Enzymes of chorismate metabolism In *Ailanthus altissima* (Mill.) Swingle cell cultures

BY

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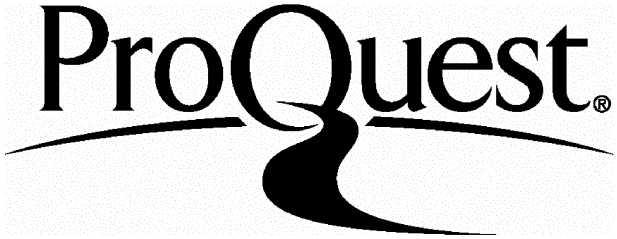

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Abbreviations

5MT	D,L-5-methyltryptophan
AS	anthranilate synthase
ASI	anthranilate synthase α-subunit
ASII	anthranilate synthase β-subunit
cAMP	cyclic adenosine monophosphate phosphodiesterase
CM	chorismate mutase
CM-F	unregulated chorismate mutase
CM-P	chorismate mutase:prephenate dehydratase
CM-R	regulated chorismate mutase
CM-T	chorismate mutase:prephenate dehydrogenase
DAHP synthase	3-deoxyarabinoheptulosonate-7-phosphate synthase
DHQ	dehydroquinate
DTT	dithiothreitol
EDTA	ethylenediamine tetra-acetic acid
EPSP synthase	5-enolpyruvylshikimate-3-phosphate synthase
InGPS	indole-3-glycerol phosphate synthase
ISC	isochorismate synthase
M&S	Murashige and Skoog
NAS	normalized alignment score
PABA	*p*-aminobenzoate synthase
PEG	polyethylene glycol
PEP	phosphoenolpyruvate
phe	L-phenylalanine
PMSF	phenylmethylsulphonyl fluoride
PRAI	phosphoribosylanthranilate isomerase
PRT	anthranilate phosphoribosyltransferase
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
TEMED	*N,N,N',N'*-tetramethylethylenediamine
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
trp	L-tryptophan
tyr	L-tyrosine
FPLC	Fast protein liquid chromatography

Abstract

The thesis incorporates the results of an investigation of chorismate utilising enzymes in *Ailanthus altissima* (Mill.) Swingle cell cultures. These enzymes are anthranilate synthase, chorismate mutase and isochorismate synthase.

The activity of the three enzymes was measured in callus and cell suspension cultures of the plant, but only anthranilate synthase (AS) and chorismate mutase (CM) were detected. A time course study was carried out to determine the growth and the enzymes' activities. The study showed that AS measured after use of a Sephadex PD-10 column, was active through the cell growth cycle, giving a maximum activity within 11 and 17 days, whereas CM activity increased after 23 days. CM activity was 150x that of AS.

Two AS enzymes were isolated from callus and cell suspension cultures of the same cell line. The enzymes were purified by 10% and 18% polyethylene glycol precipitation (PEG) followed by separation on a Q-Sepharose (Pharmacia) column using FPLC with a discontinuous NaCl gradient. Further protein purification was achieved with a Mono Q column (Pharmacia) with discontinuous NaCl gradient to yield both isoenzymes. The two isoenzymes showed different regulation by tryptophan.

One of the AS enzyme present was characterised. The molecular weight of the enzyme using gel filtration was 77±3 kDa, and the K_m respect to the substrates L-glutamine and chorismate, and the cofactor Mg^{+2} were determined as 0.050mM, 72µM and 0.18mM, respectively. An optimum pH between 7.3 and 8, and an optimum temperature of 35°C were also determined.

Two CM enzymes were also detected, but they were not characterised.

The activities and regulations of AS and CM enzymes are discussed and compared with other data from higher plants and microorganisms.

1. *Ailanthus altissima*

INTRODUCTION

A. altissima (Mill.) Swingle belongs to the family Simaroubaceae which consists of six subfamilies with 32 genera and over 170 arboreous or shrubby species [Fo et al 1992, Melchior 1964]. *Ailanthus* is classified in the subfamily Simarouboideae which contains most of the pharmacologically active species of the family. This subfamily is known to contain β-carbolines, canthin-6-one alkaloids and quassinoids [Simao et al 1991, Fo et al 1992].

A. altissima, known as *A. glandulosa* Desf., *A. giraldii* Dode and tree of heaven, originated from China, but it has been introduced into many countries including India, Japan and Australia as well as into Europe and North America, where has been established as an ornamental tree in cities. The tree is large and deciduous, usually 15 to 21 metre high with a trunk of 60 to 90 cm diameter. It has a rounded head of branches, the leaves are pinnate, 30 to 45 cm long and consist of 15 to 30 leaflets. The leaves of the male plant have foetid odour. Flowers are in terminal panicles and fruits form attractive orange to red clusters in August and September [Mabberley 1990, Roberts 1991].

USES OF *AILANTHUS ALTISSIMA* IN TRADITIONAL MEDICINE

The medicinal properties of *A. altissima* have been known for centuries and throughout the Far East the plant is used in the treatment of enteric infections. The fruits, the roots or stem have been used in the treatment of dysentery or leucorrhea, dyspepsia, bronchitis, ophthalmic diseases, snake bites, anaemia, a wide range of other gastrointestinal ailments and as taeniafuge [Chopra et al 1956, Khan et al, 1982, Roberts 1991]. This plant is also noted for anthelmintic, insecticidal and antibacterial properties [Ohmoto et al 1976, Varga et al 1980, 1981, Roberts 1991].

SECONDARY METABOLITES FROM *AILANTHUS ALTISSIMA*

Alkaloids, quassinoids and quinones have been reported as occurring in the wood, root bark and bark of *A. altissima* [Anderson et al 1983].

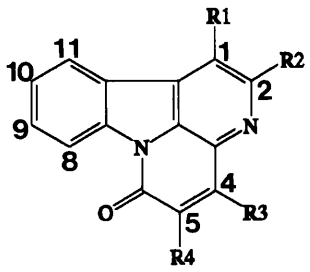
Alkaloids

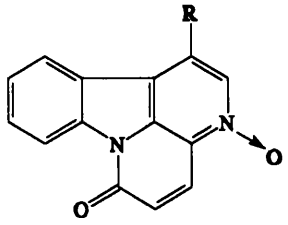
Two groups of indole alkaloids were isolated from the wood and root bark: the canthin-6-ones and the β-carbolines. Examples of the structures of canthin-6-one and β-carboline alkaloids present in *A. altissima* are shown in Fig. 1.1. For the canthin-6-one alkaloids R can be either a methoxy group or a hydrogen; R_1 can be a hydrogen, a hydroxy or a methoxy group; R_2 and R_3 are a hydrogen or a hydroxyl group and R_4 can be a hydrogen, a hydroxy, a methoxy or a hydroxymethyl group. For the β-carbolines, R_1 can be a methoxy, acetoxy, vinyl, hydroxy or hydroxyethyl, carboxyethyl or acetamide group; R_2, R_3 and R_4 can be a hydrogen or a methoxy group [Varga et al 1980, 1981; Ohmoto et al 1981a, b; Ohmoto and Koike 1984; Crespi-Perellino et al 1986a, b; Souleles and Waigh 1984; Souleles and Kokkalou 1989; Khan and Shamsuddin 1981].

The biological activity of some of these compounds has been tested [Bray et al 1987]. The major constituent canthin-6-one, has some antibacterial and antifungal activity against *Staphylococcus aureus, Klebsiella penumoniae* and *Mycobacterium smegmatis* [Mitscher et al 1972].

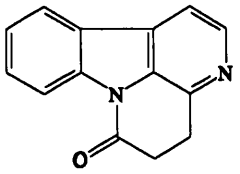
Canthin-6-one, 1-methoxycanthin-6-one and canthin-6-one-3N-oxide were found to have marked cytotoxic activity against guinea pig ear keratinocytes (GPK) [Anderson et al 1983], but in a previous report [Cordell et al 1978], canthin-6-one, 1-methoxycanthin-6-one and 5-methoxycanthin-6-one were not significantly active against KB cells. Structure activity relationships against KB cells have been investigated and it has been suggested that either hydroxylation or methoxylation of the alkaloids at C-11 or C-10 is required for cytotoxicity

Fig. 1.1 Examples of secondary metabolites from *A. altissima*

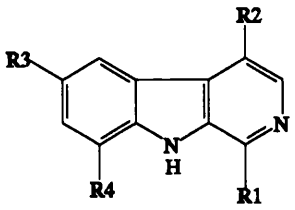




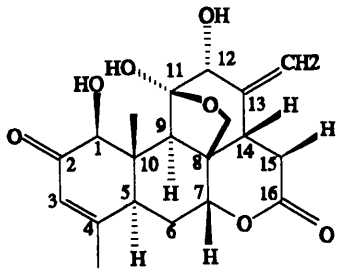
CANTHIN-6-ONE NUCLEUS



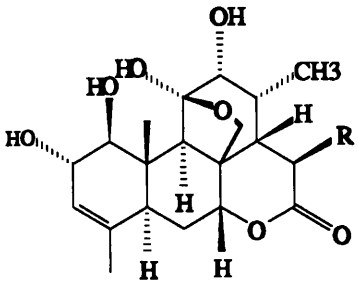
4,5-DIHYDROCANTHIN-6-ONE



B-CARBOLINE NUCLEUS

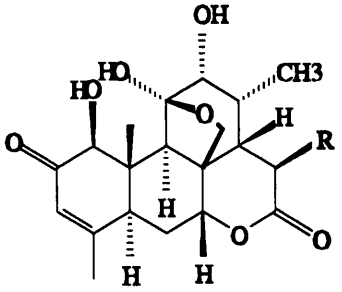


AILANTHONE



CHAPARRIN R=H

GLAUCARUBOL R=OH



AILANTHINONE R=OCOCH(CH3)CH2CH3

GLAUCARUBINONE R=OCOC(CH3)(OH)CH2CH3

[Fukamiya et al 1986, 1987]. Other studies in the lymphocytic leukaemia system from mice (P388) showed that oxygenation at C-1 and C-11 contributed significantly to the antileukaemic activity [Fukamiya et al 1986].

Studies on structure activity relationships for canthin-6-ones and β-carboline alkaloids and their inhibitory effects on cAMP phosphodiesterase have been performed [Sung et al 1984]. The canthin-6-ones with a hydroxymethyl group and an oxygen atom at C-5 or at both C-4 and C-5 and the β-carboline derivates with a hydroxymethyl group exerted a strong inhibitory effect on c-AMP. Some compounds found in *A. altissima* were tested for inhibition, but only 1-(1-hydroxy-2-methoxy)-ethyl-4-methoxy-β-carboline and 5-hydroxymethylcanthin-6-one had some inhibitory activity.

In addition to anti-cancer activity, 4,5-dihydrocanthin-6-one which has been only isolated from cell cultures of this species exhibits CNS depressant properties [Crespi-Perellino et al 1988].

Quassinoids

Quassinoids are the major constituents of the bark of *A. altissima*. The major quassinoid present is ailanthone, but to date, more than 20 quassinoids have been isolated from various parts of the tree [Tang and Eisenbrand 1992]. Many of these quassinoids are characterised by an oxygen bridge between C-11 and C-20. A few examples are given in Fig. 1.1.

The activity of the aqueous tea from the bark and fruit of *A. altissima* used in traditional medicine for the treatment of dysentery can be attributed to ailanthone, which exerts potent anti-amoebic activity against *Entamoeba histolytica in vitro* and *in vivo* [De Carneri and Casinovi 1986]. Other constituents which show anti-amoebic activity when compared with metronidazole are ailanthinone and glaucarubinone [Gillin et al 1982, Wright et

al 1988]. Tests have also shown that these two quassinoids are active against *Plasmodium falciparum in vitro* and *P. berghei* in mice but both were highly toxic, making their development as anti-malarial drugs unlikely [O'Neill et al 1986].

Quassinoids are also potentially a source of new anticancer agents [Suffness and Douros 1980]. Ailanthinone, glaucarubinone and 13(21)-dehydroglaucarubinone were found to have anti-cancer activity against the lymphocytic leukaemia system from mice (P388) and carcinoma cells of the nasopharynx (9KB) [Ogura et al 1977, Suffness and Douros 1980]. Studies in structure activity relationship suggest that the oxygen bridge at C-20 and C-11 or C-20 and C-13, and an ester function at C-15 are necessary for optimal anti-cancer activity [Cassady and Suffness 1980]. Unfortunately these quassinoids are significantly cytotoxic. The cytotoxicity may be related to the hydroxyl group at C-1 and so it is hoped that compounds with low cytotoxicity may be produced [Suffness and Douros 1980].

Quinones

The quinones that have been isolated from *A. altissima* include 2,6-dimethoxybenzoquinone [Souleles and Kokkalou 1989] and [3,3-dimethylallyl]-quinol-2-one [Anderson et al 1983].

SECONDARY METABOLITES FROM *AILANTHUS ALTISSIMA* CELL CULTURES

Cultures of this plant have been of particular interest because of their ability to produce high yields of canthin-6-one, for example 100 fold greater than the whole plant [Anderson et al 1987] in contrast with the very poor production of quassinoids [Jaziri et al 1987]. Alkaloid yields of 100 mg/l of which 62.5 mg/l was 1-methoxycanthin-6-one, 20 mg/l was canthin-6-one and the remainder was 1-hydroxycanthin-6-one have been obtained [Anderson et al 1983]. Other

authors reported an average of 400 mg/l of alkaloid from which 90% was canthin-6-one, and the second most abundant alkaloid was 1-methoxycanthin-6-one [Crespi-Perellino et al 1986a, b]. Other alkaloids that were not previously found in nature were isolated from these cultures: 2-hydroxycanthin-6-one, 4-hydroxycanthin-6-one and 4,5-dihydrocanthin-6-one. Also a yield of 50 mg/l of canthin-6-one was reported by other authors [Aragozzini et al 1988]. All these yields were obtained under different culture conditions (culture medium, temperature and light).

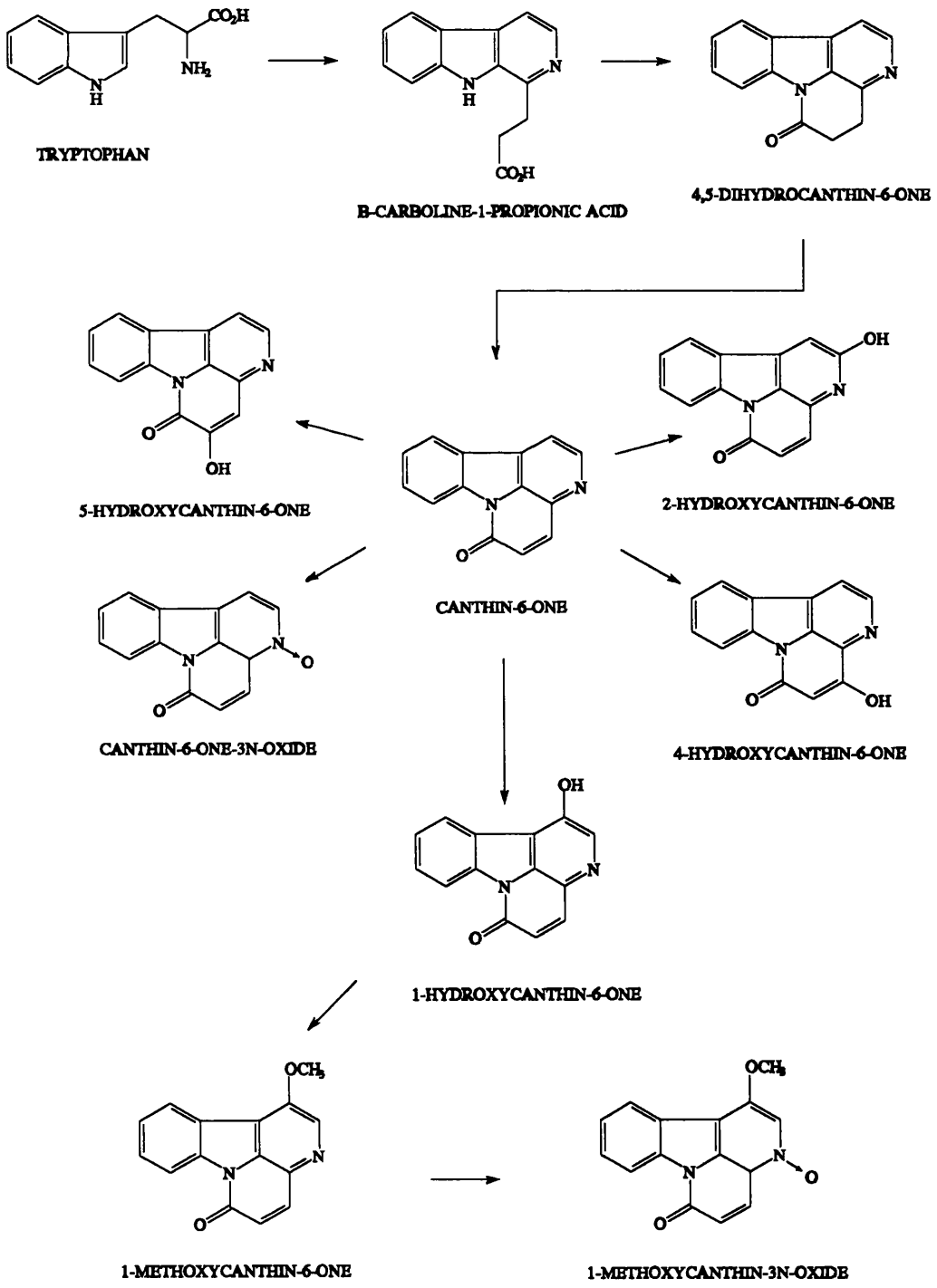
Under different culture conditions the yields for quassinoids, particularly ailanthone, were generally low and averaged only about 15 μg /100 g fresh weight [Jaziri et al 1987, Yeoman 1993].

Cell cultures of *A. altissima* are reported to produce the coumarins scopoletin and isofraxidin but in very low yield [Hay 1987, Roberts 1991, Osoba and Roberts 1994].

AILANTHUS ALTISSIMA CELL CULTURES IN THE STUDY OF THE BIOSYNTHESIS OF CANTHIN-6-ONE ALKALOIDS

Initially it was suggested that canthin-6-one could be derived from a β-carboline intermediate, to which a C-2 unit derived from either acetate or pyruvate is added, or from tryptamine, to which a C-4 is incorporated [Hegnauer 1973]. Latter the cell cultures of *A. altissima* were used to investigate the biosynthesis of canthin-6-one alkaloids. Crespi-Perellino et al [1986a, b] suggested that tryptophan, which is derived from the shikimic pathway, is the precursor of these alkaloids. They confirmed the route via 4,5-dihydrocanthin-6-one (Fig. 1.2) feeding (DL)-[methylene ^{14}C]-tryptophan, (L)-[methylene ^{14}C]-tryptophan, labelled β-carboline-1-propionic acid and 4,5-dihydrocanthin-6-one to suspension cultures and finding the labelling of the canthin-6-one alkaloids. Anderson et al [1986] and Hay [1987] also carried out experiments involving the incorporation of labelled tryptophan.

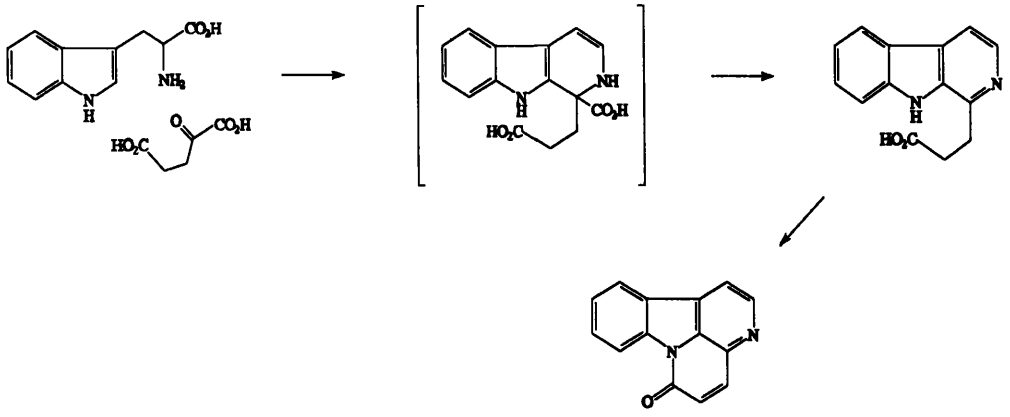
Fig. 1.2 Proposed pathway for the biosynthesis of canthin-6-one alkaloids in *A. altissima* cell suspension cultures



The role of tryptamine in the biosynthesis of these alkaloids was also investigated. In some experiments there was not found incorporation of labelled tryptamine into canthin-6-one [Crespi-Perellino et al 1986a, b] but in others, there was reported the incorporation of ^{14}C-tryptamine into the alkaloids [Hay 1987]. Subsequent experiments showed that tryptamine is not incorporated into the alkaloids, and also there were not significant levels of the enzyme responsible for the conversion of tryptophan to tryptamine (tryptophan descarboxylate) [Yeoman 1993].

The role of α-ketoglutarate in the biosynthesis of these alkaloids was also investigated. Aragozzini et al [1988] fed labelled sodium acetate to cell cultures of *A. altissima* and demonstrated a labelling pattern of canthin-6-one, that is consistent with the involvement of α-ketoglutarate as an intact precursor. It is believed that two instant acetate units are incorporated (Fig. 1.3], but as a result of the descarboxylation step, only one of these is present in canthin-6-one.

Fig. 1.3 Role of α-ketoglutarate in canthin-6-one alkaloid biosynthesis in cell suspension cultures of *A. altissima*



2. SHIKIMIC PATHWAY

INTRODUCTION

Many compounds derive from the shikimic pathway. Some few examples are the plant hormone indole acetic acid (IAA), lignin for structural support and folic acid for plant growth, chloramphenicol, phenazines, alkaloids, vitamins K_2 in microorganisms and vitamin K_1 in plants, phytoalexins and cyanogenic compounds for plant defense, and chromagens which provide brilliant colours in flowers and fruits. The three aromatic aminoacids tryptophan, phenylalanine and tyrosine, in addition to their role in protein biosynthesis, are also the precursors of many secondary compounds. Due to mammals lack of the shikimic pathway, the enzymes of this metabolic route are ideal targets for the development of herbicides [Kishore and Shah 1988]. For example glyphosate (N-phosphomethyl glycine) is a potent inhibitor of 5-enolpyruvylshikimate-3-phosphate synthase (the enzyme which catalyses the sixth step in this pathway) and is the active compound of the commercial herbicide ROUNDUP®.

Shikimic acid was the first intermediate in this metabolic pathway to be established, and this led to the pathway's name. Originally shikimic acid was obtained from *Illicium religiosum* Sieb. in 1885, and the name of the compound was derived from the Japanese name of the plant: shikimi-no-ki [Haslam 1974]*. This pathway was elucidated first in bacteria and fungi, using auxotopic mutants, tracer studies and ^{13}CNMR studies [Gilchrist and Kosuge 1980]. Evidence of common reaction sequence in plants and algae was also achieved with tracer studies and isolation of the intermediates [Dewick and Haslam 1969]. Final confirmation of the pathway in microorganisms and plants has been reached with the isolation of the pathway enzymes.

PRE-CHORISMATE PATHWAY

A condensation between two products of carbohydrate metabolism, phosphoenolpyruvate and D-erythrose-4-phosphate, initiates the pre-chorismate pathway. The first intermediate, product of the mentionated step, is 3-deoxy-D-

* references there in

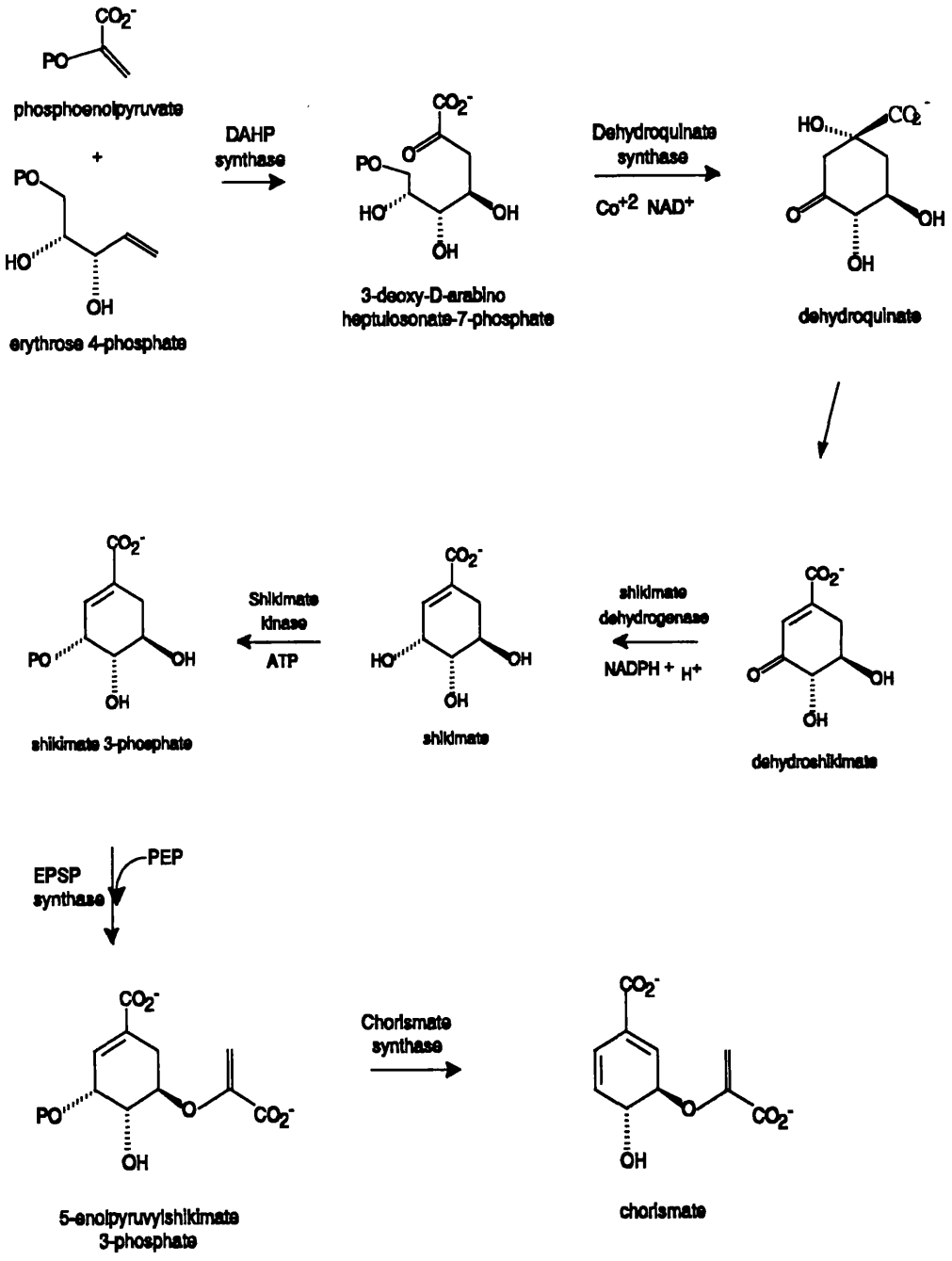
arabinoheptulosonate-7-phosphate which cyclises to form 7-dehydroquinate, which is transformed to 5-dehydroshikimate and to shikimate. Once shikimate is phosphorylated, it reacts with phosphoenolpyruvate to produce 5-enolpyruvylshikimate-3-phosphate, which is converted to chorismate (Fig. 2.1, Mann 1987). Almost 25% of the carbon fixed by green plants is routed through this pathway. All the enzymes involve in the biosynthesis of chorismate have been isolated and characterised from microorganisms and plants [Kishore and Shah 1988].

3-Deoxyarabinoheptulosonate-7-phosphate (DAHP) synthase

Erythrose 4-phosphate formed by the pent ose phosphate pathway is condensed with one molecule of phosphoenolpyruvate that is formed in the last stage of glycolysis by the first enzyme of the pathway: 3-deoxyarabinoheptulosonate-7-phosphate (DAHP) synthase. The microbial DAHP synthase has been found to be monofunctional or bifunctional. For example both *Escherichia coli* and *Salmonella typhimurium* contain three independent isoenzymes, all of them feedback regulated by L-phenylalanine (phe), L-tyrosine (tyr) and L-tryptophan (trp), respectively [Schoner and Herrmann 1976]; and in *Bacillus subtilis* chorismate mutase and DAHP synthase activities are in a single protein, which is feedback inhibited by chorismate and prephenate [Nakatasukasa and Nester 1972]. The fungal DAHP synthase enzymes are similar to the bacterial enzymes, *Neurospora crassa* contains three isoenzymes, which are also inhibited by the aromatic aminoacids phe, tyr and trp, respectively [Nimmo and Coggins 1981]. In *Saccharomyces cerevisiae* two isoenzymes were reported, one of which was feedback inhibited by phe and the other by tyr [Lingens et al 1967].

The number of reported DAHP synthase isoenzymes varies among different plant species. Two isoenzymes have been reported in mung bean (*Vigna radiata*) [Rubin et al 1982], tobacco [*Nicotiana tabacum*], while in pea (*Pisum sativum*) [Rothe et al 1976] and in carrot (*Daucus carota*) [Suzich et al 1985],

Fig. 2.1 Biosynthesis of chorismate



three isoenzymes were detected. The properties of the two or three isoenzymes were different.

Within the plants where two isoenzymes were reported, the proteins were designated DAHP-Mn and DAHP-Co. The plastid-localized enzyme, DAHP-Mn needed specifically Mn^{2+} for activity, was activated by dithiothreitol (DTT) and inhibited by L-arogenate, while the cytoplasmic, DAHP-Co utilised either Mg^{2+}, Mn^{2+} or Co^{2+}, was inhibited by DTT and caffeic acid, but was insensitive to L-arogenate [Rubin et al 1982, Rubin and Jensen 1985, Ganson et al 1986, Jensen 1986a, b].

Dehydroquinate synthase

The conversion of 3-deoxy-D-arabinoheptulosonate-7-phosphate to dehydroquinate is the first cyclization step in this pathway and is carried out by dehydroquinate (DHQ) synthase. The enzyme has been purified as a single protein in *E. coli*, but in *B. subtilis* the enzyme is a complex with chorismate synthase [Frost and Knowles 1984, Hasan and Nester 1977]. In *S. cerevisiae* and in other yeasts, this activity is catalysed by a pentafunctional enzyme that catalyses five subsequent reactions of the prechorismate pathway [Braus 1991] The enzyme shows NAD^+ and Co^{2+} dependence in bacteria and the fungal enzyme requires Zn^{2+} for its activity.

