Characterisation of Thermophilic Esterases

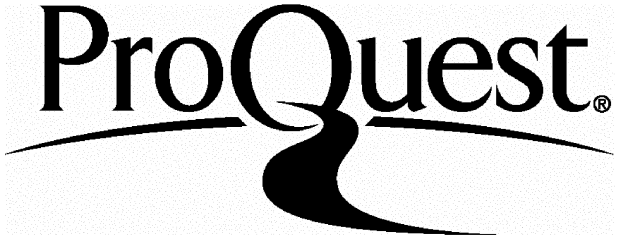
Andrew N. P. Wood

Submitted for PhD in Biochemistry

Faculty of Life Sciences

Department of Biochemistry and Molecular Biology

University College London



Abstract

Seventy two strains of thermophilic *Bacilli* (optimum growth temperatures 55°C-75°C) were screened for esterase activity. 23 strains showed some esterase activity when assayed with various *p*-NP substrates. The growth characteristics and nutritional requirements of six of these strains were investigated. An esterase activity obtained from one of the strains (Tok19 A1) was purified 5133-fold to electrophoretic homogeneity with 26 % recovery. The purified esterase had a specific activity of 2032 $\mu molmin^{-1}$ mg^{-1} based on the hydrolysis of *p*-nitrophenyl caproate at pH 7.0 and 30°C. The apparent molecular mass was 50,000 +/- 2,000 Da from SDS/polyacrylamide gel electrophoresis and 45,000 +/- 3000 Da from gel filtration. Native polyacrylamide gels stained for esterase activity showed three bands. The isoelectric points were estimated to be 5.7, 5.8 and 6.0. Forty amino acid residues were sequenced at the N-terminus. The sequence showed no degeneracy suggesting that the three esterases are functionally identical carboxylesterases differing by a limited number of amino acids. The enzyme showed maximum activity at pH 7.0 and was very stable at pH 6.0-8.9 with optimum stability at pH 6.0. At this pH and 60°C the half life was 170 hours. Esterase activity was totally inhibited by PMSF, *p*-CMB, eserine and TPCK but not by EDTA. The esterase obeyed Michaelis-Menten kinetics in the hydrolysis of *p*-nitrophenyl esters, but both V_{max} and K_M were protein-concentration dependent. The esterase was also stabilised by both homogeneous and heterogeneous protein-protein interactions. The stability of the esterases increased by more than 300 fold when the esterase concentration was increased from 2$\mu g\ mL^{-1}$ to 10$\mu g\ mL^{-1}$ or when BSA was added to a concentration of 1$mg\ mL^{-1}$. The esterase was activated by up to 115% in 1-2% v/v propanol and the half-life in 35% methanol v/v was 20 hours at 30°C. In immiscible linear alcohols C_5-C_{10} (50% v/v) the

esterase retained 100% of its activity after 24 hours at 40°C. The esterase also appeared to exhibit high levels of transferase activity, even in the presence of low concentrations of aliphatic alcohols.

The esterase was covalently immobilised to a glyoxyl agarose gel and was 10,000, 1,200 and 900 fold more stable than the soluble esterase at pHs 5, 7 and 9 respectively. The immobilised esterase also retained 70% of its initial activity after incubation in 50% DMF or DMSO at 30°C for 7 days.

The esterase had a broad substrate specificity hydrolysing linear and branched esters, aromatic esters (including indoxyl and naphthyl esters) and sugar acetates such as β-D-xylose tetraacetate.

Acknowledgments

The author wishes to acknowledge the Biotechnology and Biological Science Research Council (UK) for a research grant and Unilever PLC. (Bedford) for CASE support, Hispanager S.A. Burgos, Spain for the donation of cross-linked agarose gels and their glyoxyl derivatives, Dr. Don Cowan for his supervision and encouragement, Dr. Roberto Fernandez-Lafuente for his help with the immobilisation of the esterase, Dr. Alisdair Macrae (Unilever), Dr. Mike Orford and Paul Shadbolt for their scientific advice and their excellent technical support, Dr. Brian Coles for the N-terminal amino acid sequencing, Frances Pearl for sequence alignment, Alison and Gizzmo Taylor for their warmth, support and encouragement, my mother and the hand that fed me.

Publications

Wood, A. N. P., Cowan, D. A. and Fernandez-Lafuente, R. (1995) Purification and Partial Characterisation of a novel Thermophilic Carboxylesterase with High Specific Activity at Low Temperatures. *Enzyme Microbial Technology* (in press)

Wood, A. N. P., Cowan, D. A. and Fernandez-Lafuente, R. (1995). Characterisation of a Novel Thermophilic Esterase as an Industrial Catalyst. *Applied Biochemistry and Biotechnology* (in press)

Fernandez-Lafuente, R., Wood, A. N. P. and Cowan, D. A. (1995) Hyperstabilisation of Thermophilic Enzymes by Multipoint Covalent Attachment. *Enzyme Microbial Technology* (in press)

Fernandez-Lafuente, R., Wood, A. N. P. and Cowan, D. A. (1995) Reducing Enzyme Conformational Flexibility by Multi-point Covalent Immobilisation. *Biotechnological Techniques* (in press)

Abbreviations and symbols

Å	angstrom
Abs	absorbance (-log Transmitance)
BSA	Bovine serum albumin ()
°C	degrees Celsius
CCl	*Candida cylindracea* lipase
N-CBZ	N-carbobenzoxy
Da	daltons
DEAE	diethylaminoethyl
DFP	diisopropyl fluorophosphate
DMF	dimethylformamide
DPC	diethylpyrocarbonate
EDTA	ethylenediaminetetra-acetic acid
e.e.	enantiomeric excess
eserine	physostigimine
g	gram
GPC	gel permeation chromatography
HIC	hydrophobic interaction chromatography
h	hours
$HgCl_2$	mercury^{II} chloride, mercuric chloride
ief	isoelectric focusing
K	Kelvin
kJ	kilojoule
kDa	kilodaltons
K_M	Michaelis constant
k_{cat}	catalytic constant

k	equilibrium constant
k_d	dissociation constant
M	molar
mL	milliliter
mM	millimolar
min	minutes
nm	nanometers
Mr	relative molecular mass
PAGE	polyacrylamide gel electrophoresis
paraoxon	diethyl *p*-nitrophenyl phosphate
PEG	polyethylene glycol
PCR	polymerase chain reaction
PMSF	phenylmethanesulphonyl fluoride
p-CMB	parahydroxymercuribenzoate
p-NP	para-nitrophenyl
TDAM	thermophilic defined agar medium
TDLM	thermophilic defined liquid medium
TPCK	tosyl-L-phenylalanine chloromethyl ketone
u.v.	ultra violet
V_{max}	rate of enzyme-catalysed reaction at infinite substrate concentration
μ	micro

Abbreviations for amino acids

Amino acid	One-letter symbol
Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cysteine	C
Glutamine	Q
Glutamic acid	E
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

Contents

Lists of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Reaction Schemes and Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 1

Introduction

1.1 Organic solvent biocatalysis

Biocatalysts (enzymes) function under moderate conditions - normally at temperatures of 20 - 100 °C, pH values of 4 - 10 and at atmospheric pressure. They are uniquely suitable for the preparation of optically active compounds because they are built of only L-amino acids and consequently their active centers constitute a dissymmetric environment which is likely to distinguish between the enantiomers (Cambou and Klibanov, 1984a). This ability is especially important when only one isomer, specifically one stereoisomer, has the required biological activity and the other(s) are either inactive or have undesirable activity. For example, L- and D-carvone isomers have different tastes of spearmint and dill/caraway, respectively; only L-monosodium glutamate has taste-enhancing properties (Cheetham, 1993). By comparison, chemical processes are usually nonselective and much less environmentally friendly, particularly when reagents are used in stoichiometric amounts, or require heavy metal catalysts.

It is the culmination of properties: mild reaction conditions coupled with their reaction specificity, regiospecificity and stereospecificity that make enzymes applicable to a wide range of industrial processes.

In the past decade, enzymes have been successfully applied in non-conventional media. Earlier beliefs that practical applications for enzymes were essentially limited to water-based chemistry and the incompatibility of enzymes and organic media were disproved by several reports of enzyme catalysis in non-aqueous solutions in 1984-86 (Zaks and Klibanov, 1984a; Zaks and Klibanov, 1984b; Zaks and Klibanov, 1985; Luisi, 1985; Klibanov, 1986; Inada *et al.*, 1986). It is now clear that both enzymes and whole cell biocatalysts can function in systems far removed from simple dilute aqueous solutions. A major proportion of the reaction mixture can be an organic liquid, a gas phase, or a high-

pressure supercritical fluid. In many cases the water content in the reaction system may be microscopic, and may only contain the water bound to the biocatalyst.

Many reviews have been published on the activity of biocatalysts in non-conventional media and their applications in chemical synthesis at both laboratory and industrial scale (Arnold, 1990; Bruce, and Daugulis, 1991; Dordick, 1992; Gupta, 1992; Klibanov, 1990; Mattiasson, and Aldercreutz, 1991; Nakamura, 1990).

The value of nonaqueous biocatalysis to the advancement of enzyme technology cannot be underestimated. Tremendous advances have been made in the use of enzymes for synthetic applications ranging from chiral building blocks to biodegradable polymers. Furthermore, as discussed below, when placed in microaqueous solvents, enzymes display remarkable novel properties such as enhanced stability (Zaks and Klibanov, 1984; Ahern and Klibanov, 1985; Klibanov, 1986) and altered specificity (Zaks and Klibanov, 1984; Zaks and Klibanov, 1986;).

The most remarkable feature about the effect of a solvent on an enzyme is that the environment can be used to alter the "natural" activity and specificity of the catalyst. This approach to catalyst design has been termed "solvent engineering".

With the advancement of biocatalysis in non conventional media a new technical term, "microaqueous", has been proposed (Yamane *et al.*, 1988). This term implies that the system is neither aqueous, nor nonaqueous, nor anhydrous. In between the two extremes of aqueous and anhydrous, there is a state where the system only contains a small amount of water. This is the state that is implied by the term "microaqueous". The word "microaqueous" is more applicable than terms such as "in low water-activity media" (Larreta-Garde *et al.*, 1987), "in nearly anhydrous solvent" (Klibanov, 1987), "in an organic solvent containing a little amount of water" (Fukui, 1987).

Table 1.1 Shows some of the potential advantages and disadvantages of biocatalysis in microaqueous environments (compiled from Lilly and Woodley, 1985 and Dordick, 1989).

Table 1.1 Some of the potential advantages and disadvantages of biocatalysis in microaqueous environments

Advantages
High reactor reaction concentrations.
High reactor product concentrations.
Reduction in reaction/product inhibition.
Ease of biocatalyst separation.
Ease of product separation.
High oxygen solubility in organic solvents.
Less risk of microbial contamination.
Possibilities of thermodynamically unfavorable reactions.
Lipase-catalysed esterifications and transesterifications in non-aqueous media may show higher enantioselectivity than the corresponding hydrolysis in water (Klibanov, 1990; Dordick, 1992).
Disadvantages
Catalyst denaturation or inactivation by the organic phase.
Need for adequate rates of mass transfer between the liquid phases.
Increasing complexity of the reaction system.
Toxicity of organic media and environmental/economic consequences of disposal.

The major disadvantage with organic solvent biocatalysis is the denaturation of the biocatalyst either by the organic solvent or at the organic aqueous interface. However this problem can be overcome, in some instances, by immobilising the biocatalyst in the aqueous phase (Section 1.7) or by the use of surfactants and the formation of reverse micelles (Castro, and Cabral, 1988; Luisi, 1988; Martinek, 1989).

The fundamental understanding of enzymatic catalysis in non-conventional media lags behind the technological achievements. In part this reflects less experience with these systems, in part their greater complexity. However, some useful predictions can be made by physico-chemical analysis, especially based on thermodynamics and have been the subject of an excellent review (Halling, 1993).

1.1.1 The role of water in organic solvent biocatalysis

Evidence suggests that an enzyme molecule in aqueous solutions becomes fully hydrated when surrounded by a few layers of water (Zaks and Klibanov, 1988b). These layers of water act as a buffer between the enzyme surface and the bulk reaction medium. It is highly unlikely that the enzyme will be affected by the bulk aqueous phase unless a significant alteration of the enzymes microenvironment is caused by the bulk reaction medium (Zaks and Klibanov, 1985; Fukui *et al.*, 1987). Therefore, if this layer of essential water were somehow to be maintained then it should be possible to replace the remaining bulk water with organic solvents without any adverse effects on the enzyme. This has been shown to be the case in studies of various hydrophobic (non polar) solvents (Deetz and Rozzell, 1988; Zaks and Klibanov, 1985).

The critical facet of using enzymes in organic solvents is the amount of water in the molecular environment of the active enzyme. Enzymes cannot function under totally anhydrous conditions. The chemical and physical properties an enzyme exhibits largely

depend on the direct or indirect role of water in all noncovalent interactions (e.g., electrostatic, hydrogen bonding, Van der Waals, and hydrophobic) that help maintain the enzyme in a catalitically active conformation (Shultz and Schirmer, 1979). The complete replacement of water with an organic solvent distorts the native protein structure and destroys enzymic activity (Dordick, 1989). However, water can be present as a very small percentage of the overall reaction volume (Zaks and Klibanov, 1988b). Water has several effects in enzymic systems: as well as maintaining the enzymes' catalytically active conformation (Riva, 1988). Too little water results in poor hydration of the enzyme and corresponding poor catalysis while too much can decrease product yield in reactions such as esterification. Water is also required for most enzyme inactivation processes such as proteolysis and in particular those of thermal inactivation (Ahern and Klibanov, 1988). The critical role of water in most aspects of protein function has been reviewed by Rupley and Careri, (1991).

The water in a reaction system may not be associated solely with the enzyme but may also be present in other parts of the system, for example, associated with an immobilisation support or an organic phase. Thermodynamic water activity (a_w) describes the mass action effects of water on hydrolytic equilibria, and also the distribution of water between the various phases that can compete in binding water. Because enzyme catalytic activity is very sensitive to the hydration of the enzyme, a_w often predicts an unchanging optimum as other aspects of the system are changed. It has been argued that a_w should be measured and/or controlled in these systems, whether the primary aim is to study the effects of water or of other changes (Halling, 1992; Halling, 1993). The critical concept when using water activities is that at equilibrium (even in multiphase systems), the activity of any component will be equal in each phase (even

though its concentration may be very different), provided that the same standard reference state is used for each phase.

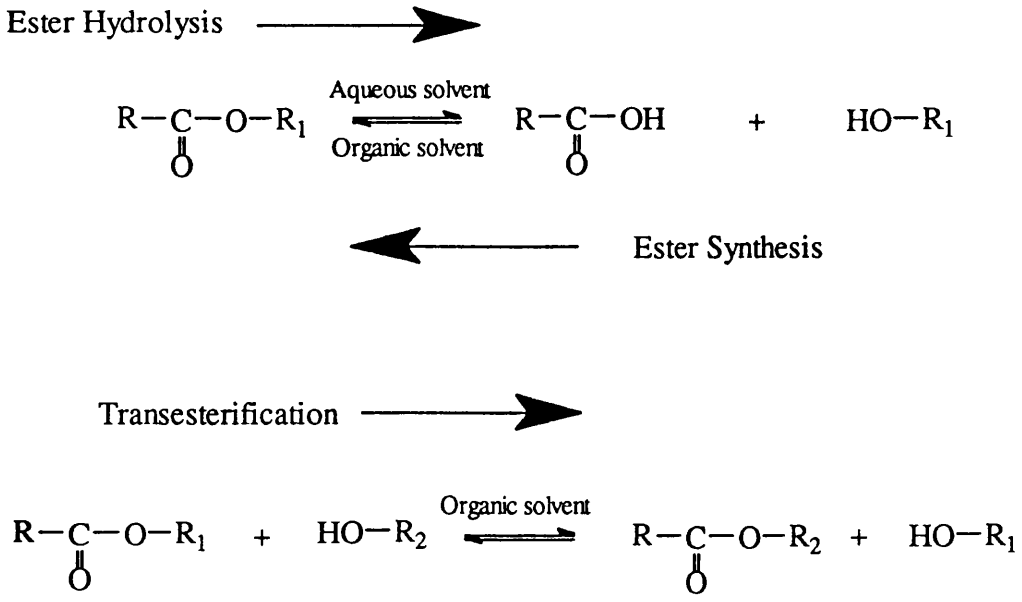
1.1.2 The control of water activity in microaqueous solvents

Silica particles have been used to control (buffer) a_w (Halling and Macrae, 1982; Goldberg *et al.*, 1988). However salt hydrates (for example a mixture of $Na_2HPO_4.7H_2O$ and $Na_2HPO_4.12H_2O$ gives a water activity of 0.74) are thought to be better as a_w buffers for the direct addition to the reaction mixture. They have been used in several enzymes and solvent systems (Kvittingen *et al.*, 1992; Kuhl and Halling, 1991; Yang and Robb, 1993; Sjursnes and Anthonsen, 1993) and proved effective donors of water over the temperature range 20 - 40°C (Robb *et al.*, 1993). In theory these salt hydrates have the advantage of giving a perfect buffering action; a_w remains completely constant while at least a trace of each hydrate is present.

1.1.3 The effect of water on hydrolytic equilibria

An important reason for choosing microaqueous solvents is to shift the equilibrium position of hydrolytic reactions to allow hydrolases such as lipases and esterases to catalyse a variety of synthetic reactions (Scheme 1.1). These reactions may include esterifications, transesterifications, interesterifications (acyl exchange), thiotransesterifications and oximolysis (Zaks and Klibanov, 1985).

Scheme 1.1 The reversal of hydrolytic equilibria in microaqueous solvents



Cassells and Halling (1988) found that the equilibrium position of the above reactions (Scheme 1.1) was not affected by a reduction in the volume of the water from 50% to 1% (over which range a_w remains close to 1). However, it has been shown that when the a_w was reduced below 1 there was a significant shift in favor of the synthetic products (Casselles and Halling. 1988; Blanco *et al.*, 1992; Chahid *et al.*, 1992; Bloomer *et al.*, 1992).

It can therefore be seen that the use of microaqueous solvents can increase the yields of synthetic reactions with little interference from hydrolytic side-reactions which constitute a major problem in aqueous reaction media (Adlercreutz, 1993).

Hydrophobic interactions are one of the driving forces of enzyme-substrate binding. In aqueous solutions lipophilic substrates are "expelled" from the water into the enzymes active site. In nonaqueous media this driving force will no longer exist, and there will be a change in the balance of binding substrates to enzymes whose energy is used in catalysis (Klibanov, 1986). For example, in water chymotrypsin hydrolyses N-acetyl-L-phenylalanine ethyl ester 100 times faster than it does the ethyl ester of N-acetyl-L-

histidine because of the hydrophobicity of the former substrate. Klibanov (1986) found that in octane the substrate specificity was reversed and the histidine derivative became a better substrate.

1.1.4 Enzyme stability in organic solvents

Until recently all biological evidence based on activity pointed to the fact that most organic solvents, in high concentrations, were enzyme denaturants. It has long been known (Bourquelot and Brindel, 1911; Tan and Lovrein, 1972) that modest concentrations of certain solvents such as glycerol (Butler, 1979), ethylene glycol (Bradbury and Jakoby, 1972) and short chain aliphatic alcohols and ketones, have the opposite effect and have been useful in enzyme stabilisation (Takemori, 1967; Asakura, 1978).

Water is required for enzyme inactivation processes inducing chemical changes. This includes proteolysis the hydrolysis of peptide bonds adjacent to aspartate residues, deamidation of asparagine and glutamine, the hydrolysis of asparagine resulting in either L-aspartyl or L-isoaspartyl residues depending on which amide linkage in the proposed sucinimide intermediate is hydrolysed and destruction of disulfide linkages via base-catalysed β-elimination (Clarke, 1985; Zaks and Klibanov, 1984; Ahern and Klibanov, 1985; Schulz and Schrimer, 1979).

Each process requires water and therefore should be suppressed in an micro-aqueous environment. This has been confirmed in experiments demonstrating that upon dehydration, enzymes become remarkably stable to heat (Zaks and Klibanov, 1984a). For example, in water, porcine pancreatic lipase spontaneously inactivates at 100°C whereas in a tributyrin-heptanol mixture containing 0.8% water its half life at 100°C was more than 12 hours.

Deetz and Rozzel (1988) measured the stability of horse liver alcohol dehydrogenase (HLADH) in butyl acetate at 25°C as a function of water concentration. In dry butyl acetate, the half-life of HLADH was greater than 100 hours: however as the concentration of water approached saturation in the solvent (~2% v/v) the stability of the enzyme decreased.

Enzymes are stable in a variety of miscible and immiscible organic solvents. However, the exact conditions necessary for the maintenance of catalytic activity in each enzyme system have to be determined empirically.

1.1.5 The activity of enzymes in aqueous organic media

The amount of water required for enzyme activity appears to be both solvent and enzyme dependent. The nature of the solvent is crucial for maintaining the layer of essential water around the enzyme molecule. The most hydrophobic solvents, such as hydrocarbons, are the most effective at maintaining this essential layer of water (Klibanov, 1986). The more hydrophilic the solvent, the higher its affinity for water hence it is more likely to strip the "essential" water layer away from the enzyme (Zaks and Klibanov, 1985; Zaks and Klibanov, 1988b). The percentage of water in the reaction media then has to be increased in order to maintain catalytic activity (Luisi, 1985). In some enzymes, such as porcine pancreatic lipase, the necessary water appears so tightly bound to the enzyme molecule that they are catalytically active in both hydrophobic and hydrophilic solvents (Klibanov, 1986). Lipases and horseradish peroxidase were from 10% to 100% as active in organic media as they were in water (Zaks and Klibanov, 1985) and similar results were obtained for several polyethylene glycol modified enzymes (Takahashi *et al.*, 1984a; Takahashi *et al.*, 1984b).

Tan and Lovrein (1972) studied ten enzymes in binary solvents at 25°C. Under the reaction conditions they found that the enzymes were more active (by a factor of 1.5 to 3 times) than they were in water alone. Occasionally they were markedly more active (by a factor of 10 or more). In no cases were they less active in the binary mixture than in water unless a critical mole fraction region was exceeded (in this case 0.01 to 0.05 mole fraction of organic compound). Studies of three enzymes, HLADH, mushroom tyrosinase and yeast alcohol oxidase showed that they were barely active in anhydrous solvents. However with the addition of a few percent of water their activity increased to the same order of magnitude as in the aqueous solution (Kazandjian and Klibanov 1985). It is often difficult to directly compare activities in aqueous and organic media because comparable rate data for aqueous catalysis is not available. With lipases for example, rate constants for esterifications and transesterifications are difficult to determine since hydrolysis predominates. Where comparisons were possible it was shown that both yeast and porcine pancreatic lipases accelerated the rate of transesterifications in hexadecane by approximately 1.5-3 x 10^6 fold relative to the uncatalysed reaction (Zaks and Klibanov, 1985).

The influence of cosolvents on the hydrolysis of aromatic esters by lipase PS from *Pseudomonas cepacia* has been studied (Bosetti *et al.*, 1993). The lipase displayed the highest enzymatic activity in solvents completely miscible (log P<0) or completely immiscible (log P>1.5) in water. It was shown that the enzyme activity depended on the amount of water in the reaction mixture and the log P value of the solvents. The log P values showed a linear correlation with the initial reaction rate: increasing with increasing log P (Gubicza and Szakacs-Schmidt, 1993). The activity of the enzyme in solvents with a high log P can be explained in terms of the maintenance of the essential water around the enzyme.

1.1.6 "Tuning" enzymes for organic solvent biocatalysis

It has been shown through site-directed mutagenesis on subtilisin BPN' that the reactivity and substrate specificity of the enzyme were strongly dependent on the polarity of the substrate, polarity of the active-site mutation (for example the enzyme became more reactive towards polar substrates when less polar amino acids were replaced by polar amino acids) and the solvent. (Dordick, 1993).

Furthermore polar mutations improved the intrinsic activity of subtilisin in apolar solvents (Dordick, 1993). In addition to protein engineering, enzyme function can be improved by making the enzyme environment more polar i.e. freeze drying subtilisin in the presence of salts such as KCl, resulted in significantly improved catalytic efficiencies- up to 600 fold in hexane (Dordick, 1993).

Claims of "pH memory"; i.e., that lyophilised enzymes placed in microaqueous solvents appeared to "remember" the pH of the latest aqueous solution to which they were exposed (Zaks and Klibanov, 1985) have recently been shown to be due to the freeze dried buffer and not interactions on a molecular scale (Blackwood *et al.*, 1993) as previously thought. However these effects can be very dramatic. For example, the transesterification reaction between N-acetyl-L-phenylalanine ethyl ester and propanol in octane was accelerated 300-fold if, instead of a commercial sample of the protease subtilisin, the same quantity of subtilisin precipitated from pH 7.8 (optimum for enzymatic activity in water) was used (Klibanov, 1986).

As well as "tuning" the enzyme, data has been presented suggesting that the structure of the solvent could influence the enzyme properties through direct solvent protein interaction (Ottolina *et al.*, 1993). The effects of solvent geometry of the chiral terpenes (R) and (S)-carvone (2-methyl-5(1-methylethenyl)-2-cyclo-hexene-1-one) on the activity and regioselectivity of enzymatic transesterifications were studied. The kinetics of the

transesterification between vinyl butyrate and hexanol catalysed by Lipase PS, Lipoprotein lipase or subtilisin were found to be different in the two solvents. With Lipoprotein Lipase, for example, the K_M was the same whereas V_{max} was higher in (S)- than in (R)-carvone, suggesting a non-competitive inhibition by the (R)-solvent. The solvent chirality also influenced enzyme regioselectivity when methyl hydroquinone dibutyrate and di-(2,2,2 trifuoroethyl)-itaconate were the substrates.

1.1.7 Selection of solvents for synthesis and the use of log P

Several factors must be taken into account in determining which solvent is most appropriate for a given reaction. The single most important factor in solvent selection is maintaining the catalytic activity of the enzyme. Another factor is that the selected solvent should be inert to the reaction. Additional factors which may influence the choice of solvent, include solvent density and viscosity, surface tension, toxicity, flammability, waste disposal and cost.

Brink and Tramper (1985) studied the effects of more than 30 solvents on the microbial epoxidation of propene and 1-butene in an aqueous/immiscible solvent biphasic system. They concluded that the suitability of a water-immiscible organic solvent can be predicted by evaluating the polarity and the molecular size of the solvent. Retention of a high catalytic activity was favoured by the solvent having a low polarity and high molecular weight. Laane *et al.*, (1985) concurred with the conclusions of Brink and Tramper although they proposed the use of log P.

Laane replotted the data of Brink and Tramper and also that of Playne *et al.*, (1983) and found a substantial correlation between activity and solvent polarity when log P was used. Generally values of log P in excess of 4 (decanol, hexadecane, diphthalate esters, etc.) supported high catalytic activity retention in the propene epoxidation reaction.

Solvents with log P values below 2 (short chain alcohols, water-miscible solvents, short chain esters and ethers etc.) were less unsuitable for biocatalysis. It is now generally accepted that hydrophobic solvents have a less negative affect on the structure and function of enzymes and hydrophilic solvents strip water from the enzyme reducing its activity (Wehtje *et al.*, 1993).

N.B. It must be noted that the majority of this work has been conducted using whole cell systems rather than free enzymes.

1.1.8 Supercritical fluids and gases as media for enzymic reactions

Supercritical (SC) fluids have received much attention in the past 10 years as possible media for enzymic reactions (Hammond *et al.*, 1985; Min *et al.*, 1988; Pasta *et al.*, 1989; Randolph *et al.*, 1988; Steytler *et al.*, 1991; Vermue *et al.*, 1992; Russell *et al.*, 1993; Koenig *et al.*, 1993) due to the advantages associated with their use:

- Their low viscosity and low surface tension favour efficient mass transfer.
- Diffusivities in the gas phase are orders of magnitude higher than in water. Furthermore whereas the diffusivities in water increase as a linear function of temperature, they increase in proportion to temperature at the power of 1.5 in case of gases. Gas phase reactions are thus less sensitive to diffusional limitations especially at elevated temperatures.
- They are fully miscible with other gases, such as oxygen, so that virtually any desired concentration can be achieved to carry out homogeneous or heterogeneous chemical reactions (Hammond *et al.*, 1985).
- Efficient separation by raising the temperature or decreasing the pressure.

- The ability to control the solubility of solutes by regulating both temperature and pressure (McHugh and Krukonis, 1986).
- As non toxic compounds SC fluids such as carbon dioxide and freons are alternatives for toxic and hazardous organic solvents during biotransformations.
- Enzymes in dehydrated forms are more resistant to thermoinactivation (Ahern and Klibanov, 1985) allowing the use of elevated temperatures, prohibited in aqueous solution, with substantial increases in reaction rates according to the Arrhenius Law.

Carbon dioxide is the most commonly used SC fluid, reaching supercritical status at 31°C and 73 bar. It has found application in the large scale extraction of caffeine and hop resins and as a solvent in chromatography (Koenig *et al.*, 1993). Many enzymes have a limited stability in SC CO_2 but from the relatively few published papers it can be inferred that the stability of enzymes is not significantly affected by contact with SC CO_2 but predominantly by the water content, pressure, and temperature (Koenig *et al.*, 1993). However immobilisation has been shown to stabilise enzymes in SC CO_2 (Knez and Habulin, 1992; Verume *et al.*, 1992).

The physical properties of supercritical fluids can be adjusted over a range which can significantly affect enzyme activity. In particular, the dielectric constant of fluroform can be predictably altered with small changes in pressure. For example at 600psi the physical properties of fluroform resemble those of propane at its vapour pressure, whilst when above 4000psi, fluroform is more like methylene chloride. It has been shown that changes in dielectric constants of conventional solvents can be used to manipulate enzyme activity and specificity (Russell *et al.*, 1993).

1.1.9 Enantioselectivity: the effect of temperature, water, solvent and chemical modification

The ability of an enzyme to choose one or other enantiomer (resolution) is termed enantioselectivity and is defined by the enantiomeric ratio, E (Equation 1 Sih and Chen, 1984).

$$E = R/S = (k_{cat}/K_M)_R/(k_{cat}/K_M)_S$$

Resolution of racemic alcohols, acids and esters via hydrolase-catalysed esterifications and transesterifications in microaqueous organic solvents is a well known procedure, especially for compounds unstable or poorly soluble in water (Langrand *et al.*, 1985; Chen and Sih, 1989; De Amici *et al.*, 1989; Santaniello *et al.*, 1992). However it is only recently that the effects of the reaction conditions (nature of the organic solvent, enzyme form, temperature and water concentration) on enzyme enantioselectivity have started to be investigated.

Temperature

It is commonly perceived that enzymatic reactions exhibit optimal stereoselectivity at low temperatures. This is because at temperatures below their optimal activity the structures of enzymes are known to be more rigid than at higher temperatures and steric hindrance results in large groups being excluded from the active site. However both an increase (Lam *et al.*, 1986; Willaert *et al.*, 1988; Keinan *et al.*, 1986) and a decrease (Boutelje *et al.*, 1988) of the selectivity with a decrease in temperature have been observed. It has also been reported that the temperature had no effect on the enantioselectivity (Barton *et al.*, 1990). However after studying Barton's results it was reported that there was a slight increase in enantioselectivity at lower temperatures (Holmberg and Hult, 1991). The theory of temperature effects on stereochemistry was reviewed by Phillips, (1992).

The increase in stereoselectivity with temperature is highlighted in the following examples. Secondary alcohol dehydrogenase (SADH) from the thermophilic bacterium, *Thermoanaerobacter ethanolicus* exhibited activity with a wide range of ketones, normally forming (S)-alcohols with enantiomeric purity. With 2-butanone, the preferred product was (S)-2-butanol below 26°C, and (R)-2-butanol at temperatures above 26°C (Pham and Phillips, 1990). In contrast, in the reduction of 2-pentanone, the formation of (S)-2-pentanol decreased in enantiomeric purity as the reaction temperature was increased (Pham and Phillips; 1990, Zheng and Phillips, 1992).

Thus decreasing the temperature is one way to modulate the enantiomeric excess from such enzyme–catalysed reactions. However conducting enantioselective resolutions at very low temperatures may be uneconomic for industrial purposes.

Water content of the reaction

The effect of water concentration on enantioselectivity is somewhat contradictory since both increases (Holmberg and Hult, 1990; Kitaguchi *et al.*, 1990; van der Lugt *et al.*, 1992) and decreases (Wickli *et al.*, 1992) in enzyme enantioselectivity as a function of water content in the reaction medium have been reported. In the above studies water concentrations rather than water activities were quoted, whereas it has been demonstrated that it is water activity (a_w) rather than concentrations that determines enzyme properties (Goderis *et al.*, 1987; Klibanov, 1989; Aldercreutz, 1991; Valivety *et al.*, 1992; Halling, 1993). Bovara and co-workers (1993) investigated water activity in enantioselectivity and found it to have no influence in the esterification of racemic sulcatol with vinyl acetate with either Lipase PS from *Pseudomonas cepacia* or lipoprotein lipase from *Pseudomonas sp.*

Solvent effects

Fitzpatrick and Klibanov (1991) found that enzymatic transesterification reactions catalysed by subtilisin in organic media can have a dramatically altered stereoselectivity in solvents of different polarity. The addition of 25-50% DMSO improved the stereoselectivity of esterase-catalysed hydrolysis of methyl alkyl dimethylmalonates (Bjorkling *et al.*, 1986). In contrast, Jones and Mehes, (1979) reported that chymotrypsin exhibited diminished enantiospecificity for the hydrolysis of phenylalanine esters in the presence of organic solvents.

The solvent effects on the esterification of phenoxypropionic acids with n-butanol using *Candida cylindracea* lipase have been studied (Ueji *et al.*, 1992). The enantioselectivity was shown to be inverted in non-polar solvents such as carbon tetrachloride from that in polar solvents such as acetone. For example, in carbon tetrachloride there was a 48% e.e of the R enantiomer of the butyl ester (a useful herbicide) whereas in acetone there was a 39% e.e of the S enantiomer. When the mole fraction of acetone in carbon tetrachloride was investigated the ratios of the R enantiomers in their ester products decreased with an increase in the mole fraction of acetone. The inversion of the enantioselectivity from "R" to "S" occurred around 0.5 mole fraction of acetone, and also the conversion decreased and the esterification became significantly slower. The change in enantioselectivity was attributed to a conformation of the lipase arising from specific interactions between the solvent and the lipase (Ueji *et al.*, 1992).

Bosetti *et al.*, (1993) also studied the effects of cosolvents on the enantioselectivity of lipase catalysed resolution of isopropylidene glycerol (an important building block for the preparation of enantiomerically pure biologically active compounds, such as β-blockers). They found that cosolvents had a marked influence on the hydrolysis of the aromatic

esters, and was correlated to the percentage of the added solvents and to the nature of the organic media expressed through their physical and chemical properties (log P, ε, μ). The lipase displayed the highest enzymatic activity in solvents miscible (logP<0) or immiscible (logP>1.5) in water. The enzyme stereoselectivity was inversely related to the logP of the solvent. In a similar study involving the esterification of 2-substituted propionic acids it was shown that the logP values showed a linear correlation with the enantioselectivity as well as the initial reaction rate (Gubicza and Szakacs-Schmidt, 1993). The enantioselectivity slightly decreased while the reaction rates increased with increasing log P values.

It has been shown that shifts in substrate specificity as well as in enantioselectivity take place when enzymes are suspended in microaqueous solvents (Zaks and Klibanov, 1984b; Martinek and Semenov, 1981a, b and c). Elimination of bulk water resulted in the increased rigidity of the enzyme structure with the inability to accommodate large substrates. This was also shown in the transesterification reaction involving porcine pancreatic lipase in microaqueous and anhydrous tributyrin (Zaks and Klibanov, 1984b). Only the "wet" lipase catalysed the transesterification between tributyrin and tertiary alcohols whereas the smaller secondary and primary alcohols were substrates for either enzyme preparation.

However in the systematic investigation into the influence of the nature of organic solvents on the resolutions of several primary and secondary alcohols catalysed by: PPL, lipase PS, Lipo protein lipase, *chromobacterium viscosum* lipase and *mucor miehei* lipase no correlation between enantioselectivity and physico-chemical properties of the solvents was found. A rationale based on the formation of solvent-enzyme complexes was proposed to explain these results. This rationale was also supported by the finding

that enantiomeric solvents showed different effects on enzyme activity and regioselectivity (Carrea *et al.*, 1993; Ottolina *et al.*, 1993).

Chemical modification

The chemical modification of enzymes has been a valuable tool for investigating the nature of the active site residues and to identify those amino acids that participate in catalysis and substrate binding. This technique has been used to alter enzyme specificity (Kaiser *et al.*, 1985) and to improve the enantioselectivity of *Candida cylindracea* lipase (B-form) (Gu and Sih, 1992). The lipase was treated with the classical chemoselective reagent tetranitromethane (TNM) which nitrated specific tyrosyl residues. The chemically modified TNM-lipase showed remarkably improved enantioselectivity in the hydrolysis of a series of aryloxypropionic and arylpropionic esters, the enantioselectivity E was raised from 1.5 (native enzyme) to 33. The marked improvement in enantioselectivity could be attributed to the change in the k_{cat} values for the S-enantiomer which decreased over ten-fold as the TNM concentration was increased. In contrast, the k_{cat} values for the R-enantiomer decreased only two-fold under the same conditions. It has also been shown that the enantioselectivities of *H. lanuginosa* and *R. miehi* lipases can be manipulated by site directed mutagenesis and chemical modifications of their respective ampipathic helices (Hult *et al.*, 1993).

The relatively few published papers on this subject suggest that chemical modification of enzymes as a way of altering their specificity and enantiospecificity is not generally applicable.

1.1.10 Thermophilic enzymes in organic solvents

The advantages of conducting enzymatic reactions in organic solvents have been outlined in Table 1.1, however, the problems of enzyme denaturation still persist. In response to

these problems it has been shown, in a study of proteases from ten different mesophilic and thermophilic origins, that thermophilic proteins are more stable to denaturation in organic solvents than proteins from mesophiles, under similar conditions (Owusu and Cowan, 1989).

Studies have also been conducted on the stability and activity of a thermostable malic enzyme, from *Sulfolobus solfataricus*, in denaturants and water-miscible organic solvents (Guagliardi *et al.*, 1989). The enzyme showed a higher tolerance than mesophiles to denaturation by urea, SDS and guanidine hydrochloride. The malic enzyme retained over 75% of its activity at 25°C after incubation in 10% of 2-propanol, 50% DMF, 50% methanol and 15 % ethanol.

Immobilised thermolysin has been used in a stirred-tank reactor for the continuous synthesis of (benzyloxycarbonyl)-L-aspartyl-L-phenylalanine methyl ester (the precursor of the synthetic sweetner aspartame) in ethyl acetate at 40°C. The enzyme showed remarkable stability and retained 70% of its activity after 302 hours incubation (Nakanishi *et al.*, 1985). An esterase from a thermophilic Bacillus has been used to conduct the transesterification of *p*-nitrophenyl esters in methanol and dry ethyl acetate. The esterase was fully thermostable in both solvents at 44°C. At 88°C the esterase activity decreased by 90% in water:methanol (10% v/v) and 30% in dry ethyl acetate after 4 hours (Owusu and Cowan, 1990).

It is apparent that biocatalysts isolated from thermophilic bacteria and archaebacteria are more resistant to common protein denaturants and organic solvents than similar biocatalysts from mesophiles (Owusu and Cowan, 1989; Nakanishi *et al.*, 1985; Guagliardi *et al.*, 1989; Fontana, 1988). Therefore, an enzyme isolated from a thermophilic organism, with its inherent stability in organic solvents, would be the obvious choice for organic solvent biocatalysis.

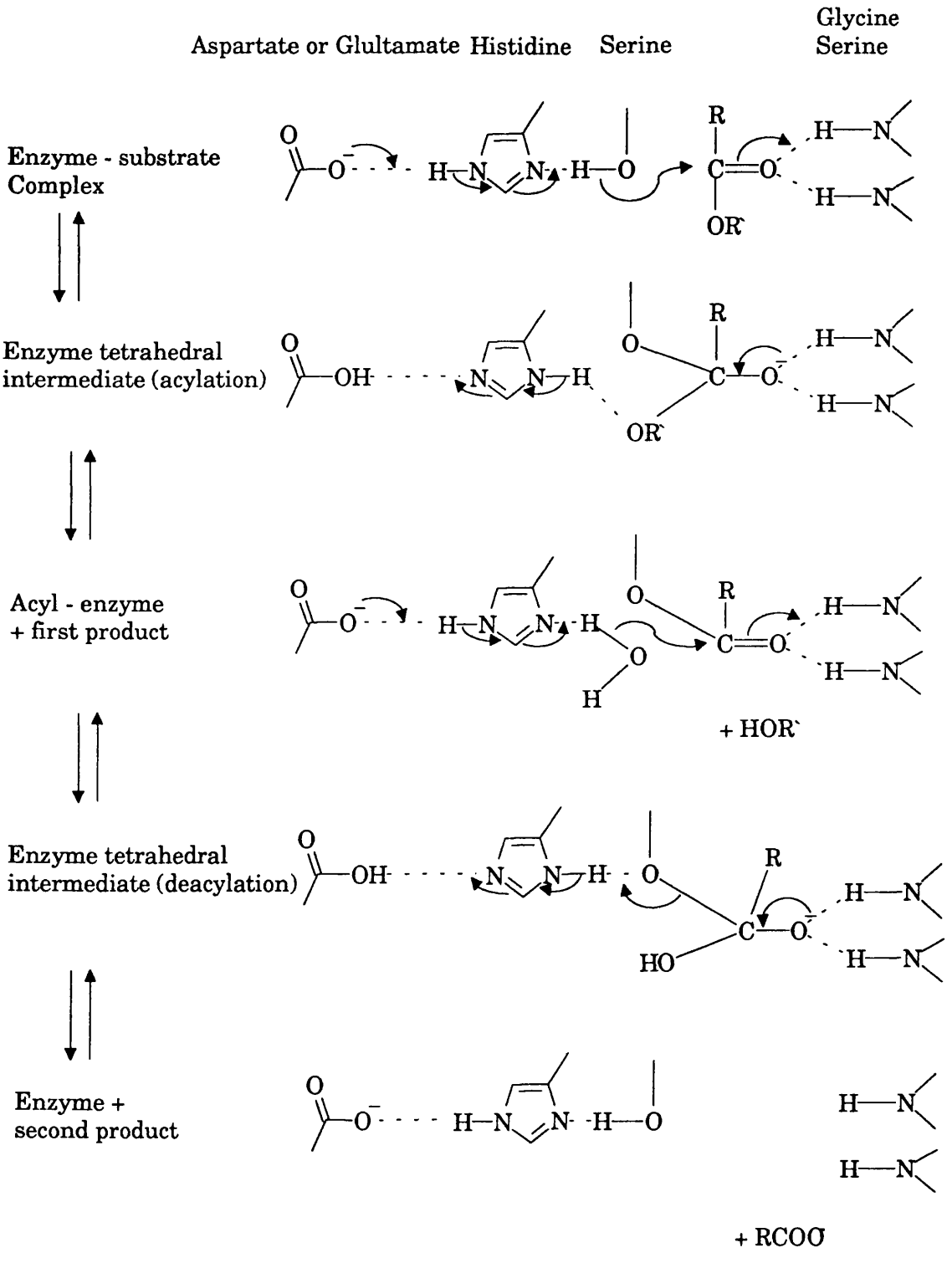
1.2. Catalytic mechanism of esterases and lipases

The catalytic mechanism of the esterases has not been elucidated but it is probably similar to that of the lipases and to a lesser extent the serine proteases. This assumption is based on a number of facts:

The inactivation by the inhibitors diethyl *p*-nitrophenyl phosphate (paraoxon), PMSF, diisopropyl fluorophosphate (DFP) and physostigimine (eserine) indicate the involvement of a serine residue in the enzyme activity of a number of mesophilic and thermophilic esterases (Matsunaga *et al.*, 1974; Nakagawa *et al.*, 1984; Sobek and Gorisch, 1988; Owusu and Cowan, 1991; Manco *et al.*, 1994). Where tested, these enzymes were also inhibited by diethylpyrocarbonate suggesting the possible involvement of a histidine residue in the active site. Taken together these data suggest that these esterases contain a catalytic triad Ser....His.....Asp/Glu. This catalytic triad is identical to that proven for the lipases and serine proteases (Brady *et al.*, 1990; Winkler *et al.*, 1990; Schrag *et al.*, 1991) whose 3D structure has recently been elucidated by X-ray crystallography.. A catalytic triad Ser....His.....Glu has also been proven in acetylcholinesterase (Sussman *et al.*, 1991).

The replacement of Glu for Asp in the lipases and acetylcholinesterase indicates that Asp is not an absolute requirement for function as was originally thought after studies with the serine proteases. The Postulated catalytic mechanism for α-chymotrypsin-catalysed hydrolysis involving the Ser.....His.....Asp triad is represented in Scheme 1.2.

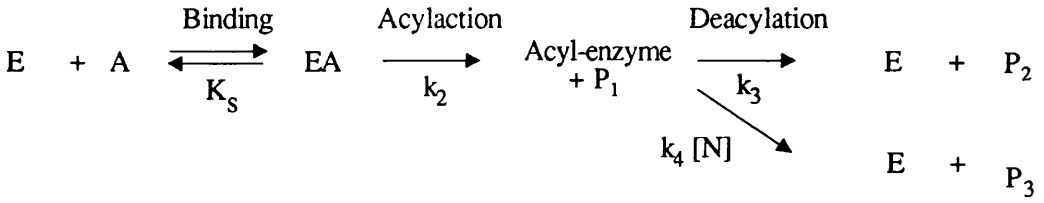
Scheme 1.2. Postulated mechanism for α-chymotrypsin-catalysed hydrolysis of an ester



The substrate is represented as RCOOR`.

Further evidence for the similarity of the catalytic mechanism comes from the fact that the hydrolysis of peptide and esterase bonds through a nucleophilic attack by the serine residue, which is activated via the Glu/Asp-His couple and the accompanying action of the oxyanion hole (Scheme 1.2.) occurs via an acyl-enzyme tetrahedral transition state (Kraut, 1977). The hydrolysis of esters by esterases including PLE (Greenzaid and Jencks, 1971) horse liver esterase and beef liver esterase (Goldberg and Fruton, 1970) have also been shown to proceed via acyl-enzyme, presumably tetrahedral, transition states according to Scheme 1.3.

Scheme 1.3. Two step mechanism for ester hydrolysis



E = Enzyme, A = Substrate, P = Product, N = Nucleophile (for example an alcohol in transesterification)

Scheme 1.3 (assuming an ester is being hydrolysed by an esterase) involves a rapid binding step with a dissociation constant K_s, an acylation step k_2, and release of the first product P_1 (an alcohol) hydrolysis of the acyl-enzyme (deacylation) k_3 with release of the second product P_2 (a carboxylic acid), and reaction of the acyl-enzyme with a nucleophile such as methanol (as in tranesterification), k_4[N] to give a product P_3 (an ester).

Sequence data also confirms that lipases and esterases have similar catalytic sites. All lipases sequenced share sequence homologies including a highly conserved region His-X-Y-Gly-Z-Ser-W-Gly or Y-Gly-His-Ser-W-Gly (W, X, Y and Z denote unspecified amino acid residues) (Antonian, 1988). This sequence is located on a common core consisting

of a well conserved β sheet, also present in the structure of esterases (the α/β-hydrolase fold) (Ollis *et al.*, 1992).

This catalytic triad Ser....His....Glu/Asp is chemically analogous to, but structurally different from, that of the serine proteases. The difference in specificity is highlighted by the fact serine proteases and mammalian esterases can hydrolyse amide and ester bonds (Krish, 1971; Alichanidis *et al.*, 1986; Stoops *et al.*, 1969) whereas bacterial esterases have been shown not to have any amidase activity (Owusu and Cowan, 1991; Manco *et al.*, 1994; Higerd and Spizizen, 1973; Sobek and Gorisch, 1989).

The chemistry of ester and amide hydrolysis is very similar (March, 1985). It therefore follows that the catalytic mechanisms of the esterases, lipases and serine proteases are probably very similar and that the structures of the inferred tetrahedral intermediate complexes of the lipases and esterases will be reminiscent of that found in serine proteases. However, structural difference in the lipases and serine proteases mean that the activation process of the lipases and serine proteases are very different. In the serine proteases the interactions at the oxy-anion hole are preformed whereas in lipases they are generated by the activation process involving large conformational changes (Brzozowski *et al.*, 1991, Tilbeurgh *et al.*, 1993; Derewenda *et al.*, 1993).

1.2.1 Lipases

True lipases, formally, referred to as triacylglyceride ester hydrolases (EC 3.1.1.3), hydrolyse ester bonds in neutral lipids. They are a sub-class of the esterase group in that their natural substrates are generally insoluble in water and their activity is maximal only when the enzyme is adsorbed to the oil-water interface (where the substrate concentration is higher than in water (Sarada and Desnulle, 1958; Entressangles and Desnulle, 1968)). This unique property is known as interfacial activation (Derewenda

and Sharp, 1993). Another common distinction is that lipases are able to hydrolyse C_{14}-triglyceryloeate (Macrea, 1983)

Because lipases usually catalyse reactions at lipid-water interfaces, the observed rates of reaction are strongly influenced by the available interfacial area (Desnuelle, 1961). Theoretical interpretations of the activation of lipases by interfaces have been attempted by a number of authors. These interpretations can be divided into two groups: 1) those which assume that the substrates are activated by the presence of an oil/water interface, and 2) those which assume that the lipase undergoes a conformational change to an activated form upon contact with an oil/water interface (Verger and Haas, 1976). Explanations for the former type of activation involve: higher concentrations of substrates in the vicinity of the interface than in the bulk of the oil (Brockman *et al.*, 1973), more suitable conformations of the lipid molecules and better orientation of the scissile ester bond (Wells, 1974; Brockerhoff, 1970; Mattson and Volpenhein, 1969; Shah and Schulman, 1967), and reduction in the water shell around the lipid molecules in water (poor hydration of lipid molecules), thereby avoiding ester-bond shielding in the neighbourhood of oil/water interfaces (Brockerhoff, 1968; Entressangles and Desnuelle, 1974). Explanations for the latter type of activation involve the existence of separate adsorption and catalytic sites for the lipase such that the lipase becomes catalytically active only after binding at the interface (Verger *et al.*, 1973; Brockerhoff, 1973; Desnuelle *et al.*, 1960) or a conformational change of the lipase upon approaching the oil/water interface (Entressangles and Desnuelle, 1974; Sarada and Desnuelle, 1958) that might be due to the high degree of order in such regions (Desnuelle *et al.*, 1960; Rothfield and Romero, 1971; James and Augenstein, 1966; Dawson, 1969), or reorientation of the amphipathic α-helix that covers the active site (Winkler *et al.*, 1990).

Although no significant conformational difference between the native and inhibited forms of phospholipase A2 were observed (Scott *et al.*, 1990), through X-ray crystallography it has been concluded that interfacial activation in lipases will generally be associated with specific structural changes at the active site (Brzozowski *et al.*, 1991).

In lipases the catalytic triad has been shown by X-ray crystallography to be buried under a 'lid' composed of a short helical fragment (amphipathic helix) of a long surface loop stabilised by extensive hydrophobic and electrostatic interactions (Winkler *et al.*, 1990; Brady *et al.*, 1990) where it is inaccessible to solvent (Malcata *et al.*, 1992). The catalytic action of the lipases can be viewed as a two stage process as exemplified by the activation of the human lipase-procolipase complex. The 'lid' is displaced through interfacial activation of the human lipase-procolipase complex by mixed micelles revealed by X-ray crystallography (Tilbeurgh *et al.*, 1993) (colipase is a cofactor necessary for the binding of the pancreatic enzyme to the oil water interface in the presence of amphiphiles such as bile salts). This reorganisation of the lid provokes a second conformational change in an active site loop, which in turn creates the oxyanion hole (induced fit) and the substrates ester bond/s is subsequently hydrolysed by a mechanism very similar to that of the action of the serine proteases (Malcata *et al.*, 1992). In this active conformation the amphipathic helix in lipases is open (Kazlauskas, 1993). The scale of the conformation change is best exemplified by *Rhizomucor miehi* lipase (Rml). Upon inhibition the amphipathic helix in Rml rolls away from the active site and across the molecules surface, moving its centre of gravity 8 Å whilst rotating about its axis by almost 180° (Brzozowski *et al.*, 1991, Derewenda *et al.*, 1992). As a result of this movement a large hydrophobic surface approximately 750 Å (Brockman *et al.*, 1973) becomes exposed. In the lipase from *Geotrichum candidum*, probably two surface helices have to move to give full access to the active site serine (Schrag *et al.*, 1991).

The specificities of lipases have been classically divided into five major types (Jensen *et al.*, 1990): Lipid class, positional, fatty acid, stereochemical and combinations thereof, these have been reviewed by Malcata *et al.*, (1992).

1.2.2 Esterases

There is little doubt as to the catalytic mechanism (hydrolysis of esters probably through a Ser.....His.....Glu/Asp triad). However whether esterases are activated by a mechanisms similar to that of the lipases or whether the substrate is hydrolysed at a preformed active site is still of some doubt. Some esterases including pig liver esterase (PLE) (Barker and Jencks, 1969) and the Tok19 A1 esterase purified in this thesis have been shown to be activated at high substrate concentrations and a low concentration of micelles (reminiscent of true lipases). However, neither was able to hydrolyse the lipase substrate C_{14}-triglyceryloeate.

1.3 A physiological role for bacterial esterases ?

Carboxylesterases have been found in a number of bacterial, mesophilic, thermophilic, thermoacidophilic archaebacteria and mammalian sources. The role of acetylcholine esterase has been elucidated. Acetylcholine is a neuro transmitter, nerve impulse leads to a release of acetylcholine where it combines with receptor molecules producing a depolarisation of the postsynaptic membrane, which is propagated along the electrically excitable membrane of the second nerve cell. The acetylcholine messenger is hydrolysed by acetylcholinesterase, and the polarisation of the postsynaptic membrane is restored. It is difficult to envisage a role for an acetylcholine type esterase in a bacterial cell.

A metabolic role for PLE is unclear however, it may be involved in a regulatory mechanism:

- PLE is activated by high concentrations of substrates (Barker and Jencks, 1969).

- It is activated by presence of a low concentration of micelles and the binding of PLE to a lipid may induce long chain activity.
- It is activated and deactivated by substrates and short chained alcohols binding to the activator site but very little is known about the mechanism by which activation is transmitted from the activator to the catalytic site or about the function of this activation.
- Carboxylesterases have also been shown to catalyse the transfer of the acyl group to alcohols (Greenzaid and Jencks, 1971; Wynne and Shalitin, 1972; Lombardo and Guy, 1981) or amines (Franz and Krish, 1968; Goldberg and Fruton, 1970; Alt *et al.*, 1975; Sobek and Gorish, 1989) this suggests a possible role in the preparation of synthons.
- Mutants of an esterase on the outer membrane of *Pseudomonas aeruginosa* were able to grow on Tween 80 as a sole carbon source. This suggests a possible role for this esterase in the utilisation of acyl esters as carbon sources (Ohkawa, 1979).

The role of bacterial esterases is unclear and detailed molecular studies are necessary to elucidate the evolutionary relationships of these enzymes.

1.4 Esterases and lipases in organic solvent biocatalysis

The total synthesis of complex substances catalysed by enzymes is a very difficult endeavour and therefore it seems inferior to conventional chemical syntheses if the latter are at all possible. On the other hand, chemical means usually lack stereopecificity and thus are not generally applicable to large scale, economical, and efficient production of optically active compounds. It would appear that the optimal way to produce complex chiral molecules in an optically active state is to employ a chemico-enzymatic approach: enzymes are used to prepare small optically active molecules ("synthons") which are then combined by traditional chemical methods. In order to implement this strategy,

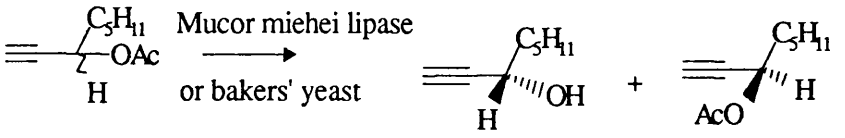
simple (preferably cofactor-independent) and reliable commercially available enzymes are required. Hydrolases undoubtedly represent the prime choice as they possess broad substrate specificity and at the same time strict stereoselectivity (Cambou and Klibanov, 1984).

Esterases and lipases perform a number of industrially useful reactions including hydrolyses, esterifications, transesterifications and interesterifications.

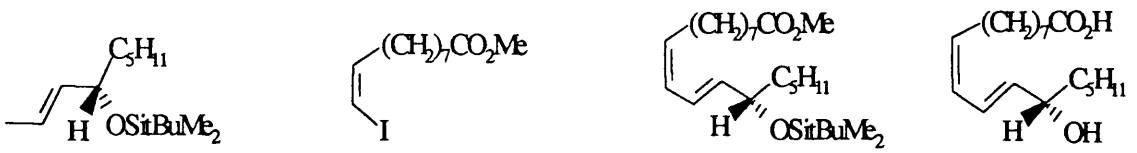
1.4.1 Hydrolysis

An example of classical chemistry coupled with the enantioselectivity of an enzyme is the preparation of intermediates for the synthesis of an antifungal antibiotic. Racemic acetate **1** is hydrolysed by *Muchor miehei* lipase and by lyophilised whole-cells of bakers yeast to give optically active (S)-alcohol **2** (enantiomeric excess 80-97%) with a 50% conversion (Scheme 1.4). The alcohol **2** has been converted into the ninyltin compound **3** and coupled to the iodoalkane **4** to give a late stage intermediate **5** to the natural product coriolic acid **6**, a fungicide (Chan *et al.*, 1990).

Scheme 1.4. The preparation of coriolic acid



1 2



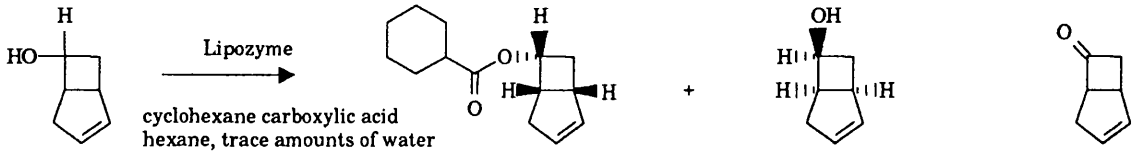
3 4 5 6

Optically active glycidyl derivatives are useful C3-synthons for the preparation of a number of industrially important chemicals. Porcine pancreatic lipase catalysed enantioselective hydrolysis of R/S glycidyl butyrate, followed by tosylation to give R(-) and S(+) glycidyl tosylate is an attractive route for the preparation of high optical purity glycidyl derivatives (Scheme 1.5). These derivatives are industrially important building blocks for a wide variety of optically active pharmaceuticals, agrochemicals and other products (Scheme 1.5 Apllications) (Martin *et al.*, 1993; Kloosterman *et al.*, 1993).

1.4.2 Esterifications

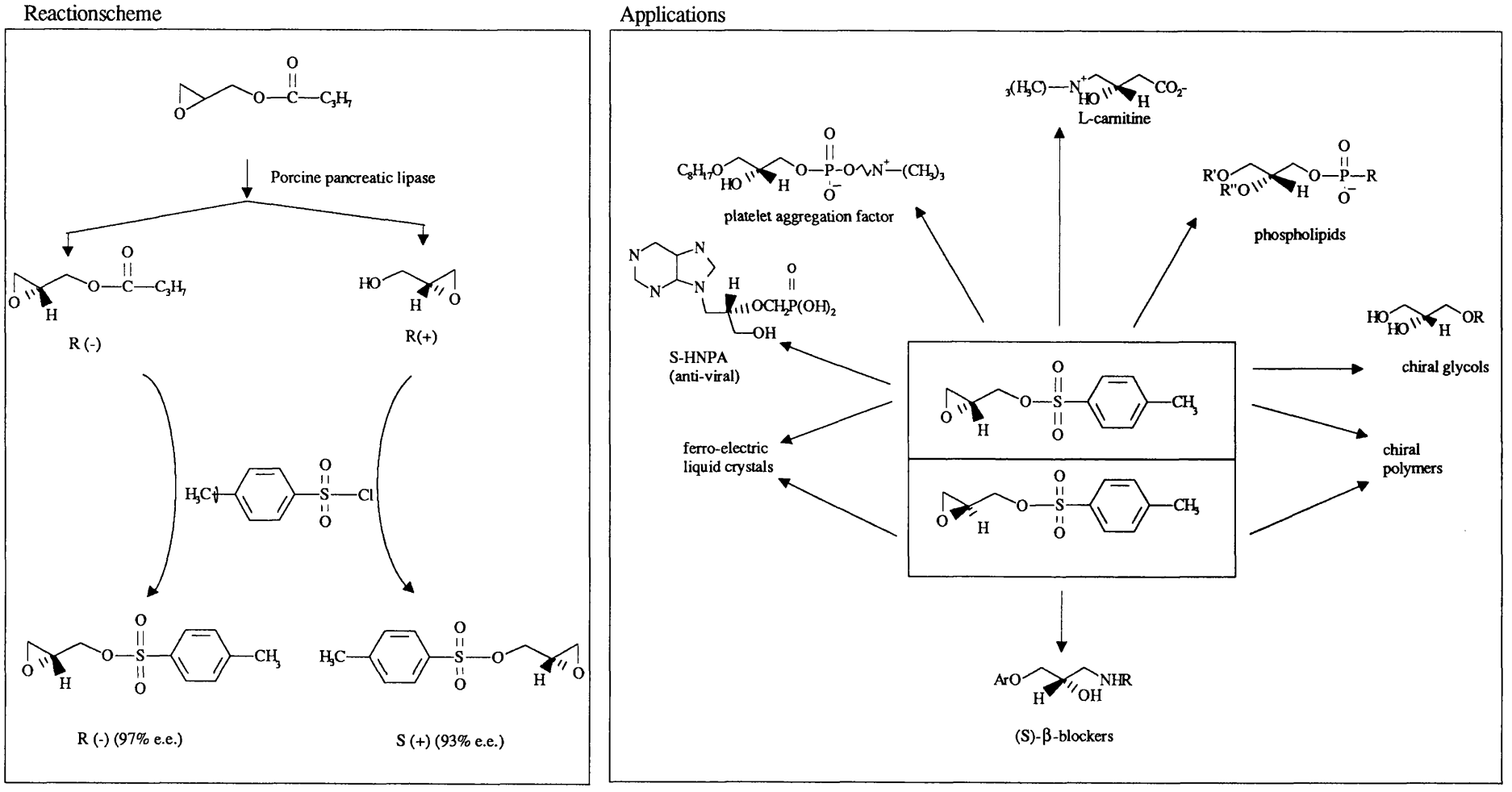
Precursors of prostaglandins and prostanoids (membrane associated biological lipids which modulate hormones and have been implicated in the inflammatory response in preventing peptic ulcers and opening bronchial and nasal passages) have been synthesised by enzyme catalysed esterification (Cotterill *et al.*, 1988). The alcohol **1** is enantioselectively acylated using cyclohexane carboxylic acid to give the ester **2** in a state of high optical purity with 50% conversion and the optically active alcohol was recovered (Scheme 1.6). The ester and the alcohol can be separated (e.g. by distillation) and the two compounds can be converted into the two enantiomers of bicyclo[3.2.0]hept-2-en-6-one **3**, a precursor of prostaglandin.

