC1 (supressor of actin mutations 1, homolog)-like; suppressor of actin 1	67509	6.84	2	68	3
27668505	similar to hypothetical protein DKFZp761D0211 [Homo sapiens]	42207	4.84	2	79	6
27691916	similar to RIKEN cDNA 0610010I20 gene [Mus musculus]	25408	6.13	2	84	9
27696657	similar to RIKEN cDNA 1500009M05 [Mus musculus]	15454	9.68	2	69	15
27716317	similar to succinate dehydrogenase complex, subunit B, iron sulfur (Ip); iron-sulfur subunit [Homo	32607	8.96	2	61	6
27712686	similar to Succinyl-CoA ligase [GDP-forming] beta-chain, mitochondrial precursor (Succinyl-CoA synt	83427	7.78	2	77	2
13592103	sulfite oxidase	54606	5.79	2	57	3
6678049	synaptosomal-associated protein 23; syndet; synaptosomal-associated protein, 23kD; synaptosomal-ass	23531	4.88	2	73	10
25742698	tumor-associated calcium signal transducer 1	35869	6.03	2	101	11
136726	UDP-glucuronosyltransferase 1-6 precursor, microsomal (UDPGT) (UGT1*6) (UGT1-06) (UGT1.6) (UGT1A6)	60720	8.91	2	49	3
26335437	unnamed protein product [Mus musculus]	78620	8.38	2	59	2
162723	vacuolar H+-ATPase	56784	5.63	2	81	4

1783347	organic cation transporter	66913	8.08	1	58	2
627754	phosphate carrier protein precursor, mitochodrial, splice form B - bovine	40434	9.37	1	65	3
2117518	aldehyde dehydrogenase [NAD(P)] (EC 1.2.1.5) - rat	55013	7.14	1	64	2
1083589	alpha adducin - rat	80691	5.91	1	55	1
1805280	alpha II spectrin [Homo sapiens]	285689	5.22	1	60	<1
267413	Aquaporin-CHIP (Water channel protein for red blood cells and kidney proximal tubule) (Aquaporin 1)	29040	7.7	1	65	3
17978459	ATP synthase, H+ transporting, mitochondrial F1F0 complex, subunit e	8249	9.34	1	46	16
20376826	barttin	34044	4.36	1	41	4
595917	capping protein alpha 1 subunit [Mus musculus]	32902	5.34	1	43	3
111503	cell surface glycoprotein OX47 precursor - rat	34052	5.35	1	48	3
5453603	chaperonin containing TCP1, subunit 2 (beta); chaperonin containing t-complex polypeptide 1, beta s	57794	6.01	1	74	2
307331	differentially expressed protein	29338	5.62	1	60	4
1706611	Elongation factor Tu, mitochondrial precursor (EF-Tu) (P43)	49852	7.26	1	46	2
545439	Erp61, GRP58=stress-inducible luminal endoplasmic reticulum protein [mice, MOPC-315, Peptide Partia	2112	3.71	1	71	73
545439	Erp61, GRP58=stress-inducible luminal endoplasmic reticulum protein [mice, MOPC-315, Peptide Partia	2112	3.71	1	67	73
71882	GTP-binding regulatory protein Gs alpha-S1 chain (adenylate cyclase-stimulating) - mouse	40671	5.34	1	59	3
16758758	lectin, mannose-binding, 1; endoplasmic reticulum-golgi intermediate compartment protein 53 [Rattus	58206	5.92	1	42	2
204570	major beta-hemoglobin	16097	7.88	1	45	8
53450	manganese superoxide dismutase [Mus musculus]	24890	8.8	1	51	6
7514011	membrane protein - rat	28321	9.72	1	58	4
6981196	meprin 1 alpha	86168	5.6	1	55	1
7657347	mitochondrial carrier homolog 2 [Homo sapiens]	33936	8.25	1	53	2
1182011	NAD+-isocitrate dehydrogenase, alpha subunit [253]	37233	5.72	1	50	3
189308	nucleobindin	53698	5.15	1	64	2
31543719	organic anion transporter			1		
190201	porin	35624	5.87	1	42	3
238427	Porin 31HM [human, skeletal muscle membranes, Peptide, 282 aa]	30737	8.63	1	57	3