This enzyme has been highly purified from *Pisum sativum* [Pompliano et al 1989]. The enzyme requires also Co^{2+} and NAD^+ as co-factors. Other divalent ions, including Mg^{2+}, Mn^{2+} and Zn^{2+}, could not replace Co^{2+}, except Cu^{2+} but only at a low concentration (0.1 mM), otherwise the enzyme is inhibited [Yamamoto 1977].

Dehydroquinate dehydratase and shikimate oxidoreductase

Dehydroquinate dehydratase, also known as DHQ hydrolyase, and shikimate

oxidoreductase, known as shikimate dehydrogenase, usually reside in a bifunctional enzyme in plants [Mousdale et al 1987, Boudet and Lecussan 1974, Koshiba 1978]. In addition of the bifunctional enzyme, an isoenzyme of dehydroquinate dehydratase without shikimate oxidoreductase activity, has been reported in monocots of the families Juncaceae, Gramineae and Cyperaceae [Boudet et al 1977].

The number of reported isoenzymes of these enzymes in different species is variable. It seems that the number depends on the source of plant tissue and its development stage [Koshiba 1978, Fiedler and Schultz 1985, Mousdale et al 1987].

The bifunctional enzyme from pea was inhibited by chloride. The same anion and several other organic acid anions inhibited the monofunctional dehydroquinate dehydratase from *E. coli* (in this bacteria the two activities, DHQ dehydratase and shikimate oxidoreductase, are present in two different monofunctional enzymes), and the same enzyme activity from the *arom* pentafunctional enzyme from *Neurospora crassa* [Chaudhari et al 1986].

Shikimate kinase

Phosphorylation in position 3 of shikimate is catalysed by shikimate kinase (ATP:shikimate-3-P-transferase). Two isoenzymes are present in *S. typhimurium* and *E. coli.* [Ely and Pittard 1979, Berlyn and Giles 1969], but the functional significance of their existence is not known. The enzyme has been isolated and purified from chloroplast of spinach (*Spinacia oleracea*) and shown to be very unstable [Schmidt et al 1990]; and was partially purified from sorghum and mung bean seedlings [Bowen and Kosuge 1978, Koshiba 1979]. The enzyme required a divalent metal cation for activity, being Mg^{2+} the most effective. There was no feedback inhibition by any of the aromatic aminoacids, but both products of the reaction, shikimate 3-phosphate and ADP inhibited the enzyme.

5-Enolpyruvylshikimate-3-phosphate (EPSP) synthase

This enzyme has been one of the most intensively studied plant enzymes because it is the herbicidal target of glyphosate [Amrhein et al 1980]. It has been purified to homogeneity from bacteria, fungi and plants [Lewendon and Coggins 1983, Boocok and Coggins 1983, Steinrücken and Amrhein 1984, Mousdale and Coggins 1984, Steinrücken et al 1986, Padgette et al 1987].

Studies in the location of EPSP synthase showed that the enzyme on glyphosate-adapted cells was located in the plastids [Smart and Amrhein 1987]. Different isoenzymes have been reported, but their location was not determine [Jensen 1986b, Ream et al 1988].

Chorismate synthase

Chorismate synthase (5-enolpyrubylshikimate-3-phosphate phospho-lyase) catalyses the last common step in the biosynthesis of the three aromatic aminoacids in microorganisms and plants: the convertion of EPSP to chorismate. Although chorismate synthase activity has been detected in plants [Mousdale and Coggins 1986], the first enzyme was isolated from a cell culture of *Corydalis sempervirens* [Schaller et al 1990]. The enzyme has also been purified and characterised from *E. coli* [Morell et al 1967, White et al 1988], *Neurospora crassa* [Welch et al 1974, White et al 1988], *Bacillus subtilis* [Hasan and Nester 1978] and *Euglena gracilis* [Schaller et al 1991]. The enzyme from plant as well as microorganisms is oxygen sensitive. For example the enzyme activity from pea was detected only in the presence of reduced flavin.

CHORISMATE METABOLISM

Chorismate is the last common intermediate in the main stem of the shikimic pathway. At least five biochemical pathways utilise chorismate for the production of essential metabolites: tryptophan, phenylalanine and tyrosine, p-

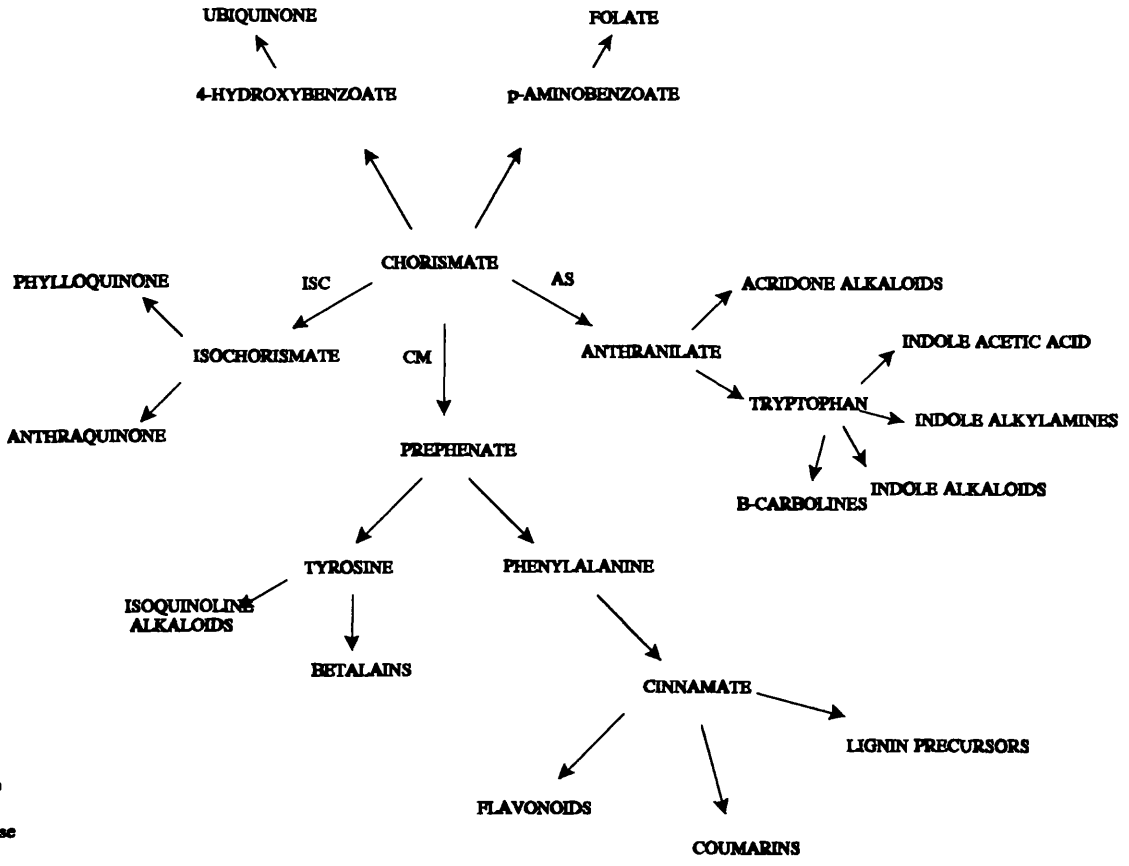
aminobenzoate and the folate group, isochorismate and 4-hydroxybenzoate with the ubiquinones (Fig. 2.2).

The conversion of chorismate into isochorismate is catalysed by isochorismate synthase, which utilises Mg^{2+} as cofactor. Isochorismate is the precursor of naphthoquinones and anthraquinones. Little work has been done on isochorismate synthase (ISC). As a result of this just a few years ago the first ISC was purified to homogeneity from *Escherichia coli* [Lui et al 1990], and the first report of purification from higher plants was from *Rubia tinctorum* L.[Poulsen 1993].

Biosynthesis of tryptophan

All the information indicates that tryptophan is produced from chorismate in plants by the same sequence (Fig. 3.3, p. 45). The first committed step to tryptophan biosynthesis in the pathway is the conversion of chorismate to anthranilate, reaction that is catalised by anthranilate synthase. After this reaction, a phosphoribosyl moiety is added to anthranilate (anthranilate phsphoribosyltransferase), and the product is transformed by an Amadori rearrangement into a 1-deoxy-ribulose 5- phosphate (phosphoribosyl anthranilate isomerase). This compound cyclizes to indole-3-glycerol phosphate (indole glycerol phosphate synthase) which is transformed into tryptophan by tryptophan synthase. In fungi and algae, several of the enzymatic activities are contained on the same polypeptide, while in plants the activities are contained in different monofunctional enzymes [Gilchrist and Kosuge 1980, Hankins et al 1976, Crawford 1989]. Important groups of secondary metabolites derived from L-tryptophan are methylated L-tryptophans, calycanthus alkaloids, β-carbolines, cinchona alkaloids and indole acetic acid (IAA).

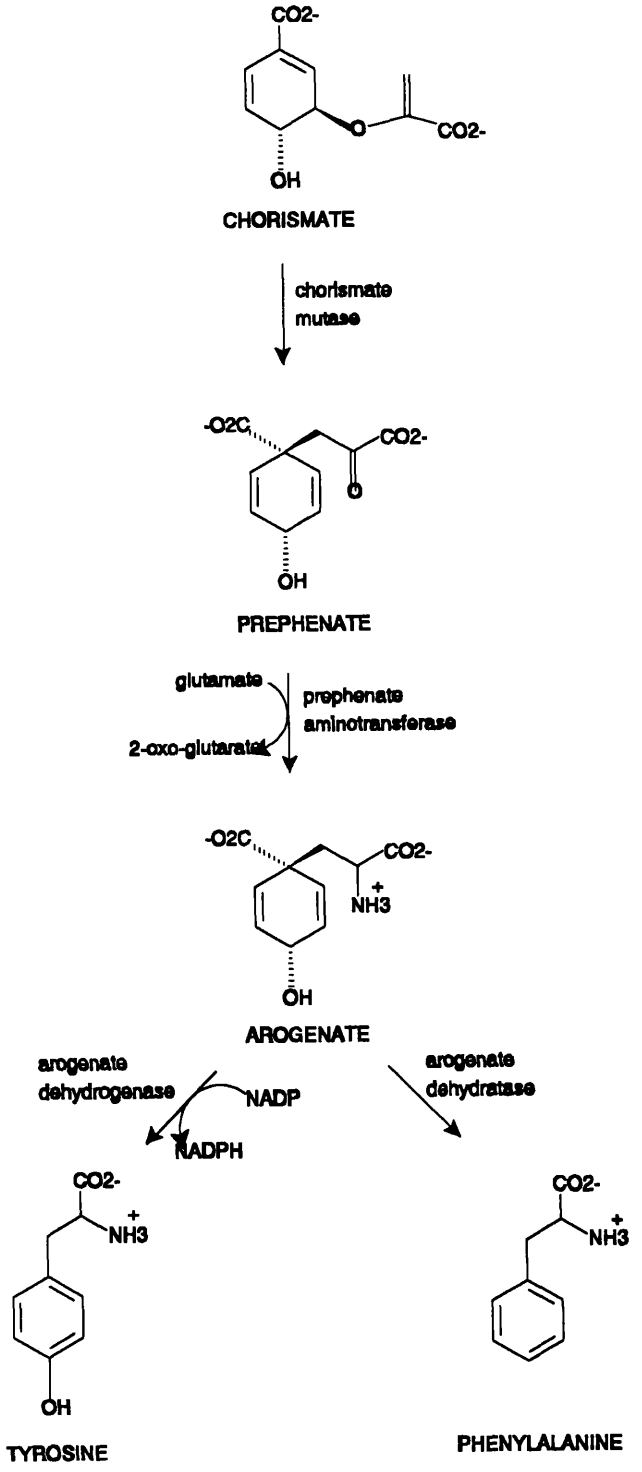
Fig. 2.2 Schematic diagram of some secondary metabolites obtained from chorismate



Biosynthesis of phenylalanine and tyrosine

Several pathways for the conversion of chorismate into phenylalanine and tyrosine exist and the pathway that is used depend on the organism. In plants as well as many microorganisms phenylalanine and tyrosine are produced via a common non- aromatic amino acid precursor: arogenate (Fig. 2.3), but also the aromatic keto-acid route is present in plants. Early work in the branches of these aromatic aminoacids sought to confirm the presence of the aromatic keto-acid routes established in enteric bacteria [Umbarger 1978, Garner and Herrmann 1983]. In these routes prephenate is aromatized to either phenylpyruvate or p-hydroxyphenylpyruvate, which are converted to phenylalanine or tyrosine, respectively (Fig. 2.3) The arogenate routes differ from the aromatic keto-acid routes only in the order of the aminotransferase and aromatization reactions, being prephenate the last common intermediate between phenylalanine and tyrosine in the keto-acid route, whereas arogenate is at the branch point in the arogenate route.

Fig. 2.3 Proposed pathway for the biosynthesis of phenylalanine and tyrosine in most plants



3. ANTHRANILATE SYNTHASE IN MICROORGANISMS AND PLANTS

INTRODUCTION

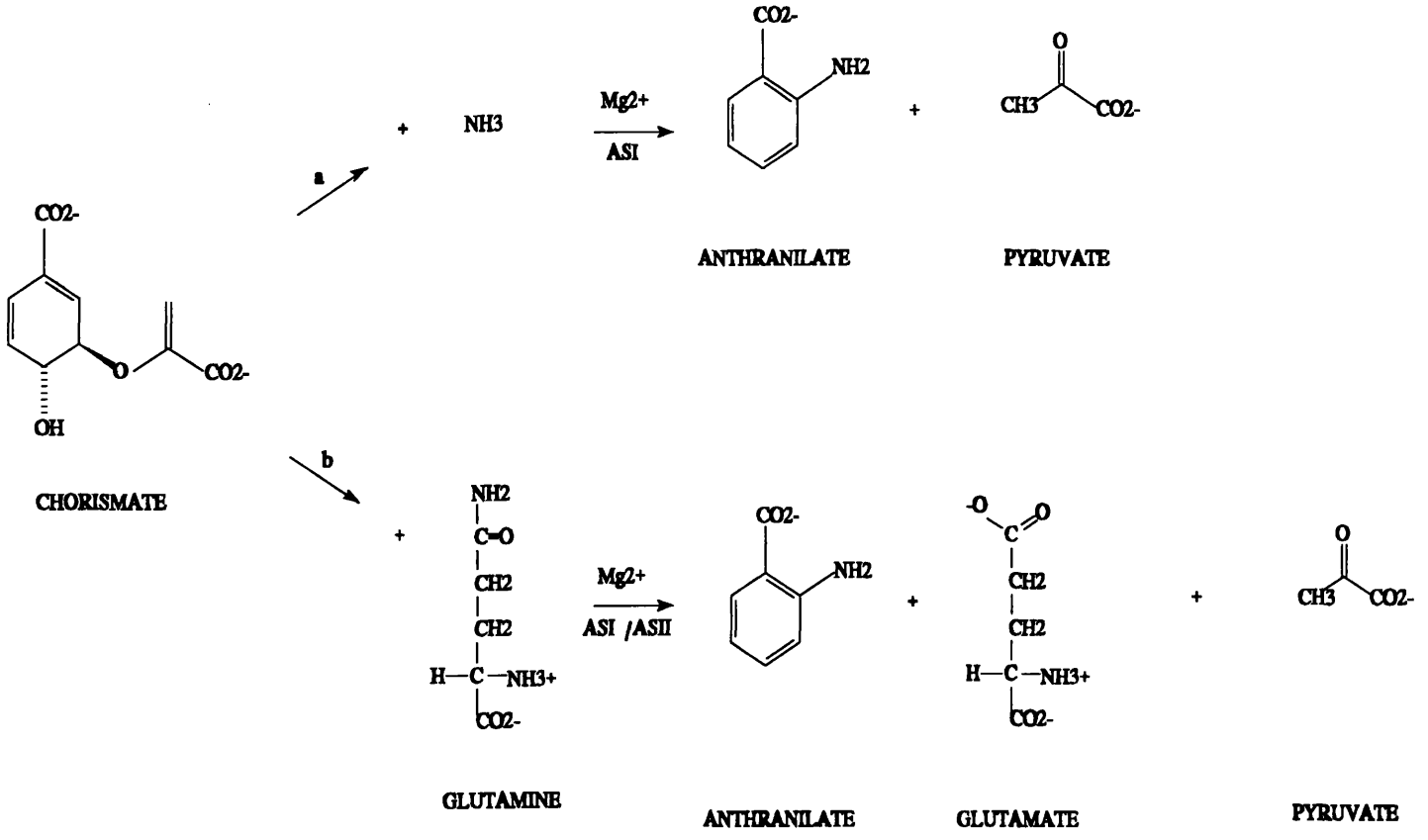
Anthranilate synthase (EC 4.1.3.27) catalyses the first committed step in the sequence of reactions which lead to the biosynthesis of tryptophan from chorismate. Tryptophan is utilised in microorganisms, as in plants, as a substrate for protein biosynthesis, however in some plants it also has an important role as precursor of secondary metabolites many of which have biological activity. In almost all microbial species, anthranilate synthase (AS) is an oligomer of nonidentical subunits designated ASI (α-subunit or component I) and ASII (β-subunit or component II). In some organisms the subunits are associated to give an αβ dimer and in others an $\alpha_2\beta_2$ tetramer [Kawamura et al 1978].

The AS α-subunit is encoded by the genes *trpE and TRP2* in prokaryotics and eukaryotics, respectively, but due to AS enzyme complex in its β-subunit sometimes contains other enzymes of tryptophan biosynthesis, other genes, besides *trpG* and *TRP3* can encode the multifunctional subunit [Hütter et al 1986].

It is believed that in plants the enzyme has also two subunits, but the enzyme complex does not contain other functionality. The presence of AS isoenzymes has been detected in cell cultures of tobacco and potato, but only in the cyanobacteria *Pseudomonas aeruginosa*.

ASI catalyses an NH_3-dependent synthesis of anthranilate (Fig. 3.1 route a) and ASII binds glutamine (Fig. 3.1 route b) facilitating the transfer of the amide function of glutamine to ASI. The utilisation of glutamine by anthranilate synthase therefore requires both subunits ASI and ASII, whereas use of ammonia requires only ASI [Zalkin 1985].

Fig. 3.1 Formation of Anthranilate from Chorismate



OCCURRENCE OF ANTHRANILATE SYNTHASE

Microorganisms

AS enzymes have been purified and characterized from a number of microorganisms and in many cases the enzyme complex catalyses other reactions of tryptophan biosynthesis. (Table 3.1, Table 3.2) Anthranilate synthase is associated with anthranilate phosphoribosyltransferase (PRT) in bacteria, with indole-3-glycerol phosphate synthase (InGPS) in yeast and with phosphoribosylanthranilate isomerase : indole-3-glycerol phosphate synthase (PRAI:InGPS) in fungi. For example, in *Neurospora crassa*, AS is fused to indole-3-glycerol phosphate synthase and phosphoribosylanthranilate isomerase, where the AS subunits associate to form an $\alpha_2\beta_2$ tetramer with the molecular weight of 310000 [Hulett and DeMoss 1973]. The trifunctional β-subunit of the AS complex of *N. crassa* has been purified from a mutant which produces no detectable α-subunit and a molecular weight of 200000 was determined using gel filtration [Walker and DeMoss 1983]. The activity of the glutamine-dependent anthranilate synthase was measured adding a partially purified preparation of free α-subunit.

Another three enzyme complex containing anthranilate synthase, phosphoribosyl anthranilate isomerase and indole-3-glycerol phosphate synthase was partially purified from a *Claviceps* species, strain SD 58, and showed a molecular weight of 400000 estimated using gel filtration [Mann and Floss 1977].

In species of genera *Escherichia*, *Salmonella*, *Klebsiella*, *Citrobacter* and *Enterobacter*, AS and anthranilate phosphoribosyltransferase (PRT) activities reside in a single, large complex enzyme [Zalkin 1985]. An example is the *Escherichia coli* enzyme which has a small subunit of 20000 molecular weight (ASII) and another of 45000 molecular weight (ASI), where these subunits associate to form an $\alpha_2\beta_2$ tetramer with molecular weight of 260000 [Ito and

Table 3.1 Occurrence of monofunctional anthranilate synthase in microorganisms

Organism	M_r	Structure	Separation/Purity	Refs
Aeromonas formicans	220000	-	crude	Largen and Belser 1975
Proteus morganii	140000	$\alpha_2\beta_2$	crude	Largen and Belser 1975
Proteus vulgaris	140000	$\alpha_2\beta_2$	crude	Largen and Belser 1975
Erwinia carotavora	140000	$\alpha_2\beta_2$	crude	Largen and Belser 1975
Erwinia hafniae	140000	$\alpha_2\beta_2$	crude	Largen and Belser 1975
Enterobacter liquefaciens	140000	$\alpha_2\beta_2$	crude	Largen and Belser 1975
Bacillus subtilis	84000	αβ	crude	Kane and Jensen 1970 Patel et al 1974
Bacillus licheniformis	α : >100000 β : 24000		crude	Patel et al 1974
Bacillus coagulans	α : 80000 β : 18000		crude	Patel et al 1974
Bacillus pumilis	86000	αβ	crude	Patel et al 1974 Hoch and Crawford 1973
Bacillus mascerans	α : 62000 β : 24000		crude	Patel et al 1974
Bacillus alvei	90000	αβ	crude	Patel et al 1974 Hoch and Crawford1973 Catena and DeMoss 1970
Chromobacterium violaceum	86000		crude	Wegman and Crawford 1968
Staphylococcus aureus	65000		crude	Procter and Kloos 1973
Serratia marcescens	141000	$\alpha_2\beta_2$	Purified to homogeneity: Protamine sulphate/$(NH_4)_2SO_4$/DEAE-cellulose/ECTEOLA-cellulose/Hydroxylapatite	Zalkin and Hwang 1971 Robb et al 1971 Largen et al 1976
Pseudomonas putida	75000	αβ	Purified to homogeneity[1]	Kawamura et al 1978 Queener et al 1973
P. aeruginosa	79000	αβ	crude	Crawford 1975
P. acifovorans	155000	$\alpha_2\beta_2$	crude	Queener and Gunsalus 1970
P. multivorans	150000	$\alpha_2\beta_2$	crude	Crawford 1975
P. testosteroni	155000		crude	Queener and Gunsalus 1970
Acinetobacter calcoaceticus	86000	αβ	$(NH_4)_2SO_4$/Sephadex G100	Twarog and Liggins 1970 Sawula and Crawford 1973
Clostridium butyricum	84000	$\alpha_2\beta_2$	crude	Baskerville and Twarog 1972 Baskerville and Twarog 1974
Bacillus caldotenax	α : 54000		Partially purified[2]	Shiratsuchi and Sato 1992
Serratia marinorubra	140000	αβ	crude	Largen and Belser 1975
Euglena gracilis	80000		Protamine sulphate/$(NH_4)_2SO_4$/DEAE cellulose/Sephadex G-200	Lara and Mills 1973

[1]ASI: $(NH_4)_2SO_4$/Sephadex G-100/DEAE cellulose/Preparative electrophoresis. ASII: $(NH_4)_2SO_4$/Sephadex G-100/DEAE cellulose/Calcium phosphate chromatography.
[2]Streptomycin sulphate/$(NH_4)_2SO_4$/DE-5PW chromatography.

Table 3.2 Occurrence of multifunctional anthranilate synthase in microorganisms

Organism	M_r	Structure	Separation/Purity	Refs
Salmonella typhimurium	280000	$\alpha_2\beta_2$	$(NH_4)_2SO_4$/Matrex gel Orange A	Bauerle et al 1987 Nagano and Zalkin 1970 Henderson and Zalkin 1971 Grieshaber and Bauerle 1974
Enterobacter aerogenes	170000	$\alpha_2\beta_2$	partial purification[1]	Egan and Gibson 1972
Citrobacter freundii	250000	$\alpha_2\beta_2$	crude	Largen and Belser 1975
Citrobacter ballerupensis	250000	$\alpha_2\beta_2$	crude	Largen and Belser 1975
Enterobacter cloacae	250000	$\alpha_2\beta_2$	crude	Largen and Belser 1975
Erwinia dissolvens	250000		crude	Largen and Belser 1975
Saccharomyces cerevisiae	130000	$\alpha_2\beta_2$	Purified to homogeneity[2]	Prantl et al 1985
Escherichia coli	260000	$\alpha_2\beta_2$	*$(NH_4)_2SO_4$/DEAE-cellulose/DEAE-cellulose	Ito and Yanofsky 1969
Neurospora crassa	α : 76000 β : 84000		Purified to homogeneity[3,4]	Gaertner and DeMoss 1969 Hulett and DeMoss 1973 Keesey et al 1981
Claviceps spec. SD58	400000	αβ	Purified to homogeneity[5]	Mann and Floss 1977

*Purified to homogeneity

[1]Protamine sulphate/$(NH_4)_2SO_4$, Sephadex G-200, DEAE cellulose

[2]Protamine sulphate/$(NH_4)_2SO_4$/Ethylamino Sepharose/Hydroxyapatite/DEAE Sepharose.

[3]$(NH_4)_2SO_4$/Sephadex G-25/DEAE cellulose/Sephadex G-75/DEAE cellulose/Sephadex G-200.

[4]$(NH_4)_2SO_4$/Anthranilic acid-agarose chromatography/DEAE cellulose/Gel filtration/Preparative electrophoresis.

[5]Protamine sulphate/$(NH_4)_2SO_4$/DEAE cellulose/Hydrosyapatite/Sephadex G-200/DEAE cellulose.

Yanofsky 1969].

Anthranilate synthase from *Saccharomyces cerevisiae* is also a bifunctional enzyme with indole-3-glycerol-phosphate synthase which shows an αβ structure with molecular weight of 130000 [Prantl et al 1985].
In *Bacillus*, *Proteus, Serratia, Aeromonas formicans, Enterobacter liquefaciens, Chromobacterium violaceum, Staphylococcus aureus, Acinobacter calcoaceticus, Clostridium butyricum,* some species of *Pseudomonas* and *Erwinia*, AS is not associated with another tryptophan biosynthetic enzyme [Largen and Belser 1975, Kane and Jensen 1970, Wegman and Crawford 1968, Procter and Kloos 1973, Twarog and Liggins 1970, Patel et al 1974]. The AS subunits are associated in a tetramer in *Serratia marcescens* (ASI= 60000 and ASII is 21000, AS= 141000 [Zalkin and Hwang 1971]), *Pseudomonas multivorans* and *P. testosteroni* (both enzymes' complexes are ~150000 [Crawford 1975, Queener and Gunsalus 1970]) and *Clostridium butyricum* (89000 [Baskerville and Twarog 1972]).

Both AS subunits isolated from *Pseudomonas putida* have been obtained in pure form: ASI has a molecular weight of 63400 and ASII of 18000 [Queener et al 1973]. Latter the molecular weight of ASII was redetermined using sodium dodecyl sulphate-gel electrophoresis giving a molecular weight of 21800 and the aminoacid sequence determination gave a value of 21684 [Kawamura et al 1978]. Less purified preparations of AS from *P. aeruginosa* and *P. acifovorans* have also been studied, and the AS enzymes found to have molecular weights of 79000 and 155000 [Crawford 1975, Queener and Gunsalus 1970], which values suggest αβ and $\alpha_2\beta_2$ structures, respectively. *Acinetobacter calcoaceticus* also shows an AS enzyme with αβ structure and molecular weight of 86000 [Sawula and Crawford 1973].

The eukaryote *Euglena gracilis* has the α and β-subunits fused (*Mr*= 80000 [Lara and Mills 1973]).

Plants

AS has been investigated from other plants but mainly in crude cell free extracts, and in most cases it has only been partially purified (Table 3.3). The enzyme has been detected in leaves of wheat (*Triticum aestivum*) [Singh and Widholm 1974], corn (*Zea mays*) [Singh and Widholm 1975, Hankins et al 1976], pea (*Pisum sativum)* [Hankins et al 1976], potato (*Solanum tuberosum*) [Carlson and Widholm 1978], tobacco (*Nicotiana tabacum*) [Brotherton et al 1986] and *Datura innoxia* [Ranch and Widholm 1983], and from the seeds of walnut (*Juglans regia*) [Grosse 1977]. AS has been studied in cell cultures of carrot (*Daucus carota*) [Widholm 1974, 1971, 1972a, 1972b], tobacco [Widholm 1974, 1971,1972a, 1972c, Belser et al 1971], rice (*Oriza sativa*), tomato (*Lycopersicon esculentum)* and soybean (*Glycine max*) [Widholm 1971], *Dioscorea deltoidea* [Kutáček et al 1981], *Cinchona succirubra* [Schmauder et al 1985], and *Ruta graveolens* [Hertel et al 1988a, Hertel et al 1988b].

The molecular weights of the AS enzyme of crude extracts from corn and peas were 95500 and 95300, respectively [Hankins et al 1976]. In tobacco, the tryptophan-resistant (trp-resistant) form of AS had a molecular weight of 200000, and the molecular weight of the tryptophan-sensitive (trp-sensitive) AS was 150000 [Brotherton et al 1986]. Latter the molecular weight of the enzyme from corn was redetermined and a value of 150000 was reported [Brotherton et al 1986] which is in contrast with that previously reported. All the molecular weights were determined using gel filtration chromatography. The molecular weight of AS isolated from cell cultures of *Ruta graveolens* was found to be 220000 [Hertel et al 1991].

Anthranilate synthase has been purified to a single protein only from cell suspension cultures of *Catharanthus roseus*. The AS enzyme was found to be a tetramer consisting of two large and two small subunits with molecular weights of 67000 and 25500 ± 500, respectively. The molecular weight of the AS tetramer determined by gel filtration was 143000 ± 500 and the optimum pH

Table 3.3 Occurrence of anthranilate synthase in plants

Organism	*Mr*	Structure	Separation/Purity	Refs
*Catharanthus roseus**	143000	$\alpha_2\beta_2$	Purified to homogeneity[1]	Poulsen et al 1993
Pisum sativum+	95300		Partially purified[2]	Hankins et al 1976
Zea mays+	95500		Partially purified[2]	Hankins et al 1976
*Nicotiana tabacum**+	200000		$(NH_4)_2SO_4$/Sephacryl S-200	Brotherton et al 1986
*Ruta graveolens**	220000		Partially purified[3]	Hertel et al 1991

*Cell cultures
+Plant
[1]Polyethylethyleneglycol/Q-Sepharose/Orange A/Mono-Q/Superose 6.
[2]Protamine sulphate/$((NH_4)_2SO_4$/DEAE cellulose/Sephadex G-150.
[3]$(NH_4)_2SO_4$/DEAE Sephacel/Sephadex G-200.

was between pH 7.5 and 8.3 [Poulsen et al 1993].