Scheme 1.6. The lipozyme resolution of precursors of prostaglandins and prostanoids



1 2 3

Scheme 1.5. The resolution of optically active glycidyl derivatives and their applications

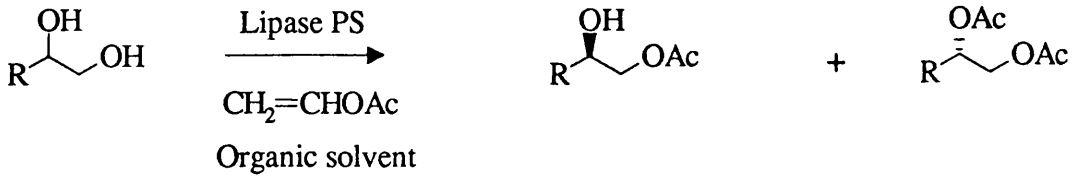


As well as the established advantages of performing reactions in organic solvents the enantioselectivity of lipases/esterases has been shown to increase in organic media. For example, the enantioselectivity of the esterification of 2-(p-chlorophenoxy)propionates in organic media was four to five fold higher than the corresponding hydrolytic reaction in water (Kirchner *et al.*, 1985). Similarly, the enantiomeric excess (ee) of the product, (S)-3-methylglutarate monomethyl ester, was higher when the product was prepared by enzymatic acylation by the corresponding anhydride in organic solvent than by enzymatic hydrolysis of the diester in water (Yamamoto *et al.*, 1988).

1.4.3 Transesterifications

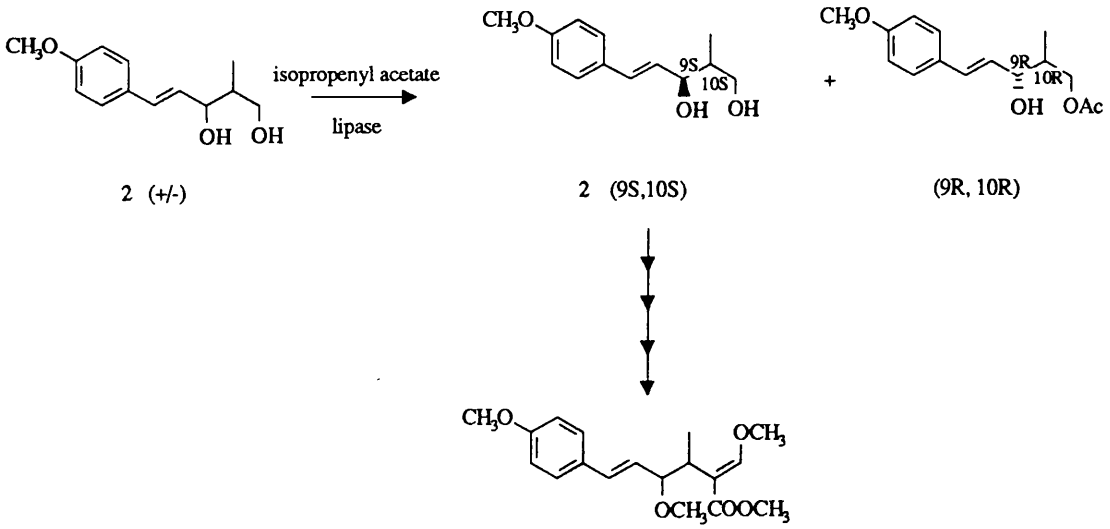
A group of compounds that are of interest as pharmaceuticals, agricultural intermediates, and liquid crystalline compounds are enantiomerically-pure aliphatic 1,2-diols (Theil *et al.*, 1993a). Lipases catalyse the regioselective acylation of aliphatic 1,2-diols at the primary hydroxyl group (Cesti *et al.*, 1985) but with low enantioselectivity (Janssen, 1991; Theil, 1991). This problem however was overcome by protecting the primary hydroxy moiety with a bulky group (Chen *et al.*, 1990; Goergens and Schneider, 1991; Kim and Choi, 1992). A method has recently been developed (Theil *et al.*, 1993a, b and c) which does not require the protection of the primary hydroxy moiety. A diglyceride was esterified using Lipase PS in the presence of vinyl acetate (Scheme 1.7). Using this method enantiomerically pure 3-aryloxy-propane-1,2-diols for instance were rapidly prepared. Both the nature of the solvent and the R substituent had a significant effect on the enantioselectivity.

Scheme 1.7. The esterification of aliphatic 1,2-diols with vinyl acetate



A further example of classical chemistry coupled with the enantioselectivity of an enzyme is the preparation of intermediates for the synthesis of an antifungal antibiotic. A diol which is an intermediate in the synthesis of oudemansin X, an antibiotic isolated from mycelial cultures of *Oudemansiella radicata* which exhibits several antifungal activities has been resolved. When (+/-) 2 was subjected to enantioselective acetylation using immobilised lipase "Amano P" from *Pseudomonas sp.* for (16hr), 97% e.e of (9S, 10S) was obtained in 27% yield. On recrystallisation of (9S, 10S) 2 optically pure (>99% e.e) (9S, 10S) was obtained. 4 chemical steps result in the fungicide oudemansin X (Scheme 1.8) (Akita *et al.*, 1993).

Scheme 1.8. The resolution of 1,2-diols for the synthesis of oudemansin X

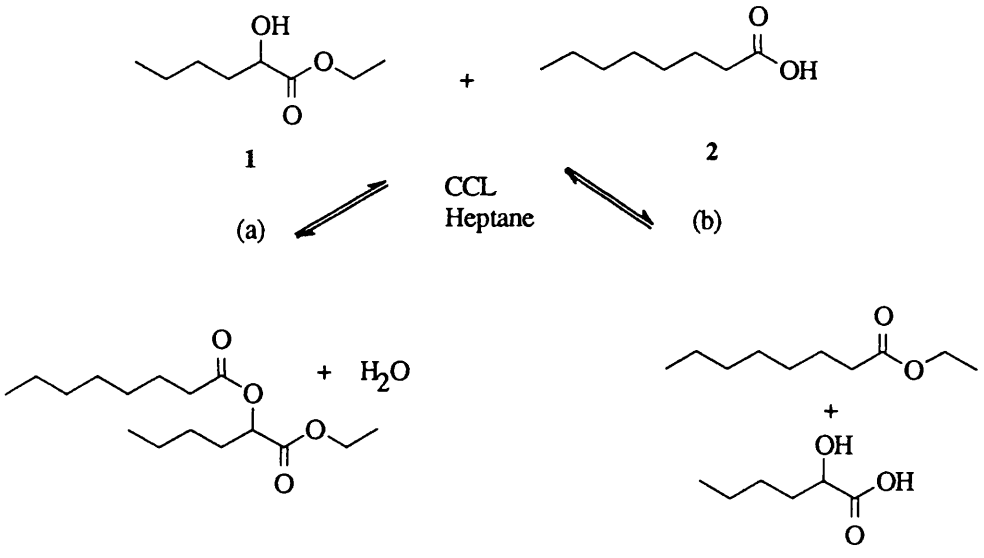


1.4.4 Competition between esterification and transesterification

The correct choice of substrate has a bearing on whether esterification or transesterification reactions occur. This was highlighted with the esterification/transesterification of hydroxy acid esters (Engel *et al.*, 1991). Hydroxy acid esters and their corresponding acetoxy derivatives are the prominent optically active constituents of various tropical fruits (Tressl and Engel, 1984; Tressl *et al.*, 1985). Lipase from *C. cylindracea* catalysed two reactions between racemic hydroxy acid esters and octanoic acid in heptane: esterification of the hydroxy group, leading to acyloxy acid esters, and transesterification via acidolysis, liberating the free hydroxy acids (Scheme 1.9).

The rate of esterifcation and transesterification were dependent on the chain length of the acid **2** and the distance between the hydroxyl moiety and the carbonyl group in the ester **1**. As the chain length of the acid increased from acetic acid to octanoic acid the relative esterification rate increased from 5 % to 100%. As the distance between the hydroxyl moiety and the carbonyl group increased there was a shift in favour of esterification, the enantioselectivity decreased and there was change in chirality of the major product.

Scheme 1.9. The esterification/transesterification of racemic hydroxy acids



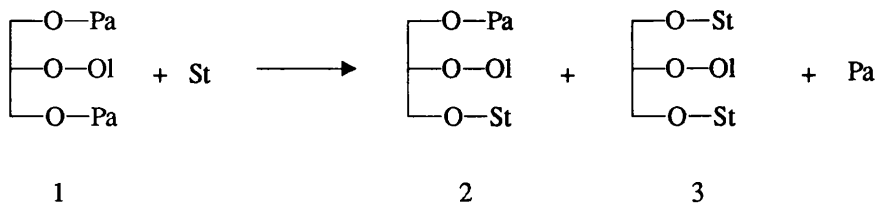
Langrand *et al.*, (1988) studied the production of isoamyl and geranyl flavour esters using both direct esterifcation and transesterification in n-heptane with 0.1% w/v water and 0.2% w/v water respectively. 13 commercially available lipases were used, in general the conversions were higher for transesterification than for direct esterification, and conversions after 24 hours at 37°C were generally greater than 90%.

1.4.5 Interesterifications

A common important type of esterification is acyl exchange, or more commonly known as interesterification. Interesterifications have been used predominantly in the food industry to modify the composition and physical properties of triglycerides hence are more the province of lipases rather than true esterases. Unlike conventional transesterification reactions interesterifications require some water, in addition to that required for hydration and activation of the enzyme. The interesterification process consists of two steps: the first is a hydrolysis of an acyl group from a triglyceride backbone to form a free glyceryl hydroxy group, followed by the esterification or transesterification of the free hydroxyl with the donor fatty acid or fatty acid ester. The water content of the reaction must be controlled. Interesterification is effectively suppressed in water by the competing hydrolysis reaction. However too little water prevents the initial hydrolysis.

For example, a 1, 3-specific lipase from *Rhizopus niveus* has been used to catalyse the interesterification of (Palm oil) 1,3-dipalmitoyl-2-oleoyl glycerol **1** with either steric acid or tristearine (Matsuo, 1981). The resultant fats,1-palmitoyl-2-oleoyl glycerol **2** and distearoyl-2-oleoyl glycerol **3** (Scheme 1.10), are the major components of cocoa butter. Pa = palmitic acid, St = steric acid, and Ol = oleic acid.

Scheme 1.10. The interesterification of palm oil with either steric acid or oleic acid



1 2 3

The modification of triglycerides has also been used in the manufacture of margarine s, to produce a lower melting temperature product. By exchanging saturated acyl moieties in existing margarines with unsaturated fatty acids, melting temperatures have been lowered by up to 30°C (Hansen and Huge-Jensen, 1985).

1.4.6 Novel strategies in enzyme kinetic resolution processes

New strategies for the enhancement of the enantiomeric purity of the products by enzymatic kinetic resolution were presented by Rakels and co-workers at the European Symposium on Biocatalysis, Graz Austria (1993). The concept behind their ideas was that two kinetic resolutions occurring simultaneously, either by a single or by two co-operating enzymes leads to vastly improved enantiomeric excess and was demonstrated in the following model reactions:

1) "Parallel resolution": The substrate enantiomer yielding the unwanted product enantiomer is itself selectively converted by the same enzyme to another product. The selectivity of the CCL-catalysed production of (R)-2-chloropropionic acid butylamide by amidation of racemic 2-chloropropionic acid methyl ester reaction was enhanced by simultaneous hydrolysis of the (R)-ester. This exploited the fact that CCl is (R)-specific for the amidation and (S)-specific for hydrolysis.

2) "Consecutive resolution":The substrate enantiomer yielding the desired product enantiomer is itself the preferred product of another, preceding enantioselective

reaction. Racemic 2-chloropropionic acid methyl ester was selectively hydrolysed by carboxylesterase NP, yielding enantiomerically enriched (S)-2-chloropropionic acid, which in turn was selectively converted by DL-dehalogenase.

3) "Tandem resolution". An enzyme catalyses two reactions in series. The production of (R)-glycidyl acetate by PPL catalysed esterification of racemic glycidol with butyl acetate was remarkably improved by using glycidyl butyrate, the enantiomerically-enriched glycidol consequently being esterified to glycidyl acetate.

1.4.7 Industrial uses of lipases for the production of flavours and fragrances

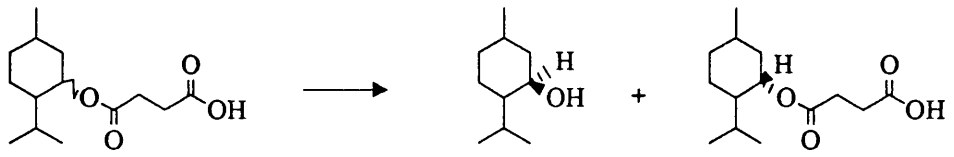
Natural flavour esters extracted from plant materials are often either too scarce or expensive for commercial use. Those manufactured by traditional synthetic methods result in expensive final products due to the purity and stereospecificity required (Welsch *et al.*, 1989). The market value of synthetic esters is less than that of esters from natural sources. Esters produced from natural substrates by biocatalysis (considered "natural" by some regulatory agencies) could satisfy the increasing commercial demand. The production of "true" flavours and fragrances is very complex, since at least 350 molecules, including over 100 esters, are thought to contribute to the taste and smell of strawberries (Cheetham, 1993).

Ethyl butyrate, a natural aroma of fruits such as strawberry and pineapple and a flavour enhancer, was used as a model system to study the effects of substrate concentrations in the esterification of ethanol and butyric acid with *Candida cylindracea* lipase as the biocatalyst (Gillies *et al.*, 1987). Ester synthesis increased as the molarity of ethanol was increased to 0.4M, but higher concentrations were inhibitory. Ester synthesis was optimum at this concentration of alcohol and between 0.25 - 0.5 M butyric acid. This model system was subsequently used to produce a further 10 flavour esters.

A Hungarian patent has been filed (Hung. Pat. APPl. No. P 92 02542 PCT/HU 92/00038) which details a new process for the production of flavour esters from C_2 - C_6 aliphatic carboxylic acids and alcohols using Lipozyme IM 20. The reaction rate depended strongly on the alcohol:acid molar ratio and the initial water content of the reaction mixture. Acid and alcohol consumed in the reaction were supplemented continuously at the rate of their consumption, and the water content of the reaction was maintained at 0.4% throughout. Under the optimum reaction conditions 75-95% ester yields were reported and reaction inhibition by acetic acid was observed.

L-Menthol has been prepared by the stereoselective hydrolysis of DL-menthylsuccinate by gel entrapped cells of *Rhodotorula minuta var. taxenis* in water-saturated n-heptane (Scheme 1.11) (Omata *et al.*, 1981). Menthol is a compound having a peppermint flavour and it is used extensively in the food and pharmaceutical industries, only L-menthol, out of the eight isomers possible because of the three chiral centres of the menthol molecule, has the desirable combination of mint taste and cooling sensation (Cheetham, 1993). The advantage of performing the reactions in organic solvents is that L-menthol is poorly soluble in aqueous buffers.

Scheme 1.11. The resolution of DL-menthyl succinate



DL-Menthyl succinate L-Menthol D-Menthyl succinate

1.5.0 Thermophily and thermostability of enzymes

1.5.1 Definitions and terminology

Growth temperatures have often been used to classify groups of microorganisms. The most common divisions were psychrophiles (-10 to +20°C), mesophiles (15 to 45°C) and thermophiles (45 to >100°C) (Herbert, 1992)

Brock (1979) defined the thermophile division and suggested that organisms with a maximum growth temperature (T_{max}) greater than 60°C be classified as thermophiles. He made this suggestion based on two main criteria. Firstly, temperatures below this barrier are common in nature but higher temperatures are usually associated with geothermal areas. Secondly, no eucaryotes are known to grow above this boundary, thus it would be exclusively a procaryotic world.

The thermophilic range has again been subdivided as regards optimum growth temperature: 60-75°C for thermophiles, 75-85°C for extreme thermophiles and greater than 85°C for hyperthermophiles. The temperature ranges do not represent absolute values for growth as there is some overlap within species for example *Bacillus stearothermophilus* can grow between 35 and 77°C whilst *Pyrodictium occultum* can grow between 80 and 110°C (Edwards, 1990a)

1.5.2 The upper limit of life

Bacterial life on earth is defined by several boundaries -10 to 110°C for temperature, 0.1 to 120 Mpa for hydrostatic pressure, 1.0 to 0.6 (corresponding to < 6 M salt) for water activity and pH 1 to 12 (Jaenicke and Zavodsky, 1990). Unequivocal data that growth exists above 100°C has been obtained for *Pyrodictium spp.* which has an optimum growth temperature of 105°C and a maximum in excess of 110°C (Stetter, 1982).

Reports of 'black smoker' bacteria growing at 250°C and 265 atmospheres in water samples from deep sea vents (Baross and Deming, 1983) have not been reproduced by other research groups. Results indicate that if these organisms exist, and if their metabolic reactions occur in an aqueous environment, they could not survive at this temperature if they were composed of biomolecules such as proteins and nucleic acids. These assumptions were based on the very rapid rate of decomposition of these biomolecules i.e. the half-lives for the hydrolysis and decomposition of Ala-Asp, Glu-Ala and ATP were all less than one second at 250°C (White, 1984).

The upper temperature limit for microbial growth has not yet been defined though it is not likely to be above 150°C (White, 1984; Daniel, 1986) and could be imposed by the thermostability of key biomolecules such as ATP, amino acids and peptides.

1.5.3 Thermophiles as sources of thermostable enzymes

The thermostability of enzymes from thermophiles is not due to their rapid resynthesis but rather their inherent stability since purified enzymes are thermostable. It is apparent from Table 1.2 that thermophilic organisms give rise to thermophilic enzymes. The increase in stability of β-galactosidase with increasing growth temperature is clearly demonstrated.

Table 1.2 The thermostability of β-galactosidase from mesophilic and thermophilic bacteria.

Source	Class	Growth temperature °C	Half-life	Reference
E. coli	Mesophile	37	1 min at 60°C	Daniel *et al.*, (1982)
B. stearothermophilus	Thermophile	60	40 min at 60°C	Daniel *et al.*, (1982)
T. aquaticus	Ex. thermophile	75	3hr at 80°C	Daniel *et al.*, (1982)
S. acidocaldarius	Ex. thermophile	85	8 hr at 80°C	Buonocore *et al.* (1980)

Thermostability is not an isolated property and also confers stability against denaturation by organic solvents (Owusu and Cowan, 1989; Guagliardi *et al.*, 1989) detergents (Guagliardi *et al.*, 1989) and proteolysis (Daniel *et al.*, 1982).

1.5.4. Intrinsic thermostability

Intrinsic thermostability in proteins is a result of stabilisation by two types of non-covalent interactions, electrostatic and hydrophobic. Electrostatic interactions include ion pairs, hydrogen bonds, weak polar interactions and Van der Waals interactions (reviewed by Burley and Petsko, 1988). Hydrophobic effects consist of Van der Waals interactions and hydration effects of non polar groups (Privalov and Gill, 1988). The importance of these interactions has been described in several manuscripts and reviews (for references see Sundaram, 1986).

Much of our understanding of the intrinsic stability of proteins has come from site directed mutagenesis (SDM) studies on T4 lysozyme and some general observations have been made.

Hydrogen bonds within proteins contribute only marginally to stability but that uncharged polar groups that lack a hydrogen-bonding partner are very destabilising ($\Delta\Delta G > 3$ kcal/mol) (Blaber, *et al.*, 1993).

Mutations which reduce the hydrophobicity of buried residues are often destabilising (Eriksson, *et al.*, 1992).

Mutations which lead to increases in cavity volume within the protein tend to be the most destabilising, whereas mutants in which the structure adjusts to minimise cavity volume tend to destabilise less (Eriksson, *et al.*, 1992). Relaxation in the protein structure offsets the energy cost of creating the cavity therefore, the amount of structural relaxation that

occurs is a major factor in determining the change in protein stability (Eriksson, *et al.*, 1993).

The individual structural responses seem to be dominated by the maintenance of the interface between the α-helices and the rest of the protein (Heinz *et al.*, 1993)

It has been shown by amide proton and hydrogen deuterium exchange rates, as well as resistance to proteolysis (Wagner and Wuthrich, 1979; Wrba *et al.*, 1990; Daniel *et al.*, 1982) and flexibility indices (Vihinen, 1987) that thermophilic enzymes are more rigid than their mesophilic counterparts at ambient temperatures but this stability becomes marginal at physiological temperatures (Daniel, 1986; Amelunxen and Murdock, 1978; Lakatos *et al.*, 1978; Harris *et al.*, 1980;Vali *et al.*, 1980). Also the same activity and catalytic mechanisms for enzymes from thermophiles and mesophiles suggests similar conformational mobility at the corresponding physiological temperatures. Thus thermophiles attain a more rigid structure at ambient temperatures as a result of the increased thermal mobility in their natural environments. It has been shown that although lactate dehydrogenases from thermophiles had more rigid structures than their mesophilic counterparts at mesophilic temperatures some local sites of flexibility were highly conserved (Vihinen, 1987). This suggests that only specific interactions were necessary for the increase thermostability rather than an overall change in amino acid composition. This is collaborated by the fact that the difference in free energy, between the β-galactosidase from *Sulfolobus* which is 3000 times more stable than the β-galactosidase from *E.coli* (Table1.2), is only 23 kJ mol^{-1}. The small variance in the values for flexibility indices (Vihinen, 1987) and free energy calculations (Kinney *et al.*, 1980) both indicate that only a small change in either the number or type of amino acids is essential for thermostability. This has been confirmed by comparing amino acid sequences from mesophiles, thermophiles and by site directed mutagenesis.

The stability of enzymes from thermophilic bacteria has been studied for many years, with the advent of gene cloning and sequencing it has been shown that the stability of thermophilic enzymes and proteins are due to subtle changes in the internal structure of the protein, which result in greater intramolecular interactions. These subtle changes can often be brought about by single amino acid changes (Ahern *et al.*, 1987). Site directed mutagenesis on yeast triosephosphate isomerase (Asn14-Thr14 and Asn78-Ile78) nearly doubled the half-life of the enzyme at 100°C and pH 6 (Ahern *et al.*, 1987). Correlations between the content of aliphatic amino acids and protein stability have also found. For example, in thermophilic malate synthetase (Sunderam *et al.*, 1980), glucosephosphate isomerase (Murmatsu and Nosoh, 1971) and glyceraldehyde-3-phosphate dehydrogenases (Hoching and Harris, 1976), the content of alanine, valine, isoleucine and leucine is higher than in respective mesophilic proteins and these presumably led to increased hydrophobic interactions. However studies on lactic acid from thermophilic and mesophilic B*acilli* have indicated an increase in the number of polar amino acid residues (e.g. arginine and lysine) at the active site (Zuber, 1979) and attributed the thermostability to an increase in the number of ionic and hydrogen bond interactions with a concomitant increase in half life and resistance to denaturants etc.

Amino acid sequence studies for thermophilic and mesophilic molecules of ferredoxin, glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase (Craik *et al.*, 1985) and thermophilic and mesophilic species of *Clostridia* were compared (Tanaka *et al.*, 1971; Devanathan, 1969). It was shown that Gly,Ser,Lys and Asp in mesophiles are generally substituted by Ala or Thr, Arg and Glu, respectively in thermophiles. The overall effect is primarily to increase internal and decrease external hydrophobicity as well as to favour the formation of helices. They also found that the thermostability increased as the number of glutamic acid residues increased. It has been indicated that

glutamic acid was the best helix promoting amino acid (Robson and Pain, 1971). This data also suggests that there is only a slight difference in energy between thermophilic and mesophilic enzymes. Energy calculations have put this enhanced stability at 5-7 kcal (20-30) kJ mol^{-1} (Kinney *et al.*, 1980). A ΔG of this order can be derived from only one or two additional salt bridges (Perutz, 1978) or several additional hydrogen bonds, (Shultz and Schirmer, 1979).

ΔG values for various intra-molecular interactions:

Hydrogen bonds (critically placed)	2-20 kJ mol^{-1}
Ion pairs	4 - 12 kJ mol^{-1}
Hydrophobic interactions	~ 3 kJ mol^{-1}

It is clear that such a gain in the stability of the enzyme will hardly require a drastic rearrangement in its structure and that the stability of thermophiles is due to subtle changes in the internal structure of the protein.

The cloning of thermophilic genes into mesophilic hosts and the subsequent expression of thermophilic enzymes clearly demonstrates the intrinsic stability of these proteins, and that thermophilic conditions were not necessary for protein folding. Although the majority of stability is gained though intrinsic factors many thermophilic enzymes derive some stability through extrinsic stabilisation.

1.5.5 Extrinsic thermostability

Extrinsic stabilisation is gained through interactions with cell constituents such as membranes, cofactors, substrates and metal ions. For example the loss of thermostability of alkaline phosphatase from *B. stearothermophilus* when released from protoplasts was attributed to stabilisation by the cell membrane (Welker, 1976). Glutamine synthases

from *B. caldolyticus* (Wedler, 1978; Merkler *et al.*, 1988) and *B. stearothermophilus* (Wedler and Hoffman, 1974) has been shown to be stabilised by the chemical binding of L-glutamate, Mn^{2+} and ATP (Wedler, 1978; Wedler and Merkler, 1985; Merkler *et al.*, 1988) and aggregation (Wedler and Hoffman, 1974) respectively. Calcium ions have been shown to stabilise a number of proteases (Sidler and Zuber, 1977; Tajima *et al.*, 1976) Table 1.3 as well as an α-amylase from *B. stearothermophilus* (Yutani, 1976).

Table 1.3. The effect of calcium ions on the thermal stability of proteases

Enzyme	Type	Number of Ca^{2+} ions	Stability $t_{1/2}$ at 80°C	Reference
Subtilisin	Mesophile	4	< 0.1h	Cowan *et al.* (1985)
Thermolysin	Thermophile	4	1 h	Cowan *et al.* (1985)
Caldolysin	Ex. thermophile	6	30 h	Cowan *et al.* (1985)

Cobalt ions have also been shown to stabilise aminopeptidase 1 from a *Bacillus stearothermophilus* (Deranleau and Zuber, 1977) and glycerophosphate dehydrogenase from *B. coagulans* has been shown to be only stable at a critical ionic strength (Amelunxen and Singleton, 1976).

Small conformational changes have been shown to stabilise several enzymes from *B. stereothermophiusi* as the temperature was increased (Magsanaga and Nosoh, 1974; Matsunaga *et al.*, 1974; Sugimoto and Nosoh, 1971; Hachimori *et al.*, 1970; Muramatsu and Nosoh, 1971). These conformational changes were observed well below the temperature of enzyme inactivation. For example, at 55°C points of inflexion were observed in the Arrhenius plots of a carboxylesterase (Matsunaga *et al.*, 1974) and at temperatures below 55°C glutamic synthetase became susceptible to thermolysin digestion (Magsanaga and Nosoh, 1974).

1.5.6 Catalytic activity of thermophilic enzymes

A common misconception concerning thermophilic enzymes is that their catalytic activities will be higher than the corresponding mesophilic enzymes at their respective physiological temperatures. This misconception stems from the Arrhenius equation; i.e., that a 10°C rise in temperature will approximately double the rate of a reaction. Therefore if the activity of a mesophile at 37°C is X, then the equivalent hyperthermophilic enzyme activity should be 32X at around 90°C. However, this is a simplification, Table 1.4 shows the specific activities of a number of proteinases isolated from thermophilic and mesophilic bacteria, the thermophilic enzymes have similar or identical specific activities to the mesophilic enzymes at their respective temperature optima. However, thermophilic enzymes are less efficient catalysts at temperatures below their temperature optima. This restriction on the activity of the thermophiles has been attributed, in part, to the restriction in the conformational flexibility demonstrated by amide proton and hydrogen deuterium exchange rates, as well as resistance to proteolysis (Wagner and Wuthrich, 1979; Wrba *et al.*, 1990; Daniel *et al.*, 1982) and flexibility indices (Vihinen, 1987).

Table 1.4. The specific activities of some thermophilic and mesophilic proteinases

Source of proteinase (and growth temperature	Specific activity (Units mg^{-1})	Assay temperature (°C)	Reference
T. aquaticus strain T351 (72°C)	35,000	75	Cowan *et al.*, (1982)
Thermus strain Tok3 (75°C)	37,500	75	Saravani, (1985)
Thermus strain 41A (72°C)	37,860	75	Cowan *et al.*, (1987)
Thermus strain RT6 (72°C)	59,900	75	Cowan *et al.*, (1987)
B. thermoproteolyticus (55°C)	17,000	37	Keay *et al.*, (1972)
B. subtilis NRRL B3411 (25°C)	22,000	37	Keay and Wildi, (1970)
B. subtilis amylosaccu (25°C)	12,800	37	Keay, (1969)
B. megaterium (25°C)	15,000	37	Keay *et al.*, (1972)
B. cereus (25°C)	20,000	37	Keay *et al.*, (1972)
Aeromonas proteolytica (25°C)	14,800	37	Keay *et al.*, (1972)

Table 1.6 taken from Cowan *et al.*, (1987).

1.5.7 Biotechnological potential and future applications of thermophiles

Cost, thermostability, the lack of commercially-available enzymes and lack of experience with these systems are probably the main obstacles in the implementation of enzymes in industry.

Thermophilic enzymes and bacteria posses several properties which can be considered advantageous in high temperature industrial processes (Table 1.5).

Table 1.5. The main advantages of high temperature industrial bioconversions

Property	Advantage in process
Thermostability	Tolerate high temperatures, long half-lives, increased resistance to denaturing agents such as organic solvents, high and low pH, detergents etc.
High optimum temperature	Little activity at low temperature, long shelf-life
General Robustness	Tolerate 'harsh' purification methods; gives better yields
Genes can be cloned into *E. coli*	A heating step makes purification easier
Chemical reaction rates	Diffusion and other chemical processes are accelerated
Solubility	Higher concentrations of poorly soluble compounds are possible
Viscosity	Decreases; mixing and pumping can be accelerated; mass transfer rate increases
Microbial Contamination	Growth of most pathogens and most environmental mesophiles is prevented. Thermophilic treatment of sewage wastes will kill off pathogenic bacteria and viruses
Biological activity in raw materials	Heating kills most interfering enzyme or microbial activities.
Operate at high temperatures	Distillation of volatile end products such as ethanol

Taken from Kristjansson, 1989.

If thermostability and the ability to operate at high temperatures is viewed as an isolated property then it is probably not feasible to replace existing enzymes or bacteria with thermophilic alternatives. This conclusion is based on several factors:

- Several industrial processes, many in the food industry are incompatible with high temperature operation. Some chemicals, raw materials and cofactors are damaged by heating.

- Commercial and environmental trends favour low temperature operation e.g. domestic detergents.
- Enzymes which possess the necessary thermostability are already in use.
- There are instances when there is no obvious advantage in increasing the reaction temperature, and it may even be detrimental, e.g. in the biosynthesis of fine chemicals, penicillins and amino acids.
- The advantages may be offset by large investment in a low temperature operation.
- Higher temperatures place a greater stress on equipment and constrain the materials that can be used.
- The solubility of gases such as oxygen generally decreases although this may be advantageous when anaerobic bacteria are being used.

However, as discussed in section 1.5 thermostability is not an isolated property, and resistance to heat denaturation imparts stability to a number of other denaturing influences (detergents, organic solvents etc.). These characteristics are those most likely to form the basis of new biotechnological applications. The implementation of thermophilic enzymes in industrial processes may only become viable when the following criteria are fulfilled.

- They need to be available in sufficient quantity with a regular supply this inevitably means that they have to be cloned.
- The cost has to be comparable (per unit activity) with mesophilic enzymes.
- They have to perform significantly better (not just thermostability).
- The investment in plant and equipment has to be minimal.
- Clear economic advantages have to be demonstrated over existing chemical synthesis and analogous mesophilic processes.

It is doubtful whether thermophilic enzymes will capture large scale industrial applications due to the criteria above however, it is in the speciality areas, making use of the properties associated with their structural stability, that they may have the best chance of implementation, the classic example being the use of DNA polymerase (Taq polymerase) in PCR. Some commercially available thermostable enzymes and their applications are shown in Table 1.6.

Enzymes isolated from the extremely thermophilic Archaea represent the most stable biocatalysts presently available. Several protein genes from Archaea have been cloned so its is possible that these will soon be commercially available (Cowan, 1991). However relatively little work has been conducted on the archaebacteria. Cowan (1991) lists the organisms and enzymes that have been studied in detail. The lack of research has been mainly due to the difficulties and cost of growing them in large scale culture (especially the anaerobes) in order to attain the high cell yields necessary for enzyme purification (Kelly and Deming, 1988).

Table 1.5. Some commercially available thermostable enzymes, organisms and their applications

Enzyme	Organism	Application
*Taq*1 restriction endonuclease	*T. aquaticus*	DNA sequencing
*Tth*111 I restriction endonuclease	*T. thermophilus*	DNA sequencing
Bst EII restriction endonuclease	*B. stearothermophilus*	DNA sequencing
DNA polymerase	*T. aquaticus*	Polymerase chain reaction in molecular biology
Thermolysin	*B. thermoproteolyticus*	Synthesis of aspartame
Pre*Taq* (protease)	*T. spp.*	DNA purification
Acetate kinase	*B. stearothermophilus*	ATP regeneration system
Lipase	*B.* spp.	No applications specified
Pullulanase	*B.* spp.	No applications specified
Leucine dehydrogenase	*B. stearothermophilus*	Measurement of leucine aminopeptidase
Alanine dehydrogenase	*B. stearothermophilus*	Measurement and synthesis of alanine
	B.Stearothermophilus	'Spore strip' sterility indicators
	B.Stearothermophilus	Measurement of penicillin in milk
DNA Ligase		Ligase chain reaction a method for detecting genetic lesions

1.6 The genus *Bacillus stearothermophilus*

The species *Bacillus stearothermophilus* was first isolated by Donk (1920) and was rationalised by Gordon and Smith, (1949). This species was most commonly found in thermally heated soils and had a pH optimum of 7.0. The major characteristics of *B. stearothermophilus* were good growth at 50 to 65°C, no growth at 28°C, variable growth at 37 and 70 °C and maximum growth temperatures of 70 to 85°C. Starch hydrolysis was positive, growth in 3% (w/v) NaCl broth was scant or negative, hydrolysis of gelatin was positive (only weak in seven strains), hydrolysis of casein was variable, reduction of nitrate to nitrite was positive (negative in ten strains). Spores were variable in size, oval, terminal to subterminal, and the spore walls were thick and stainable (i.e. gram positive). Although starch hydrolysis was one of the main criterion for characterisation as *B. stearothermophilus* strains unable to hydrolyse starch have been isolated (Daron, 1973; Epstein and Campbell, 1975). The description of *B. stearothermophilus* has now been modified in the 8th edition of Bergeys Manual (Buchanan and Gibbons, 1975): distinguishing features include a maximum growth temperature of 65 to 75°C, minimum growth temperature of 30 to 45°C, and the inability to grow on Sabouraud's dextrose agar or in 0.02% (w/v) sodium azide; starch hydrolysis was considered to be variable.

1.6.1 Growth and media for *B. stearothermophilus*

B. stearothermophilus has been grown on minimal medium with glucose or sucrose as the sole carbon source, thiamine, biotin, and nicotinic acid, arginine, histidine and isoleucine also enhanced the growth (Baker *et al.*, 1955). *B. stearothermophilus* strain NCA 1503 was continuously cultured in a 3000 Litre fermentor using a defined medium and 3 g L^{-1} dry weight of cells were harvested (Atkinson *et al.*, 1975a; Atkinson *et al.*,

1975b). This dry weight of cells was higher than that from batch or complex medium (Sergeant *et al.*, 1975).

Strains of *B. stearothermophilus* have been shown to be inhibited by a number of ions such as phosphate (Rowe *et al.*, 1975) and magnesium at temperatures above 65°C (Jurado *et al.*, 1987). The inhibition by magnesium was relieved by the addition of calcium ions (Jurado *et al.*, 1987). The growth temperature of a strain of *B. stearothermophilus* was also increased by increasing the calcium ion concentration concentration to 10 mM (Jurado *et al.*, 1987). Hydrostatic pressure has been found to affect the growth of *B. stearothermophilus* (Yayanos *et al.*, 1983). At atmospheric pressure colonies grew at temperatures between 39 and 70°C, when the pressure was increased to 45 Mpa the strain would only grow at temperatures between 54 and 67°C.

1.7 Immobilisation

The term 'immobilised enzyme' was adopted in 1971 to denote "enzymes physically confined or localised in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously" (Katchalski-Katzir, 1993). Enzymes were originally immobilised to elucidate their mechanism of action in native biological membranes (Silman, 1966). It soon became apparent that these immobilised enzymes could be employed in the construction of enzyme reactors in which the enzyme could be reused and/or the process operated continuously. Immobilisation is also becoming increasingly important for modifying the behaviour of the catalyst. For example, kinetics (Gaertner *et al.*, 1991), enantioselectivity (Gaertner *et al.*, 1991; Gu *et al.*, 1992), stability (Blanco *et al.*, 1989; Guisan *et al.*, 1993) and specificity (Ampon *et al.*, 1993) can be altered to design a more appropriate system for commercialisation. Enzyme specificity is clearly an important consideration when choosing catalysts for reactions of commercial interest. However, the cost of producing the necessary enzymes is often prohibitive. In this context immobilised enzyme preparations may be more effective since they are recoverable and often more stable than free enzymes. These observations have enabled bioengineers to construct enzyme reactors that can then be adapted to accommodate a particular biocatalytic reaction. Enzyme reactors in current use include batch-fed stirred-tank reactors, continuous stirred-tank reactors, continuous packed-bed reactors, or fluidized-bed reactors, and continuous ultrafiltration-membrane reactors (Katchalski-Katzir 1993). Immobilised-enzyme reactors have additional advantages such as ease of separation of the catalyst and products, high enzyme loads, prolonged enzyme activity, the ability to recycle products, high flow rates, and high yields of pure material.

There are also some disadvantages, for example there is usually a loss of activity during immobilisation, mass transfer may be a problem and there is also the additional cost of immobilisation.

The advantages far outweigh the limitations, as with heterogeneous catalysis in general, once diffusion (mass transfer) of the reactants is faster than the enzymic reaction it is inconsequential. This can usually be achieved by increasing the stirrer speed in the reactor. The loss of activity during immobilisation is a fraction of what would be lost if it were not immobilised. In some cases immobilised enzymes retained 100% of their activity (Blanco *et al.*, 1989) showed higher catalytic activities (Horvath, 1974; Legoy, 1980), or withstood higher concentrations of organic solvents without complete loss of activity (West and Wong, 1986; Nilsson and Mosbach, 1984; Guisan *et al.*, 1993). The additional cost of immobilisation can be offset by the automation of the process which leads to a considerable reduction in cost. For example, the optical resolution of racemic amino acids by aminoacylase led to a 40% reduction in overall production costs, compared with the conventional batch process using soluble enzyme (Katchalski-Katzir, 1993).

The first reported industrial use of immobilised enzymes was in 1967 (Tosa 1967). *Aspergillus oryzae* which produces an amino acylase was used for the resolution of synthetic, racemic DL amino acids into the corresponding optical enantiomers. A review of commercially-successful immobilised enzymes and the expected developments within the field has recently been published (Katchalski-Katzir, 1993) One of the expected developments (highlighted in this paper) was the isolation, characterisation and industrial evaluation of enzymes from thermophilic micro-organisms.

1.7.1 Enzyme immobilisation techniques

Enzymes can be immobilised by a number of techniques: adsorption onto an insoluble matrix such as DEAE-Sephadex, by single or multipoint covalent attachment to a solid matrix, by cross-linking enzyme molecules or entrapment either in a lattice or microcapsule.

Covalent coupling for the immobilisation of enzymes is based upon the formation of a covalent bond between the enzyme molecules and support material. It is therefore important that the amino acids essential to the catalytic activity of the enzyme are not involved in the linkage to the support. This may be difficult to achieve, and enzymes immobilised in this fashion may lose activity upon immobilisation. This problem may be prevented if the enzyme is immobilised in the presence of its substrates or inhibitors (Blanco and Guisan, 1989).

Multipoint covalent attachment of an enzyme to the surface of a support (Martinek *et al.*, 1977a; Martinek *et al.*, 1977b; Guisan, 1988) appears to be the most promising immobilisation procedure. Varying degrees of stability have been achieved using this method of attachment: by more than an order of magnitude (West and Wong, 1986; Clark and Bailey 1984) up to 1000 fold (Mozhaev *et al.*, 1983, Guisan *et al.*, 1993), 5,000 fold (Blanco *et al.*, 1989) and 10,000 fold (Mozhaev *et al.*, 1988; Guisan, 1988; Blanco and Guisan, 1987; Blanco and Guisan, 1989).

1.7.2 Immobilisation of whole cells

Whole cells can also be immobilised, negating the need to isolate and purify intracellular enzyme(s) and providing a mechanism for multistage bioconversions. Moreover, being in their native environment, intracellular enzymes should be less susceptible to operational denaturation. An added advantage of immobilised whole cells is that co-factor

regeneration can take place under suitable conditions. However there are several problems and disadvantages associated with the immobilisation of whole cells: diffusion of substrates across cell walls, plasma membranes or subcellular membranes may be limited, the integrity of cells and the stage of the growth cycle during which the enzyme(s) in question is produced must be maintained, and the multiplicity of enzymes present may introduce unwanted side reactions. Immobilisation of the whole cell is likely to be simpler than attempting to simulate the same balance of enzymes and associated cofactor recycle system through enzyme immobilisation (especially in organic solvents were cofactors will be difficult to dissolve).

1.7.3 The support material

A large number of support materials for enzyme immobilisation are available: they include poly (glycidyl methacrylate) discs for high performance membrane chromatography (Tennikova *et al.*, 1990; Josic *et al.*, 1992), polymer beads (Ampon *et al.*, 1993, Ramos *et al.*, 1992), chitin (Heras and Acosta, 1993) and aldehyde-agarose gels (Guisan, 1988; Ovsejevi *et al.*, 1993). It has been observed that enzymes immobilised on hydrophilic supports are more stable and show higher activities than the same enzymes immobilised on hydrophobic supports (Cuperus *et al.*, 1993). While this may be true in aqueous systems it has been shown that in microaqueous solvents there is competition between the enzyme and support for the water in the system (Reslow *et al.*, 1988; Nakamura *et al.*, 1993a; Nakamura *et al.*, 1993b). Hydrophobic supports are therefore usually used in organic solvent systems.

In order to maintain the catalytic activity of enzymes when hydrophilic supports are used higher water concentrations must be used (Reslow *et al.*, 1988). This behaviour is consistent with the hypothesis that the retention of the essential water layer around the

enzyme is critical for molecular stability. A hydrophilic support can be beneficial in microaqueous solvents for example, in esterification reactions were stochiometric amounts of water are produced as a biproduct the water partitioned towards the support and hence was not available for hydrolysis (Nakamura *et al.*, 1993a; Nakamura *et al.*, 1993b).

1.7.4 Aldehyde-agarose gels as activated supports for immobilisation-stabilisation of enzymes

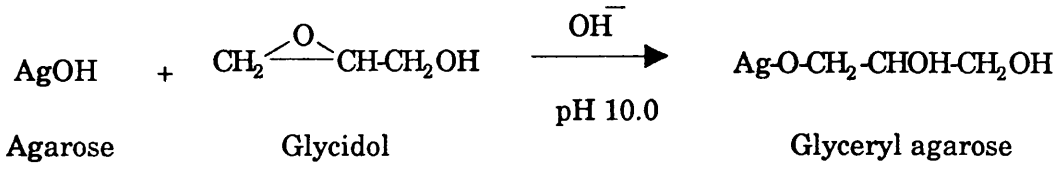
Background

Immobilisation using aldehyde-agarose involves the covalent coupling of aldehyde groups on an insoluble support to amine groups on the enzyme, either through lysine residues or terminal amino groups (Guisan, 1988). There are several advantages of immobilising through lysine residues:

- Proteins usually contain many lysine residues.
- They are not usually involved in the catalytic site.
- They usually reside on the proteins surface and are exposed to the medium.
- When unprotonated they are very reactive and act as nucleophiles, without prior activation.

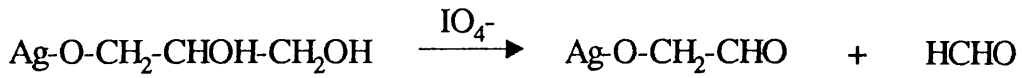
Glyoxyl-agarose gels are prepared by oxidising Sepharose 6B-CL gels using a periodate solution at pH 10 (Scheme 1.12). The aldehyde concentration per mL of gel is controlled by the addition of glycidol (2,3 epoxypropanol) at 25°C as proposed by Shainoff (1980).

Scheme 1.12. The oxidation of Sepharose 6B-CL gel using a periodate solution at pH 10



Further oxidation of the glyceryl groups is achieved using periodate, and yields glyoxyl groups (Scheme 1.13).

Scheme 1.13. The oxidation of glyceryl agarose to glyoxyl agarose



The surface density of the aldehyde groups in the activated gel, based on the surface area of Sepharose 6B-CL ($25m^2\ mL^{-1}$) (Guisan 1988) and a total glyceryl content of 70 μ moles mL^{-1} is 17 residues / 1000 Å. Assuming a protein of intermediate size, (molecular weight 90,000, Stoke's radius ≈ 40 Å) and that only 10 % of the external area can contact the support, there will be 34 aldehyde groups able to interact with the protein. The potential for multiple covalent bonds is therefore very high.

There are several advantages associated with the use of aldehyde-agarose gels

- The relatively low steric hindrance in the chemical reaction between aldehyde and amine increases the probability of multicovalent attachment.
- High chemical stability at high pHs prolongs immobilisation times with the amine groups (without prior activation) resulting in higher stabilisation factors.
- The formation of single point derivatives is fast and reversible but becomes stabilised by the formation of two or more point derivatives.

- The method is simple, reproducible and easy to control.
- The gels can be assayed and studied by spectrophotometric methods.
- They can be used in "batch reactors" because they are compressible and, therefore, resistant to mild stirring devices.

Chemical stability of the aldehyde-agarose gels

Aldehyde-agarose gels are very stable even under alkaline conditions (pH 10-11). Denaturation of the gels follows first order kinetics and the half-lives of the gels at pH 7, 8.5, 10 or 11 were 90, 30, 12 and 2 days at 25°C and 400, 85, 28 and 2 days respectively at 0°C (Guisan, 1988; Blanco and Guisan, 1989).

The stability of the gels at pHs above 10 means that they can be used for multicovalent immobilisation with enzymes which have high densities of free amine groups. However, high alkaline pHs may have a deleterious effect on the enzyme activity. An alkaline pH (above 10) is important because only unprotonated amines will react with the aldehyde groups (Guisan, 1988; Guisan *et al.*, 1993; Blanco *et al.*, 1988; Blanco and Guisan, 1989; Alvaro *et al.*, 1991).

Activity of glyoxyl-agarose immobilised enzymes

The use of reversible amine aldehyde reactions reduces the probability of deleterious torsional changes in the enzymes structure, possibly preventing losses of activity. In the immobilisation of chymotrypsin (Guisan *et al.*, 1992) and penicillin G acylase (Guisan *et al.*, 1993) by multipoint covalent attachment to glyoxyl-agarose gels, 100% and 90% of initial activity was retained respectively. Competitive inhibitors have also been used to maintain enzyme catalytic activity during immobilisation. For example, in the presence of benzamidine, trypsin retained 100% of its catalytic activity on immobilisation and was stabilised 10,000 fold compared to the soluble enzyme (Blanco and Guisan, 1988).

Variables that control the degree of amine-aldehyde agarose multipoint covalent attachments

Surface density of the aldehyde groups, pH, time and temperature all influence the degree of multipoint covalent attachment. For example, enzymes immobilised by covalent attachment become more stable as the number of covalent bonds between the support and the enzyme increases (Blanco *et al.*, 1989; Blanco and Guisan, 1988; Khmelnitsky *et al.*, 1988).

As the pH of the enzyme-support reaction medium increased, for example, increasing the pH from 7.6 - 10 the stability of trypsin derivatives increased by a factor of 10.

After the initial rapid enzyme insolubilisation of the enzyme (i.e., to give the single point derivative) longer incubation times result in the formation of multipoint derivatives (Guisan, 1988). Stabilisation of the resulting derivatives greatly increased as the contact time between the insolubilised enzyme and the activated support increased (Guisan, 1988). It is therefore clear that the formation of a multipoint attached enzyme is much slower than the initial insolubilisation. It has been proposed that after the initial insolubilisation, the remaining amine and aldehyde groups are misaligned. Since the support is rigid the rate of formation of further covalent linkages is governed by the rate of alignment which is influenced by the conformational movement of the enzyme.

The stability factor also increased as the immobilisation temperature was increased (Guisan, 1988), the maximum temperature being limited by the stability of the particular enzyme used. It has been proposed that as the temperature increases the vibrational motion of the enzyme increases, leading to increased interaction between the gel and the enzyme with a concomitant increase in the number of bonds formed.

1.7 Aims

The general aims of this project were to identify, isolate and characterise a thermostable broad specificity esterase/lipase with high activity at mesophilic temperatures. Specific aims included the development of an efficient high–resolution purification strategy, a detailed investigation of both the fundamental molecular and functional properties of the purified enzyme (s) and an investigation of the biotechnological properties of the enzyme regarding its application in industrial biotransformations.

Chapter 2

Materials and Methods

This chapter details the materials and methods used during the research conducted for this thesis. Any alterations to these procedures have been described in the text of the appropriate results chapter.

- Distilled and deionised water was used in the preparation of aqueous solutions.
- The pH values of solutions and buffers were determined at 30°C unless otherwise stated. International system (S.I.) units and abbreviations have been used throughout.
- Other abbreviations used in this chapter and elsewhere are listed separately.
- Strains were named according to the following convention used in our laboratory. Strains were given a three letter prefix (an abbreviation of the town or area of origin) a sample number and the colony number.
- All assays were performed in triplicate. Where standard error bars are not shown, the error lies within the data point.

2.1 Materials

2.1.1 Chemicals

Paranitrophenol, paranitrophenyl acetate, paranitrophenyl propionate, paranitrophenyl butyrate, paranitrophenyl valerate, paranitrophenyl caproate, paranitrophenyl caprylate, paranitrophenyl caprate, paranitrophenyl laurate, paranitrophenyl myristate, paranitrophenyl palmitate, paranitrophenyl stearate, tributyrin, methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, nonanol, decanol, 2-propanol, 2-methoxy ethanol, 1-3 propan diol, dimethyl formamide, dimethyl sulfoxide, acetone, ethylene glycol, gycerol, acetonitrile, methyl butyrate, ethyl butyrate, propyl butyrate, isopropyl butyrate, butyl butyrate, isobutyl butyrate, amyl butyrate, isoamyl butyrate, hexyl butyrate, heptyl butyrate, octyl butyrate, decyl butyrate, ethyl acetate, propyl acetate, butyl acetate, isobutyl acetate, amyl acetate, isoamyl acetate, hexyl acetate, octyl acetate, secondary butyl acetate, tertiary butyl acetate, benzyl acetate, phenyl acetate, α-naphthyl acetate, indoxyl acetate, cholesterol acetate, glucose pentaacetate, glucose pentabenzoate, 1,2,3,4,-tetra-o-acetyl-β-D-ribopyranose, β-D-xylose teraacetate, methyl propionate, methyl butyrate, methyl isobutyrate, methyl valerate, methyl caproate, methyl heptanoate, methyl benzoate, methyl 2-hydroxybutyrate, methyl *p*-hydroxybenzoic acid, methyl S(-) chloropropionic acid, methyl R(+) chloropropionic acid, methyl mandelic acid, methyl 4-methyl umberferyl propionate, methyl L-tyrosine, methyl N-acetyl-L-phenylalanine, N-carbobenzoxy glycine *p*-NP ester, N-carbobenzoxy L-alanine *p*-NP ester, N-carbobenzoxy L-valine *p*-NP ester, N-carbobenzoxy L-leucine *p*-NP ester, N-carbobenzoxy L-isoleucine *p*-NP ester, N-carbobenzoxy L-phenylalanine *p*-NP ester, N-carbobenzoxy L-tyrosine *p*-NP ester, N-carbobenzoxy L-tryptophan *p*-NP ester, N-carbobenzoxy L-proline *p*-NP ester, tributyrin, tricaproin, *p*-hydroxybenzoic acid ethyl

ester, n-butyl *p*-nitrobenzoate, ethyl *p*-nitrobenzoate, mandelic acid methyl ester, β-naphthyl propionate, (R/S) glycidyl butyrate, R(-) glycidyl butyrate, diisooctyl phthalate, N-acetyl-L-tyrosine ethyl ester, N-benzoyl-L-tyrosine ethyl ester, N-α-benzoyl L-arginine ethyl ester, caproic acid propyl ester, caprylic acid n-butyl ester, Tris-HCl, Tris Base, 2-[N-morpholino]ethanesulphonic acid, diethyl aminoethyl (DEAE-) Sepharose CL-6B, carboxymethyl (CM-) Sepharose CL-6B, polyethylene glycol 6000, phenyl-Sepharose, Q-Sepharose, Benzamidine sepharose, SDS200 molecular weight Kit, bovine serum albumin fraction V, N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid, biotin, nitrilotriacetic acid, folic acid, pyridoxine hydrochloride, riboflavin, pantothenic acid, thiamine hydrochloride, L-alanine, L-arginine, L-asparagine monohydrate, L-aspartate (monopotassium salt), L-cystine, L-glutamate hydrochloride, L-glutamine, glycine, L-hystidine hydrochloride monohydrate, L-isoleucine, L-leucine, L-lysine hydrochloride, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, L-aspartic acid, ethylenediaminetetraacetic acid, phenylmethanesulphonyl fluoride, parahydroxymercuribenzoate, tosyl-L-phenylalanine chloromethyl ketone, physostigmine, diethylpyrocarbonate were of the highest grade available and were purchased from Sigma Chemical Company, Poole, Dorset, U.K.

Concentrated hydrochloric acid, potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, sodium hydroxide, ammonium sulphate, magnesium sulphate heptahyrate, iron sulphate heptahydrate, manganese sulphate dihydrate, sodium chloride, cobalt sulphate, cobalt chloride, calcium chloride (anhydrous), calcium chloride dihydrate, zinc sulphate, copper sulphate pentahydrate, potassium aluminium sulphate, boric acid, nickel chloride hexahydrate, iron chloride trihydrate, manganese chloride, phosphoric acid, sodium nitrate, ethanol, coomassie brilliant blue G-250, D-glucose, lactose, sodium citrate, sodium sulphate, sodium succinate, citric acid, sodium

dodecylsulphate, sodium carbonate, sodium bicarbonate, mercuric chloride, were all purchased from BDH Ltd., Poole, Dorset, UK.

Nutrient agar CM3 and nutrient broth No. 2 CM67 were purchased from OXOID Basingstoke, UK.

Yeast extract was purchased from Beta-lab Surrey, UK.

Purified agar was purchased from Difco Laboratories Detroit Michigan USA.

Crosslinked 6% and 10% agarose gels and their glyoxyl derivatives containing 75 μEq aldehyde groups mL^{-1} gel were generously donated by Hispanagar S.A. Burgos, Spain.

2.1.2. Preparation of buffers and solutions

Potassium phosphate buffer pH 7.0

50 mM potassium-dihydrogen orthophosphate buffer (KH_2PO_4-NaOH) was prepared by dissolving 6.81g L^{-1} of KH_2PO_4 in 990 mL of distilled water. The pH was adjusted to pH 7.0 at 30°C using 10M NaOH and the volume made up to 1 L using distilled water.

Tris buffers

50 mM Tris-HCl, at various pH's, was prepared according to the Trizma mixing table.

MES-HCl buffers

50 mM MES pH 5.5 was prepared by weighing the correct amount of MES and adjusting the pH with 1 M HCl.

Additional buffers

Citric acid-Na_2HPO_4, HEPES-NaOH, Borate-HCl, Clark and Lubbs solution and sodium carbonate-sodium bicarbonate buffers were prepared according to Dawson *et al.*, (1986).

Solutions

Table 2.1. Trace element stock solution	g L^{-1}
Nitrilotriacetic acid	1.5
$MgSO_4.7H_20$	3.0
$MnSO_4.2H_20$	0.5
NaCl	1.0
$FeSO_4.7H_2O$	0.1
$CoSO_4$ or $CoCl_2$	0.1
$CaCl_2.2H_2O$	0.1
$ZnSO_4$	0.1
$CuSO_4.5H_2O$	0.01
$KAl(SO_4)_2$	0.01
H_3BO_3	0.01
$Na_2MoO_4.2H_2O$	0.01
$NiCl_2.6H_2O$	0.025

Use 10 mL L^{-1}

Table 2.2. Vitamin stock solution	mg L^{-1}
Biotin	0.9
Folic acid	6
Pyridoxine hydrochloride	10
Riboflavin	15
Pantothenic acid	100
Thiamine hydrochloride	15
Nicotinic acid	150

Use 10 mL L^{-1}

Media taken from Campbell and Williams, (1953).

2.1.3 Liquid and solid media

Liquid media

Nutrient broth

Nutrient broth at a concentration of 25g L^{-1} was autoclaved at 121°C for 20 minutes. In some instances yeast extract was added to a final concentration of 5g L^{-1}.

PNR minimal media

The composition of PNR minimal media is shown in Table 2.3. The PN salts and the R salts were autoclaved separately at 121°C for 20 minutes and combined under sterile conditions.

Table 2.3. PNR minimal media

PN Salts x 20		R Salts	
	g L^{-1}		g L^{-1}
KH_2PO_4	136.0	$MgSO_4.7H_2O$	80.0
NaOH	25.0	$FeSO_4.7H_2O$	2.0
$(NH_4)_2\ SO_4$	24.0	Concentrated HCl	14 mL
pH	7.0	Use 7 mL / L	
Use 50 mL / L			

Thermophilic defined liquid medium (TDLM)

The composition of TDLM is shown in Table 2.4. Tyrosine required the addition of 1 N HCl for solubilization, and tryptophan required the addition of 1 N NaOH. The components were added in the order listed to minimise precipitation (Rowe *et al.*, 1975).

Defined Medium

Defined media contained 50 mL L^{-1} of PN salts, 7 mL L^{-1} of R salt, 10 mL L^{-1} of Vitamin solution, 10 mL L^{-1} of Trace elements solution and 0.2 g L^{-1} of methionine.

Table 2.4 Thermophilic Defined Liquid Medium (TDLM)

Ingredients[a]	Amount (mL)
L-Alanine	8.4
L-Arginine.HCl	6.4
L-Asparagine.H_20	5.0
L-Aspartate (monopotassium salt)	13.0
L-Cystine	5.0
L-Glutamate.HCl	40.0
L-Glutamine	5.0
Glycine	5.0
L-Histidine.HCl.H_20	4.2
L-Isoleucine	10
L-Leucine	16.4
L-Lysine.HCl	14.0
L-Methionine	5.2
L-Phenylalanine	8.6
L-Proline	10.0
L-Serine	14.0
L-Threonine	8.4
L-Tryptophan	3.0
L-Tyrosine	5.6
L-Valine	12.6
Biotin (10 mg / 100 mL)	10.0
Thiamine.HCl (10 mg / 100 mL)	10.0
Nicotinic acid (10 mg / 100 mL)	10.0
Anhydrous $CaCl_2$ (5%)	0.1
$FeCl_3.6H_20$ (0.05%)	0.1
$ZnSO_4.7H_20$ (5%)	0.1
$MnCl_2$ 10 mM	0.1
Glucose (20%)	10
*EDTA (25 mg / 100 mL)	
Mineral salts solution	
	g L^{-1}
NH_4Cl	10
NaCl	10
$MgSO_4$	4
Use 100 mL L^{-1}	
Potassium phosphate buffer (125g of K_2HPO_4 and 30g of KH_2PO_4 per 500 mL) use 50 mL L^{-1}	
Total volume 1 L	

[a] All stock amino acid solutions were 1% (w/v). The final volume was adjusted to 100 mL with deionized water, and the solution was filter sterilized; the pH was 7.3

Media taken from Rowe *et al.*, 1975

*EDTA was subsequently added in order to prevent precipitation.

Solid media

Nutrient agar plates

Nutrient agar at a concentration of 28 g L^{-1} in dH_2O was autoclaved at 121°C for 20 minutes, in some instances yeast extract was added to a final concentration of 5g L^{-1}. After the nutrient agar had cooled sufficiently (to approximately 60°C) the nutrient agar was poured into petri dishes (under sterile conditions) and allowed to cool. Once the nutrient agar had set the plates were inverted and placed in an oven at 60°C for one hour to dry. The plates were stored in a sterile condition at 4°C.

Thermophilic defined agar medium (TDAM)

TDAM was prepared as described in Table 2.4. except that purified agar was added to a concentration of 1.5%.

PNR defined media plates

Purified agar at a concentration of 15g L^{-1} was autoclaved at 121°C for 20 minutes. When it had cooled to approximately 60°C, PN salts (50 mL L^{-1}), R salts (7 mL L^{-1}), Vitamin solution (10 mL^{-1}), Trace elements solution (10 mL^{-1}) and methionine 0.2g L^{-1} were added under sterile conditions.

2.2. General protocols

2.2.1 Cell culture

The contents of freeze dried vials were resuspended in 0.5 mL of autoclaved distilled water. 100 µL of the resuspended cells were streaked onto nutrient agar plates supplemented with 0.5% yeast extract and the remaining 400 µL were used to inoculate 10 mL volumes of nutrient broth, supplemented with 0.5% yeast extract, in 30 mL glass

universal bottles. The plates were incubated at the specified temperatures for up to 72 hours. The universal bottles were placed in orbital shakers at 130 rpm at the specified temperatures for up to 72 hours.

2.2.2 Preparation of whole cell suspensions and cell-free extracts

Whole-cell suspensions

The strains were grown for up to 72 hours after which time they were harvested (centrifuged in an MSE desktop centrifuge at 4000 rpm for 20 minutes). The supernatant was decanted and the wet weight of cells recorded. The pellets were washed and resuspended in 500 μL of KH_2PO_4-NaOH buffer at pH 7.0.

Cell-free extracts.

The resuspended cells were sonicated (Soniprep 150 by MSE) for 30 seconds using the microprobe at a power setting of 4. The cell suspension was centrifuged in a microfuge (MSE) for 10 minutes to remove the cell debris. The supernatant was retained and used immediately or stored at 4°C.

2.2.3 Optical densities of bacterial cultures

The optical density of the bacterial cultures was measured at 600 nm in a Cecil CE1020 spectrophotometer (UV-VIS single beam). The samples were diluted with deionised water to ensure that the optical density was less than 1.0. Deionised water was used as a blank.

2.2.4 Storage of bacteria

Bacterial cultures were either stored in freeze dried vials at -70°C or on nutrient agar plates, supplemented with 0.5% yeast extract, at 4°C. Cultures were re-streaked from the plates every 2 weeks.

2.3 Assays

2.3.1 Esterase activity

Esterase activity was determined using *p*-nitrophenol ester (*p*-NP-ester) substrates. Stock substrate solutions were prepared by dissolving the *p*-NP-ester in ethanol at a concentration of 100 mM. For each assay 15 µL of stock ester was added to 2.90 mL of 50 mM KH_2PO_4-NaOH buffer pH 7.0 to give a final substrate concentration of 0.5 mM. The buffered substrate was pre-incubated at 30°C in a Cecil CE1020 spectrophotometer (UV-VIS single beam) equipped with a water-heated cuvette block. After monitoring the background non-enzymic release of *p*-nitrophenol at 405 nm (ε=11900 $M^{-1}.cm^{-1}$) for 1-2 minutes, 20-100 µL of enzyme was added and the enzymic rate recorded. Enzymic rates were corrected by subtraction of the non-enzymic rate. One unit of enzyme activity is defined as the enzyme-catalysed release of 1 µmole of *p*-nitrophenol min^{-1} under the specified assay conditions.

2.3.2 Hydrolytic activity assays using an autoburette

The substrate specificity of immobilised esterase derivatives was determined using an autoburette (pH-stat system; Radiometer autoburette (ABU 80) and Standard pH meter (PHM 82). Substrates were dissolved in 3 mL DMF to give a final concentration of between 1 and 10 mM, depending on substrate solubility. 27 mL of 50 mM KH_2PO_4-NaOH buffer pH 7.0 were added. The buffered substrate was pre-incubated at 30°C in a 40 mL jacketed vessel and the reaction mixture was stirred at 300 rpm to minimise pH gradients. After monitoring the background non-enzymic release of acid, the reaction was initiated by the addition of between 0.5g and 2.5g of immobilised esterase, and the released acid was continuously titrated to pH 7.0 with 10 or 25 mM NaOH. One unit of enzyme corresponds to the release of 1 µmol of acid min^{-1} under the assay conditions.