200966	put. serine protease inhibitor	45737	8.91	1	60	2
66035	pyruvate dehydrogenase (lipoamide) (EC 1.2.4.1) alpha chain 1 precursor - rat	43853	8.35	1	42	2
4506609	ribosomal protein L19; 60S ribosomal protein L19 [Homo sapiens]	23565	11.48	1	46	4
13385656	RIKEN cDNA 0610010D20 [Mus musculus]	34964	7.62	1	45	3
5453706	similar to Caenorhabditis elegans protein C42C1.9 [Homo sapiens]	39072	7.67	1	50	3
27718935	similar to solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), membe	33159	9.55	1	46	4
54130	sodium/potassium ATPase beta subunit [Mus musculus]	31861	8.32	1	52	4
12408328	sodium-dependent high-affinity dicarboxylate transporter 3	66455	8.09	1	80	2
6677897	stromal cell derived factor receptor 1 [Mus musculus]	31446	6.11	1	53	3
6678177	syntaxin 4A (placental) [Mus musculus]	34258	5.84	1	42	3
12860092	unnamed protein product [Mus musculus]	20432	5.66	1	52	7

B. Proteins identified in brush border membrane fractions

Accesion No.	Protein Name	Mr	pI	PEP	SCR	COV
13562118	low density lipoprotein receptor-related protein 2; megalin	537697	5.03	90	3152	16
13591914	kidney aminopeptidase M; Leucine arylaminopeptidase 1	109779	5.3	54	1801	40
13928998	PDZ domain containing 1	57252	5.23	49	1352	65
462608	Moesin (Membrane-organizing extension spike protein)	67839	6.22	48	1121	46
17105370	ATPase, H+ transporting, lysosomal (vacuolar proton pump), beta 56/58 kDa, isoform 2 [Rattus norveg	56857	5.57	47	1250	51
12832989	unnamed protein product [Mus musculus] (Cytovillin) (Villin 2)	69506	5.9	39	824	32
119717	Ezrin (p81) (Cytovillin) (Villin 2)	69470	5.94	38	898	38
27710072	similar to Maltase-glucoamylase, intestinal	777951	5.23	34	967	5
33302595	Glutamyl aminopeptidase (EAP) (Aminopeptidase A) (APA)	108440	5.24	33	834	31
1586819	myosin VI:SUBUNIT=heavy chain	147614	9.01	30	1083	20
4758012	clathrin heavy chain; clathrin, heavy polypeptide-like 2 [Homo sapiens]	193260	5.48	29	1149	21
358959	ATPase alpha1,Na/K	114132	5.3	26	991	28

109893	dnaK-type molecular chaperone grp78 precursor - mouse	72491	5.12	26	915	36
114523	ATP synthase alpha chain, mitochondrial precursor	58904	9.22	26	988	39
11024674	ERM-binding phosphoprotein	39149	5.7	24	731	48
6978773	dipeptidyl peptidase 4	88689	5.87	22	668	27
17380501	Spectrin alpha chain, brain (Spectrin, non-erythroid alpha chain) (Alpha-II spectrin) (Fodrin alpha	285261	5.2	21	788	9
28436809	radixin [Homo sapiens]	68636	5.88	21	476	22
11230802	actinin alpha 4; alpha actinin 4 [Mus musculus]	105368	5.25	20	749	23
27720877	similar to Myosin VI	59947	5.46	20	778	39
71625	actin gamma - bovine (tentative sequence)	41977	5.31	19	580	46
16758040	cubilin; cubilin (intrinsic factor-cobalamin receptor)	407570	5.4	18	554	6
7106421	spectrin beta 2 isoform 2; beta-spectrin 2, non-erythrocytic; beta fodrin; spectrin G; brain spectr	251686	5.36	17	620	8
2804273	alpha actinin 4 [Homo sapiens]	102661	5.27	17	548	23
20892559	ATPase, H+ transporting, lysosomal (vacuolar proton pump), alpha 70 kDa, isoform 1 [Mus musculus]	68625	5.42	17	601	28
117164	Cytochrome P450 4A2 precursor (CYPIVA2) (Lauric acid omega-hydroxylase) (P450-LA-omega 2) (P450 K-5	58274	9.18	17	740	29
90292	alpha-adaptin C - mouse	104863	6.51	16	492	16
13994159	annexin VI	76106	5.39	16	445	26
28940	unnamed protein product [Homo sapiens] (ATP synthase beta chain, mitochondrial precursor)	57976	5.8	16	697	33
16924020	X-prolyl aminopeptidase (aminopeptidase P) 2, membrane-bound	76545	5.49	15	517	19
347019	dnaK-type molecular chaperone hsp72-ps1 - rat	71112	5.43	15	514	21
6978847	flavin-containing monooxygenase 1	60427	8.66	15	585	29
19527064	ATPase, H+ transporting, V1 subunit B, isoform 1; ATPase, H+ transporting, lysosomal (vacuolar prot	57154	5.19	14	462	20
7514017	multifunctional beta-oxidation protein 2, peroxisomal - rat	79781	8.49	14	535	23
20892559	ATPase, H+ transporting, lysosomal (vacuolar proton pump), alpha 70 kDa, isoform 1 [Mus musculus]	68625	5.42	14	375	24
17902245	EZRIN	54254	6.16	14	345	28
9506891	solute carrier family 3, member 2; antigen identified by monoclonal antibodies 4F2; solute carrier	58150	5.19	14	498	34