ISOENZYMES

Microorganisms

Two anthranilate synthase enzymes have been found in *Pseudomonas aeruginosa* and both have conventional α and β subunits [Essar et al 1990], each enzyme having a different function. One participates in tryptophan biosynthesis and is strongly inhibited by tryptophan and the genes for this enzyme have been designated as *trpE* (for α subunit) and *trpG* (for β subunit), being similar to the genes isolated from *P. putida*. The second AS, which was not inhibited by tryptophan, is encoded by the genes designated *phnA* (α subunit) and *phnB* (β subunit). This latter enzyme participates in the synthesis of the blue-green phenazine pigment, pyocyanin, which may be classified as a secondary metabolite.

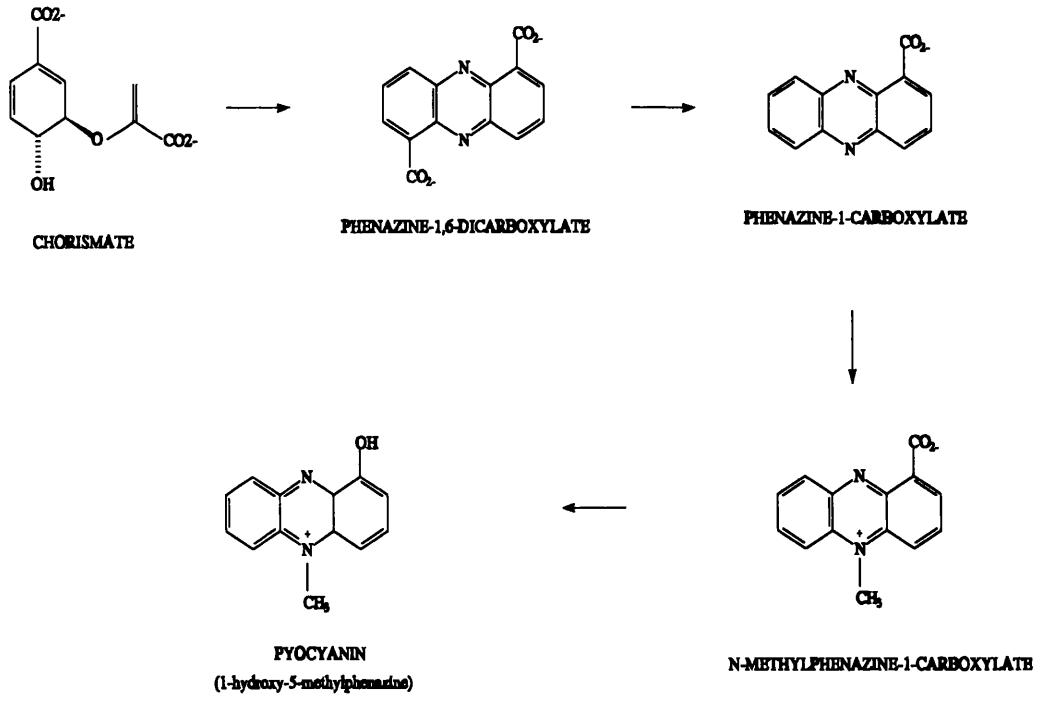
Chorismate is considered the precursor of the phenazines in all the cases investigated [Shinomiya et al 1983]. A hypothetical scheme for pyocyanin biosynthesis is in Fig. 3.2 [Leisinger and Margraff 1979]. This is the only clear example of the presence of two isoenzymes in either prokaryotic or eukaryotic organisms.

It has been reported in the literature that AS from an alkaloid-producing strain of *Claviceps paspali* was not inhibited by tryptophan, but latter the AS enzyme from *Claviceps* species strain SD58 was reported to be tryptophan inhibited [Lingens 1971]. It is not clear if the cells from which the enzyme was isolated produce alkaloid. The same strain, SD58, from an alkaloid-producing *Claviceps* yielded only AS which was inhibited by tryptophan and there was no evidence to support the presence of a second isoenzyme [Mann and Floss 1977].

Tryptophan feedback-resistant mutants of AS have been obtained in

microorganisms, for example in *E. coli* K-12, *E. coli* W and *S. typhimurium*. In *E. coli* K-12 mutants, it was established that the presence of the tryptophan-resistant AS was the result of a single mutation [Mann and Floss 1977, Gibson and Pittard 1968].

Fig. 3.2 Hypothetical Scheme for Pyocyanin Biosynthesis [Leisinger and Margraff 1979]



Plants

AS isoenzymes have also been reported in plants and these isoenzymes are mostly tryptophan inhibited.

In *Solanum tuberosum* cell suspension cultures [Carlson and Widholm 1978] which were resistant to growth inhibition by D,L-5-methyltryptophan, two AS

isoforms were separable by preparative polyacrylamide gel electrophoresis: one form was feedback-sensitive and the other feedback-resistant to tryptophan. The AS isoforms from the normal cell lines were also examined and the tryptophan-sensitive form was found to predominate. In *Nicotiana tabacum* L. cells, two forms of AS (trp-sensitive and trp-resistant) were detected [Brotherton and Widholm 1985, Brotherton et al 1986] and separated by gel filtration. The trp-sensitive was found in the organelle fraction and the trp-resistant in the cytosol. When cells were selected for resistant to growth inhibition by D,L-5-methyltryptophan (5MT) they were found to contain predominantly the tryptophan-resistant form, which was only present in small amounts in the wild-type cells. Plants regenerated from both normal and 5MT resistant cells lack detectable quantities of trp-resistant AS. Overexpression of the trp-resistant AS reappeared when callus was initiated from leaves of these plants.

Altered AS enzymes less sensitive to tryptophan feedback-inhibition have been reported in *Datura inoxia, Dioscorea deltoida, Daucus carota*, etc, but only one AS isoenzyme was detected [Singh and Widholm 1975, Ranch and Widholm 1983, Widholm 1971, Widholm 1972b, Kutáček et al 1981, Widholm 1977, Sung 1979].

The presence of two AS enzymes in *Pseudomonas aeruginosa* and in cell cultures of potato and tobacco, may support the hypothesis that two complete pathways exist for the biosynthesis of aromatic aminoacids for primary and secondary metabolism.

GENE-ENZYME RELATIONSHIP AND FUNCTIONALITY

Microorganisms

The tryptophan pathway has been studied extensively. Genes for the seven enzymatic functions of the pathway in prokaryotics (bacteria and blue-green

algae) are represented by the letters *A-G* , for example *trpE*, and in eukaryotic organisms (fungi) by numbers and capital letters, for example *TRP2* (Figure 3.3, Table 3.4) [Hütter et al 1986].

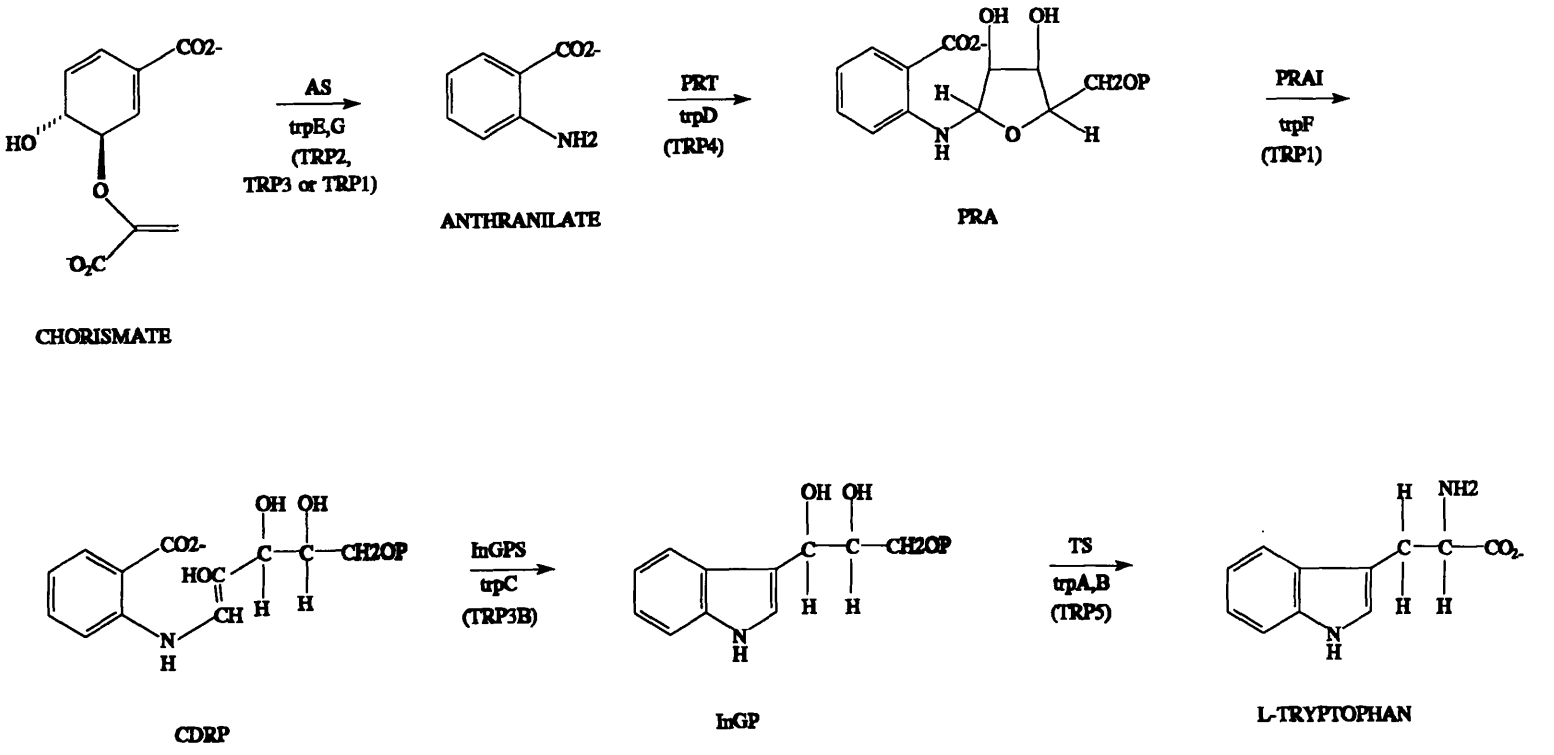
In bacteria ASI is encoded by the single gene *trpE*, however the situation for ASII is more complex.

In some bacteria ASII is encoded by a single gene, *trpG*, as is found in *Serratia marcescens* [Tso et al 1980], and in *Thermus thermophilus* [Sato et al 1988]. In other microorganisms such as *E. coli*, *Salmonella typhimurium*, and *Shigella dysenteriae, trpG* is fused to the *trpD* gene, which encodes for anthranilate phosphoribosyl transferase, the second enzyme in the tryptophan pathway [Nichols et al 1980, Yanofsky et al 1981]. *Rhizobium melitoti* presents a special case in that a single gene encodes both ASI and ASII activities. Fusion of these subunits has so far been seen only in one group of purple bacteria: the α subdivision [Crawford 1989, Bae et al 1989]. The product of this unusual *trpE(G)* gene, anthranilate synthase, is feedback inhibited by tryptophan, as happens for all known microbial anthranilate synthases with the exception of *P. aeruginosa* [Bae and Crawford 1990].

Pseudomonas putida and *P. aeruginosa* possess two genes *trpE* and *trpG* that encode the α-subunit (ASI) and the small β-subunit (ASII) of anthranilate synthase [Essar et al 1990b]. In experiments where mutants of these organisms were created [Essar et al 1990a, 1990b] the DNA sequence analysis as well as growth and enzymes assays of the strains developed showed that *trpG* is the first gene in a three-gene operon that also contains *trpD* (phosphoribosylanthranilate transferase) and *trpC* (indole-3-glycerol phosphate synthase) meanwhile the gene *trpE* is solitary.

Two anthranilate synthase gene pairs have been identified in *P. aeruginosa* [Essar et al 1990c]. After being cloned, sequenced and inactivated *in vitro*

Fig. 3.3 Biosynthesis of Tryptophan



Abbreviations: AS, anthranilate synthase; PRT, phosphoribosylanthranilate transferase; PRA, N-phosphoribosylanthranilate; PRAI, N-phosphoribosylanthranilate isomerase; CDRP, 1-(o-carboxyphenylamino)-1-deoxyribulosephosphate; InGPS, indole-3-glycerol phosphate synthase; InGP, indoleglycerol phosphate; TS, tryptophan synthase.

Table 3.4 Examples of gene-enzyme relationships and functional organization in plants and microorganisms respect to anthranilate synthase.

Organism	*Gene encoding AS synthase (subunit encoded)*	*Functionality of the enzyme complex*
plants:		
Arabidopsis thaliana	*ASA1/ASA2* (α-subunit) *ABS1/ABS2/ABS3* (β-subunti)	monofunctional?
prokaryotics:		
Escherichia coli	*trpE* (α-subunit) *trpG-trpD* (β-subunit)	bifunctional with anthranilate phosphoribosyl transferase
Serratia marcescens	*trpE* (α-subunit) *trpG* (β-subunit)	monofunctional
Rhizobium melitoti	*trpE(G)* (α and β subunits)	monofunctional
eukaryotics:		
Neurospora crassa	*TRP2* (α-subunit) *TRP1* (β-subunit)	trifunctional with PR transferase and InGP synthase
Saccharomyces cerevisiae	*TRP2* (α-subunit) *TRP3* (β-subunit)	bifunctional
Euglena gracilis	*trpE(G)*	monofunctional

by insertion of an antibiotic resistance gene, they were returned to *P. aeruginosa*, replacing the wild-type gene. One AS gene pair participates in tryptophan biosynthesis, with the known *trpE* and *trpG* genes, but the other pair, encoded by *phnA* and *phnB* participate in the synthesis of the phenazine, pyocyanin. Surprisingly these latter two genes are more closely related to *E. coli trpE* and *trpG* than *Pseudomonas trpE* and *trpG*, whereas *Pseudomonas trpE* and *trpG* are more closely related to *E. coli pabA* and *pabB*. Genes homologous to *phnA* and *phnB* were not found in *P. putida* PPG1 (a non-phenazine producer).

The anthranilate synthases in fungi are also composed of two subunits α and β encoded by the *TRP2* and *TRP3* or *TRP1* respectively. The glutamine amidotransferase domain (β-subunit) is located on different multifunctional polypeptides so that the AS complex contains either AS, N-phosphoribosylanthranilate isomerase and indole-3-glycerol phosphate synthase (*TRP1* gene), or AS and InGP synthase (*TRP3* gene) [Hütter and Niederberger 1986]. Biochemical studies have established that the first pattern of genetic control, where AS, PRA isomerase and InGP synthase are in the same polypeptide, is present in *Neurospora crassa* [Walker and DeMoss 1983, 1986], *Aspergillus nidulans* [Hütter and DeMoss 1967, Roberts 1967], *Coprinus radiatus* [Henke 1973] and *Schizosaccharomyces pombe* [Schweingruber and Dietrich 1973, Thuriaux et al 1982]. In general, the trifunctional gene encoding these activities has been widely observed in members of the classes Myxomycetes, Chytridiomycetes, Ascomycetes and some Basidiomycetes on the basis of sedimentation on sucrose gradients, resolution by classical purification techniques and genetic studies.

Enzymes' complexes with AS and InGP synthase activities are found exclusively among genera of the order Endomycetales, e.g. *Dipodascus uninucleatus*, *Endomyces bisporus* and *Saccharomyces cerevisiae* [Zalkin et al 1984, Mortimer and Schild 1985, Braus 1991].

In the algae *Euglena gracilis*, AS is encoded by only one gene *trpE(G)*, because this enzyme has both subunits, ASI and ASII, fused [Hankins and Mills 1976].

Plants

Little work has been done with plants, generally because purification and characterisation of AS is rather difficult due to its relative instability. Only the enzyme from *Catharanthus roseus* has been purified to a single protein but the genes for this enzyme in *C. roseus* have yet to be isolated [Poulsen et al 1993]. This enzyme did not contain anthranilate-5-phosphoribosylpyrophosphate phosphoribosyltransferase activity.

Arabidopsis thaliana has two genes, *ASA1* and *ASA2*, encoding the α-subunit of AS and the aminoacid sequences of the genes were similar to their microbial counterparts [Niyogi and Fink 1992]. *ASA1* is induced by wounding and bacterial pathogen infiltration, suggesting a novel role for *ASA1* in the production of tryptophan pathway metabolites as part of *Arabidopsis* defense response. This plant seems to have three very similar *ASB* genes which encode the β-subunit of AS and the most characterized of these genes, *ASB1*, encodes a β-subunit that can interact with either *ASA1* or *ASA2* in *E. coli* to provide functional glutamine-dependent AS activity. It is not known which *ASB* gene(s) is induced by pathogens [Niyogi et al 1993].

Aminoacids sequences

There have been thirty five aminoacid sequences published for the two anthranilate synthase subunits and most of them have been reviewed and their sequences compared with those of *p*-aminobenzoate synthase (PABA) [Crawford 1989]. This latter enzyme, as well as isochorismate synthase, seems to have a reaction mechanism similar to AS. It has also two dissimilar subunits which are very similar in sequence, to their AS counterparts [Goncharoff and

Nichols 1984, Kaplan and Nichols 1983]. An interesting fact is that species in three genera, *Acinetobacter*, *Bacillus*, and *Pseudomonas*, have a single β-subunit that supplies the glutamine aminotransferase activity for both AS and PABA [Buvinger et al 1981, Kane et al 1972, Sawula and Crawford 1972]. As these enzymes participate in both the conversion of chorismate to anthranilate and to p-aminobenzoate, they are called amphibolics. It is believed that species from genus *Azospirillum* also carry an amphibolic protein [Zimmer et al 1991].

To express quantitatively the degree of similarity the method of Doolittle [Doolittle 1986] was used. The normalized alignment score (NAS) is almost 10 times the percent of identical residues less the gap penalty (gap penalty is 2.5 times the weight of a residue identity). For sequences 200 residues long, similarity values greater than 160 indicate that common ancestry is probable, and values above 280 make it certain.

Values for the α-subunits of a wide range of microorganisms are given in Table 3.5, and because all these components have approximately 500 aminoacid residues, the values cited are within the range indicative of homology. There is remarkable similarity between the *trpE* aminoacid sequences of *P. savastanoi*, *P. aeruginosa* and *P. putida*. [Li et al 1974]. These sequences are on average 86% similar (values for *P. savastanoi* and *P. putida* were not included in Table 3.5).

The aminoacid sequence of ASI of *Bacillus caldotenax* has been also determinated and the conserved aminoacids among the ASI sequences of *B. subtilis* [Henner et al 1984], *Clostridium thermocellum* [Sato et al 1989], *Thermus thermophilus* [Sato et al 1988], *E. coli* [Nichols et al 1980] and *Brevis lactofermentum* [Matsui et al 1987] amount to 61 residues, most of them being found in the C-terminal half [Shiratsuchi and Sato 1991].

The *trpE* from *Haloferax volcanii* shows 29 to 30% aminoacid sequence identity

Table 3.5 Similarity (NAS) scores for AS α-subunits (Crawford 1989, Brahamska et al 1991)

Organism and Gene	*E.c. trpE*	*V.p. trpE*	*P.a. phnA*	*B.l. trpE*	*R.m. trpE*	*S.a. trpE*	*P.a. trpE*	*B.s. trpE*	*S.c. trpE*
L.bif. trpE	187		248	117				233	180
E.coli trpE		580	456	426	238	269	252	240	224
V.par. trpE			431	415	208	275	252	230	215
P.aer. phnA				392	208	247	260	225	204
B.lac. trpE					198	222	216	205	208
R.mel. trpE						208	193	201	217
S.aur. trpE							213	261	248
P.aer. trpE								319	272
B.sub. trpE									255

Abbreviations: *V.par and V.p., Vibrio parahaemolyticus; P.aer.and P.a, Pseudomonas aeruginosa; B.lac.and B.l., Brevibacterium lactofermentum; R.mel. and R.m., Rhizobium meliloti; S.aur. and S.a., Spirochaeta aurantia; B.sub. and B.s., Bacillus subtilis; S.c., Saccharomyces cerevisiae; E.c. and E.coli, Escherichia coli; L.bif. Leptospira biflexa.*

Table 3.6 Similarity (NAS) score for anthranilate synthase β-subunits (Crawford 1989)

Organism and Gene	*S.t. trpG*	*S.m. trpG*	*V.p. trpG*	*P.a. phnB*	*B.l. trpG*	*B.s. trpG*	*P.p. trpG*	*P.a. trpG*	*A.c. trpG*	*R.m. trpG*	*S.c. trpG*	*N.c. trpG*
E.coli trp(G)	965	839	622	475	290	399	383	396	317	260	332	332
S.tym. trp(G)		834	627	470	290	384	363	381	302	265	330	322
S.mar. trpG			591	396	311	401	380	401	327	267	320	302
V.par. trpG				415	347	369	367	379	362	347	342	296
P.aer. phnB					325	363	388	395	331	303	318	328
B.lac. trpG						225	270	316	278	206	265	281
B.sub. trpG							633	585	563	284	477	485
P.put. trpG								843	655	281	467	561
P.aer. trpG									649	297	472	500
A.cal. trpG										267	434	487
R.mel. trp(G)											305	251
S.cer. trp(G)												654

Abbreviations: *S.tym. and S.t., Salmonella typhimurium; S.mar. and S.m., Serratia marcescens; V.par. and V.p., Vibrio parahaemolyticus; P.aer. and P.a., Pseudomonas aeruginosa; B.lac. and B.l., Brevibacterium lactofermentum; B.sub. and B.s., Bacillus subtilis; P.put. and P.p., Pseudomonas putida; A.cal. and A.c., Acinetobacter calcoaceticus; R.mel. and R.m., Rhizobium meliloti; S.cer. and S.c., Saccharomyces cerevisiae; N.c., Neurospora crassa.*

with *E. coli* and yeast enzymes, respectively, 34% identity with *Methanobacterium thermoautotrophicum*, and comparable values with the homologous *pabB* and *phnA* genes of *E. coli* and *P. aeruginosa* [Lam et al 1992, Meile et al 1991].

E. coli and *Shigella dysenteriae* show the highest similarity comparing their *trpG* sequences, where there were no gaps and only three nonidentical aminoacids in the 195-residue polypeptide [Nichols et al 1980]. The fungi *Aspergillus nidulans* [Kos et al 1988, Mullaney et al 1985], *Aspergillus niger* [Kos et al 1988], *Penicillium chrysogenum* [Penalva and Sanchez 1987], *Phycomyces blakesleeanus* [Choi et al 1989] and *N. crassa* have very highly similar sequences (The NAS values are between 624 and 721 [Crawford 1989]).

Comparisons between other species are presented in Table 3.6 [Crawford 1989]. The *P. putida* ASII sequence exhibits identities with the NH_2-terminal end of *E. coli* and *S. typhimurium* ASII and the monofunctional ASII of *Serratia marcescens*. These results suggest that the glutamine amidotransferase region of the bifunctional AS of *E. coli* and *S. typhimurium* with the smaller subunit of *S.marcescens* and *P. putida* share the same evolutionary origin [Kawamura et al 1978, Li et al 1974]. This suggestion gives support to the idea that the bifunctional ASII polypeptides of *E.coli* and *S. typhimurium* arose by fusion of two separate genes.

The *trpG* from *H. volcanii* is 30 to 37% identical in sequence to *E.coli* and yeast homologous, respectively, and 38% similar to *M. thermoautotrophicum trpG* [Lam et al 1992].

The *A. thaliana ASA1* and *ASA2* predicted amino acid sequences were aligned with AS α subunits from *S. cerevisiae*, *E. coli*, and *B. subtilus*, along with the PABA synthase α-subunit from *E. coli* and are 30 to 36% identical to these microbial subunits and 67% identical to each other [Niyogi and Fink 1992]. The *ASB1* is 34 to 45% identical to AS β-subunit of *S. cerevisiae* and

E.coli AS and PABA synthase β-subunits [Niyogi et al 1993].

THE REACTION MECHANISM

Anthranilate synthase is available from organisms harboring plasmids with the gene for one or both subunits [Zalkin 1985], and has consequently been the focus of several mechanistic studies.

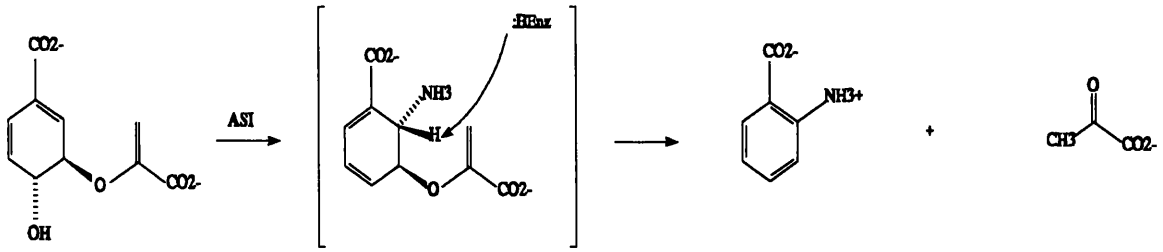
The larger α-subunit of AS is involved with the binding of chorismate and the regiospecific amination/pyruvate elimination sequence [Walsh et al 1987]. This α-subunit can synthesize anthranilate directly from chorismate and a high concentration of ammonia. The low molecular weight β-subunit possesses the glutaminase activity that hydrolyses the cosubstrate glutamine, via a γ-glutamyl-*S*-cysteinyl enzyme intermediate to release NH_3 for the amination sequence [Walsh et al 1990].

The glutamine binding site has been identified by using reactive analogs of glutamine as affinity labels which block the active site. An example is the inactivation of the multifunctional anthranilate synthase from *Neurospora crassa* that loses its glutamine-dependent anthranilate synthase activity on exposure to azaserine (*O*-diazoacetyl-L-serine) and DON (6-diazo-5-oxo-L-norleucine). The inactivation depends on the presence of the substrate chorismate, which is an essential cofactor for the inactivation reaction, is enhanced by the cofactor Mg^{+2}, and is antagonized by glutamine. The ammonia-dependent anthranilate synthase activity is lost but in less extensive rate, which indicates that the maximum expression of the ammonia-dependent domain also depends on the interaction with an active glutamine amidotranferase site [Paukert et al 1982].

The most likely intermediate in the conversion of chorismate to anthranilate is an "amino" analogue of isochorismate: *trans*-6-amino-5-[(1--carboxyethenyl)-oxy]-1,3-cyclohexadiene-1-carboxylate (Fig. 3.4). For the formation of this intermediate, It has been proposed that there is a formal syn-1,5 displacement

of hydroxide by ammonia [Policastro et al 1984]. In these magnesium dependant enzymes, the most obvious role that Mg^{2+} may play is to chelate to the 4-hydroxyl group of chorismate, making it a better leaving group. The same mechanism is proposed for isochorismate synthase [Kozlowski and Bartlett 1991], but in this case the magnesium ion is bound to the enzyme with at least one ammonia molecule in its coordination sphere. The subsequent aromatization, when pure enzyme was used, consisted in a cis elimination/aromatization route (Fig. 3.4) [Teng and Ganem 1984]. However other authors found that during aromatization of chorismate the third methyl hydrogen in pyruvate comes from a *re*-face addition of a proton from the solvent [Asano et al 1985].

Fig. 3.4 *Cis* elimination/aromatization route for the formation of anthranilate from chorismate



The synthetic *trans*-aminoenolpyruvate is a chemically and kinetically competent intermediate in the biosynthesis of anthranilate but to date has not been detected as an accumulating intermediate during enzymatic processing. Both stepwise and concerted mechanisms are possible.

Active sites

Several experiments have been done to identify the active site on the anthranilate subunits. Different chemical methods were used to probe residues

essential for ASI activity in *Serratia marcescens* [Tso and Zalkin 1981]. For example, phenylglyoxal and 1,2-cyclohexanedione modified the 2-5 arginine residues and inactivated the subunit, but in the presence of chorismate, the rate of inactivation was reduced. Analysis of the data indicated that one arginine residue is essential for activity. The histidine residues were modified also with ethoxyformic anhydride and by photooxidation, and the enzyme was also inactivated, but the substrate prevented the inactivation. The enzyme lost activity when one cysteine residue was alkylated and a tryptic peptide containing the essential cysteine residue has been isolated.

As in *S. marcescens*, *Bacillus caldotenax* showed that one arginine residue is crucial for ASI activity. The identity of this residue was established using site-directed mutagenesis and a comparative survey of aminoacid sequences disclosed that six arginine residues were conserved in seven ASI subunits from *B.caldotenax, Bacillus subtilis, Clostridium thermocellum, Thermus thermophilus, E. coli, Brevis lactofermentum* and *Saccharomyces cerevisiae* [Shiratsuchi and Sato 1992]. There was no conserved cysteine, but two histidines are conserved among these microorganisms which suggest further studies to examine the effect of substitution of these histidine residues.

In the case of ASII, the active site has been studied in *S. marcescens* [Tso et al 1980a,b] and *Pseudomonas putida* [Kawamura et al 1978]. The active site region for *S. marcescens* is virtually identical to that of the *P. putida*. An essential cysteine residue was alkylated selectively in both microorganisms: in *P. putida* the active site residue is 79, and in *S. marcescens* is residue 83. This essential residue is conserved in ASII subunits of *E. coli, S.typhimurium, S. dysenteriae, P. aeruginosa, Acinobacter calcoaceticus, N. crassa* and *Saccharomyces cerevisiae* [Zalkin et al 1984].

Latter it was found that modification of a lysyl residue in *S. marcescens* reduces the reactivity of the essential cysteinyl residue, resulting in the lost of the amidotransferase activity. The rate of lysyl modification is enhanced by

chorismate, providing evidence for the interaction of the cysteinyl and lysyl residues, and it has been suggested that the lysyl residue functions as a general acid-base to promote ionization of the cysteinyl residue [Bower and Zalkin 1982]. The lysyl residue is conserved in all the organisms noted above [Zalkin et al 1984].

The active site for tryptophan feedback inhibition has been studied in *Brevibacterium lactofementum*, where 5-fluorotryptophan-resistant mutant was isolated and the mutation site was determined [Matsui et al 1987]. The mutant has an altered AS which was fully active even in the presence of 10mM tryptophan, while the activity of the wild type AS under the same conditions was less than 1% of that lacking tryptophan. One adenine to cytosine single-base-pair substitution at codon Ser-38 (the Ser codon, AGC, was changed to an Arg codon, CGC) was the cause for the desensitization to feedback inhibition in the mutant. It was found also that the particular aminoacid sequence from Leu-35 to Ser-38, Leu-Leu-Glu-Ser, in *B. lactofermentum trpE* gene was conserved in the corresponding regions of *E. coli* [Yanofsky et al 1981], *Salmonella typhimurium* [Caligiuri and Bauerle 1991a], *Haloferax volcanii* [Lam et al 1992] and *B. subtilis* (Henner et al 1984), suggesting that this particular aminoacid region and Ser-38 are essential for the allosteric regulation of AS. Substitution guanine to adenine in the attenuator region was found to enhance expression of tryptophan genes.