2.3.3 Native PAGE (indoxyl acetate) esterase assay

Esterase activity was detected after non-denaturing PAGE using indoxyl acetate. 1 mL of a 10 mg mL^{-1} ethanolic indoxyl acetate solution was added to 100 mL of Laemmli (1970) buffer preincubated at 60°C, the gel was submerged in the solution and incubated at 60°C. Esterases are characterised by the appearance of blue bands on the gel.

2.3.4 Protein quantitation

Protein concentrations above 0.01 mg mL^{-1} were measured according to Bradford (1976). BSA (fraction V) was used as a standard. Below 0.01 mg mL^{-1}, the protein concentration was measured using the 280/205 nm absorption procedure (Peterson, 1983).

Bradford Assay and standard curves.

100 mg of Coomassie Brilliant Blue G-250 were dissolved in 50 mL of 95% ethanol. 100 mL of 85% (w/v) phosphoric acid were added and the volume made to 1L using distilled water. For the macro assay a standard curve containing 0.1 mg to 0.01 mg of BSA in 5 mL of Coomassie blue solution was used. Protein samples were diluted if necessary and 100 µL were added to 5 mL of dye reagent and mixed using a vortex mixer. After 5 minutes and before 1 hour the absorbance at 595 nm was recorded. The reagent was used as a blank. For the micro assay a standard curve containing 0.01 mg to 0.001 mg of BSA in 1 mL of Coomassie blue solution was used.

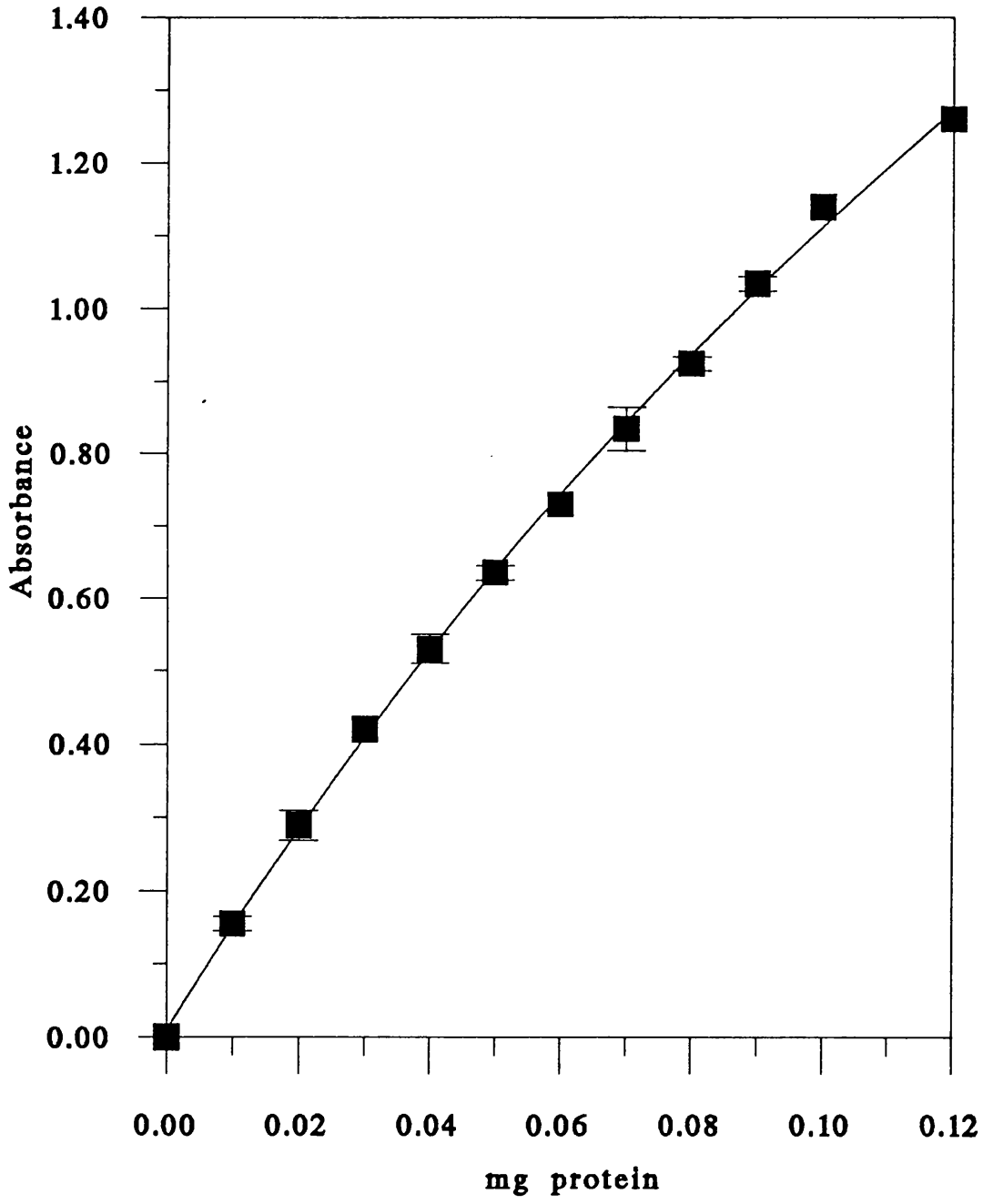
The 280/205 nm absorption procedure

Protein content was determined using a Shimadzu (UV-2101PC UV-VIS) dual-beam scanning spectrophotometer and Shimadzu CPS Temperature Controller. The spectrophotometer was controlled by an IBM PS/2 Model 35SX Computer.

The protein concentration P was calculated from the following expression:

$$P \text{ (in mg mL}^{-1}) = A_{205}/[27.0 + 120(A_{280}/A_{205})]$$

Figure 1.1 A typical Bradford standard curve (macro assay)



2.4 Initial Characterisation

2.4.1 Screening for esterase activity

The esterase activity of whole cells (100 µL), cell-free extracts (100 µL) and the supernatant (100 µL) were assayed using 0.5 mM *p*-NP propionate and 0.5 mM *p*-NP laurate.

Controls involved monitoring the background hydrolysis of the substrates in the presence of 100 µL nutrient broth supplemented with 0.5% yeast extract and boiled enzyme samples.

2.4.2 Growth curves

Strains were grown in 1 litre volumes of Nutrient Broth No. 2 supplemented with 0.5% yeast extract, in 2 L baffled flasks at 60°C and 130 rpm. 50 mL of a standard inoculum that had been growing for 12 hours was used to inoculate the flasks. 20 mL aliquots were taken each hour, and optical density and the pH were measured. The samples were centrifuged at 20,000 rpm for 20 minutes in an SS34 Sorval rotor. The pellets were resuspended in 0.75 mL 50 mM KH_2PO_4-NaOH buffer pH 7.0. The resuspended pellets were sonicated (Soniprep 150 MSE), using the microprobe, in 4 cycles of 30 seconds with 30 seconds cooling between each cycle at a power setting of 4. The samples were centrifuged in a microfuge for 2 minutes and the supernatant was retained. The supernatant was assayed for *p*-NP propionate and *p*-NP laurate activity and the protein content was measured.

2.4.3 Thermostability trials

Samples of crude cell extracts in 50 mM KH_2PO_4-NaOH buffer pH 7.0 were sealed in 1.5 mL Eppendorf tubes and heated in a water bath at 60°C, 65°C, 70°C, 75°C and 80°C.

Samples were removed at predetermined time intervals and rapidly cooled on ice. Subsequently the samples were centrifuged in a microfuge for two minutes to sediment any precipitated protein, and residual activity was assayed in 50 mM KH_2PO_4-NaOH buffer, pH 7.0 at 50°C using *p*-NP propionate as the substrate. Half-life values ($t_{1/2}$) for activity loss were estimated from plots of % initial activity versus time.

2.4.4 Development of a defined media for the aerobic growth of six strains of *B. stearothermophilus*

TDAM plates (without glucose) were supplemented with either 10 mM sodium nitrate or 10 mM ammonium sulphate and one of four sole carbon sources: glucose, lactose, sodium succinate and sodium citrate (all 10 mM). The strains were incubated at 60°C for up to 72 hours.

50 mL of TDLM containing sodium nitrate and the carbon sources: glucose, lactose, sodium succinate and sodium citrate (all 10 mM) were prepared in 100 mL baffled flasks. The flasks were inoculated from strains growing on the respective sodium nitrate plates (the initial optical density was at least 0.05). The strains were incubated at 60°C for up to 72 hours. The optical density of the cultures was monitored.

2.4.5 Defined media for the induction/repression of esterase activity in the Tok19 A1 isolate

TDAM plates were supplemented with one of three esters, tributyrin (10 mM) *p*-NP laurate (0.5 mM) and *p*-NP stearate (0.1 mM). *p*-NP stearate and *p*-NP laurate were dissolved in a minimum of ethanol and filter sterilised into the agar. The strains were incubated at 60°C for up to 72 hours.

One litre volumes of TDLM were prepared in 2.5 L baffled flasks. The flasks were supplemented with various ester substrates: tributyrin (10 mM), *p*-NP laurate (0.5 mM), *p*-NP stearate (0.1 mM). The flasks were inoculated from their respective TDAM plates.

2.5 Characterisation of the Tok19 A1 strain

Gram staining

Reagents:

A. 1% Aqueous crystal violet

B. Iodine solution: 1g of Iodine, 2g of Potassium Iodate (dissolved in 25 mL of water and made up to 100 mL.

C. Decolourisation: Acetone or Ethanol

D. Counterstain: Aqueous Safranin O (2% w/v) or dilute Carbol Fuchsin

Cells harvested from the stationary phase in the growth cycle of Tok19 A1 were stained for 2 minutes with aqueous crystal violet and 2 minutes with iodine solution. The cells were decolourised with acetone or ethanol and counterstained with aqueous safranin. The cells were air dried on a glass plate and viewed under a microscope.

Proteinase determination

Proteinase activity was determined by the hydrolysis of azocasein as described by Cowan *et al.*, 1987. 900 µL of azocasein (0.1% w/v in 100 mM HEPES-NaOH pH 7.5) was incubated at 60°C with 100 µL of enzyme for various times. The reaction was stopped by the addition of 500 µL of 15% w/v trichloroacetic acid. The assay tubes were allowed to cool for 10 minutes before being centrifuged in a MSE Microfuge for 3 minutes. The supernatant was retained and the absorbance was measured at 400 nm. The controls involved the incubation of the substrate only and subsequent addition of enzyme. One

unit of proteinase activity was defined as the volume of enzymic solution giving an absorbance change of 1.0 per minute under the specified assay conditions.

Amidase activity

Amidase activity was determined by measuring the hydrolysis of *p*-nitroacetanilide at 50°C. The reaction mixture contained 0.5 mM *p*-nitroacetanilide in 50 mM KH_2PO_4-NaOH buffer pH 7.0 and the release of *p*-nitroaniline was monitored at 405 nm (Heymann and Mentlein, 1981).

2.6 Protein purification protocols

2.6.1. Cell culture

For purification procedures 12 L of Nutrient Broth No. 2 supplemented with 0.5% yeast extract was prepared in 12 baffled 2 litre flasks. The flasks were autoclaved at 121°C for 30 minutes. When the flasks had cooled sufficiently (approximately 50°C) they were inoculated from Nutrient agar plates supplemented with 0.5% yeast extract which had been growing for 24 hours. The Tok19 A1 cells were flushed from the plates and resuspended using autoclaved distilled water. One plate was used to inoculate all the flasks. The cells were typically grown aerobically for 12 hours at 60°C at 130 rpm in orbital incubators.

2.6.2 Cell harvest

For small volumes of cells (up to 6 L) the cells were centrifuged at 9,000 rpm in a GS3 type Sorval Rotor for 30 minutes. The cell free supernatant was discarded and the cell pellets were resuspended in 50 mM KH_2PO_4-NaOH buffer pH 7.2. For volumes of cells greater than 6 L the cells were harvested and washed (50 mM Tris-HCl pH 7.2) using a microfiltration membrane (Life Sciences Laboratory 0.22 μm filter).

2.6.3 Preparing cell homogenates

Cells were lysed by the addition of 0.1 mg mL^{-1} lysozyme (incubated at 37°C for 30 minutes). 30 mL aliquots were sonicated for 30 seconds to reduce the viscosity. The resulting homogenate was centrifuged (48,000*g* for 30 minutes in a Sorvall SS34 type rotor) to remove the cell debris and the cell free supernatant was retained.

2.6.4 Protein precipitation trials

Ammonium sulphate and sodium sulphate precipitation

Solid ammonium sulphate or sodium sulphate was slowly added to the cell-free extract at 4°C on a magnetic stirrer to give a final saturation of 30%. After 30 minutes the cell-free supernatant was centrifuged at 20,000 rpm for 20 minutes in an SS34 Sorval rotor at 4°C. The supernatant was removed and returned to the stirrer at 4°C and the precipitate was retained. The precipitate was resuspended in 2 mL of 50 mM KH_2PO_4-NaOH buffer pH 7.0. Both the supernatant and resuspended pellet were assayed for activity and protein content. Ammonium sulphate or sodium sulphate was added slowly to the supernatant to give a final saturation of 60%. After a further 30 minutes the cell-free supernatant was centrifuged at 20,000 rpm for 20 minutes in an SS34 Sorval rotor at 4°C. The supernatant and the protein precipitate were retained. The precipitate was resuspended and both supernatant and the precipitate were assayed for activity and protein content. Controls involved the addition of 20 µL and 100 µL of 30% and 60 % saturated ammonium sulphate or sodium sulphate solution to the assay.

N.B. The maximum solubility of sodium sulphate is 1 part in 3.6 parts water (MERCK Index 1983), approximately equal to a concentration of 2 M.

Organic solvent (decanol) precipitation

Four 5 mL samples of cell free extract were stirred and incubated at 50°C for 5 minutes, 20 minutes, 30 minutes and 40 minutes respectively with 5 mL of n-decanol. After the respective times the samples were removed and centrifuged in a desktop centrifuge MSE at 4000 rpm for 10 minutes to separate the organic and aqueous layers. The aqueous layer was decanted and assayed for activity and protein content.

Heat precipitation

5 mL samples of cell-free extract were sealed in glass universal bottles and heated in water baths at 60°C, 65°C and 70°C. Samples were removed at predetermined time intervals and rapidly cooled on ice. The samples were centrifuged in a microfuge for 2 minutes to sediment any precipitated protein, and the supernatant was assayed for activity and protein content.

Polyethylene glycol 6000 (PEG 6000) precipitation

A 60% stock w/v of PEG 6000 was made in 50 mM Tris-HCl pH 7.0. 10 mL volumes of cell-free extract were stirred and 60% w/v PEG 6000 was added to give final concentrations of between 4% and 24% w/v. After 30 minutes the samples were centrifuged at 20,000 rpm for 20 minutes in an SS34 Sorval rotor. After centrifugation the supernatants were assayed for esterase activity and protein content. The total esterase activity and specific activity were plotted.

2.6.5 Chromatographic methods

Ion exchange chromatography

DEAE-Sepharose CL-6B and CM-Sepharose CL-6B columns were equilibrated with 5 gel volumes of 50 mM Tris-HCl, pH 8.5 and 50 mM MES pH 5.5. Samples were loaded onto the respective columns and the effluents collected. The columns were washed with a further 15 mL of the appropriate buffer and 5 mL fractions were collected. The absorbed protein was eluted from the columns using 15 mL of the appropriate buffer containing 1M NaCl. 5 mL fractions were collected. All the fractions were assayed for esterase activity.

Hydrophobic interaction chromatography (Phenyl -Sepharose)

Mini-columns were prepared containing 5 mL of Phenyl-Sepharose CL-4B. The columns were equilibrated with 5 gel volumes of 50 mM Tris-HCl pH 7.2 containing between 0 and 3M NaCl. Samples from the PEG 6000 precipitation trial were adjusted to the appropriate sodium chloride concentration and were loaded onto the respective columns. The effluents were collected and the columns were washed with further 15 mL volumes of buffer, containing the appropriate amount of NaCl. The absorbed protein was eluted from the columns using 50 mM Tris-HCl pH 7.2 and 50 mM Tris-HCl pH 7.2 containing various concentrations of ethylene glycol (v/v). 5 mL fractions were collected.

For large scale purification, hydrophobic interaction chromatography was performed in a 5 cm (d) x 30 cm (l) Pharmacia glass column containing 300 mL of Phenyl-Sepharose using the BioRad Econo Chromatography system. The Phenyl-Sepharose was equilibrated with 3 column volumes of 50 mM Tris-HCl pH 7.2 containing 1 M NaCl. A maximum of 300 mL of supernatant was loaded onto the column at a flow rate of 5 mL min^{-1}. The column was washed with 2 column volumes of 50 mM Tris-HCl pH 7.2

containing 1 M NaCl. The column was then washed successively with 2 column volumes of 50 mM Tris-HCl pH 7.2 and 50 mM Tris-HCl pH 7.2 containing 40% ethylene glycol (v/v) until no protein could be detected (at 280 nm). The column was finally washed with 50 mM Tris-HCl pH 7.2 containing 60% ethylene glycol (v/v) and the total activity peak was collected.

Q-Sepharose chromatography

Ion-exchange chromatography was performed in a pre-packed HiLoad 26/10 Q-Sepharose XK column containing 100 mL of gel. The 60% ethylene glycol peak from the phenyl-Sepharose column was diluted to less than 15% (v/v) ethylene glycol with 50 mM Tris-HCl pH 7.2 and loaded onto the Q-Sepharose column which had been equilibrated with 200 mL of 50 mM Tris-HCl pH 7.2 at a flow rate of 5 mL min^{-1}. The column was washed with 3 column volumes of 50 mM Tris-HCl pH 7.2 at 5 mL min^{-1} and the enzyme eluted with a 10 column volume linear gradient (0-0.5 M) of NaCl in 50 mM Tris-HCl pH 7.2.

Affinity chromatography (Benzamidine-Sepharose 6B)

Benzamidine-Sepharose mini-columns were prepared containing 5 mL of gel. The column was equilibrated with 3 column volumes of 50 mM Tris-HCl pH 8.0 1 M NaCl. 5 mL of esterase containing 10 units per mL of activity and 0.2 mg mL^{-1} of protein was made to 1 M NaCl and loaded onto the column. The column was washed with 15 mL of 50 mM Tris-HCl pH 8.0 1 M NaCl. Bound protein was eluted either by washing the column with 15 mL of 50 mM Tris-HCl pH 8.0 or with 1 mM para-benzamidine. 5 mL fractions were collected.

Gel filtration chromatography (Superdex 200 and Superdex 75)

Gel filtration chromatography (GPC) was performed using the Pharmacia FPLC system and Pharmacia pre-packed HiLoad Superdex 200 prep grade XK16/60 column. The column was equilibrated with 2 column volumes of 50 mM Tris-HCl pH 7.2 at a flow rate of 1 mL min^{-1}. 2 mL volumes of enzyme were loaded and eluted with 120 mL of 50 mM Tris-HCl pH 7.2 at a flow rate of 0.2 mL min^{-1}. 1 mL fractions were collected.

Gel filtration was also performed using the Pharmacia FPLC system and Pharmacia Superdex 75 column. The column was equilibrated with 2 column volumes of 50 mM Tris-HCl pH 7.2. The volume of the sample was reduced to 200 µL using an Amicon Centricon. 200 µL of enzyme were loaded onto the column and the esterase was eluted with 50 mM Tris-HCl pH 7.2 at a flow rate of 0.5 mL min^{-1}. 0.5 mL fractions were collected.

2.7. Protein characterisation protocols

2.7.1 Polyacrylamide gel electrophoresis (PAGE).

SDS and native polyacrylamide gel electrophoresis (PAGE) were performed using an LKB model 2001 electrophoresis unit in conjunction with a Bio-Rad 3000/300 power supply. 10% denaturing PAGE was performed (80 mA constant current) according to Laemmli, (1970). The enzyme samples were denatured by boiling in protein solubilisation solution (125 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) mercaptoethanol and 0.002% (w/v) bromophenol blue) in a sealed tube for 2 minutes. Non-denaturing PAGE was performed under identical conditions except that samples were not boiled, SDS was omitted from the buffers and both mercaptoethanol and SDS were omitted from the protein solubilisation solution.

SDS-PAGE protein bands were stained with Coomassie Blue G250 (0.1% w/v): 75% H_2O (v/v): 15% glacial acetic acid (v/v): 10% methanol (v/v) and destained with 75% H_2O (v/v): 15% glacial acetic acid (v/v): 10% methanol (v/v).

SDS and non-denaturing PAGE gels were prepared according to Table 2.5.

Table 2.5. SDS polyacrylamide gel constituents

	Separating gel			
% Acrylamide	7.5	10	12	15
Volume of Solution (mL)				
Acrylamide[a]	11.25	15	18	22.5
2% Running buffer[b]	22.5	22.5	22.5	22.5
Demineralised water	11.25	7.5	4.5	-
Ammonium persulphate[c]	0.5	0.5	0.5	0.5
TEMED	0.02	0.02	0.02	0.02
	Stacking gel			
Acrylamide[a]	2	2	2	2
2% Running buffer[d]	6	6	6	6
Demineralised water	4	4	4	4
Ammonium persulphate[c]	0.1	0.1	0.1	0.1
TEMED	0.01	0.01	0.01	0.01

[a] Acrylamide solution: 30% (w/v) Acrylamide, 0.8% (w/v) N,N'-Methylenebisacrylamide

[b] 2% Running gel buffer: 0.75M Tris-HCl, pH 8.8, 0.2% (w/v) SDS

[c] Ammonium persulphate: 10% (w/v) ammonium persulphate (fresh)

[d] 2% Stacking gel buffer: 0.25M Tris-HCl, pH 6.8, 0.2% (w/v) SDS

2.7.2 Molecular weight determination

Molecular weight determination from SDS PAGE

The molecular mass of the esterase was determined by comparison with the high molecular weight markers from the Sigma kit MW-SDS-200 (30,000-200,000).

The relative mobility (RF) of the protein bands is given by the expression

$$RF = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

The $\log_{10}$ of the molecular weight markers were plotted against the relative mobility (RF) of the bands. The molecular weight of the Tok19 A1 esterase was calculated from this standard curve.

Gel filtration chromatography

The Pharmacia Superdex 75 column was calibrated with molecular weight standards, from the Sigma kit MW-GF-70 (bovine lung aprotinin 6,500, horse heart cytochrome c 12,400, bovine erythrocyte carbonic anhydrase 29,000 and bovine serum albumin 66,000). Blue Dextran 2,000,000 was used to determine the void volume of the column.

2.7.3 Isoelectric point determination

Isoelectric focusing (IEF) was performed using a Pharmacia PhastSystem with preformed (IEF 3-8) PhastGels. Selected markers from the Sigma kit IEF-MI were used as standards: (*Aspergillus niger* amyloglucosidase, pI 3.55, soybean trypsin inhibitor, pI 4.55, bovine β-lactoglobulin A, pI 5.15; bovine erythrocyte carbonic anhydrase II, pI 5.85; human erythrocyte carbonic anhydrase I, pI 6.55 and horse heart myoglobin, pI 6.75, 7.15). 3 μg of protein were loaded onto the gel.

Isoelectric points were also determined using the FPLC and Pharmacia H/R Mono P column 5/20. The column was equilibrated with 25 mM Bis-Tris-HCl, pH 7.1 at a flow rate of 1 mL min^{-1} and a pre-gradient of 3 mL of polybuffer 74-HCl, pH 5.0 was established. 200µL of sample containing 10 units of esterase activity was loaded, followed by 26 mL of polybuffer 74-HCl pH 5.0. 0.5 mL fractions were collected.

2.7.4 N-Terminal amino acid sequence

An Applied Biosystems 470A amino acid Sequencer was used to determine the N-terminal amino acid sequence. Phenyl thiohydantoin derivatives were detected on an Applied Biosystems 120A HPLC.

2.7.5 Sequence alignment

The sequence of the 40 N-terminal amino acids of Tok19A1 esterase was keyed into the SWISSPROT database. 26,706 amino acid sequences in the database were searched for identity using the Fasta program (Lipman and Pearson, 1985). The highest homology sequences together with the esterase-6 sequence from *Drosophila* (Oakeshott *et al.*, 1987) and the yeast *Candida cylindracea* lipase sequence (Kawaguchi *et al.*, 1989) were aligned using Pileup (GCG) (Feng and Doolittle, 1987) which implements the general algorithm of Needleman and Wunsch, (1970).

2.7.6 Inhibitor studies

The following compounds were used for inhibition studies: ethylenediaminetetraacetic acid (EDTA), phenylmethanesulphonyl fluoride (PMSF), mercuric chloride ($HgCl_2$), parahydroxymercuribenzoate (*p*-CMB), tosyl-L-phenylalanine chloromethyl ketone (TPCK), physostigmine (eserine) and diethylpyrocarbonate (DPC)

900 µL containing ten units of enzyme in 50 mM Tris-HCl buffer pH 7.2 (were incubated with each of the inhibitors at 30°C for 30 minutes. Residual activity was determined by the standard assay.

2.7.7. Thermostability

1 mL samples of crude esterase (10 mg mL^{-1} pH 7.0), and purified esterase (2 µg mL^{-1} pH 7.0), were incubated at various temperatures between 20°C and 80°C. Samples were removed periodically, centrifuged in a microfuge for 2 minutes, and residual activity in the supernatant was determined by the standard assay. The residual activity was plotted against time and the half-lives ($t_{1/2}$; time for activity to decrease to 50% of the initial titre) of the enzyme at the various temperatures were taken from first order rate plots.

The thermostability of the purified esterase (10 µg mL^{-1}) was also studied at 56°C, 60°C, 65°C, 70°C and 75°C. 50 mM citric acid-Na_2HPO_4 buffer was adjusted to pH 6.0 at each temperature. The residual activity was plotted against time and the half-lives of the enzyme at the various temperatures were taken from first order rate plots.

2.7.8. Temperature-activity profile

50 mM KH_2PO_4-NaOH buffer was adjusted to pH 7.0 at each temperature and incubated with 0.5 mM *p*-NP valerate at temperatures between 4°C and 76°C and 0.5 mM *p*-NP propionate at temperatures between 4°C and 60°C. The background rate was monitored and subtracted as described. The $\log_e$ of the activity was plotted against 1/(absolute temperature) from which the activation energy and Q_{10} value were calculated for each substrate.

2.7.9 pH stability profile

The stability of the enzyme was studied in four buffers; 50 mM citric acid-Na_2HPO_4 pH 3.0-7.0, 50 mM HEPES-NaOH pH 7.0-9.0, 50 mM Clark and Lubbs solution pH 7.3-9.3 and 50 mM sodium carbonate-sodium bicarbonate pH 9.5-11.0. 25 units of esterase were incubated in 1 mL of each of the buffers at 50°C, 57°C, 60°C and 65°C (the pH of the buffers were confirmed after the addition of the enzyme). Samples were removed periodically and the residual activity was measured using the standard assay.

2.7.10 pH-activity profile

50 mM citric acid-Na_2HPO_4 pH 5.0-7.0, 50 mM HEPES-NaOH pH 7.0-8.0 and 50 mM borate-HCl pH 8-8.5 buffers were adjusted to various pH values at 30°C. The enzymic activity was determined at 30°C by the addition of 2 units of enzyme to 0.5 mM *p*-nitrophenyl propionate in the appropriate buffer. The hydrolysis of the substrate was measured at 348 nm, (ε=5150 M^{-1} cm^{-1}), the pH-independent isobestic wavelength of *p*-nitrophenol and its anion *p*-nitrophenolate (Lombardo and Guy, 1981).

2.7.11 Kinetics

Kinetics were performed at 50°C, using a Shimadzu (UV-2101PC UV-VIS) dual beam scanning spectrophotometer and Shimadzu CPS Temperature Controller. The spectrophotometer was controlled by an IBM PS/2 Model 35SX Computer. Initial velocity versus (substrate concentration)/(initial velocity) data were fitted to the Hanes transformation of the Michaelis-Menton equation using the Shimadzu optional Kinetics Software package (Revision 1.0) (Hanes, 1932). Kinetics were also performed in the presence of 0.2 mg mL^{-1} of bovine serum albumin at 4°C, 30°C and 50°C.

2.7.12 Activity in organic solvents

Esterase activity was assayed using the standard assay except that various concentrations (between 0 and 50% v/v) of organic solvents were added to the assay. In some cases BSA was added to a final concentration of 1 mg mL^{-1}.

2.7.13 Organic solvent stability

10 mL volumes of esterase in 50 mM Tris-HCl pH 7.0 were incubated with between 0 and 50% v/v of various solvents for between 30 minutes and 48 hours, at 30°C and at a stirrer speed of 300 rpm. Aliquots were withdrawn periodically and assayed using the standard assay except that corrections were made for the solvent carried over in the aqueous phase.

2.8 Immobilisation protocols

Esterase stability at pH 10.0

The esterase was incubated at pH 10.0 at 25°C for up to 48 hours. Samples were withdrawn periodically and assayed for residual activity

2.8.1 Preparation of esterase-glyoxyl agarose derivatives

10 mL volumes of glyoxyl agarose gels were added to 40 mL of enzyme preparation in 50 mM of different buffers pH 10 at 25°C. This "immobilisation suspension" was stirred gently and the pH was monitored throughout. Control suspensions ("blank derivatives"), identical to the immobilisation suspension but containing inert agarose gels (glyoxyl-agarose gels completely reduced with borohydride (Blanco and Guisan, 1989)) were also incubated under the same conditions. Aliquots of supernatant and whole suspension were withdrawn at intervals and their activities were assayed as described

below. Where specified, pH (from 9.7 to 10.5), temperature (from 20 to 37°C) and gel volumes (from 10 to 40 mL) were altered.

After a specified contact periods between the immobilised enzyme and activated agarose gel, derivatives were reduced with an excess of sodium borohydride (sodium borohydride was added to give a final concentration of 1 mg mL^{-1} and the suspension was stirred for 30 minutes at room temperature). The reduced esterase derivatives were washed with an excess of 0.1 M sodium phosphate buffer at pH 7 and with distilled water.

2.8.2 Immobilised esterase assay

The esterase activity of the soluble and immobilised derivatives was determined using *p*-nitrophenol propionate. Stock substrate solutions were prepared by dissolving the *p*-NP-ester in ethanol at a concentration of 100 mM. Assays were performed in a 1 cm pathlength cell fitted with a magnetic stirrer. The assay mixture contained 2 mL of 50 KH_2PO_4-NaOH buffer at pH 7.0 and 10 µL of *p*-NP propionate and was preincubated at 30°C in a Cecil spectrophotometer (UV-VIS single beam), with a water-heated cuvette block. After monitoring the background non-enzymic release of *p*-nitrophenol at 405 nm (ε=11900 $M^{-1}\cdot cm^{-1}$) for 1-2 minutes, 100 µL of soluble or immobilised derivative suspension. Correction for non-enzymic hydrolysis involved the subtraction of the background rate. (Where indicated, cosolvents and substrates were added to the reaction mixture). When necessary, corrections of extinction coefficient was performed in these cases. One unit of enzyme activity is defined as above (section 2.3.1).

2.8.3 Stability in the presence of organic cosolvents

Esterase derivatives were filtered and washed with 50 mM Tris-HCl buffer, pH 7.0 and 1.8g (2.5 mL) aliquots were suspended in 12.5 mL of the same buffer. Specified volumes of different cosolvents were added. The initial activity was measured immediately (less than 15 seconds after the addition of cosolvent) and the derivatives were incubated in 40 mL jacketed vessels at 30°C. The reaction mixture was stirred at 300 rpm. Aliquots were withdrawn periodically and assayed using the derivative assay. No pH corrections were carried out.

Chapter 3

Screening and Initial Characterisation

This chapter details the screening for a thermophilic esterase with high thermostability, high activity at mesophilic temperatures and broad substrate specificity. The initial characterisation, including substrate specificity, growth characteristics, thermostability and nutritional requirements of esterases from six strains of thermophilic *Bacilli,* has been reported.

3.1 Initial Screening

A complete list of the thermophilic *Bacillus* strains screened for esterase activity is given in Appendix 1.

No extracellular or whole-cell esterase activity was detected for any of the 72 strains of *Bacillus* under the assay conditions. 23 of the homogenised cell free supernatants showed *p*-NP propionate (C_3) activity and several hydrolysed *p*-NP laurate (C_{12}). In no instance was the activity towards *p*-NP laurate higher than the activity towards *p*-NP propionate. The 23 strains were subsequently screened with a wider range of *p*-nitrophenyl substrates. The results are summarised in Table 3.1.

The highlighted strains were those chosen for further analysis due to their ability to hydrolyse both *p*-NP-propionate(C_3) and *p*-NP myristate (C_{14}). The strain G18 A7 was included as it had the highest *p*-NP laurate (C_{12}) specific activity. The activity tended to decrease with increasing chain length although in some instances the *p*-NP valerate activity was higher than the *p*-NP propionate activity. Only one of the screened strains (Tok19 A1) had measurable *p*-NP stearate (C_{18}) activity.

3.2 Growth curves

Graphs of optical density, *p*-NP propionate activity per 100 mL and *p*-NP laurate activity per L during culture growth, for six strains of thermophilic *bacillus* isolates G18 A7, Fur6 A3, Rot34 A7, Tau8 A3, Tau10 A1 and Tok19 A1, are shown in Figures 3.1-3.6. The biomass and esterase activity for each strain was calculated in order to determine the optimum time to harvest the *Bacillus* cells and to determine whether the esterase activities were constitutive or were under regulative control. Both the *p*-NP propionate

and the *p*-NP laurate activity were measured as one or more esterases might be present in the cells.

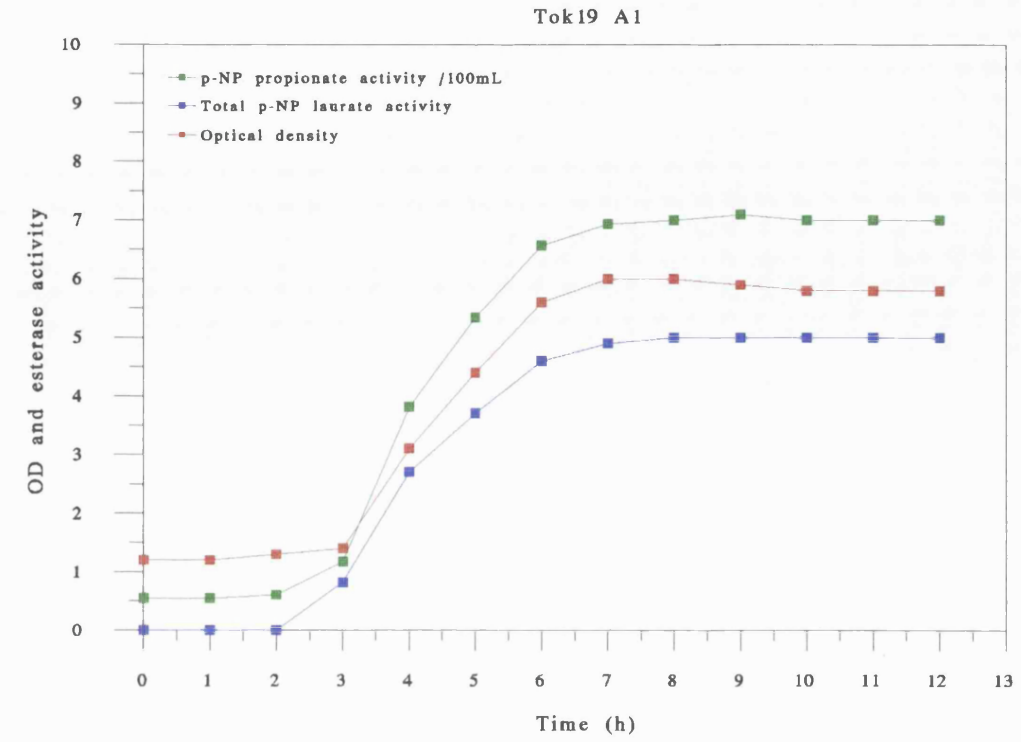
Table 3.1. The *p*-nitrophenyl substrate specificities of thermophilic esterases

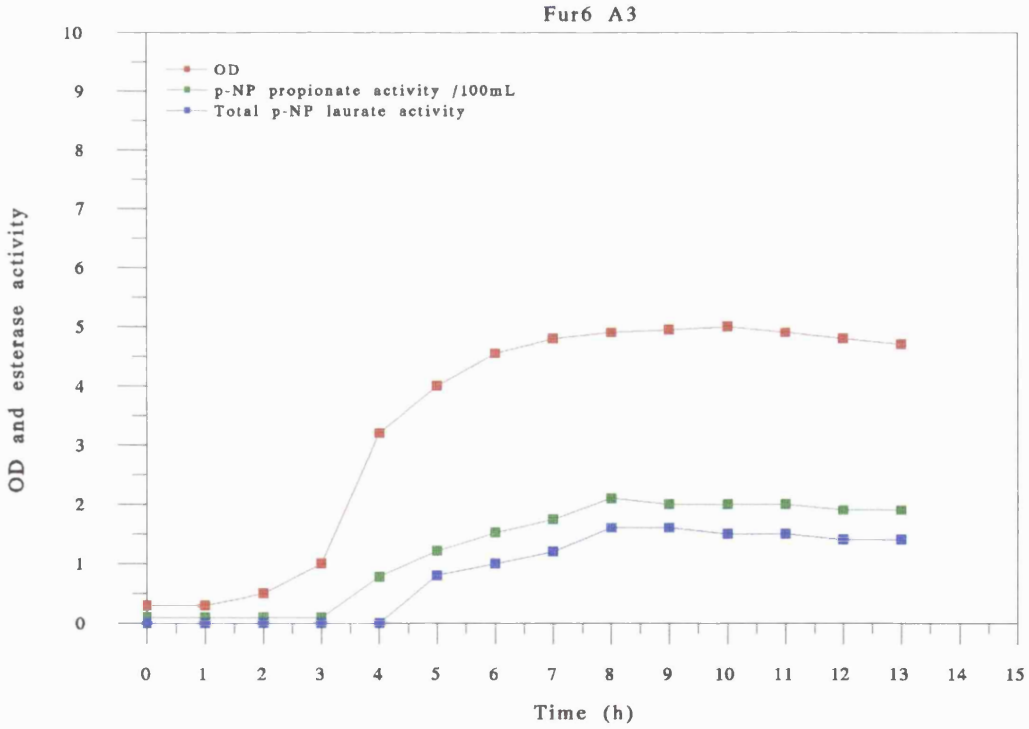
	Specific activity (Units mg^{-1} x 10^2)				
Strain	*p*-NP propionate	*p*-NP valerate	*p*-NP laurate	*p*-NP myristate	*p*-NP stearate
G18 A1	2	2	1		
G18 A2	32	40	1		
G18 A3	17	15			
G18 A5	38	41			
G18 A6	12	3	2		
G18 A7	27	35	16		
G18 A8	8	6	1		
G18 A9	12	14			
G18 A10	9	11			
Fur6 A3	120	108	8	2	
Fur9 A1	12	14			
Fur13 B2	5	4			
Fur15 A2	6	3			
Prf1 A3	5	5			
Rot34 A7	20	20	4	1	
Tau1 A5	47	42	4		
Tau2 A2	24	28			
Tau3 A1	7				
Tau3 A2	19	17	2		
Tau8 A3	258	279	9	2	
Tau10 A1	185	179	15	1	
Tok4 A3	27	12			
Tok19 A1	134	107	4	2	1

Where no data are shown, esterase activity was undetectable (below the estimated detection limit of 0.01 Units mg^{-1}.

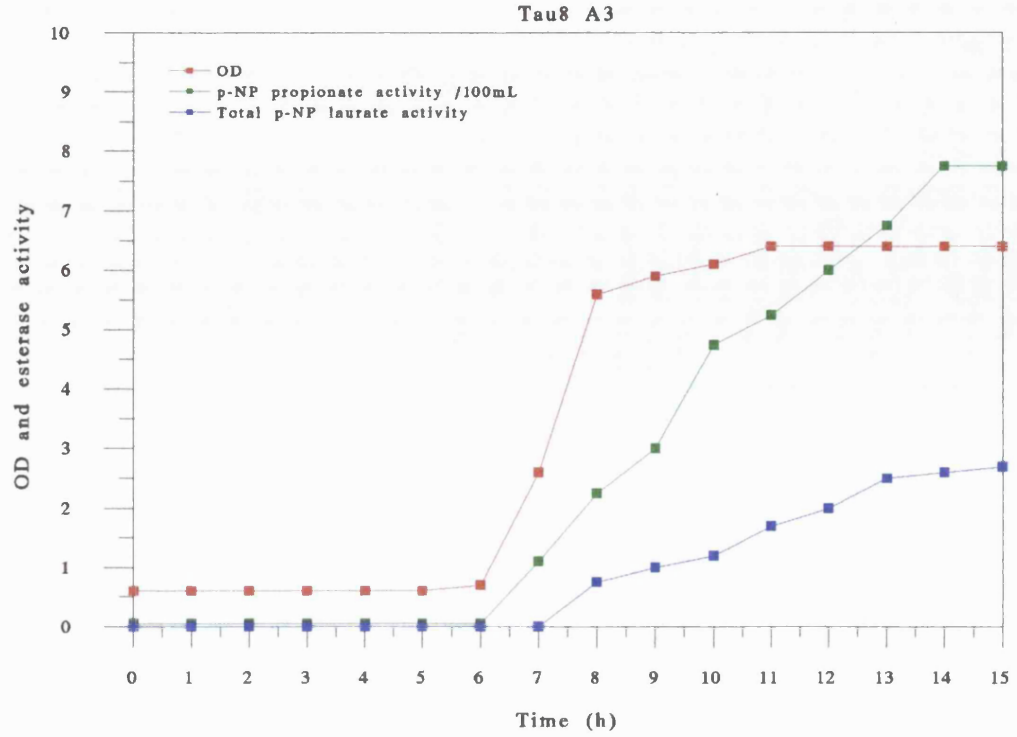
Growth curves were determined at 60°C, 130 rpm and pH 7.0 in 2 L baffled flasks containing nutrient broth supplemented with 0.5% yeast extract. Optical density was measured at 600nm (section 2.2.3 page 94). Esterase activity was measured using *p*-NP propionate and *p*-NP laurate at 30°C (section 2.3.1 page 95).

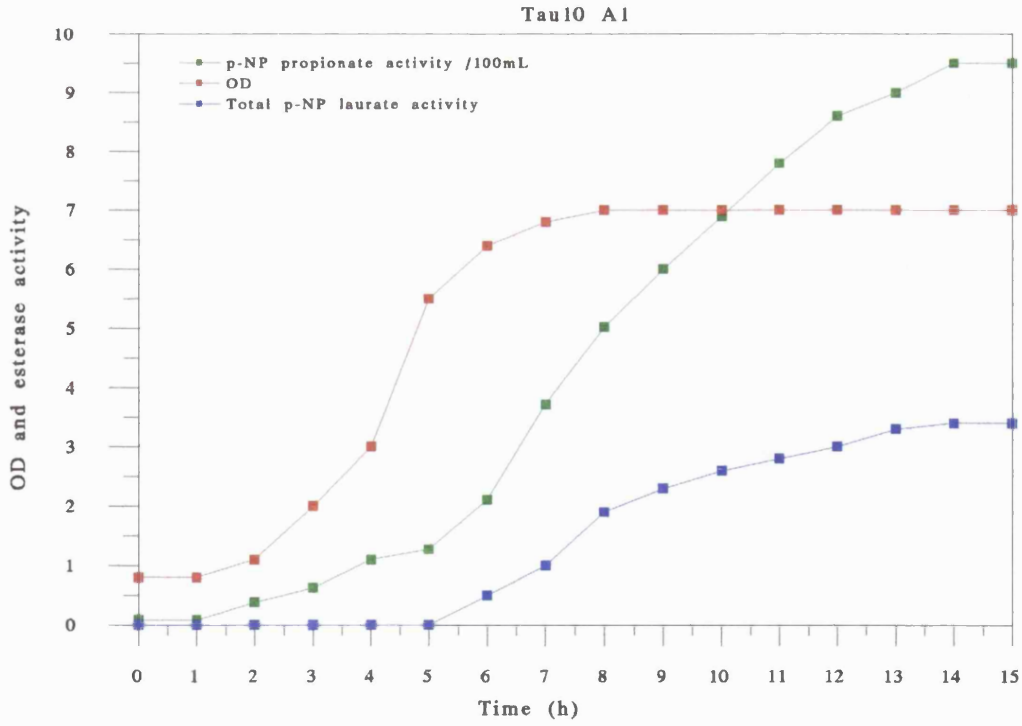
Figures 3.1 and 3.2. Growth curves for the isolates Tok19 A1 and Fur6 A3



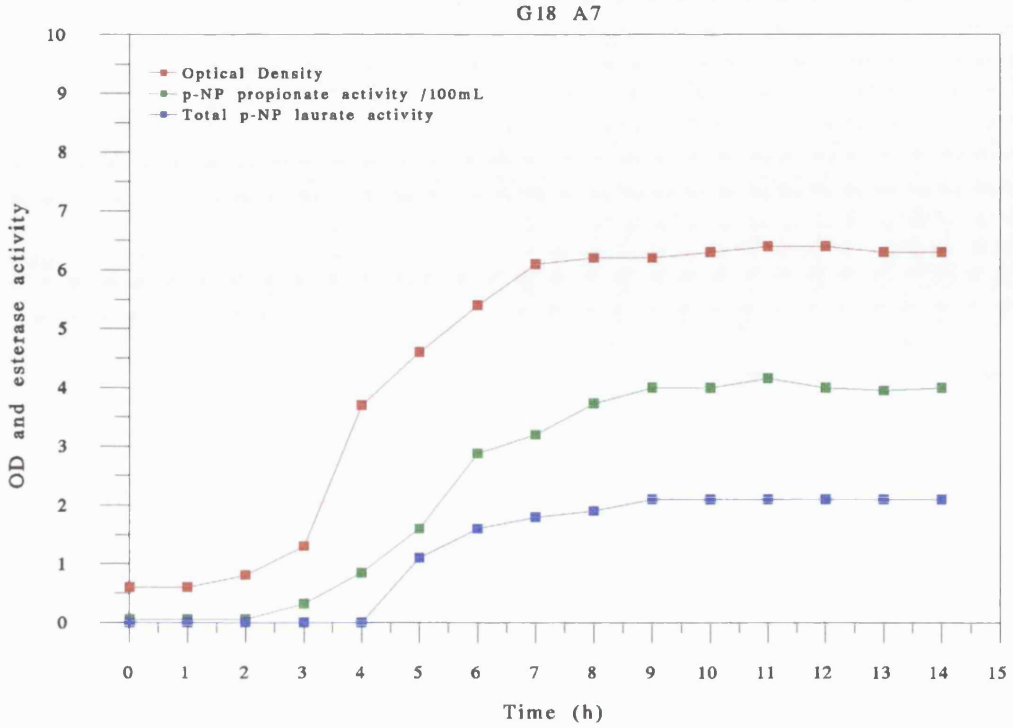


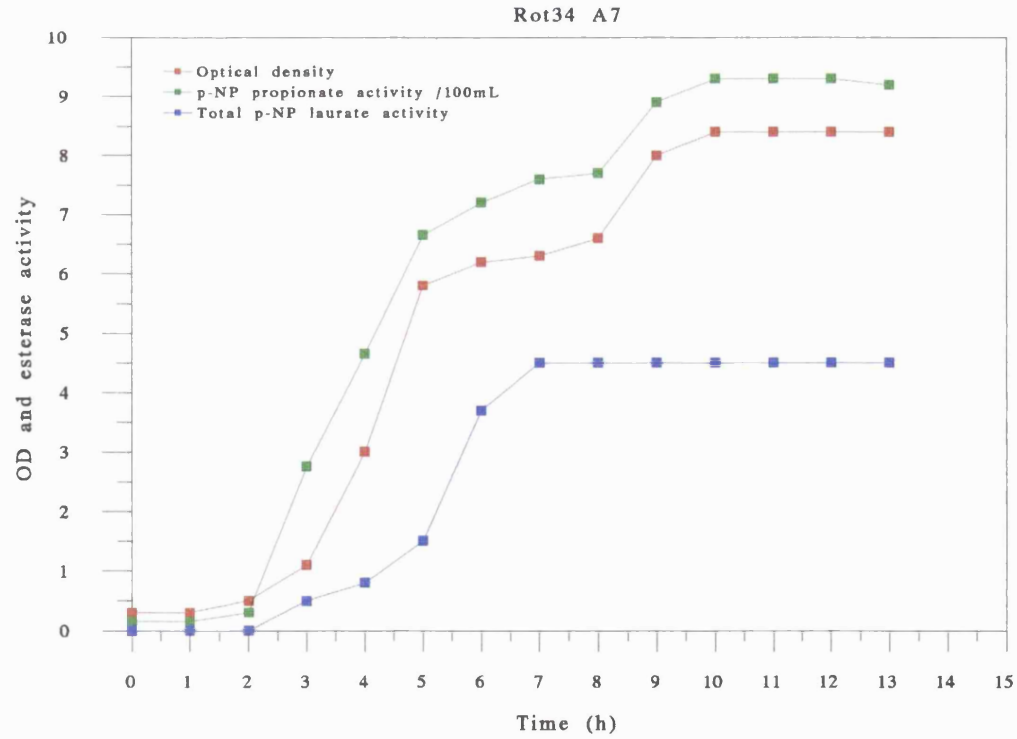
Figures 3.3 and 3.4. Growth curves for the isolates Tau8 A3 and Tau10 A1





Figures 3.5 and 3.6. Growth curves for the isolates G18 A7 and Rot34 A7





The pH, measured hourly throughout the growth curves, remained constant in the region of 7.0 - 7.2. A linear relationship between optical density (OD) and the wet weight of cells for all the strains was determined where an OD of 1.0 corresponded to approximately 1g of wet cells per litre of cell culture . The optical densities, and hence cell yield, in the stationary phase varied considerably between isolates (see Table 3.2). Whereas Rot34 A7 produced approximately 8g of cells per litre, Fur6 A3 yielded 5g per litre. These values, though of little significance on the 1 litre scale, would become very important on a 100-1000 L industrial scale. All the isolates, except Tok19 A1 and Rot34 A7, had typical growth curve profiles (Figures 3.1-3.6). The OD at the beginning of stationary phase for Tok19 A1 decreased slightly. This decrease might be attributed to an artifact which reflects the change in the ratio of biomass: optical density (Pirt, 1988). The growth curve for Rot34 A7 appeared to undergo diauxic growth (the sequential utilisation of substrates), the classical example of this is the successive utilisation of glucose and lactose by *E. coli*. No quantitative data on substrate concentrations was obtained.

The esterases from Tok19 A1, Fur6 A3 and G18 A7 appeared to be expressed constitutively i.e. they were expressed continually throughout the growth cycle (Figures 3.1-3.6). The esterases from the other three strains (Rot34 A7, Tau8 A3 and Tau10 A1) were expressed in late exponential phase and hence appeared to be inducible. The *p*-NP propionate and *p*-NP laurate activity curves for these strains were not parallel and the *p*-NP propionate and *p*-NP laurate activities for the strain Rot34 A7 showed biphasic and monophasic profiles respectively suggesting that these strains contain at least 2 esterase activities. The *p*-NP propionate and *p*-NP laurate activity curves were monophasic and parallel suggesting that a single esterase was hydrolysing both substrates.

Strains G18 A7 (Owusu and Cowan, 1991) and Fur6 A3 (V.K. Ramana, unpublished results) have been shown to contain single esterase activities. The *p*-NP laurate activities for the six strains were between 14 and 40 fold lower than the *p*-NP propionate activities and were in the range of 1.6 - 5.0 units L^{-1} of cell culture. Table 3.2 shows the biomass (g L^{-1}) and total esterase yield per litre of nutrient broth, based on the hydrolysis of *p*-NP propionate at 30°C.

The esterase activity increased to early stationary phase and there was no decrease in the total esterase activity after 15 hours incubation. Cultures could thus be harvested at any time during the stationary phase with no loss in total yields of activity. The specific growth rate of the strains was comparable with the specific growth rate of *B. stearothermophilus* strain LLD-15 (μ_{max} = 1.6h^{-1}) on an optimised defined medium (Amartey *et al.*, 1991).

Table 3.2 Biomass yields and esterase activity per litre of cell culture.

Isolate	Biomass (g (wet cells) L^{-1})	Esterase activity (Units L^{-1})	Specific growth rate (μ_{max}) (h^{-1})	Doubling time (h)
Tok19 A1	6.0	70	1.0	1.0
Fur6 A3	5.1	20	2.0	0.5
G18 A7	6.5	40	0.7	1.4
Rot34 A7	7.9	90	1.0	1.0
Tau8 A3	6.5	75	1.3	0.8
Tau10 A1	7.0	80	2.0	0.5

Biomass and the total esterase activity (based on the hydrolysis of *p*-NP propionate at 30°C) were calculated from at least 3 different cultures (average values are shown, error ± 2.5%)

3.3 Thermostability Trials

Preliminary esterase thermostability trails were conducted on crude cell free homogenates from cultures harvested in stationary phase. The thermostability profiles for the crude esterases at 65°C, 70°C, 75°C and 80°C are shown in Figures 3.7-3.10. The half-lives of the esterases at the various temperatures are summarised in Table 3.3.

At 60°C, which is near the optimum growth temperature of all but one of the organisms, there was no loss of esterase activity after twenty four hours. The half-life of the Tok19 A1 esterase at 60°C was 50 hours. At 80°C, no esterase activity could be detected in any of the strains after 20 minutes incubation and the half-lives were all less than 5 minutes. At 65°C, the thermostabilities of the esterases were significantly different (Table 3.3). At 75°C the esterase from Tok19 A1 and Tau10 A1 were twice as stable as the esterases from the other strains. The low stability of the esterases above 65°C was not due to proteolysis since the denaturation followed first order kinetics (Figure 3.11).

Table 3.3 Half-lives of the esterases from crude homogenates

	Half-lives $t_{1/2}$ (min)				
Organism	60°C	65°C	70°C	75°C	80°C
Tok19 A1	3000	420	90	23	<5
Tau 10A1	n.d.	192	100	7	<5
Fur6 A3	n.d.	192	160	16	<5
G18 A7	n.d.	430	190	9	<5
Tau 8A3	n.d.	96	75	8	<5
Rot 34A7	n.d.	290	35	9	<5

n.d. not determined

Half-life values ($t_{1/2}$) for activity los were estimated visually from plots of % residual activity against time (pages 124 and 125).

Figure 3.7 Thermostability profiles of the crude esterase preparations at 65°C

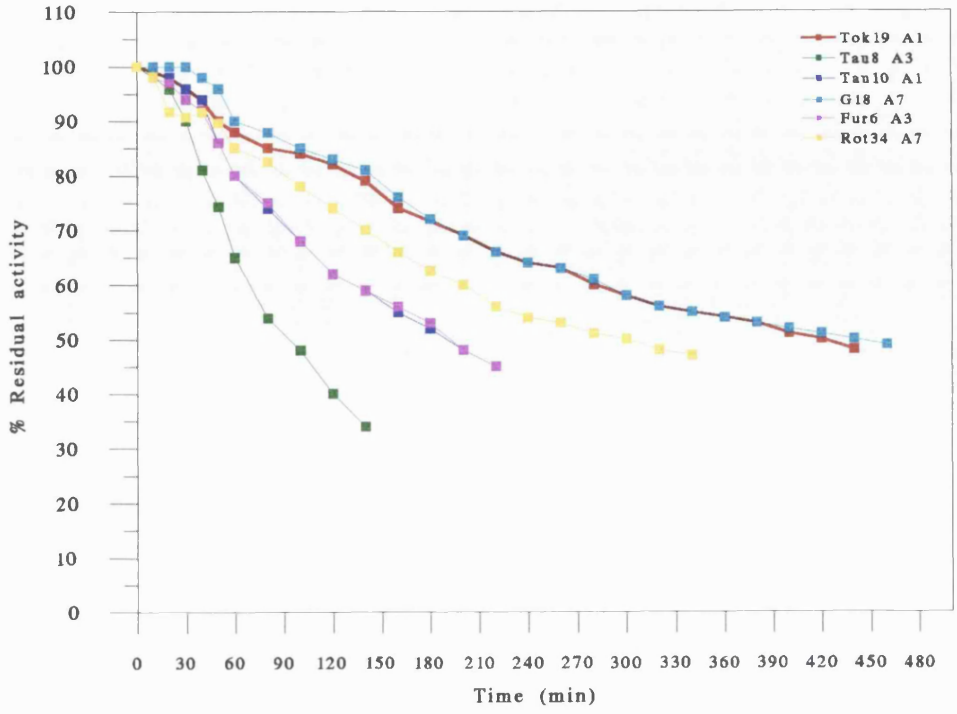
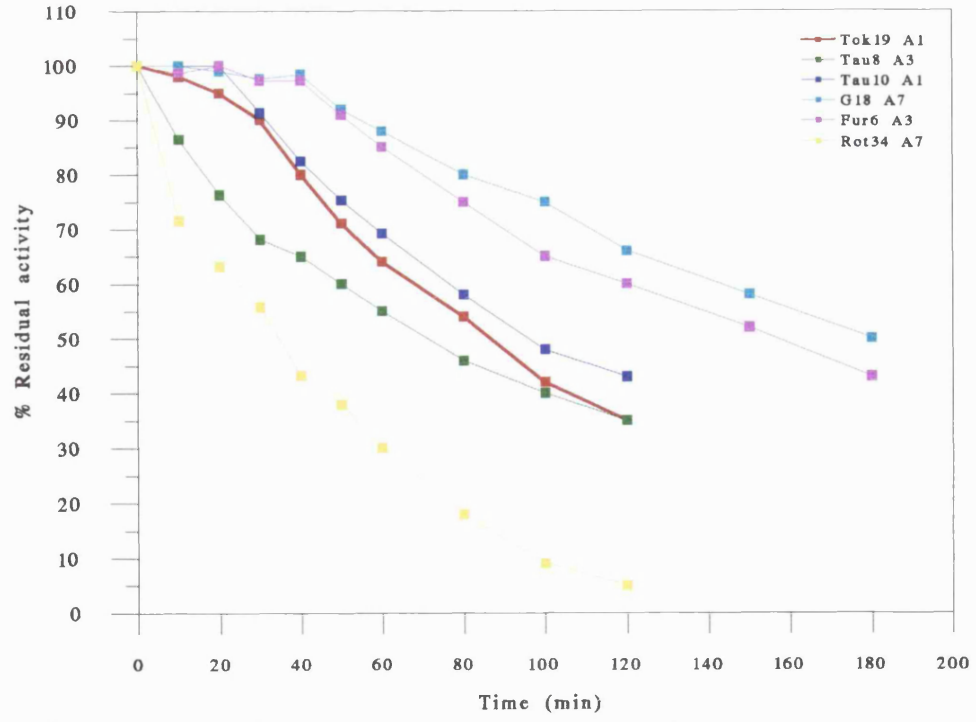


Figure 3.8 Thermostability profiles of the crude esterase preparations at 70°C



Crude cell extracts were heated at the stated temperatures (section 2.4.3 page 98). Aliquots were withdrawn periodically and assayed using the standard assay (section 2.3.1 page 95).

Figure 3.9 Thermostability profiles of the crude esterase preparations at 75°C

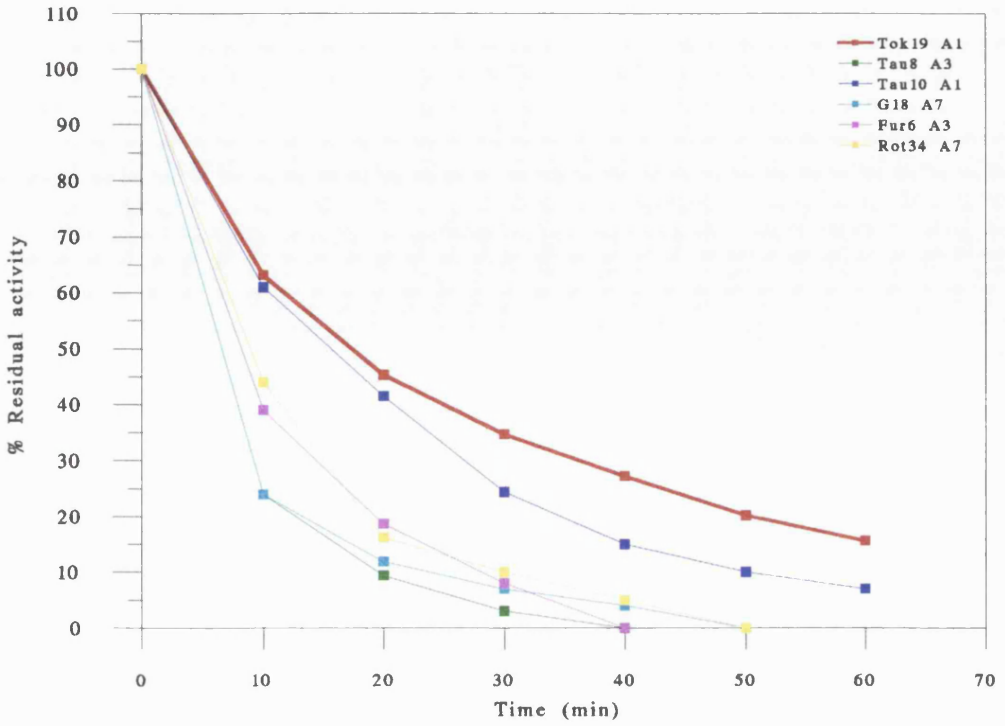


Figure 3.10 Thermostability profiles of the crude esterase preparations at 80°C

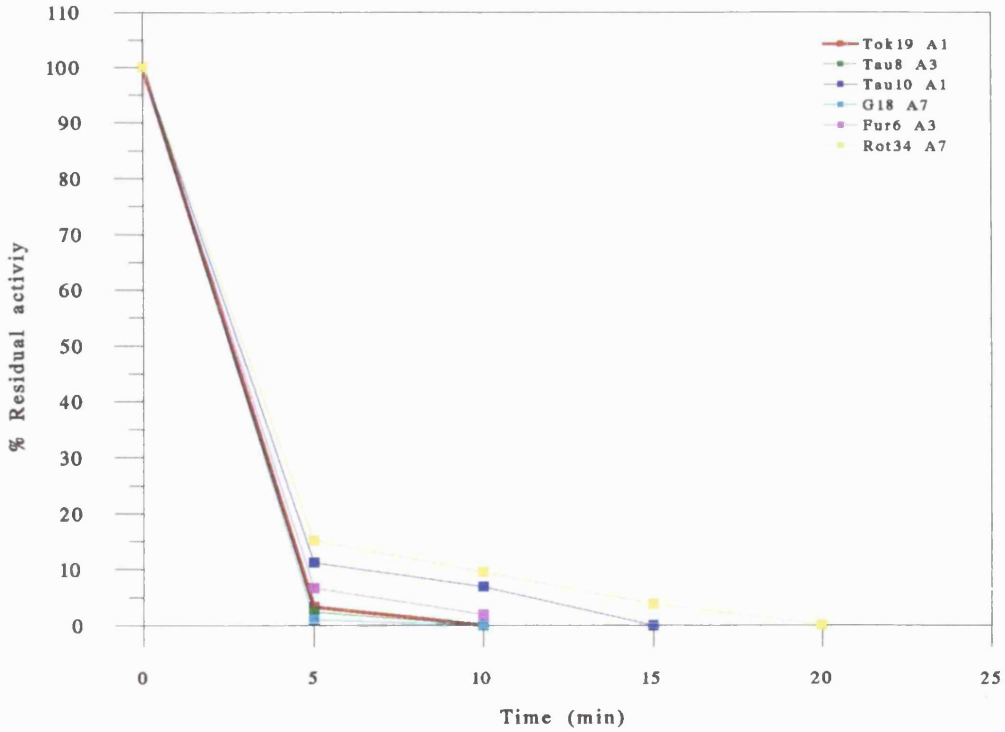
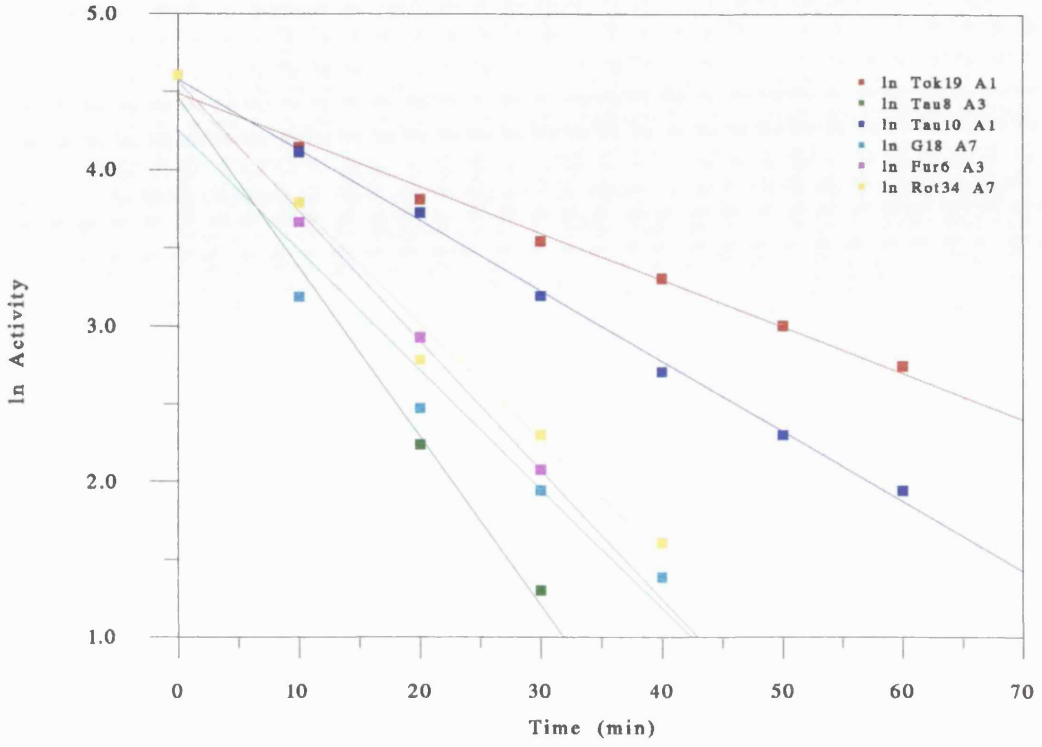


Figure 3.11 First order thermostability $\log_e$ plots for the crude esterase preparations at 75°C



It is noted that the thermostability of enzymes in crude extracts may not reflect the thermostability of the purified enzyme for a variety of reasons: the pH of the samples and the ionic strengths in the crude and purified samples may be different, protein-protein and protein-carbohydrate interactions might lead to enhanced stability while the presence of proteases will have a deleterious effect on the stability. An esterase from a thermophilic Bacillus G18 A7 was shown to have significantly enhanced stability in the purified state compared to the crude homogenate (Owusu and Cowan, 1991). It has since been shown that the properties of the purified esterase from Tok19 A1 are affected by protein-protein interactions (Section 5.1.6).