19424318	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	79179	9.28	13	411	18
6753138	ATPase, Na+/K+ transporting, beta 1 polypeptide; sodium/potassium ATPase beta subunit [Mus musculus	35571	8.83	13	372	26
87528	dnaK-type molecular chaperone HSPA5 precursor - human	72185	5.03	12	469	23
22219429	Chain M, Ap2 Clathrin Adaptor Core	50006	9.6	12	257	27
17105334	folate hydrolase; N-acetylated alpha-linked acidic dipeptidase	84715	7.04	11	277	15
16359229	ribophorin I [Mus musculus]	68657	6.02	11	348	21
806754	Na,K-ATPase beta subunit	35270	8.84	11	262	22
109533	alkaline phosphatase (EC 3.1.3.1) precursor - mouse	57794	6.52	11	380	23
13990959	a4 subunit isoform [Mus musculus] (ATPase, H+ transporting, lysosomal V0 subunit A isoform 4; ATPase, H+ transporting, lysosomal (vacu)	96341	5.94	10	360	10
13277651	Akap2 protein [Mus musculus]	88337	5.17	10	390	13
462608	Moesin (Membrane-organizing extension spike protein)	67839	6.22	10	275	13
6755863	tumor rejection antigen gp96; tumor rejection antigen (gp96) 1 [Mus musculus]	92703	4.74	10	432	13
57806	unnamed protein product (gamma-glutamyl transpeptidase)	61913	7.21	10	397	14
6755863	tumor rejection antigen gp96; tumor rejection antigen (gp96) 1 [Mus musculus]	92703	4.74	10	409	14
13928828	cytochrome P450, 4a10	58766	9.24	10	363	16
3157995	DOC-2 p59 isoform (mitogen-responsive phosphoprotein; DOC-2 p82 isoform)	58957	5.66	10	384	21
7706262	vacuolar ATPase subunit H [Homo sapiens]	56399	6.07	10	296	25
22096350	Alpha enolase (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase) (NNE) (Enolase 1)	47513	5.84	10	379	26
71620	actin beta - rat	42066	5.29	10	234	30
6981450	ATP-binding cassette, sub-family D (ALD), member 3; Peroxisomal membrane protein 1 [Rattus norvegic	75780	9.32	9	159	11
21361142	PDZ domain containing 1 [Homo sapiens]	57379	5.36	9	228	15
18373323	UDP glycosyltransferase 1 family, polypeptide A6	60837	8.91	9	334	17
32189394	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide; ATP synthase, H+ transpo	56525	5.26	9	294	24
27705116	similar to RIKEN cDNA 1200011D11 [Mus musculus]	18155	4.72	9	363	38
6981236	myosin, heavy polypeptide 9; Myosin, heavy polypeptide 9, non-muscle	227566	5.49	8	221	5
404105	'human alpha-catenin' [Homo sapiens]	100702	6.06	8	250	10