Another interesting study was done on a hybrid complex containing one catalytically active, feedback-insensitive to tryptophan and one catalytically inactive, feedback-sensitive mutant *trpE* subunit from *Salmonella typhimurium*, where the binding of a single inhibitor molecule to one *trpE* subunit was sufficient for the propagation of a conformational change that affects the active site of the companion unit [Caligiuri and Bauerle 1991b].

UTILITY OF BIFUNCTIONAL ENZYME FOR THE CLASSIFICATION OF BACTERIA

Since bifunctional proteins are the result of relatively infrequent genetic events that are faithfully conserved, they are reliable markers to define phylogenetic clusters [Ahmad and Jensen 1986].

The bifunctional enzyme anthranilate synthase-anthranilate phosphoribosyl transferase is present in the lineage shared by the genera *Escherichia, Salmonella, Citrobacter, Klebsiella* and *Enterobacter*, but is absent in other enteric bacteria from genera *Erwinia, Serratia, Proteus, Morganella* and *Hafnia* [Henderson et al 1970, Largen and Belser 1975 and Zalkin 1980]. The presence or absence of this enzyme has been used to separate two enteric clusters and it has been used to postulate that aerogenic and anaerogenic strains of *Enterobacter agglomerans* belong to different groups [Ahmad and Jensen 1989]. The strains assigned to *E. agglomerans* have been isolated mainly from plants, soil, foodstuffs, and human and animal sources [Richard 1985], but it was proposed that the strains isolated from clinical sources be designated *E. agglomerans*, because the conformity with the genus *Enterobacter*, and those isolated from plant lesions and surfaces be placed in the genus *Erwinia* [Ewing and Fife 1972]. It is difficult to distinguish strains from different sources due to the diversity in this group of organisms, that is why it has been suggested that the strains can be grouped with the genus *Erwinia* or *Enterobacter* depending upon whether bifunctional AS:PRT is absent or present [Ahmad and Jensen 1989]. For example, *Enterobacter agglomerans* ATCC 29915 (aerogenic) possesses the bifunctional enzyme, whereas *E. agglomerans* ATCC 27155 (anaerogenic) and *Erwinia herbicola* 33243^T lack AS:PRT. It was showed that the last two types of strains belong to the same genomic species [Beji et al 1988] and together with the fact of the absence of the bifunctional enzyme, the exclusion of the *E. agglomerans* from the genus *Enterobacter* has been suggested [Ahmad and Jensen 1989].

4. CHORISMATE MUTASE IN MICROORGANISMS AND PLANTS

INTRODUCTION

Chorismate mutase (EC 5.4.99.5) catalyses the conversion of chorismate to prephenate and plays a key role in the biosynthesis of the essential aromatic aminoacids tyrosine and phenylalanine. The catalysed reaction is unique in nature because it is the only example in primary metabolism of a pericyclic process, a Claisen rearrangement [Walsh et al 1990].

Microorganisms are diverse in the arrangement and variety of enzymes comprising the terminal pathways of tyrosine and phenylalanine. They can synthesize these aminoacids via phenylpyruvate or arogenate utilising either bifunctional chorismate mutases (CM-P, CM-T, CM-DAHP synthase) or monofunctional chorismate mutases (CM-F, CM-R) (Fig. 4.1) [Ahmad and Jensen 1988, Xia and Jensen 1992]. In plants, only monofunctional forms of chorismate mutase have been isolated and they differ in their regulation by tyrosine and phenylalanine. It is possible to find from one to three chorismate mutase isoenzymes (CM-1, CM-2 and CM-3) [Poulsen and Verpoorte 1991].

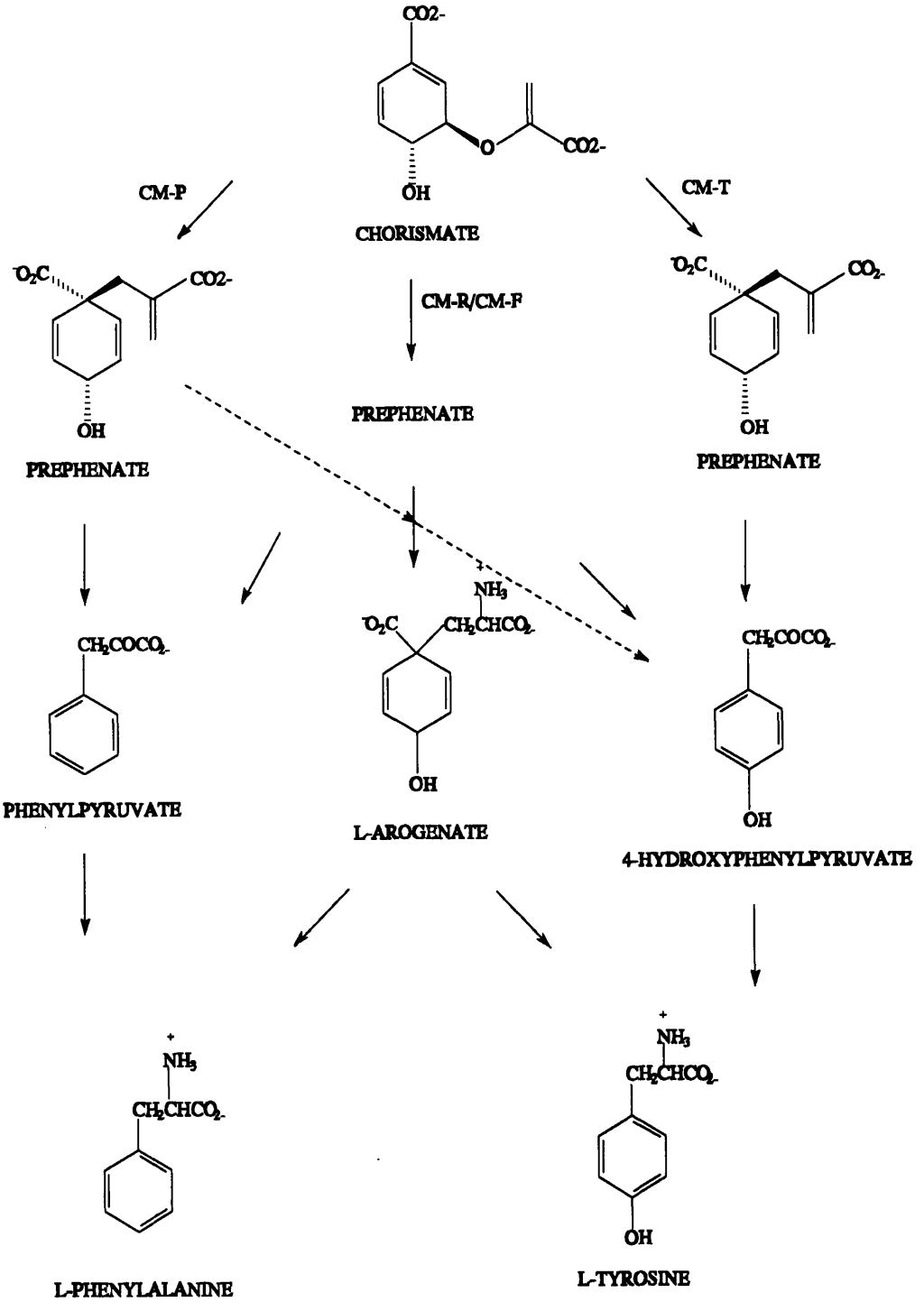
OCCURRENCE OF CHORISMATE MUTASE

Microorganisms

Chorismate mutase has been isolated and characterised from many microorganisms (Table 4.1) and in all the cases up to date, excepting *Bacillus subtilis* [Rajagopalan et al 1993] and *Streptomyces aureofaciens* [Görisch and Lingens 1974], the enzyme is a dimer with similar or identical subunits and molecular weights between 23000 and 91000 (*Acinobacter calcoaceticus*) [Byng et al 1985, Ahmad et al 1988].

Different isoenzymes can be found in microorganisms, three bifunctional, CM-P, CM-T and CM-DAHP synthase, which contain besides the chorismate mutase activity, prephenate dehydratase, prephenate dehydrogenase and 3-deoxy-D-

Fig. 4.1 Different arrangements of phenylalanine and tyrosine biosynthesis in microorganisms. (CM-DAHP synthase is not include)



Dotted arrow indicates that prephenate can be used for the synthesis of 4-hydroxyphenylpyruvate

Table 4.1 Occurrence of chorismate mutase in microorganisms

Microorganism	Enzyme	M_r	Structure	Separation/purity	References
Candida maltosa	CM-F	63000	-	G-25/DEAE cellulose	Bode et al 1985
Pichia guilliermondii	CM-F	-	-	DEAE cellulose	Bode et al 1989
Saccharomyces cerevisiae	CM-R	-	homodimer	Mono-Q/Mono-Q/Sephadex G-75	Schmidheini et al 1990
Bacillus subtilis ATCC 6051	CM-F	44000	homotrimer	DEAE-cellulose/DEAE-cellulose/Gel filtration/Mono-Q/Gel filtration/$(NH_4)_2SO_4$/Ethylamino sulphate/Ethylamino Sepharose	Gray et al 1990
Erwinia herbicola[1]	CM-F	34000	homodimer	DEAE cellulose/Hydroxyapatite/Gel filtration/ DEAE cellulose	Xia et al 1993
Streptomyces aureofaciens	CM-F	63000	-	$(NH_4)_2SO_4$/Sephadex G-200/ Hydroxyapatite/Sephadex G-100/Preparative disc electrophoresis/Sucrose gradient electrophoresis	Görisch and Lingens 1974 Görisch 1987
Arxula adeninivorans SBUG 724 *Brettanomyces anomalus* SBUG 289 *Candida albicans* SBUG 182 *Candida parapsolosis* SBUG 523 *Candida tropicalis* SBUG 590 *Candida utilis* SBUG 576 *Cryptococcus uniguttulatus* SBUG 305 *Debaryomyces hansenii* SBUG 528 *Hansenula bimundialis* SBUG 117 *Hansenula fabianii* SBUG 131 *Hansenula henricii* SBUG 155 *Hansenula polymorpha* SBUG 500 *Kluyveromyces marxianus* SBUG 702 *Rhodotorula aurantiaca* SBUG 244 *Rhodotorula glutinis* SBUG 63 *Saccharomycopsis capsularis* SBUG 241 *Schizosaccharomyces octosporus* SBUG 575 *Sporobolomyces salmonicolor* SBUG 549 *Trigonopsis variabilis* SBUG 703 *Yarrowia lipolytica* SBUG 130	CM	-	-	DEAE cellulose	Bode and Birnbaum 1991

[1] Expressed in *E. coli*

Table 4.1 Occurrence of chorismate mutase in microorganisms (continuation)

Microorganism	Enzyme	M_r	Structure	Separation/purity	References
Serpens flexibilis ATCC 29606	CM-P	-	-	DEAE cellulose	Ahmad and Jensen 1987a
Azomonas insignis ATCC 29360 *Azotobacter paspali* ATCC 23833 *Azotobacter vinelandii* ATCC 17962	CM-P	-	-	DEAE cellulose	Byng et al 1986
Neisseria gonorrhoeae ATCC 27630	CM-P	-	-	DEAE cellulose	Berry et al 1987
Aerobacter aerogenes	CM-T	76000	dimer	Protamine sulphate/$(NH_4)_2SO_4$/AMP-Sepharose/ Hydroxyapatite	Heyde and Morrison 1978
Acinobacter calcoaceticus	CM-P CM-F	91000 23000	homodimer	Streptomicin sulphate/$(NH_4)SO_4$/DE52/DE52 Phenylalanine Sepharose/Sephadex G-150/ Hydroxyapatite DEAE-cellulose/Gel filtration	Ahmad et al 1988 Byng et al 1985
Escherichia coli	CM-P CM-T	40000 88000	homodimer[2] dimer	Streptomycin sulphate/Phenyl-Sepharose/ Hydroxyapatite/Sepharosyl-phenylalanine Streptomycin sulfate/$(NH_4)_2SO_4$/Sepharose-AMP/ DEAE cellulose/Hydroxyapatite	Ma and Davidson 1985 Gething et al 1976 Sampathkumar and Morrison 1982[3]
Citrobacter freundii ATCC 29935 *Shigella dysenteriae* ATCC 11456a *Enterobacter aerogenes* ATCC 13048 *Enterobacter agglomerans* ATCC 29915 (aerogenic strain) *Cedecae davisae* ATCC 33431 *Kluyvera ascorbata* ATCC 33433 *Hafnia alvei* ATCC 13337 *Edwardsiella tarda* ATCC 15947 *Yersinia enterocolitica* ATCC 9610 *Proteus vulgaris* ATCC 29905 *Providencia alcalifaciens* ATCC 9886 *Morganella morganii* ATCC 2583	CM-P CM-T	- - - - - - - - - - - - - -	- - - - - - - - - - - - - -	DEAE cellulose	Ahmad et al 1990
Proteus mirabilis ATCC 29906 *Aeromonas hydrophila* ATCC 14715 *Alteromonas putrefaciens* ATCC 8071	CM-P CM-T	- - -	- - -	DEAE cellulose	Ahmad and Jensen 1988

[2]The enzyme can be a mixture of dimer, tetramer and probably octamer because undergoes a concentration-dependet self-association [Baldwin et al 1981]

[3]Other purification procedures have been reported: Streptomycin sulphate/$(NH_4)_2SO_4$/Blue Dextran-Sepharose/DEAE-Sepharose [Hudson et al 1984], Streptomycin sulphate/$(NH_4)_2SO_4$/Blue A gel/Sepharose-AMP [Turnbull et al 1990]

Table 4.1 Occurrence of chorismate mutase in microorganisms (continuation)

Microorganism	Enzyme	M_r	Structure	Separation/purity	References
Salmonella enteritidis ATCC 13076 *Enterobacter cloacae* ATCC 13047 *Enterobacter agglomerans* ATCC 27155 (anaerogenic strain)	CM-F CM-P CM-T	- - -	- - -	DEAE cellulose	Ahmad et al 1990
Serratia marcescens ATCC 13880	CM-F CM-P CM-T	- - -	- - -	DEAE cellulose	Ahmad and Jensen 1988
Serratia rubidaea ATCC 27614	CM-F CM-P CM-T	40000 - -	homodimer - -	DEAE cellulose/Hydroxylapatite/DEAE cellulose/ Bio-Gel P150 DEAE cellulose DEAE cellulose	Xia and Jensen 1992 Ahmad et al 1990 Ahmad et al 1990
Erwinia herbicola ATCC 33243 *E. carotovora* ATCC 15713 *E. amylovora* ATCC 15580 *E. milletiae* ATCC 33261 *E. chrysanthemi* ATCC 11663 *E. tracheiphila* ATCC 33245	CM-F CM-P CM-T	- - - - - -	- - - - - -	DEAE cellulose/Hydroxyapatite	Ahmad and Jensen 1988
Salmonella typhimurium ATCC 15277	CM-F CM-P CM-T	- - -	- - -	DEAE cellulose	Ahmad and Jensen 1988

arabino-heptulosonate 7-phosphate synthase activities respectively, and two monofunctional enzymes, CM-F and CM-R. These two monofunctional enzymes differ in their regulation by the aromatic aminoacids phenylalanine and tyrosine: CM-F is unregulated, and CM-R is not.

Plants

Chorismate mutase has been characterised from many plants and the presence of three isoenzymes has been detected in several species (Table 4.2). The molecular weights of the enzymes range between 36000 (CM-2, *Vigna radiata*) and 84000 (CM-1, *Papaver somniferum*). Besides the data in Table 4.2, a comparison of chorismate mutase isoenzyme patterns in a variety of plants was made after separating the isoenzymes by disc electrophoresis (Woodin et al 1978). All anthophyta tested, except some members of the Lotoideae subfamily of the Leguminosae, contained three isoenzymes, while vascular but primitive plants such as pine showed two isoenzymes. Most algae, both blue-green and green, contain one chorismate mutase [Jensen and Pierson 1975, Jensen et al 1974, Weber and Bock 1970].

ISOENZYMES

Microorganisms

The five chorismate mutase isoenzymes that are found in microorganisms, provide alternative enzymatic routes for the biosynthesis of phenylalanine and tyrosine (Fig. 4.1). The chorismate mutase: prephenate dehydratase (CM-P) catalyses the formation of prephenate, which is transformed into phenylpyruvate through the action of the same bifunctional enzyme (prephenate dehydratase component, PDT). The phenylpyruvate is converted by a separate enzyme into L-phenylalanine, which inhibits the two activities of CM-P. This bifunctional enzyme, CM-P is present in two of the three major superfamilies of gram-negative bacteria, the superfamily B (includes enteric genera, *Oceanospirillum,*

Table 4.2 Occurrence of chorismate mutase in plants

Plant	Enzymes	M_r	Structure	Separation/purity	References
Brassica juncea (callus)[1]	CM-1 CM-2	- -	- -	G-25/DEAE cellulose/$(NH_4)_2SO_4$	Sharma et al 1993
Brassica rapa (fleshy roots)	CM	-	-	Crude	Schmidt et al 1991
Spinacia oleracea (roots)	CM-1 CM-2	59000 48000	- -	G-25/DEAE Sepharose	Schmidt et al 1991
Papaver somniferum (seeds and seedlings)	CM-1 CM-2	84000 80000	dimer dimer	DEAE-cellulose/Sephadex G200/ Hydroxyapatite	Benesova and Bode 1992
Sorghum bicolor (seedlings)	CM-1 CM-2	56000 48000	- -	G-25/DEAE-cellulose/ω-Aminohexyl-agarose/ Red A gel/L-tryptophan agarose G-25/DEAE-cellulose/Blue A gel/ ω-Aminohexyl agarose/Synchropak AX-310	Singh et al 1985
Medicago sativa (plant and seedlings)	CM-1 CM-2 CM-3	46000 58000 69000	- - -	Protamine sulphate/$(NH_4)_2SO_4$/Sephadex G-100/ Electrophoresis	Woodin and Nishioka 1973
Pisum sativum (seedlings)	CM-1	-	-	DEAE-celullose	Cotton and Gibson 1968
Vigna radiata (beans)	CM-1 CM-2	50000 36000	- -	$(NH_4)_2SO_4$/Hydroxyapatite/DEAE-cellulose/ Sephadex G-100/ultrafiltration $(NH_4)_2SO_4$/DEAE-cellulose/ultrafiltration/ Sephadex G-100	Gilchrist and Connely 1987
Nicotiana silvestris (Leaf tissue and cell suspension)	CM-1 CM-2	52000 65000	- -	G-25/DEAE-cellulose/Hydroxyapatite/ gel filtration	Goers and Jensen 1984a,b
Daucus carota (cell suspension)	CM	-	-	Crude extract	Widholm 1974
Solanum tuberosum (tubers and leaves)	CM-1 CM-1 CM-2	- 55000 52000	- - -	Blue A/Tryptophan agarose G-25/DEAE-cellulose	Kuroki and Conn 1988 Kuroki and Conn 1989 Morris et al 1989
Quercus pedunculata (leaves)	CM-1 CM-2	45000 -	- -	$(NH_4)_2SO_4$/Sephadex G-50/Hydroxyapatite gel	Gadal and Bouyssou 1973
Ruta graveolens L (Suspension cultures)	CM-1 CM-2	56000 45000	- -	$(NH_4)_2SO_4$/DEAE-sephacel/Sephadex G-150	Hertel et al 1991

[1]CM-1 is absent in root-forming callus

Zanthomonas, fluorescent pseudomands and *Acinobacter*) and the superfamily A (includes for example *A. eutrophus*) [Ahmad and Jensen 1986, 1988, Davidson 1987, Jensen and Fischer 1987].

Chorismate mutase: prephenate dehydrogenase (CM-T) is involved in the formation of prephenate which is utilised preferentially for the biosynthesis of L-tyrosine. Prephenate is transformed into 4-hydroxyphenylpyruvate via the action of the cyclohexadienyl dehydrogenase (CDH) component of the same bifunctional enzyme. The CM-T is inhibited by tyrosine and it has been detected only in the enteric bacteria: *Aeromonas* and *Alteromonas* [Ahmad and Jensen 1986].

The CM-DAHP synthase is present in gram-positive bacteria *Bacillus* [Lorence and Nester 1967] and *Brevibacterium* (*Corynebacterium*) [Shiio and Sugimota 1979].

The monofunctional chorismate mutase, CM-F, catalyses the formation of prephenate which is utilised for biosynthesis of L-tyrosine or L-phenylalanine, via either phenylpyruvate or L-arogenate. This enzyme lacks allosteric control and is found in gram-negative bacteria (although it is not present in half of the genera of enteric bacteria [Ahmad et al 1990], in gram-positive *B. subtilis* [Gray et al 1990] and *S. aureofaciens* [Görisch and Lingens 1974] and in cyanobacteria. Organisms from superfamily B possess either a monofunctional CM-F or the CM-T protein, but not both [Ahmad and Jensen 1987b].

The monofunctional CM-R exhibits allosteric inhibition by L-phenylalanine and/or L-tyrosine and allosteric activation by tryptophan, and is characteristic of eukaryotic microorganisms such as *Saccharomyces cerevisiae* [Schmidheini et al 1989], *Euglena gracilis* [Byng et al 1981], *Neurospora crassa* [Baker 1966] and *Claviceps paspali* [Lingens et al 1967].

Chorismate mutase activity was checked in several other yeast species (Table

4.1) where the detected enzymes were localized exclusively in the cytosol and were not classified as CM-F or CM-R. All the enzymes were activated by tryptophan, but the enzyme from *C. anomalous, C. albicans, C. tropicalis, C. uniguttulatus, D. hansenii* and *H. henricii* was not inhibited by either phenylalanine or tyrosine. The rest of the enzymes were inhibited by phenylalanine and/or tyrosine excepting *Y. lipolytica* which showed a small activation in the presence of phenylalanine and tyrosine [Bode and Birnbaum 1991].

Plants

In plants it is possible to find from one to three monofunctional chorismate mutase isoenzymes. (Table 4.2). For example one CM enzyme was isolated from pea (*Pisum sativum*) [Cotton and Gibson 1968], but latter a second isoenzyme was found [Woodin et al 1978]. The presence of only one CM was also reported for potato (*Solanum tuberosum*) [Kuroki and Conn 1988] however later these researchers detected three isoenzymes [Kuroki and Conn 1989]. It is possible that the absence of a second CM isoenzyme in those plants where only one CM enzyme has been reported, may be due to unstability of the second isoenzyme or a lack of separation when the enzymes have been partially purified. Nevertheless it is possible also that the presence of only one enzyme means only one active pathway for aromatic aminoacids.

Where two isoenzymes have been detected, that is the case for most plants, one of them is activated by tryptophan and regulated by tyrosine or phenylalanine, and the other one is not regulated. When three isoenzymes are presented the situation is more complex because the isoenzymes show different patterns of regulation. For example in alfalfa, all the three forms are inhibited by *p*-coumarate, CM-1 and CM-3 are inhibited by tyrosine and phenylalanine and activated by tryptophan; CM-1 and CM-2 are inhibited by other secondary metabolites such as caffeate and chlorogenate and CM-3 is activated by 3,4-dimethoxycinnamate and inhibited by ferulate [Woodin and

Nishioka 1973].

In *Brassica juncea*, the undifferentiated callus showed the presence of two enzymes of chorismate mutase, as well the presence of two isoenzymes for DAHP synthase [Sharma et al 1993], whereas the root-forming callus only showed one enzyme for CM and one for DAHP synthase. These enzymes were similar to the unregulated isoenzymes in normal *B. juncea* callus. An explanation may be that in the phase of rapid cell division and proliferation of undifferentiated callus the presence of both isoenzymes correlates with higher requirement for amino acids. On the other hand, the inactivation of one of the isoenzymes might be attributed to a higher content of products of phenoloxidase reactions and other tanning products [Schmidt et al 1991]. *Papaver somniferum* seedlings showed similar situation to *B. juncea*: the CM-2 activity decreased with the age, and for five-day-old seedlings only CM-1 activity was detected [Benesova and Bode 1992].

Beside the information in Table 4.2, there has been an examination of the CM isoenzymes patterns in other plants [Singh et al 1986]. Only one enzyme was found after protein separation on DEAE-cellulose in *Avena sativa, Brassica oleracea, Eschscholtzia california* and *Pennisetum typhoides*. The enzyme from these plants was always activated by tryptophan and inhibited by phenylalanine and tyrosine. *Amaranthus hypochondriacus, Hordeum vulgare, Hoya carnosa, Medicago sativa, Sroghum bicolor, Spinacia oleracea, Xerosicyos danguyi* and *Zea mays* contained two isoenzymes, the regulated CM-1 and the unregulated CM-2.

GENE-ENZYME RELATIONSHIP AND FUNCTIONALITY

Microorganisms

Only few genes encoding chorismate mutase activity have been cloned and sequenced, and maybe this is because the chorismate mutases comprise a set

of functionally related proteins that show little sequence similarity to each other. This absence of similarity stands in contrast to other enzymes of the aromatic aminoacid biosynthesis, specially to other chorismate-utilising enzymes [Gray et al 1990].

The *ARO7, aroH* and *aroQ* encode for the monofunctional chorismate mutases and *pheA* and *tyrA* encode the bifunctional enzymes CM-P and CM-T.

The *ARO7* gene from *Saccharomyces cerevisiae* encodes CM-R has been cloned and sequenced [Ball et al 1986, Schmidheine et al 1989], but no significant homology between the monofunctional yeast chorismate mutase and the corresponding domains of the two bifunctional *E. coli* enzymes was found. In other yeast enzymes *ARO3, ARO4* and *TRP4* , gene products share significant homology with their *E. coli* counterparts.

The CM-F enzyme from *B. subtilis* is encoded by the gene *aroH*. This gene has been cloned and sequenced [Gray et al 1990], but the derived aminoacid sequence has no significant similarity [Doolittle 1986] to any sequence in the GenBank and EMBL data bases. For example the *aroH* gene was found to be slightly similar to a short region of the amino-terminal (N-terminal) portion of the two *E. coli* bifunctional enzymes [Hudson and Davidson 1984], and no similarity was detected with the monofunctional enzyme of *Saccharomyces cerevisiae* [Schmidheini et al 1989].

The gene *aroQ* for CM-F from *Erwinia herbicola* was also cloned. This gene exhibited no obvious resemblance to the gene for the *Bacillus subtilis* monofunctional chorismate mutase protein [Xia et al 1993].

The *pheA* aminoacid sequence which encodes the bifunctional CM-P from *Pseudomonas stutzeri*, showed homology in the N-terminal portions with CM-P and CM-F from *E. coli*. From the group of three aminoacid residues reported to be associated with the activities of the CM-P from *E. coli* the cysteine residue

216 and threonine residue 278 have been conserved, while the cysteine residue 374 was substituted by serine [Fischer et al 1991].

The aminoacid sequences of *tyrA* and *pheA* have significant similarities in their N-terminal portions only, with 22 of the first 56 residues identical [Maruya et al 1987]. This homologous region reflects not only the chorismate mutase domain but a common ancestry [Davidson and Hudson 1987, Turnbull and Morrison 1990].

Plants

The chorismate mutase cDNA from *Arabidopsis thaliana* is the only chorismate mutase cDNA isolated from a plant. The identity of the deduced aminoacid sequence is 41% to the CM sequence from *Saccharomyces cerevisiae*, but the N-terminal portion has no homology to the *S. cerevisiae.* Hardly any similarity was found to known sequences of bacterial chorismate mutases [Eberhard et al 1993].

REACTION MECHANISM

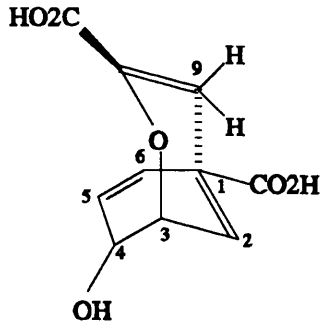
The chorismate to prephenate rearrangement has been extensively studied mechanistically because it is a rare example of an enzyme-catalysed pericyclic reaction, however there is still no consensus in the details of the mechanism of the enzymatic process [Delany et al 1992].

Several mechanisms have been proposed for the chorismate mutase-catalysed reaction or for the analogous thermal reaction based in isotope effects [Addadi et al 1983, Guilford et al 1987, Delany et al 1992), molecular orbital calculations [Andrews et al 1973, 1977], kinetic studies [Andrews et al 1973, Copley and Knowles 1985], reactivities of substrate analogues [Gajewsky et al 1987, Pawlak and Berchtold 1988, Pawlak et al 1989] and transition states analogues [Bartlett et al 1988, Clarke et al 1987, 1990]. The mechanism where C_3-O bond

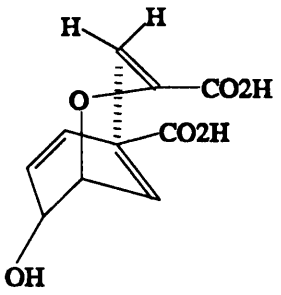
breakage precedes C_9-C_1 bond formation is supported by the data available (Fig. 4.2). The non-enzymic process is believed to proceed via a concerted, asynchronous reaction [Dewar 1984] in which bond breaking is far in advance of bond making at the transition state [Adadi et al 1983]. In contrast, for the mechanism of the enzyme-catalysed rearrangement it can be either through an asynchronous concerted rearrangement or through a dissociate mechanism involving the formation of discrete intermediates.

It has also been established that, for both enzymic and non-enzymic processes, the chorismate mutase catalysis proceeds via a chairlike transition state rather than a boatlike transition state (Fig. 4.2) [Andrews et al 1977, Sogo et al 1984, Asano et al 1985].