3.4 Designing a defined medium for the control of esterase synthesis.

Introduction

The absence of defined and minimal media restricts the genetic and physiological studies of thermophilic organisms. Defined media are critical in order to elucidate the effects of changes in media composition on cell growth and product formation, for the calculation of mass balances, and to facilitate the isolation of auxotrophic mutants.

For many thermophiles, defined and minimal media have not been elucidated due to their fastidious vitamin and amino acid requirements (Baker *et al.*, 1960). It is because of these reasons that thermophilic bacteria are often grown on nutrient broth or the growth medium is supplemented with tryptone, yeast extract and Brain-Heart-Infusion (Cleverdon *et al.*, 1949; Amelunxen, 1966; Sargeant *et al.*, 1971; Kuhn *et al.*, 1979).

The nutritional requirements of a number of thermophilic bacteria, including various strains of *B. stearothermophilus*, have been studied and found to be complex (Cleverdon *et al.*, 1949; Campbell *et al.*, 1952; Kuhn *et al.*, 1979; Amartey *et al.*, 1991). When the vitamin requirements of these *B. stearothermophilus* were studied, biotin was found to be essential for growth for all the strains (Cleverdon *et al.*, 1949, Campbell *et al.*, 1952, O'Brien and Campbell, 1956; Kuhn *et al.*, 1979; Amartey *et al.*, 1991). Other vitamins such as niacin (Campbell, 1952; Cleverdon, 1949), thiamine (Campbell, 1952; Cleverdon, 1949; O'Brien and Campbell, 1957) and nicotinic acid (O'Brien and Campbell, 1957) have also been shown to be essential for growth. Methionine has been shown to be an essential amino acid (O'Brien, 1956; Kuhn *et al.*, 1979; Amartey *et al.*, 1991) in every case where the amino acid requirements were investigated. Other amino acids such as histidine, isoleucine and valine (Rowe *et al.*, 1975) and isoleucine, leucine, valine, histidine and arginine (O'Brien and Campbell, 1956) have also been shown to be

essential for the growth of strains of *B. stearothermophilus*. Amino acid-requiring mutants for arginine, threonine and tryptophan have been isolated on solid media (Rowe *et al.*, 1975).

Given the heterogeneous nature of the thermophilic *Bacilli*, it cannot be assumed that specific nutritional requirements identified for one strain may be generalised to other strains. The purpose of this study was not to define or optimise a medium for maximum growth of a single strain but rather to find a medium that would support the growth of several strains of thermophilic *Bacilli*. The medium should also be suitable for subsequent optimisation of esterase expression.

The thermophilic defined medium of Rowe *et al.*, (1975) was chosen for these studies since the use of defined media in agar relievesmuch of the tedium associated with shake flasks. In shake flasks, oxygen supply may become limiting and pH control is far from optimal, also the use of buffers to control pH has been shown to reduce growth rates considerably (Kuhn *et al.*, 1979; Rowe *et al.*, 1975).

The defined liquid media of Campbell *et al.*, (1953) would have been used in these studies since it supported the growth of eight strains of *B. stearothermophilus*. However in the solid form (1.5% agar), this medium failed to support growth (Rowe *et al.*, 1975) and it has been suggested that the growth obtained in these studies on defined media were due to the carryover of nutrients from the complex inoculum (Rowe *et al.*, 1975).

The abbreviations TDLM and TDAM (Thermophilic Defined Liquid Medium and Thermophilic Defined Agar Medium respectively) have been used throughout this thesis. Both TDLM and TDAM supported the growth of the six strains of *B. stearothermophilus* although the wet cell yield was approximately 0.8 g L^{-1} in all cases and the *p*-NP propionate activities were in the range of 5 - 10 units L^{-1}. The total activity

per litre from nutrient broth supplemented with 0.5 % yeast extract had been in the order of 20 - 90 units L^{-1}. Consequently the carbon, nitrogen, amino acid and vitamin requirements of the strains were investigated in order to increase the cell yield and esterase production.

3.4.1. The growth of *B. stearothermophilus* on different carbon and nitrogen sources

In order to determine the carbon and nitrogen requirements of the six strains, a preliminary investigation was performed using various compounds, in TDAM, as sole nitrogen or carbon sources. The carbon sources were chosen because of their role in the major metabolic pathways.

A visual interpretation of the growth of the isolates was made after 72 hours incubation at 60°C. The results are summarised in Tables 3.5 and 3.6.

Table 3.4. Ammonium Sulphate as the nitrogen source.

Strain	Tok19 A1	Tau8 A3	Tau10 A1	G18 A7	Rot34 A7	Fur6 A3
Carbon Source						
D-Glucose	+++	-	++	++	+++	+
Fructose	++	-	++	++	+	-
Glycerol	+++	-	++	++	++	+
Sodium citrate	++	-	+	++	++	-
Sodium succinate	++	+	++	++	++	-

- no growth, + poor growth, ++ growth, +++ very good growth

For experimental details see section 2.4.4

Table 3.5. Sodium nitrate as the nitrogen source.

Strain	Tok19 A1	Tau8 A3	Tau10 A1	G18 A7	Rot34 A7	Fur6 A3
Carbon Source						
D-Glucose	+++	++	+++	+++	+++	+++
Fructose	+++	++	++	++	+	++
Glycerol	+++	++	+++	++	+++	++
Sodium citrate	++	++	++	++	++	++
Sodium succinate	+++	++	+++	++	+++	++

- no growth, + poor growth, ++ growth, +++ very good growth N.B. There was no growth on TDAM containing no nitrogen or carbon source.

For experimental details see section 2.4.4

The growth of the strains using sodium nitrate as a nitrogen source generally exceeded that using ammonium sulphate (Tables 3.4 and 3.5). It is possible that the ammonium sulphate was in excess and excess ammonia is known to inhibit the production of certain amino acids (Kinoshita, 1972). Tau8 A3 and Fur6 A3 did not grow or grew very poorly in the presence of 10 mM ammonium sulphate although they had grown in TDLM and on TDAM. It is suggested that either these isolates cannot metabolise NH_3^+ or NH_3^+ at a concentration of 10 mM may be inhibitory. When sodium nitrate was used as the nitrogen source the isolates generally grew well on carbon sources involved in both glycolysis and the citric acid cycle. It has been reported that a strain *of B. stearothermophilus* (NCA 1503, ATCC 7954) did not grow on citric acid cycle intermediates (Rowe *et al.*, 1975). This inability to utilise lactate, acetate, citrate, α-ketoglutarate and succinate was attributed to the organisms' inability to transport these carbon sources across its cytoplasmic membrane. A previous report had demonstrated the presence of all the enzymes of this cycle in strain NCA 1503 (Rowe *et al.*, 1973).

Since growth using sodium nitrate as the sole nitrogen source, exceeded that using ammonium sulphate, 50 mL of TDLM containing sodium nitrate and the carbon sources

were prepared in 100 mL baffled flasks. The flasks were inoculated from the sodium nitrate plates to give an initial optical density of at least 0.05 and incubated at 60°C for up to 72 hours. The optical density of the cultures was checked periodically. The isolates failed to grow on any of the medium except that containing glucose. It was unclear why the isolates did not grow in the other liquid medium, since the strains grew well on the agar medium. The growth on the agar medium did not result from the carry over of the complex medium as the strains grew after repeated sub-culture. It has already been suggested that some strains which grew well on solid media will not grow in shake-flasks in identical liquid media (Kuhn *et al.*, 1979) because oxygen supplies can become limiting also pH is far from optimal and buffers have been shown to reduce growth rates considerably. However some growth under these conditions would be expected. The pH of the media was checked and found to be in the region of 7.2. The pH of nutrient broth is typically 7.1 and the strains grew well on nutrient broth (growth curves Figures 3.1-3.6). It is doubtful that oxygen transfer in TDLM should be any different than in nutrient broth where the strains grew very well.

The effects of omitting biotin, L-methionine and nicotinic acid from the TDLM and TDAM media were investigated. These three were selected because they had been shown by a number of authors (Cleverdon *et al.*, 1949; Campbell *et al.*, 1952; O'Brien and Campbell, 1956; Kuhn *et al.*, 1979; Amartey *et al.*, 1991) to be essential for the growth of several strains of *B. stearothermophilus*.

None of the isolates grew in the absence of L-methionine and biotin, and Tau8 A3 and Tau10 A1 would not grow in the absence of nicotinic acid. Hence L-methionine and biotin appear to be essential for the growth of *B. stearothermophilus*.

3.4.2. Induction of esterase activity

Several esters (tributyrin, *p*-NP laurate and *p*-NP stearate) were investigated for their ability to induce esterase activity in the six isolates.

When the isolates were grown in TDLM supplemented with the carbon sources there was no increase in the specific activity of the esterase indicating that it/they had not been induced (they may have been produced constitutively). The strains grew particularly well in the presence of 10 mM tributyrin (maximum OD=1.5). The strains produced approximately 20 units (based on the hydrolysis of *p*-NP propionate) of esterase activity per litre of cell culture, a two-fold increase over TDLM alone but there was no increase in specific activity. This increase in activity was attributed to an increase in biomass rather than induction of the esterase. Tributyrin was subsequently shown to be suitable as a sole carbon source, unlike *p*-NP laurate and *p*-NP stearate.

3.4.3. Conclusions

All the isolates were found to have a nutritional requirement for biotin and methionine, and two, Tau8 A3 and Tau10 A1, also required nicotinic acid. None of the TDLM supported the same level of growth as nutrient broth supplemented with 0.5% yeast extract. The maximum esterase yield from 1 L of TDLM was approximately 10 units. This was increased to 20 units when tributyrin was added at a concentration of 10 mM, whereas in nutrient broth supplemented with 0.5% yeast extract the maximum esterase yield was approximately 90 units. In no instance were the esterases of the six strains enhanced by the addition of ester substrates.

3.5. The isolate Tok19 A1

The scope of the project did not allow work with all six isolates. The esterase from the isolate Tok19 A1 was chosen for further purification and characterisation. The esterase had a broad substrate specificity (hydrolysing both *p*-NP propionate and *p*-NP stearate) under the assay conditions. The half-life of the crude esterase was 50 hours at 60°C. The strain produced approximately 70 units of esterase activity per litre of nutrient broth (based on the hydrolysis of *p*-NP propionate at 30°C). However the isolate only produced 10 units of esterase activity per litre of TDLM, increased to 20 units per litre when TDLM was supplemented with 10 mM tributyrin

3.5.1. Tok19 A1 morphological characteristics.

The organism, designated Tok19 A1, was originally isolated from a sediment sample collected from the Tokaanu thermal region, New Zealand (Cowan, unpublished results). The bacterium was found to be a Gram-positive, aerobic, rod shaped, sporulating, motile, obligate thermophilic microorganism with an optimum growth temperature of 60°C (T_{min}< 55°C and T_{max}<65°C) in nutrient broth supplemented with 0.5% yeast extract. The growth curve at 60°C is shown in Figure 3.1. The strain grew on glucose, fructose, glycerol, citrate, succinate, lactose and tributyrin. Morphological characteristics are consistent with the growth characteristics of *B. stearothermophilus* NCA 1503 (Sharp *et al.*, 1991) (except that strain NCA 1503 did not grow on citrate) and a provisional taxonomic designation as *Bacillus stearothermophilus.*

3.5.2 The growth of Tok19 A1 at various glucose concentrations

In attempts to increase biomass levels, Tok19 A1 was grown on TDLM supplemented with glucose (1 mM, 5 mM, 10 mM 25 mM and 50 mM).

Tok19 A1 grew poorly on glucose concentrations from 1 mM to 25 mM (Figures 3.12-3.15). Growth was inhibited at a glucose concentration of 50 mM. Glucose concentrations in the region of 1% (approximately 50 mM) are known to be toxic to many micro-organisms. The optical density in the stationary phase increased with increasing glucose concentration from 0.8 at 1 mM glucose to 1.2 at 10 mM glucose and decreased slightly to 1.1 with 25 mM glucose (Figures 3.12-3.15). 10 mM glucose gave the highest total esterase activity when assayed with *p*-NP propionate while total activity decreased at 25 mM glucose. Specific activity of crude cell extracts from 25 mM glucose were consistently lower than the crude cell extracts from other media (Table 3.6). It was concluded that glucose concentrations above 10 mM may repress Tok19 A1 esterase expression.

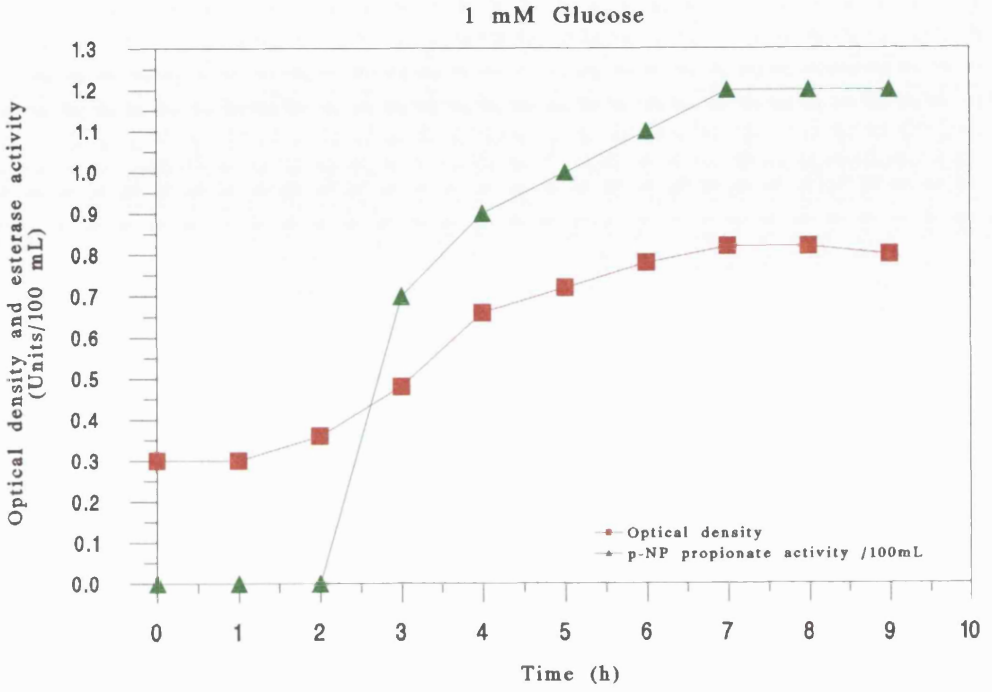
After 9 hours the remaining cells were harvested by centrifugation. The corresponding specific activity, wet weight of cells and total esterase activity per litre of culture medium for each glucose concentration were compared with other growth media (Table 3.6). TDLM supplemented with 10 mM and 25 mM glucose resulted in a two-fold increase in biomass over TDLM alone, the same as TDLM supplemented with 10 mM tributyrin. The number of esterase units (based on the hydrolysis of *p*-NP-propionate at 30°C) per gram of wet cells for all the glucose media was approximately the same (14-15 units per litre) (Table 3.6). However 1 L of nutrient broth supplemented with 0.5% yeast extract gave 5-6 g of wet cells (70 esterase units) whereas 1 L TDLM supplemented with 10 mM glucose yielded approximately 1.4 g wet cells L^{-1} (21 esterase units).

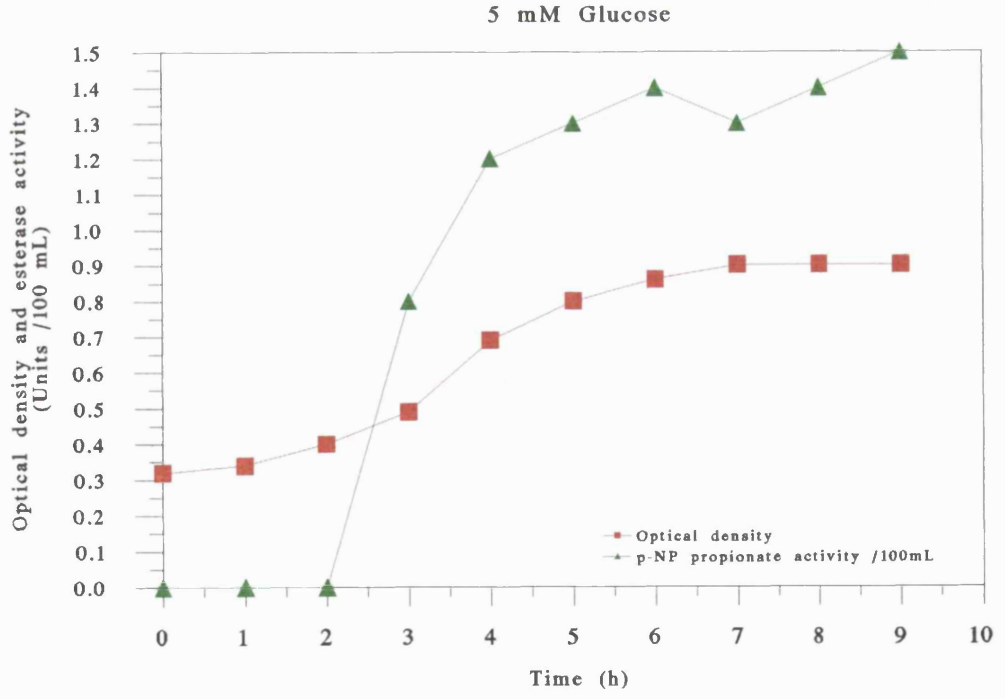
Table 3.6 Biomass and esterase activity of the Tok19 A1 isolate in various growth media

Growth medium	Wet weight of cells (gL^{-1})	Activity per gram of wet cells (Units g^{-1})	Specific activity (Units mg^{-1})	Total activity (Units L^{-1})
Nutrient Broth and 0.5% Yeast Extract	5-6	12-14	0.3*	70
TDLM	0.7	14	0.57	10
TDLM + 10 MM Tributyrin	1.3	15	0.65	19
TDLM + 1 mM Glucose	0.8	14	0.61	12
TDLM + 5 mM Glucose	1.1	14	0.58	15
TDLM + 10 mM Glucose	1.4	15	0.60	21
TDLM + 25 mM Glucose	1.3	11	0.44	14

* This value is for lysozyme lysed cells compared with sonicated cells in the other samples

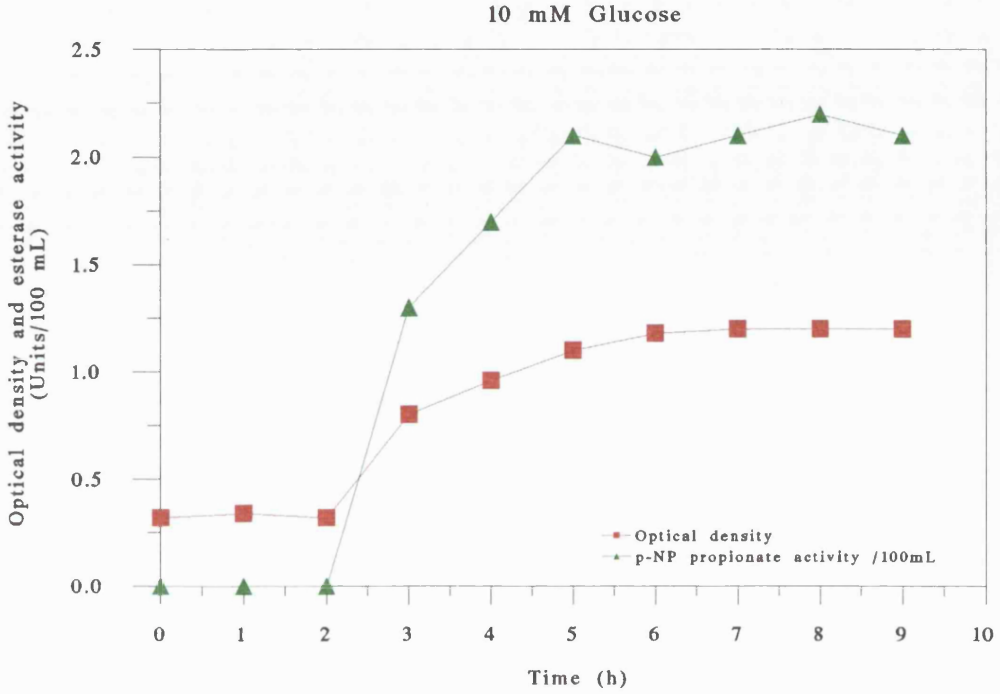
Figures 3.12 and 3.13 Growth curves for Tok19 A1 grown on Glucose as the sole carbon source

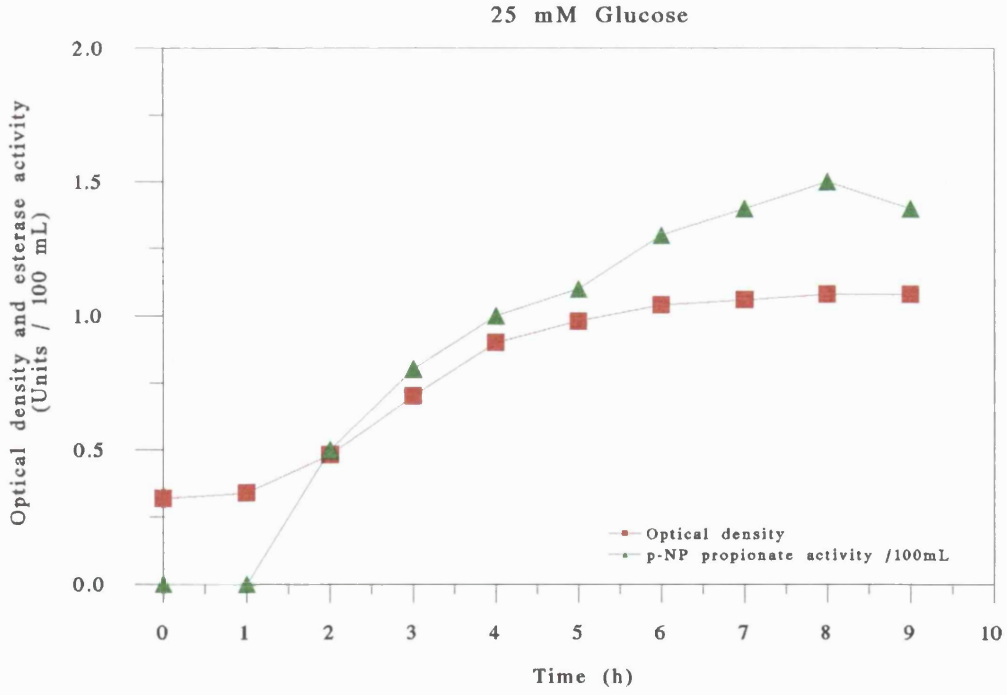




The strain Tok19 A1 was grown on TDLM containing various concentrations of glucose. Optical density was measured at 600nm (section 2.2.3 page 94). Esterase activity was measured using the standard assay (section 2.3.1 page 95).

Figures 3.14 and 3.15 Growth curves for Tok19 A1 grown on Glucose as the sole carbon source





Chapter 4

Purification of the Tok19 A1 esterase

This chapter details the development of methods used to purify the esterase from the thermophilic *Bacillus* strain Tok19 A1.

4.0 Introduction

One litre volumes of Tok19 A1 cells were grown in nutrient broth supplemented with 0.5 % yeast extract in 2 L baffled flasks. The flasks were incubated in a rotary incubator at 60°C for 12 hours at 130 rpm. The cells were harvested by centrifugation (see section 2.6.2) and used immediately or stored at -70°C . Frozen cells were thawed and a cell free homogenate was prepared by sonication (see section 2.6.3) and further centrifugation (see section 2.6.3). The crude cell free extracts were assayed for esterase activity and protein content. Several precipitation techniques were tried and the results are detailed below.

Samples of crude cell extract, when electrophoresed on non denaturing PAGE and stained for esterase activity using indoxyl acetate, showed three low-mobility and two high-mobility bands. When the bands were scanned using the Joyce-Loebl Chromoscan 3, the relative intensities were typically 40, 30 and 10 for the three low mobility bands and 10 for each of the high mobility bands. During subsequent purification steps, the removal of the two-high mobility bands and the separation of the three low-mobility bands were important objectives (see below).

4.1 Protein precipitation techniques

4.1.1 Ammonium sulphate precipitation

When the pellets and supernatant from the ammonium sulphate trial were assayed the total Tok19 A1 esterase activity had apparently increased compared to the original sample. Ammonium sulphate at concentrations of 30% (w/v) and 60% (w/v) were assayed in the absence of enzyme and the ammonium sulphate was found to significantly increase the background hydrolysis of *p*-NP propionate. 100 µL of 60% ammonium

sulphate (w/v) increased the background hydrolysis of the substrate 9 fold, equivalent to an esterase concentration of 6 μmol min^{-1} mL^{-1}. The effect of various concentrations of ammonium sulphate on the *p*-NP propionate assay are shown in Table 4.1.

Table 4.1 Background hydrolysis of *p*-NP propionate in the presence of ammonium sulphate

Volume (μL)	% ammonium sulphate (w/v)	Background hydrolysis Δ (Abs) min^{-1}
100	30	1.10
100	60	1.30
20	30	0.72
20	60	0.90
100	dH_2O	0.14
Background *p*-NP propionate		0.14

For experimental detail see section 2.6.4.

The high background rates made the interpretation of the ammonium sulphate precipitation trials very difficult since it was not known whether the observed activity was due to esterase activity or ammonium sulphate. 100 μL of 60 % sodium sulphate was found to have no effect on the background hydrolysis of *p*-NP propionate hence the increased background absorbance was probably due to the ammonium ion. Several buffers and reagents (e.g. glycine) have subsequently been shown to affect the *p*-NP propionate assay. The ammonium sulphate trials were suspended in favour of other precipitation methods.

4.1.2 Sodium sulphate precipitation

Sodium sulphate did not increase the background hydrolysis of the *p*-NP propionate and further precipitation trials were conducted using this salt. The results of the sodium sulphate precipitation are detailed in Table 4.2

Table 4.2 Sodium sulphate precipitation

Sodium sulphate saturation level	Activity (Units mL^{-1})	Protein content (mg mL^{-1})	Volume (mL)	Specific activity (Units mg^{-1})	% Enzyme activity	Purification factor
supernatant	2.88	1.60	14.0	1.80	100	-
30 % supernatant	2.66	1.20	10.2	2.22	67.3	1.2
30 % pellet	2.20	2.02	4.9	1.09	26.7	0.6
60 % supernatant	2.47	0.6	8.2	4.12	50.2	2.3
60 % pellet	2.13	2.17	3.5	0.98	18.5	0.54

For experimental detail see section 2.6.4.

The precipitates formed very loose pellets and consequently the volumes quoted for the pellets were larger than would otherwise have been expected. The volume of supernatant associated with the pellet contributed to its high activity. Nevertheless the specific activity increased and up to 2.3 fold purification was obtained with 50% of the activity retained.

Further precipitation trials were performed with other precipitation systems to investigate whether the enzyme yield could be increased.

4.1.3 Organic solvent precipitation

Organic solvents such as acetone have been used to purify esterases (Matsunaga *et al.*, 1974; Alder and Kistiakowsky, 1961). Hydrophobic organic solvents such as decanol are known to be less detrimental to enzyme activity than more hydrophilic solvents (Reslow *et al.*, 1988; Laane *et al.*, 1985; Brink and Tramper, 1985).

Decanol was added to crude cell free homogenates, to a concentration of 50 %, and incubated at 50°C for up to 40 minutes. After the incubation period the samples were centrifuged and the aqueous layer was assayed for esterase activity and protein content. The results of the trials are detailed in Table 4.3.

Table 4.3 Organic solvent precipitation trial using 50% decanol at 50°C

Time (min)	Activity (Units mL^{-1})	Protein Content (mg mL^{-1})	Volume (mL)	Specific Activity (Units mg^{-1})	Purification factor	% increase in Enzyme Activity
0	1.05	0.65	30	1.61	-	100
5	1.31	0.55	28.2	2.38	1.48	117
20	1.30	0.50	27.6	2.60	1.62	114
30	1.49	0.42	25.2	3.55	2.21	119
40	1.53	0.42	24.0	3.64	2.26	117

For experimental detail see section 2.6.4.

When the samples had been centrifuged three distinct layers could be seen: an upper layer (decanol), an intermediate solid layer probably consisting of denatured protein and a lower aqueous layer. The total protein content of the aqueous layer after 40 minutes incubation with decanol was approximately 50 % of the original value. The total esterase

activity increased. by approximately 17% during the trial, this could be due to a variety of factors:

- an inhibitor of esterase activity could have been precipitated during the course of the experiment
- a conformational change in the Tok19 A1 esterases structure may have occurred
- Tok19 A1 esterase catalysed transesterification (between *p*-NP propionate and decanol to form decyl-propionate) could have occurred.

It has been subsequently shown (section 6.1) that this increase in Tok19 A1 esterase activity is almost certainly the result of a transesterification reaction.

4.1.4 Precipitation (temperature denaturation)

Introduction

Denaturation of proteins by heat can be described as a first-order process. Thus the rate constant K_{den} can be related to temperature by

$$\frac{d \ln K_{den}}{dT} = \frac{E_{act}}{RT^2}$$

where E_{act} = energy of activation = $\Delta H^{\#}$ + RT

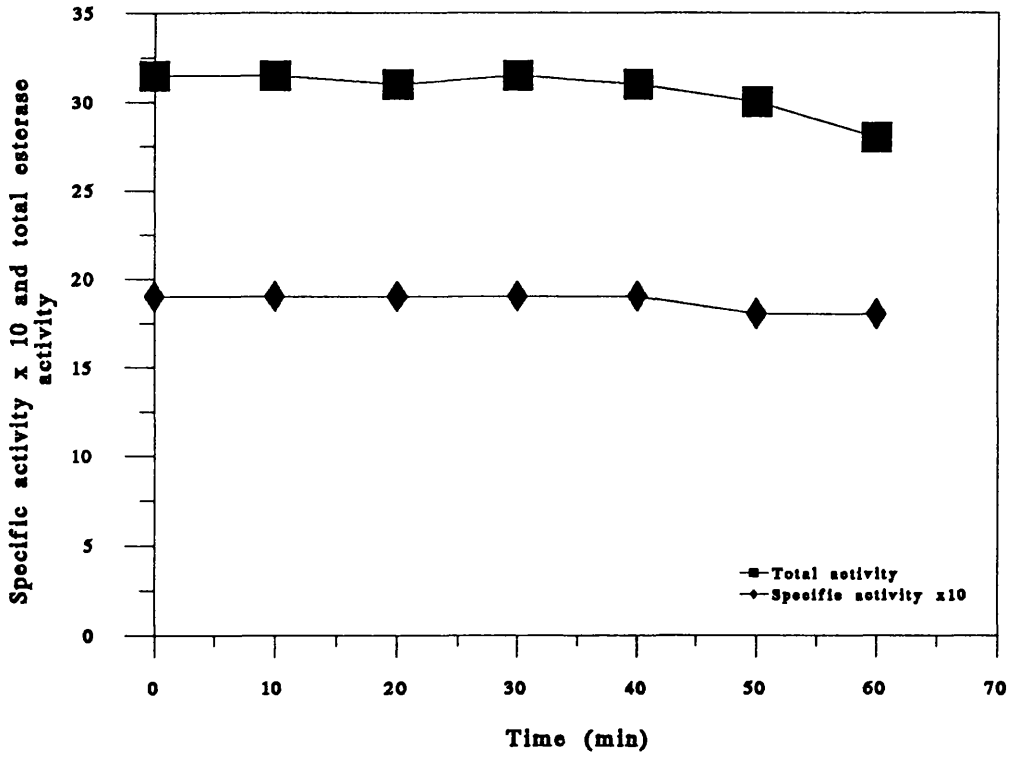
$\Delta H^{\#}$ = enthalpy of activation

Different proteins have different energies of activation, and the mid points and the shapes of their denaturation curves will therefore vary widely. Consequently, it is possible to choose a temperature that will completely denature one protein whilst leaving 95% of another protein unaffected. Heat precipitation has been used to purify a number of esterases (Sobek and Gorisch, 1989; Murata, 1987).

Results

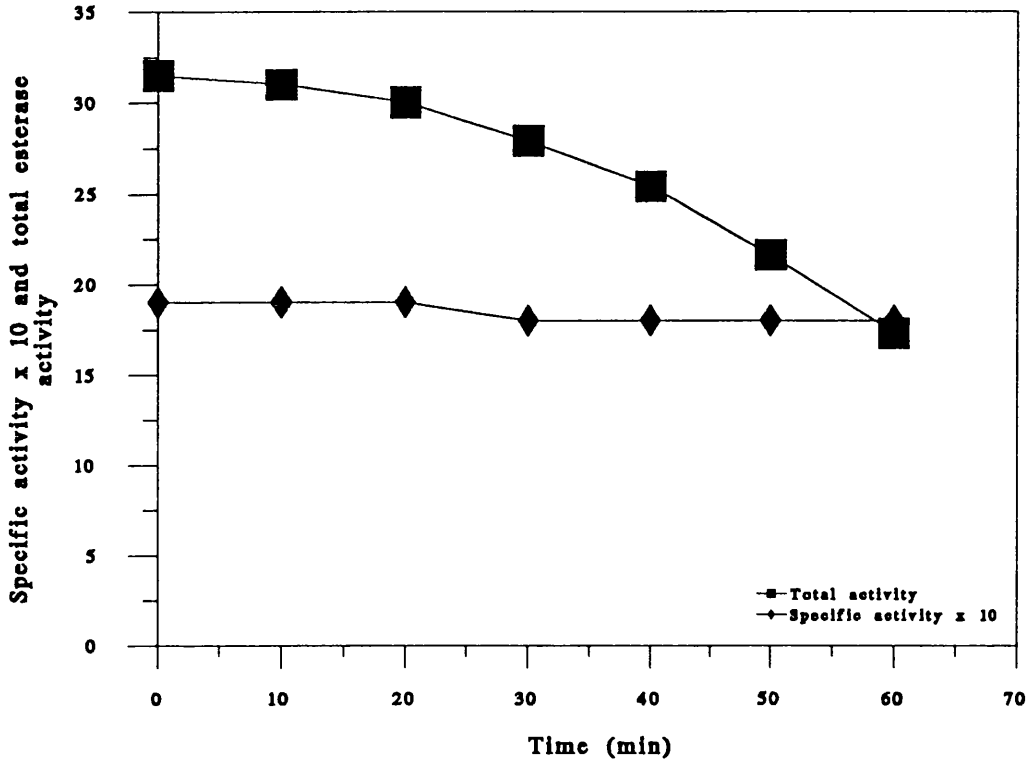
When a solution of crude cell extract was heated at 60°C there was no decrease in the activity or protein content after 60 minutes. At 65°C and 70°C the Tok19 A1 esterase activity and protein concentration decreased at the same rate, maintaining a constant specific activity (Figures 4.1 and 4.2). Since, at 70°C, there was a rapid decrease in Tok19 A1 esterase activity, precipitation at higher temperatures was not investigated. In view of these results heat precipitation was not used as a purification method.

Figures 4.1 The relationship between the total esterase activity and specific activity at 65°C



5 mL samples of cell-free extract were sealed in glass universal bottles and heated in water baths at 60°C, 65°C and 70°C (section 2.6.4 page 103). Esterase activity was determined using the standard assay (section 2.3.1 page 95) and protein content (section 2.3.4 page 96).

Figures 4.2 The relationship between the total esterase activity and specific activity at 70°C



For experimental details see section 2.6.4

4.1.5 Poly ethylene glycol (PEG) 6000 precipitation

Introduction

Water soluble nonionic polymers, in particular polyethylene glycol (PEG), have been used for the selective precipitation of proteins. The advantages of using PEG stem from its benign chemical properties (unlike acetone and other organic precipitating agents, PEG has little tendency to denature proteins even at high concentrations) and its relatively low viscosity when compared to other polymers. Another advantage of PEG over ethanol or ammonium sulphate is the length of time required for the precipitated proteins to equilibrate and achieve a physical state suitable for large scale centrifugation (Ingham, 1990).

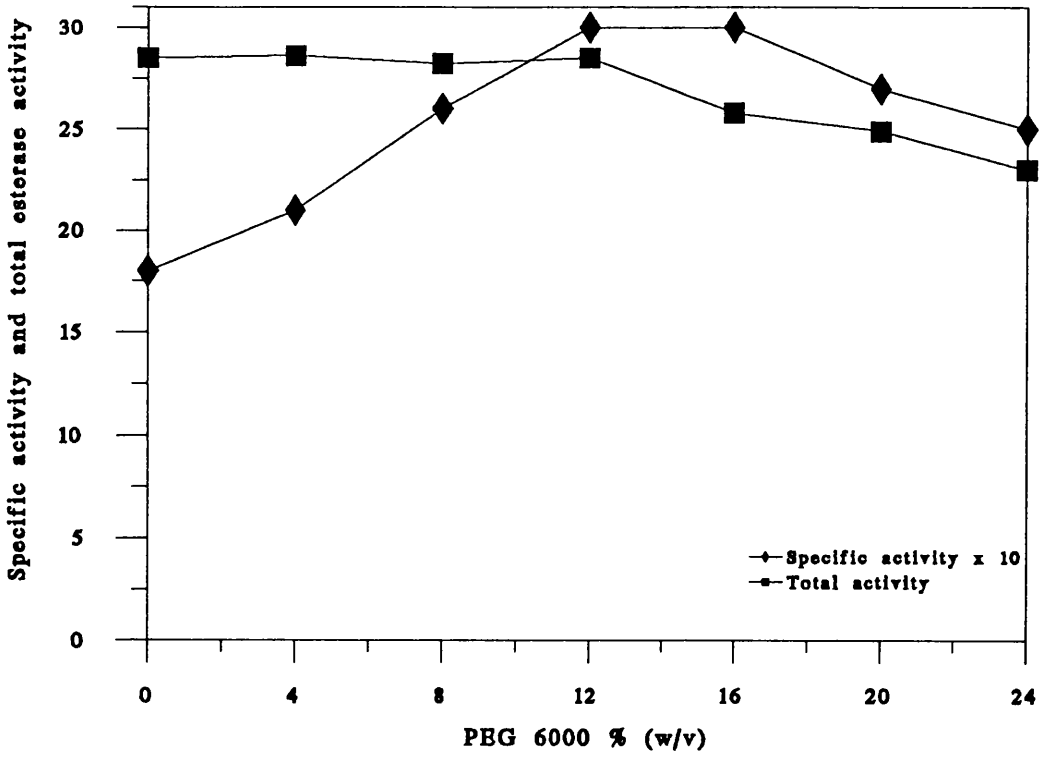
60% w/v PEG 6000 was added to 10 mL volumes of cell free extracts to give final concentrations of between 4% and 24% w/v. After 30 minutes the samples were centrifuged at 20,000 rpm for 20 minutes in an SS34 Sorval rotor. After centrifugation the supernatants were assayed for esterase activity (section 2.3.4 page 96) and protein content (section 2.3.4 page 96).

Results

The protein/PEG pellets were very difficult to resuspend and no protein content and activity values were obtained for the pellet fractions. The supernatants retained 100% of their activity in the presence of up to 12% w/v PEG 6000 (Figure 4.3). 12 % w/v PEG and 16 % w/v PEG gave the highest specific activity of 3.0. The sample at 12 % w/v PEG retained 100% activity whereas the activity at 16% w/v PEG decreased by 10%. The increase in specific activity corresponded to an increase in the purification factor of 1.7.

The only disadvantage of this otherwise simple purification method proved to be the sample volume increases of 26%.

Figure 4.3. The relationship between esterase activity and total protein in the presence of PEG 6000.



Summary of the precipitation methods

Ammonium sulphate interfered with the esterase assay in a non linear manner making accurate activity quantitation difficult. This interference appeared to be due to the ammonium ion. While sodium sulphate did not interfere with the assay, the salt was only soluble to a concentration of approximately 2 M. This concentration caused protein precipitation but the precipitated protein would not pellet. Organic solvent precipitation gave high specific activities with a concomitant increase in the purification factor, and an apparent increase in esterase activity. This increase is almost certainly due to a transesterification process rather than the removal of an inhibitor or activation of the esterase through a change in conformation. There was no increase in the specific activity of samples after heat precipitation trials at 60^{o}C and above. PEG 6000 precipitation was found to be a simple reproducible method, giving purification factors of up to 1.7, and was used in further purification studies.

4.2 Chromatography

4.2.1 Ion exchange chromatography

Since the isoelectric point of the esterase was unknown, the binding properties of two different ion exchangers were investigated. Esterase samples from the PEG precipitation were adjusted to the appropriate pH and loaded onto DEAE-Sepharose CL-6B and CM-Sepharose CL-6B columns, equilibrated with 5 column volumes of Tris-HCl, pH 8.5 and MES buffer, pH 5.5 respectively.

The results from these trials are shown in Tables 4.4 and 4.5.

Table 4.4 DEAE- Sepharose at pH 8.5

Fraction	Volume	Activity (Units mL^{-1})			Total Activity (Units)	% Enzyme eluted
Enzyme loaded	5 mL	2.88			14.4	-
Effluent	5 mL	0.00			0.00	0
Buffer wash	3 x 5 mL	0.00	0.00	0.00	0.00	0
NaCl eluate	3 x 5 mL	1.93	0.55	0.00	12.4	86

Table 4.5 CM-Sepharose at pH 5.5

Fraction	Volume	Activity (Units mL^{-1})			Total Activity (Units)	% Enzyme eluted
Enzyme loaded	5 mL	2.48			12.40	-
Effluent	5 mL	0.98			4.9	61
Buffer wash	3 x 5 mL	1.36	0.13	0.00	7.45	0
NaCl eluate	3 x 5 mL	0.00	0.00	0.00	0.00	0

For experimental details see section 2.6.5

It can be clearly seen that the enzyme bound to the DEAE-Sepharose column at pH 8.5 but did not bind to the CM-Sepharose column at pH 5.5. Approximately 14% of the enzyme activity could not be eluted from the DEAE column even in 2 M NaCl. It was assumed that this activity had been denatured through non-specific binding to either the Sepharose matrix or DEAE groups or precipitation on the column.

A column was prepared using 40 mL of DEAE-Sepharose and esterase activity was eluted using a linear 0 - 1.0 M NaCl gradient over 10 column volumes. The resolution of the column was very poor (the adsorbed protein was only resolved into two distinct peaks) and the purification factor increased by only 2 fold. Consequently, this step was suspended in favour of hydrophobic interaction chromatography.

4.2.2 Hydrophobic interaction chromatography (HIC)

Introduction

Hydrophobic interaction chromatography (HIC) developed as a consequence of affinity chromatography. Whereas relatively few proteins bind to short immobilised aliphatic chains at low salt concentrations, HIC can be extended to cover all proteins, since hydrophobic interactions increase in strength with increasing salt concentration.

Once the protein of interest has been adsorbed to a column the protein may be eluted using a number of different procedures: decreasing the ionic strength in the column, decreasing the temperature, by the inclusion of an organic solvent in the elution buffer, by the inclusion of polyols (especially ethylene glycol) in the elution buffer, using non ionic detergents such as Tween 80 and Triton 100 and lowering the pH.

Results

Samples from the PEG 6000 precipitation trial were loaded onto phenyl-Sepharose and octyl-Sepharose mini columns equilibrated with 50 mM Tris-HCl, pH 7.2. The Tok19 A1 esterase did not bind to the octyl-Sepharose column and only approximately 80% bound to the phenyl-Sepharose column. The binding capacity of 20 mg per mL of gel (100 mg per 5 mL) was not exceeded. The salt (NaCl) concentration was increased to 1M NaCl to increase the hydrophobic interactions. However, when the PEG 6000 sample was made to 1M NaCl proteins visibly precipitated. The sample was centrifuged and approximately 97% of the initial esterase activity remained in the supernatant. The specific activity increased from 2.8 to 3.4 Units mg^{-1}. Consequently NaCl was added to the PEG 6000 sample to a concentration of 1M. At 1M NaCl, all the esterase bound to the phenyl-Sepharose column. Under similar conditions the esterase would not bind to the octyl-Sepharose column.

Approximately 20 % of the esterase activity was eluted from the phenyl-Sepharose column with a decreasing salt gradient from 1 M NaCl to 0 M NaCl.

Methanol (1-10 % v/v) in 50 mM Tris-HCl pH 7.2 was used to elute the remaining esterase but protein precipitated on the column and only 10 % of the initial esterase activity was recovered.

Various concentrations of ethylene glycol (20-80% v/v) in 50 mM Tris-HCl pH 7.2 were used to elute the esterase. No esterase was eluted at 20 and 40 % ethylene glycol. Approximately 60% of the total esterase activity was eluted with 60% ethylene glycol. No further esterase could be eluted even when the ethylene glycol concentration was increased to 80%.

To improve the efficiency of purification the esterase was eluted in step-wise manner. The highest esterase specific activity (~70 Units mg^{-1}) was achieved when the esterase

was loaded onto the phenyl-Sepharose in 1 M NaCl and eluted as follows (see Table 4.6):

Step 1. 50 mM Tris-HCl pH 7.2 / 1 M NaCl

Step 2. 50 mM Tris-HCl pH 7.2

Step 3. 50 mM Tris-HCl pH 7.2 / 40 % ethylene glycol (v/v)

Step 4. 50 mM Tris-HCl pH 7.2 / 60 % ethylene glycol (v/v)

Table 4.6 Stepwise elution of the Tok19 A1 esterase from the phenyl-Sepharose column

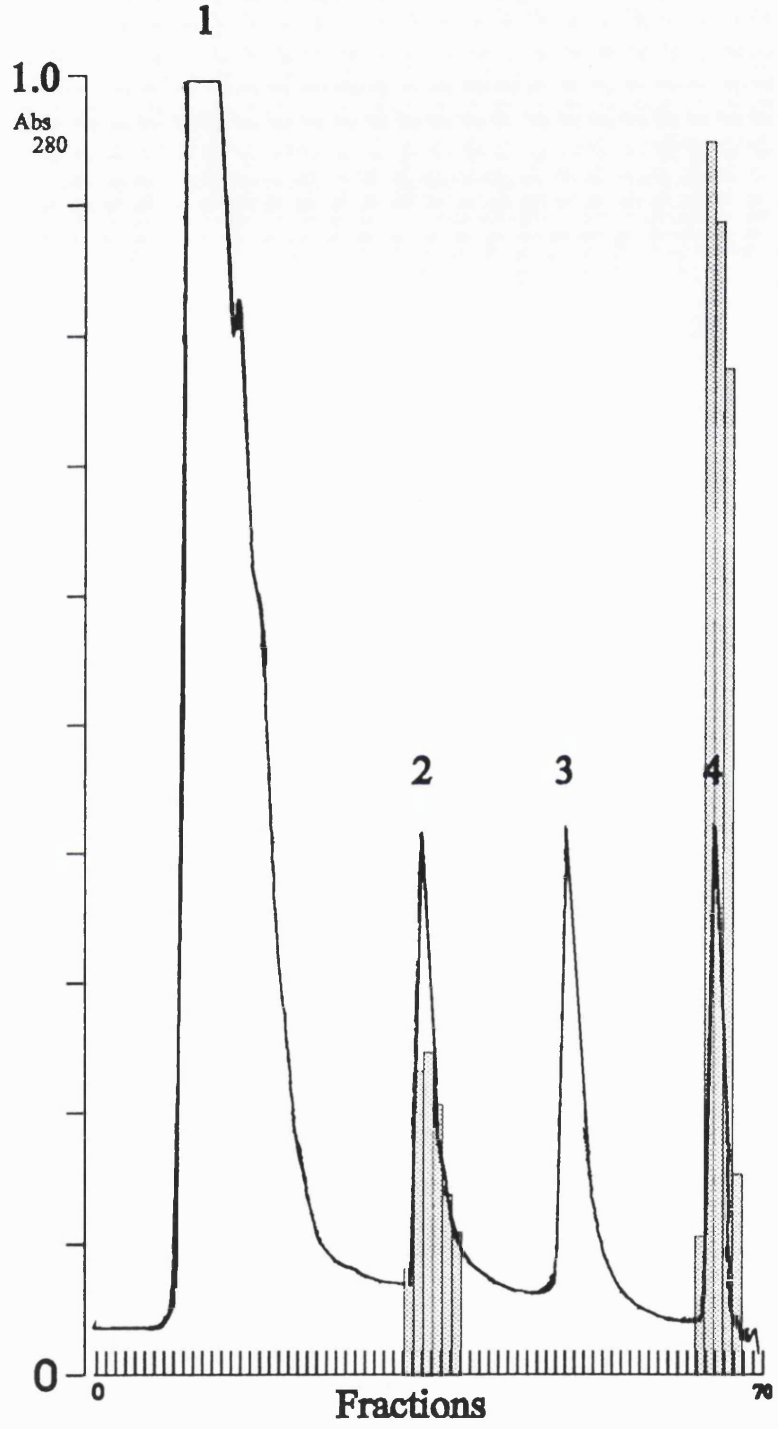
Fraction	Volume	Activity (Units mL^{-1})			Total Activity (Units)	Total Protein (mg)	Specific Activity (Units mg^{-1})
Enzyme loaded	5 mL	3.36			16.8	12.7	1.32
Effluent	5 mL	0.00			0.00	8.00	-
Step 1	3 x 5 mL	0.00	0.00	0.00	0.00	1.17	-
Step 2	3 x 5 mL	0.81	0.00	0.00	4.05	1.63	2.49
Step 3.	3 x 5 mL	0.00	0.00	0.00	0.00	1.75	-
Step 4.	3 x 5 mL	1.91	0.18	0.00	10.45	0.15	69.67

For experimental details see section 2.6.5

Approximately 20 % of the total esterase activity was eluted in the second buffer wash, 50 mM Tris-HCl pH 7.2, and a further 60% was eluted in buffer wash 4, 50 mM Tris-HCl pH 7.2 / 60% ethylene glycol (v/v). For large scale purification, a 30 cm (l) by 5 cm (d) Pharmacia column containing 300 mL of phenyl-Sepharose was used. A typical U.V. absorbance trace from a phenyl-Sepharose column is shown in Figure 4.4.

Figure 4.4 U.V. absorbance trace from a typical phenyl-Sepharose column.

Shaded areas indicate esterase distribution.



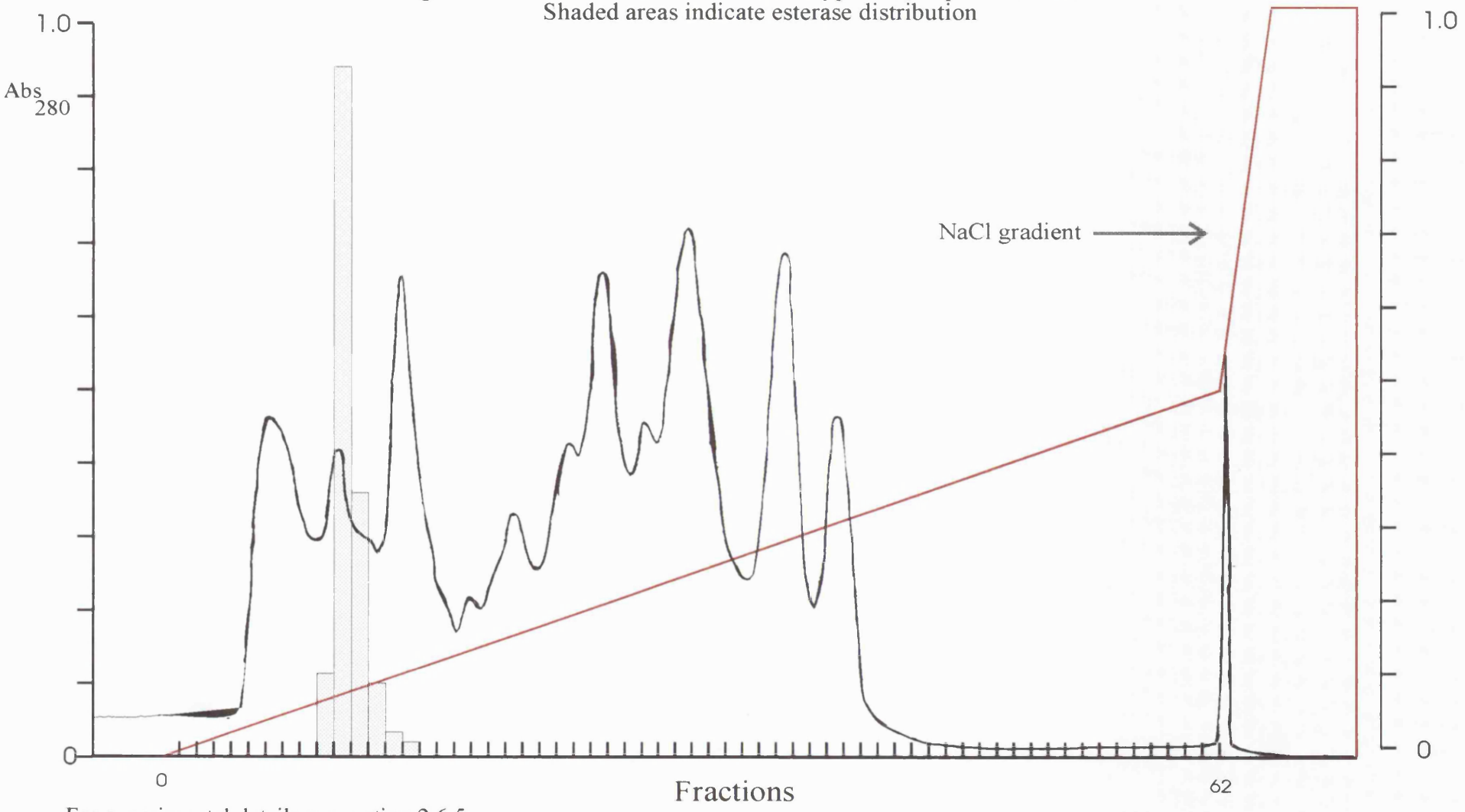
For peaks 1 (load and wash) ,2 (50 mM Tris-HCl pH 7.2 wash) and 3 (50 mM Tris-HCl pH 7.2 / 40% ethylene glycol wash) the full scale deflection (FSD) was set to 2.0, for peak 4 (50 mM Tris-HCl pH 7.2 / 60% ethylene glycol wash) the FSD was set to 0.1. Only fractions 63 to 68 were routinely collected. Shaded regions represent esterase activity

The Tok19 A1 isolate was subsequently shown to contain 5 esterase activities on native PAGE when stained with indoxyl acetate: 3 low mobility esterases and two high mobility esterases (Section 4.5.1). The 20% esterase activity eluted from the column in step 2 contained the two high-mobility esterases and the 60% esterase activity eluted in step 4 contained the three low-mobility esterases.

4.2.3 Q-Sepharose chromatography

As it was previously shown that the esterase activity bound effectively to DEAE-Sepharose (Table 4.4) a pre-packed Q-Sepharose column was used for ion-exchange chromatography. Esterase activity would not bind to the Q-Sepharose column at an ethylene glycol concentration greater than 15 % v/v. The resolution from the Q-Sepharose column far exceeded that from the DEAE-Sepharose column (Figure 4.5). The esterase activity was typically eluted in a 48 mL volume at a salt concentration of 0.10 M NaCl. The specific activity of the eluted esterase was typically 300 Units mL^{-1} which corresponded to a purification factor of approximately 1000.

Figure 4.5 U.V. absorbance trace from a typical Q-Sepharose column.
Shaded areas indicate esterase distribution



For experimental details see section 2.6.5.

4.2.4 Gel filtration chromatography (Superdex 200)

Introduction

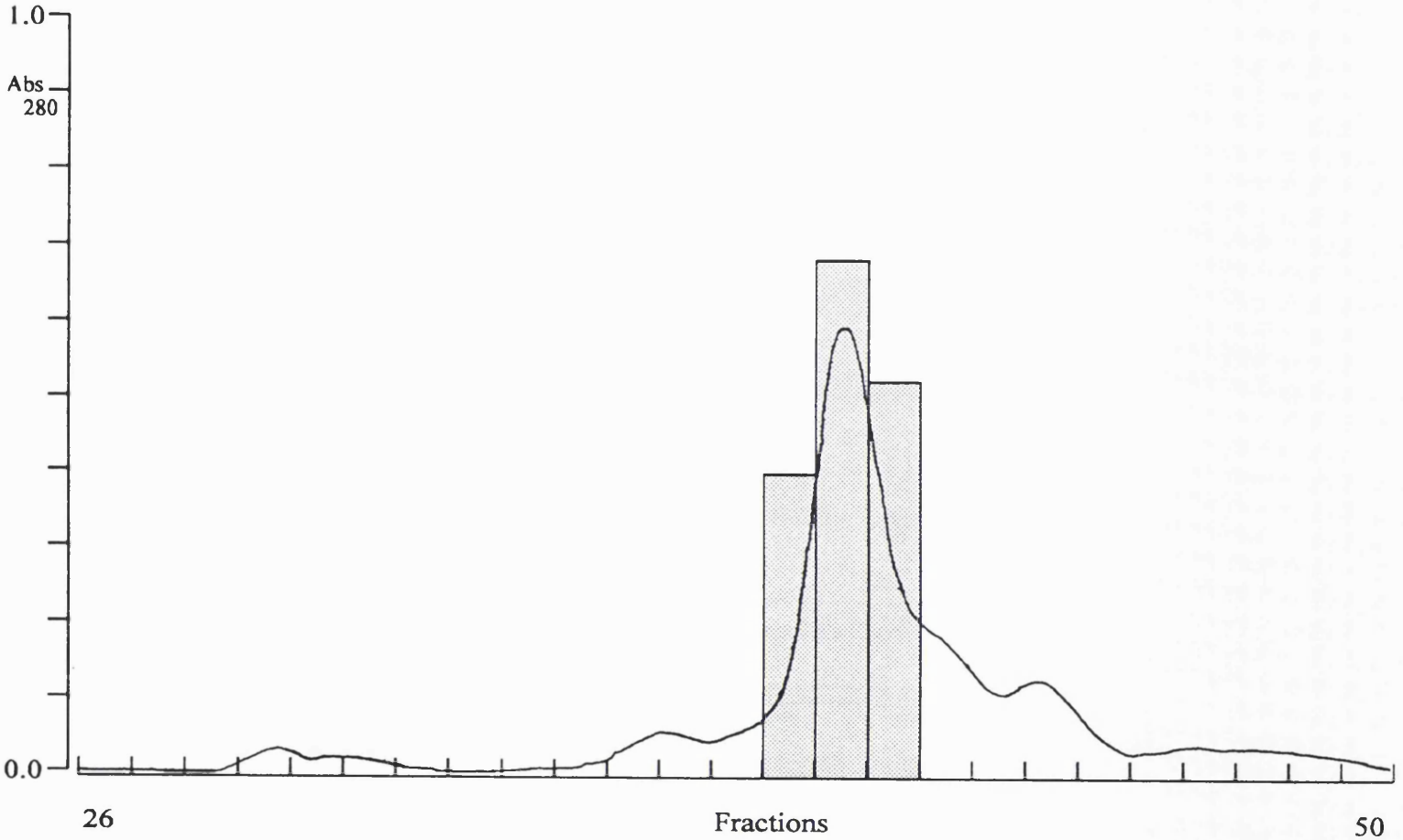
Gel filtration is one of the principle techniques used in protein purification. Superdex gels can be used for both the rapid separation of proteins and for molecular weight determination.

Results

Both Superdex 200 and Superdex 75 columns were used to purify the esterase. A U.V. trace from a typical Superdex 200 column is shown in Figure 4.6. Only the elution from 52 to 100 mL of the 140 mL is shown, the first 40 mL corresponding to the void volume of the column. 2 mL fractions were collected and the esterase was typically eluted in a single peak over 6 mL with a retention volume of approximately 86 mL. In some instances (as in Figure 4.6) the esterase peak had a shoulder and a second gel filtration step was required to remove a minor contamination (see Lane 5 Figure 4.8).

When a second gel filtration step was required, active fractions from the Superdex 200 column were pooled (6 mL), reduced to 200 μL using an Amicon Centricon 10 Microcentor, and loaded onto a Superdex 75 column. The esterase activity was eluted from the Superdex 75 column as a single peak over 1 mL at an elution volume of 10 mL ± 0.2 mL (Figure 4.7).