601865	aminopeptidase M	109569	5.34	8	295	10
8392872	adaptor protein complex AP-1, beta 1 subunit	105377	5	8	292	10
303618	phospholipase C-alpha [Homo sapiens]	57065	6.23	8	257	13
123928	Epoxide hydrolase 1 (Microsomal epoxide hydrolase) (Epoxide hydratase)	52719	8.59	8	202	15
339647	thyroid hormone binding protein precursor	57468	4.82	8	318	16
4759140	solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulatory factor 1 [Homo sapiens]	39130	5.55	8	210	18
300172	cystine, dibasic, and neutral amino acid transporter {clone D2H} [human, kidney, Peptide, 663 aa]	76574	5.55	7	117	5
10880126	X-prolyl aminopeptidase 2, membrane-bound; X-prolyl aminopeptidase 2 (aminopeptidase P); aminoacylp	76132	5.69	7	151	6
12831209	unconventional myosin Myr2 I heavy chain	118815	9.41	7	248	6
4557469	adaptor-related protein complex 2, beta 1 subunit; adaptin, beta 2 (beta); clathrin-associated/asse	105398	5.22	7	247	7
6679891	alpha glucosidase 2, alpha neutral subunit [Mus musculus]	109791	5.75	7	128	7
111948	dipeptidyl-peptidase IV (EC 3.4.14.5), membrane-bound form precursor - rat	91610	5.88	7	235	9
179212	Na+ K + ATPase alpha subunit	82706	5.08	7	190	9
72222	heat shock protein 90-beta [validated] - human	83584	4.97	7	230	9
2331224	Diphor-1 (PDZ domain containing 1)	52617	5.31	7	196	14
693933	2-phosphopyruvate-hydratase alpha-enolase; carbonate dehydratase [Homo sapiens]	47421	7.01	7	278	18
15805031	eukaryotic translation elongation factor 1 alpha 2	50460	9.1	7	234	19
20898514	RIKEN cDNA 4732495G21 gene [Mus musculus]	42319	5.3	7	81	19
1040689	Human Diff6,H5,CDC10 homologue [Homo sapiens]	47069	6.95	7	252	23
27710072	similar to Maltase-glucoamylase, intestinal	777951	5.23	6	129	<1
4507195	spectrin, beta, non-erythrocytic 1 isoform 1; Spectrin, beta, nonerythrocytic-1 (beta-fodrin) [Homo	275259	5.41	6	178	3
13990959	a4 subunit isoform [Mus musculus] (ATPase, H+ transporting, lysosomal V0 subunit A isoform 4; ATPase, H+ transporting, lysosomal (vacu)	96341	5.94	6	156	7
6678573	villin [Mus musculus]	93256	5.72	6	253	8
8393886	solute carrier family 22 member 6; organic cationic transporter-like 1	61468	9.21	6	113	8
12018248	low affinity Na-dependent glucose transporter (SGLT2)	73940	8.04	6	270	10

5764661	DAL1P [Mus musculus] (type II brain 4.1 minor isoform)	79856	5.41	6	131	10
31092	elongation factor 1-alpha (AA 1-462) [Homo sapiens]	50437	9.1	6	181	11
27715409	similar to hypothetical protein MGC37700 [Mus musculus]	62331	6.16	6	234	12
6681153	dolichyl-di-phosphooligosaccharide-protein glycotransferase [Mus musculus]	49211	5.52	6	257	13
522193	vacuolar H+-ATPase 56,000 subunit	56792	5.57	6	287	15
340188	H+ -ATPase C subunit	40099	6.96	6	190	16
4538856	54 kDa vacuolar H+(+)-ATPase subunit [Sus scrofa]	56286	6.18	6	294	17
625305	myosin heavy chain nonmuscle form A - human	227799	5.53	5	139	3
28510667	expressed sequence AW544947 [Mus musculus]	116806	9.45	5	71	4
6678790	mannosidase 2, alpha 1 [Mus musculus]	132247	8.17	5	150	4
6677905	golgi apparatus protein 1; selectin, endothelial cell, ligand [Mus musculus]	137524	6.45	5	180	5
27477041	adaptor-related protein complex 2, alpha 2 subunit; adaptin, alpha B; clathrin-associated/assembly/	104807	6.53	5	133	6
7672737	hsp-70-related intracellular vitamin D binding protein [Saguinus oedipus]	71569	6.06	5	172	8
683793	calnexin	65314	4.48	5	135	9
13929034	solute carrier family 27 (fatty acid transporter), member 32; solute carrier family 27 (fatty acid	71447	8.82	5	174	10
4557337	argininosuccinate synthetase [Homo sapiens]	46682	8.36	5	162	10
6680027	glutamate dehydrogenase [Mus musculus]	61640	8.05	5	242	10
189308	nucleobindin	53698	5.15	5	274	11
6166042	Cytochrome P450 2C23 (CYPIIC23) (Arachidonic acid epoxygenase)	57024	8.68	5	196	14
6754976	peroxiredoxin 1; proliferation-associated gene A; osteoblast specific factor 3; macrophage 23 Kd st	22390	8.26	5	85	16
4502695	cell division cycle 10; cell division cycle 10 (homolog to CDC10 of S.cerevisiae); CDC10 protein ho	49041	8.85	5	222	18
8922712	hypothetical protein FLJ10849 [Homo sapiens]	49652	6.36	5	153	18
7304963	chloride intracellular channel 4 (mitochondrial) [Mus musculus]	28939	5.44	5	103	32
27733113	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	169912	8.83	4	105	2
4503131	catenin (cadherin-associated protein), beta 1, 88kDa; catenin (cadherin-associated protein), beta 1	86069	5.53	4	69	3
6644384	sodium bicarbonate cotransporter	122228	6.4	4	49	3