Fig. 4.2 Possible transition states in the chorismate mutase reaction



CHAIR



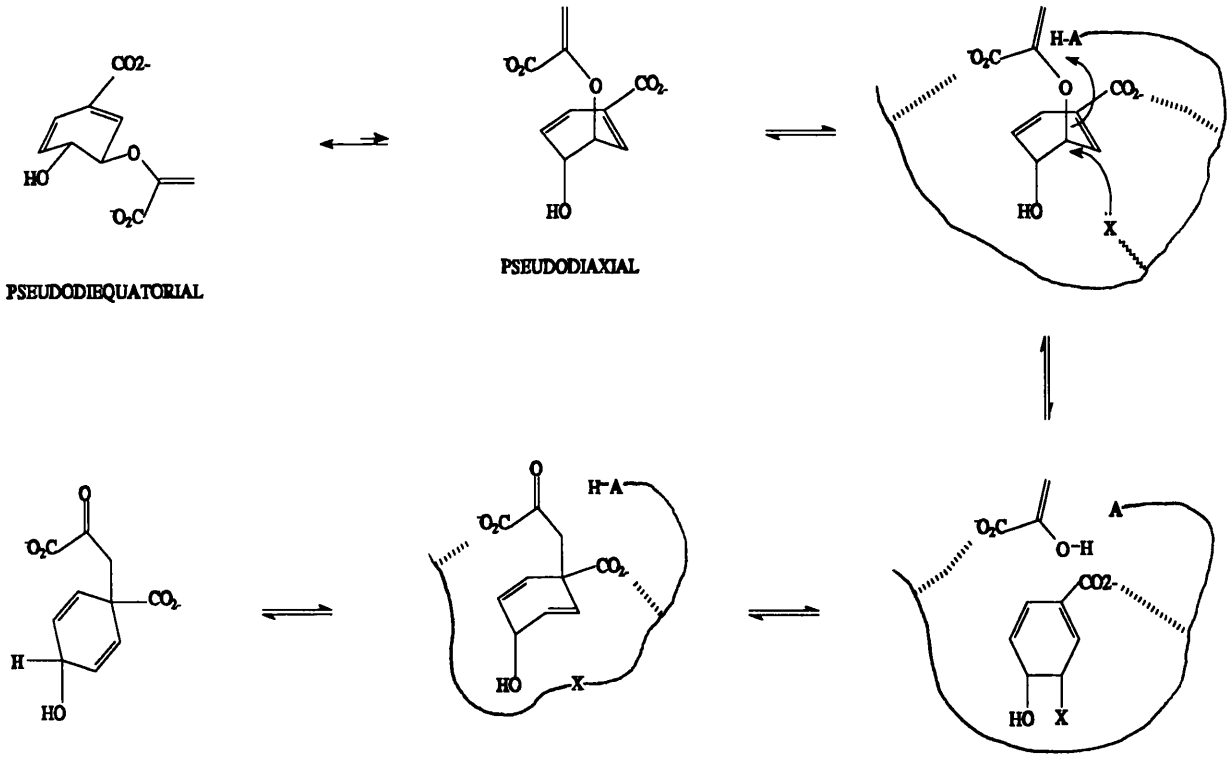
BOAT

Analysis by ^{1}H NMR in aquous solution at 25°C established that while the pseudodiequatorial conformer of chorismate predominates, as it is expected, some 10-40% of the molecules exist in the pseudodiaxial conformer, that is the species set up for the 3,3-rearrangement [Copley and Knowles 1987].

For example, one mechanistic proposal involves acid catalysed protonation of

the vinyl ether oxygen with attack by an enzyme nucleophile at C-5 to give a transient intermediate. The enolpyruvate fragment attacks at the C-1 position by a possible *SN2'* route [Guilford et al 1987]. The existence of the nucleophile has not been established (Fig. 4.3).

Fig. 4.3 Proposed mechanism for chorismate mutase catalyzed 3,3-rearrangement of chorismate

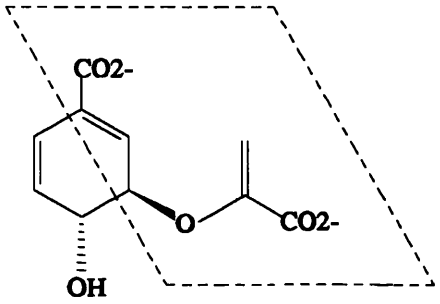


It is important to mention that while the non-enzymic rearrangement is unusually fast in aqueous solution ($t_{1/2}$= 15h for chorismate at 30°C), chorismate mutase accelerates the process $2 \cdot 10^6$ fold (37°C, pH=7.5) [Andrews et al 1973].

The structural features that are essential for enzyme catalysis has been

established. Neither the 5,6-olefinic nor the 4-OH group is absolutely necessary, but the enzyme requires besides the allyl vinyl ether, the two carboxylate groups for active site binding and catalysis [Pawlak et al 1989, Ife et al 1976, Delany et al 1992]. In summary the structural requirements are indicated in the dotted box in Fig. 4.4

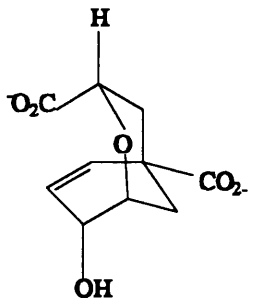
Fig. 4.4 Substrate recognition requirements for chorismate mutase



Several inhibitors of chorismate mutase, mainly adamantane derivates and bicyclic diacids, have been synthesized [Bartlett and Johnson 1985, Clarke et al 1987, Bartlett et al 1988, Nakagawa et al 1989, Clarke et al 1990, Wood et al 1992].

The oxabicyclic compound in Fig. 4.5, 3-*endo*, 8-*exo*-8-hydroxy-2-oxabicyclo[3.3.1]non-6-ene-3,5-dicarboxylic acid, is the highest affinity inhibitor of chorismate mutase known [Bartlett et all 1988] and it binds to the enzyme roughly 250 times more tightly than chorismate itself. This mimic was used to produce two antibodies that catalysed the rearrangement of chorismate to prephenate. One antibody, 11F1-2E11, catalyzes the reaction with a rate of acceleration of 10^4 fold over the uncatalysed reaction [Jackson et al 1992] and the other 1F7 achieves 10^2 fold acceleration [Hilvert et al 1988, Hilvert and Nared 1988]. Several experiments have been done with these antibodies to alucidate how they function [Hilvert and Hill 1991, Tang et al 1991, Bowdish et al 1991, Jackson et al 1992, Benkovic 1992, Campbell et al 1993].

Fig. 4.5 Highest affinity inhibitor of chorismate mutase



Active sites

Several experiments have been done to identify the active sites on the chorismate mutase enzyme. For example for yeast *Saccharomyces cerevisiae* it is proposed that the tyrosine 234 residue (Tyr-234) is located at or near the allosteric activation site for tryptophan [Ramilo et al 1993]. Also for the same yeast a single point mutation in which threonine at position 226 has been replaced by isoleucine, generated a mutant that was not feedback regulated by any of the aromatic aminoacids and was permanently in the activated state [Schmidheini et al 1990]. Kinetic studies established that the site of allosteric activation is separate from the active site [Schmidheini et al 1990].

In the case of the bifunctional chorismate mutase enzymes, experiments to determine the active site have been performed, but it took some time to conclude if the two functionalities had a common active site. For example for chorismate mutase: prephenate dehydrogenase (CM-T) from *E. coli* and *A. aerogenes*, chemical modification of sulfhydryl groups of cysteine was associated with parallel loss of both enzymes activities [Koch et al 1972, Heyde 1979] suggesting for some authors that it was possible that the two activities were catalysed at a single site or for others, that there was a close spatial relationship between the sites for the two functions [Hudson et al 1983, 1984, Christopherson and Morrison 1983]. Also kinetic data obtained with CM-T from

A. aerogenes were consistent with the two reactions occurring at a single site or at two separate sites with similar kinetic properties [Heyde and Morrison 1978] and experiments with *E. coli* using substrate analogues indicated that the sites overlap [Christopherson et al 1983]. The separability of CM-T functions remained in doubt until molecular genetic approaches established the genetic separability of mutase and dehydrogenase components in *E. coli* [Maruya et al 1987]. Also the chorismate mutase component of CM-T from *E. herbicola* was removed by deletion of the 5' terminus of the *tyrA* gene *in vitro* and a new monofunctional prephenate dehydrogenase was reported [Xia et al 1992]. Evidence in support with the molecular genetic approaches was obtained also from kinetic studies and experiments with substrate analogues [Turnbull and Morrison 1990]. Some years before these findings a mutant enzyme lacking prephenate dehydrogenase but retaining chorismate mutase activity was obtained, but the separability of the active sites still was not clear [Rood et al 1982].

For chorismate mutase:prephenate dehydratase has been shown by a variety of procedures to have separate sites [Duggleby et al 1978]. For example a sulphydryl group was concluded to be essential for the prephenate dehydratase activity [Gething and Davidson 1977a, b], the active sites were subject to differential inhibition and selective chemical activation [Schmit et al 1970, Baldwin and Davidson 1983], but since mutants lacking only chorismate mutase or dehydratase activity have been isolated [Schmit et al 1970, Dayan and Sprinson 1971, Baldwin and Davidson 1981, Stewart et al 1990], it has been clear that the two catalytic functions of the CM-P enzyme are spatially distinct.

Mutations in the *pheA* gene of *E. coli* and *Erwinia herbicola* have been obtained in which there is loss of allosteric control [Nelms et al 1992, Xia et al 1992].

UTILITY OF BIOCHEMICAL-PATHWAY CHARACTERISTICS TO FINE-TUNED PHYLOGENETIC POSITION OF CERTAIN MICROORGANISMS

Phylogenetic trees may be constructed using modern nucleic acid sequencing techniques, but it is sometimes useful to use characteristics of aromatic aminoacid pathways as a basis to fine-tune the phylogenetic position of certain organisms.

This criteria has been used for investigating the phylogenetic position of enteric bacteria. The method suggests three major clusters: enterocluster 1 which possesses a gene fusion *trpG-trpD* encoding anthranilate synthase:anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase and includes the genera *Escherichia, Shigella, Citrobacter, Salmonella, Klebsiella* and *Enterobacter*; enterocluster 2 which lacks the *trpG-trpD* gene fusion but contains the overflow to L-phenylalanine and consist of genera *Serratia* and *Erwinia*; enterocluster 3 which lacks both the *trpG-trpD* gene fusion and the overflow to L-phenylalanine and includes the genera *Cedecea, Kluyvera, Edwardsiella, Hafnia, Yersinia, Proteus, Providencia,* and *Morganella*. This method of classification was compared with the partial trees based upon sequences and the results are generally consistent [Ahmad et al 1990].

Other fine-tuning with *Serpens flexibilis* and the results showed that this organism clusters with *P. stutzeri, P. mendocina, P. alcaligenes* and *P. pseudoalcaligenes* within the group Ia psedomonad cluster, because this organism lacks the overflow pathway to phenylalanine [Ahmad and Jensen 1987b].

AIMS AND OBJECTIVES

The aim of this project was to investigate the regulation of three chorismate utilising enzymes in *Ailanthus altissima* cell cultures. To fulfil this, some objectives were proposed:

1. Examine the activities of anthranilate synthase, chorismate mutase and isochorismate synthase throughout the growth cycle of cell suspension cultures.

2. Investigate the possible presence of isoenzymes from anthranilate synthase and chorismate mutase and their regulation by the aromatic amino acids.

3. Isolate and characterise anthranilate synthase from cell cultures to compare this enzyme with other enzymes from plants and microorganisms.

5. MATERIALS AND METHODS

List of Reagents

β-amylase	Sigma
2,4-dichlorophenoxyacetic acid	Flow Laboratories
2,4-dinitrophenylhydrazine	Sigma
30%acrylamide/0.8%bisacryalmide	Boehringer
Acetic acid	Fisons
Agar	Imperial Laboratories
Alcohol dehydrogenase	Sigma
Ammonium persulphate	LKB Bromma
Anthranilate	Sigma
Apoferritin	Sigma
Barium chorismate	Sigma
Bovine albumin	Sigma
Carbonic anhydrase	Sigma
Coomasie Brilliant Blue G-250	LKB- Bromma
Domestos	Lever Brothers Ltd.
DTT	BDH
EDTA	BDH
Ethanol	BDH
Glycerol	BDH
Glycine	Sigma
H_3PO_4	BDH
HCl	Fisons
Isopropanol HPLC grade	BDH
Kinetin	Flow Laboratories
L-glutamine	Sigma
Leupeptin	Sigma
Methanol HPLC grade	BDH
$MgCl_2$	Sigma
Murashige and Skoog medium	Imperial Laboratories
NaCl	BDH

Phenylpyruvate	Koch-Light Laboratories
PMSF	Sigma
Polyethylene glycol 6000	BDH
Polyvinylpyrrolidone	Sigma
Prestained SDS molecular weight markers (SDS-7B)	Sigma
Q-Sepharose	Pharmacia
sodium dodecylsulphate	Sigma
Sucrose	BDH
TEMED	Sigma
Tris	Sigma
Triton X-100	BDH
Tryptophan	Sigma

DEVELOPMENT OF *AILANTHUS ALTISSIMA* CELL CULTURES

Plant material

Fruits of *Ailanthus altissima* (Mill.) Swingle (Simaroubaceae) were gathered from Brunswick Square in 1991 (London).

Development of cell cultures

The seeds were removed from the fruits and soaked in 20ml of tap water for 12 hours at room temperature. They were sterilized with 70% ethanol: water solution for two minutes. The ethanol was then rinsed thoroughly from seeds using 4 x 20ml of distilled water. After, a 7.5% (v/v) solution of commercial Domestos, which contains 5-15% of hypochlorite, was added. The Domestos was removed after 20 minutes using 5 x 20ml of sterile distilled water. About 20 seeds were placed aseptically on damp Whatman no.1 in each square sterile plastic dish (10 x 10cm). The dishes were sealed and left to germinate in dark at 22 ± 1°C. The germination began after 7 days under these conditions.

The sterile seedlings (approx. 2 weeks old) were placed aseptically on the surface of 30ml of Murashige and Skoog (M&S) 1:0.2:5 medium [Murashige and Skoog 1962, Anderson et al 1983] solidified with agar (2% w/v) in 9cm Petri dishes. The M&S 1:0.2:5 medium, which was prepared from 4.71g/l of Murashige and Skoog basal salts and with pH 5.8 adjusted previously to sterilization, contained 5% (w/v) sucrose and 1mg/l of 2,4-dichlorophenoxyacetic acid and 0.2mg/l of kinetin. Once the callus developed, the cells were transferred aseptically to the solid medium at least 4 times before transferring them to liquid medium. Cell suspension cultures were grown in M&S 1:0.2:5 on a gyratory shaker at 120rpm (Lh Fermentation). Subculturing was done every 3 or 4 weeks. Callus cells and cell suspension cultures were always in continuous light at 22 ± 1°C.

ENZYMES ASSAYS

Anthranilate synthase [Poulsen et al 1991a]

To 125µl of desalted homogenised tissue preparation, 125µl of solution containing 0.1MTris-HCl (pH 7.5), 1mM barium chorismate, 20mM L-glutamine and 10mM $MgCl_2$ was added. After incubation for 1h at 30°C the reaction was stopped by the addition of 62 µl of 1M H_3PO_4. Blanks were made by adding the acid before the incubation. The samples were centrifuged at 10000rpm for 5 min and analysed by HPLC. The HPLC analysis was carried out using an Altex Isocratic Liquid Chromatograph (model 330) with a 20µl valve-loop injector, equipped with a Kratos FS290 fluorescence detector. The excitation wavelength was 340nm and the emission wavelength 400nm. The analysis were done at room temperature on a 4.0mm (id) x 250mm LiChrosorb RP-Select B, 5µm particle size (Merck), with a mobile phase of 50mM H_3PO_4 in water-methanol (65:35, v/v), pH 2,5 at a flow rate of 0.7ml/min. A guard column was always used and the eluent was filtered and degassed under vacuum.

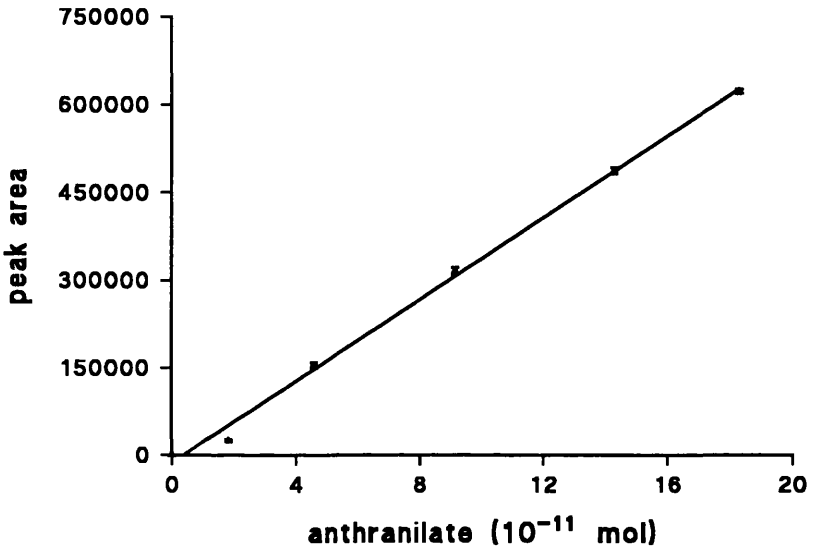
A calibration curve of anthranilate mol versus area was obtained (Fig. 5.1) The quantification was made using a Midas 16 Vr 2.27 calibrated integrator program on an Opus computer.

Isochorismate synthase [Poulsen et al 1991b]

A 125µl volume of desalted enzyme preparation was added to 125µL of solution containing 0.1M Tris-HCl (pH 7.5) and 15mM $MgCl_2$. The reaction began with the addition of barium chorismate (1mM in all incubation sample) and It was stopped after 1h incubation at 30°C by the addition of 62 µl MeOH-*sec*-BuOH (1:1). Blanks were made by addition of MeOH-*sec*-BuOH before incubation. The samples were centrifuged at 10000rpm for 5 min and analysed by HPLC. The HPLC system was the same as for the AS assay, except the machine was equipped with a spectrophotometric detector (Altex analytical optical unit) at

280nm, instead of the fluorescence detector.

Fig. 5.1 Calibration curve of anthranilate mol versus peak area for determination of anthranilate synthase activity using HPLC assay



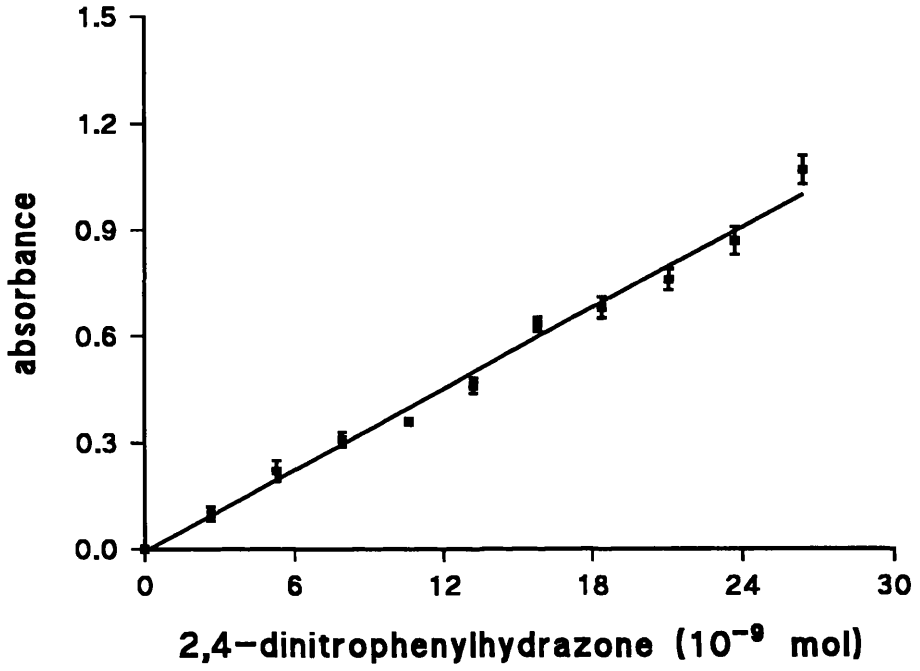
Chorismate mutase [Görisch 1978, Poulsen and Verpoorte 1992]

The assay was run in microtitreplates.

To 50µl of desalted enzyme 50µl of solution containing 0.1M Tris-HCl (pH 7.5), 1mM barium chorismate, 1mM tryptophan was added. The reaction was stopped by the addition of 25µl 4M HCl after 30min incubation at 30°C. The blanks were made by adding the acid before the incubation. After 15min at room temperature 30µl of 2,4-dinitrophenylhydrazine (1mg/l in 2M HCl) was added. After 15min, 50µl of saturate solution of sodium etoxide was added; after 2min, 50µl more of EtONa solution were added. The adsorption of the 2,4-dinitrophenylhydrazone was measured at 450nm in a microplate reader (Minireader II, Dynatech Laboratories).

A calibration curve of 2,4-dinitrophenylhydrazone of phenylpyruvate versus corresponding absorbance was plotted (Fig. 5.2) and the hydrazone levels of the unknown samples were found by interpolation.

Fig. 5.2 Calibration curve of phenylpyruvate 2,4-dinitrophenylhydrazone mol versus optical density measured at 450nm

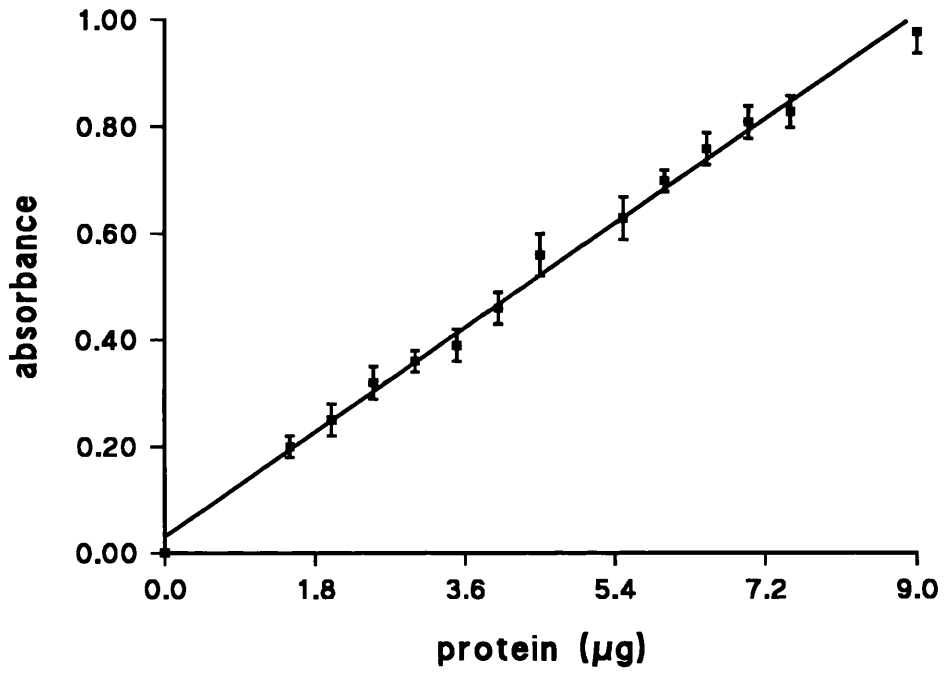


Protein determination

The protein content was determined in triplicate using the Bradford method [Bradford 1976] in microtiterplates. Preparation of the reagent was made dissolving 50mg of Coomassie Brilliant Blue G-250 in 25ml of 95%(v/v) ethanol and adding 50ml of 85% (w/v) of phosphoric acid afterwards. The volume of the mixture was adjusted to 250ml and left to stand over night. The reagent was filtered before use. Bovine serum albumin (BSA) was used as standard to construct a protein calibration curve.

Determination of the protein in the samples was achieved adding to a 10µl volume of enzyme extract, 90µl of extraction buffer and 100µl of concentrated Bradford reagent. The absorbance values were read at 595nm on a Minireader II Dynatech Laboratories.

Fig. 5.3 Example of the calibration curve used for protein determination



TIME COURSE STUDY

Cell suspension cultures of *A. altissima* (β_3) were filtered aseptically and 3g fresh weight was inoculated into each of 30 flasks (40ml medium per flask, M&S 1:0.2:5 medium). Samples were harvested by filtration under vacuum in triplicate every three days throughout the growth cycle. The final fresh weight was determined.

Enzyme extraction [Poulsen et al 1991a]

The harvested cells were immediately frozen in liquid nitrogen. For each gram of fresh weight cells 0.1g of polyvinylpyrrolidone (PVP) and 1ml of extraction buffer were added. The extraction buffer consisted in 0.1 M of Tris-HCl at pH 7.5, with 10% glycerol, 1mM EDTA, 1mM DTT, 10mM leupeptin, 0.2mM PMSF and 10mM L-glutamine.

After thawing, the mixture was centrifuged at 12000g for 25 min. The supernatant was desalted on Sephadex G-25 (Pharmacia PD-10 columns) equilibrated with the extraction buffer. The activity of the enzymes AS, CM and ISC was measured in the samples.

PURIFICATION OF ANTHRANILATE SYNTHASE

A. altissima cell suspension cultures were harvested 10 days after subculturing by filtration under vacuum. The cells were immediately frozen in liquid nitrogen and homogenized in a coffee maker (Moulinex) for 30s. After this point all the operations were carried on at 4°C.The cells were extracted as it is indicated before in **Enzyme extraction**, and after centrifugation at 12000g, the supernatant was purified using the following methods.

Polyethylene glycol precipitation

To the supernatant after centrifugation, finely ground polyethylene glycol 6000 (PEG) was added slowly to a final concentration of 10% (w/v). During the addition, the supernatant was stirred with a magnetic stirrer. After the addition of PEG, the enzyme solution was stirring for 25 min and the solution was centrifuged at 12000g for 30 min. To the supernatant additional PEG was added to give a final concentration of 18% (w/v) and after stirring for 25 min, the precipitated protein was obtained by centrifugation under the same conditions as before. The pellet was dissolved in extraction buffer.

Q-Sepharose anion exchange chromatography

The dissolved pellet was centrifuged for 25 min at 12000g and the supernatant was applied to a column packed with Q-Sepharose (2.7 x 9.9 cm) equilibrated with extraction buffer. The column which was linked to a Pharmacia FPLC system, was washed with 120ml of buffer and the column was eluted at 1ml/min with 50ml of buffer and a linear salt gradient from 0% to 20% of the same buffer containing 1M NaCl. After the column was eluted with 25ml, keeping constant the salt concentration at 20%, the linear salt gradient was continued with 50 ml of eluent to reach 1M NaCl. Fractions of 3ml were collected with an automated fraction collector (Pharmacia, Frac-100) and the proteins eluted from the column were detected by a UV detector (Pharmacia single path monitor UV-1) which was set at 280nm and linked to a Pharmacia chart recorder. The AS active fractions were desalted on PD-10 columns equilibrated with the extraction buffer.

Mono-Q column anion exchange chromatography

The desalted fractions were injected to a Mono Q HR 5/5 column (Pharmacia), equilibrated previously with buffer containing: 0.1M Tris-HCl, pH 7.5, 10% glycerol, 10mM L-glutamine 10 µM leupeptin and 1mM DTT. The column was washed with 5ml and eluted with the last buffer, using a 11ml linear salt gradient from 0% to 15% 1M NaCl. This concentration was kept for 5ml, and the linear salt gradient was continued with 5ml to reach 20% of 1M NaCl. Again the concentration was kept constant during 5ml and with 9ml the linear salt gradient was continued to reach 1M NaCl. The flow rate was 0.5ml/min and fractions of 0.5 ml were collected. The proteins eluted from the column were monitored using the same detection system described for the Q-Sepharose column.

Superose 12 HR 10/30 column

The AS active fractions were applied 200µl per run to a Superose 12 HR 10/30 column (Pharmacia), linked to Pharmacia FPLC system and equilibrated with the buffer containing: 0.1M Tris-HCl, pH 7.5, 10% glycerol, 10mM L-glutamine and 1mM DTT. The proteins were eluted from the column at a flow rate of 0.45ml/min and fractions of 0.5ml were collected.

CHARACTERISATION OF ANTHRANILATE SYNTHASE ENZYME

Characterisation of AS was made utilising enzyme fractions from the Mono-Q column. The characterisation involved determination of conditions required for optimum enzyme activity, kinetic properties of the enzyme substrates, inhibition studies and molecular weight determination.

Molecular weight determination

Two methods were used for molecular weight determination: gel filtration and sodium dodecylsuphate polyacrylamide gel electrophoresis (SDS-PAGE).

Gel filtration method

The molecular weight of the enzyme was determined by comparing the elution volume of the enzyme on a Pharmacia Superose 12 HR 10/30 column with the elution volumes of molecular weight markers which had previously been run on the same column, under the same conditions. The molecular weight markers used include carbonic anhydrase, bovine albumin, alcohol dehydrogenase, β-amylase and apoferritin of molecular weights 29000, 66000, 150000, 200000 and 443000 respectively.

Solutions of 1mg/ml of each molecular weight marker and a mixture containing all of them with the same concentration were prepared in the running buffer

(0.1M Tris-HCl, pH 7.5, 10% glycerol, 10mM L-glutamine and 1mM DTT) and 200 µl of the solutions were injected and the proteins were eluted at a flow rate of 0.4 ml/min. The elution volumes of each of the molecular weight markers were determined and a calibration curve of molecular weight versus the elution volume of the markers was plotted. 200 µl of the active fractions collected from Mono-Q column were injected on the column under the same conditions as the markers. The elution volume of the active fraction was determined and the molecular weight of the enzyme was obtained from the calibration curve.

SDS-PAGE method

Vertical SDS-PAGE was performed simultaneously on a mixture of molecular weight markers, and on samples of anthranilate synthase enzyme using 7.5% polyacrylamide minigel of 1 mm thickness.

The 7.5% polyacrylamide minigels were prepared from a mixture containing 5ml of 1,5M Tris-HCl buffer pH 8.8, 2,5ml of 30% (w/v) acrylamide/0.8% (w/v) bisacrylamide solution, 5ml of water and 100µl of 10% (w/v) sodium dodecylsulphate (SDS). This mixture was degassed and 50µl of 10% (w/v) ammonium persulphate and 5µl of TEMED were added to the solution, before pouring it into a cast where the gel was left standing until it set. A saturated solution of butanol in water was added in the top of the gel meanwhile the gel solidified. After an hour when the resolving gel was set a 4% stacking gel was set in the cast. The stacking gel was prepared from a solution containing 1.25ml of 0.5M Tris-HCl buffer pH 6.8, 666µl of 30% (w/v) acrylamide/0.8% (w/v) bisacrylamide solution, 3ml of water and 50µl of 10% (w/v) SDS solution. This mixture was degassed and 50µl of 10% (w/v) ammonium sulphate and 10µl of TEMED were added. At this point the mixture was poured in the cast and the gel comb was placed on the top. When the gel was set, approximately 15min. later, the gel comb was removed to reveal the wells formed for sample application.