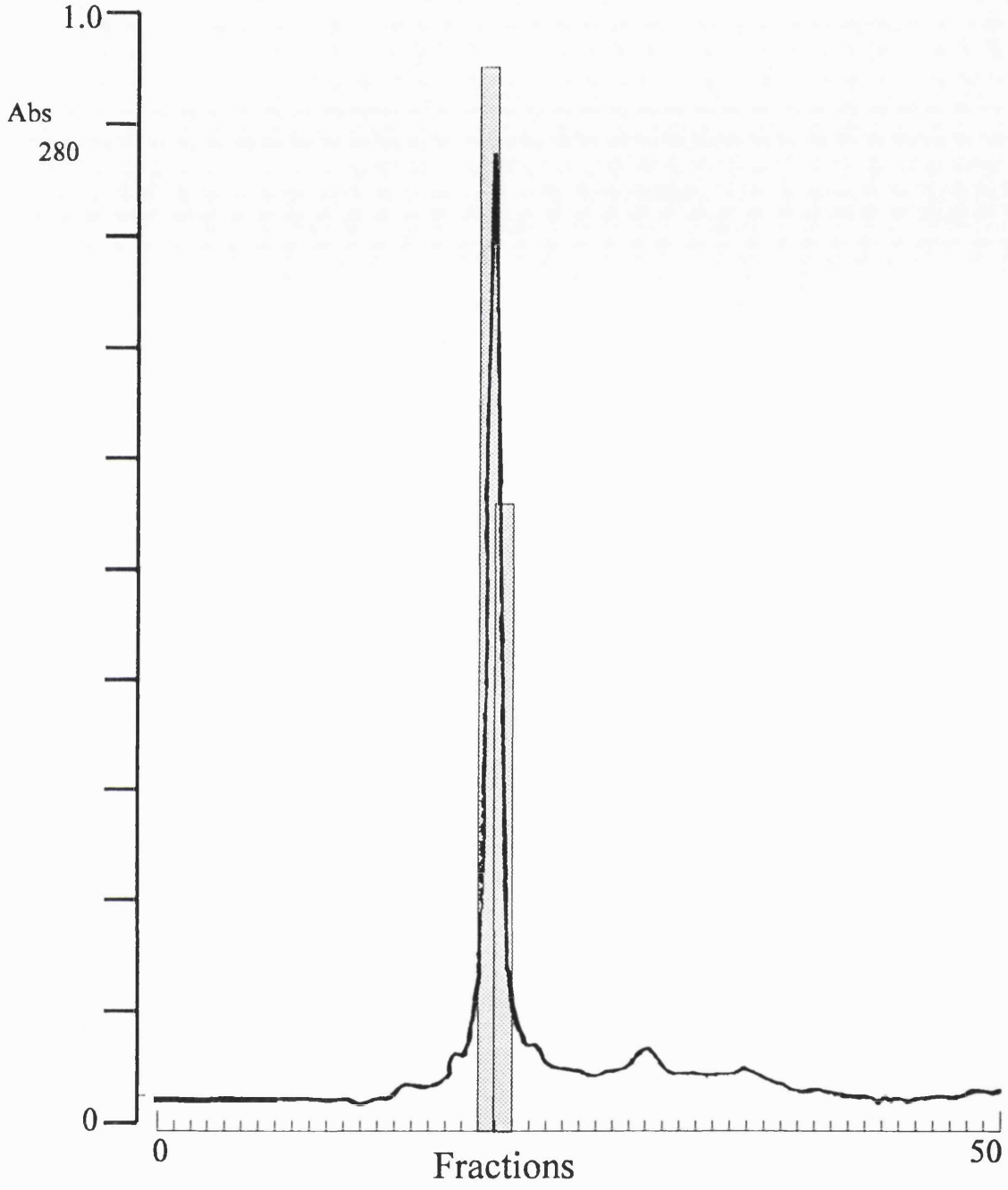
Figure 4.6 U.V. absorbance trace from a Superdex 200 column.
Shaded areas indicate esterase distribution.



For experimental details see section 2.6.5

Figure 4.7 U.V. absorbance trace from a typical Superdex 75 column.

Shaded areas indicate esterase distribution



For experimental details see section 2.6.5

4.3 Summary of the purification protocol

A typical purification protocol, using the optimised steps reported above, is as follows. Cells from 12 L of culture (see section 2.6.1) were harvested in stationary phase and washed (50 mM Tris-HCl pH 7.2) using a microfiltration membrane (Life Sciences Laboratory 0.22 μm filter) and peristaltic pump. A final cell yield of 0.3g wet weight of cells mL^{-1} was typically obtained. The cells were lysed by the addition of 0.1 mg mL^{-1} lysozyme (incubated at 37°C for 30 minutes) after which 30 mL aliquots were sonicated for 30 seconds to reduce the viscosity. The resulting homogenate was centrifuged (48,000*g* for 30 minutes) to remove cell debris and the cell-free supernatant retained.

Polyethylene glycol (PEG) 6000 was added to the supernatant to a final concentration of 12% w/v (from a stock solution of 60 % w/v), and NaCl was added to a final concentration of 1 M. The supernatant was centrifuged (48,000*g* for 20 minutes) and the pellet discarded.

Hydrophobic interaction chromatography was performed in a 5 cm (d) x 30 cm (l) Pharmacia glass column containing 300 mL of Phenyl-Sepharose using the BioRad EconoChromatography system. The Phenyl-Sepharose column was equilibrated with 50 mM Tris-HCl pH 7.2 containing 1 M NaCl. A maximum of 300 mL of supernatant was loaded onto the column at a flow rate of 5 mL min^{-1}. The column was washed with 2 column volumes of 50 mM Tris-HCl pH 7.2 containing 1 M NaCl. The column was then washed successively with 2 column volumes of 50 mM Tris-HCl pH 7.2 and 50 mM Tris-HCl pH 7.2 containing 40% ethylene glycol (v/v) until no protein could be detected (at 280 nm) in the eluate. The column was finally washed with 50 mM Tris-HCl pH 7.2 containing 60% ethylene glycol (v/v) and the total activity peak was collected.

The 60% ethylene glycol peak was diluted to less than 15% (v/v) ethylene glycol with 50 mM Tris-HCl pH 7.2 and loaded onto a prepacked Pharmacia HiLoad 26/10 Q-Sepharose HP column equilibrated with 200 mL of 50 mM Tris-HCl pH 7.2. The column was washed with 3 column volumes of 50 mM Tris-HCl pH 7.2 at 5 mL min^{-1} and the enzyme eluted with a 10 column volume linear gradient (0-0.5M) of NaCl in 50 mM Tris-HCl pH 7.2. The esterase activity peak (48 mL) was collected and the volume reduced to ~2mL using either an Amicon ultrafiltration cell with a YM 30 membrane or an Amicon Centriprep 30.

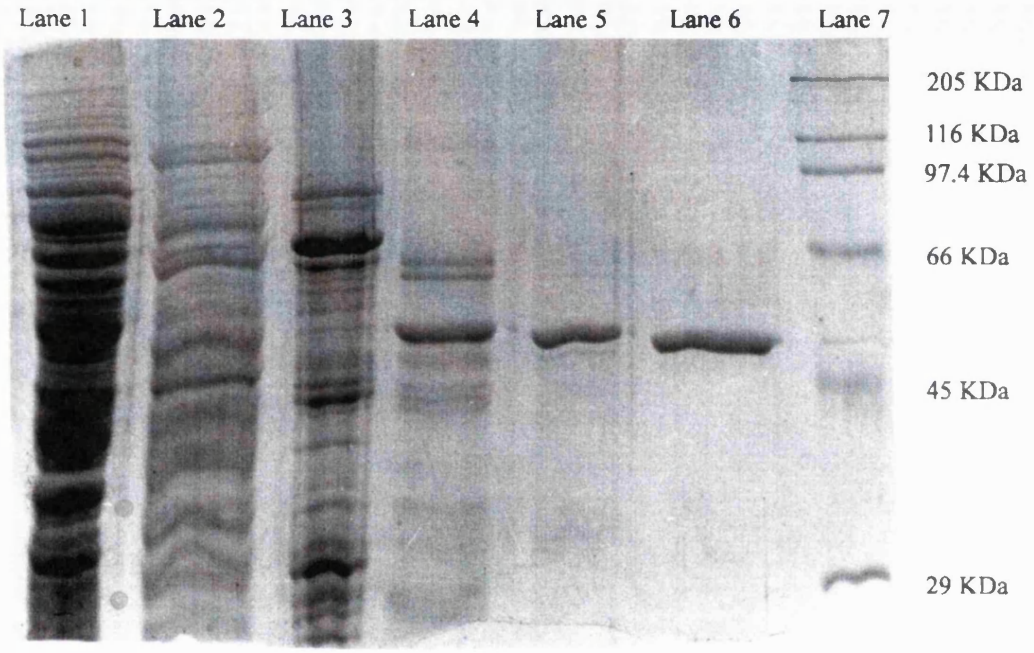
Gel filtration chromatography was performed using the Pharmacia FPLC system and a Pharmacia HiLoad Superdex 200 column. The column was equilibrated with 2 column volumes of 50 mM Tris-HCl pH 7.2 and 2 mL of enzyme were loaded. The enzyme was eluted with 50 mM Tris-HCl pH 7.2 at a flow rate of 0.2 mL min^{-1}. In some instances a second gel filtration step was performed to remove a minor contamination (Figure 4.8. Lane 5). The fractions from Superdex 200 containing esterase activity were pooled (6 mL) and reduced to 200 µL using an Amicon Centricon 10 Microcentor. Gel filtration was performed again using the Pharmacia FPLC system and Pharmacia Superdex 75 column. The column was equilibrated with 2 column volumes of 50 mM Tris -HCl pH 7.2. 200 µL of enzyme were loaded and the esterase was eluted with 50 mM Tris-HCl pH 7.2 at a flow rate of 0.5 mL min^{-1}. 0.5 mL fractions were collected.

Samples were retained from all the steps in the protocol. Activity and protein content was measured in each case and the samples were electrophoresed on SDS PAGE (Figure 4.8).

The purification results are summarised in Table 4.7. Esterase activity from Tok19 A1 was typically purified 5133-fold to electrophoretic homogeneity with 26 % recovery of

the total esterase (Table 4.7). The purified esterase had a specific activity of 1540 units mg^{-1}, based on the hydrolysis of *p*-NP propionate at pH 7.0 and 30°C.

Figure 4.8 SDS PAGE



Lane 1, Cell free extract; Lane 2, 12% PEG 6000 (w/v) and 1 M NaCl precipitation; Lane 3, Phenyl-Sepharose; Lane 4, Q-Sepharose; Lane 5, Superdex 200; Lane 6, Superdex 75; Lane 7, SDS Molecular weight markers

Table 4.7 Purification protocol for Tok19 A1 carboxylesterase

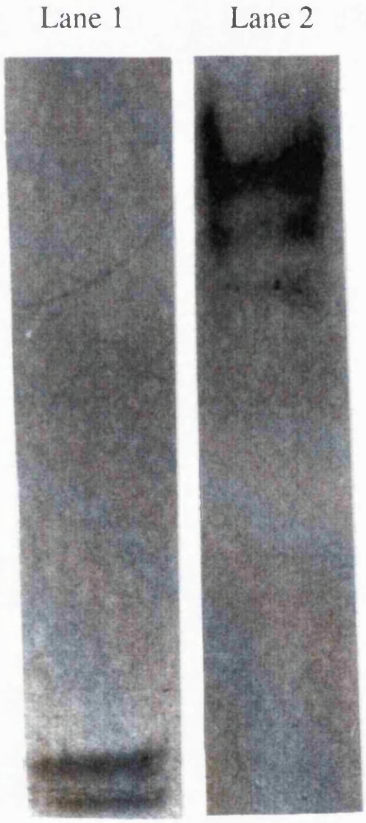
Procedure	Volume (mL)	Activity (Units mL^{-1})	Protein (mg mL^{-1})	Total Activity (Units)	Specific Activity (Units mg^{-1})	Yield %	Purification Factor
Crude	280	3.0	10.0	840	0.30	100	1
12% PEG/1M NaCl	340	2.3	5.1	782	0.45	93	1.5
Phenyl-Sepharose	56	12.4	0.5	694	24.8	83	83
Q-Sepharose	48	11.2	0.038	538	295	64	983
Superdex 200	4	53.9	0.035	216	1540	26	5133
Superdex 75	1	162	0.105	162	1543	19	5143

For details see the experimental section.

4.4 Analysis of the different forms of the esterase

Native PAGE electrophoresis of the esterase peaks from phenyl-Sepharose, indicated that three low mobility esterase bands had been separated from two high mobility esterase bands (Figure 4.9). The relative intensities of the three low mobility bands remained constant throughout the purification protocol. The two high-mobility esterases had low specific activities and narrow substrate specificities and were discarded.

Figure 4.9 Separation of the high and low-mobility Tok19 A1 esterases.



Lane 1, High mobility esterases; Lane 2, Low mobility esterases.

For experimental details see section 2.3.3.

4.4.1 Analysis of the esterases during the growth cycle of the Tok19 A1 strain

10 mL samples of actively growing cultures were removed periodically throughout the Tok19 A1 growth cycle and the crude cell extract electrophoresed on native PAGE. The samples showed that the five esterases were not apparently growth-phase dependant and were expressed equally during the exponential and stationary phases (data not shown).

4.4.2 Esterase heterogeneity

Multiple forms of carboxylesterase with different chromatographic, electrophoretic, or catalytic properties occur in many micro-organisms (Bott, 1971; Matsunaga *et al.*, 1974; Krisch, 1971; Barker and Jencks, 1969; Owusu and Cowan, 1989). In some cases this has been attributed to the dissociation of an active high-molecular-weight esterase complex into low-molecular-weight active monomers (Owusu and Cowan, 1989; Greenzaid and Jencks, 1971). For example, it was observed that a crude mesophilic esterase sample incubated at 23°C for between 5 minutes and 22 hours progressively dissociated into a faster moving band, presumably an active monomer (Greenzaid and Jencks, 1971). However, when a sample of Tok19 A1 crude cell extract was incubated at 70°C for 45 minutes there was no change in the electrophoretic properties of the bands (data not shown).

It is unlikely that the three low-mobility esterases are products of chemical or proteolytic degradation since the electrophoretic behaviour and relative proportions were consistent under a variety of different preparation procedures, and from different stages of the purification sequence. Heterogeneous association with non-enzymic components (other proteins, lipids etc.) is also unlikely since relative mobilities are independent of the level

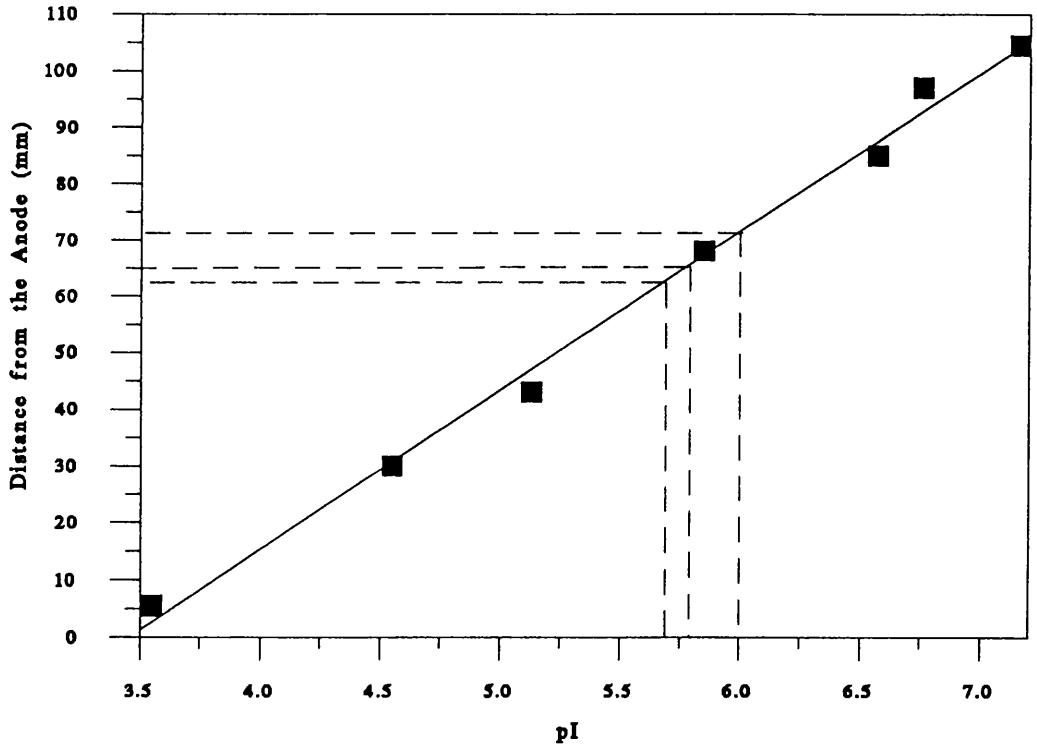
of purification. Homogeneous association is also unlikely because the protein concentration in native PAGE analysis did not have any influence on the electrophoretic behaviour of the bands and was 40 fold lower than in gel filtration chromatography (where no dimers or multimers were observed). Furthermore, no degeneracy was observed for any amino acid in the first 40 residues of the N-terminal sequence (see section 5.0). It is therefore suggested that these three low mobility esterases are highly related carboxylesterases with very slightly differing amino acid compositions. It thus seems likely that the three active esterase bands observed, in the purified sample, are discrete but highly homologous polypeptides (i.e., isozymes or isoforms) although the possibility of post-translational modifications cannot be excluded.

4.5 Isoelectric Point Determination of the Esterases

The isoelectric points of the three low mobility esterases from the electrophoretically pure enzyme preparation were determined on the Pharmacia Phast System.

The relative distances of the three esterases from the anode were 62.5 mm, 65 mm and 71 mm, corresponding to isoelectric points of 5.7,5.8 and 6.0 respectively. The standard curve and the relative mobilities of the three esterases are shown in Figure 4.10.

Figure 4.10 The standard curve for the pH 3.0-7.5 isoelectric focusing gel and the relative mobilities of the three esterases.



For details see the experimental section 2.7.3

4.6 Separation of the three esterases

It was previously demonstrated that the three low mobility Tok19 A1 esterases could not be separated on the basis of size since they were eluted in a single peak from both a Superdex 200 and 75 gel filtration columns. Three further methods of separating the esterases on the basis of differences in their charge characteristics were attempted.

Mono Q chromatography

The esterases were eluted in a single peak from the Q-Sepharose column (section 4.2.4). Samples of purified esterase, at pHs between 5.5 and 7.5, were applied to a Pharmacia HR 5/5 Mono Q column, in an attempt to selectively bind one or two of the three esterases while eluting the remaining esterase/s with the buffer wash. No esterase bound to the column until the pH of the sample was increased to 7.0. At pH 7.0 and above 100% of the esterase activity bound to the column and the esterase was eluted in a single peak.

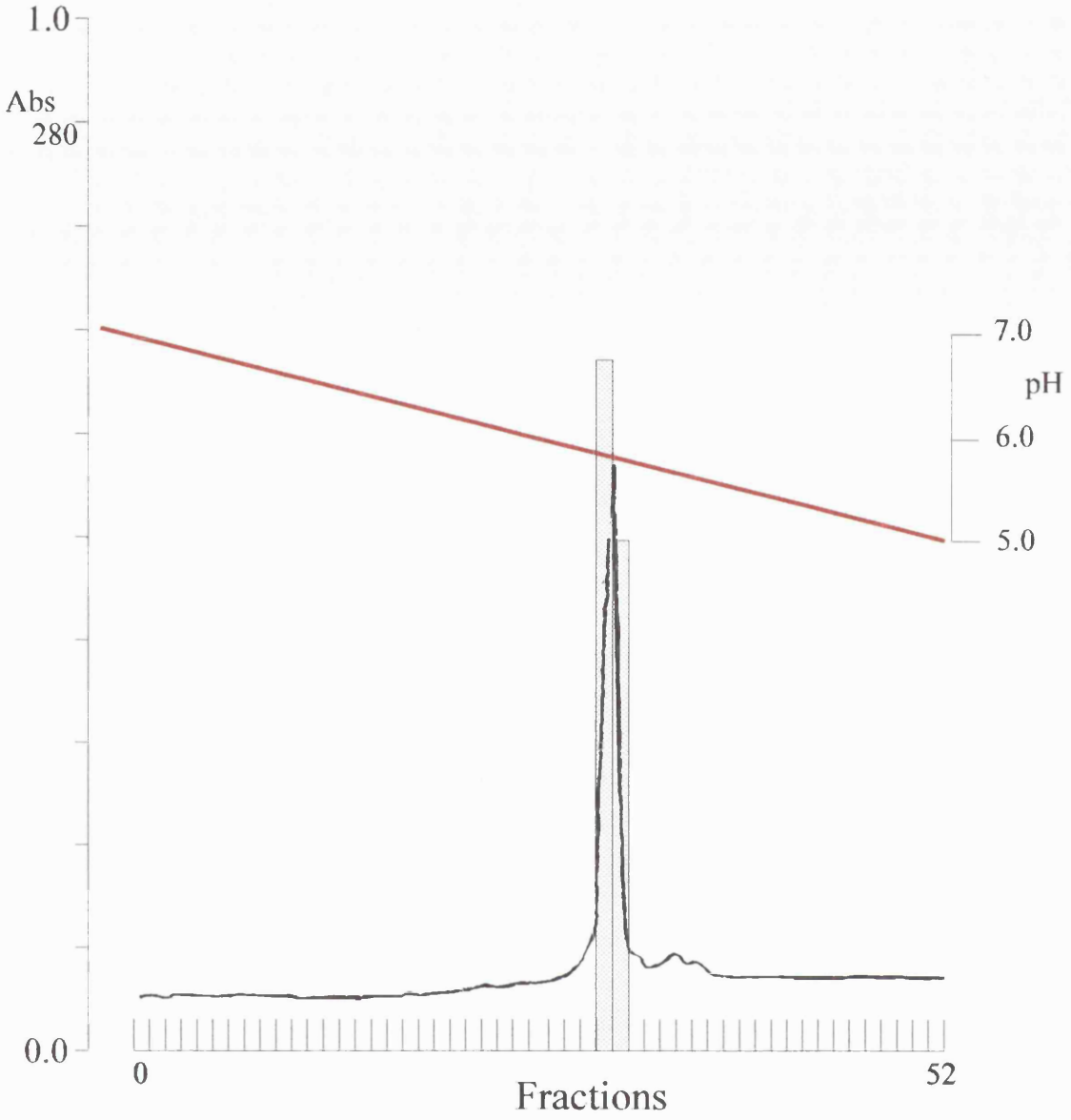
Mono P chromatography (chromatofocusing)

Chromatofocusing using a Mono P column is claimed to be highly selective (i.e. being able to separate biomolecules with pI differences of less than 0.02 pH units (Pharmacia technical bulletin)).

100 µL of esterase (15 Units ~ 10 µg protein) was loaded onto the Mono P column equilibrated with 25 mM bis-Tris, pH 7.1, HCl and eluted using Polybuffer 74, pH 5.0, HCl. The esterase was eluted in a single peak over 1.0 mL with an isoelectric point corresponding to 5.8. The U.V. absorbance trace from the column is shown in Figure 4.11.

Figure 4.11 Mono P chromatography

Shaded areas indicate esterase distribution



For experimental details see section 2.7.3.

It is not clear why the Mono P column did not separate the three esterases (it is possible that the esterases have strong homogeneous attractions at or near their pI values, thus forming aggregates which were eluted as a single peak).

Gel elution from native PAGE

100 units of esterase were loaded onto a native gel and electrophoresed. Due to the similar relative mobilities of the three esterases the gel was electrophoressed for 12 hours before staining with indoxyl acetate and excision of the bands from the gel. The excised gel fragments were placed in a GE 200 SixPac Gel Eluter and eluted into 4X TAE (Tris-acetic acid-EDTA buffer) rather than 4X Laemmli buffer (Laemmli buffer contains glycine and has been shown to affect the *p*-NP propionate assay). Less than 1% of the total esterase activity could be eluted from the gel. It was thought that staining the gel with indoxyl acetate may have inhibited the esterases. A second gel was briefly stained with Coomasie blue R 250 the bands were excised and rapidly destained (5% methanol v/v: 5% acetic acid v/v: 90% water v/v). The yield of activity after elution was again very low. To assess the possibility that Coomassie Blue might have bound to the proteins reducing their yields or that the destaining may have denatured the enzyme, two esterase samples were electrophoresed side by side on a native gel. The reference lane was excised and stained, then realigned with the rest of the gel in order to locate the esterase bands. The unstained bands were excised and the esterase eluted. However, yields of esterase activity were less than 1% of the total. The low yields of enzyme prevented further investigation

Summary

Because of the similarity of all the properties of these esterases (their co-elution from 4 different types of chromatography columns) and bearing in mind the objective of using these enzymes as an industrial catalyst, this separation may well be considered non cost-effective. Thus further studies on this esterase activity were conducted using the purified Tok19 A1 esterase preparation containing the three forms.

Chapter 5

Characterisation

This chapter details the characterisation of the Tok19 A1 esterase with respect to structural and functional properties.

5.0 N-Terminal sequence and the sequence alignment of the Tok19 A1 esterase

40 amino acids at the N-terminus of the Tok19 A1 esterase were sequenced (see section 2.7.4). The sequence was aligned with over 25,000 N-terminal regions of bacterial and eukaryotic esterases from the SWISS-FASTA library and 60 sequences with the highest statistical scores were recorded. These 60 sequences had a sequence identity of over 25 % with the Tok19 A1 esterase.

The recorded sequences showed highly variable degrees of homology (Table 5.1). For example, 50% sequence identity was observed with residues 8-51 of bovine acetylcholinesterase (Doctor *et al.*, 1990) and residues 38-84 of human acetylcholinesterase (Soreq *et al.*, 1990). Percent similarity (% similarity includes both exact matches and the replacement of an amino acid with a similar one) for these alignments was greater than 70%. It was notable that residues 10-12 (LRG), 23-25 (GIP) and 34-40 (RRFLPPE) of the Tok19 A1 esterase sequence showed very high similarity (often 100%) to the human (Soreq *et al.*, 1990) and bovine (Doctor *et al.*, 1990) esterases, esterase-6 from *Drosophila* (Oakeshott *et al.*, 1987), yeast *Candida cylindracea* lipase (Kawaguchi *et al.*, 1989) and a number of other eukaryotic esterases (Rachinsky *et al.*, 1990; Ozols, 1989; Takagi *et al.*, 1988) (Figure 5.1.). Several amino acids appeared to be conserved throughout all the sequences.

Figure 5.1 Sequence alignment of the Tok19 A1 esterase

```
                1                                                  50                                                  100
Tok19 A1 EST    ..........  ..........  ..........  .......MI VETRYGRLRG ..V....VNG GVFVWKGIPY AKAPVGKRRF LPPE
ACES-HUMAN      MRPPQCLLHT PSLASPLLLL LLWLLGGGVG AEGREDAELL VTVRGGRLRG ..IRLKTPGG PVSAFLGIPF AEPPMGPRRF LPPEP.KQPW SGVVDATTFQ
ACES-BOVINE     ..........  ..........  ..........  .EGPEDPELL VMVRGGELRG ..LRLMAPRG PVSAFLGIPF AEPPVGPRRF LPPEP.KRPW PGVLNATAFQ
ACES-MOUSE      MRPPWYPLHT PSLAFPLLFL LLSLLGGGAR AEGREDPQLL VRVRGGELRG ..IRLKAPGG PVSAFLGIPF AEPPVGSRRF MPPEP.KRPW SGVLDATTFQ
EST2-RABBIT     ..........  ..........  ..........  ...ZDSASPI RNTHTGQVRG SLVHVEGTDA GVHTFLGIPF AKPPLGPLRF APPEP.AEAW SGVRDGTSLP
EST1-RAT        ..........  .....MWLCA LVWASLAVCP IWGHPSSPPV VDTTKGKVLG KYVSLEGFTQ PVAVFLGVPF AKPPLGSLRF APPEP.AEPW SFVKNTTTYP
DM.6 EST        ..........  ..........  ..........  ..SDTDDPLL VQLPQGKLRG ...RDNGSYY SYESYESIPY AEPPTGDLRF EAPEPTKQKW SDIFDATKTP
C.c. LIPASE     ..........  ..........  ..........  ......APTA TLANGDTITG LNAIINE... ...EFLGIPF AEPPVGNLRF KDPVPYSGSL DG.QKFTLYG
Conserved amino acids                                              G                             A PP G
```

The sequence alignment was calculated using Pileup (GCG) (Feng and Doolittle 1987). Underlining indicates sequences of very high homology.

Table 5.1. Sequence identity with 40 N-terminal amino acids of Tok19 A1 esterase.

Sequence	ACES HUMAN	ACES BOVINE	ACES MOUSE	EST-2 RABBIT	D.M.-6 ESTERASE	EST-1 RAT	C. c. LIPASE	P. f. LIPASE	A. c. ESTERASE	B.a ESTERASE	S. a. ESTERASE
% Identity to Tok19 A1 Esterase	50	50	47.7	43.5	42.5	39.1	26.1	18	12	26.6	7

The percentage identity was calculated using Pileup (GCG) (Feng and Doolittle, 1987). Tok19 A1 ESTERASE, esterase from Tok19 A1; ACES-HUMAN (Soreq *et al.*, 1990), human acetylcholinesterase; ACES-BOVINE (Doctor *et al.*, 1990), bovine acetylcholinesterase from fetal serum; ACES-MOUSE (Rachinsky *et al.*, 1990), mouse acetylcholinesterase; EST2-RABBIT (Ozols, 1989), rabbit liver esterase 2; EST1-RAT (Takagi *et al.*, 1988), rat liver esterase 1; DM 6 ESTERASE (Oakeshott *et al.*, 1987) , esterase-6, from *Drosophila*; C. c. LIPASE (Kawaguchi *et al.*, 1989), asporogenic yeast *Candida cylindracea* lipase; P.f LIPASE (Kugimiya *et al.*, 1986), *Pseudomonas fragi* lipase, A. c. ESTERASE (Reddy *et al.*, 1989), *Acinetobacter calcoaceticus*. B. a. ESTERASE (Manco *et al.*, 1994), *Bacillus acidocaldarius* and S. a. ESTERASE (Sobek and Gorisch, 1989), *Sulfolobus acidocaldarius*. It should be noted that the alignment with the *Sulfolobus acidocaldarius* and *Bacillus acidocaldarius* sequences do not extend to the high homology region at positions 34-40.

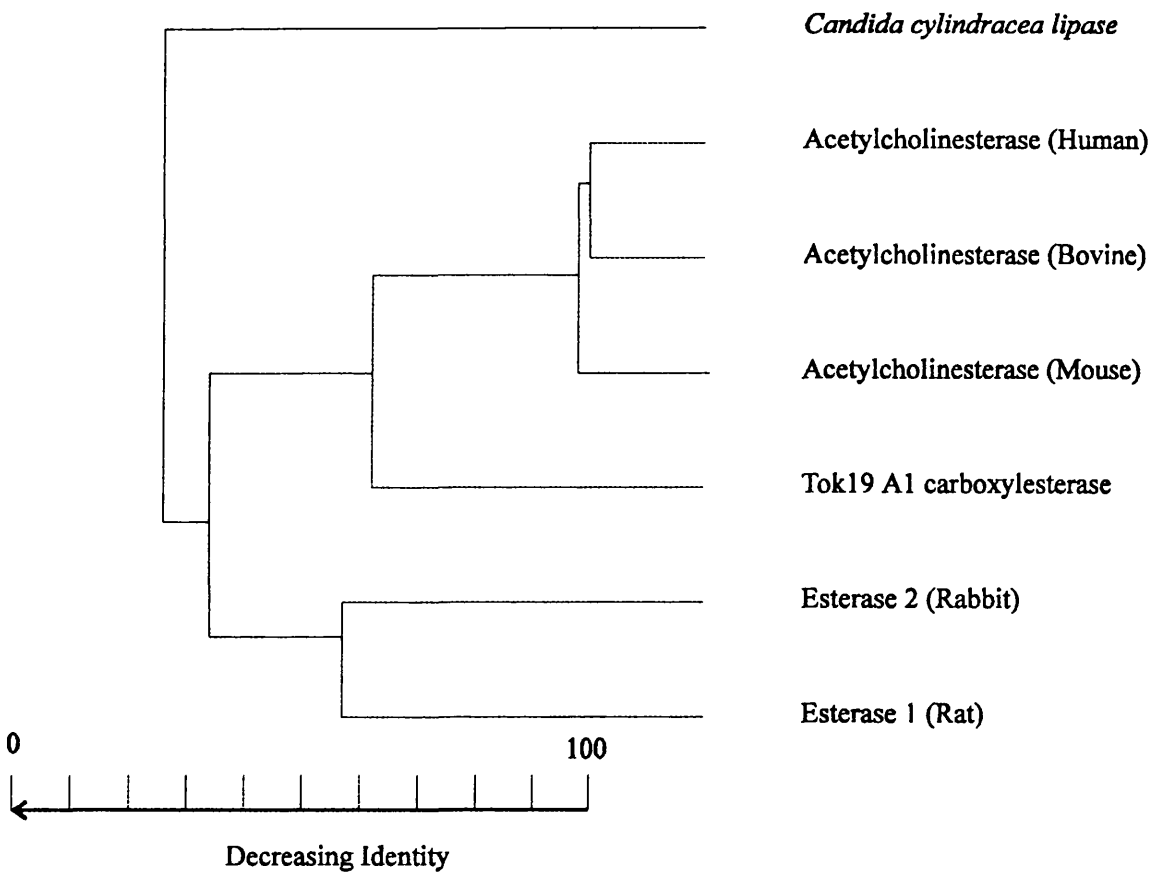
The Tok19 A1 esterase appears to have a higher sequence identity to the mammalian esterases than the bacterial esterases and lipases, possibly suggesting a common molecular ancestry (note that the majority of sequences extracted from the data base were from eukaryotic sources). The Tok19 A1 esterase sequence was also compared with several microbial lipase and esterase sequences, e.g. *Pseudomonas fragi* lipase (Kugimiya *et al.*, 1986), *Acinetobacter calcoaceticus* esterase (Reddy *et al.*, 1989), 18 amino acids of the esterase from *Sulfolobus acidocaldarius* (Sobek and Gorisch, 1989) and 19 amino acids of the carboxyl esterase from *Bacillus acidocaldarius* (Manco *et al.*, 1994). Sequence identity with the N-terminal regions of these microbial esterases was generally much lower than the eukaryotic sequences (Table 5.1).

A phylogenetic tree (Figure 5.2) was generated using PILEUP GCG (Feng and Doolittle, 1987). The sequences used to generate the tree were those with the highest statistical score, while the *Candida cylindracea* lipase was included as a "negative control" (*C. cylindracea* lipase has a 26.1 identity with the Tok19 A1 esterase). It is difficult to speculate as to the genetic origin of the Tok19 A1 esterase as the majority of its sequence is unknown. However it could be argued that the similarity with the eukaryotic esterases is due to functional identity and/or convergent evolution.

Arguments that thermophiles evolved from mesophilic species and vice versa have been presented (Brock, 1967). It is now generally accepted that when prokaryotic type cells arose (during the Archean era 3.5-2.9 x 10^9 years ago) water temperatures on earth were cool to warm but not hot (Brock, 1967). Thus there is no need to assume a primary origin for the thermophiles even though thermal environments existed. (Zeikus, 1979). However, primaeval organisms were probably more similar to one of the three phenotypic groups of the archaea, most of which are thermophilic or hyperthermophilic. It is thus likely that the primaeval organism was thermophilic, from which bacterial and

eukaryotic cell types evolved and adapted to the more extensive mesophilic niches. Moderately thermophilic bacteria such as *Bacillus* and *Thermus* do not root deeply in the phylogenetic tree and thus are thought to have evolved latterly from a mesophilic bacterial progenitor.

Figure 5.2 A phylogenetic tree generated using PILEUP, showing the relationship between the Tok19 A1 esterase and a number of microbial and eukaryotic esterases



5.1 Characterisation

5.1.1 Introduction

It has been stated (Cheetham, 1993) in a review on the use of biotransformations for industrial processes that "enzymes need to be resistant to inhibition by substrates and products, resistant to inactivation by proteases, and they must be thermostable and stable in organic solvents when required. In addition, the bioprocess must use microorganisms that are safe and acceptable to the regulatory authorities, that can be grown easily on cheap media, and that possess constitutive enzyme activities. An ideal biocatalyst is manufactured easily and is stable under the conditions of use. It should have high stereospecificity, but a broad substrate specificity so that it can be potentially useful for a range of related reactions".

The Tok19 A1 esterase fulfils many of the above criteria. A relatively cheap medium has already been developed (see section 3.5) and the Tok19 A1 esterase is expressed constitutively. The thermostability, organic solvent stability and the substrate specificity of the Tok19 A1 esterase have been extensively studied and the results are detailed in the following chapters.

5.1.2 Molecular weight determination

The molecular weight of the Tok19 A1 esterase (Lane 6 Figure 4.9) was determined from SDS-PAGE using SDS molecular weight standards (Table 5.2) from which a standard curve was generated (Figure 5.3). Native molecular weight values were also obtained using gel filtration chromatography.

$$\text{The relative mobility } R_f = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

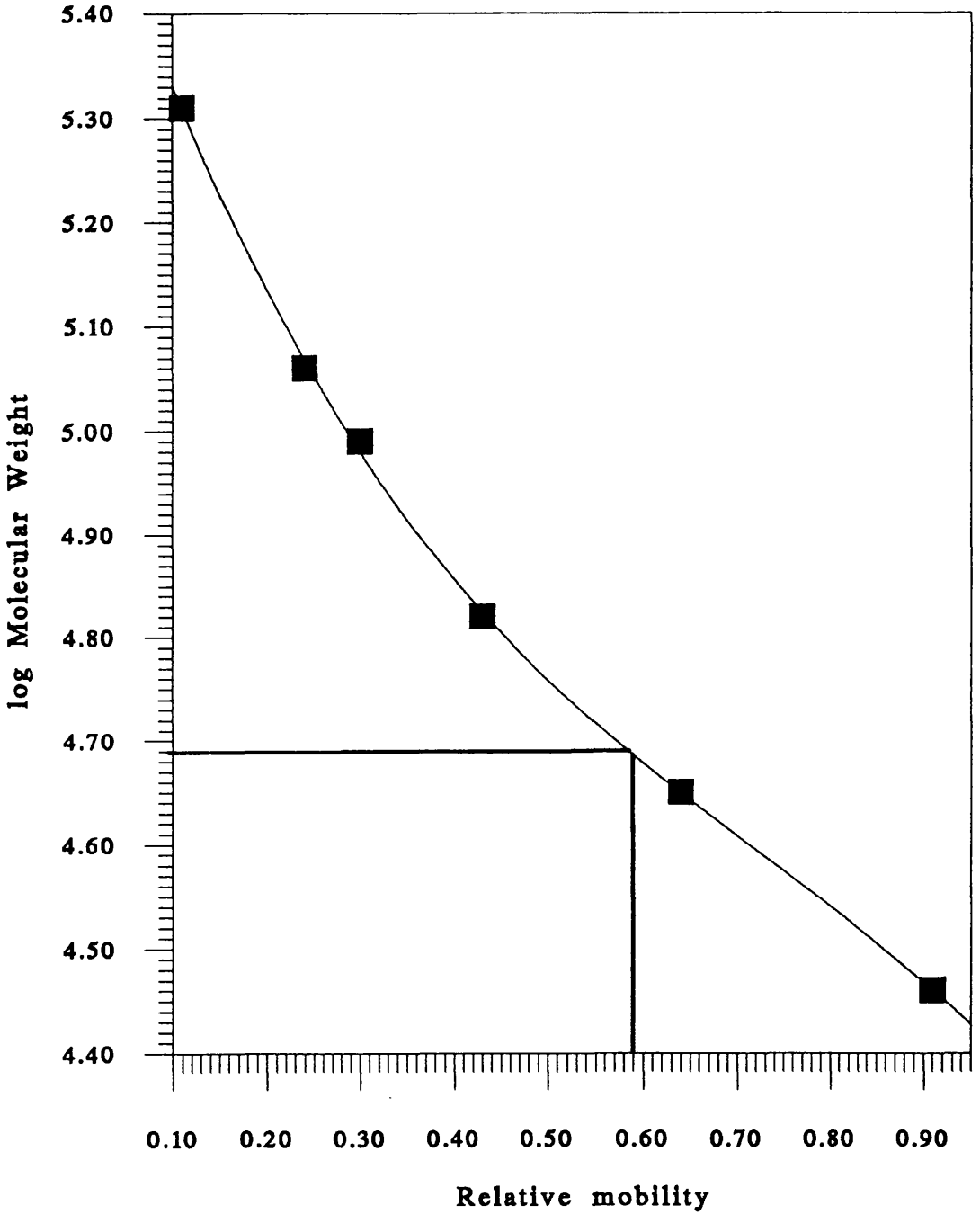
Table 5.2 Molecular weight and relative mobilities of the SDS standards and Tok19 A1 esterase

Marker	Molecular Weight	Relative Mobility	$\log_{10}$ Mw
Myosin	205,000	0.11	5.31
β-Galactosidase	116,000	0.24	5.06
Phosphorylase b	97,400	0.31	4.99
Albumin (Bovine)	66,000	0.43	4.82
Albumin (Egg)	45,000	0.64	4.65
Carbonic Anhydrase	29,000	0.91	4.46
Esterase	49,000	0.59	4.69

SDS-PAGE analysis of the purified esterase (step 6, Table 4.7) produced a single protein band with a molecular mass of approximately 49,000 +/- 2,000 Da. (Figure 4.8. Lane 6 section 4.3).

The electrophoretically-pure esterase was eluted from a Superdex 75 GPC column in a single-activity peak with an elution volume (Ve) of 10 mL (± 0.2 mL) (Figure 4.7). The column had previously been calibrated with molecular weight markers and a standard curve was plotted (Figure 5.4). An elution volume of 10 mL (± 0.2 mL) corresponded to a molecular mass of 48,000 ± 3,000 Da.

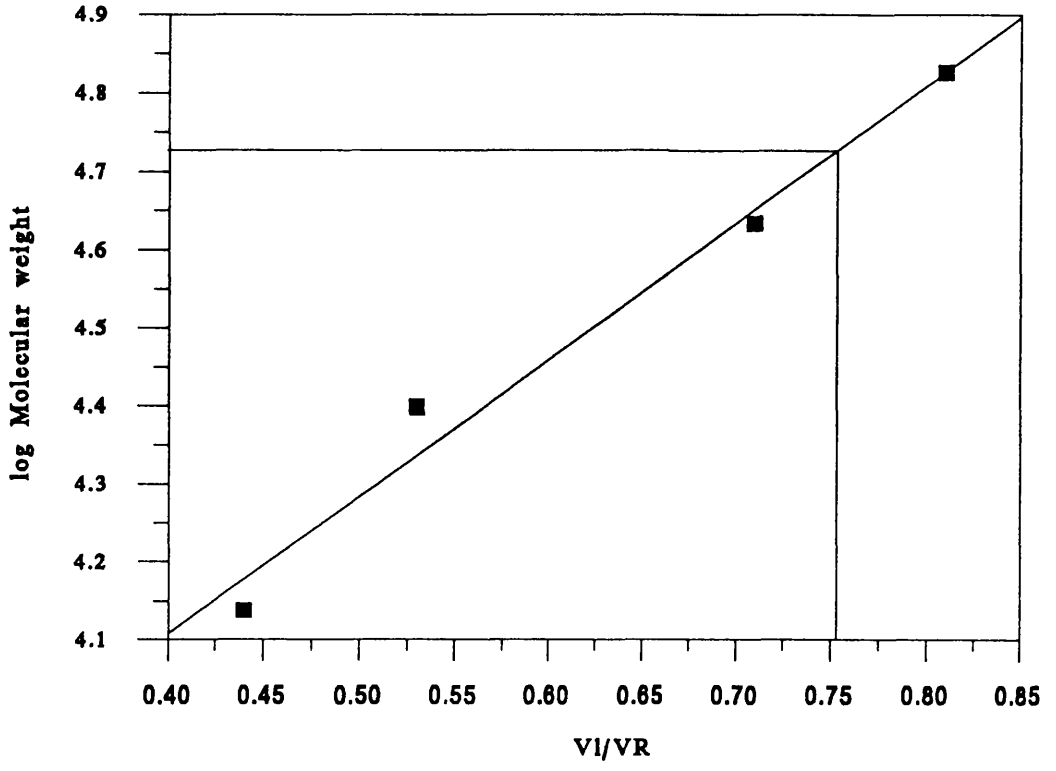
Figure 5.3 Standard curve for the determination of the molecular weight of the Tok19 A1 esterase



The standard curve was generated using the migration of the molecular weight markers Sigma kit MW-SDS-200 (30,000-200,000). The $\log_{10}$ of the molecular weight markers were plotted against the relative mobility (R_f) of the bands. (Figure 4.8 page 160).

The Pharmacia Superdex 75 column was calibrated with molecular weight standards, from the Sigma kit MW-GF-70 (bovine lung aprotinin 6,500, horse heart cytochrome c 12,400, bovine erythrocyte carbonic anhydrase 29,000 and bovine serum albumin 66,000) at a flow rate of 0.5 mL min^{-1}. Blue Dextran 2,000,000 was used to determine the void volume of the column.

Figure 5.4 Superdex 75 standard curve



The chromatographic and electrophoretic results suggested that the esterase was a monomeric protein with a molecular mass of 45,000-51,000 Da. Other esterases from *Bacillus stearothermophilus* have molecular weights of 38,000-45,000 Da (Matsunaga *et al.*, 1974) and 42,000-47,000 Da (Owusu and Cowan, 1991) while the esterase from the thermoacidophilic eubacterium *Bacillus acidocaldarius* has a molecular mass of 36,500 Da (Manco *et al.*, 1994) and the thermophilic esterase from the thermoacidic archaeon *Sulfolobus acidocaldarius* is a homotetramer with a molecular mass of 128,000 Da (Sobek and Gorisch, 1988).

5.1.3 Inhibition characteristics

Simple inhibition studies are traditionally used to identify and categorise the active site characteristics of enzymes. While these studies do not provide detailed information on the catalytic mechanism *per se*, comparisons with the behaviour of well characterised enzyme mechanisms are valuable in assigning the general catalytic group.

50 mM EDTA did not inhibit the enzyme (Table 5.3), implying that a metal ion is not essential for maintenance of catalytic activity. The esterase was strongly inhibited by PMSF, mercuric chloride, eserine and diethylpyrocarbonate. PMSF and mercuric chloride typically inhibit enzymes by covalent interaction with active site serine residues. *p*-CMB, which typically inhibits sulfhydryl groups, strongly inhibited the esterase. TPCK, an irreversible inhibitor which alkylates hystidines, also strongly inhibited TOK19 A1 esterase activity.

Table 5.3. Inhibition of Tok19 A1 esterase.

Concentration mM	EDTA	PMSF	Mercuric chloride	Eserine	Diethyl-pyrocarbonate	*p*-CMB	TPCK
50	100	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10	n.d.	n.d.	0	n.d.	n.d.	n.d.	0
1	n.d.	2	10	3	0	0	0
0.5	n.d.	10	45	20	12	12	24
0.1	n.d.	n.d.	57	50	45	76	60

Percentage activity remaining after incubation of 10 units of esterase with the stated concentrations of inhibitors for 30 minutes at 30°C. The residual activity was measured using *p*-NP propionate. n.d. not determined.

In common with other esterases from *B. stearothermophilus* (Owusu and Cowan, 1991; Matsunaga *et al.*, 1974) Tok19 A1 esterase appears to contain both serine and sulfhydryl groups in the active site whereas the esterase from *Sufolobus acidocaldarius* only appears to contain a serine group (Sobek and Gorisch, 1988). Together these data suggest that Tok19 A1 esterase might possess a catalytic mechanism related to that of the serine hydrolases.

5.1.4 Thermostability

In order for the Tok19 A1 esterase to be considered a good industrial catalyst it must have a high thermostability at a range of operating temperatures and conditions. The thermostability of the Tok19 A1 esterase was consequently studied at temperatures between 55°C and 80°C, at a variety of protein concentrations and in the presence of BSA, a potential stabilising agent.

Figure 5.5 shows the stability of the esterase at various protein concentrations and temperatures. At temperatures of 55°C and below the half-life of the Tok19 A1 esterase was greater than 170 hours in all samples. The thermostability of the pure esterase (at 60°C) could be enhanced 7-fold by the addition of 1 mg mL^{-1} of BSA and 20-fold by increasing the purified esterase concentration from 2 μg mL^{-1} to 10 μg mL^{-1}, at which point stability was similar to that of the crude Tok19 A1 esterase preparation. No further increase in the stability of the Tok19 A1 esterase was noted when the esterase concentration was higher than 10 μg mL^{-1}. This data suggests that the enzyme may be stabilised by both homogenous and heterogeneous protein-protein interactions. In light of these data, all further stability experiments were conducted using enzyme concentrations of 10 μg mL^{-1}.

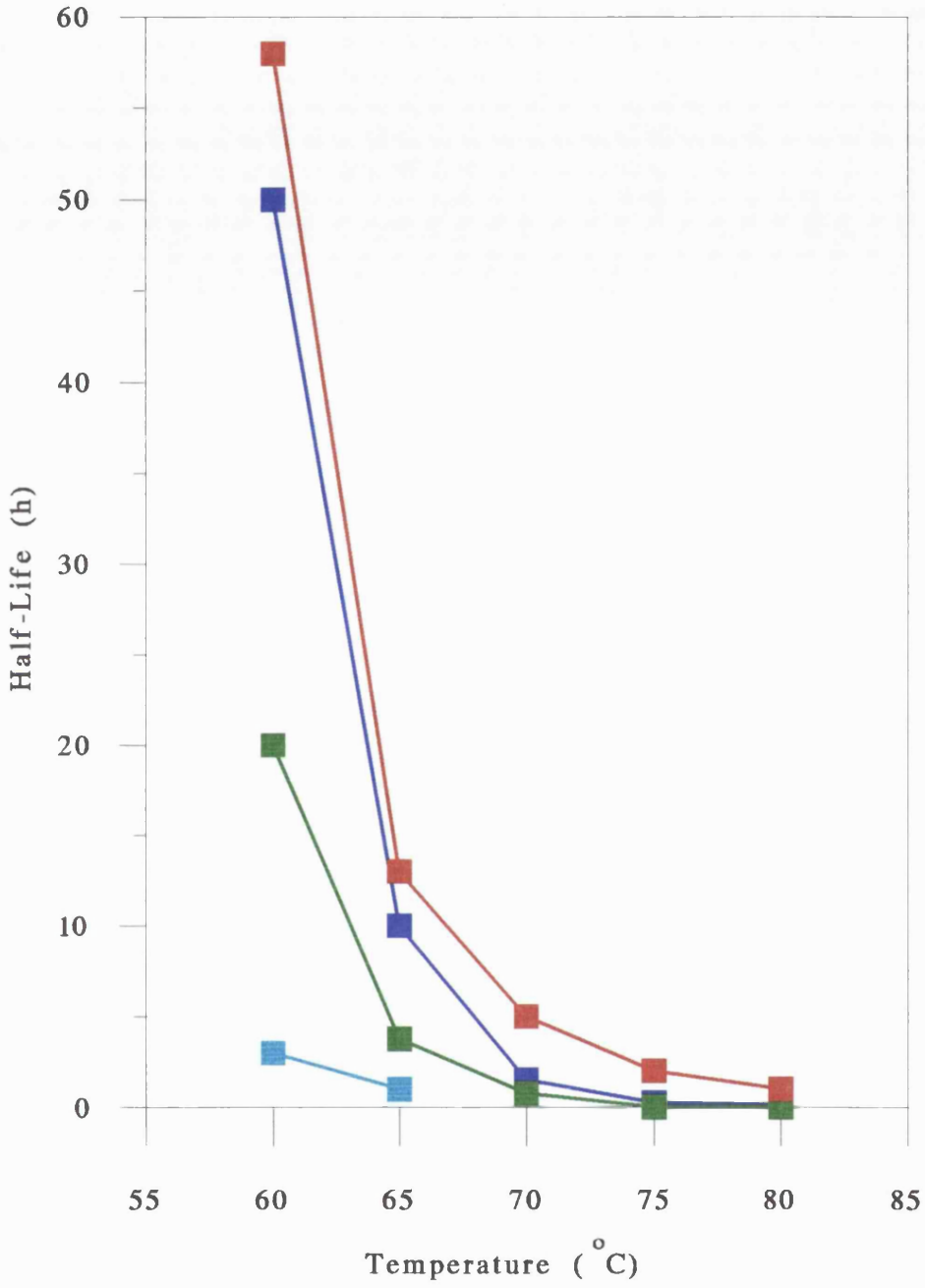
No loss of activity was observed when the esterase was incubated at 56°C (pH 6.0) for 7 days (Figure 5.6). Activity loss at higher temperatures was first order with respect to time (see Figures 5.7a, b, c and d.) and the half-life results, taken from the first order natural log plots, are summarised in Table 5.4. The half-life at 60°C (the optimum growth temperature of the organism) was 170 hours at pH 6.0. At temperatures above 65°C the Tok19 A1 esterase showed a dramatic drop in thermostability. The predicted melting temperature (t_m) was 62°C.

Table 5.4. Thermostability of Tok19 A1 esterase

Temperature (°C)	56	60	65	70	75
Half-life $t_{1/2}$ (h)	>170	170	23	2.4	0.9

The data are the thermostability half-lives, at pH 6.0 and an enzyme concentration of 10 μg mL^{-1}, taken from the first order plots.

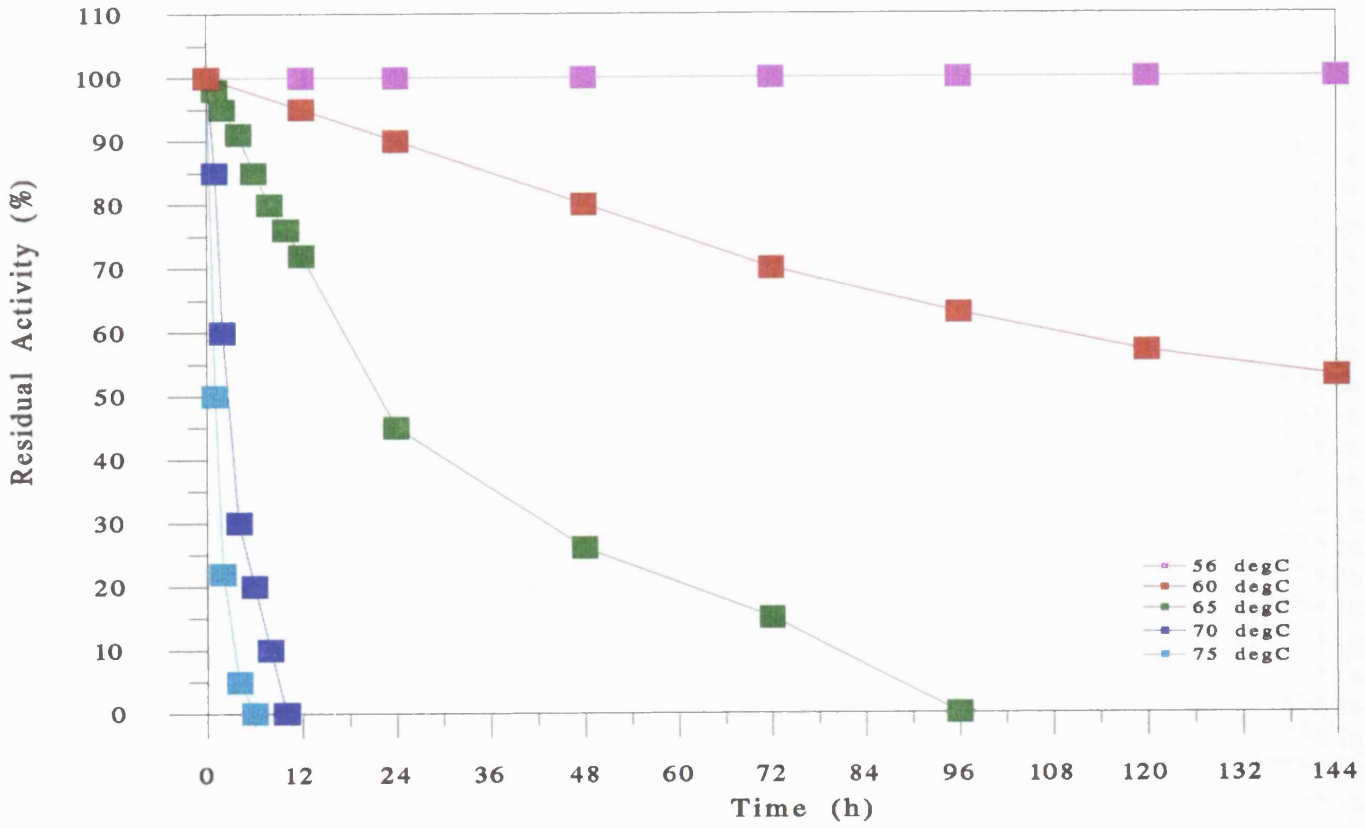
Figure 5.5. Relationship between protein concentration and thermostability for the Tok19 A1 esterase.



Data taken from thermostability profiles at 60°C, 65°C, 70°C 75°C and 80°C. ■ Pure esterase (10 μg mL^{-1}), ■ Crude esterase (cell free extract 10 mg mL^{-1}), ■ Pure esterase (2 μg mL^{-1}) supplemented with 1 mg mL^{-1} BSA, ■ Pure esterase (2 μg mL^{-1}).

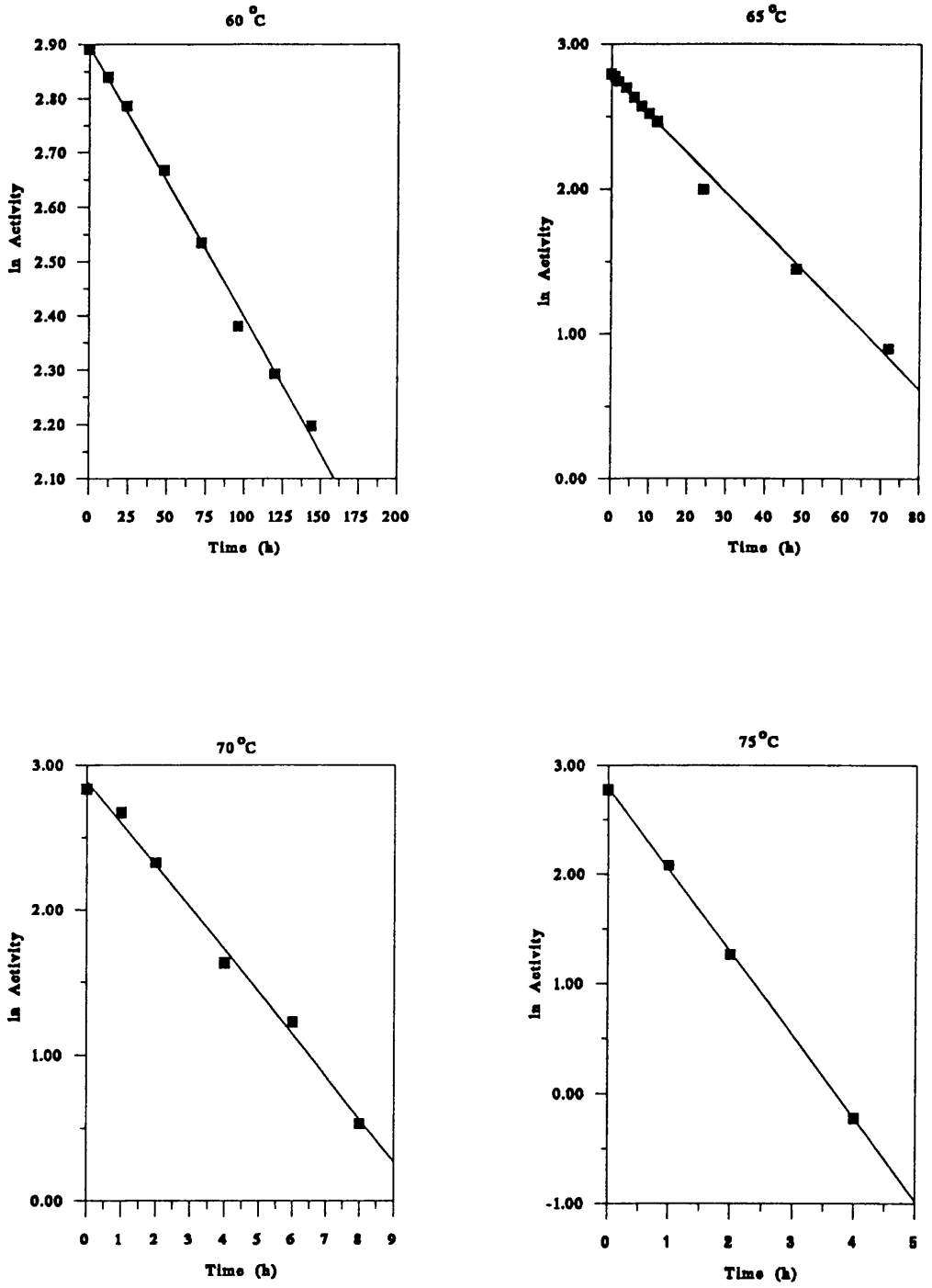
For experimental details see section 2.7.7.

Figure 5.6 Thermostability profiles for the Tok19 A1 esterase.



The purified esterase (10 μg mL^{-1}) was incubated at 56°C, 60°C, 65°C, 70°C and 75°C in 50 mM citric acid-Na_2HPO_4 buffer adjusted to pH 6.0 at each temperature (section2.7.7 page 110). The residual activity was plotted against time and the half-lives of the enzyme at the various temperatures were taken from first order rate plots (page 183).

Figures 5.7a, b, c and d Thermostability First order rate plots for the Tok19 A1 esterase



Data taken from thermostability profiles at 60°C, 65°C, 70°C and 75°C (at pH 6.0 and a protein concentration of 10 μg mL^{-1})

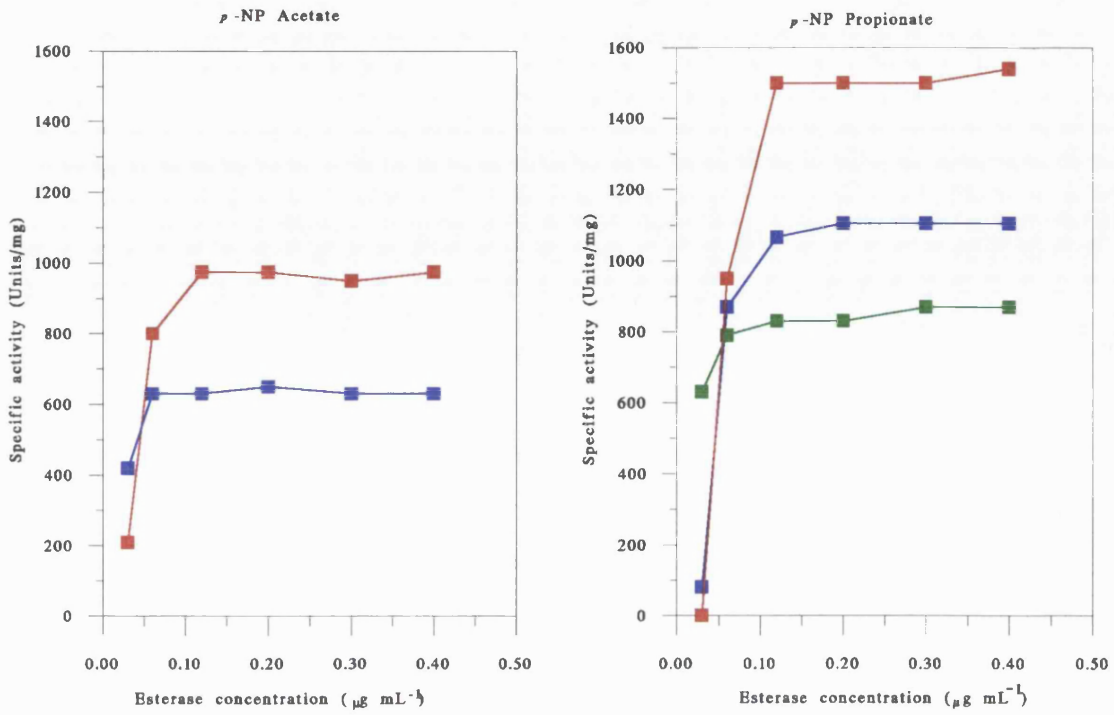
5.1.5 The effect of the enzyme concentration and substrate concentration on specific activity

The specific activity of the Tok19 A1 esterase was strongly dependant on the enzyme concentration below 0.1 μg mL^{-1} and 0.15 μg mL^{-1} with *p*-NP acetate and *p*-NP propionate respectively, but became independent above these values (Figures 5.8a and b). This effect could not be attributed to thermal denaturation since no change in activity values were observed when the enzyme was incubated in the same conditions in the absence of substrate and subsequently assayed with 0.09 mM *p*-NP acetate. In light of these data, all activity assays were conducted using 0.3 μg mL^{-1} of Tok19 A1 esterase.