13928930	solute carrier family 15, member 2; solute carrier family 15 (H+//peptide transporter), member 2 [Ra	82009	8.25	4	63	4
205632	Na,K-ATPase alpha-1 subunit	114504	5.27	4	101	4
2224709	KIAA0384 [Homo sapiens] (catenin src [Mus musculus])	105457	6.34	4	80	4
6005944	villin 1; Villin-1 [Homo sapiens]	93093	5.99	4	152	4
1363274	cell surface antigen RB13-6 - rat (phosphodiesterase I)	100962	5.98	4	86	5
13786160	organic anion transporter; organic anion transporter 3	59718	9.1	4	86	5
15487264	villin-like protein [Mus musculus]	97531	5.92	4	72	5
27806351	villin 2 [ezrin] [Bos taurus]	68832	6.06	4	99	5
8394307	solute carrier family 3, member 1	78913	5.48	4	112	6
5174735	tubulin, beta, 2 [Homo sapiens]	50255	4.79	4	156	13
135395	Tubulin alpha-1 chain (Alpha-tubulin 1)	50804	4.94	4	116	14
4502695	cell division cycle 10; cell division cycle 10 (homolog to CDC10 of S.cerevisiae); CDC10 protein ho	49041	8.85	4	131	14
120707	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (38 kDa BFA-dependent ADP-ribosylation substrate)	36098	8.43	4	124	15
1619606	aldolase B	40035	8.66	4	137	16
464496	Protein-tyrosine phosphatase, non-receptor type 12 (Protein-tyrosine phosphatase G1) (PTPG1)	88893	5.4	3	51	<1
11560059	aminopeptidase A	108499	5.19	3	80	3
2696709	RST [Mus musculus] (solute carrier family 22 (organic cation transporter)-like 2 [Mus musculus])	60806	8.45	3	51	3
205660	NADPH-cytochrome P-450 oxidoreductase	77285	5.3	3	82	4
2492741	Estradiol 17 beta-dehydrogenase 4 (17-beta-HSD 4) (17-beta-hydroxysteroid dehydrogenase 4) (HSD IV)	79891	8.77	3	97	4
26331764	unnamed protein product [Mus musculus]	70789	9.16	3	104	4
27721231	similar to plastin 1 (I isoform) [Homo sapiens]	79831	5.48	3	83	4
30023556	ATP-binding cassette transporter ABCG2	73428	9.25	3	60	4
177207	4F2 antigen heavy chain	58049	5.2	3	79	5
27670717	similar to hypothetical protein MGC33971 [Homo sapiens]	78357	6.25	3	156	5
27708570	similar to villin [Mus musculus]	70946	6	3	159	5
2852699	A kinase anchor protein [Mus musculus]	96458	5.22	3	187	5
6981544	solute carrier family 34, member 1; Solute carrier family 34 (sodium phosphate), member 1 [Rattus n	69347	9.01	3	81	5
1136741	KIAA0002 [Homo sapiens]	59035	5.75	3	174	6