The enzyme samples applied to the gel were treated according to the Laemlli method [Laemlli 1970]. The Laemlli buffer was double strength and contained 125mM Tris-HCl pH 6.8, 4% (w/v) SDS, 20% glycerol (v/v), 2% DTT and 0.0025% (w/v) bromophenol blue. For each volume of the enzyme samples equal volume of Laemlli buffer was added and the solutions were immersed in boiling water for 10min. The mixture of prestained SDS molecular weight markers contained α_2-macroglobulin, β-galactosidase, fructose-6-phosphate kinase, pyruvate kinase, fumarase, lactic dehydrogenase and triosephosphate isomarase with apparent molecular weights of 190000, 125000, 88000, 65000, 56000, 38000 and 33500, respectively. They were prepared according to Sigma Product Information (Information sheet MWM-105A). 20 μl of enzyme sample and 10μl of molecular weight markers solution were applied to each well in the gel.

The gel was run on a vertical electrophoresis unit using a Consort E702 Microcomputer electrophoresis power supply (Jencons Ltd.) with a constant current of 40mA and at 21°C. The gel running buffer contained 25mM Tris-HCl, 192mM glycine and 0.1% SDS, and was at pH 8.3. The gel was developed approximately in an hour.

The developed gel was transferred into the Coomassie Blue stain solution (0.3% Coomassie Blue G-250, 50% methanol and 1% glacial acetic acid) and was allowed to stand for 30 min with constant stirring. After the gel was transferred into detaining solution (10% acetic acid and 10% isopropanol), where it was allowed to stand until the background of the gel became clear.

6. RESULTS

ENZYME ASSAYS

Anthranilate synthase

Anthranilate synthase was assayed according to Poulsen et al [1991a], but with a modification which used an incubation mixture of 250µl instead of 500µl.

The crude anthranilate synthase enzyme was very unstable and the enzyme lost 20% of its activity in 2 hours when it was kept on ice in Tris-HCl buffer without additives. Initially, cells were extracted with 0.1M Tris-HCl buffer at pH 7.5 containing 10% glycerol, 1mM EDTA, 1mM DTT and 10mM leupeptin. This buffer with the additives was enough to stabilise the enzyme for one working day, but when the crude enzyme was needed for further separation, it was necessary to add PMSF (0.2mM) and L-glutamine (10mM).

The calibration curve of anthranilate mol versus peak area was obtained with standard solutions of anthranilate, and the graph was linear in the range used (Fig. 5.1).

It was necessary to remove low molecular weight substances on Sephadex G-25 (Pharmacia PD-10 columns) before the assay was done, otherwise no enzyme activity could be detected.

Isochorismate synthase

Isochorismate synthase was assay according to Poulsen et al (1991b) with modifications which used an incubation mixture volume of 250µl instead of 500µl and an extraction buffer as for the anthranilate synthase assay (including glutamine and PMSF).

Crude enzyme extracts and concentrated extracts (after 18% PEG precipitation) were tested, but this enzyme was not active in the cell line tested.

Chorismate mutase

The chorismate mutase assay was taken from Görisch [1978] and Poulsen and Verpoorte [1992]. Initially the assay was run as they described it, but with the the enzyme extracts a precipitate was formed. Different solutions of Triton X-100 were used to dissolve the precipitate but without success. Finally, NaOH was substitued by a saturated solution of EtONa and when the assay was performed there was no precipitation. A linear graph was obtained for the calibration curve of 2,4-dinitrophenylhydrazone of phenylpyruvate versus corresponding absorbance when EtONa was used to increase the pH of the solution.

TIME COURSE STUDY OF *A. ALTISSIMA* CELL SUSPENSION CULTURES

A. altissima cell suspension cultures produce relatively high yields of the canthin-6-one alkaloids and also low yields of the coumarins scopoletin and isofraxidin [Hay 1987, Osoba 1993]. Due to the presence of these secondary metabolites, *A. altissima* served as a model system for studying the activity of anthranilate synthase and chorismate mutase.

To investigate the activity of these two enzymes a detailed time course study was carried out for cell suspension cultures. To select the cultures for this experiment, cell suspension cultures of different ages β_1, β_2, β_3 and β_4 (β_x, where x means the number of transfers) were transfered under sterile conditions: 5g fresh weight were inoculated into 40ml of M&S 1:0.2:5 medium. These cultures were maintained for a period of 29 days and were harvested in triplicate. The final fresh weight was determined and the cells were inmediately frozen in liquid nitrogen and extracted. Once the cells were extracted and processed on a Sephadex G-25 column, the activity of the enzymes AS, CM

and ISC was measured in the samples. The experiment was done twice and the results are summarised in Table 6.1.

Cell suspension cultures β_3 and β_4 showed the highest anthranilate synthase and chorismate mutase activities, but activity for isochorismate synthase was not detected at any time.

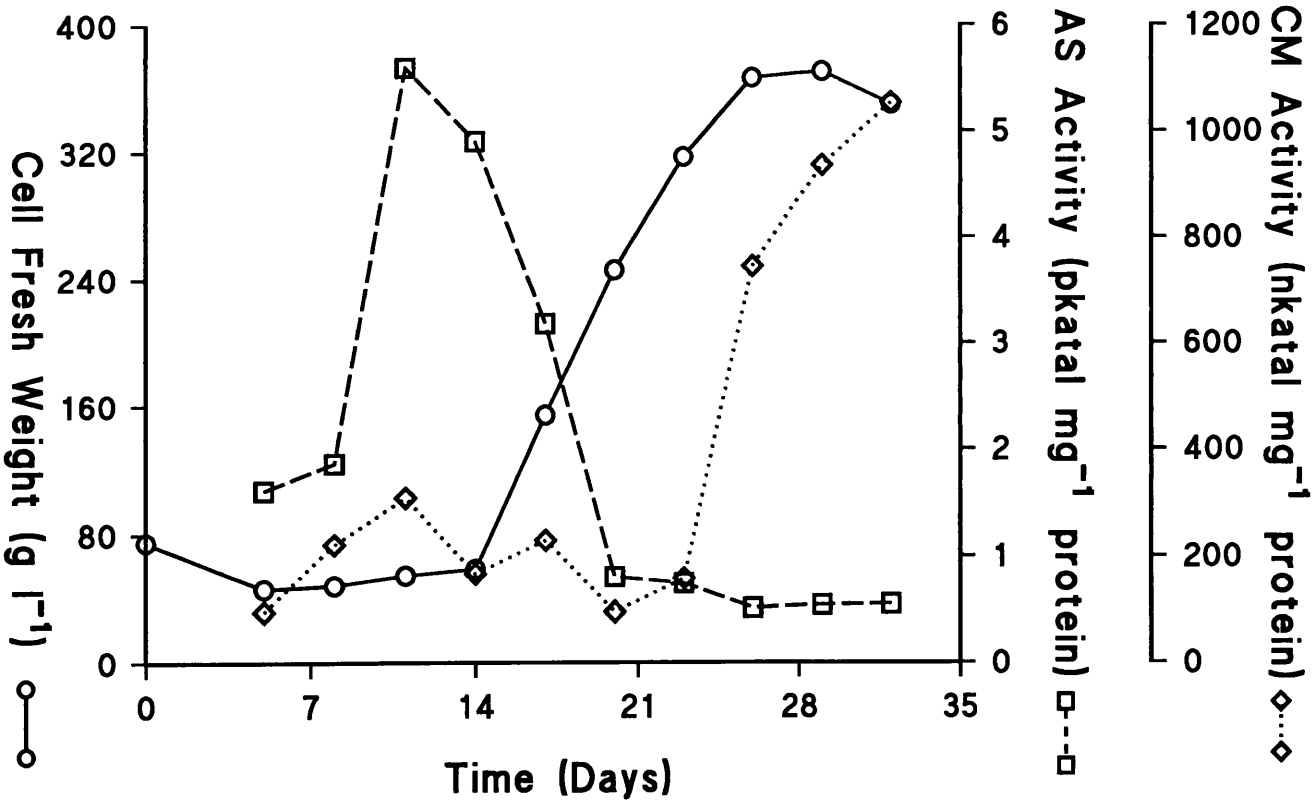
β_3 cell suspension cultures were selected for the time course study. The cultures were filtered aseptically and 3g fresh weight was inoculated into each of 30 flasks containing 40ml of M&S 1:0.2:5 medium per flask. The cultures were harvested in triplicate every three days, and they were assayed for AS, CM and ISC activity. The results are shown in Figure 6.1.

Table 6.1 Activity of anthranilate synthase and chorismate mutase in cell suspension cultures of *A. altissima* of different ages

Cell culture	AS activity (pkatal/mg protein)		CM activity (nkatal/mg protein)	
	Experiment I	Experiment II	Experiment I	Experiment II
β_1	37.5	40.1	1.12	1.23
β_2	61.1	58.6	1.22	1.15
β_3	68.5	70.6	1.35	1.27
β_4	80.4	75.6	1.61	1.55

The cells multiplied quite rapidly after day 14, undergoing almost a five fold increase in fresh weight over the growth cycle. The activity of AS showed a maximum between days 8 and 17, while the activity of CM increased after day 20. The activity of CM was 150x the activity of AS. ISC activity was not detected during the growth cycle.

Fig. 6.1 Time course study of chorismate mutase and anthranilate synthase activity In *Ailanthus altissima* cell suspension cultures.



PURIFICATION OF ANTHRANILATE SYNTHASE

In every instance callus cultures showed more AS activity than the suspension cultures. The activity of the callus was usually twice and sometimes up to four times the activity of the suspension cultures.

The suspension cultures used for purification were always harvested between day 10 and 15 of the growth cycle, and the callus cultures were harvested between 10 and 23 days. Once the cells were harvested, they were immediately frozen in liquid nitrogen and ground. When the harvested cells were not used for extraction the same day, after being frozen they were stored at -80°C and they mantained AS activity for two months.

Cells frozen in liquid nitrogen were ground, extracted using the extracted buffer and after thawing, the mixture was centrifuged. The supernatant was used for further purification.

A series of procedures were explored before reaching the final purification scheme, and the efficacy of each procedure was estimated on the basis of the enzyme activity, the levels of protein and the specific activity of the enzyme after each step.

Piksi agarose A6XL columns (Affinity Chromatography Ltd)

The efficiency of agarose A6XL columns was determined. The columns were equilibrated with extraction buffer and equal volumes of enzyme extract were applied on each column. The columns were eluted with the same buffer and the activity of AS and the protein levels were determined in the collected fractions. The samples collected showed the activity presented in Table 6.2.

The results were not reproducible: sometimes AS activity was not detected with the Yellow ™ 1 column, and also the AS activity detected with Blue 2 column

was higher than with Blue™ 1 column, and almost the same value as with Sephadex G-25 column. For the rest of the columns, if some AS activity was detected, it was not significant.

Because Yellow™ 1 column showed the higher protein level without significant AS activity, and columns Blue™ 1 and Blue 2 showed the higher AS activity, they were selected for further experiments. Crude enzymes extracts were loaded into these three columns and the extracts were eluted first with extraction buffer, and latter with extraction buffer containing 0.2, 0.5 or 1M NaCl respectively. AS activity and protein levels were determined for the samples eluted with both systems. The results of one experiment are showed in Table 6.3. When the experiment was repeated there was no reproducibility in the

Table 6.2 Recovery of AS activity from A6XL columns

Column	Protein level (µg)	Total enzyme activity (pmol anthranilate)
Sephadex G-25	605	267
Red™ 1	401	-
Red 2	523	-
Orange™ 1	545	-
Orange 2	350	-
Orange 3	475	-
Yellow™ 1	592	5
Yellow 2	478	-
Green™ 1	472	-
Blue™1	441	163
Blue 2	548	20

Table 6.3 Recovery of AS activity from agarose Blue™ 1, Blue 2 and Yellow™ 1 A6XL columns

Columns	Sephadex G-25		Blue™ 1		Blue 2		Yellow™ 1	
Eluent	AS activity (pmol anthranilate)	Protein (µg)	AS activity (pmol anthranilate)	Protein (µg)	AS activity (pmol anthranilate)	Protein (µg)	AS activity (pmol anthranilate)	Protein (µg)
extraction buffer*	950	1804	584	1670	262	1368	238	1574
extraction buffer + 0.2M NaCl	-	-	ND	-	ND	100	ND	80
extraction buffer + 0.5M NaCl	-	-	ND	105	ND	200	ND	204
extraction buffer + 1M NaCl	-	-	ND	110	ND	280	ND	212

*Extraction buffer: 0.1M Tris-HCl pH 7.5, 10% glycerol, 1mM EDTA, 1mM DTT, 10mM leupeptin, 0.2mM PMSF and 10mM L-glutamine.

ND: not detected

- : not done

results. The enzyme activity was variable for the three agarose column, and no activity was detected in the fractions eluted with buffer containing NaCl.

Ammonium sulphate and polyethylene glycol precipitations

Ammonium sulphate and polyethyleneglycol precipitations of proteins from the crude extract were investigated. A precipitate was obtained with 55% saturation of $(NH_4)_2SO_4$ and also with 10% and 18% PEG. Anthranilate synthase activity and protein levels were determined (Table 6.4).

Polyethylene glycol precipitation gave a good and reproducible fractionation with a higher enzyme activity than $(NH_4)_2SO_4$ precipitation. For this reason, PEG was selected for further experiments to find the correct amount of the organic polymer for a maximal yield (Table 6.5).

The results were reproducible and from the values obtained, precipitation with 18% PEG was shown to be the optimum procedure to precipitate anthranilate synthase.

Table 6.4 Anthranilate synthase activity after $(NH_4)_2SO_4$ and PEG precipitation

Precipitating agent	55% $(NH_4)_2SO_4$	PEG	
		10%	18%
Total enzyme activity (pkatal)	1.93	-	5.51
Protein (µg)	2802	250	1849

Table 6.5 Anthranilate synthase activity after precipitation of protein with different amounts of PEG

% PEG (w/v)	16	17	18	19	20	22
Total enzyme activity (pkatal)	0.31	0.32	0.46	0.41	0.23	0.20
Protein (µg)	2070	2080	2480	2480	2530	2870

Comparison between agarose A6XL and PEG precipitation

A comparison between polyethylene glycol precipitation and Blue™ 1 and Blue 2 agarose columns was done. Polyethylene glycol was shown to be the adequate step because it concentrated the protein and there was no loss of specific activity (Table 6.6).

Table 6.6 Recovery of anthranilate synthase activity after 18% PEG precipitation and agarose A6XL columns

Step	Total activity (pkatal)	Total protein (mg)	Specific activity (pkatal/mg protein)
G-25 column	1.82	59.79	0.030
Blue™ 1	1.08	39.88	0.027
Blue 2	0.43	36.86	0.012
18% PEG	0.52	13.38	0.039

Q-Sepharose column

The reconstitued PEG precipitate was applied to a Q-Sepharose column. The same extraction buffer was used to elute the proteins from the column with a

flow rate of 1ml/min and a constant gradient from 0 to 1M NaCl. The same procedure was followed using 20mM triethanolamine as eluent buffer at the same pH, 7.5, and with the same additives as the extraction buffer. No differences were found. Figure 6.2 shows the elution profile of proteins from this column when extraction buffer was used as eluent.

In two separate experiments, the dissolved pellet from PEG precipitation was applied into Blue™ 1 or Blue 2, and the active fractions from the columns were applied also into the Q-Sepharose column. The results from these experiments were compared with a sequence where no agarose A6XL columns were used, but no advantages were found with the use of the agarose A6XL columns.

Orange A column (Amicon)

After the Q-Sepharose column, it was decided to use an Orange A column for further purification. The active fractions from the Q-Sepharose column were pooled and concentrated by ultrafiltration in an Amicon 8200 unit equipped with a YM-10 (10kDa cutoff) ultrafiltration membrane. The concentrated pool was desalted on PD-10 columns equilibrated with 0.1M potassium phosphate (pH 7.0), 10% glycerol, 1mM EDTA, 1mM DTT, 10mM leupeptin, 0.2mM PMSF and 10mM L-glutamine. The desalted fractions were applied to the Orange A column and the flow, 1ml/min, was stopped for 2 hours. The column was washed with two column volumes of the potassium phosphate buffer and the proteins were eluted with a flow rate of 0.33ml/min and a linear salt gradient from 0 to 1M KCl in the same buffer (Figure 6.3). The AS activity in the collected samples was determined. A summary of the purification steps is shown in Table 6.7.

After Orange A column, the active fractions were pooled, concentrated and desalted. The desalted pool was applied on a Mono-Q column that was equilibrated with extraction buffer. The flow rate was 0.5ml/min and the proteins were eluted with a linear salt gradient from 0 to 1M NaCl. The collected

Figure 6.2 Elution profile of the partially purified *Ailanthus altissima* anthranilate synthase enzyme from Q-Sepharose column

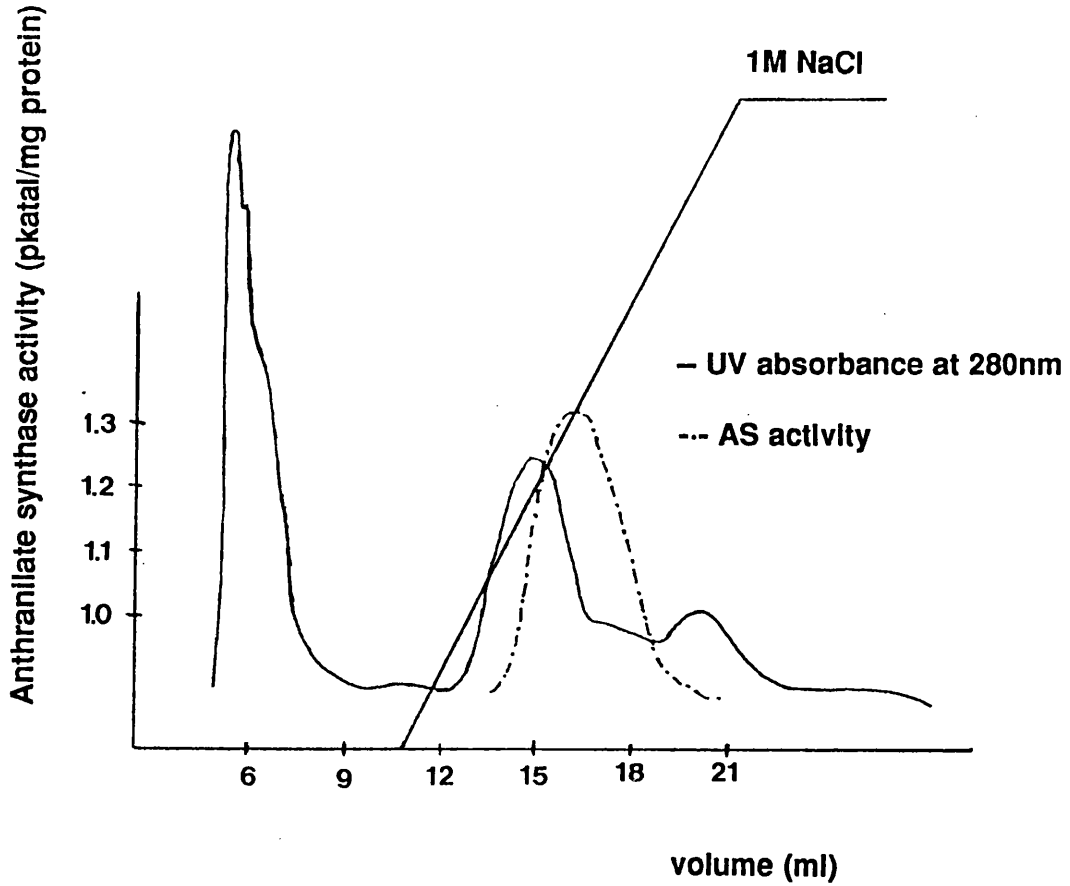


Figure 6.3 Elution profile of the partially purified *A. altissima* anthranilate synthase enzyme from Orange A column

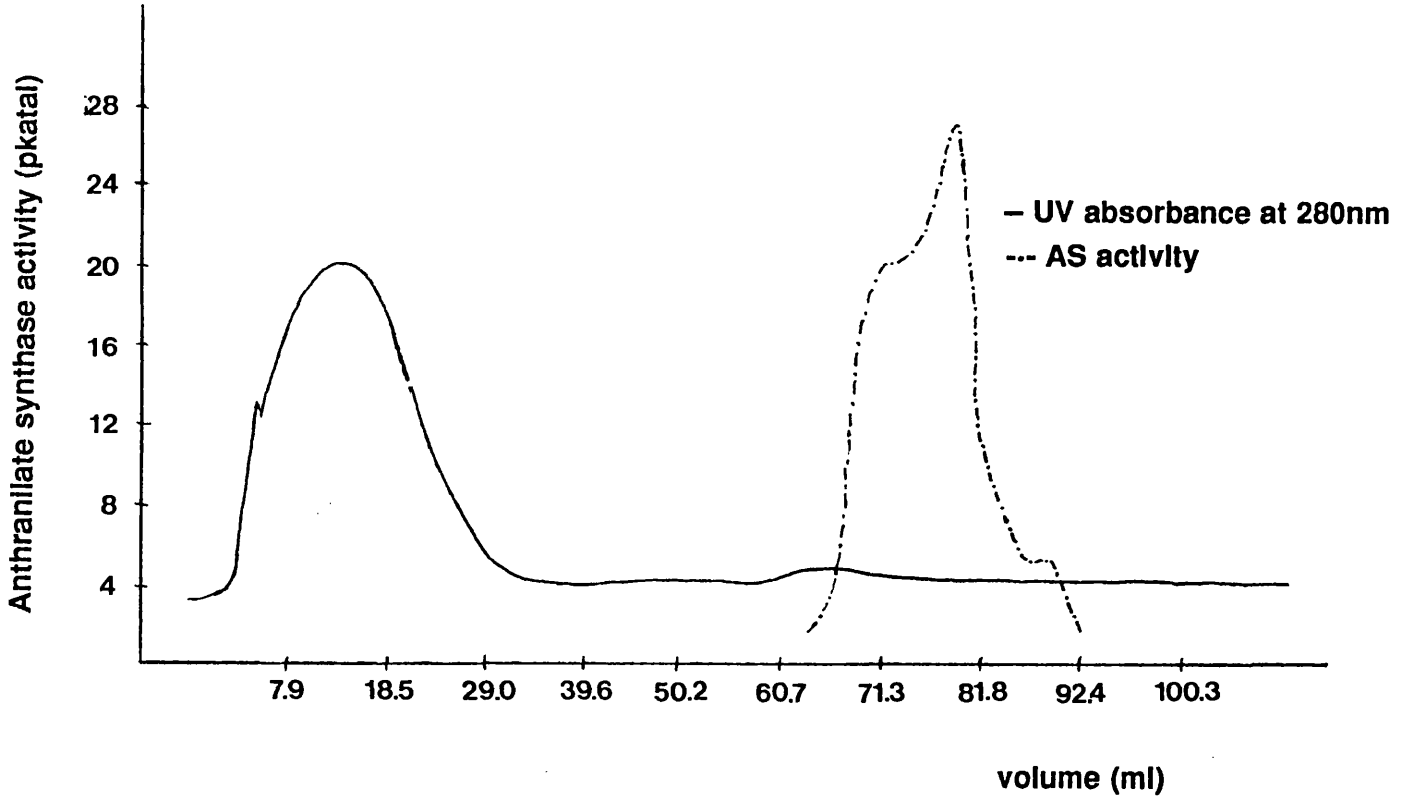


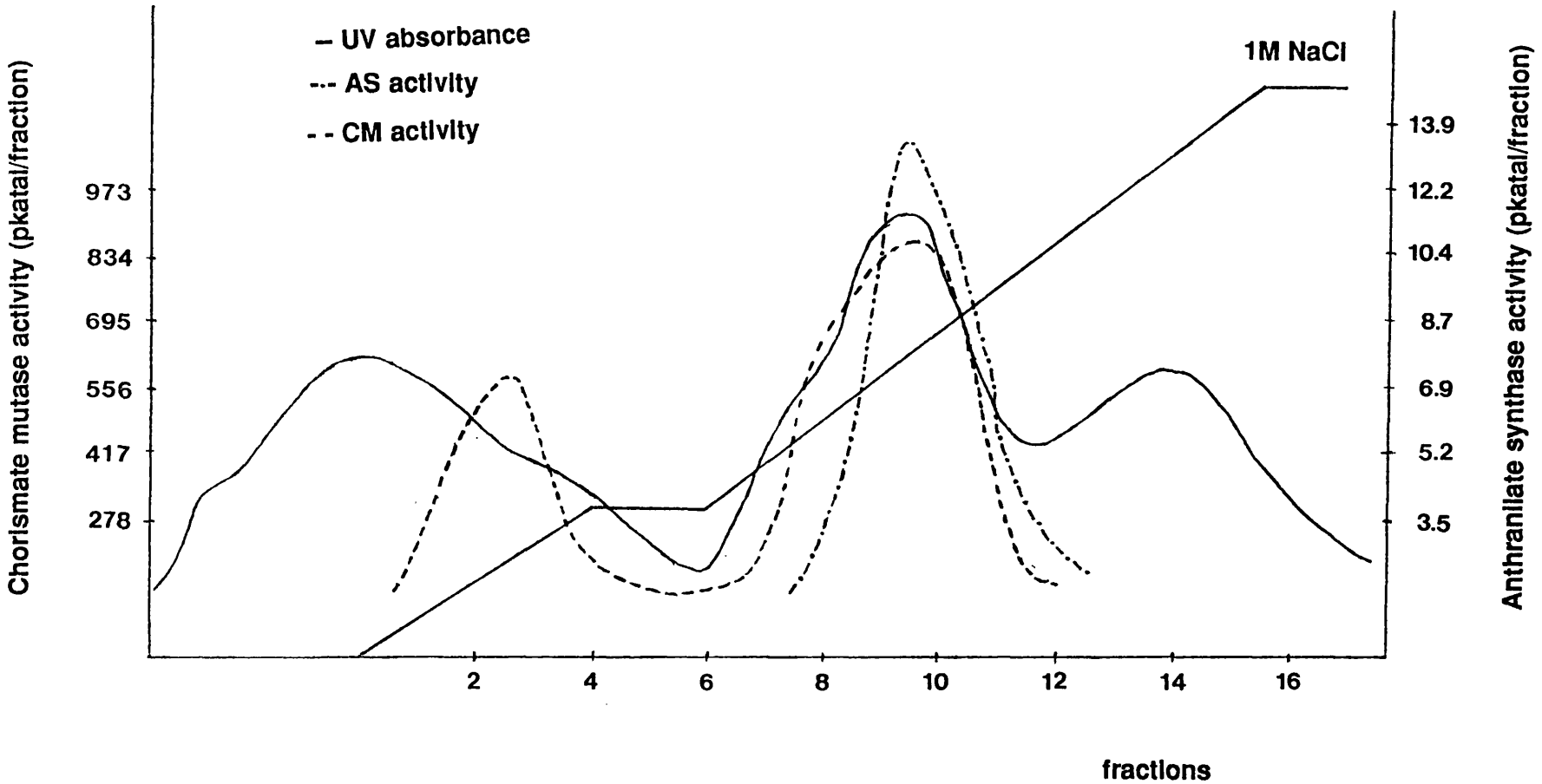
Table 6.7 Summary of AS partial purification from 270g callus cells

Fraction	Total protein (mg)	Total Activity (nkatal)	Specific Activity (pkatal/mg protein)	Purification factor	Yield (%)
crude extract	54.25	14.65	270.05	1	100
PEG precipitation	2.92	1.52	520.55	1.9	10
Q-Sepharose	2.04	11.49	5632.35	20.9	78
Orange A	0.91	2.23	2450.55	9.07	15

fractions were checked for AS activity, but the activity of the fractions was very low. The loss of specific activity during the elution of the proteins through the Orange A caused a problem in that not enough activity was available to continue with the enzyme purification. For this reason Orange A column was rejected from the purification scheme, and more experiments were done with the Q-Sepharose column to improve this step. The Q-Sepharose column was eluted using discontinuous salt gradients. The proteins were first eluted with a linear salt gradient and the gradient was stopped at different concentrations between 18% and 35% of buffer containing 1M NaCl. The concentration selected in each run was kept constant during determined volume; after the linear gradient was continued to reach 1M NaCl. After several experiments it was found that the best procedure was the one where the column was eluted with a linear salt gradient from 0 to 20% of buffer containing 1M NaCl, keeping constant the salt concentration at 20% and continuing with the linear salt gradient to 1M NaCl. Figure 6.4 shows the elution profile of proteins.

Chorismate mutase activity was determined in the fractions obtained from the Q-Sepharose column. Two peaks with CM activity were detected, and one of them overlaps the peak for AS activity.

Fig. 6.4 Elution profile of *A. altissima* anthranilate synthase and chorismate mutase enzymes from Q-Sepharose column



Mono-Q column

To continue with AS purification, the active fractions from the Q-Sepharose column were pooled and applied to a Mono-Q column. This column, previously equilibrated with extraction buffer, was run using a linear salt gradient from 0 to 0.5M NaCl (Figure 6.5).

The running conditions for the Mono-Q column were modified during several experiments. The linear salt gradient was interrupted at different salt concentrations, and the activities of AS and CM enzymes were checked every time. At the end, the best resolution in the column was reached using a linear salt gradient from 0 to 15% of buffer containing 1M NaCl, keeping the 15% salt concentration constant, continuing with the linear gradient up to 20%, keeping the 20% salt concentration constant and continuing with the gradient to reach in the running buffer a salt concentration of 0.5M NaCl (Figure 6.6).

At this stage, two anthranilate synthase enzymes were detected in callus and cell suspension cultures, but they were not always present. On one of the occasions where the two AS isoenzymes were detected, each one was injected again onto the Mono-Q column, and only the injected peak was detected.

In Figure 6.6 AS-b shows higher activity than AS-a, but this was not always the case. The CM enzyme detected during fractionation on the Mono-Q column was not detected every time.