One possible explanation for this behaviour is that high substrate concentrations may distort the enzyme structure and reduce catalytic activity, while protein-protein interactions may prevent substrate induced conformational changes. Data presented above suggests that the enzyme is stabilised by protein-protein interactions. There is evidence for a competitive effect between protein-protein interactions and substrate-protein interactions; i.e. when the substrate concentration was increased, an increase in protein concentration was necessary to maintain the same apparent activity.

The interaction of the Tok19 A1 esterase molecule with a hydrophobic surface (e.g. a second esterase molecule or BSA) in order to induce a conformational change resulting in activation would also be consistent with these data. This behaviour is reminiscent of true lipase activation, where interaction with a lipid micelle surface exposes the active site by displacement of the amphipathic helix (Tilberg *et al.*, 1993; Brzozowski *et al.*, 1991).

Figures 5.8a and b The effect of enzyme concentration and substrate concentration on the enzyme activity.



■ 0.7 mM substrate, ■ 0.14 mM substrate and ■ 0.09 mM substrate

The specific activity of the Tok19 A1 esterase was determined at 30°C using *p*-NP acetate and *p*-NP propionate. The esterase concentration in the assay was varied between 0.05 μgmL^{-1} and 0.5 $\mu g\ mL^{-1}$.

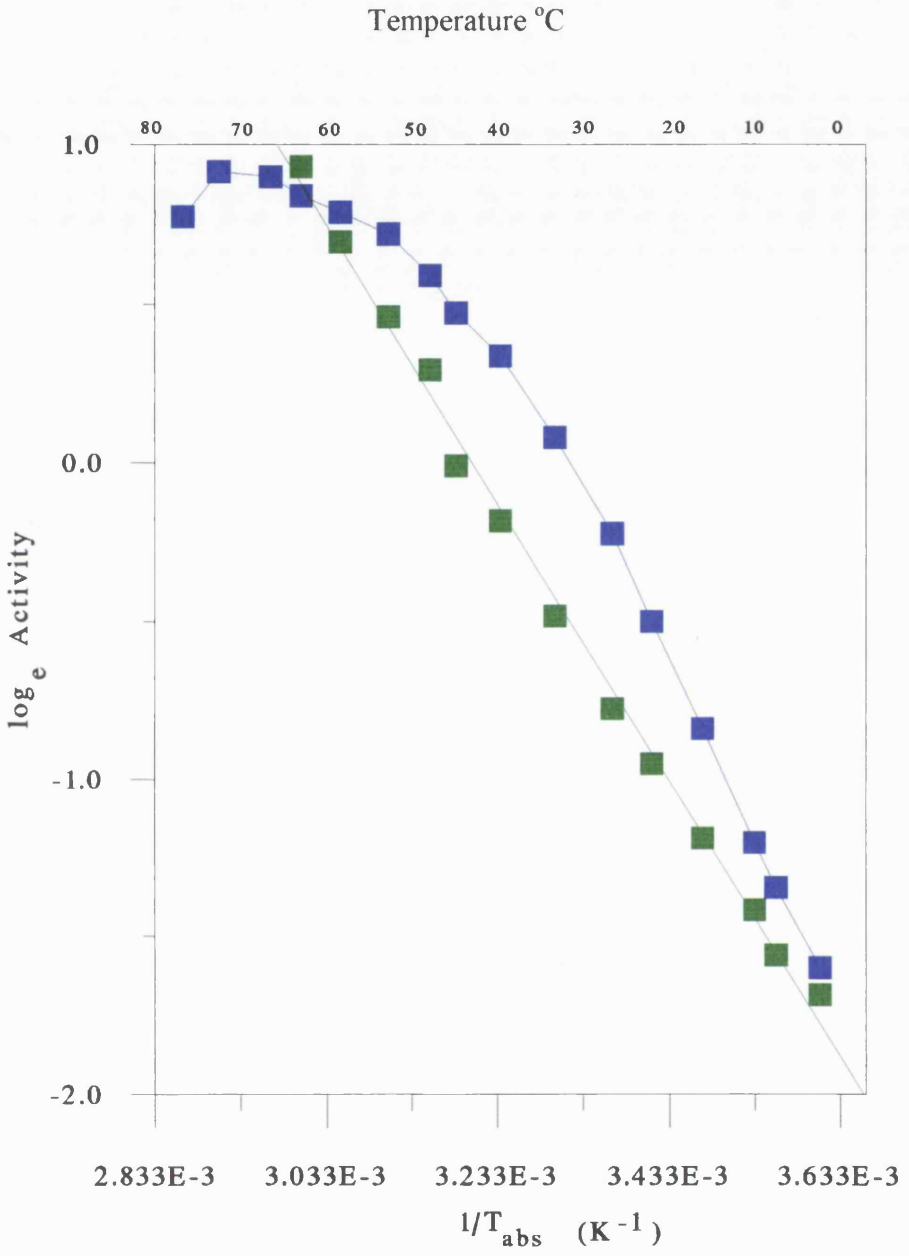
5.1 6 Temperature-activity profile

The temperature-activity profiles (expressed as Arrhenius plots) for the esterase using *p*-NP propionate and *p*-NP valerate are shown in Figure 5.9. Under the specified assay conditions and using *p*-NP propionate as the substrate, the activity of the enzyme increased linearly to 60°C, above which the background hydrolysis of the substrate was too rapid to obtain reliable data. The Arrhenius plot between 4°C and 60°C showed no obvious inflexion. The activation energy estimated from the slope of the plot was -36.4 kJ mol^{-1} and the increase in the activity for successive 10°C rises between 4°C and 60°C (Q_{10}) was 1.63 (± 0.07; n = 3).

The Arrhenius plot for the Tok19 A1 esterase using *p*-NP valerate as the substrate was only linear between 4°C and 36°C (see Figure 5.9). The activation energy was -44.9 kJ mol^{-1} and Q_{10} over the same temperature range was 1.88 (± 0.04 n=3). The activity of the enzyme increased in a non-linear fashion with temperature between 36°C and 72°C. At higher temperatures the enzyme was observed to precipitate in the reaction mixture with a concomitant decrease in activity and consequent slope inversion in the Arrhenius plot.

The loss of the linearity in the Arrhenius plot may be attributed to the distortion of the enzyme structure by *p*-NP substrates as previously discussed (section 5.1.6) with the distortion increasing with increasing acyl chain length. The enzyme was apparently stable in the presence of *p*-NP propionate up to a temperature of 60°C, but was fully stable in the presence of *p*-NP valerate only up to a temperature of 36°C.

Figure 5.9. Temperature-activity profiles for the Tok19 A1 esterase expressed as Arrhenius plots



50 mM KH_2PO_4-NaOH buffer pH 7.0 was incubated with 0.5 mM *p*-nitrophenyl valerate ■ at temperatures between 4°C and 76°C and 0.5 mM *p*-nitrophenyl propionate ■ at temperatures between 4°C and 60°C. The $\log_e$ of the activity at each temperature was plotted against 1/(absolute temperature).

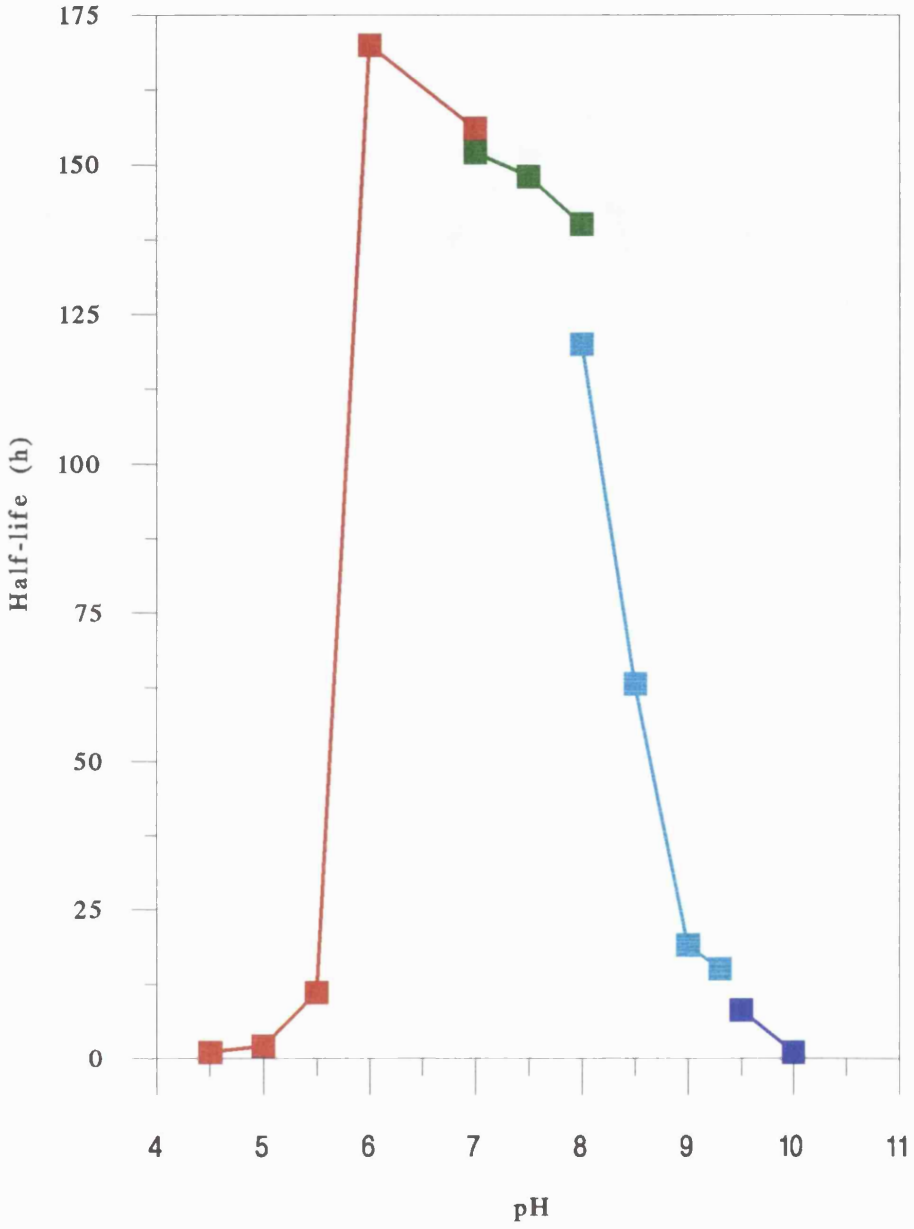
5.1.7 pH Stability

The stability of the Tok19 A1 esterase was very high over a broad range of pH between 6.0 and 8.5, with optimum stability in citric acid-Na_2PO_4 buffer at pH 6.0 (the half-life was 170 hours at 60°C) (Figure 5.10). At pH 6.0 and a temperature of 56°C the half-life was greater than 7 days. However, at the same temperature the enzyme was rapidly irreversibly denatured below pH 4.5 and in carbonate buffer at pH 9.5. The enzyme showed a sharp decrease in stability at pHs below the isoelectric point. For example, the enzyme was 85 times more stable at pH 6.0 than pH 5.0 but only 1.2 times more stable at pH 6.0 than pH 8.0.

5.1.8 pH-activity profile

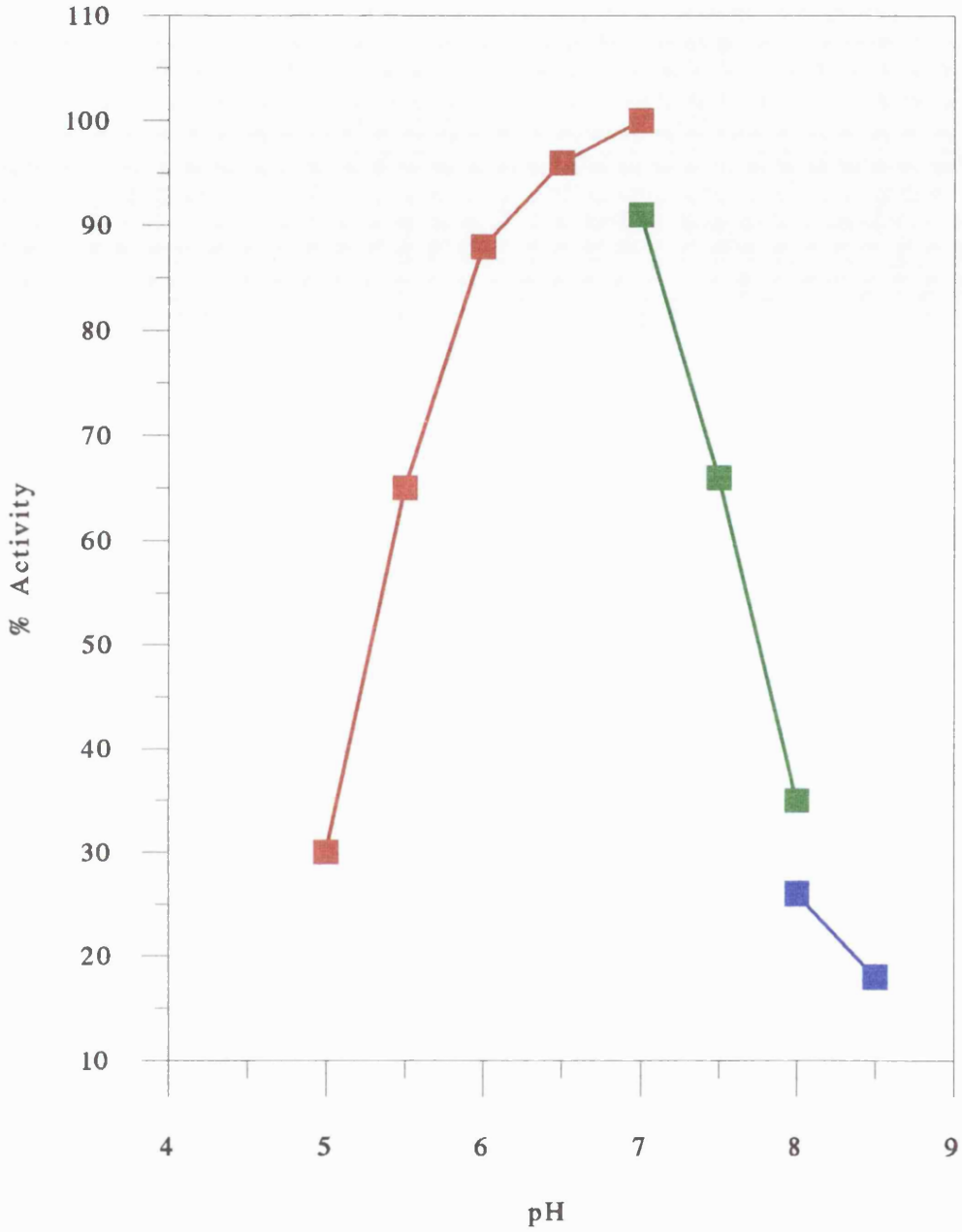
The pH profile for the Tok19 A1 esterase (Figure 5.11.) was relatively narrow with maximal activity at pH 7.0. The relatively rapid decrease in activity at alkaline pH was not due to denaturation of the enzyme since the esterase was more stable at alkaline pH than acid pH (Figure 5.10.).The pH optimum of the Tok19 A1 esterase is significantly lower than that reported for G18A7 esterase (9.0-9.5) (Owusu and Cowan, 1991) but the same as the esterase from strain NCA2184 (Matsanaga *et al.*, 1974). The esterase from *S. acidocaldarius* had a broad optimum between pH 7.5 and 8.5 (Sobek and Gorisch, 1988).

Figure 5.10. The influence of pH on the stability of the Tok19 A1 esterase



Data are the half life of the Tok19 A1 esterase after incubating 25 units of esterase in the following buffers at 60°C: 50 mM citric acid-Na_2HPO_4 buffer (pH 4.5-7.0) ■, 50 mM HEPES buffer (pH 7.0-8.0) ■, 50 mM Clark and Lubbs solution (pH 8.0-9.3) ■ and 50 mM sodium carbonate-sodium bicarbonate (pH 9.5-11.0) ■. The residual enzyme activity was assayed in 50 mM KH_2PO_4-NaOH buffer (pH 7.0) at 30°C using *p*-NP propionate as the substrate.

Figure 5.11 pH activity profile for the Tok19 A1 esterase



Enzyme activity was determined in; 50 mM citric acid-Na_2HPO_4 buffer ■ (pH 5-7), 50 mM HEPES buffer (pH 7-8) ■ and 50 mM borate-HCl buffer ■ (pH 8-8.5) at 30°C using *p*-NP propionate as the substrate.

5.1.9 Substrate specificity

Acyl-chain specificity

Tok19 A1 esterase hydrolysed a broad range of *p*-NP substrates between *p*-NP acetate and *p*-NP laurate (Table 5.5). Substrate affinity increased with increasing acyl chain length, the affinity for *p*-NP laurate being 5 times greater than for *p*-NP acetate. The esterase had a broad maximum k_{cat} between *p*-NP propionate and *p*-NP caprylate, as have the esterases reported from other thermophilic bacilli (Owusu and Cowan, 1991; Matsanaga *et al.*, 1974; Manco *et al.*, 1994). When the acyl chain length of the substrate was increased above C_6 there was a gradual decrease in the enzyme activity, the activity towards *p*-NP laurate being 30% of the maximum. The specificity constant k_{cat}/K_M showed a maximum for *p*-NP-caprylate. The esterase from *Sulfolobus* (Sobek and Gorisch, 1988) was also able to hydrolyse *p*-NP laurate (unlike the esterases from G18A7 (Owusu and Cowan, 1991) and NCA2184 (Matsanaga *et al.*, 1974)).

Enzyme activity decreased with increasing substrate concentration. This has been attributed to micelle formation of the substrates (Matsunaga *et al.*, 1974), but no substrate activation of the Tok19 A1 esterase was observed. This is in contrast to the results with PLE where large increases in enzyme activity were noted at high substrate concentrations (Barker and Jencks, 1969; Greenzaid and Jencks, 1971)

As shown in Table 5.5, the K_M values for esters decreased as the acyl chain length increased, but the k_{cat} values were maximal for *p*-NP caprolate and decreased as the size of the acyl moiety increased. Fatty acids have been shown to be competitive inhibitors for goat intestinal esterase (Malhorta 1966) and an esterase from *Bacillus stearothermophilus* (Matsunaga *et al.*, 1974). One possible explanation for the low k_{cat} values of the higher esters might be that the anions of the larger acids produced as a

result of the enzyme reaction may not be efficiently removed from the enzyme active site due to their higher affinity for the site. This would, therefore, result in a competitive inhibition by fatty acids and a greater inhibition by the longer acids (Matsunaga *et al.*, 1974).

Table 5.5. The influence of the *p*-NP ester acyl chain length on esterase kinetic parameters

Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}mM^{-1}$)
p-NP-Acetate (C_2)	0.055	750	13636
p-NP-Propionate (C_3)	0.050	1174	23480
p-NP-Butyrate (C_4)	0.041	1375	33537
p-NP-Valerate (C_5)	0.031	1532	49419
p-NP-Caproate (C_6)	0.021	1550	73810
p-NP-Caprylate (C_8)*	0.012	1250	104167
p-NP-Caprate (C_{10})*	0.011	800	72727
p-NP-Laurate (C_{12})*	0.010	555	55500

Enzyme activity was determined at 50°C in 50 mM KH_2PO_4-NaOH, pH 7.0. Initial-velocity data was analysed as described by Hanes 1932. The maximum substrate concentration was 0.5 mM, * in these cases 0.1 mM

The Tok19 A1 esterase was also able to hydrolyse a number of N-carbobenzoxy amino acid para-nitrophenyl esters (Table 5.6).

Table 5.6 The relative activity of the Tok19 A1 esterase towards some N-carbobenzoxy amino acid para-nitrophenyl esters

Substrate	Relative % Activity
p-NP propionate	100
N-CBZ glycine	175
N-CBZ-L-alanine	20
N-CBZ-L valine	1
N-CBZ-L leucine	2
N-CBZ-L isoleucine	0
N-CBZ-L tyrosine	51
N-CBZ-L phenylalanine	8
N-CBZ-L tryptophan	0
N-CBZ-L proline	1
α-N-CBZ-L asparagine	0*
α-N-CBZ-L lysine	0*

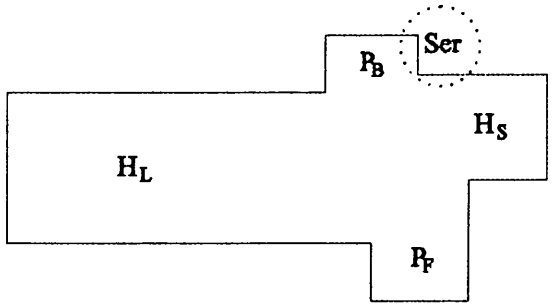
The enzyme activity was determined at 50°C in 50 mM KH_2PO_4-NaOH buffer pH 7.0. A substrate concentration of 0.1 mM was used. * Enzyme activity was determined at 18.5°C.

The esterase had high activity towards N-CBZ glycine *p*-NP ester, while the addition of a methyl group to the amino acid side chain (i.e. alanine) resulted in a 9 fold decrease in esterase activity. Further increases in side chain length (i.e. valine, leucine and isoleucine derivatives) further decreased activity. Of the three amino acid derivatives with aromatic side chains N-CBZ-L-tyrosine *p*-NP ester was the best substrate. The removal of the

hydroxyl group from the aromatic ring of N-CBZ-L-tyrosine *p*-NP ester (giving N-CBZ-L-phenylalanine *p*-NP ester) resulted in a 6 fold decrease in activity of the esterase. N-CBZ-L-tryptophan *p*-NP ester and the basic amino acids α-N-CBZ-L-asparagine *p*-NP ester and α-N-CBZ-L-lysine *p*-NP ester (assayed at 18.5°C since the background hydrolysis of these substrates at 50°C was very rapid and no reliable data could be obtained) were not substrates for the esterase.

From the substrate specificity data it is proposed that Tok19 A1 esterase contains two relatively large hydrophobic pockets at the active site, one of which is able to accommodate the relatively large para-nitrophenyl group and a second which is able to accommodate groups such as n-C_2 - n-C_{12} acyl chains or a phenol group (as N-CBZ-L-tyrosine *p*-NP ester). Several models of the active site of pig liver esterase implicate a large and a small hydrophobic binding site (Lam *et al.*, 1986; Zemlicka and Craine, 1988; Sabbioni and Jones, 1987; Toone *et al.*, 1990) (Figure 5.12)

Figure 5.12 Active-site model for pig liver esterase



H_L, H_S Large and small hydrophobic pockets, P_B, P_F polar back and front pockets (Toone *et al.*, 1990).

It has been suggested that hydrophobic groups bind preferentially in the smaller site in order to maximise hydrophobic interactions until they exceed its dimensions, whereupon

they are obliged to locate in the larger pocket (Sabbioni and Jones, 1987). The activity of the Tok19 A1 esterase was decreased by over 8-fold when N-CBZ-glycine *p*-NP ester was replaced by N-CBZ-L-alanine *p*-NP ester. These substrates differ only in that a methyl group has replaced one of the α-carbon protons, suggesting that Tok19 A1 esterase has a region of topological constraint in the region of the α-carbon. Several reports (Zemlicka and Craine, 1988; Toone *et al.*, 1990) have postulated a model for the active site of pig liver esterase with two relatively small polar pockets at right angles to two larger hydrophobic pockets (Figure 5.12). The results above might be consistent with such a structure.

5.1.01 The kinetic behaviour in the presence of BSA

The apparent k_{cat} of the Tok19 A1 esterase was increased by approximately 30% in the presence of 0.2 mg mL^{-1} bovine serum albumin (BSA) (Table 5.7), while K_M was decreased significantly. Further increases in the concentration of BSA did not change either the k_{cat} or K_M. This data appears to support the conclusion that Tok19 A1 esterase readily undergoes heterogeneous protein-protein associations, probably via hydrophobic interaction (BSA is known to be a relatively hydrophobic protein (Bigelow, 1967). These associations appear to cause some conformational change in the protein, both the increase in k_{cat} and decrease in K_M being consistent with a small but significant change in conformational flexibility.

Temperature was also shown to affect substrate affinity (Table 5.7). Over a 46°C temperature range (4°C to 50°C), K_M decreased by a factor of 1.5. This change is in accord with the accepted relationship between the strength of hydrophobic interactions and temperature (Brandts, 1967). As the temperature increases hydrophobic bond strength increases (up to around 70°C) resulting in tighter binding between the substrate

and the Tok19 A1 esterase, and subsequent decrease in K_M. In parallel as temperature increases, conformational flexibility will increase, probably reducing the interaction between the substrate and the enzyme and favouring an increase in K_M. It therefore appears that the hydrophobic effects are more significant than the conformational flexibility. This is supported by the BSA effects; i.e. that the conformational restriction caused by association with BSA means that the temperature effects are less obvious.

Table 5.7. The influence of bovine serum albumin on the kinetic parameters of the Tok19 A1 esterase

Temperature °C	K_M (mM)		k_{cat} (s^{-1})		k_{cat}/K_M (s^{-1} mM^{-1})	
	No BSA	BSA	No BSA	BSA	No BSA	BSA
4	0.075	0.050	110	160	1467	3200
30	0.060	0.030	360	520	6000	17300
50	0.050	0.020	1174	1490	23480	74500

Enzyme activity was determined in the presence and absence of 0.2 mg mL^{-1} bovine serum albumin at 4°C, 30°C and 50°C in 50 mM KH_2PO_4-NaOH, pH 7.0. *p*-NP propionate was used as the substrate. Initial-velocity data was analysed as described by Hanes, 1932.

Chapter 6

Further characterisation of the native and immobilised Tok19 A1 esterase

This chapter details the activity and stability of the Tok19 A1 esterase in a number of miscible and immiscible organic solvents. The immobilisation of the Tok19 A1 esterase, a brief characterisation of the immobilised enzyme and its organic solvent stability and substrate specificity are included.

6.0 Introduction

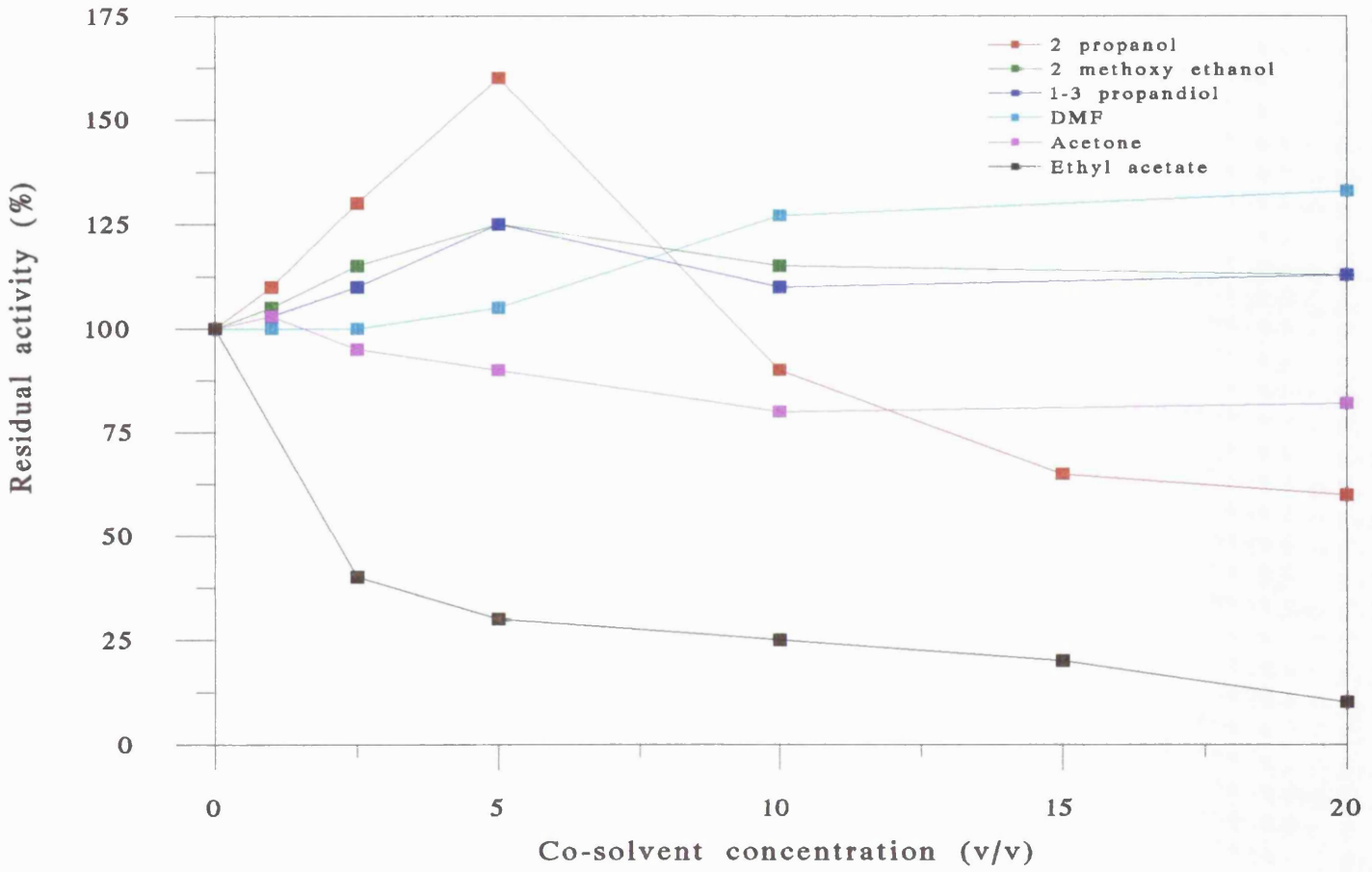
In the previous chapter, the basic functional properties of the Tok19 A1 esterase were determined. In this chapter, the characterisation of this enzyme from a more technological point of view will be discussed.

6.1 Activity of the Tok19 A1 esterase in the presence of organic solvents.

An increase in the rate of the enzymic *p*-NP liberation (from *p*-NP propionate) was detected when primary alcohols were added to the reaction mixture at low concentrations (Figure 6.1). The initial increase in activity (activation) at low solvent concentrations was followed by a subsequent decrease in activity at higher concentrations. The activation increased as the chain length of the alcohol increased from methanol to propanol (50, 90 and 115% respectively). However butanol only activated the enzyme by 30%. The amount of alcohol needed to cause the maximum activation decreased with increasing chain length of the alcohol (i.e. 10, 6, 1.75 and 1.5% respectively for methanol, ethanol, propanol and butanol).

Both the increase and decrease in activity were related to the chain length of the alcohol. For example propanol promoted the highest activation but was also most deleterious on enzyme activity at high concentrations (i.e., it was able to denature/inhibit the esterase at a concentration of only 3%). The addition of BSA to the reaction mixture promoted a further increase in the activation (Figure 6.1), possibly suggesting that different mechanisms of "activation" were involved.

Figure 6.2 Activation and stabilization by alcoholic and non-alcoholic solvents.



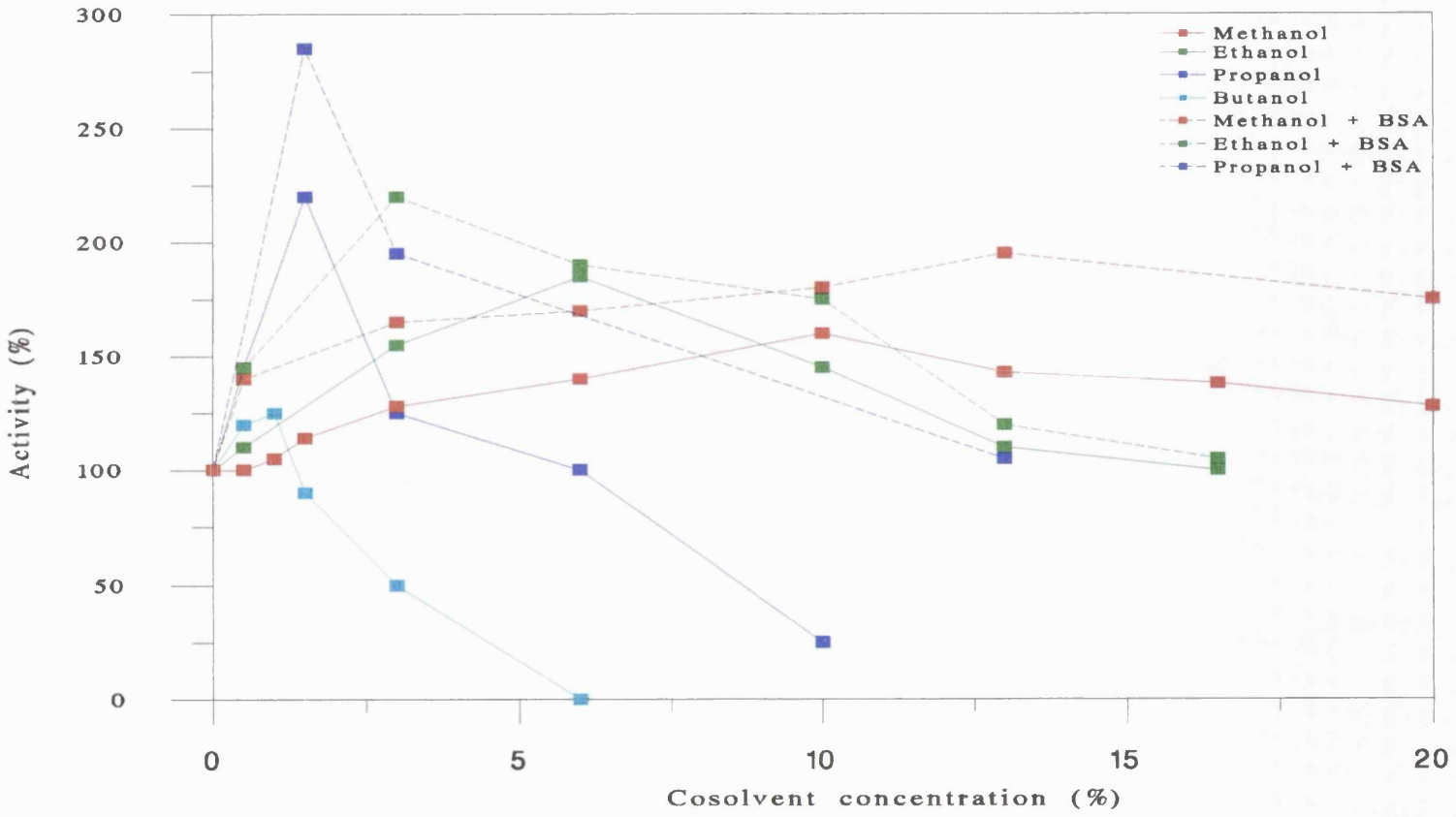
Esterase activity was determined using the standard asay (section 2.3.1 page 95) except that various concentrations (v/v) of organic alcohols were added. In some instances BSA was also added to a final concentration of 1 mg mL^{-1}.

However, when higher alcohol concentrations (2-4% v/v) were used the total activity decreased but the BSA-induced esterase activity was approximately constant. The decrease in the activation at an alcohol concentration of 2-3% (v/v) could be reversed by doubling the substrate concentration. The alcohol-induced activation was also observed using *p*-NP butyrate as the substrate. The inhibition at higher alcohol concentrations 5-10% (v/v) was reduced by 50% in the presence of *p*-NP butyrate. This might be attributed to the higher affinity of the esterase for *p*-NP butyrate (Table 5.5).

Secondary alcohols also activated the esterase at low solvent concentrations, although activation was lower than with the primary alcohols. A decrease in activity at higher solvent concentrations was also observed (Figure 6.2). This decrease in activity at higher cosolvent concentrations was also reversed by increasing the substrate concentration from 0.5 mM to 1.5 mM, suggesting that these co-solvents act as competitive inhibitors at higher concentrations. This activation and inhibition effect has also been observed with pig liver esterase (PLE) (Greenzaid and Jencks, 1971) who attributed the decrease in activity at moderate concentrations of organic solvents to competitive inhibition.

Non alcoholic cosolvents (acetone and ethyl acetate) reduced esterase activity at cosolvent concentrations of less than 5% v/v. 5% v/v DMF showed no apparent effect but a concentration of 10% resulted in a significant increase (approximately 30%) in the enzymic release of *p*-nitrophenol (Figure 6.2.). This might be attributed to a conformational change in the enzyme induced by the cosolvent. Increasing the substrate concentration reversed the esterase inhibition by non-alcoholic cosolvents. For example, the inhibition by 5% v/v acetone could be reversed by a 2-fold increase in the substrate concentration.

Figure 6.1 Activation by methanol, ethanol, propanol, butanol and BSA



Esterase activity was determined using the standard asay (section 2.3.1 page 95) except that various concentrations (v/v) of alcoholic and non-alcoholic solvents were added.

The activation and subsequent inhibition of mesophilic and thermophilic esterases by organic solvents has been reported by a number of authors (Barker and Jencks, 1969; Stoops *et al.*, 1969; Greenzaid and Jencks, 1971; Sobek and Gorisch, 1989).

Barker and Jencks, (1969) observed an activation of up to 75 % with a range of non alcoholic solvents in the PLE-catalysed cleavage of *p*-NP acetate. Greenzaid and Jencks, (1971) followed this earlier work with a more extensive study of the effect of nucleophiles on the activity of PLE. Methanol showed the highest activation (550 %), and the activation decreased with the increasing chain length of the alcohol. All the alcohols screened with phenyl acetate as the substrate caused an increase in the rate of esterase-catalysed hydrolysis except ethylene glycol (which had no effect) and trifluoroethanol which inhibited the reaction.

The initial activation of PLE was followed in all cases by a decrease in the enzymes activity. The alcohol which gave the highest activation generally had the most deleterious effect on the enzyme activity at high alcohol concentrations as was the case with the Tok19 A1 esterase.

In studies of a thermostable carboxylesterase from *Sulfolobus acidocaldarius* Sobek and Gorisch, (1989) observed activation in the presence of alcohols. The esterase-catalysed cleavage of *p*-NP acetate increased with the increasing chain length of the alcohol, a result similar to Tok19 A1 esterase but in contrast to the studies with PLE.

Several explanations have been postulated for this activation and subsequent decrease in enzyme activity in the presence of organic solvents. Barker and Jencks (1969), based on their early work with non alcoholic solvents, suggested that PLE had an activator site which was distinct from the active site. Stoops *et al.*, (1969) independently reached the same conclusion from the observations that the esterase-catalysed hydrolysis of phenyl butyrate was accelerated by benzene. The occurrence of two active sites on mammalian

esterases has been assumed by several enzymologists (Krish, 1966; Horgan, 1966; Horgan, 1969). It was also concluded that the maximum rate of hydrolysis was dependent upon the nature of the activator, the substrate and the PLE preparation being examined (Barker and Jencks, 1969).

Allen and Abeles (1989) studied the rate of dissociation and association of PLE-inhibitor complex in the presence of organic solvents and hydrophobic substrates and concluded that the activation effect of organic solvents was the result of two separate binding sites, a catalytic site and an effector site. No compounds were found which effected the catalytic reaction but did not effect the association or dissociation of the enzyme-inhibitor complex. It was suggested that occupation of the effector/activator site resulted in a more flexible open form of the enzyme so that entry and exit from the active site was facilitated (Botts and Morales, 1953).

Allen and Abeles (1989) also concluded that the occupation of the activator/effector site could decrease as well as increase the rate of ester hydrolysis. Their results also indicated that an aromatic or hydrophobic structure and a carbonyl group were required for optimal interaction with the effector site.

The results of Sobek and Gorish (1989), who studied a thermostable carboxyl esterase from *Sulfolobus acidocaldarius*, indicated the existence of a second binding site in addition to the catalytic site which was capable of binding the secondary alcohol propan-2-ol. The existence of a hydrophobic binding site for primary alcohols was also indicated by the increase in transesterifcation with increasing alkyl chain length.

A second explanation for this activation effect, applying only to alcohols, is that the activation is due solely to transesterification. This explanation was used by Greenzaid and Jencks (1971) to explain the increase in the hydrolysis of phenyl acetate in the presence of methanol. They concluded that the increase in enzyme catalysed hydrolysis

represented the conversion of phenyl acetate to methyl acetate with methanol acting as a nucleophile. Sobek and Gorish (1989) concluded that although the carboxylesterase from *Sulfolobus acidocaldarius* possessed a second binding site the difference between the rate of release of *p*-NP and the rate of acid formation represented the esterase-catalysed transesterification of *p*-NP acetate. It is obvious that the transesterification theory cannot explain the activation by organic solvents such as acetone and dioxan (Barker and Jencks, 1969) and benzene (Stoops *et al.*, 1969).

Preliminary studies using immobilised Tok19 A1 esterase in pH-stat experiments have shown that there was no increase in the rate of propionic acid production from the hydrolysis of *p*-NP propionate in the presence of concentrations of propanol between 0.5 and 3.0% (v/v). However spectrophotometric studies (using the immobilised esterase) under essentially identical assay conditions showed that the rate of *p*-NP released increased by 220 %. It would appear from these results that the apparent "activation" of the Tok19 A1 esterase is due to transesterification.

It has already been shown that homogeneous and heterogeneous protein-protein interactions have a significant effect on the stability and kinetics of the Tok19 A1 esterase (Table 5.7, Figure 5.5 and Figure 6.1) and that the Tok19 A1 esterase also showed enhanced catalytic activity in the presence of alcohols, acetone, and BSA. It is therefore possible that Tok19 A1 esterase also possesses a second binding site and interaction with this binding site causes the observed changes in the kinetic parameters. The Tok19 A1 esterase was strongly inhibited by sulfhydryl reagents (Table 5.3) although sulfhydryl groups have not been shown to be present in the active sites of several lipases (Winkler *et al.*, 1990; Scott *et al.*, 1990; Brady *et al.*, 1990) which have a similar catalytic mechanism to the carboxylesterases. Although sulfhydryl groups appear to be essential for maintenance of the catalytic activity of a number of mesophilic

(Tombo *et al.*, 1987; Bott, 1971; Nakagawa *et al.*, 1984) and thermophilic esterases (Matsunaga *et al.*, 1974; Owusu and Cowan, 1989) only one inference has been made as to its role in the catalytic mechanism. It has been postulated that the sulfhydryl group in the thermophilic esterase strain NCA 2184 may play some role in maintaining the serine residue in the reactive state (Matsunaga *et al.*, 1974). The possibility that this esterase may have two ester binding sites with the sulfhydryl group residing in one of these sites with occupancy of this site (either by a second substrate molecule or an organic solvent) being an absolute criterion for the esterase activity was not ruled out (Matsunaga *et al.*, 1974). It would be convenient to postulate the "missing" sulfhydryl group as being part of a second binding site although the action of sulfhydryl groups is usually to act as nucleophiles. Allen and Abeles (1989) inferred that this second binding site was hydrophobic, and it is difficult to envisage a role for a sulfhydryl group in a hydrophobic binding site.

It has also been postulated that the activator/modulator site could be filled by a second substrate molecule (at high substrate concentrations) and this in turn could cause activation of PLE (Barker and Jencks, 1969; Greenzaid and Jencks, 1971). This would be a another possible explanation for the apparent increase in the specific activity of the Tok19 A1 esterase at various substrate concentrations (Figures 5.8a and b), and further evidence for the existence of a second binding site on the Tok19 A1 esterase.

In all cases the initial activation was followed by a decrease in activity as the solvent concentration was increased. This was attributed by Greenzaid and Jencks (1971) to competitive inhibition. The decrease in activity (inhibition) has also been attributed to the activator binding to the catalytic site, at moderate to high concentrations, thus preventing binding of the substrate (Barker and Jencks, 1969). A similar inhibition was observed

with n-butyl alcohol and 2-butanone in the hydrolysis of ethyl butyrate (Ocken, 1967; Levy and Ocken, 1967).

6.2 Stability in the presence of organic solvents.

6.2.1 Monophasic systems.

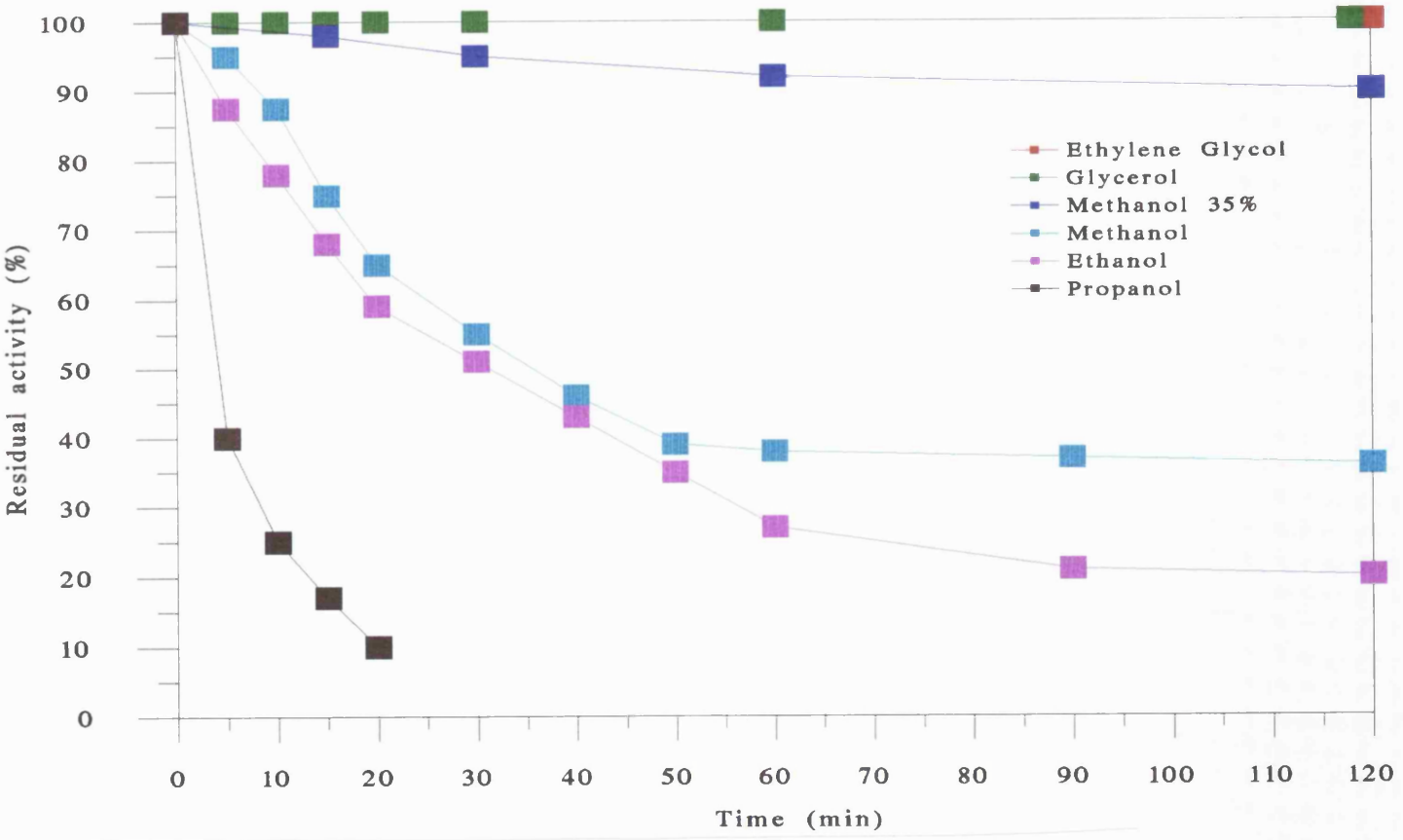
Figure 6.3 shows the Tok19 A1 esterase stability in monophasic aqueous-organic solvent systems. The esterase was very stable in 50% v/v polyols (ethylene glycol and glycerol), retaining 100% of its activity after incubation at 30°C for 7 days.

High concentrations (e.g. 50% v/v) of aliphatic alcohols such as methanol, ethanol or propanol significantly reduced the esterase stability. Decreasing the alcohol concentration significantly increased the enzyme stability. For example, Tok19 A1 esterase was approximately 35 times more stable in 35% methanol than in 50% methanol (Table 6.1). The deleterious effect on the enzyme stability could be positively correlated with the acyl chain length of the alcohol.

Table 6.1 Half-life of the Tok19 A1 esterase at 30°C

Solvent (v/v)	Half-life at 30°C (h)
50% Ethylene glycol	>168
50% Glycerol	>168
35% Methanol	20
50% Methanol	0.6
50% Ethanol	0.5
50% Propanol	0.1

Figure 6.3 The stability of Tok19 A1 esterase in the presence of miscible organic solvents



10 mL volumes of esterase in 50 mM Tris-HCl pH 7.0 were incubated with between 0 and 50% v/v of various solvents for between 5 minutes and 2 hours, at 30°C and at a stirrer speed of 300 rpm. Aliquots were withdrawn periodically and assayed using the standard assay (section 2.3.1 page 95) except that corrections were made for the solvent carried over in the aqueous phase.

6.2.2 Biphasic solvent systems

The stability of the Tok19 A1 esterase was very high in biphasic solvent systems at 30°C when stirring rates of 300 rpm were used (Figure 6.4 and Table 6.2). With the exception of butanol ($t_{1/2}$ = 4 hours) the Tok19 A1 esterase retained 100% of its activity after incubation for 24 hours in 1:1 biphasic mixtures of buffer and C_5-C_{10} alcohols.

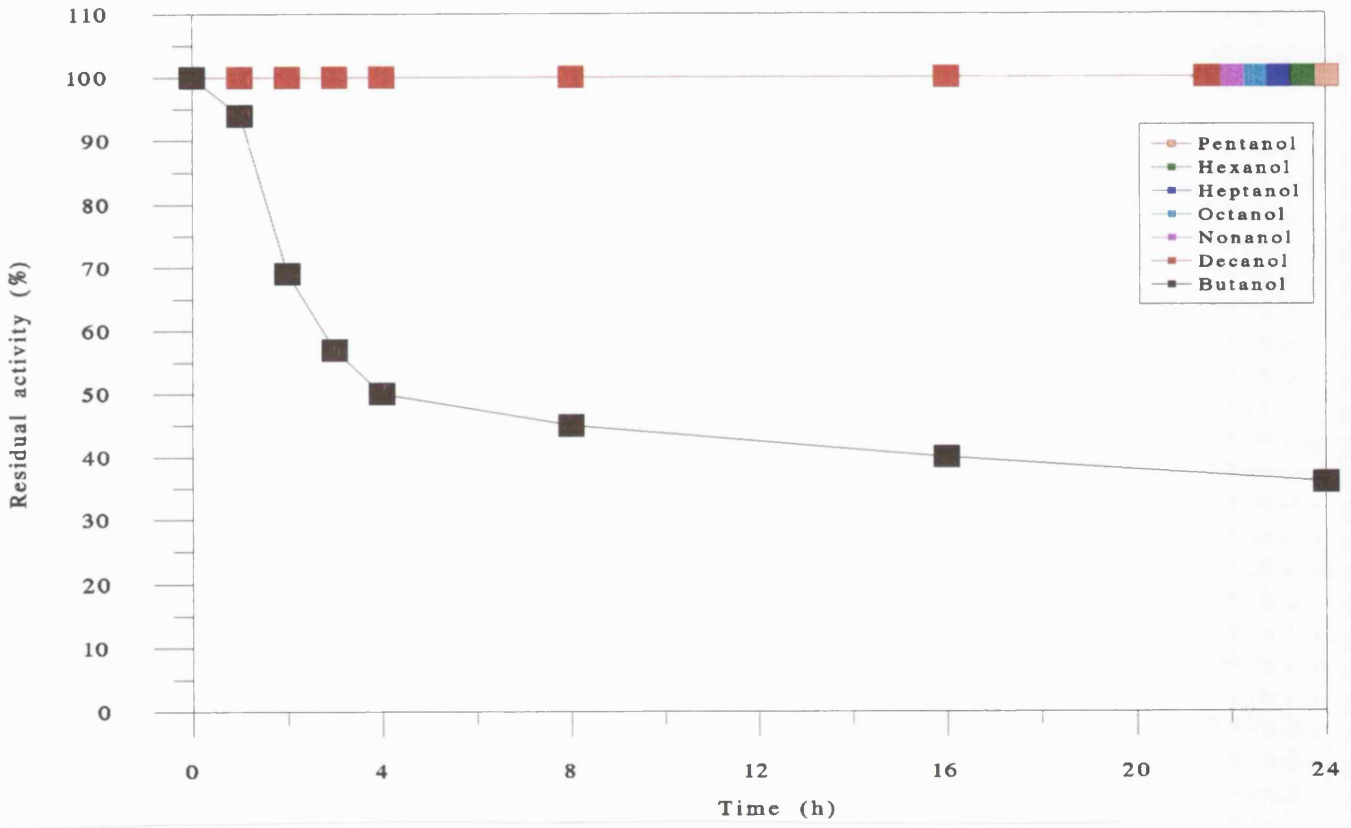
Table 6.2. The half-life of the Tok19 A1 esterase at various temperatures and 50% solvent (v/v).

Solvent	Half-life at 30°C (h)	Half-life at 40°C (h)	Half-life at 50°C (h)	Half-life at 60°C (h)
Butanol	4	n.d.	n.d.	n.d
Pentanol	>24	>24	n.d	30
Hexanol	>24	>24	n.d	n.d.
Heptanol	>24	>24	n.d.	n.d.
Octanol	>24	>24	n.d.	n.d.
Nonanol	>24	>24	n.d.	n.d.
Decanol	>24	>24	60	25

n.d. not determined. For experimental details see section 2.7.13

The decrease in enzyme stability in the presence of butanol may be explained by the higher solubility of this alcohol (12-13%) in the aqueous phase. Where the organic phase has a high solubility in the aqueous phase, the deleterious effect on the enzyme stability was positively correlated with the alcohol chain length (see results above for the shorter chained alcohols). However, the solubility of pentanol and longer chained alcohols appeared to be sufficiently low to preclude any direct effect on the enzyme structure.

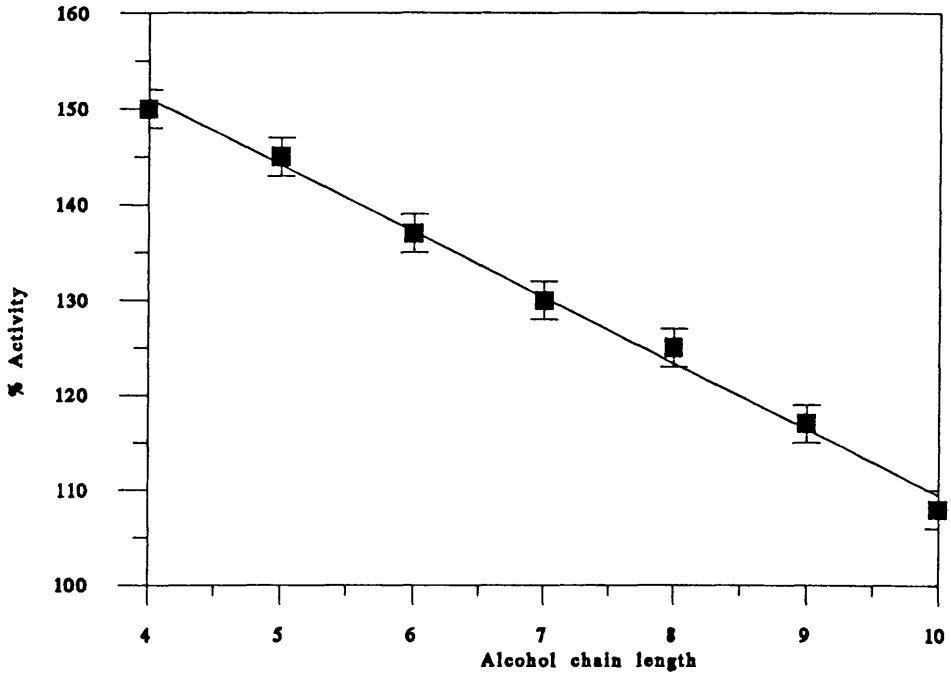
Figure 6.4 Stability of Tok19 A1 esterase in the presence of immiscible organic solvents at 30°C



10 mL volumes of esterase in 50 mM Tris-HCl pH 7.0 were incubated with 50% v/v of immiscible organic solvents for 24 hours, at 30°C and at a stirrer speed of 300 rpm. Aliquots were withdrawn periodically and assayed using the standard assay (section 2.3.1 page 95) except that corrections were made for the solvent carried over in the aqueous phase.

When aliquots of the aqueous phase were taken (between 5 minutes and 24 hours incubation) from the biphasic solvent systems and assayed for esterase activity an increase in the release of *p*-nitrophenol was observed. This activation decreased linearly with the increasing chain length of the alcohol from butanol to decanol (Figure 6.5).

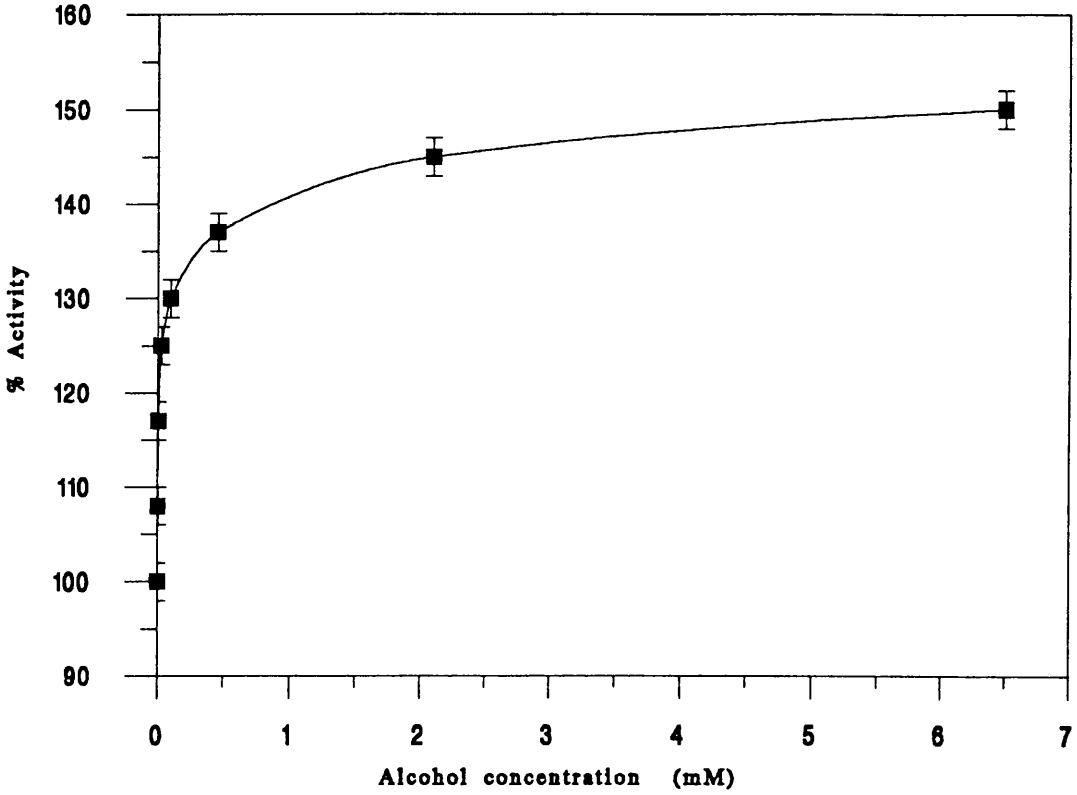
Figure 6.5 The increase in the activity of the Tok19 A1 esterase after incubation at 30°C with long chained alcohols



The increase in activity could be due to one of two factors: a transesterification reaction between *p*-NP propionate and the cosolvent dissolved in the aqueous phase (the only added cosolvent was that dissolved in the aqueous phase after the stability trials in the bioreactor and subsequently carried over into the assay mixture) or a conformational change in the enzyme resulting in a more catalytically active enzyme (it has already been suggested that 10% DMF caused a conformational change in the Tok19 A1 esterase; Figure 6.2).

The activation was not as dramatic as with miscible alcohols. However, the activation with butanol was similar to that observed before (Figure 6.1) although the volume of butanol in the assay was 40 fold lower than in the previous study. A plot of the increase in activity against the concentration of solvent in the assay was not linear; Figure 6.6.

Figure 6.6 The increase in activity of the Tok19 A1 esterase as a function of the concentration of alcohol in the assay.



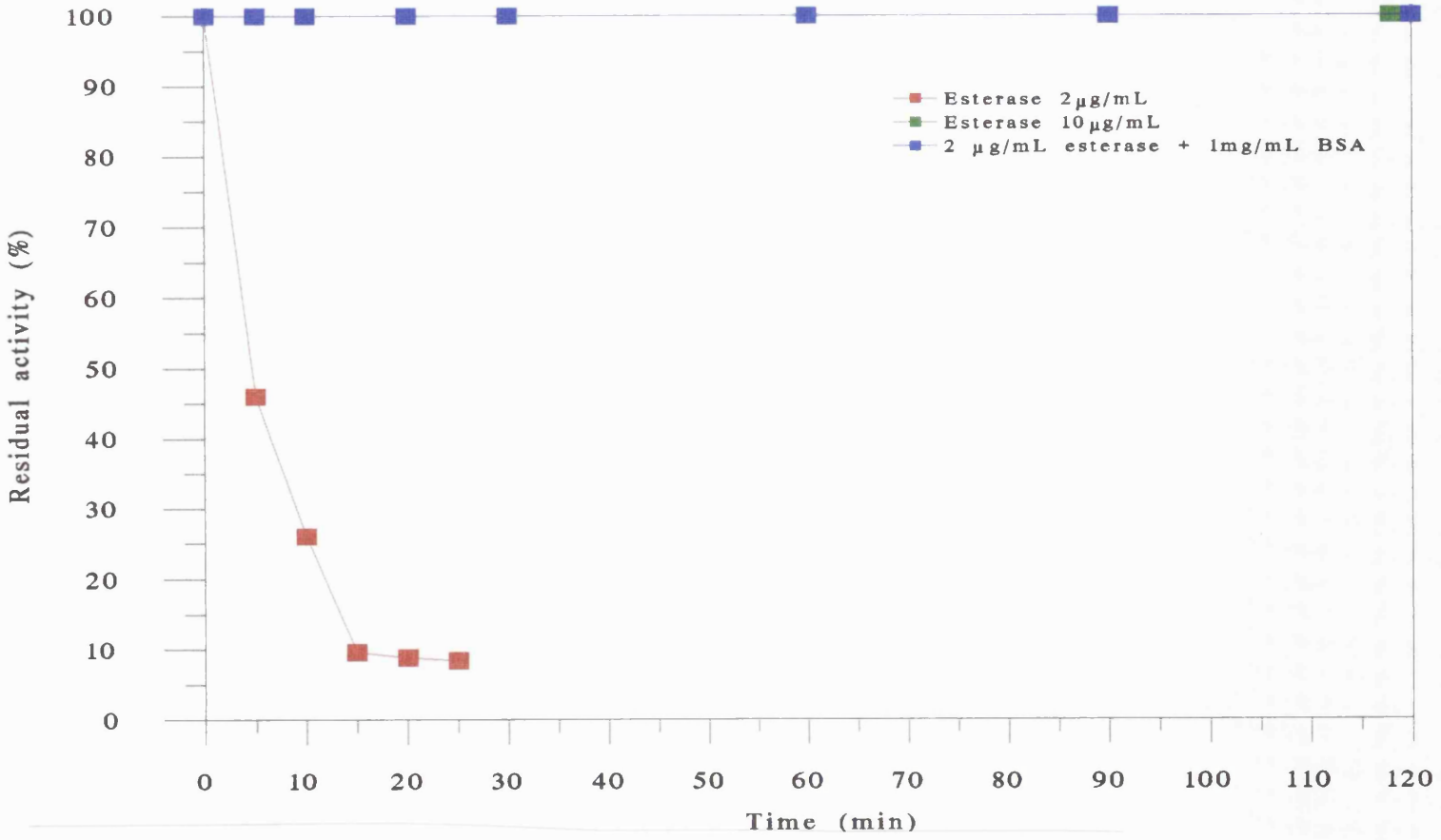
It has already been shown that the activation with the miscible primary and secondary alcohols is probably due to transesterification, but it is difficult to attribute the activation seen here to transesterification when the alcohol concentrations are as low as 1.6 μM for decanol. This alcohol concentration is similar to the enzyme concentration in the assay and 300 fold less than the substrate concentration (0.5 mM). A transesterification reaction between *p*-NP propionate and decanol would result in the formation of decyl

propionate. From the substrate specificity trials (section 6.4) decyl propionate would not be a good substrate for the Tok19 A1 esterase.