27687171	similar to assembly protein 50 - human	43951	6.47	3	78	6
14010881	UDP-glucuronosyltransferase	61455	7.95	3	122	7
21338248	aminopeptidase [Bos taurus]	19924	4.4	3	51	7
6753074	adaptor protein complex AP-2, mu1; adaptor-related protein complex AP-2, mu1; clathrin-associated A	49965	9.57	3	59	7
11024643	5 nucleotidase; 5' nucleotidase, ecto	64384	6.51	3	72	8
2529707	Hpast [Homo sapiens] (EH-domain containing protein testilin [Homo sapiens])	60722	6.49	3	98	8
11693172	calreticulin	48137	4.33	3	55	9
4759140	solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulatory factor 1 [Homo sapiens]	39130	5.55	3	92	9
7656991	coronin, actin binding protein, 1C; coronin, actin-binding protein, 1C; coronin 1C [Homo sapiens]	53899	6.65	3	98	9
1185280	glutathione S-transferase [Sus scrofa]	25490	8.87	3	50	10
20981679	6-phosphogluconate dehydrogenase, decarboxylating	53619	6.8	3	137	10
27733107	similar to multidrug resistance-associated protein [Homo sapiens]	58550	7.17	3	78	10
1710248	protein disulfide isomerase-related protein 5 [Homo sapiens]	46512	4.95	3	185	11
37492	alpha-tubulin [Homo sapiens]	50810	5.02	3	180	11
111503	cell surface glycoprotein OX47 precursor - rat	34052	5.35	3	93	12
307331	differentially expressed protein	29338	5.62	3	131	13
1526539	14-3-3 zeta [Mus musculus]	27908	4.7	3	63	14
28336	mutant beta-actin (beta'-actin) [Homo sapiens]	42128	5.22	3	70	17
4758988	RAB1A, member RAS oncogene family; RAB1, member RAS oncogene family [Homo sapiens]	22891	5.93	3	57	20
92339	GTP-binding protein rab1B - rat	22347	5.55	3	68	21
14193488	ezrin binding protein 50 [Cricetulus griseus]	14589	4.6	3	160	25
1805280	alpha II spectrin [Homo sapiens]	285689	5.22	2	48	<1
27721389	similar to hypothetical protein KIAA0678 - human (fragment)	306705	7.76	2	60	<1
27683737	similar to myosin VIIb [Mus musculus]	168398	9.38	2	86	1
72475	multidrug resistance protein 1a - mouse (fragment)	121533	8.86	2	105	1
1083802	sodium-chloride transporter, Thiazide-sensitive - rat	111445	7.98	2	161	2
12836071	unnamed protein product [Mus musculus]	92180	6.17	2	48	2
19924057	mucin and cadherin-like; mu-protocadherin	91262	4.69	2	102	2
2897946	prostate-specific membrane antigen [Homo sapiens]	84480	6.52	2	97	2
34758	enkephalinase (AA 1-743) [Homo sapiens]	85397	5.54	2	78	2

35336	dipeptidyl peptidase iv [Homo sapiens]	88947	5.67	2	79	2
4314340	Human alpha-adaptin A homolog [AA 159-977] [Homo sapiens]	90845	5.89	2	59	2
4507031	solute carrier family 5 (sodium/glucose cotransporter), member 1; Human Na+/glucose cotransporter 1	74305	7.82	2	63	2
6981196	meprin 1 alpha	86168	5.6	2	96	2
127805	SODIUM/GLUCOSE COTRANSPORTER 1 (NA(+)/GLUCOSE COTRANSPORTER 1) (HIGH AFFINITY SODIUM-GLUCOSE COTRAN	73886	7.44	2	57	3
187512	microsomal epoxide hydrolase (EC 3.3.2.3)	36842	6.46	2	52	3
20908689	RIKEN cDNA 4632401C08 [Mus musculus]	72403	5	2	128	3
27661930	solute carrier family 22 (organic cation transporter)-like 2	90500	9.31	2	56	3
27666274	similar to membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 5); protein associated wit	77390	5.86	2	89	3
28487424	similar to apical iodide transporter , putative [Homo sapiens] [Mus musculus]	68933	8.52	2	83	3
28570190	Na+ dependent glucose transporter 1	52141	8.86	2	58	3
291868	ATPase	68677	5.35	2	63	3
4038352	breast cancer resistance protein [Homo sapiens] (ATP-binding cassette transporter ABCG2)	72897	8.85	2	55	3
729927	Long-chain-fatty-acid--CoA ligase 2 (Long-chain acyl-CoA synthetase 2) (LACS 2)	78900	6.81	2	56	3
7304885	annexin A11; annexin XI [Mus musculus]	54419	7.53	2	100	3
7513988	high-affinity carntine transporter, CT1 - rat	62797	7.59	2	56	3
1053142	LX1 (organic cation transporter OCT1A)	62452	6.22	2	72	4
106054	gamma-glutamyltransferase (EC 2.3.2.2) type 1 precursor, short splice form - human	39737	8.66	2	90	4
12053195	hypothetical protein [Homo sapiens] (Protein kinase C and casein kinase substrate in neurons protein 2)	51663	5.26	2	83	4
123576	47 kDa heat shock protein precursor (Collagen-binding protein 1) (Colligin 1)	46352	8.27	2	55	4
16758174	flavin-containing monooxygenase 3	60606	8.44	2	100	4
27684065	similar to NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial precursor (Complex I-75Kd)	45829	5.12	2	64	4
3641398	NADP-dependent isocitrate dehydrogenase [Homo sapiens]	46944	6.34	2	104	4
4758412	polypeptide N-acetylgalactosaminyltransferase 2; UDP-GalNAc transferase 2 [Homo sapiens]	65433	8.63	2	52	4
6005733	phosphatidylinositol-binding clathrin assembly protein; clathrin assembly lymphoid-myeloid leukemia	70879	7.22	2	91	4