To complete the purification scheme up to this point was difficult due to the instability of the enzymes. When the work was begun, there was no difficulty in obtaining enough activity to work with the enzymes, and the cells would keep at -80°C for at least two months, but this situation changed. Due to failure of the air conditioner system in the room where the cultures were kept, the cells were exposed to extreme changes in temperature, and after this stress

Fig. 6.5 Elution profile of *A. altissima* anthranilate synthase from Mono-Q column

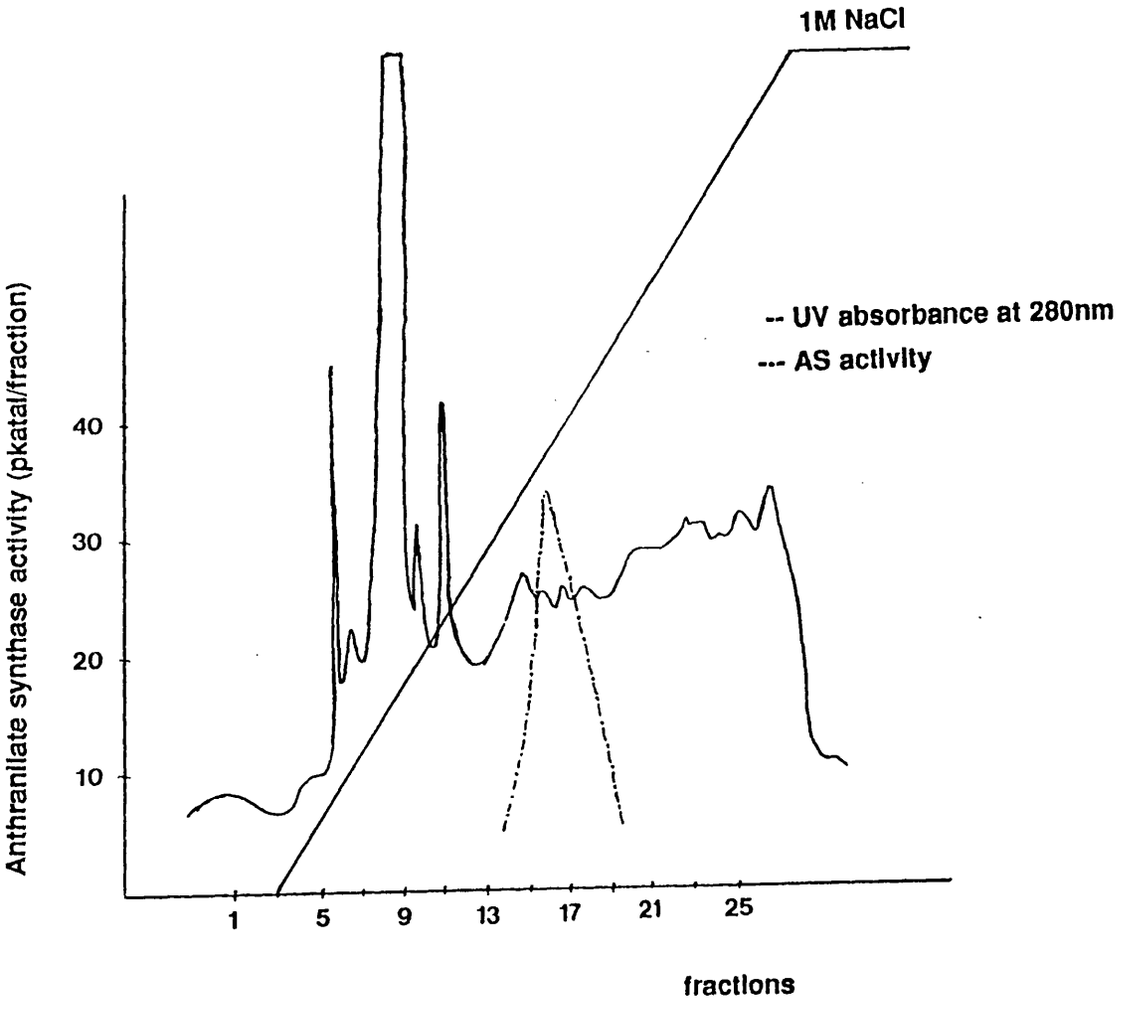
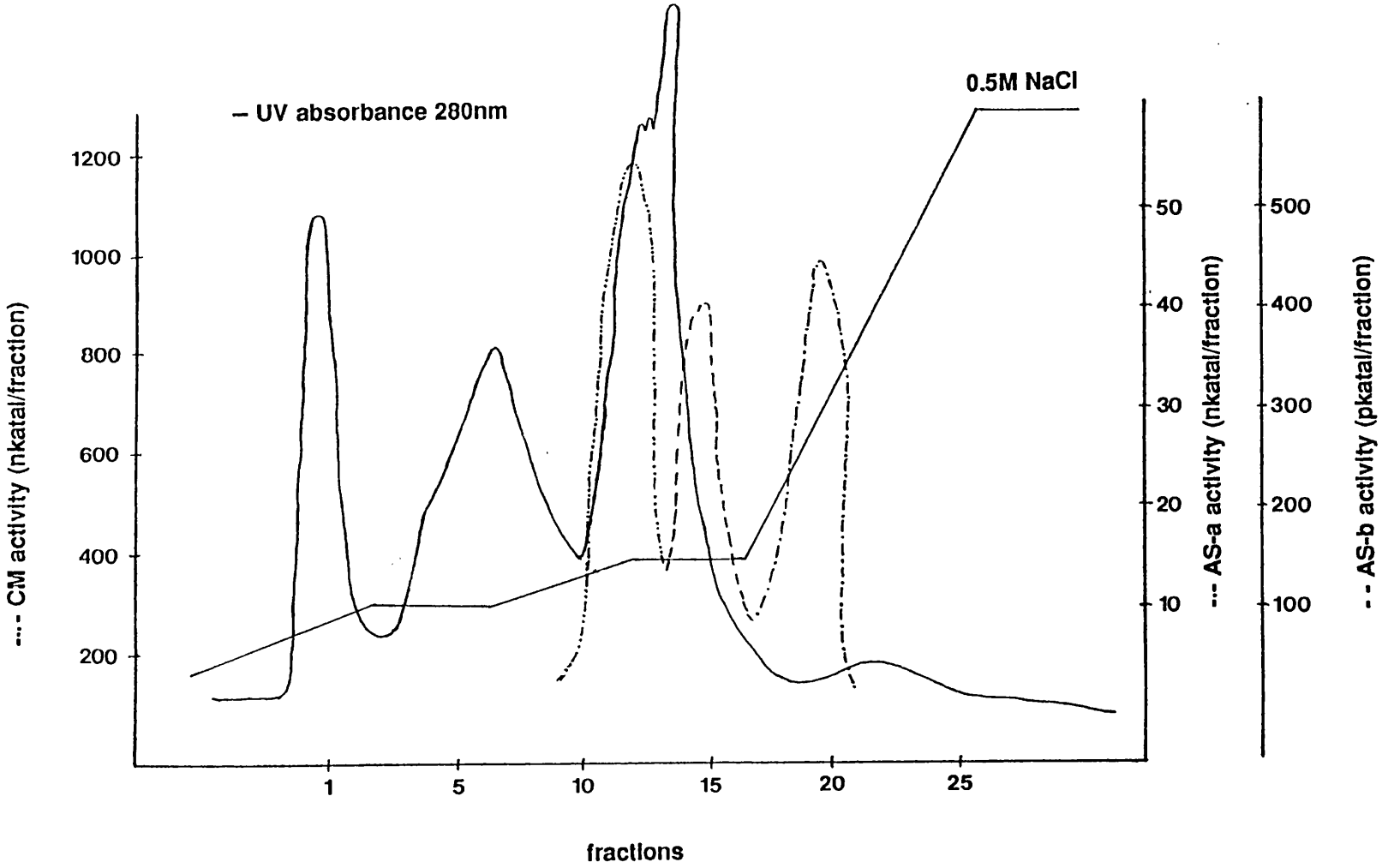


Fig. 6.6 Elution profile of proteins of *A. altissima* from Mono-Q column



the cells showed a different behaviour in terms of CM and AS activities. Frequently, the cells had no enzyme activity, or only one or the other, or the activity was not sufficient, or the enzymes were more fragile and decomposed during the attempt to purify them.

In Tables 6.8 and 6.9 there is a summary of the purification of AS in two different experiments. From this data it is possible to see the variation in anthranilate synthase activity from one preparation to another and also the absence of chorismate mutase in the cells used for one of the experiment.

Table 6.8 Purification of AS from *A. altissima* cell suspension cultures

Purification step	Total protein (mg)	Total activity (pkatal)	Specific activity (pkatal/mg protein)	Yield (%)	Purification (Fold)
Crude extract	36.47	2538	69.59	100	-
PEG	7.08	118.8	16.78	4.7	0.24
Q-Sepharose	3.90	2232	572.31	87.9	8.22
Mono-Q:					
AS-a	0.56	1095	1955.36	43.1	28.1
AS-b	0.31	955	3080.65		

Table 6.9 Purification of AS from *A. altissima* cell suspension cultures

Purification step	Total protein (mg)	Total activity (pkatal)	Specific activity (pkatal/mg)	Yield (%)	Purification (fold)
Crude extract	152.11	950	6.25	100	-
PEG	31.10	152	4.9	16	0.78
Q-Sepharose	17.08	753	44.1	79	7.1
Mono-Q:					
AS-a	1.3	321	246.9	33.8	39.5
AS-b	1.80	349	193.9		
CM-b	1.51	2115	1410		

Superose 12 HR 10/30 column

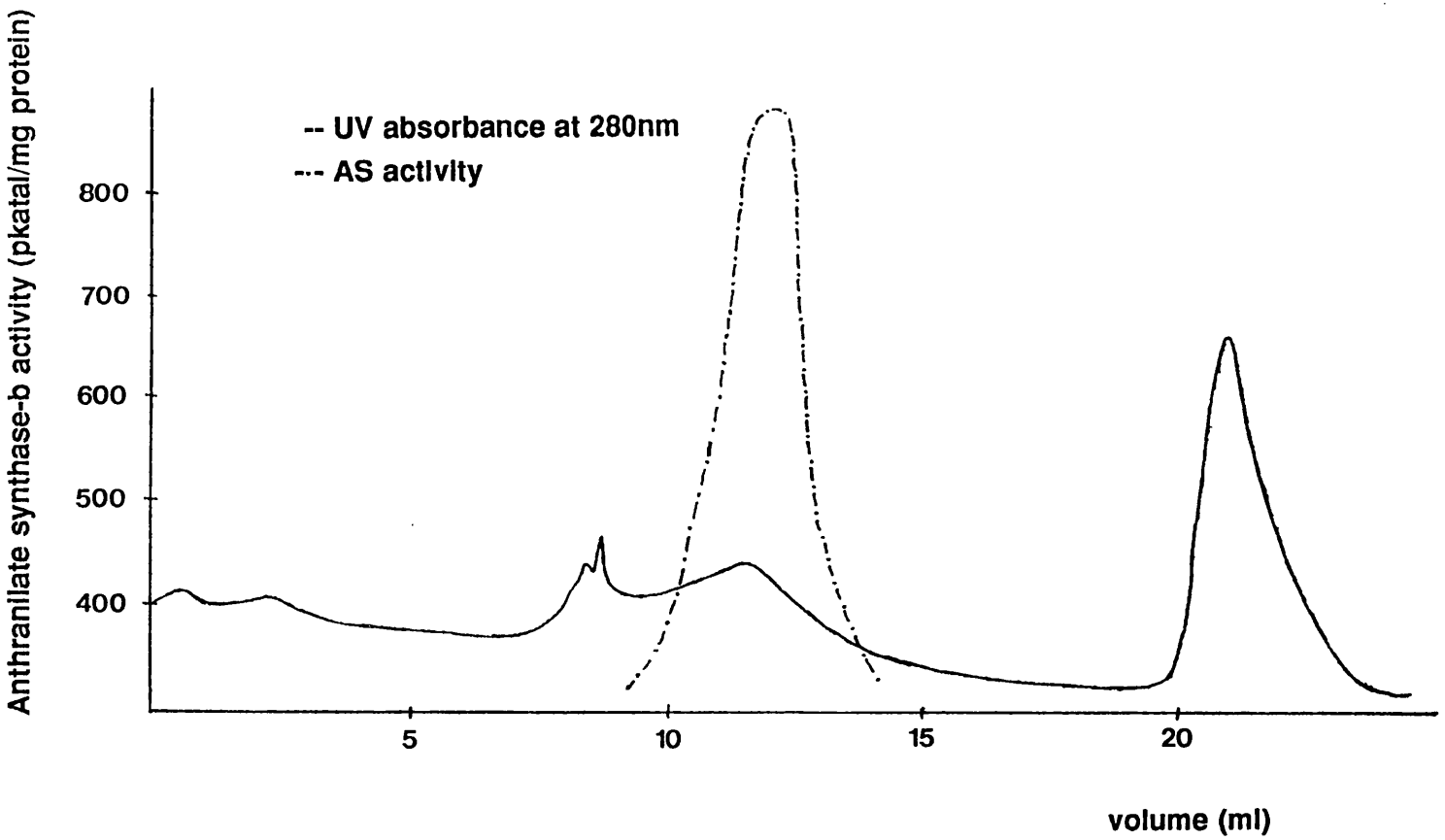
Some of the AS active fractions from the Mono-Q column were applied to a Superose 12 HR column. This column was previously equilibrated with buffer containing 0.1M Tris-HCl, pH 7.5, 10% glycerol, 10mM L-glutamine and 1mM DTT. The elution profile of AS-b from this column is shown in Figure 6.7.

CHARACTERISATION OF ANTHRANILATE SYNTHASE

Characterisation of anthranilate synthase-b involved the determination of conditions required for optimum enzyme activity, the kinetic properties of the enzyme substrates, inhibitor studies and molecular weight determination.

The experiments for the characterisation were performed on the enzyme obtained from the Mono-Q column.

Fig. 6.7 Elution profile of *A. altissima* anthranilate synthase-b from Superose 12 HR 10/30 column



Optimum conditions for enzyme activity

The conditions investigated for optimal enzyme activity include optimum incubation time, temperature and pH. Each of this conditions were individually investigated for their effect on enzyme activity. During the study of each condition, one condition was varied while the others were kept constant. Two sets of experiments with triplicate samples were performed for each condition.

Optimum incubation time and optimum temperature

Enzyme samples from the Mono-Q column were assayed for periods of time ranging from 20 to 100 minutes. For each time the incubation temperature was changed from 10 to 40°C, and the incubations were carried out in standard incubation mixes. Results of enzyme activity versus incubation time at different temperatures were plotted as shown in Figure 6.8. With the information obtained from this graph it was possible to select the optimum incubation time, which is where there was maximal enzyme activity within the linear region of the graph. This time was found to be 60 minutes. A temperature of 35°C was found to be the temperature at which maximum activity occurred (optimum temperature). Because temperatures above 35°C were deleterious to enzyme activity, temperatures of 30°C were employed for standard assays.

Activation energy

The activation energy of the enzyme obtained from the Mono-Q column was determined using the Arrhenius equation which relates specific rate constant to temperature. The data used for this calculation were those obtained at an incubation time of 60min and at temperatures of 20, 25, 30 and 35°C. Figure 6.9 shows the Arrhenius plot for the anthranilate synthase-b enzyme. An activation energy of 66kJ/mol was found.

Fig. 6.8 Effect of temperature on anthranilate synthase-b activity at different incubation times

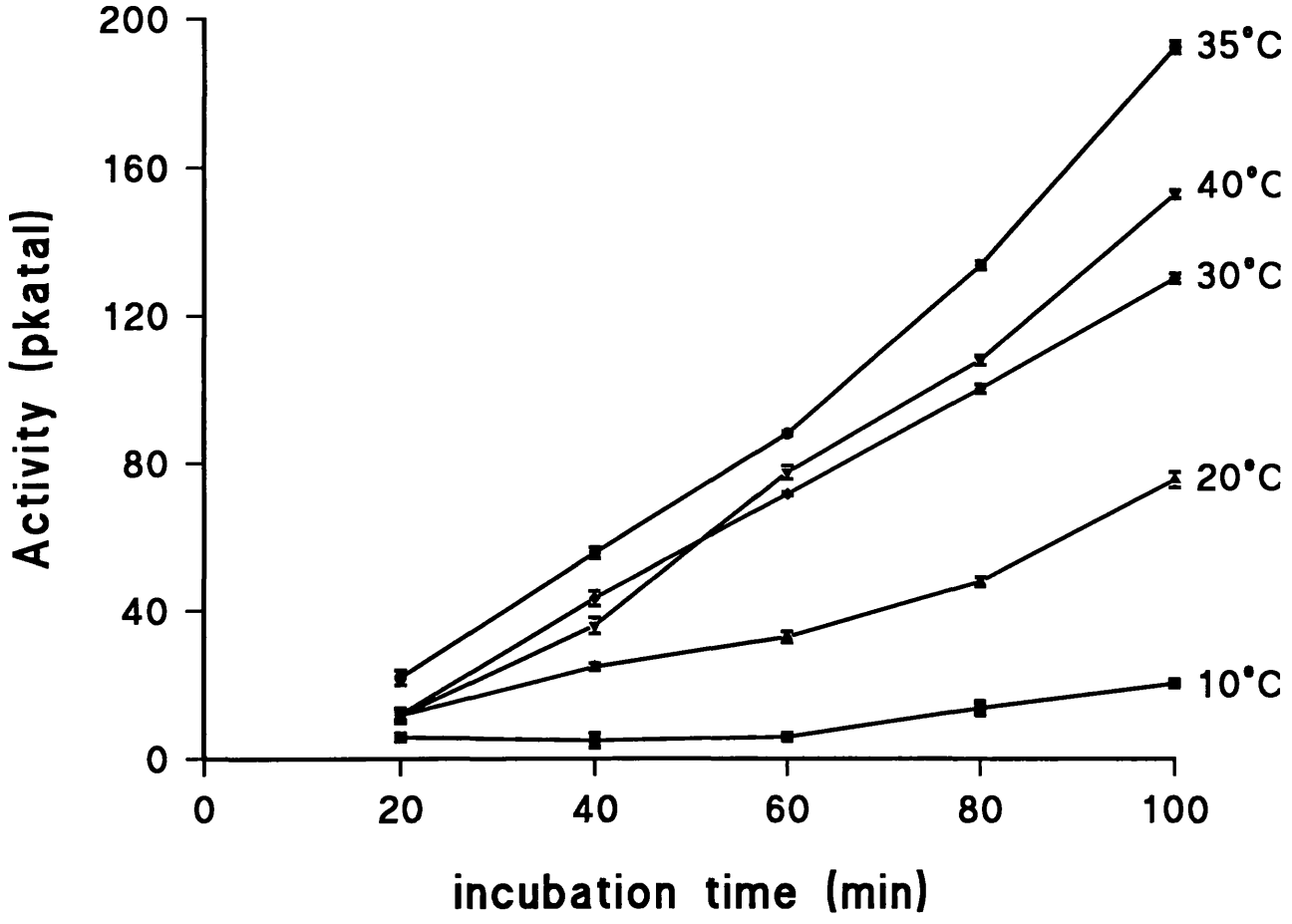
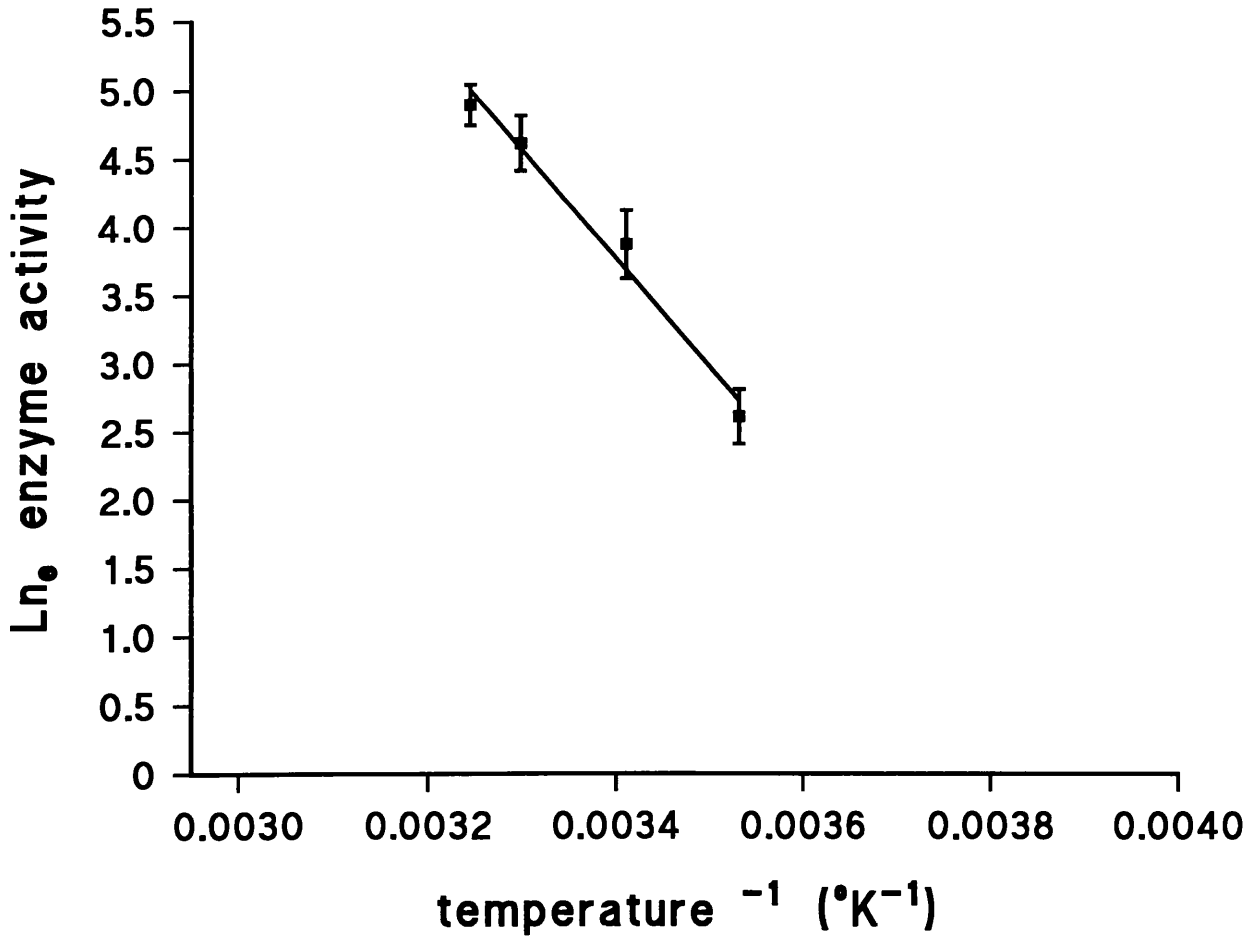


Fig. 6.9 Arrhenius plot for anthranilate synthase-b enzyme



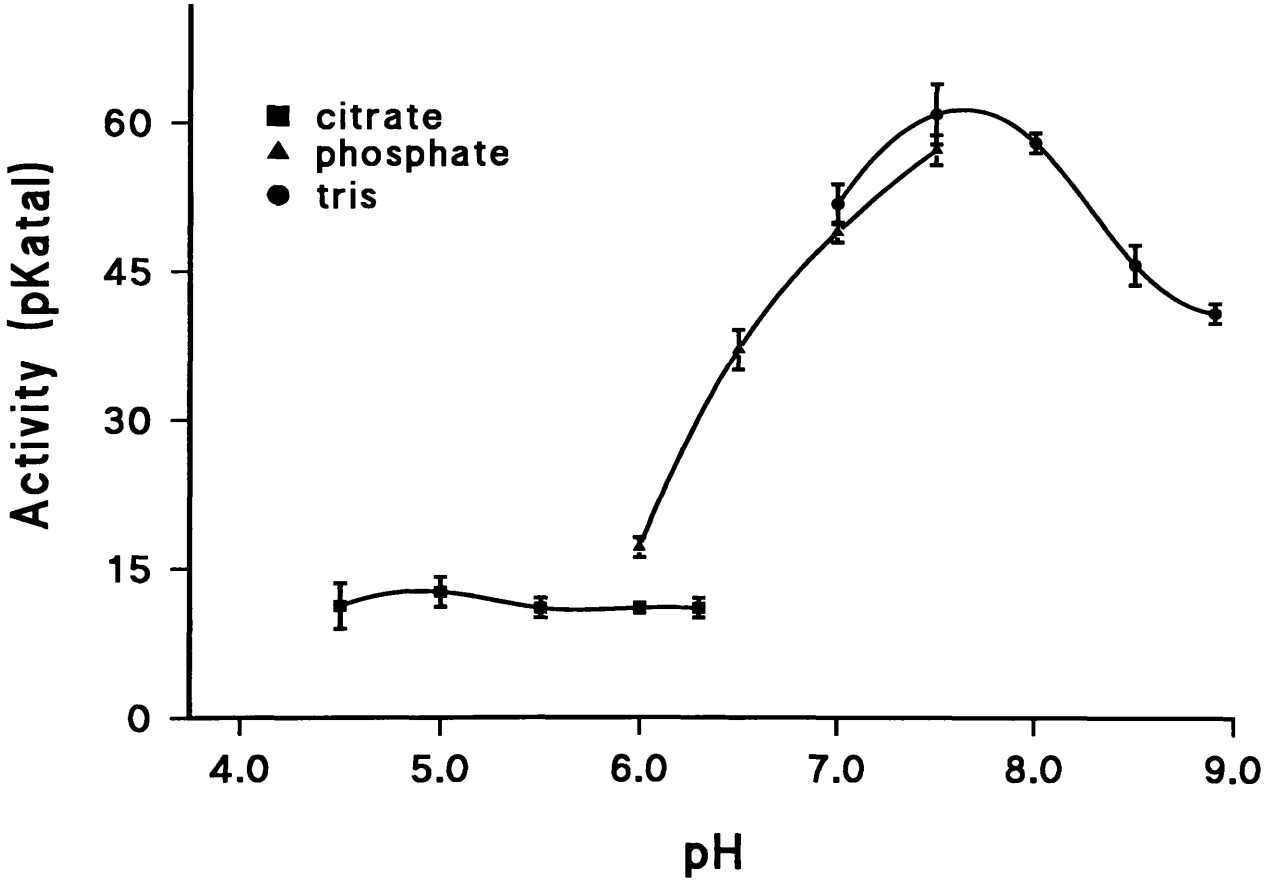
Optimum pH

To determine the optimum pH, enzyme solutions were incubated at 30°C and at pH levels from 4.5 to 9 using three different buffer systems, citrate, phosphate and Tris, in order to keep the enzymes solutions to the required pH. The assay components $MgCl_2$ and L-glutamine were solubilized in 0.1M of each buffer used and chorismate was dissolved in water. The assay was performed in the same way described in **Materials and Methods** with the exception that a volume of 25µl of enzyme solution from Mono-Q column was used per assay. To keep the total incubation volume of 250µl, 100µl of 0.1M from the corresponding buffer was added. Results of enzyme activity versus pH are plotted in Figure 6.10.

At lower pH levels below 6 and higher than 8, the activity of the enzymes is low. This situation agrees with the fact that pH changes affect the ionic character of the amino and carboxylic acid groups on the protein, and therefore affect the catalytic site and conformation of the enzyme. The bell-shaped pH profile implies that ionizing groups change their state of protonation as the pH raised. The pH optima fell between 7.3 and 8.

It was also observed that the enzyme activity was lower in citrate than in phosphate buffer at the same pH and that enzyme activity in phosphate buffer was slightly lower than in Tris buffer at pH values of 7 and 7.5.

Fig. 6.10 Effect of pH on anthranilate synthase-b enzyme activity



Michaelis-Menten constants for the enzyme substrates and cofactor

The Michaelis-Menten constants, (K_m), for the enzyme substrates, chorismate and L-glutamine, and for the cofactor Mg^{2+}, were determined.

The most commonly used method for the determination of K_m utilises the Lineweaver-Burk equation which is the the double reciprocal of the equation postulated by Michaelis-Menten.

Lineweaver-Burk equation

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}}$$

Michaelis-Menten equation

$$V = \frac{V_{max}[S]}{[S] + K_m}$$

where V is the reaction rate and (S) is the substrate concentration.

The plots corresponding to this two equations were obtained for the substrates and cofactor.

The K_m for chorismate was determined by carrying out assays in triplicate for samples which contained a fixed concentration of $MgCl_2$ and L-glutamine, 10mM and 20mM, respectively, and different concentrations of chorismate ranging from 12µM to 150µM. The same experiment was repeated and the average values were plotted in Figure 6.11. The values for K_m and V_{max} are: 72µM and 93 respectively.