It has been postulated that the Tok19 A1 esterase possesses a second binding site and that substrate, homogeneous and heterogeneous protein, and alcohol binding to this site may cause activation of the esterase through a conformational change in its structure. It is possible that during the stability trial the proposed binding sites on the esterases were occupied by alcohol molecules. If this mode of activation is to be believed the activator site must be relatively hydrophobic.

Further evidence exists for the theory of a second binding site. When low concentrations of the Tok19 A1 esterase (2 $\mu g\ mL^{-1}$) were incubated in the presence of 50% hexanol at 30°C and a stirrer speed of 300 rpm the esterase activity was rapidly denatured/inhibited (the half-life was less than 5 minutes). Increasing the esterase concentration to 10 $\mu g\ mL^{-1}$ or the addition of 1 $mg\ mL^{-1}$ of BSA increased the esterase stability 300 fold (Figure 6.7). It is possible that the binding of a second esterase molecule or a molecule of BSA to the activator/inducer site prevented the binding of the alcohol and maintained the activity of the esterase.

Figure 6.7 The effect of enzyme concentration and BSA on the stability of the Tok19 A1 esterase in 50% hexanol (v/v)



Esterase at a concentration of 2 µg mL^{-1}, 10 µg mL^{-1} and 2 µg mL^{-1} + 1 mg mL^{-1} of BSA was incubated at 30°C in 50% hexanol (v/v) at a stirrer speed of 300 rpm. Aliquots were withdrawn periodically and assayed using the standard assay (section 2.3.1 page 95) except that corrections were made for the solvent carried over in the aqueous phase.

6.3 Immobilisation of the Tok19 A1 Esterase

The Tok19 A1 esterase was immobilised in order to improve its stability in miscible organic solvents which are frequently used in organic synthesis and to facilitate the investigation of the substrate specificity of the esterase. The dispersion of the enzyme on a soluble support may also decrease protein-protein interactions which have been shown to effect the properties of this enzyme.

Glyoxyl agarose gels were chosen as the support material because they yielded highly stabilised enzyme derivatives with little loss of activity (Guisan, 1988; Blanco and Guisan, 1989; Otero *et al.*, 1991; Guisan *et al.*, 1993). Several properties should be evaluated before starting the immobilisation,for example, the effects of temperature and pH on the esterase.

6.3.1 Esterase stability at pH 10

The immobilisation of enzymes on glyoxyl-agarose gels must be performed at alkaline pH (around pH 10 (Blanco *et al.*, 1988)). The Tok19 A1 esterase stability at this pH was therefore a critical factor.

The esterase retained 100% of its activity after incubation for 4 hours at 25°C and pH 10.0. Longer incubation periods significantly decreased the activity (60% of the initial activity remained after 48 hours). Extended incubation periods are generally necessary to generate multi-point covalent linkages between enzyme and support, during which the immobilisation process may also induce deleterious conformational changes (Blanco *et al.*, 1988).

Alcoholic co-solvents and substrates were screened in order to determine their effect on the esterase stability under immobilisation conditions. Competitive inhibitors or substrates (Blanco and Guisan, 1988) as well as polyols (sorbitol, glycerol, ethylene

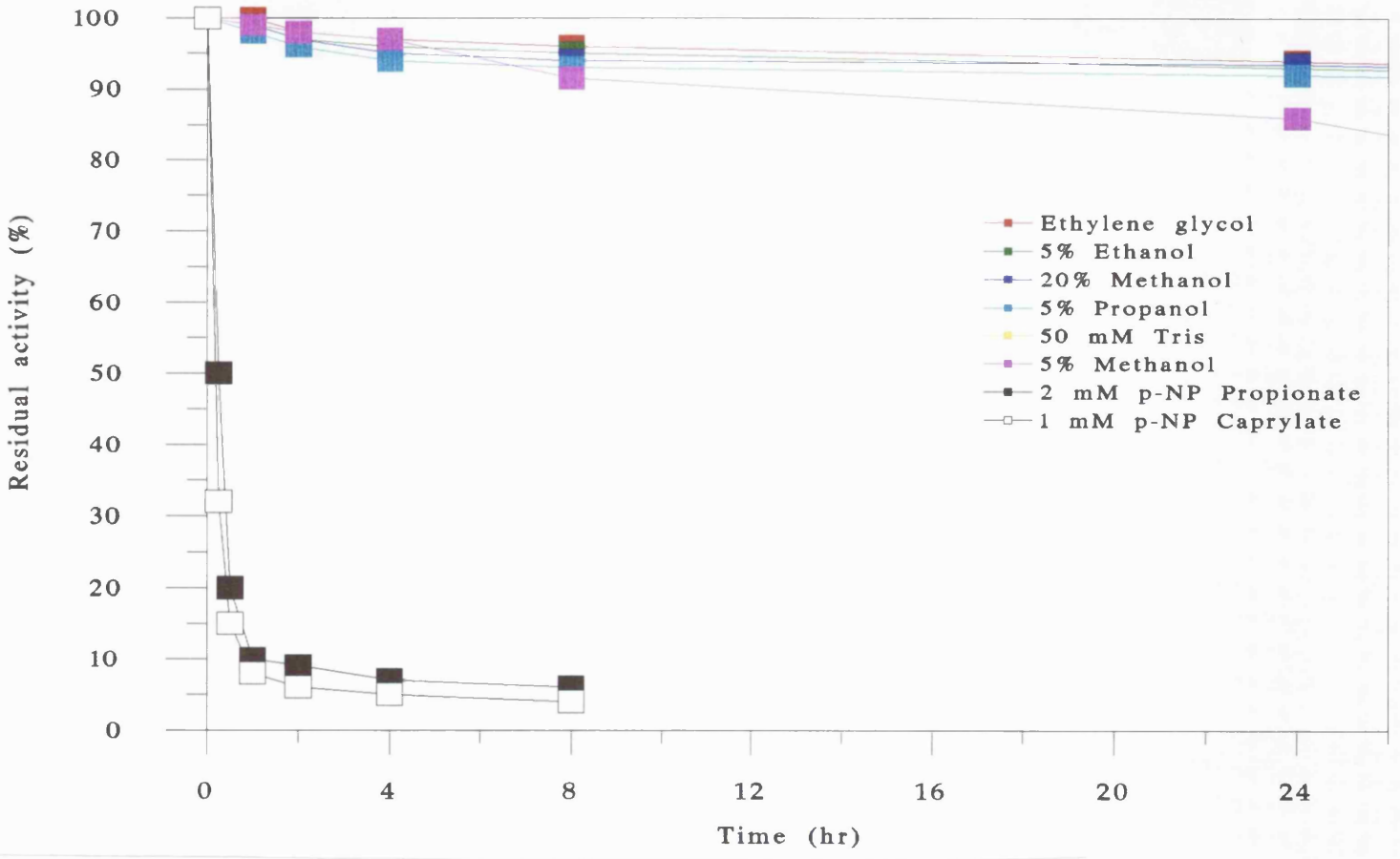
glycol and PEG) (Asther and Meunier, 1990) have been used to prevent the distortion of the active centre of enzymes and improve activity/stability characteristics (Alvaro 1991). Low concentrations of alcohols, which have been observed to act as competitive inhibitors of the Tok19 A1 esterase, enhanced the Tok19 A1 esterase stability at pH 10.0. 5% v/v propanol or ethanol (or 20% v/v methanol) prevented enzyme inactivation during a 24 hour incubation period at pH 10.0 and 25°C (Figure 6.8). These results support previous data which suggest that the esterase has a higher affinity for longer chain alcohols. The esterase also retained over 90% of its initial activity after incubation in 50% of glycerol or ethylene glycol (v/v) for 48 hours at pH 10.0 (Figure 6.8). However, the presence of ester substrates (2 mM *p*-NP propionate or 1 mM *p*-NP caprylate) resulted in a dramatic decrease in enzyme activity over a relatively short incubation period (Figure 6.8). The destabilising effect of *p*-nitrophenyl esters has been described in section 5.1.6.

6.3.2. Immobilisation of the esterase in the presence of Tris buffer

The immobilisation of the esterase was initially conducted in the buffer used for the esterase purification (50 mM Tris-HCl). However, no immobilisation was observed, even when the gel/immobilisation suspension was 1:1 or the pH increased to 10.5.

This failure was attributed to the presence of the Tris salt, the amino groups of which may compete with the enzyme for reaction with the matrix aldehyde groups. The Tris concentration was therefore decreased from 50 mM to 20, 10, 5, 1 and 0.1 mM by dialysis against 50 mM sodium phosphate. When the concentration of Tris was reduced to 10 mM, 25% of the esterase was immobilised after 180 minutes, but only when the Tris concentration was reduced to 1 mM was quantitative immobilisation of the enzyme achieved after 1 hour (Figure 6.9).

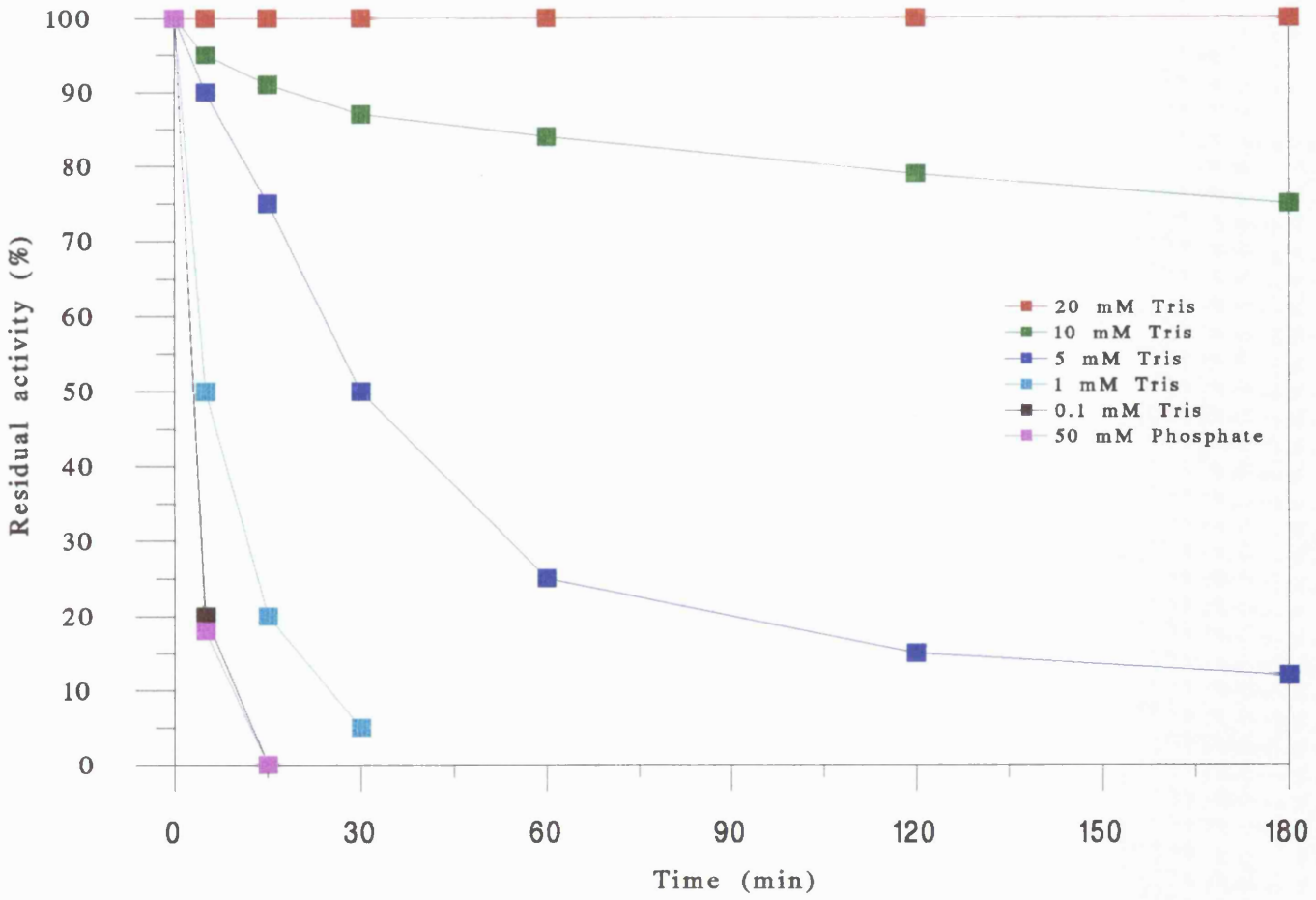
Figure 6.8The effect of solvents and substrates on the stability of the Tok19 A1 esterase at pH 10.0



The esterase was incubated with various concentrations of solvents (v/v) and substrates (mM) for upto 48 hours at pH 10 and 25°C. Aliquots were withdrawn periodically and assayed using the standard assay (section 2.3.1 page 95) except that corrections were made for the solvent carried over in the aqueous phase.

The Tris-HCl content of the esterase samples after purification was reduced by dialysis against 50 mM phosphate buffer pH 7.0 and the esterase was subsequently immobilised to glyoxyl-agarose gels (section 2.8.1 page 112). During the immobilisation aliquots of supernatant were removed and assayed for residual activity using the standard assay (section 2.3.1 page 95).

Figure 6.9 Immobilization of the Tok19 A1 esterase in the presence of Tris-HCl



When multipoint derivatives were required, (with a consequent increase in the immobilisation times) even this low concentration of Tris (1 mM) might compete for the aldehyde groups and decrease the rate of immobilisation. 50 mM phosphate buffer pH 7.0 was therefore substituted for the Tris-HCl buffer in the final enzyme purification step. This substitution had no detectable effect on the purification protocol.

6.3.3 Preparation of single-point immobilised enzyme derivatives

A single-point non-distorted immobilised derivative was required for the substrate specificity trials since pH-stat determination of esterase activity required large quantities of esterase which was impractical.

After the preliminary studies, the esterase was immobilised on glyoxyl agarose using standard conditions designed to generate non-distorted (i.e., limited covalent bonds) enzyme derivatives (Guisan, 1988). These conditions included a low reaction temperature (15°C), and a short reaction time (less than one hour). Two derivatives with different levels of immobilised enzyme were prepared (10 and 50 *p*-NP propionate units of esterase per mL of support). Volume/gel volume ratios of 4:1 (low loading derivative) and 20:1 (high loading derivative) were used respectively. Quantitative immobilisation was achieved in 15 minutes and 50 minutes respectively. Both derivatives were chemically reduced with borohydride after 50 minutes of immobilisation. Activity losses of approximately 10% for the low load derivative and 50% for the high load derivative were determined using the standard immobilisation assay. However, when the substrate concentration in the assay was doubled to 1.0 mM, 90 % of the initial activity could be detected. This indicated that the apparent loss of activity at a substrate concentration of 0.5 mM was partially a consequence of substrate diffusion limitations. Thus, it was

consequence of substrate diffusion limitations. Thus, it was assumed that the immobilized activity of the two derivatives were 9 units mL^{-1} and 45 units mL^{-1} respectively.

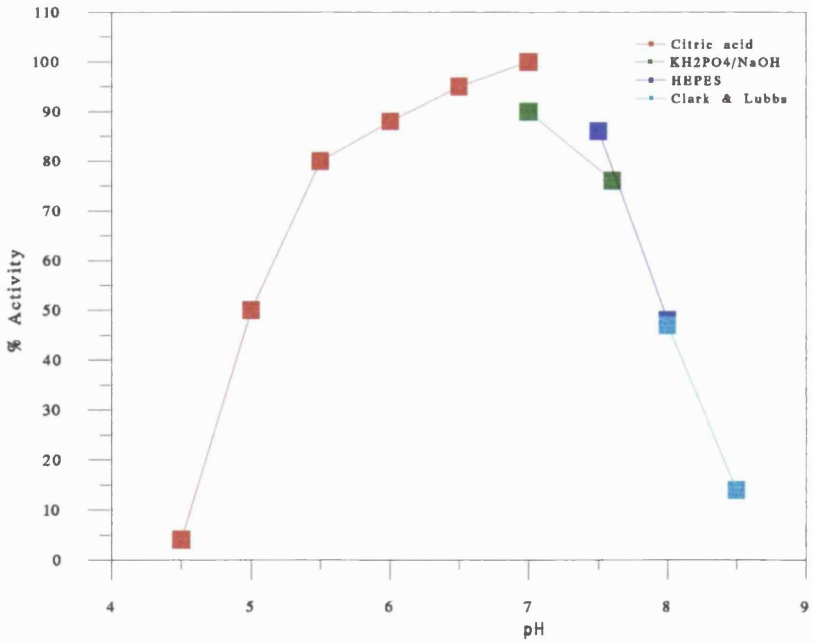
6.4 Characterization of the immobilized esterase

In order to determine whether the single point derivative could be used for substrate specificity studies, its physical properties were characterized and compared with the soluble enzyme.

6.4.1 pH activity profile

The pH profile of the esterase derivative (Figure 6.10) was essentially identical to the pH profile of the soluble enzyme (Figure 5.11) although it had a slightly broader maximum. It was therefore assumed that the esterase catalytic site had not been chemically modified during the immobilization procedure.

Figure 6.10 pH profile for the single point immobilized Tok19 A1 esterase derivative



6.4.2 Stability of the immobilized esterase derivative

The stability of the low-loading derivative with respect to temperature and in the presence of organic cosolvents was compared with that of the soluble enzyme. In both cases, the stability factor was approximately 10 to 12-fold with respect to the soluble enzyme. This suggested that some degree of multi-point covalent attachment was obtained under the reaction conditions, and that this imparted some degree of conformational restriction. The results imply that the deleterious effect of the organic cosolvent was a direct consequence of protein unfolding rather than aggregation.

As the "non-distorted" derivatives and soluble enzyme had essentially identical properties, the "non-distorted" derivatives were used to determine the substrate specificity of the Tok19 A1 esterase.

N.B. The stability factor is defined as the ratio of two half-lives under essentially identical conditions. For example if the half-lives of the soluble enzyme and immobilised enzyme at 70°C and pH 7 were 10 minutes and 120 minutes respectively, the stability factor would be 12.

6.4.3 Derivative activity in organic cosolvents

The addition of low concentrations of alcohols during the spectrophotometric determination of immobilised esterase activity with *p*-NP substrates resulted in the apparent "activation" (based on *p*-nitrophenol release) (see section 6.1). However, experiments carried out using a pH-stat which titrates the liberated acid, showed no enzyme activation in the presence of 0.1-3% propanol. These results suggested that the "activation" observed using the rate of *p*-nitrophenyl release was the result of transesterification reactions, in which the alcohols acted as nucleophiles (see section 6.1).

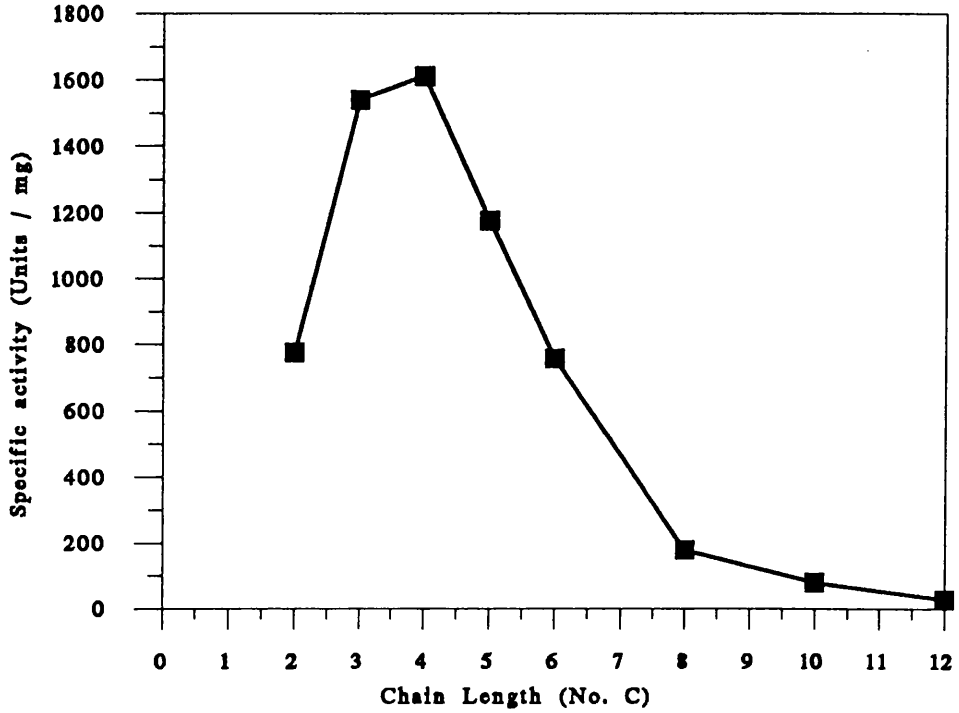
Apparently, the short chain alcohols (methanol-propanol) did not cause any inhibition since the rate of hydrolysis observed in the pH stat experiments was exactly the same as in the absence of alcohol. Thus, the effect of both the rate of esterase hydrolysis and alcoholysis reactions was observed by measuring the spectrometric *p*-nitrophenyl release. Quantitation of the activation induced by 1% v/v propanol indicated that the ratio of transesterification to hydrolysis was greater than one.

6.4 4. Substrate specificity of the immobilised esterase derivative

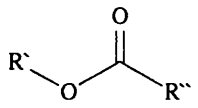
The broad specificity of this esterase with respect to the acyl (acidic) chain of *p*-NP esters has been presented in section 5.1.10. These experiments were repeated (Figure 6.11) using the immobilised enzyme derivatives at 30°C in 10 % (v/v) DMF (see section 2.3.2). Under these conditions, the maximum *p*-NP substrate activity was obtained using *p*-NP butyrate, whilst the *p*-NP caprylate activity was 50% lower than when the soluble enzyme was used. The differences between these results and those previously reported for the soluble enzyme at 50°C might be due to several facts:

- The effect of temperature on enzyme activity (Arrhenius plots have been shown to be different for different substrates (section 5.1.6)).
- The change in specificity might be attributed to a conformation change in the enzyme induced by 10% DMF (section 6.1).
- The procedure for obtaining a single-point esterase derivative was followed (Blanco and Guisan, 1989). Some degree of multipoint attachment may have been achieved which could have resulted in conformational restrictions. It has been suggested that multipoint derivatives resulted in increased rigidity of the esterase and its inability to hydrolyse large substrates (section 6.8).

Figure 6.11. Substrate specificity of the immobilised Tok19 A1 esterase in the hydrolysis of some *p*-NP substrates



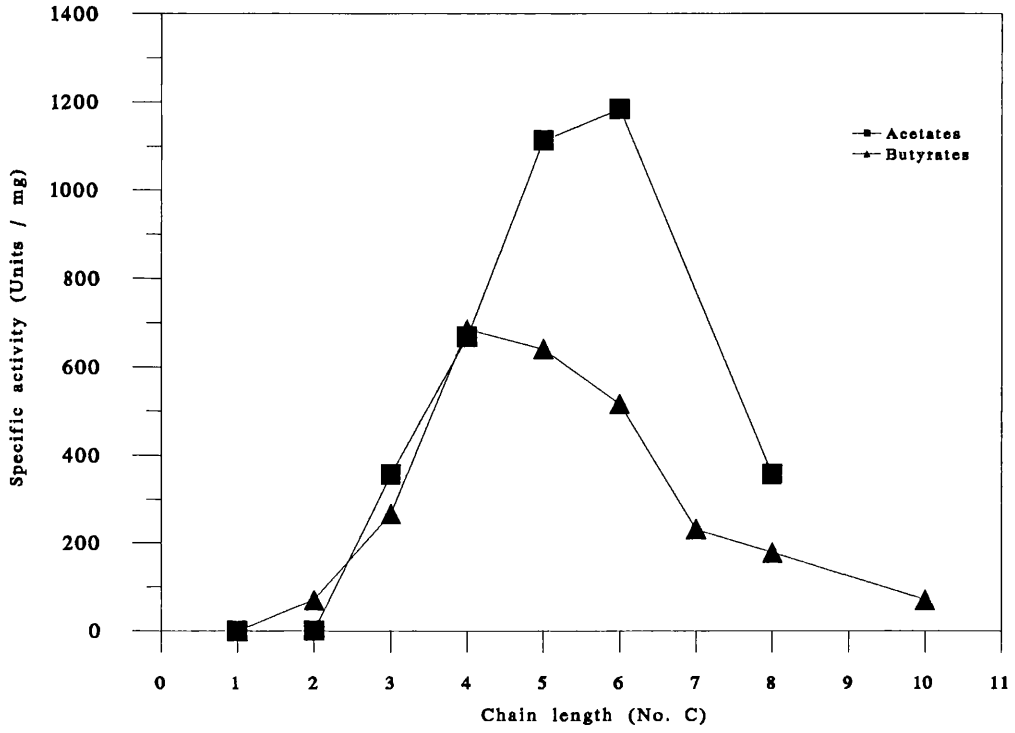
The specificity of the esterase is described in terms of the alcohol (acyl) moiety R` and the acid (carboxyl) moiety R``.



N.B. The structures of the substrates are shown in Appendix 2.

The rate of hydrolysis of aliphatic acetates is presented in Figure 6.12. Methyl and ethyl acetates were not hydrolysed by the enzyme under the assay conditions. When acyl was a linear or iso alcohol an increase in the activity was detected as the length of the acyl chain increased up to C_6. A decrease in the activity was observed with octyl acetate, perhaps as result of steric hindrance or a distortion in the enzyme structure induced by this moderately long and apolar compound.

Figure 6.12 Substrate specificity of the immobilised Tok19 A1 esterase in the hydrolysis of linear acetates and butyrates



Isobutyl and isoamyl acetates had very similar activities to propyl and butyl acetates respectively (Table 6.3) indicating that the methyl branch plays little or no role in the binding of the acyl group to the enzymes' active site.

Primary and secondary butyl acetates were hydrolysed at very similar rates while tertiary butyl acetate was not a substrate for the esterase. This may be due to the steric hindrance induced by the bulky tertiary group (Table 6.3).

Table 6.3 Substrate specificity of the immobilised Tok19 A1 esterase in the hydrolysis of normal and isoacetates

Substrate	R` =	Immobilised esterase derivative (Units mL^{-1} of gel)	Specific activity (Units mg^{-1})
Ethyl	CH_3CH_2	50	0
Propyl	$CH_3CH_2CH_2$	10	356
Butyl	$CH_3CH_2CH_2CH_2$	10	668
Isobutyl	$(CH_3)_2CHCH_2$	10	360
Amyl	$CH_3CH_2CH_2CH_2CH_2$	10	1113
Isoamyl	$(CH_3)_2CHCH_2CH_2$	10	713
Hexyl	$CH_3CH_2CH_2CH_2CH_2CH_2$	10	1184
Octyl	$CH_3CH_2CH_2CH_2CH_2CH_2CH_2CH_2$	10	356

When the alcohol chain length was C_4 and below, the acetate and butyrate activities were very similar (Figure 6.12). The specific activity increased with increasing chain length, reaching a maximum with butyl butyrate. Tok19 A1 esterase was able to hydrolyse ethyl substrates as the size of the carboxyl moiety increased from acetate to butyrate, perhaps as a consequence of the higher affinity of the esterase for butyrate (Table 6.4). Comparisons of the activities of isopropyl, isobutyl and isoamyl butyrates with ethyl, propyl and butyl butyrates (Table 6.4) confirmed that the methyl branch plays little or no role in the binding of the acyl group to the enzymes' active site.

Table 6.4 Substrate specificity of the immobilised Tok19 A1 esterase in the hydrolysis of normal and isobutyrates

Substrate	R` =	Immobilised esterase derivative (Units mL^{-1} of gel)	Specific activity (Units mg^{-1})
Ethyl	CH_3CH_2	50	71
Propyl	$CH_3CH_2CH_2$	10	267
Isopropyl	$(CH_3)_2CH$	50	53
Butyl	$CH_3CH_2CH_2CH_2$	10	685
isobutyl	$(CH_3)_2CHCH_2$	10	303
Amyl	$CH_3CH_2CH_2CH_2CH_2$	10	641
Isoamyl	$(CH_3)_2CHCH_2CH_2$	10	614

The relative binding affinities of the acyl and carboxyl sites can be crudely assessed by comparisons of substrate pairs (e.g. ethyl butyrate and butyl acetate; 71 U mg^{-1} and 668 U mg^{-1} respectively). The importance of "full" occupancy of the acyl (alcohol binding site) is emphasised by the absence of detectable activity with methyl ester derivatives, including short and medium acyl chains, aromatic, branched aliphatic and amino acyl groups (Table 6.5).

Table 6.5 Methyl, ethyl and miscellaneous substrates not hydrolysed by the immobilised esterase

Substrate
Methyl propionate
Methyl butyrate
Methyl isobutyrate
Methyl valerate
Methyl caproate
Methyl heptanoate
Methyl benzoate
Methyl 2-hydroxybutyrate
p-hydroxybenzoic acid methyl ester
p-hydroxybenzoic acid ethyl ester
S(-) chloropropionic acid methyl ester
R(+) chloropropionic acid methyl ester
Mandelic acid methyl ester
L-tyrosine methyl ester
N-acetyl-L-phenylalanine methyl ester
n-butyl *p*-nitrobenzoate
Ethyl *p*-nitrobenzoate
N-acetyl-L-tyrosine ethyl ester
N-benzoyl-L-tyrosine ethyl ester
N-α-benzoyl-L-arginine ethyl ester
Diisooctyl phthalate
Phenyl benzoate
Phenyl salicylate
γ butyrolactone
Triglyceryloleate
4-methyl umbelliferyl propionate**

** The soluble esterase hydrolysed this substrate on native PAGE

Aromatic esters were the favoured substrates for the Tok19 A1 esterase (Table 6.6), the specific activity approached 6,000 and 6800 Units mg^{-1} with phenylacetate and β-naphthyl propionate respectively. Separating the aromatic ring from the alcohol by a methyl group (benzyl acetate) reduced the specific activity by 80%. Acetate esters containing complex aromatic rings (e.g., indoxyl) were also readily hydrolysed. The presence of a para-nitro group on the phenyl ring, (*p*-NP acetate) which might be expected to favour hydrolytic cleavage by electron withdrawal, reduced enzyme activity by nearly 90%. Together these data suggest that the acyl site in the active center of the enzyme is large, hydrophobic and relatively non-specific. The carboxyl site appears to be more extensive than the alcohol site. From the studies with the *p*-NP substrates the acid site also appears to be large and hydrophobic, accommodating n-C_{12} acyl chains. This site appears to be laterally smaller than the alcohol site since it could not accommodate large aromatic groups such as benzoate or *p*-nitrobenzoate (Table 6.5).

Table 6.6 Miscellaneous substrates hydrolysed by the immobilised esterase

Substrate	Immobilised esterase derivative (Units mL^{-1} of gel)	Specific Activity (Units mg^{-1})
phenyl acetate	10	5857
p-NP acetate	10	775
β-naphthyl propionate	10	6765
benzyl acetate	10	1050
indoxyl acetate	10	3187
α-naphthyl acetate	10	4095

The Tok19 A1 esterase was also able to hydrolyse glycidyl butyrates, compounds which are commonly used in the manufacture of β-blockers, optically active pharmaceuticals, agrochemicals and other products.

Tributyrin (2,400 Units mg^{-1}) and tricaproin (89 Units mg^{-1}) were also substrates for the Tok19 A1 esterase (Table 6.7) however, the esterase did not hydrolyse the lipase substrate triglyceryloleate.

Table 6.7 Miscellaneous substrates hydrolysed by the immobilised esterase

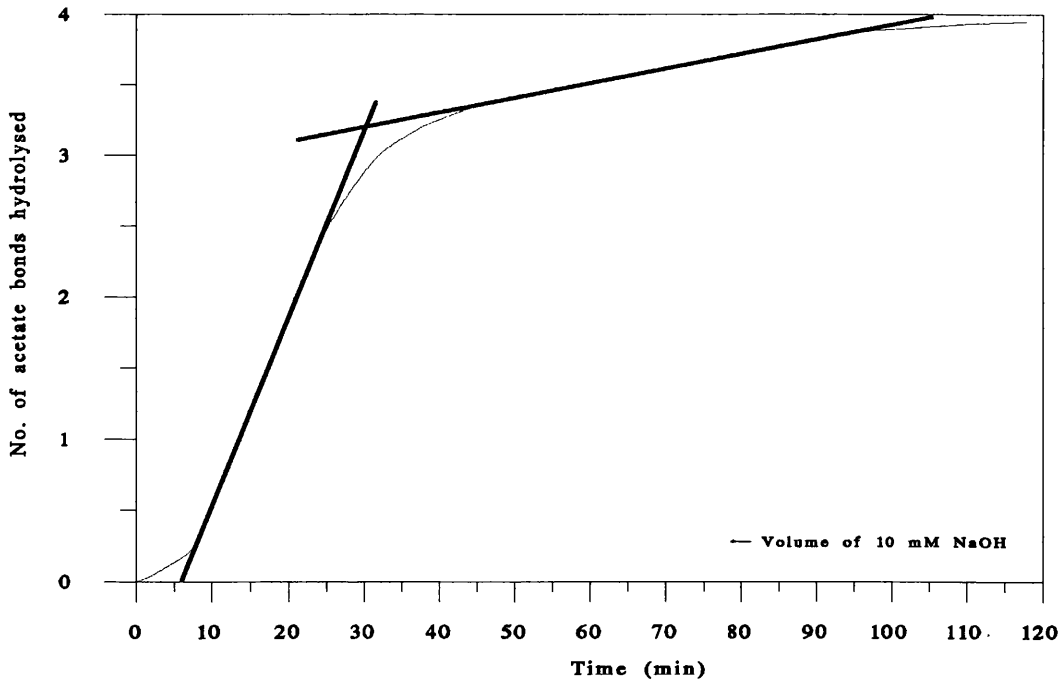
Substrate	Immobilised esterase derivative (Units mL^{-1} of gel)	Specific Activity (Units mg^{-1})
(R/S) glycidyl butyrate	10	445
(R-) glycidyl butyrate	10	490
caproic acid propyl ester	10	1362
caprylic acid n-butyl ester	10	400
secondary butyl acetate	10	800
tributyrin	10	2400
tricaprylin	50	90

Tok19 A1 esterase was able to hydrolyse several acetate derivatives of different sugars (Table 6.8) suggesting that this enzyme could be used to regioselectively deacylate and acylate sugar esters. The exact regiospecificity of the sugar hydrolysis has not been determined. The total hydrolysis of β-D-xylose tetraacetate occurred at two distinct rates with three acetate molar equivalents being released approximately 13 fold faster than the fourth molar equivalent (Figure 6.13). This would provide some potential for kinetic separation of regiospecific products.

Table 6.8 Sugars hydrolysed by the immobilized esterase

Substrate	Immobilized esterase derivative (Units mL^{-1} of gel)	Specific activity (Units mg^{-1})
1,2,3,4,-tetraacetyl-β-D-ribofuranose	50	62
1,2,3,4,-tetra-o-acetyl-β-D-ribopyranose	50	107
β-D-xylose tetraacetate	10	535

Figure 6.13 Total hydrolysis of β-D-xylose tetraacetate



The total hydrolysis of 1 mM β-D-xylose tetraacetate was determined using an autoburette section 2.3.2. The molar equivalent of acetate was calculated from the amount of 10mM NaOH required to neutralize the liberated acetic acid.

6.5 Multipoint covalent immobilisation to aldehyde-agarose gels

Instability of the soluble and single point immobilised esterase in miscible organic solvents might be a possible obstacle to the implementation of the enzyme as an industrial catalyst. As a possible means of enhancing esterase stability in organic solvents a multipoint covalent immobilised derivative was prepared.

6.5.1 The support

As discussed in the introduction (section 1.7.4), a key point in the formation of multipoint covalent derivatives is the "surface contact" between the support and the protein surface. Two different supports were used to investigate the surface contact (glyoxyl 6% and 10% BCL agarose) under otherwise identical conditions. Glyoxyl agarose 10 BCL has smaller pores than 6% agarose, and this may lead to an increase in the surface contact between the enzyme surface and the support.

10% agarose gels gave the highest stability factor (Table 6.9). The residual enzyme activity was approximately the same in both cases. These results agreed with those reported for Penicillin G acylase (Guisan *et al.*, 1993). 10% BCL agarose was therefore chosen for further experimentation.

Table 6.9 The effect of the support

Agarose Gel	Residual Activity (%)	Stability Factor
6% BCL	70	150
10% BCL	75	200

6.5.2 Temperature

The critical step in multipoint covalent attachment is the slow correct alignment between the reactive groups on the support and the reactive groups on the enzyme surface (Blanco and Guisan, 1989).

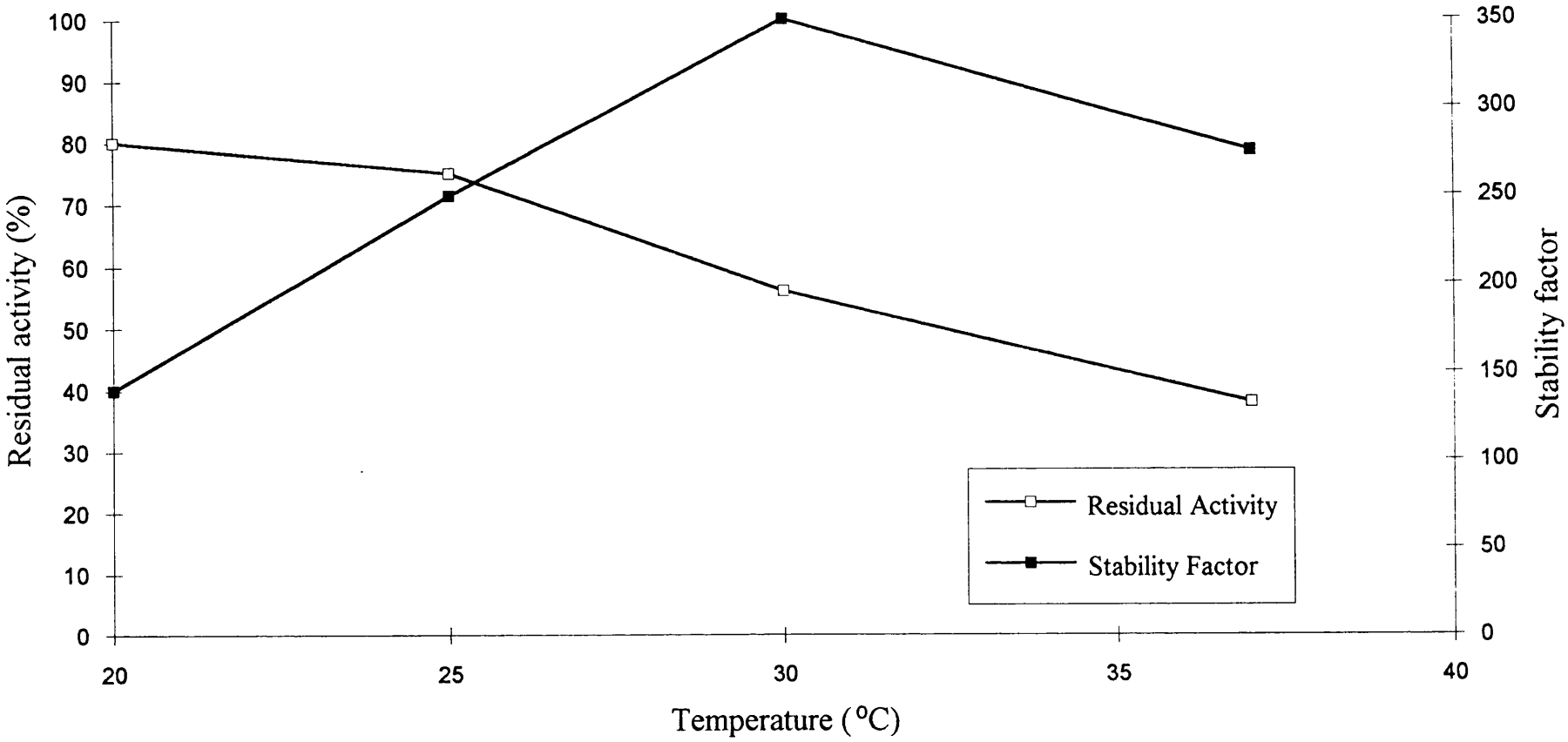
Temperature is one of the most important variables in protein-support multiinteraction, because it controls the "flexibility" of the protein, increasing the potential for new protein-support linkages (Blanco and Guisan, 1989). The influence of the immobilisation temperature on the enzyme derivative properties was investigated using incubation times of 3 hours.

As the temperature was increased from 20°C to 30°C, the stability factor increased (from 140 to 350) while the residual activity decreased from 80% to 60% (Figure 6.14). At 37°C the residual enzyme activity decreased to 35% and the stability factor was 225. Chemical modification of critical amino acids, immobilisation in an unfavourable orientation (limiting access to the active site) or large numbers of enzyme-matrix interactions resulting in a very rigid enzyme conformation could explain the results obtained from immobilisation at 37°C.

Thus, 25-30°C was chosen as the optimal temperature range for the preparation of multipoint enzyme derivatives.

The esterase was immobilised to glyoxyl-agarose gels at pH 10 according to section 2.8.1 page 112 except that the temperature was altered as specified. The immobilised esterase activity was assayed using the standard immobilisation assay (section 2.8.2 page 113). The stability factor of the immobilised esterase was determined by comparing its thermostability with the thermostability of the single point immobilised derivative at pH 7.0 and 70°C.

Figure 6.14 The effect of temperature during the enzyme-support reaction on the activity/stability relationships



6.5.3 Contact time between the enzyme and the activated support

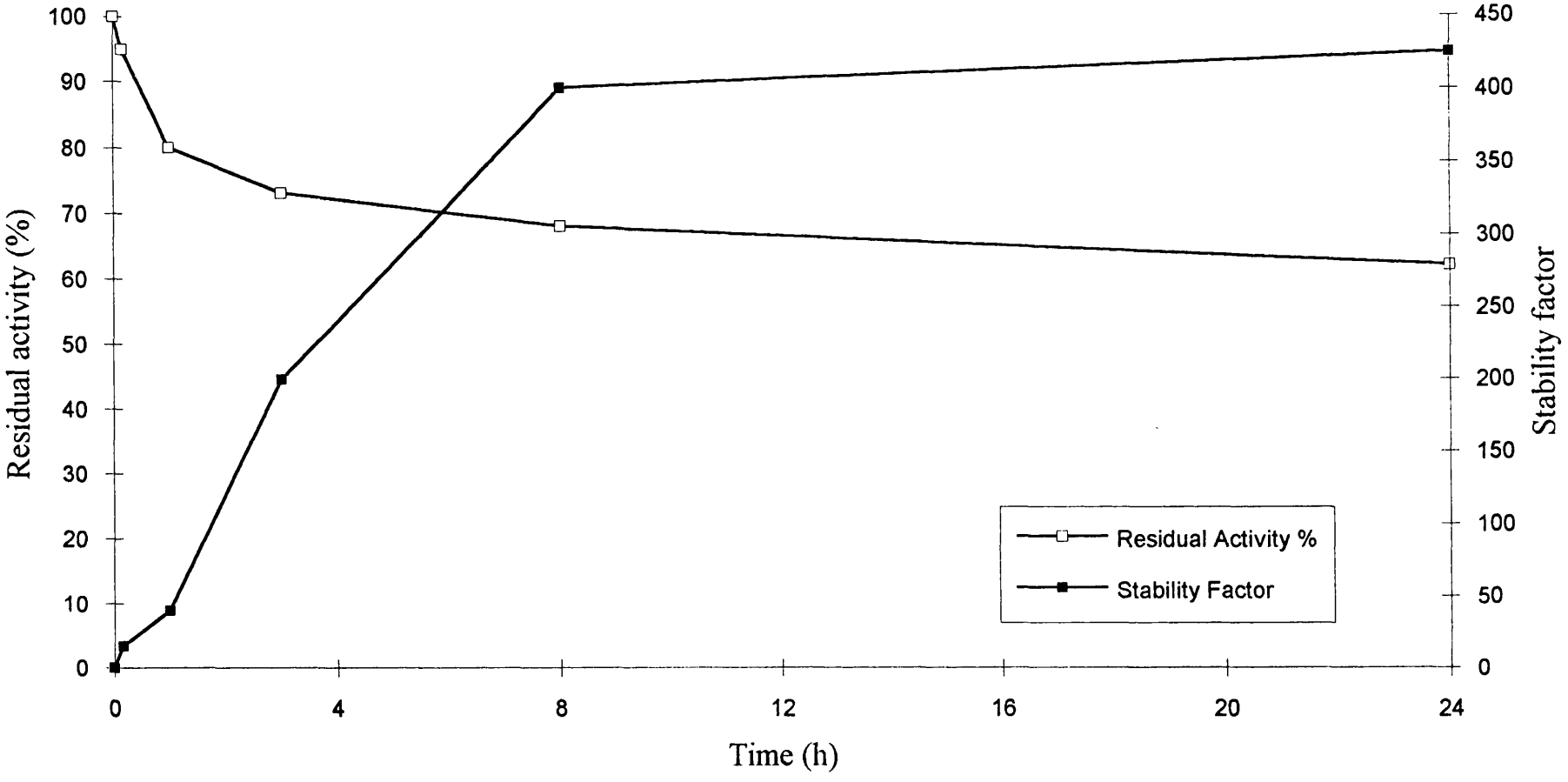
It was previously shown (section 6.2.3) that the esterase was completely immobilized to glyoxyl agarose 10 CBL within 10 minutes under standard conditions (50 mM phosphate buffer, pH 10.05 and 25°C). After 10 minutes the derivative could be reduced (to give the single point immobilisation) or allowed to continue reacting with the remaining active groups, and subsequently reduced with borohydride (to give varying degrees of multipoint immobilisation). Different derivatives were therefore prepared by varying the enzyme-support reaction time between 10 minutes and 24 hours.

The activity and stability characteristics of the resulting immobilized enzyme derivatives are shown in Figure 6.15. Increasing the reaction time increased the immobilized derivative stability (e.g. the stability factor increased from 12 to 400 on increasing the reaction time from 10 minutes to 8 hours, with only a 35% loss in activity). Reaction times longer than 8 hours did not significantly increase the immobilized Tok19 A1 esterase stability and resulted in a further decrease in enzyme activity.

Experiments conducted at 30°C gave poorer activity and stability relationships (e.g., after 3 hours of reaction at 30°C, the residual activity was 60% and the stabilization factor was 350 (Figure 6.14)).

The esterase was immobilised to glyoxyl-agarose gels at pH 10.0 according to section 2.8.1 page 112 except that the enzyme support reaction time was altered as specified. The immobilised esterase activity was assayed using the standard immobilisation assay (section 2.8.2 page 113). The stability factor of the immobilised esterase was determined by comparing its thermostability with the thermostability of the single point immobilised derivative at pH 7.0 and 70°C.

Figure 6.15 The effect of the enzyme-support reaction time on the activity/stability relationships



6.5.4 pH

The effect of pH during immobilization was studied (Figure 6.16). At pH 9.7, Tok19 A1 esterase retained 80% of its initial activity which was higher than in the standard pH (pH 10.0), but was only 140 fold more stable than the single point immobilized esterase. pH values above 10.1 significantly decreased the derivative stability and activity. The decrease in stability and activity under these high pHs might be attributed to chemical modification of critical amino acids, immobilization in an unfavorable orientation (limiting access to the active site) or large numbers of enzyme-matrix interactions resulting in a very rigid enzyme conformation. The "optimal" pH was found to be 10.05.

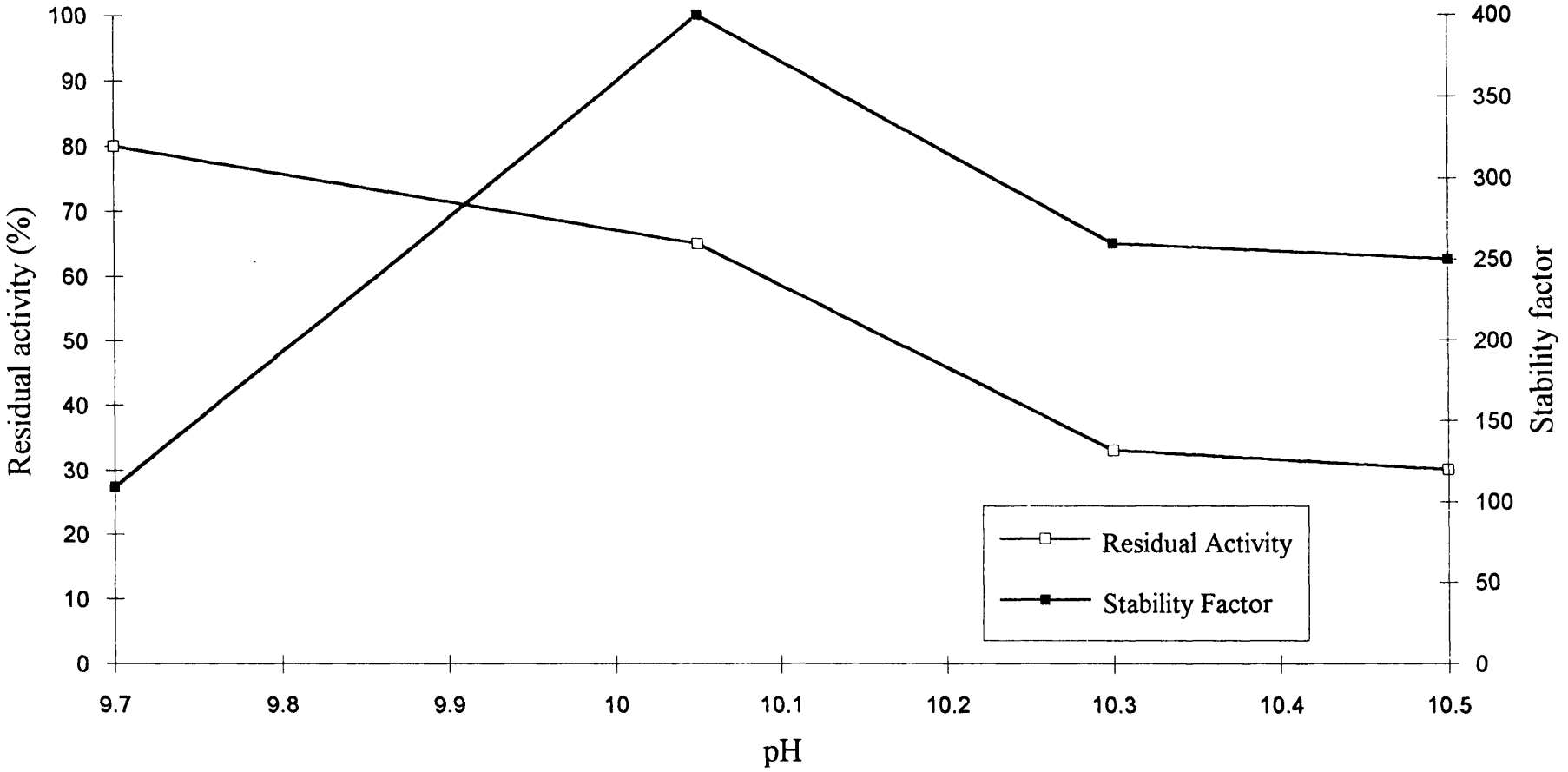
6.5.5 Presence of inhibitors

Several alcohols increased the stability of the native Tok19 A1 esterase at pH 10.0 (Figure 6.8). Figure 6.12 shows that immobilized derivatives prepared in the presence of propanol and butanol retained higher activity (10 and 20% respectively) than in their absence, while the stability factor decreased by 200. Methanol did not stabilise the enzyme structure, the residual activity of immobilized derivatives prepared in its presence decreased by 10%.

Alcohols with moderately long acyl chains protected the enzyme during the immobilization process. However, immobilized derivatives prepared in the presence of these alcohols had a much lower stability factor.

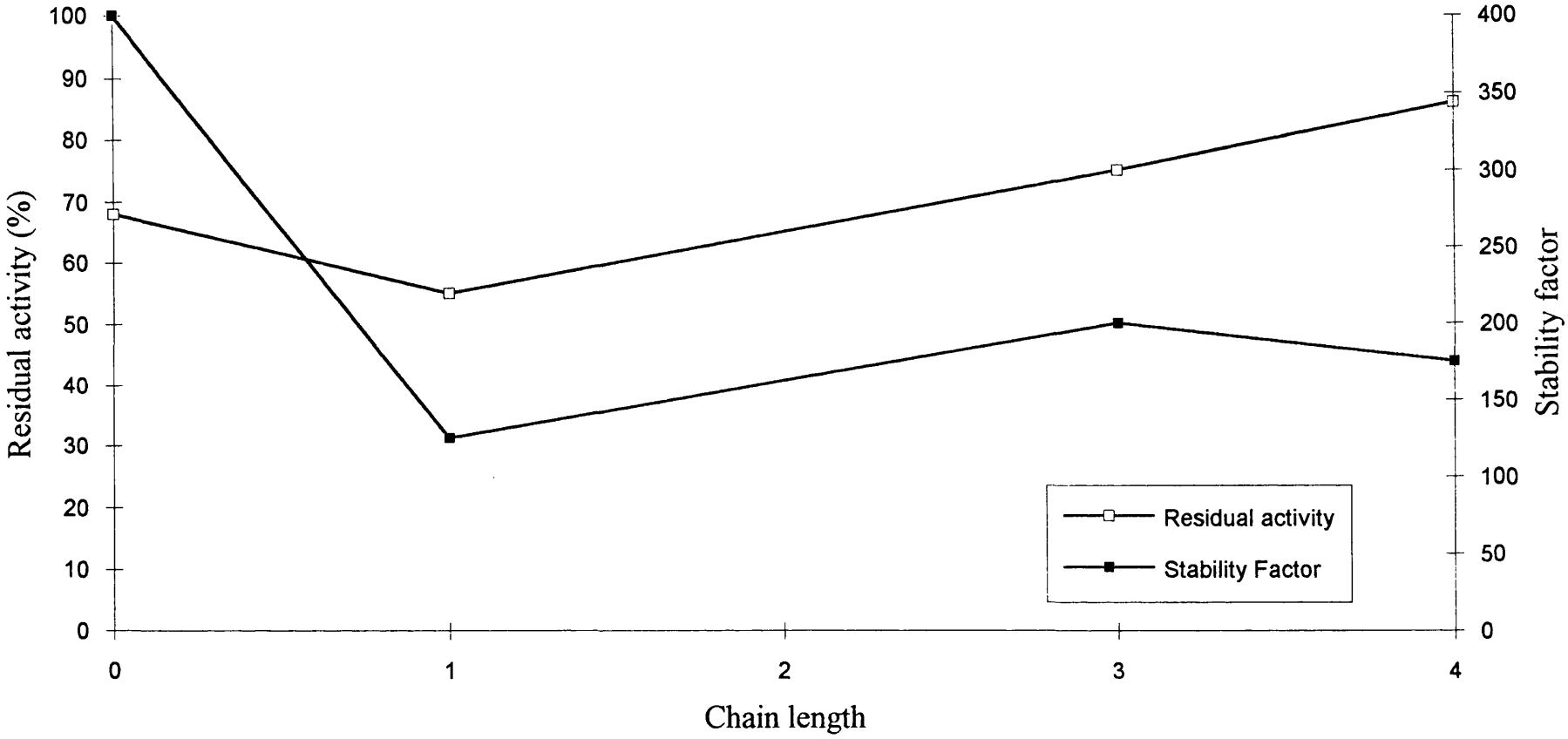
The esterase was immobilised to glyoxyl-agarose gels according to section 2.8.1 page 112 except that the pH was altered as specified. The immobilised esterase activity was assayed using the standard immobilisation assay (section 2.8.2 page 113). The stability factor of the immobilised esterase was determined by comparing its thermostability with the thermostability of the single point immobilised derivative at pH 7.0 and 70°C.

Figure 6.16 The effect of pH during enzyme support reaction on the activity/stability relationships



The esterase was immobilised to glyoxyl-agarose gels according to section 2.8.1 page 112 except that methanol, ethanol, propanol and butanol were added to the immobilisation reaction. The immobilised esterase activity was assayed using the standard immobilisation assay (section 2.8.2 page 113). The stability factor of the immobilised esterase was determined by comparing its thermostability with the thermostability of the single point immobilised derivative at pH 7.0 and 70°C.

Figure 6.17 The effect of alcohols during the enzyme-support reaction on the activity/stability relationships



6.5.6 Optimal reaction conditions

"Optimal conditions" for the preparation of were as follows: agarose 10 BCL, 25°C, 8 hour reaction time, pH 10.05. Whilst retaining 65% of the initial esterase activity, these reaction conditions resulted in an immobilised esterase derivative 600-fold more stable (at pH 7) than the "single point covalent attached derivative".

6.6 pH stability of the "optimal" esterase derivative

Figure 6.18 shows the pH stability profiles of the "optimal derivative" at 70°C. Maximum stability, as shown for the soluble enzyme (section 5.1.8 Figure 5.10), was found at pHs near to neutrality. The stability of the multipoint immobilised esterase derivatives at pH 5 or 9, were 3 and 22 fold lower than at pH 7 respectively, in contrast with the behavior of the soluble enzyme which was 75 and 8 fold lower than at pHs 5 and 9 respectively.

The stability factor of the multipoint derivative at pH 5 was similar to that obtained at pH 7 (Table 6.10). However, aggregation of the soluble enzyme under acid pH and moderately high temperatures meant that the operational stability factor with respect to the soluble enzyme (at a concentration of 2 $\mu g\ mL^{-1}$) was 10,000. At pH 9, the stability factor with respect to the soluble enzyme was much lower (900).

The "optimal" immobilised esterase derivative (section 6.5.6 page 238) was incubated at pH 5.0, 7.0 and 9.0 at 70°C. Aliquots were withdrawn at the specified times and the residual immobilised esterase activity was assayed using the standard immobilisation assay (section 2.8.2 page 113).

Figure 6.18 pH stability profiles for the multipoint derivative at 70 °C

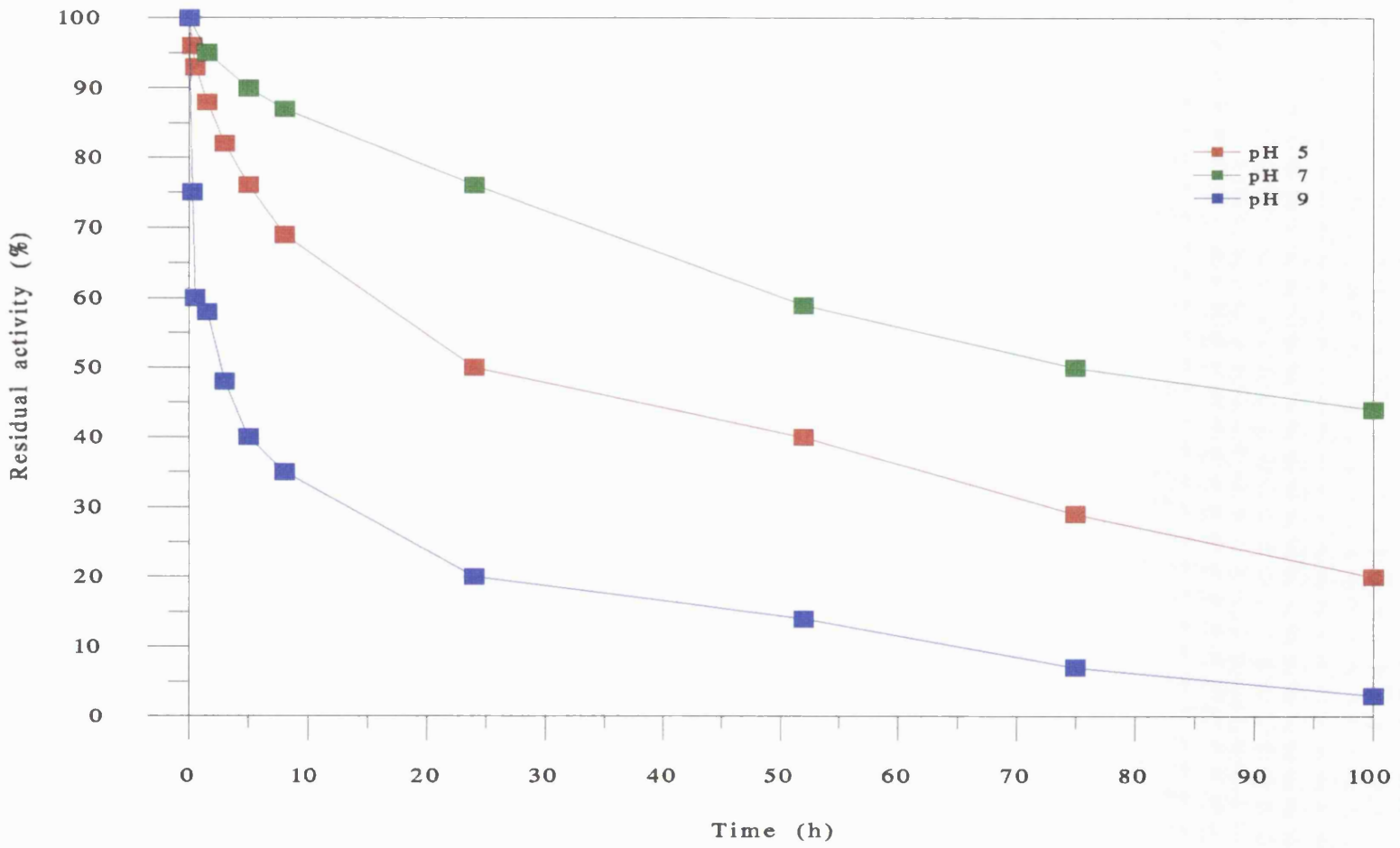


Table 6.10 Stability of "optimal" multipoint derivatives at different pHs

Stability factor of multipoint derivatives		
	with respect to	
pH	("soluble enzyme")	("single point derivative")
9	900	450
7	1,200	600
5	10,000	550

6.7 Stability in organic cosolvents

6.7.1 Alcohols

The effect of several alcohols (which were possible substrates in transesterification reactions) on the stability of the Tok19 A1 esterase derivatives was investigated.