14192935	aldehyde dehydrogenase family 1, member A1; Aldehyde dehydrogenase 1; aldehyde dehydrogenase 1, sub	54994	7.94	2	81	5
162723	vacuolar H+-ATPase	56784	5.63	2	49	5
6009628	brain carboxylesterase hBr3 [Homo sapiens]	62584	6.65	2	80	5
89602	H+H+-exporting ATPase (EC 3.6.3.6) polypeptide IV, vacuolar - bovine	31814	5.22	2	39	5
114395	Sodium/potassium-transporting ATPase beta-1 chain (Sodium/potassium-dependent ATPase beta-1 subunit	35578	8.83	2	55	6
16359053	Similar to RIKEN cDNA 2010309H15 gene [Homo sapiens]	44378	6.76	2	63	6
1729977	Transketolase (TK)	68342	7.23	2	48	6
179950	catalase	51550	7.83	2	39	6
2494386	Liver carboxylesterase 4 precursor (Carboxyesterase ES-4) (Microsomal palmitoyl-CoA hydrolase) (Kid	62634	6.29	2	44	6
31952	alpha-subunit (AA 1-394) [Homo sapiens]	46079	5.59	2	92	6
129902	Phosphoglycerate kinase 1 (Primer recognition protein 2) (PRP 2)	45098	8.3	2	96	8
267413	Aquaporin-CHIP (Water channel protein for red blood cells and kidney proximal tubule) (Aquaporin 1)	29040	7.7	2	87	8
307331	differentially expressed protein	29338	5.62	2	50	8
126889	Malate dehydrogenase, cytoplasmic	36625	6.16	2	72	9
27661336	similar to alpha glucosidase II alpha subunit; likely ortholog of mouse G2an alpha glucosidase 2, a	24692	5.96	2	51	9
54130	sodium/potassium ATPase beta subunit [Mus musculus]	31861	8.32	2	70	9
6981146	lactate dehydrogenase B; Lactate dehydrogenease B	36874	5.7	2	65	9
313014	vacuolar proton ATPase [Homo sapiens]	26271	8.45	2	121	10
609308	cytoplasmic chaperonin hTRiC5	22059	5.52	2	142	10
131794	Ras-related protein Rab-5A	23872	8.32	2	49	11
1710027	Ras-related protein Rab-5C	23669	8.64	2	50	11
6647578	Membrane associated progesterone receptor component 1 (Acidic 25 kDa protein) (25-DX)	21699	4.45	2	37	11
10198602	kidney-specific membrane protein	25495	6.22	2	69	13
238427	Porin 31HM [human, skeletal muscle membranes, Peptide, 282 aa] (Voltage-dependent anion-selective channel protein 1 (VDAC-1) ()	30737	8.63	2	69	15
27687833	similar to mannosidase 2, alpha 1 [Mus musculus]	17636	7.78	2	66	17
420272	GTP-binding protein rab14 - rat	24078	5.85	2	58	20
27680919	hypothetical protein XP_223116	144597	8.99	1	46	<1