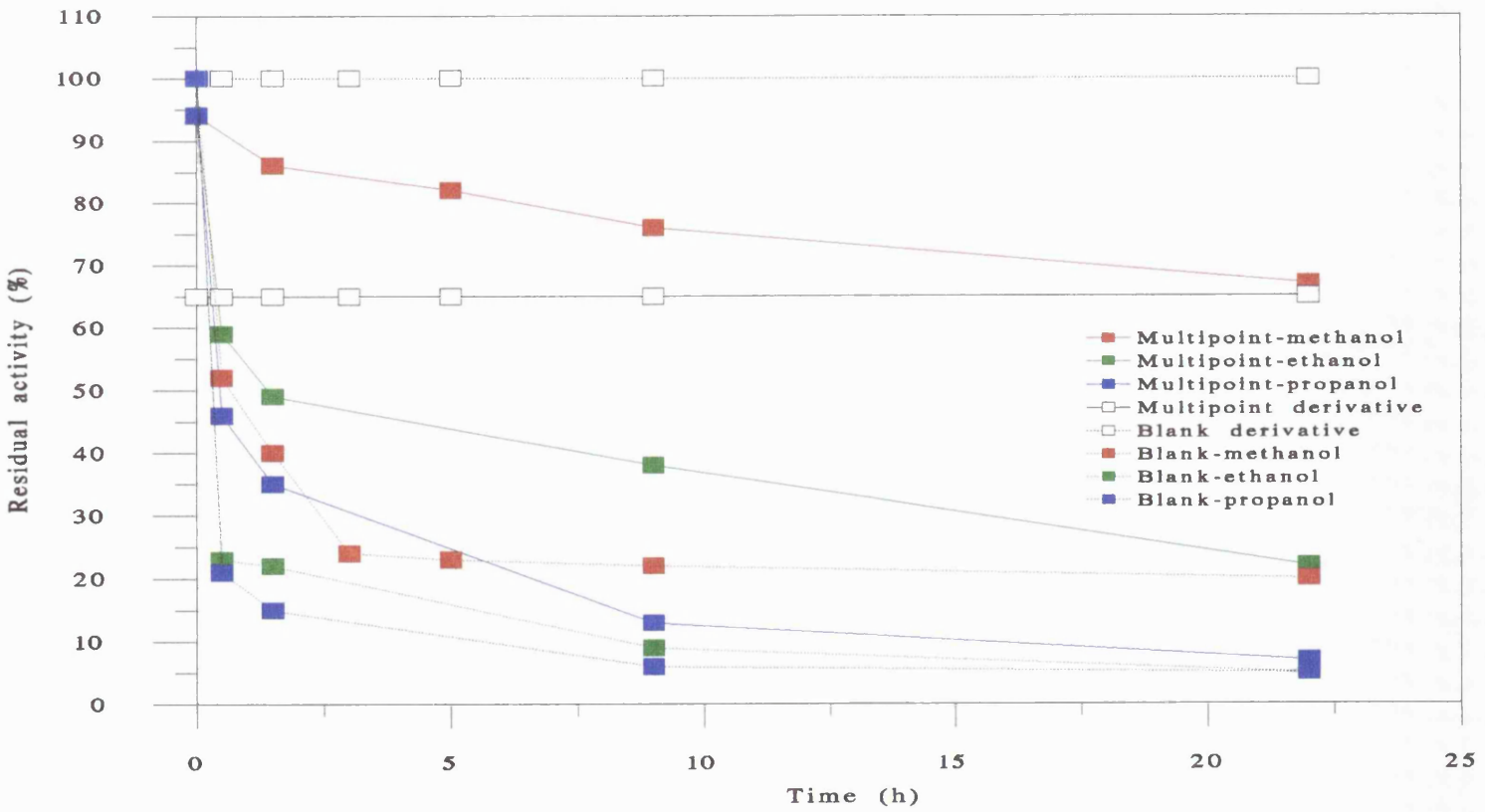
Figure 6.19 shows that the stability of the derivatives at 30°C in these miscible alcohols was poor, except in the presence of 50 % methanol ($t_{1/2}$ > 24 hours). The rate of inactivation was positively correlated with alcohol chain length.

When the Tok19 A1 esterase was immobilised by multipoint covalent attachment, it retained 65% of its activity and was stable in 50 mM Tris-HCl pH 7.0 at 30°C for over 24 hours (Figure 6.19). However, when the activity of the multipoint derivative was assayed (within 15 seconds of the addition of the alcohols) the activity increased from 65% to 94% (Figure 6.19).

The "optimal" immobilised esterase derivative and a blank derivative (section 6.5.6 page 238) were incubated in the pressence of various organic alcohols at a concentration of 50% v/v. Aliquots were withdrawn at the specified times and the residual immobilised esterase activity was assayed using the standard immobilisation assay (section 2.8.2 page 113).

N.B. a blank derivative is an immobilised derivative that has been immobilised in exactly the same conditions as the immobilised derivative that it is being compared to except that the glyoxyl-agarose gel was completely reduced with sodium borohydride before immobilisation.

Figure 6.19 Stability of the multipoint derivative in the presence of alcoholic cosolvents



□ Residual activities of the multipoint and blank derivatives after incubation in 50 mM Tris-HCl pH 7.0 at 30°C in the absence of solvent

This increase in activity of the multipoint derivative was unexpected since multipoint covalent attached enzyme derivatives are usually more insensitive to changes in the medium (Guisan *et al.*, 1991). The "blank derivative" prepared under essentially identical conditions showed no increase in activity. The increase in activity of the multipoint derivative was approximately equal to the activity lost during the immobilisation. This result suggested than the increased activity of the stabilised derivative may have resulted from a conformational change induced by the presence of high concentrations of organic cosolvents. The esterases' structure may have been altered from the partially distorted structure (produced by multipoint covalent attachment) to a new enzyme structure with an activity close to that of the native enzyme.

6.7.2 Other miscible organic solvents

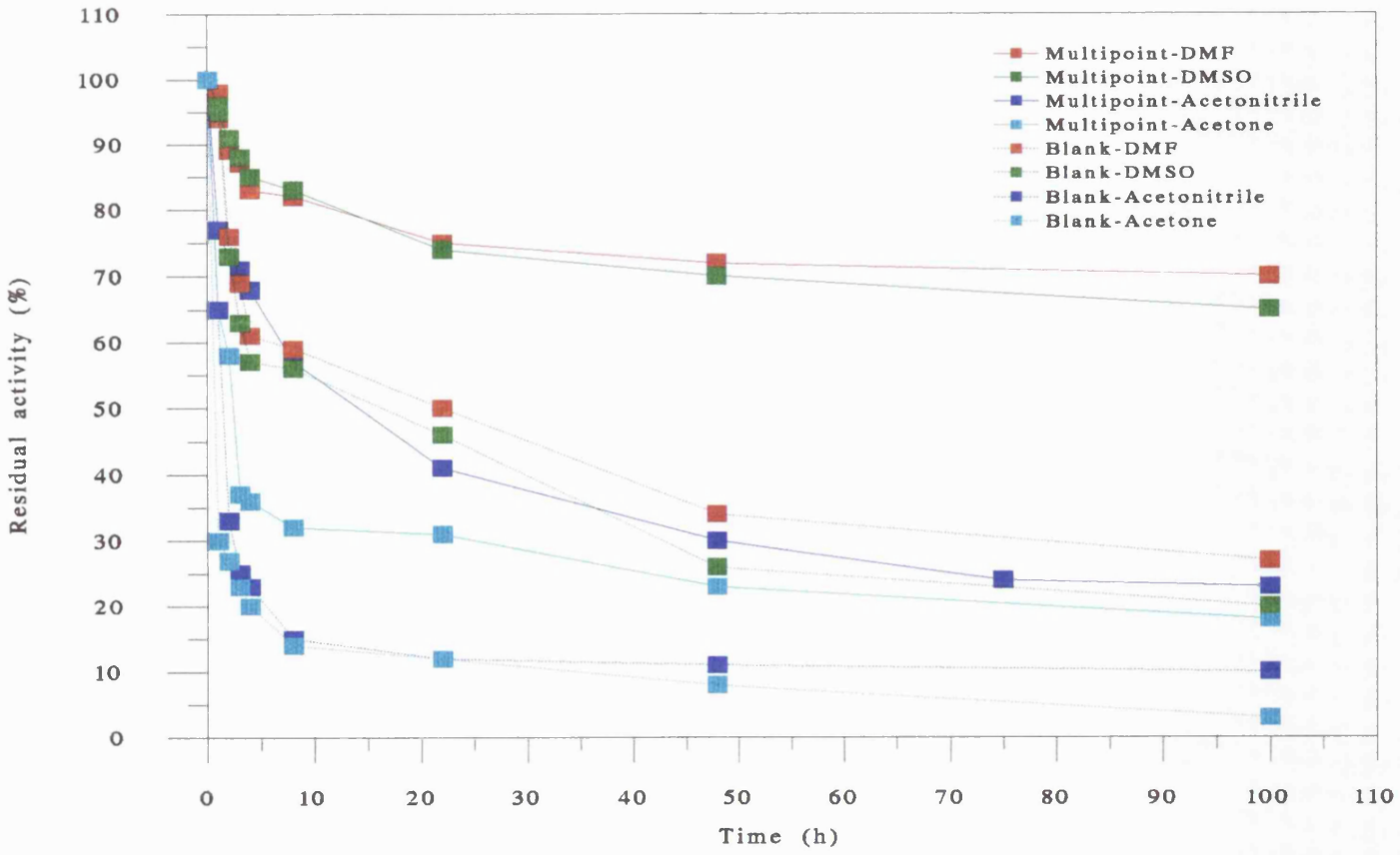
The multipoint immobilized derivative retained nearly 70% of its initial activity after incubation in 50% DMF or DMSO for 7 days (Figure 6.20). Acetonitrile and acetone had a more deleterious effect on the enzyme activity, the half-lives being 15 and 2 hours respectively.

The results above indicate the importance of choosing the correct cosolvent for organic solvent biocatalysis. For example, the "blank" and single-point derivative were more stable when they were incubated in 50% DMF than when the multipoint derivative was incubated in 50% acetonitrile, acetone and propanol. DMF and DMSO have proved useful solvents in biosynthesis, they are able to increase the pKs of carboxylic acid, a very important parameter in thermodynamically controlled synthesis (Fernandez-Lafuente *et al.*, 1991) and DMSO has also been used to improve the stereoselectivity of esterase-catalysed hydrolysis (Bjorkling *et al.*, 1986).

The "optimal" immobilised esterase derivative and a blank derivative (section 6.5.6 page 238) were incubated in the pressence of various non-alcoholic solvents at a concentration of 50% v/v. Aliquots were withdrawn at the specified times and the residual immobilised esterase activity was assayed using the standard immobilisation assay (section 2.8.2 page 113).

N.B. a blank derivative is an immobilised derivative that has been immobilised in exactly the same conditions as the immobilised derivative that it is being compared to except that the glyoxyl-agarose gel was completely reduced with sodium borohydride before immobilisation.

Figure 6.20. Stability of the multipoint derivative in the presence of non-alcoholic cosolvents



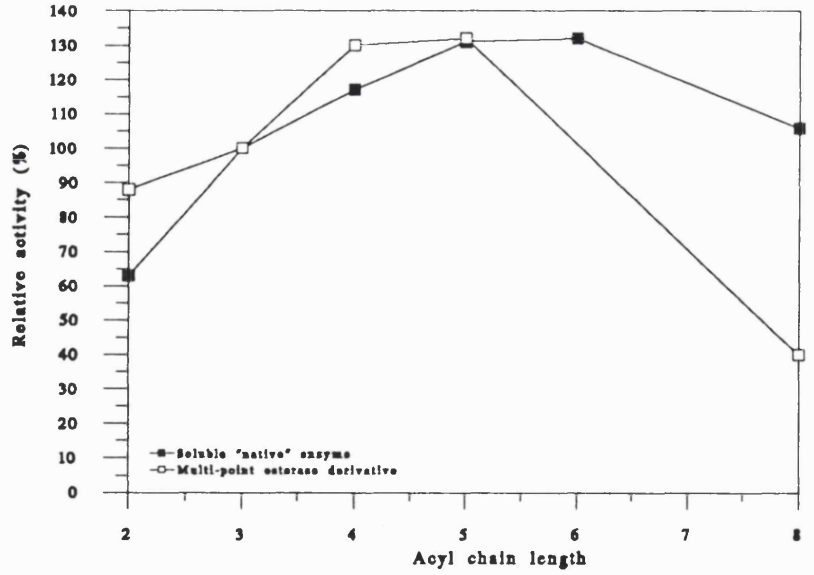
For experimental details see section 2.8.4.

6.8 Reducing enzyme conformational flexibility by multipoint covalent immobilisation

A highly stabilised multipoint derivative (immobilised for 30 hours at 30°C, compared with 8 hours at 25°C) was prepared. The multipoint derivative was approximately 830 fold more stable to thermal denaturation than the "blank derivative" at 72°C.

Figure 6.21 shows a comparison of the relative activities of the soluble esterase and the multipoint immobilised esterase derivative on C2 to C8 *p*-nitrophenyl ester substrates. The data suggests that the substrate specificities are not identical. For example, where the activities of the soluble esterase and multipoint immobilised esterase derivative were normalised (by selecting the activity to *p*-NP propionate as 100%), the ratios of the C3/C8 activities were 1 and 2.5 respectively. The decreased specificity for *p*-NP caprylate might be explained in terms of the increased rigidity of the esterase and its inability to accommodate this large substrate.

Figure 6.21 Relative activity of soluble "native" enzyme and multipoint esterase derivative



6.9 Conclusions

Enzymes from micro-organisms have numerous industrial, medical and environmental applications. These applications often involve subjecting the enzymes to extremes of pH and temperature, denaturants such as organic solvents and detergents as well as increased ionic strength and pressure. Hence there is a continual need to isolate micro-organisms and enzymes which can function under these conditions.

The potential of thermophilic micro-organisms and enzymes, and the advantages of microaqueous solvent and high temperature industrial bioconversions in biotechnology are clearly recognised Tables 1.1 and 1.4. During the past few years the numbers of commercially available thermophilic enzymes has increased substantially (Table 1.5).

The inherent stability of thermophilic enzymes in organic solvents and denaturants such as urea and SDS, coupled with the ability of some enzymes to conduct stereoselective and novel reactions in microaqueous organic solvents, makes them attractive for industrial processes.

However, there is a conflict between stability and activity. For example, an enzyme from a hyperthermophile may have excellent stability at 20°C but its activity at that temperature will generally be very low. The ideal enzyme would combine thermostability and an inherent stability towards denaturation by organic solvents, pH and denaturants such as urea and SDS with a high activity at low temperatures.

The thermophilic esterase from *Bacillus* strain Tok19 A1 was purified 5133-fold to electrophoretic homogeneity and contained three iso-forms, separable only by isoelectic focusing and native PAGE. Size-dependent chromatographic properties were identical, no heterogeneity was detected in the first 40 N-terminal residues, and kinetic and stability data were consistent with a single active site. It was concluded that the three

forms differed only in a limited number of non-essential residues. For subsequent use of this enzyme as an industrial catalyst, further separation of these forms must be considered uneconomic, and the purification could be terminated after the phenyl-Sepharose step.

This esterase fulfilled some of the criteria necessary for effective application in biotransformations, including: good thermostability and high activity at mesophilic temperatures. At 50°C between pH values of 5.5 and 8.5, no activity loss was observed over long periods. A decrease in stability below the pI of the esterase could be correlated with the precipitation of the enzyme rather than with inherent pH instability. Despite a "temperature optimum" at over 60°C, the specific activity at 30°C was very high (2032 *p*-NP caproate Units mg^{-1}). The high specific activity of the esterase, with a broad range of substrates (Tables 5.5, 5.6, 6.3, 6.4, 6.6, 6.7 and Figures 6.11, 6.12) is a further potential advantage.

The activity of the esterase in low concentrations of organic solvents was very high. The Tok19 A1 esterase was very stable in immiscible organic solvents and was able to catalyse transesterification reactions with a number of primary and secondary alcohols.

The Tok19 A1 esterase immobilised rapidly to glyoxyl agarose gels with little loss of activity and a significant increase in functional stability.

While single point immobilisation does not increase the rigidity of the enzyme structure, it may modify operational stability by preventing protein-protein interactions. A comparison of the soluble and single-point immobilised enzymes showed that the low stability of the esterase at pHs below its isoelectric point was due to aggregation rather than protein unfolding. In contrast, multipoint covalent attachment increased the stability of the enzyme in denaturing conditions which induced both aggregation and protein unfolding. The multipoint immobilised esterase derivative showed enhanced stability in

organic solvents, retaining 70% of initial activity in 50% DMSO and DMF v/v after 7 days.

The improved thermostability, pH stability and solvent stability of the multipoint immobilised enzyme in the presence of organic cosolvents suggested that multipoint immobilised esterase derivatives may be appropriate as industrial catalysts for biotransformation processes.

The apparent differences in substrate specificity between a highly stabilised multipoint immobilised derivative and the native enzyme supported the suggestion that multipoint immobilisation enhances the overall conformational rigidity of the enzyme structure.

The immobilisation protocol used in this study yielded much better activity/stability relationships than other immobilisation methods used previously with other thermophilic enzymes (Nanalov *et al.*, 1993; Wilson *et al.*, 1993).

References

Adlercreutz, P. (1991) Eur. J. Biochem **199**, 609-614

Adlercreutz, P. and Clapes, P. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria L21

Ahern, T.J. and Klibanov, A.M. (1985) Science **228**, 1280-1284

Ahern, T.J. and Klibanov, A.M. (1988) Methods Biochem. Anal. **33**, 91-127

Ahern, T.J. Casal, J.I., Petsko, G.A. and Klibanov, A.M. (1987) Proc. Natl. Acad. Sci. USA **84**, 675-679

Akita, H., Umezawa, I., Nozawa, M. and Hagumo, S. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria P103

Alichanidis, E., Wrathall, J.H.M. and Andrews, A.T. (1986) J. Dairy Res. **53**, 259-264

Allada, S.R. (1984) Ind. Eng. Chem. Process Des. Dev. **23**, 344-348

Allen, K.N. and Abeles, R.H. (1989) Biochemistry **28**, 135-140

Alvaro, G. Fernandez-Lafuente, R. Blanco, R.M. and Guisan, J.M. (1991) Enzyme Microb. Technol. **13**, 1-5

Amelunxen, R.E. and Lins, M. (1969) Arch. Biochem. Biophys. **125**, 765-770

Amelunxen, R.E. and Murdock, A.L. (1978) Crit. Rev. Microbiol. **6**, 343-393

Amelunxen, R.E. and Singleton, R. (1976) In: Enzymes and Proteins from Thermophilic Microorganisms, (Ed., H. Zuber) Birkauser Verlag, Basel, pp 107

Amelunxen, R.E. (1966) Biochim. Biophys. Acta. **122**, 175-181

Amartey, S.A., Leak, D.J. and Hartley, B.S. (1991) Biotech. Lett. **13**, 621-626

Ampon, K., Basri, M. Salleh, A.B., Wan-Yunus, W.Z. and Razak, C.N.A. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria P130

Antonian, E. (1988) Lipids **23**, 1101-1106

Arnold, F.H. (1990) Trends in Biotechnol. **8** 244-249

Asada, M., Hamaguchi, S., Kutsuki, H., Nakamura, Y., Takahashi, H., Takahara, K., Shimada, Y., Ohashi, T. and Watanabe, K., European Patent Application. Application number 86104352.9, Publication number 0197474

Asakura,T. (1978) J. Biol. Chem. **253**, 6423-6429

Asther, M. and Meunier, J-C, (1990) Enzyme Microb. Technol. **12**, 902-905

Atkinson, A., Evans, C.G.T. and Yao, R.G. (1975a) J. Appl. Bacteriol. **38**, 301

Atkinson, A., Banks, G.T., Bruton, C.J., Comer, M.J., Jakes, R., Kamalagharan, T., Whitaker, A.R., and Winter, G.P., (1975b) J. Appl. Biochem. **1**, 247

Baker, H., Hunter, S.H. and Sobotka, H. (1955) Ann. N.Y. Acad. Sci. **62**, 349-379

Baker, H., Frank, O., Pather, I., Black, B., Hunter, S.H. and Sobotka, I. (1955) Can. J. Bacteriol. **124**, 279-285

Baker, H., Frank, O., Pasher, I., Black, B., Hunter, S.H. and Sobotka, I. (1960) Can. J. Microbiol. **6**, 557-563

Barker, D.L. and Jencks, W.P. (1969) Biochemistry **8**, 3879-3889

Baross, J.A. and Deming, J.W. (1983) Nature **303**, 423-426

Barton, M.J., Hamman, J.P., Fichter, K.C. and Calton, G.J. (1990) Enzyme Microb. Technol. **12**, 577-583

Bigelow, C.C. (1967) Theroet. Biol. **16**, 187-211

Bjorkling, F., Boutelje, J., Gatenbeck, S., Hult, K., Norin, T. and Szmulik, P. (1986) Bioorg. Chem. **14**, 176-181

Blaber, M., Lindstrom, J.D., Gassner, N., Xu, J., Dirk, W.H. and Matthews, B.W. (1993) Biochemistry **32**, 11363-11373

Blackwood, A.D., Moore, B.D. and Halling, P.J. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria P18

Blanco, R.M. and Guisan, J.M. (1988) Enzyme Microb. Technol. **10**, 227-232

Blanco, R.M. and Guisan, J.M. (1989) Enzyme Microb. Technol. **11**, 360-366

Blanco, R.M., Calvete, J.J and Guisan, J.M. (1989) Enzyme Microb. Technol. **11**, 353-359

Blanco, R.M., Rakels, J.L.L., Guisan, J.M. and Halling, P.J. (1992) Biochim. Biophys. Acta **11**, 56 67-70

Bloomer, S., Adlercreutz, P. and Mattiasson, B. (1992) J. Am. Oil. Soc. **69**, 966-973

Bockerhoff, H. (1970) Biochim. Biophys. Acta **212**, 92-101

Bott, K.F. (1971) J. Bacteriol. **108**, 720-732

Botts, J. and Morales, M. (1953) Trans. Faraday Soc. **49**, 696-701

Bourquelot, E. and Brindel M. (1911) J. Pharm. Chem. **4**, 385-390

Boutelje, J., Hjalmarsson, M., Hult, K., Lindback, M. and Norin, T. (1988) Bioorg. Chem. **16**, 364-375

Bossetti, A., Bianchi, D. Cesti, P. and Golini, P. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria P15

Bovara, R., Carrea, G., Ottolina, G. and Riva, S. (1993) Biotech. Lett. **15**, 169-174

Bradbury, S.J. and Jakoby, W.B. (1972) Proc. Natl. Acad. Sci. USA **69**, 2373-2378

Bradford, M. M. (1976) Anal. Biochem. **72**, 248

Brady, L. and Brzozowski, A.M. (1990) Nature **343**, 767-770

Brandts, J.F. (1967) in: Thermobiology Chapter 1 (Ed., A.H. Rose) Academic press, London, p 25

Brink, L.E.S. and Tramper, J. (1985) Biotechnol. Bioeng. **27**, 1258-1269

Brock, T.D. (1967) Science **158**, 1012-1016

Brock, T.D. (1978) in Thermophilic Microorganisms and Life at High Temperatures (Ed., M.P. Starr) Springer-Verlag, New York pp 92-179

Brockerhoff, H. (1968) Biochim. Biophys. Acta **159**, 296-303

Brockerhoff, H. (1973) Chem. Phys. Lipids **10**, 215-222

Brockman, H. L. and Law, J.H. and Krezdy, F.J. (1973) J. Biol. Chem. **248**, 4965-4970

Brzozowski, A.M., Derewenda, U., Derewenda, Z.S., Dodson, G.G., Lawson, D., Turkenburg, J.P., Bjorkling, F., Huge-Jensen, B., Patkar, S.A. and Thim, L. (1991) Nature **251**, 491-494

Bruce, L.J. and Daugulis, A.J. (1991) Biotechnol. Prog.**7**, 116-124

Buchanan, R.E. and Gibbons, N.E. (1975) The genus *Bacillus*, in Bergey's Manual of Determinative Bacteriology, 8th ed., pg. 529

Buonocore, V., Sgambati, O., De Rosa, M., Esposito, E., and Gambacorta, A. (1980) J. Appl. Biochem. **3**, 390

Burley, S.K. and Petsko, G.A. (1988) Adv. Prot. Chem. **39**, 125-186

Butler, L.G. (1979) Enzyme Microb. Technol. **1**, 253-259

Cambou, B. and Klibanov, A.M. (1984a) J. Am. Chem. Soc. **106**, 2687-2692

Cambou, B. and Klibanov, A.M. (1984b) Biotechnol. Bioeng. **26**, 1449-1454

Campbell, L.L., Jnr. and Williams, O.B. (1953) J. Bact. **65**, 141-145

Carrea, G. Ottolina, G. Riva, S. and Secundo, F. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria L6

Carrington, T.R. (1971) Proc. R. Soc. London, Ser. B **179**, 321-334

Cassels, J.M. and Halling, P.J. (1988) Enzyme Microb. Technol. **10**, 486-491

Castro, M.J.M. and Cabral, J.M.S. (1988) Biotech Adv. **6**, 151-167

Cesti P., Zaks, A. and Klibanov, A.M. (1985) Appl. Biochem. Biotechnol. **11**, 401-406

Chahid, Z., Montet, D., Pina, M. and Graille, J. (1992) Biotechnol. Lett. **14**, 281-284

Chan, C., Cox, P.B. and Roberts, S.M. (1990) Biocatal. **3**, 111-118

Cheetham , P.S.J. (1993) TiBTech. **11**, 478-488

Chen, C.-S. and Sih, C.J. (1989) Angew. Chem. Int Ed. Eng. **28**, 695-707

Chen, C.-S., Liu, Y.-C. and Marsella (1990) J. Chem. Soc. Perkin. Trans. **I** 2559-2561

Clark, D.S. and Bailey, J. E. (1984) Biochim. Biophys. Acta **788**, 181-188

Clarke, S. (1985) Annu. Rev. Biochem. **54**, 479-506

Cleverdon, R.C., Pelczar, M.J. and Doetsch, R.N. (1949) J. Bacteriol. **58**, 523-526

Cotterill, I.C., Macfarlane, E.L.A. and Roberts, S.M. (1988) J. Chem. Soc. Perkin Trans. **1**, 3387-3389

Cowan, D.A., Daniel, R.M. and Morgan, H.M. (1982) Chem. N.Z. **46**, 122-125

Cowan, D.A., Daniel, R.M. and Morgan, H.M. (1985) Trends Biotechnol. **3**, 68-72

Cowan, D.A., Daniel R.M. and Morgan H.W. (1987) Int. J. Biochem. **8**, 741-743

Cowan, D.A. (1991) Enzymes from Thermophilic Archaebacteria:Current and Future Applications in Biotechnology Biochem. Soc. Symp. 58, 149-169

Craik, G.S., Largman, C., Fletcher, T., Roczniak, S., Barr, P.J., Fletterick, R. and Rutter, W.J. (1985) Science **228**, 291-297

Cuperus, F.P., Bouwer, S.Th., Derksen, J.T.P. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria P13

Daniel, R.M., Cowan, D.A., Morgan, H.W. and Curran, M.P. (1982) Biochem. J. **207**, 641-644

Daron, H.H. (1973) J. Bacteriol **116**, 1096-1100

Dawson, R.M.C. (1969) Methods Enzymol. **14**, 633-648

Dawson, R.M.C., Elliot, D.C., Elliot, W.H. and Jones, K. M. (1986) Data for Biochemical Research, 3rd ed. Oxford University Press p.316

De Amici, M., De Micheli, C., Carrea, G. and Spezia, S. (1989) J. Org Chem. **54**, 2646-2650

de Goede, A.T.J.W., Bencckhuijsen, W., van Rantwijk, F., Matt. L., and Van Bekkum, H. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria L20

Deetz, J.S. and Rozzell, J.D. (1988) TiBTech **6**, 15-19

Deranleau, D.A. and Zuber, H. (1977) Int. J. Pept. Protein Res. **9**, 243-249

Derewenda, Z.S. and Sharp, A.M. (1993) TiBS **18**, 21-25

Derewenda, U., Brozozowski, A.M., Lawson, D. and Derewenda, Z.S., (1992) Biochemistry **31**, 1532-1541

Desnuelle, P., Sarda, L. and Ailhaud, G. (1960) Biochim. Biophys. Acta **37**, 570-571

Desnuelle, P. (1961) Adv. Enzymol. **23**, 129-160

Devanathan, T., Akagi, J.M., and Hersh, R.T. (1969) J. Biol. Chem. **244**, 2853-3860

Doctor, B.P., Chapman, T.C., Christner, C.E. Deal, C.D., De La Hoz, D.M., Gentry, M.K., Ogert, R.A., Rush, R.S., Smyth, K.K. and Wolfe, A.D. (1990) FEBS Lett. **266**, 123-127

Donk, P.J. (1920) J. Bacteriol **5**, 373-382

Dordick, J.S. (1989) Enzyme Microb. Technol. **11**, 194-198

Dordick, J.S. (1992) Biotechnol. Prog. **8**, 259-267

Dordick, J.S. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria L5

Edwards, C. (1990a) in Microbiology of extreme Environments,

Edwards, C. (1990b) in Microbiology of extreme Environments, (Ed. C.Edwards) Open University Press Milton Keynes pp 22-24

Engel, K.-H., Bohnen, M. and Dobe, M. (1991) Enzyme Microb. Technol. **13**, 655-660

Entressangles, B. and Desnuelle, P. (1968) Biochim. Biophys. Acta **159**, 285-295

Entressangles, B. and Desnuelle, P. (1974) Biochim. Biophys. Acta **341**, 437-446

Epstein, L. and Campbell, L.L. (1975) Appl. Microbiol. **29**, 219-222

Eriksson, A.L., Baase, W.A., Zhang, X-J., Heinz, D.W., Blaber, M., Baldwin, E.P. and Mathews, B.W. (1992) Science **255**, 178-183

Eriksson, A.L., Baase, W.A., and Mathews, B.W. (1993) J. Mol. Biol. **229**, 747-769

Feng, D.F. and Doolittle, R.F. (1987) J. Mol. Evol. **25**, 351-360

Fernandez-Lafuente, R., Rosell, C.M. and Guisan, J.M. (1991) Enzyme Microb. Technol. **13**, 898-905

Fontana, A. (1988) Biophys. Chem. **29**, 181-193

Freeze, H and Brock. T.D. (1970) J. Bacteriol. **98**, 289-297

Franz, W. and Krisch, K. (1967) Hoppe-Seyler's Z. Physiol. Chem. **349**, 1413-1419

Fukui, S., Tanaka, A. and Iida, T. (1987) in Biocatalysis in Organic Media. (Eds., C. Laane, J. Tramper and M. Lilly) Elsevier. Amsterdam/New York pp 21-41.

Geartner, H., Watanabe, T., Sinisterra, J.V. and Puigvert, A.J. (1991) J. Org. Chem. **56**, 3149-3153

Gill, S.J. (1985) J. Phys. Chem. **89**, 3758-3761

Gillies, B., Yamazaki, H., and Armstrong, D.W. (1987) Biotech. Lett. **9**, 709-714

Godberg, M. I., and Fruton, J. S. (1970) Biochemistry **9**, 3371-3380

Goderis, H.L., Ampe, G., Feyten, M.P., Fouwe, B.L., Guffens, W.M., Van Cauwenbergh, S.M., and Tobback, P.P. (1987) Biotechnol. Bioeng. **39**, 258-266

Goergens, U. and Schneider, M.P. (1991) J. Chem. Soc. Chem. Commun. 1064-1066

Gordon, R.E. and Smith, N.R. (1949) J. Bacteriol. **58**, 327-332

Goodman, R.E. and Pederson, D.M. (1976) Can. J. Microbiol. **22**, 817-822

Greenzaid, P. and Jencks, W.P. (1971) Biochemistry **10**, 1210-1222

Gu, Q-M., and Sih, C.J. (1992) Biocatal. **6**, 115-126

Guagliardi, A., Manco, G., Rossi, M. and Bartolucci, S. (1989) Eur. J. Biochem. **183**, 25-30

Gubicza, L. and Szakacs-Schmidt, A. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria P16

Guisan, J.M. and Blanco, R.M. (1987), Ann. N.Y. Acad. Sci. **501**, 67-72

Guisan, J.M. (1988) Enzyme Microb. Technol. **10**, 375-382

Guisan, J.M., Alvaro,G., Fernandez-Lafuente, R., Rosell, C.M., Garcia, J.L. and Tagliatti, A. (1993a) Biotechnol. Bioeng. **42**, 455-464

Guisan, J.M., Blanco, R.M., Fernandez-Lafuente, R., Rosell, C.M., Alvaro, G. and Bastida, A. (1993b) in: Proteins Stability and Stabilisation (Ed., W.J.J. van den Tweel) Elsevier Science Publ. pp 55-62.

Gupta, M.N. (1992) Eur. J. Biochem. **203**, 25-32

Hachimori, A., Muramatsu, N. and Nosoh, Y. (1970) Biochim. Biophys. Acta **206**, 426-437

Halling P.J. (1993) Thermodynamic Predictions for Biocatalysis in Non-Conventional Media Therory, Tests and Recommendations for Experimental Design and Analysis. Department of Bioscience and Biotechnology, University of Strathclyde, Glasgow G1 1XW.

Halling P.J. (1992) Biotechnol. **6**, 271-276

Hammond, D.A., Karel, M. Klibanov, A.M. and Krukonis, V.J. (1985) Appl. Biochem. Biotechnol. **11**, 393-400

Handbook of Chemistry and Physics, 57th ed. (1976-1977), (Ed. R.C. West) CRL Press, C726-C732.

Hanes, C.S. (1932) Biochem. J. **26**, 1406-1421

Heinz, D.W., Baase, W.A., Dahlquist, F.W. and Matthews, B.W. (1993) Nature **361**, 561-564

Heras, A. and Acosta, N. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria P131

Herbert R. A. (1992) TiBTech**10**, 395-402

Heymann, E. and Mentlein, R. (1981) Methods Enzymol. **77**, 533-544

Higerd, T. B. and Spizezen, J. (1973) J. Bacteriol. **114**, 1184-1192

Hoching, J.D. and Harris, J.I. (1976) in Enzymes and Proteins from Thermophilic Microorganisms (Ed. H. Zuber) Birkhauser Verlag Basel p 107

Holmberg, E. and Hult, K. (1990) Biocatal. **3**, 243-251

Holmberg, E. and Hult, K. (1991) Biotech. Lett. **13**, 323-326

Horgan, D.J., Webb, E.C. and Zerner, B. (1966) Biochem. Biophys. Res. Commun. **2**, 18-25

Horgan, D.J., Dunstone, J.R., Stoops, J.K., Webb, E.C. and Zerner, B. (1969), Biochemistry **8**, 2006-2012

Hult, K., Holmquist, M. and Martinelle, M. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria L4

Inada, Y., Nishimura, H., Takahashi, K. and Yoshimoto , H. (1986) TiBTech, **7**, 190-194

Jaenicke, R. and Zavodszky, P. (1990). FEBS Lett. **268**, 344-349

James, L.K. and Augenstein, L.G. (1966) Adv. Enzymol. **28**, 1-40

Janssen, A.J.M., Klunder, A.J.H. and Zwanenburg, B. (1991) Tetrahedron **47**, 7409-7416

Jensen, R.G., Galluzzo, D.R. and Bush, V.J. (1990) Biocatal. **3**, 307-316

Jones, J.B. and Mehes, M.M. (1979) Can. J. Chem. **57**, 2245-2248

Josic, D., Reusch, K., Baum, O. and Reutter, W., (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria P129

Jurado, A.S., Santana, A.C., Da Costa, M.S., and Madeira, V.M.C. (1987) J. Gen. Microbiol. **133**, 507-510

Kaiser, E.T., Lawrence, D.S. and Rokita, S.E. (1985) Ann. Rev. Biochem., **54**, 565-595

Katchalski-Katzir, E. (1993) TiBTech **11**, 471-478

Kawaguchi,Y., Honda, H., Taniguchi-Morimura, J. and Iwasaki, S. (1989) Nature **341**, 164-166

Kazandjian, R.Z. and Klibanov, A.M. (1985) J. Am. Chem. Soc. **107**, 5448-5450

Keay, L. (1969) Biochem. Biophys. Res. Commun. **36**, 257-265

Keay, L. and Wildi, B.S. (1970) Biotechnol. Bioeng. **12**, 179-212

Keay, L., Moseley, M.H., Anderson, R.G., O'Connor, R.G. and Wildi, B.S (1972) Biotechnol. Bioeng. Symp. **3**, 63-92

Keinan, E., Hafeli, F.V., Seth, K.K. and Lamed, R.J. (1986) J. Am. Chem. Soc. **108**, 162-169

Kelly, R.M. and Deming, J.W. (1988) Biotechnol. Prog. **4**, 47-62

Khmelnitsky, Yu.L. Levashov, A. V., Klyachko, N.L. and Martinek, K. (1988) Enzyme Microb. Technol. **10**, 710-724

Kim, M.-J. and Choi, Y.K. (1992) J. Org. Chem. **57**, 1605-1610

Kinney, J.L. (1980) J. Biophys. **32**, 17-20

Kirchner, G., Scollar, M.P. and Klibanov, A.M. (1985) J. Am. Chem. Soc. **107**, 7072-7080

Krisch, K. (1963) Biochem. Z. **337**, 546-552

Krisch, K. (1966) Biochim. Biophys. Acta **122**, 265-273

Krisch, K. (1971) in The Enzymes, Vol. 5 3rd ed. (Ed. Boyer, E.D.) Academic Press, London, pp 43-69

Kitaguchi, H., Itoh, I. and Ono, M. (1990) Chem. Lett. 1203-1206

Klibanov, A.M. Samokhin, G.P., Martinek, K. and Berezin, V. (1977) Biotechnol. Bioeng. **19**, 1351-1361

Klibanov, A.M. (1986) Chemtech. **16**, 354-359

Klibanov, A.M.. (1987). In Enzyme Engineering 8. Volume 501. (Eds. A. Laskin, K. Mosbach, D. Thomas and L. Wingard, Jr.): Ann. N.Y. Acad. Sci. New York. p129

Klibanov, A.M. (1989) Trends Biochem. Soc. **14**, 141-146

Klibanov, A.M. (1990) Acc. Chem. Res **23**, 114-120

Kloosterman, M., Elferink, V.H.M., van Iersel, J., Roskam, J.H., Meyer, E.M., Hulshof, L.A. and Sheldon, R.A. (1993) TiBTech **11**, 432-437

Knez, Z. and Habulin, M. (1992) Proceeding 5th International Symposium on Biocatalysis in non conventional media (Eds. J. Tranper, M.H. Vermue, H.H. Beeftink, U. von Stocker.) Elsevier Amsterdam pp 401-406

Koenig, E., Nogoceke, E. and Menge, U. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria P29

Kraut, J. (1977) Annu. Rev. Biochem. **46**, 331-358

Kristjansson J.K. (1989) TiBTech **7**, 349-353

Kugimiya, W., Otano, Y., Hashimoto, Y. and Takagi, Y. (1986) Biochem. Biophys. Res. Commun. **141**, 185-190

Kuhl, P. and Halling, P.J. (1991) Biochim. Biophys. Acta **1078**, 326-328

Kuhn, H., Friederich, U. and Fiechter, A. (1979) Eur. J. Appl. Microbiol. Biotech. **6**, 341-349

Kvittingen, L., Sjursnes, B., Anthonsen, T. and Halling, P.J. (1992) Tetrahedron **48**, 2793-2802

Laane, C. Boerens, S. and Vos, K., (1985) TiBTech **3**, 251-252

Laemmli, U.K.. Nature (1970) **227**, 680-685

Lakatos, S., Halasz, G. and Zavodszky, P. (1978) Biochem. Soc. Trans. **6**, 1195-1197

Lam, L.K.P., Hui, R.A.H.F. and Jones, J.B. (1986) J. Org. Chem. **51**, 2047-2050

Langworthy, T.A., Brock, T.D., Castenholtz, R.W., Esser, A.F., Johnson, E.J., Oshima, T., Tsuboi, M.T., Zeikus, J.G. and Zuber, H. (1979) in: Stratergies of Life in Extreme Environments (Ed. M. Shilo) Verlag Chemie. New York. pp 489-502

Langrand, G., Secchi, M., Buono, G., Baratti, J. and Triantaphylides, C. (1985) Tetrhedron Lett. **26**, 1857-1860

Langrand, G., Triantaphylides, C. and Baratti, J. (1988) Bitechnol. Lett. **10**, 549-554

Larreta-Garde, V., Xu, Z., Biton, J. and Thomas, D. (1987). In Biocatalysis in Organic Media. (Eds., C. Laane, J. Tramper and M. Lilly.) Elsevier. Amsterdam/New York. pp 247-251.

Legoy,M. Biochemie (1980) **62**, 341-345

Levy, M., and Ocken, P. (1967) Fed. Proc. **26**, 816-820

Lilly, M.D. and Woodley, J.M. (1985). in Biocatalysis in Organic Syntheses (Eds., J. Tramper, H.C. van der Plas and P. Linko.) Elsevier, Amsterdam, pp 179-192

Lipman, D.J. and Pearson, W.R. (1985) Biochemistry **227**, 1435-1441

Lombardo, D. and Guy, O. (1981) Biochim. Biophys. Acta **657**, 425-437

Luisi, P.L. (1985) Angew. Chem. Int. Ed. **24**, 439-450

Luisi, P.L. (1988) Biochim. Biophys. Acta **947**, 209-246

Macrae, A.R.J. (1983) J. Am. Oil Soc. **60**, 243A-246A

Magsanaga, A. and Nosoh, Y. (1974) Biochim. Biophys. Acta **365**, 208-214

Malcata F. X., Reyes, H.R. Garcia H.S., Hill C.G. and Amundson C.H. (1992) Enzyme Microb. Technol. **14**, 426-446

Malhortra, O.P. and Philip, G. (1966) Biochem. Z. **346**, 386-390

Manco, G., Gennaro, S., Rosa, M. and Rossi, M. (1994) Eur. J. Biochem. **221**, 965-972

March, J. (1985) in Advanced Organic Chemistry: reactions, mechanisn and structure 3rd Edition (Publishrd by Wiley, Chichester,) pg 335

Martin, A.A., Offhauss, U. and Wagner, J. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria P87

Martinek, K., Klibanov, A.M., Goldmacker, V.S. and Berezin, I.L. (1977) Biochem. Biophys. Acta **485**, 1-24

Martinek, K. and Semenov, A.N (1981a) J. Appl. Biochem. **3**, 93-126.

Martinek, K. and Semenov, A.N (1981b) Biochim. Biophys. Acta **658**, 76-101

Martinek, K. (1989) Biochim. Biophys. Acta **981**, 161-172

Mateles, R.I. and Battat, E. (1974) Appl. Microbiol. **28**, 901-906

Matsunaga, A., Koyama, N. and Nosoh, Y. (1974) Arch. Biochem. Biophys. **160**, 504-513

Matsuo, T (1981) US Patent 4,268,527

Mattiasson, B. and Aldercreutz, P. (1991) TiBTech. **9**, 394-398

Mattson, F.H. and Volpenhein, R.A. (1969) J. Lipid Res **10**, 271-276

McHugh, M. and Krukonis, V. (1986) in Supercritical Fluid Extraction; Principles and Practice. Butterworth, Boston, pp 1-95.)

MERCK Index 10th Edition. (Eds. Windholz, M., Budavari, S., Blumetti, R. F. and Otterbein, E.S.) Published by MERCK & Co. INC. Rahway, N.J. USA 1983

Merkler, D.J., Srikumar, K., Marches-Ragona, S.P. and Wedler, F.C. (1988) Biochim. Biophys. Acta **952**, 101-106

Min Chi, Y., Nakamura, K. and Yano, T. (1988) Agric. Biol. Chem. **52**, 1541-1550

Mozahaev,V,V. Siksnis, V.A., Torchilin, V.P. and Martinek, K. (1983) Biotechnol. Bioeng. **25**, 1937-1942

Muramatsu, N. and Nosoh, Y. (1971) Arch. Biochem. Biophys. **144**, 245-252

Murata, K., Sato, N., Rhee, H-i., Watanabe, K. and Kimura, A. (1987) Agric. Biol. Chem. **51**, 1901-1907

Nakagawa, A., Tsujita, T. and Okuda, H. (1984) J. Biochem. **95**, 1047-1054

Nakamura, K. (1990) TiBTech. **8**, 288-292

Nakamura, K., Takano, S. and., Ohno, A (1993a) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria L32

Nakamura, K., Kinoshita, M. and Ohno, A. Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria P11

Nakanishi, K., Kamikubo, T. and Matsuno, R. (1985) Biotechnol. **3**, 359-464

Nanalov, R.J., Kambourne, M.S. and Emanuiloda, E.I. (1993) Biotechnol. Appl. Biochem. **18**, 409-416

Needleman, S.B. and Wunsch, C.D. (1970) J. Mol. Biol. **48**, 443-453

Nilsson, K. and Mosbach, K. (1984) Biochem. Bioeng. **26**, 1146-1154

Oakeshott, J.G., Collet, C., Phillis, R.W., Nielsen, K.M., Russell, R.J., Chamers, G.K., Ross, V. and Richmond, R.C. (1987) Proc. Natl. Acad. Sci. **84**, 3359-33639.

O'Brien, R.T. and Campbell, L.L., Jr. (1956) Bacteriol. Proc., 46-50

O'Brien, R.T. and Campbell, L.L., Jr. (1957) J. Bacteriol. **73**, 522-525

Ohkawa, I., Shiga, S., and Kageyama, M. (1979) J. Biochem. **86**, 643-656

Omata,T. (1981) Eur. J. Appl. Microbiol. Biotechnol. **11**, 199-206

Ottolina, G., Carrea, G. Gianinetti and Riva, S. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria P14

Otero, C., Ballesteros, A. and Guisan, J.M. (1991) Appl. Biochem. Biotechnol. **19**, 163-175

Ovsejevi, K., Brena, B. Batista-Viera, F. and Carlsson, J. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria P132

Owusu, R.K. and Cowan, D.A. (1989) Enzyme Microb. Technol. **11,** 568-574.

Owusu, R.K. and Cowan, D.A. (1990) Enzyme Microb. Technol. **12**, 374-377

Owusu, R.K. and Cowan, D.A. (1991) Enzyme Microb. Technol. **13,** 158-163

Ozols, J. (1989) J. Biol. Chem. **264**, 12533-12539

Pasta, P., Mazzola, G. Carrea, G. and Riva, S. (1989) Biotech. Lett. **2**, 643-648

Peterson, G.L.B. (1983) Methods Enzymol. **91**, 95-119

Perutz, M.F. (1978) Science **201**, 1187-1189

Pham, V.T. and Phillips, R.S. (1990) J. Am. Chem. Soc. **112**, 3629-3631

Phillips, R.S. (1992) Enzyme Microb. Technol. **14**, 417-419

Pirt, S.J. (1988) in Principles of Microbe and Cell Cultivation (Ed., S.J. Pirt) John Wiley, New York pp 24-25

Playne, M. J. and Smith, B.R. (1983) Biotechnol. Bioeng. **25**, 1251-1255

Privalov, P.L. and Gill, S.J. (1988) Adv. Prot. Chem. **39**, 193-231

Rachinsky, T.L., Camp, S., Li, Y., Ekstroem, T.J., Newton, M. and Taylor, P. (1990) Neuron **5**, 317-327

Rakels, J.L.L, Straathof, A.J.J. and Heijnen J.J. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria L32

Ramos, M.C., Gil, M.H., Garcia, F.A.P., Cabral, J.M.S. and Guthrie, J.T. (1992) Biocatal. **6**, 223-234

Randolph, T.W., Blanch, H.W. and Prausnitz, J.M. (1988) AIChE J. **34**, 1354-1360

Reddy, P.G. Allon, R., Mevarech, M., Mendelovitz, S., Sato, Y. and Gutnick, D.L. (1989) Gene **76**, 145-152

Reslow, M. Adlercreutz, P. and Mattiasson, B. (1988) Eur. J. Biochem. **172**, 573-578

Riva, S. (1988) J. Am. Chem. Soc. **110**, 584-589

Robb, D.A., Yang, Z. and Halling, P.J. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria L7

Robson, E. and Pain, R.H. (1971) J. Mol. Biol. **58**, 237-240

Rothfield, L.I. and Romeo, D. (1971) in Structure and Function of Biological Membranes (Rothfield, L.I. ed.) Academic Press, New York, pp 251-284

Rowe, J.J., Goldberg, I.D. and Amelunxen, R.E. (1973) Can. J. Microbiol. **19**, 1521-1523

Rowe, J.J., Goldgerg, I.D. and Amelunxen, R.E. (1975) J. Bacteriol. **124**, 279-282

Rupley, J.A. and Careri, G. (1991) Adv. Protein Chem. **41**, 37-172.

Russell, A.J. Beckman, E.J. and Kamat S.V. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria L13

Sabbioni, G. and Jones, B. J. (1987) J. Org. Chem. **52,** 4565-4570

Santaniello, E., Ferraboschi, P., Grisenti, P. and Manzocchi, A. (1992) Chem. Rev. **92**, 1071-1140

Sarada, L. and Desnuelle, P. (1958) Biochim. Biophys. Acta **30**, 513-521

Saravini, A. (1985) D.Phil. thesis, University of Waikato, Hamilton, New Zealand

Sargeant, K., East, D.N., Whitaker, A.R. and Elsworth, R. (1971) J. Gen. Microbiol. **65**, iii-iv

Schrag, J.D., Li, Y, Wu S. and Cygler, M. (1991) Nature **351**, 761-764.

Scott, D.L. White, S.P., Otwinowski, Z., Yuan, W., Gelb, M.H. and Sigler, P.B. (1990) Science **250**, 1541-1546

Shainof, J.R. (1980) Biochem. Biophys. Res. Commun. **95**, 690-695

Silman, I.H. and Katchalski, E. (1966) Annu. Rev. Biochem. **35**, 773-908

Sjursnes B. J. and Anthonsen, T. (1993) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria L11

Sleytler, D.C., Moulson, P.S. and Reynolds, J. (1991) Enzyme Microb. Technol. **13**, 221-226

Sobek, H. and Gorisch, H. (1988) Biochem. J. **250**, 453-458

Sobek, H. and Gorisch, H. (1989) Biochem. J. **261** 993-998

Soreq, H., Ben-Aziz, R., Prody, C.A., Seidman, S., Gnatt, A., Neville, L., Lieman-Hurwitz, J., Lev-Lehman, E., Ginzberg, D., Lipidot-Lifson, Y. and Zakut, H. (1990) Proc. Natl. Acad. Sci. **87**, 9688-9692

Stetter, K.O. (1982) Nature **300**, 258-260

Stoops, J.K., Horgan, D.J., Runnegar, M.T.C., de Jersey, J., Webb, E.C. and Zerner, B. (1969) Biochemistry **8**, 2026-2033

Sugimoto, S. and Nosoh, Y. (1971) Arch. Biochem. Biophys. **235**, 210-221

Sundaram, T.K. (1986) In Thermophiles: General, Molecular and Applied Microbiology (Ed. T.D. Brock). John Wiley & Sons, New York, pp 75-106

Sundaram, T.K., Chell, R.M. and Wilkinson, A.E. (1980) Arch. Biochem. Biophys. **199**, 515-525

Sussman, J. L. Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L. and Silman, I. (1991) Science **253**, 872-879

Tajima, M., Urabe, L., Utani, K. and Okada, H., (1976) Eur. J. Biochem. **64**, 243-247

Takahashi, K., Nishimura, H., Yoshimoto, T., Saito, Y. and Inada, Y. (1984a) Biochem. Biophys. Res. Commun. **121**, 261-265

Takahashi, K., Ajima, A.,Yoshimoto, T. and Inada, Y., (1984b) Biochem. Biophys. Res. Commun. **125**, 761-766

Takagi, Y., Omura, Y. and Go, M.. (1988) J. Biochem. **104**, 801-806

Takemori, S. (1967) Nature **215**, 417-419

Tan, K.H. and Lovrein, R. (1972) J. Biol. Chem. **247**, 3278-3285

Tanaka, M., Haniu, M., Matsueda, G., Uasunobu, K.T., Himes, R.H., Akagi, J.M., Barnes, E.M. and Devanathan, T. (1971) J. Biol. Chem. **246**, 3958-3965

Tennikova, T.B., Belenkii, B.G. and Svec, F. (1990) J. Liq. Chrom. **13**, 63

Theil, F., Weidner, J., Ballschuh. S., Kunath, A. and Schick, H. (1991) Tetrahedron Asymmetry **2**, 1031-1035

Theil, F. and Bjorkling, F. (1993a) Biotechnol. Lett. **15**, 605-608

Theil, F., Weidner, J., Ballschuh. S., Kunath, A. and Schick, H. (1993b) Abstract of the European Symposium on Biocatalysis (Eds., D.H.G. Crout, S.M. Roberts, H. Griengl and K. Faber) Graz, Austria L39

Theil, F., Weidner, J., Ballschuh. S., Kunath, A. and Schick, H. (1993c) Tetrahedron Lett. **34**, 305-309

Tilbeurgh, H., Egloff, M., Martinez, C., Rugani, N., Verger, R. and Cambillau, C. (1993) Nature **362,** 814-820

Tombo, G.M.R., Schar, H.P. and Ghisalba, O. (1987) Agric. Biol. Chem. **51**, 1833-1838

Toone, E.J. Werth, M. J. and Jones B. J. (1990) J. Am. Chem. Soc. **112,** 4946-4952

Tosa, T., Mori, T., Fuse, N. and Chibata, I. (1967) Biotechnol. Bioeng. **9**, 603-615

Tressl, R., Engel, K.-H., Albrecht, W. and Bille-Abdullah, H. (1985) in Characterisation and Measurement of Flavour compounds (Bills, D.D. and Mussinan, C.J., eds) ACS Symposium Series No. 289, American Chemical Society, Washington, DC, pp 43-60.

Tressl, R. and Engel, K.-H. (1984) in Analysis of Volatiles (Schreier, P., ed.) Walter de Gruyter, Berlin, New York, pp 323-342

Ueji, S., Fujino, R., Okubo, N. Miyazawa, T., Kurita, S., Kitadani, M. and Muromatsu, A. (1992) Biotecnol. Lett. **14**, 163-168

Ulrich, T.J., McFeters G. A. and Temple, K. L. (1972) J. Bacteriol., **110**, 691

Vali, Z., Kilar, F., Lakatos, S., Venyaminov, S.A. and Zavodszky, P. (1980) Biochim. Biophys. Acta **615**, 34-47

Valivety, R.H., Halling, P.J., and Macrea, A.R. (1992) Biochim. Biophys. Acta **118**, 218-222

Van der Lugt, J.P., Elfrink, H., Evenaar, J. and Doddema, H.J. (1992) in Microbial Reagents in Organic Synthesis (Servi, S., Ed.) Kluwer Aced. Publ. Dordrect, pp 261-272

Verger, R., Mieras, M.C.E. and Haas, G.H. (1973) J. Biol. Chem. **248**, 4023-4034

Verger, R. and Haas, G.H. (1976) Ann. Rev. Biophys. Bioeng. **5**, 77-117

Verume, M. H., Tramper, J., D-Jong, J. P. J. and Oostrom, W. H. M. (1992) Enzyme Microb. Technol. **14**, 649-655

Vihinen, M. (1987) Prot. Eng. **1**, 477-480

Wagner, G. and Wuthrich, K. (1979) J. Mol. Biol. **130**, 31-37

Wedler, F.C. and Hoffman, F.M. (1974) Biochemistry **13**, 3215-3221

Wedler, F.C., (1978) in: Biochemistry of Thermophily, (Friedman, S.M., Ed.) New York, pg 325

Wedler, F.C. and Merkler, D.J. (1985) Curr. Top. Cell. Regul. **26**, 263-270

Wehtje, E., Adlercreutz, P. and Mattiasson, B. (1993) Biocatal. **7**, 149-153

Welker, N.E., (1976) in: The Survival of Vegetative Microbes, (Gray, T.G.R and Postgate, J.R. Eds.), Cambridge University Press, London, pg 241

Wells, M.A. (1974) Biochemistry **13**, 2248-2257

Welsch, F.W., Murray, W.D. and Williams, R.E. (1989) CRC. Crit. Rev. Biotechnol. **9**, 105-109

West, J.B. and Wong, G.H. (1986) J. Org. Chem. **51**, 2728-2735

White, R.H. (1984) Nature **310**, 430-432

Wickli, A., Schmidt, E. and Bourne, J.R. (1992) in Biocatalysis in Non-Conventional Media (Eds. Tramper, J., Verume, M.H., Beeftink, H.H. and von Stokar, U.), Elsevier, Amsterdam, pp 577-584

Willaert, J.J., Lemiere, G.L., Joris, L.A., Lepoivre, J.A. and Alderweireldt, F.C. (1988) Bioorg. Chem. **16**, 223-231

Wilson, S.A., Peek, K. and Daniel, R.M. (1993) Biotechnol. Bioeng. **42**, 225-231

Winkler, F.K., D'Arcy, A. and Hunziker, W. (1990) Nature **343**, 771-774

Wrba, A., Schweiger, A., Schultes, V., Jaenicke, R. and Zavodszky, P. (1990) Biochemistry **29**, 7584-7592

Wynne, D., and Shalitin, Y. (1972) Eur. J. Biochem. **31**, 554-560

Yamamoto, K., Nishioka, K., Oda, J. and Yamamoto, Y. (1988) Tetrahedron Lett. **29**, 1717-1720

Yamane, T., Kojima, Y., Ichira, T., and Shimizu, S. (1988) Ann. N. Y. Acad. Sci. 282-293

Yang, Z. and Robb, D.A. (1993) Biotechnol. **7**, 37-42

Yayanos, A.A., Van Boxtel, R. and Deitz, A.S. (1983) Appl. Environ. Microbiol. **43**, 1357-1363

Yutani, K. (1976) in: Enzymes and Proteins from Thermophilic Microorganisms, (Zuber, H., Ed.), Birkauser Verlag, Basel, pg 91

Zaks, A. and Klibanov, A.M. (1984a) Science **224**, 1249-1251

Zaks, A. and Klibanov, A.M. (1984b) J. Am. Chem. Soc. **106**, 2687-2692

Zaks, A. and Klibanov, A.M. (1985) Proc. Natl. Acad. Sci. USA **82**, 3192-3196

Zaks, A. and Klibanov, A.M. (1986) J. Am. Chem. Soc.**108**, 2767-2768

Zaks, A. and Klibanov, A.M. (1988a) J. Biol. Chem. **263**, 3194-3201

Zaks, A. and Klibanov, A.M. (1988b) J. Biol. Chem. **263**, 8017-8021

Zeikus, J.G. (1979) Enzyme Microb. Technol. **1**, 243-251

Zemlicka, J. and Craine, L. E. (1988) J. Org. Chem. **53**, 937-942

Zheng, C. and Phillips, R.S. (1992) J. Chem. Soc. Perkin. **1**, 1083-1084

Zuber, H. (1979) in: Stratergies of Microbial Life in Extreme Environments (Ed. M. Shilo) Verlag Chemie. New York pp 393-415

Appendix 1. Thermophilic *Bacillus* strains screened for esterase activity

The following 72 bacterial strains, with the provisional taxonomic designation of *Bacillus stearothermophilus*, with optimum growth temperatures in the region of 50°C - 75°C were screened for esterase activity.

Strain	Growth temperature °C
But5 A1	50
Fur5 B2	60
Fur6 A1	60
Fur6 A2	60
Fur6 A3	70
Fur6 A4	60
Fur6 B2	60
Fur9 A1	60
Fur12 A2	60
Fur13 B2	60
Fur14 A1	60
Fur14 A2	60
Fur15 A2	60
Fur15 A3	60
G18 A1	70
G18 A2	60
G18 A3	60
G18 A4	55
G18 A5	70
G18 A6	65
G18 A7	70
G18 A8	65
G18 A9	70
G18 A10	70

Strain	Growth temperature
	°C
Laf4 A1	70
Laf4 A2	60
Laf4 A3	60
Laf4 B2	60
Laf4 B3	60
PrF1 A2	60
PrF1 A3	60
PrF1 A13	60
PrF1 A14	73
Rec2 A2	70
Rec2 A3	75
Rot11 B1	60
Rot34 A2	72
Rot34 A6	70
Rot34 A7	75
Rot34 A8	65
Tau1 A5	70
Tau1 A6	70
Tau2 A2	70
Tau2 A3	70
Tau2 A4	75
Tau3 A1	65-70
Tau3 A2	72
Tau3 A3	70
Tau4 A1	70
Tau5 A3	65
Tau8 A2	60-65
Tau8 A3	60
Tau8 A4	55
Tau10 A1	60

Strain	Growth temperature °C
Tok4 A3	70
Tok5 A3	70
Tok6 A2	65
Tok12 A1	70
Tok12 A2	70
Tok12 A3	70
Tok19 A1	60
Tok19 A2	60

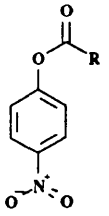
The following strains had been isolated on 2% olive oil plates and were all tributyrin positive (Cowan D. A. unpublished results).

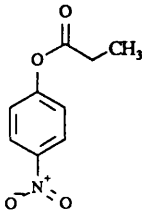
Strain	Growth temperature °C
Laf4 A2	60
Laf4 A3	60
Laf4 B2	60
Laf4 B3	60
Fur5 B2	60
Fur6 A4	60
Fur6 B2	60
Fur12 A2	60
Fur13 B2	60
Rot11 B1	60

All the strains had previously been isolated from several New Zealand thermal sites and stored in freeze dried vials at -70^{o}C (Cowan D. A. unpublished results).

Appendix 2. Chemical structures

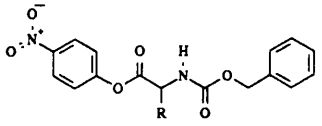
p-NITROPHENYL ESTERS





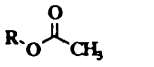
p-nitrophenyl propionate

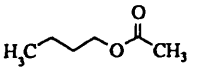
N-carbobenzoxy amino acid para-nitrophenyl ester



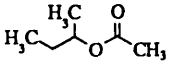
R = the amino acid side chain

ACETATES

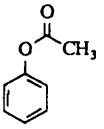




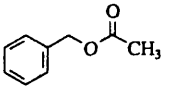
butyl acetate



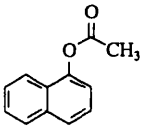
2°butyl acetate



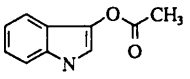
benzyl acetate



phenyl acetate

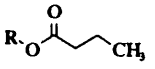


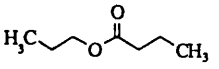
α-naphthyl acetate



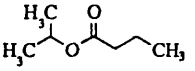
indoxy acetate

BUTYRATES



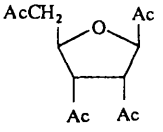


propyl butyrate

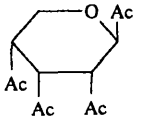


isopropyl butyrate

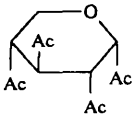
SUGAR ESTERS



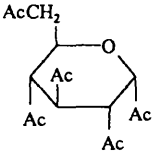
1,2,3,5-tetraacetyl-β-D-ribofuranose



1,2,3,4,-tetra-o-acetyl-β-D-ribopyranose

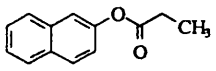


β-D-xylose tetraacetate

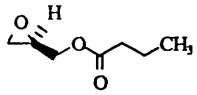


glucose pentaacetate

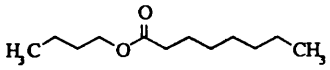
MISCELLANEOUS SUBSTRATES



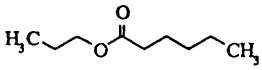
β-naphthyl propionate



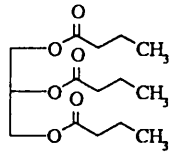
glycidyl butyrate



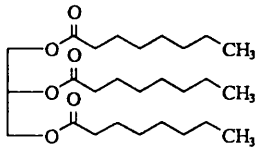
caprylic acid n-butyl ester



caproic acid n-propyl ester

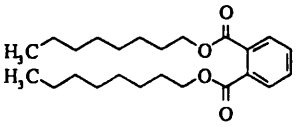


tributyrin



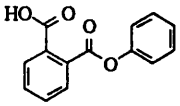
tricaprylin

MISCELLANEOUS SUBSTRATES

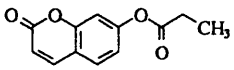


diisooctyl phthalate

gamma butyrolactone

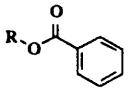


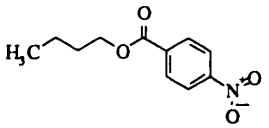
phenyl salicylate



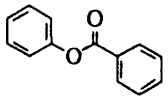
4-methyl umberferyl propionate

BENZOATES



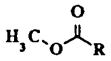


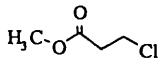
n-butyl paranitobenzoates



phenyyl benzoate

METHYL ESTERS





chloropropionic acid methyl ester