10190666	ATPase, H+ transporting, lysosomal V0 subunit a isoform 4; vacuolar proton pump 116 kDa accessory s	97207	5.75	1	45	1
106109	glucuronosyltransferase (EC 2.4.1.17) - human	60831	6.88	1	45	1
14249308	hypothetical protein MGC13047 [Homo sapiens]	63614	6.47	1	53	1
27658450	similar to cadherin 16 [Mus musculus]	94401	4.59	1	51	1
27819651	nicastrin	79035	5.51	1	42	1
28878	argininosuccinate lyase (AA 1 - 464) [Homo sapiens]	52095	6.29	1	59	1
22074648	retinoic acid inducible protein 3 [Mus musculus]	48986	7.56	1	106	2
400621	Sodium- and chloride-dependent creatine transporter 1 (CT1)	71748	6.97	1	58	2
4505237	palmitoylated membrane protein 1; membrane protein, palmitoylated 1 (55kD); erythrocyte membrane pr	52492	6.91	1	39	2
88214	Na+/K+-exchanging ATPase (EC 3.6.3.9) alpha chain - human (fragment)	86964	5.03	1	71	2
19743875	fumarate hydratase precursor; fumarase; Leiomyomatosis and renal cell cancer [Homo sapiens]	54773	8.85	1	50	3
238482	long chain alpha-hydroxy acid oxidase=FMN-dependent alpha-hydroxy acid-oxidizing enzyme EC 1.1.3.1	39502	7.9	1	39	3
2792500	clathrin assembly protein short form	64844	9.02	1	48	3
28488992	hypothetical protein XP_289590 [Mus musculus]	46416	8.05	1	48	3
435487	aldehyde dehydrogenase (NAD+) [Homo sapiens]	51367	5.83	1	75	3
9910242	GK001 protein [Homo sapiens]	56065	4.8	1	39	3
184462	chaperonin-like protein	43358	6.19	1	53	4
19527236	RIKEN cDNA 1110014L17 [Mus musculus]	26233	8.33	1	40	4
2465729	TFAR15 [Homo sapiens]	24642	8.58	1	49	4
25090044	Aldehyde dehydrogenase family 7 member A1 (Antiquitin 1)	25313	7.72	1	54	4
28336	mutant beta-actin (beta'-actin) [Homo sapiens]	42128	5.22	1	47	4
806754	Na,K-ATPase beta subunit	35270	8.84	1	43	4
996057	gp25l2 [Homo sapiens]	25277	6.4	1	39	5
1927215	ERS-24 [Cricetulus griseus] (SEC22 vesicle trafficking protein-like 1 [Mus musculus])	24824	8.88	1	60	6
234746	RAS-related protein MEL [Homo sapiens]	23753	9.35	1	48	6
27664498	similar to ribosomal protein L15, cytosolic [validated] - rat	16219	10.56	1	47	6
66313	glutathione peroxidase (EC 1.11.1.9) I - rat	22486	7.66	1	42	6
1633081	Catechol O-Methyltransferase	24960	5.11	1	68	7

576133	Chain A, Glutathione S-Transferase Yfyf (Class Pi) (E.C.2.5.1.18) Complexed With Glutathione Sulfon	23634	8.13	1	37	7
7415414	AQP-CHIP [Canis familiaris]	29009	6.96	1	39	7
27705116	similar to RIKEN cDNA 1200011D11 [Mus musculus]	18155	4.72	1	45	8
9790225	calcium binding protein P22 [Mus musculus]	22418	4.97	1	57	8
27685747	similar to Pantetheinase precursor (Pantetheine hydrolase) (Vascular non-inflammatory molecule 1) (	15921	6.05	1	65	9
70637	ubiquitin - Mediterranean fruit fly	8446	6.56	1	53	21

Definitions:

Accesion No: gene identifier number as reported in the NCBI protein database

Mr and pI: the theoretical molecular weight and isoelectric point based on the reported amino acid sequence.

PEP: number of peptides that derived MS/MS spectra for sequence determination of the nemed protein.

SCR: Mascot score. In general, scores above 40 are significant. When scores bellow this threshold were found, the MSMS spectra was reinterpreted manually.

COV: sequence coverage of the identified protein in percentage.

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