To obtain the K_m for L-glutamine the concentrations of chorismate and $MgCl_2$ were kept constant at 1mM and 10mM, respectively, and the concentration of L-glutamine was changed in a range from 0.15 to 0.50 mM. For each concentration, samples in triplicate were assayed, and the experiment was also repeated. The results were plotted as it is shown in Figure 6.12. The values

Fig. 6.11 Anthranilate synthase-b activity at different chorismate concentrations

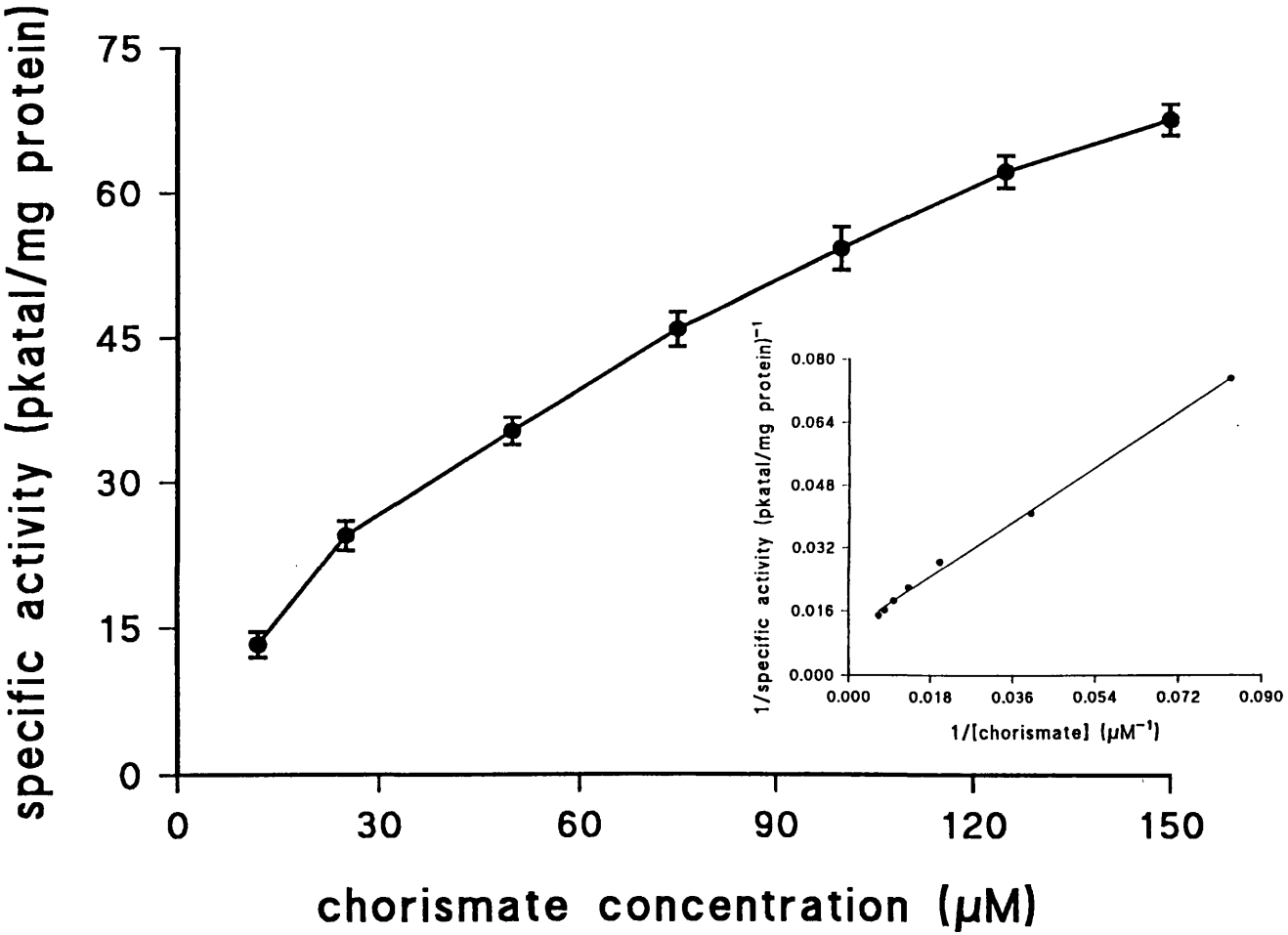
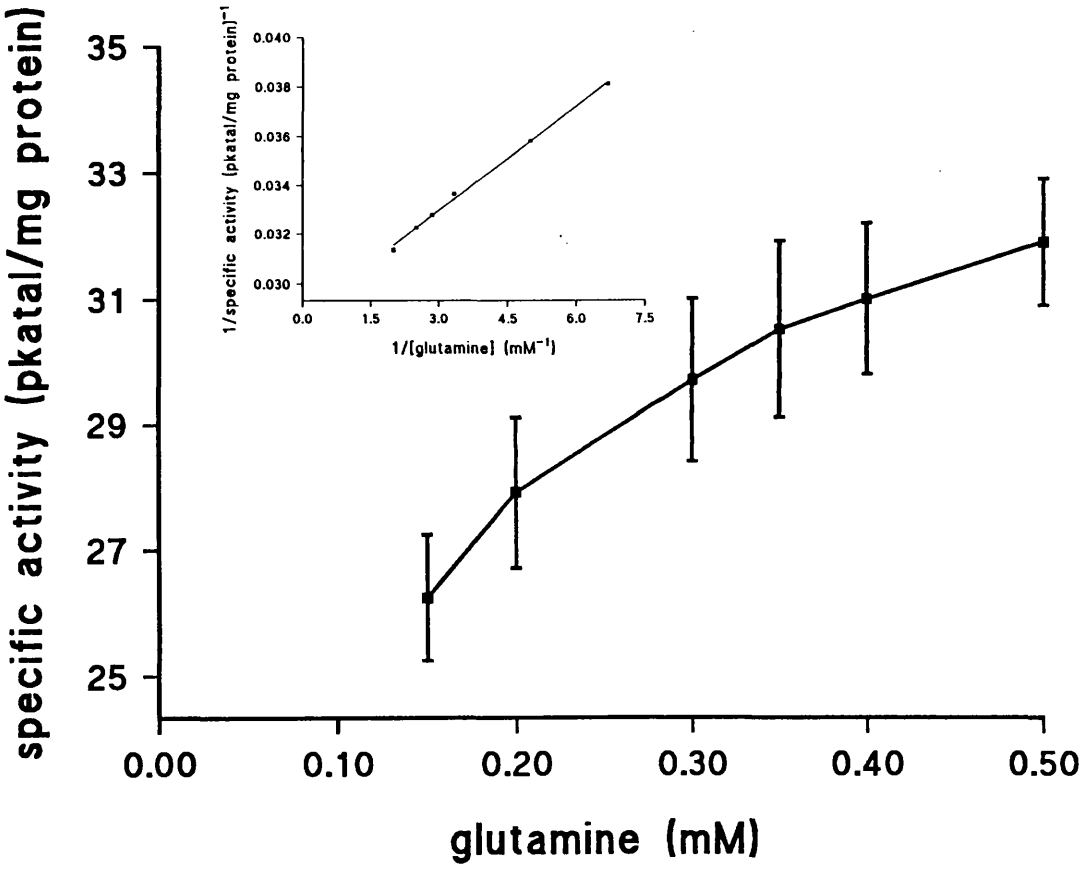


Fig. 6.12 Anthranilate synthase-b activity at different L-glutamine concentrations



obtained from the Lineweaver-Burk plot are: 0.050mM for K_m and 35 for V_{max}.

For $MgCl_2$, the determination of the K_m and V_{max} was done by carrying out assays, in triplicate, with fixed concentrations of chorismate and L-glutamine, 1mM and 20mM, respectively. Different $MgCl_2$ concentrations ranging from 0.10 to 0.60mM were used. After repeating the experiments, the results were plotted and the Figure 6.13 was obtained. Values of 0.18mM for K_m and 45 for V_{max} were calculated from the graph.

Feedback inhibition by tryptophan

The activity of anthranilate synthase-b was measured at various concentrations of chorismate and at four fixed concentrations of L-tryptophan: 2.5μM, 5μM, 10μM and 20μM; L-glutamine and $MgCl_2$ were kept constant at 20mM and 10mM respectively. Figure 6.14 shows the effect of chorismate concentration on AS activity in the absence and presence of varying tryptophan concentrations. The presence of tryptophan caused a change to sigmoid curves. When the reciprocal activity was plotted against tryptophan concentration a concave upward curvature was observed (Dixon plot, Fig. 6.15).

From the data obtained it was possible to calculate the K_i, the inhibition constant, that is a measure of the affinity of the enzyme for the inhibitor (I). A value of 1.5 μM was obtained.

Other inhibitors

Anthranilate synthase-b was also inhibited by D-tryptophan and tryptamine. A concentration of 100μM of D-tryptophan inhibited the enzyme by 38%, meanwhile 100μM of tryptamine inhibited the AS-b activity by 27%. When L-tryptophan was used to inhibit the enzyme, a 20μM solution resulted in 97% inhibition.

Fig. 6.13 Anthranilate synthase-b activity at different $MgCl_2$ concentrations

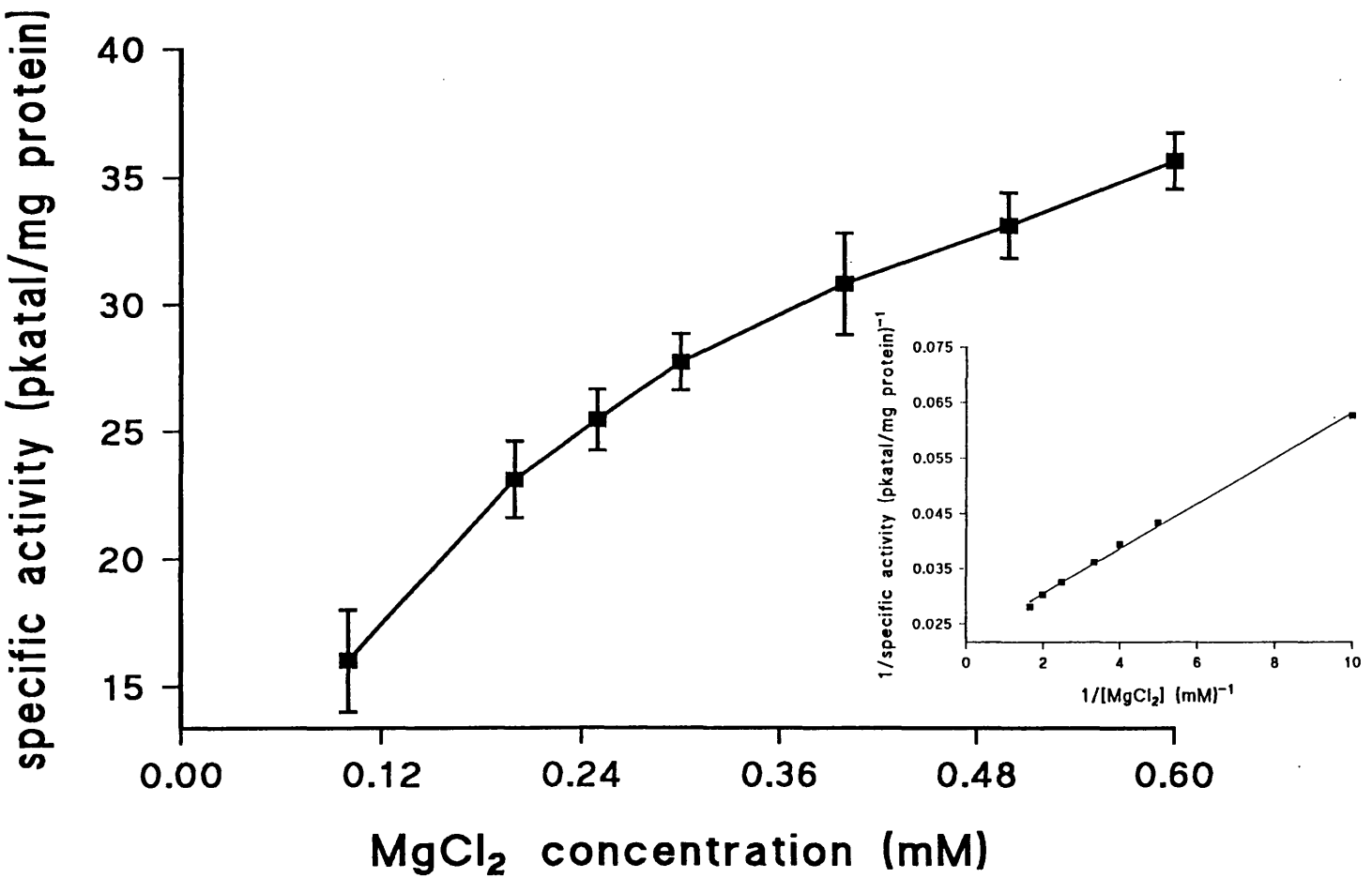


Fig. 6.14 Anthranilate synthase-b activity at different chorismate and tryptophan concentrations

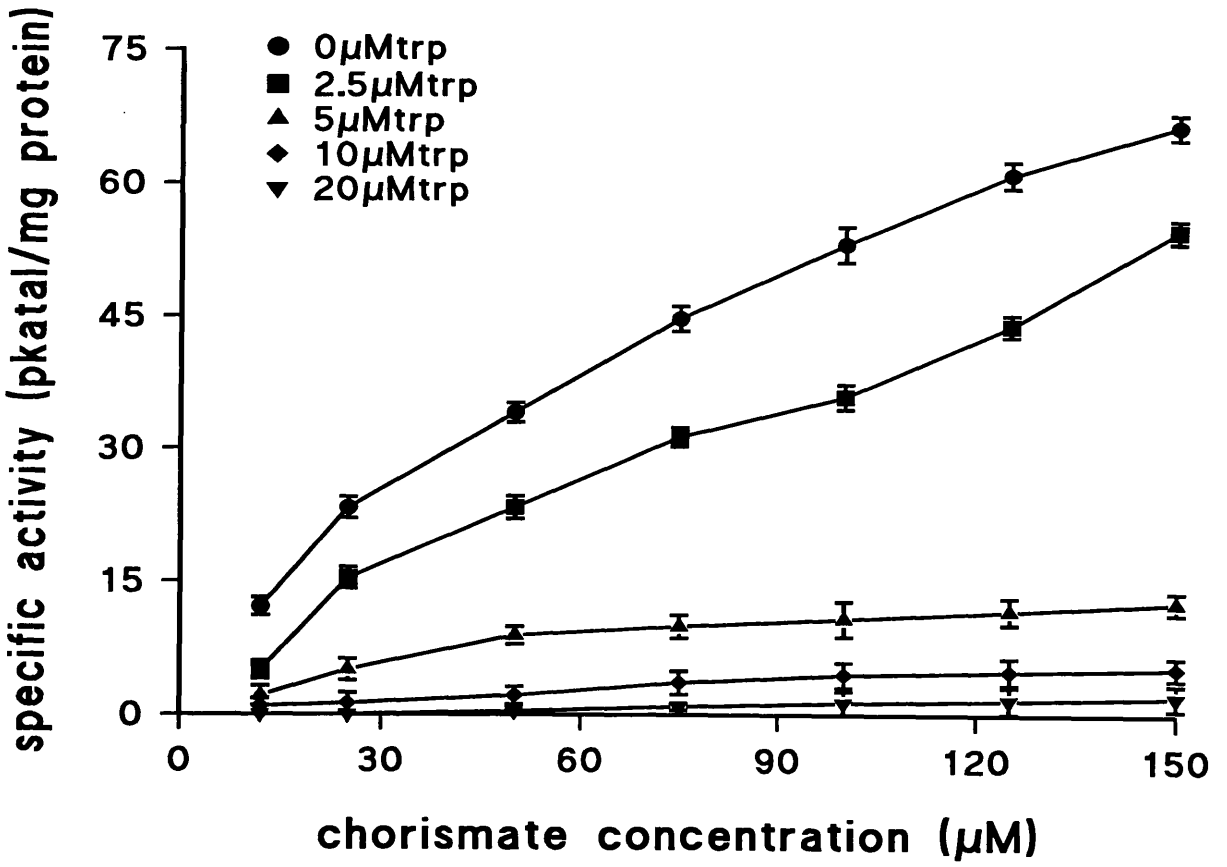
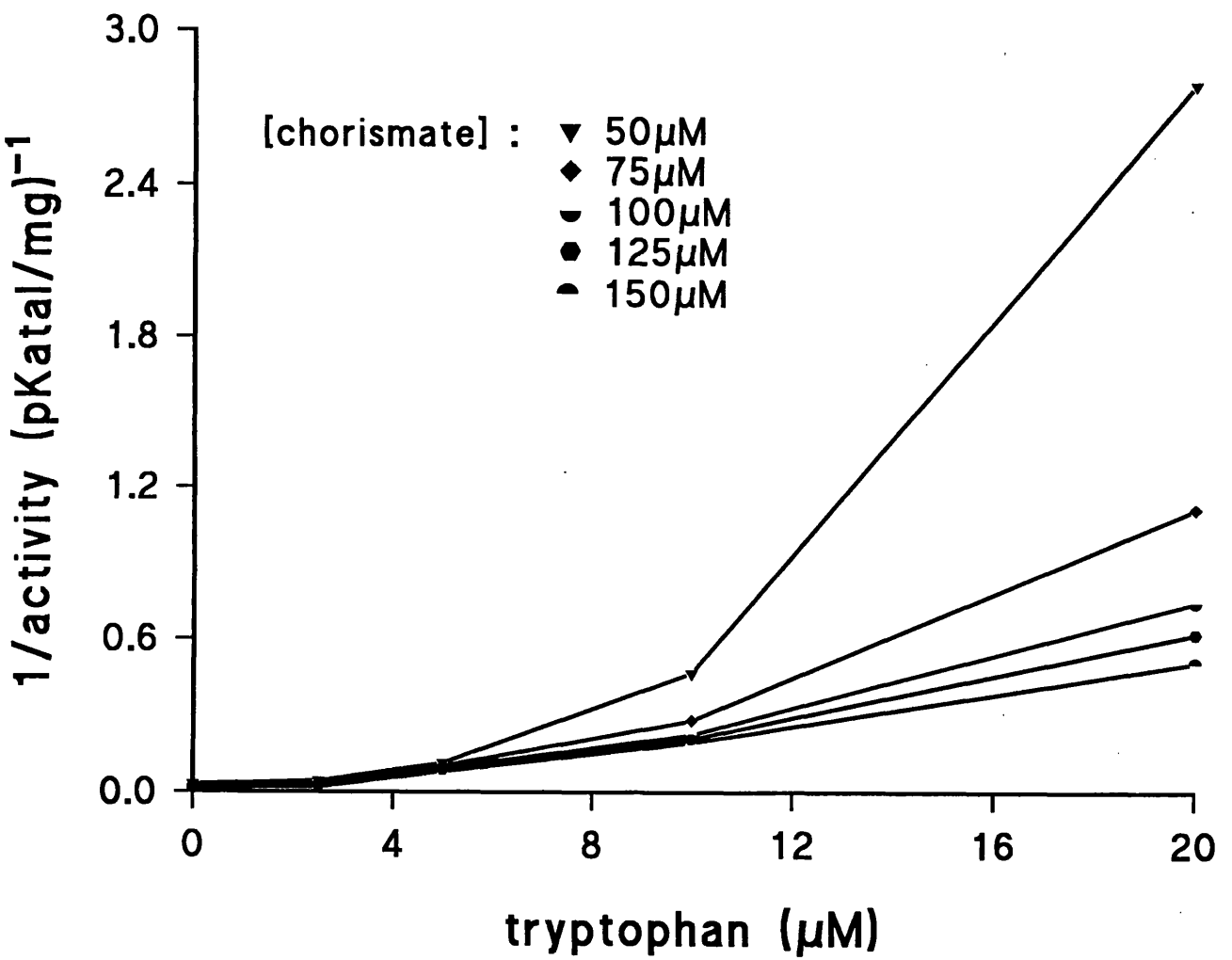


Fig. 6.15 Tryptophan inhibition of anthranilate synthase-b activity



Tyrosine, phenylalanine and indole acetic acid were tested at concentrations of 100μM, but they did not show inhibitory effects.

The inhibition of anthranilate synthase-a by L-tryptophan was also tested and 66% inhibition was obtained with 20μM of tryptophan.

Determination of the molecular weight of anthranilate synthase-b

Figure 6.7 (p. 110) shows the elution profile of proteins from the Superose column. The enzyme activity coincided with a single protein peak, suggesting that the enzyme had been purified to a single protein. The peak at approximately 21ml gave no absorbance with Bradford reagent. It is possible that this peak corresponded to some of the additives that were used in the buffer used to dissolve the enzyme and were not present in the running buffer.

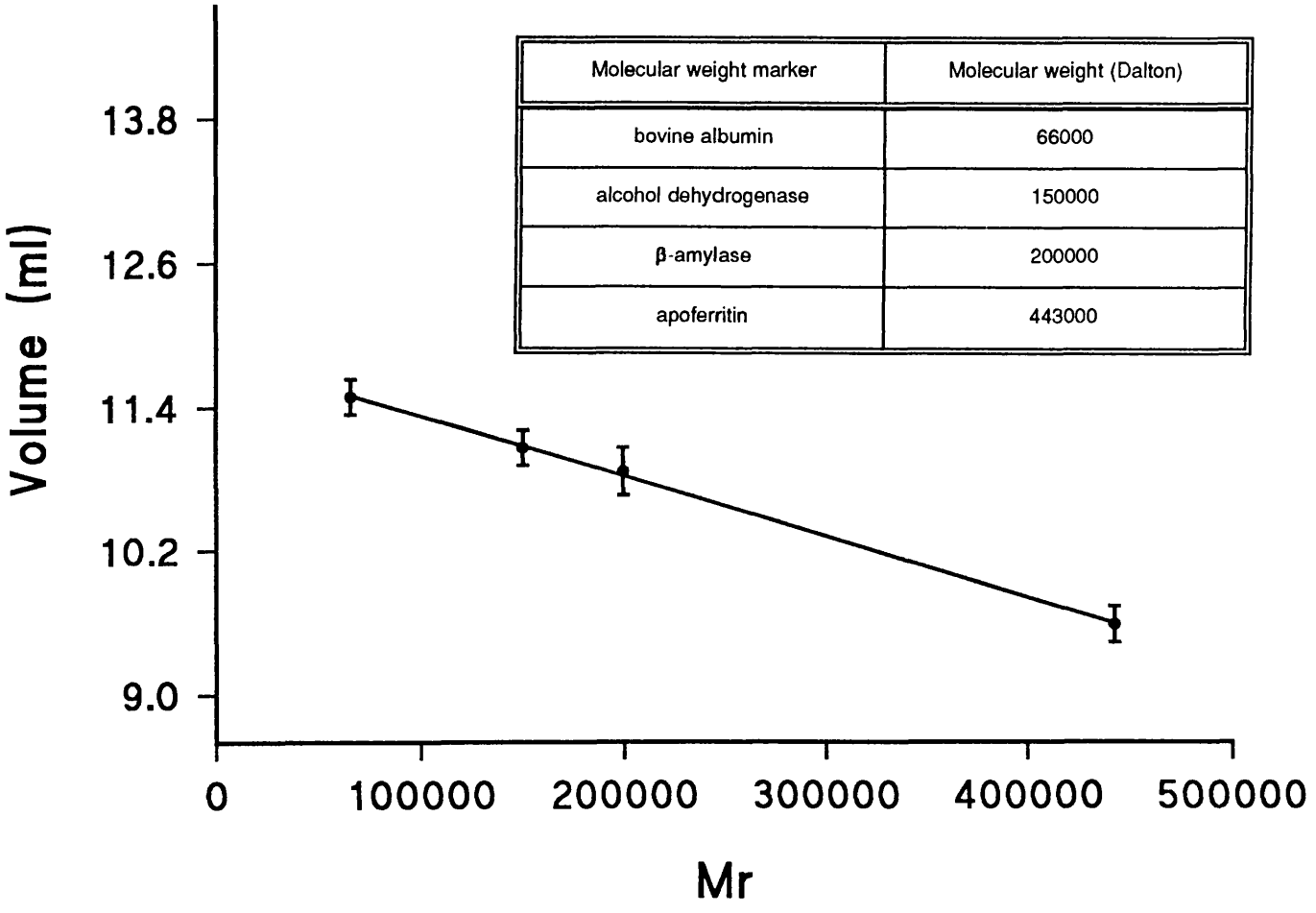
To determine the molecular weight of the enzyme, molecular weight markers were injected on to the column for purposes of calibration (see **Materials and Methods**). After this, 200μl of enzyme solution were injected under the same conditions. After the elution volume of each of the markers was determined, a calibration curve of molecular weight of the markers versus the elution volume was plotted (Fig. 6.16).

The molecular weight of the enzyme was subsequently determined as 77 ± 3 kDa by interpolation from the calibration curve.

Vertical SDS-PAGE was also used to determine the molecular weight of the enzyme. The experimental procedure involved is explained in **Materials and Methods**.

Figure 6. 17 shows the migratory profile of the mixture of protein standards as well as one of the fractions containing AS-b from Mono-Q column. In this figure only a single protein band can be seen, but in reality at least one other band

Fig. 6.16 Calibration curve for determination of Mr using Superose 12 HR 10/30 column

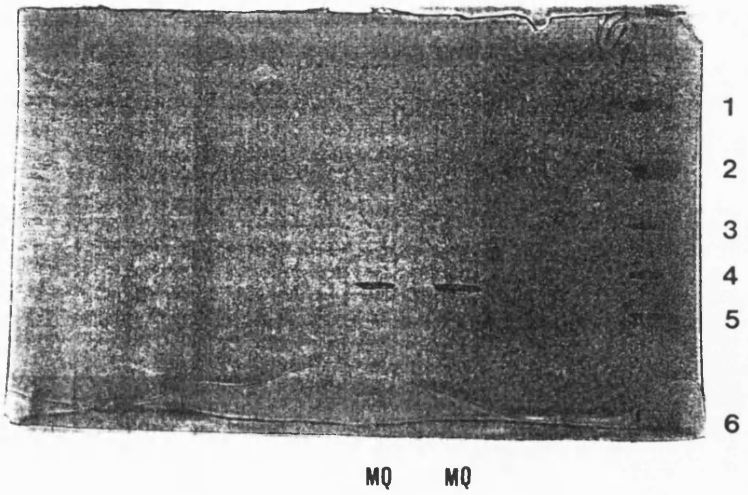


Molecular weight marker	Molecular weight (Dalton)
bovine albumin	66000
alcohol dehydrogenase	150000
β-amylase	200000
apoferritin	443000

could be observed in the original gel. Several attempts were performed to clarify this situation, running vertical SDS-PAGE electrophoresis for fractions of the Superose column, but due to low quantity it was not possible to observe any band from those fractions.

A calibration curve of the molecular weights of each of the markers versus the distance migrated by each marker from the cathodic end of the gel was plotted, and the molecular weight of the single protein band was determined by interpolation. The molecular weight corresponding to this band is 60 ± 1 kDa. The other band that was mentioned before showed a molecular weight of 37 ± 1 kDa.

Fig. 6.17 Migration profile of anthranilate synthase-b during SDS-PAGE



	Molecular weight marker	Molecular weight (Dalton)
1	α_2-macroglobulin	190000
2	β-galactosidase	125000
3	fructose-6-phosphate kinase	88000
4	pyruvate kinase	65000
5	fumarase	56000
6	lactic dehydrogenase	38000

MQ: fraction from Mono-Q column

7. DISCUSSION AND CONCLUSIONS

It is estimated that 60% of the total energy flow in a plant cell can pass through the shikimic pathway [Jensen 1986]. Many compounds derive from this pathway, chromagens that give the brilliant colours in flowers and fruits, lignins for structural support, phytoalexins for plant defense and many other secondary metabolites, are a few examples. The end products of this pathway, tryptophan, tyrosine and phenylalanine, besides their role in protein biosynthesis, are the substrates that give origin to many secondary compounds.

For example, higher plants produce alkaloids from all three aromatic aminoacids: indole alkaloids in plants such as *Catharanthus roseus* which derived from tryptophan [Pennings et al 1989], isoquinoline alkaloids derived from L-tyrosine in *Papaver somniferum* [Hay et al 1988], and tropane alkaloids derived in part from phenylalanine in species of *Datura stramonium* [Leete 1979].

Despite the importance of the secondary metabolism in plants, relatively little is known about the processes regulating the synthesis of these metabolites.

Cell cultures are valuable experimental material for studies on the regulation of product formation and on biosynthesis, besides their potential as producers of secondary metabolites. Plant cell cultures possess certain advantages over differentiated plants for biosynthetic studies. The cultures can be grown under standard conditions for short growth cycles, are not subject to seasonal variations and are less complex in organisation than the entire plant. However, sometimes cultured cells produce inferior yields of the desired metabolites in comparison to the whole plant and may not produce the same secondary metabolites as the parent plant. In spite of these inconsistencies, the use of cell cultures has made possible the isolation and study of enzymes involved in different metabolic pathways.

Ailanthus altissima cell suspension cultures produce high yields of canthin-6-one alkaloids, up to 100 fold greater than the whole plant, and produce the

coumarins scopoletin and isofraxidin, but in very low yield. For these reasons this plant is a good model for the study of enzymes, especially those involved in the biosynthesis of canthin-6-one alkaloids which are derived from tryptophan.

Plant cell cultures of *A. altissima* have been very useful in the study of the biosynthetic pathway leading to the production of canthin-6-one alkaloids. The role of tryptamine and α-ketoglutarate in the biosynthesis of these alkaloids was investigated using labelled compounds (see Chapter 1, p. 20).

In this project two chorismate utilising enzymes, anthranilate synthase and chorismate mutase were examined in cell cultures of *A. altissima.*

TIME COURSE STUDY

The activities of CM, AS and ISC were studied during the growth cycle of cell suspension cultures. Before this experiment, cells were selected for the most rapid growth and greatest enzyme activity by testing the activity of the enzymes in cell suspension cultures of different ages. During the growth cycle, the cells underwent almost 5 fold increase in fresh weight (Fig. 6.1, p. 94) which coincides with values previously reported [Anderson et al 1985, 1987, Hay 1987, Osoba 1994].

Isochorismate synthase activity was not detected in the cultures used in all the recorded experiments. Isochorismate, the product of the reaction catalysed by this enzyme, is the precursor of anthraquinones and phylloquinone. Anthraquinones have not been reported either in cell cultures or the parent plant *A. altissima* and it is not known if phylloquinone is a vitamin in cell suspension cultures [Poulsen and Verpoorte 1992], hence the lack of ISC activity in the cultures can be justified. Another possibility is that if there was an active ISC enzyme, its activity was below the detection limit of the assay: 5 pmol [Poulsen et al 1991].

During the time course study, anthranilate synthase activity was always detected throughout the growth cycle, but there was a maximum between days 8 and 17. This result coincides with the fact that the alkaloid level in cell suspension cultures increases after day 20 [Osoba 1994]. On the other hand, CM activity, which was also active throughout the growth cycle, increased after day 20, the activity of this enzyme being 150x the activity of AS enzyme. In a previous experiment, coumarin production reached maximum levels early in the growth cycle, at day 12 and at day 28 [Osoba 1994].

The activities of AS and CM enzymes have been tested at the same time in several plant cell cultures. In all cases the activity of CM was measured in nkatal/mg protein, whereas AS activity was measured in pkatal/mg protein. In a comparison of the specific activities of these two enzymes from cell suspension cultures of *Catharanthus roseus, Tabernaemontana divaricata, Tabernaemontana pandacaqui, Cinchona robusta, Rubia tinctorum* and *Euonymus europaeus*, the specific activity of CM was up to a factor of 1000 times higher than the specific AS activity [Poulsen and Verpoorte 1992]. This agrees with the finding that the phenylalanine pathway is very active. In the same laboratory they found that in *C. robusta*, where CM enzyme was very active, phenylalanine lyase was also very active and the cultures produce large amounts of phenolic compounds. In *C. roseus* cultures, where CM activity was very low, there was also very low activity of phenylalanine lyase and very low production of phenolic compounds.

PURIFICATION OF ANTHRANILATE SYNTHASE

Anthranilate synthase has been investigated in plants, but mainly in crude cell free extracts, and it has been isolated and characterised from cell suspension cultures of *Catharanthus roseus* (Table 3.3, Chapter 3). The enzyme has also been purified and characterised from many microorganisms (Table 3.1, 3.2, Chapter 3).

The specific activity of AS in plants is low as compared with that of microorganisms. Neglecting the differences in assay conditions, the specific activities of the AS enzymes from plants have been reported to be between 0.1 pkatal/mg protein in cell suspension cultures of *Tabernaemontana divaricata* [Poulsen et al 1991] and 33-150 pkatal/mg protein in callus tissue of *Nicotiana tabacum* [Belser et al 1971]. On the other hand in microorganisms the reported AS enzyme activities vary from 12 pkatal/mg protein in *Euglena gracilis* [Hankins and Mills 1976] to 1833 pkatal/mg protein in *Serratia marcescens* [Zalkin and Hwang 1971]. In general the specific activity of AS enzyme extracted from microorganisms is higher than the one from plant source. In the plant studied in this project, high specific activities of 270 pkatal/mg protein for callus tissue, and 70 pkatal/mg protein for cell suspension cultures were obtained. This values show that AS enzyme activity in *Ailanthus altissima* is high relative to the specific activities of this enzyme in other plants.

The buffers normally used for the extraction of the enzyme either from plants or microorganisms, are Tris-HCl and potassium phosphate. When the enzyme was purified or partially purified 0.1M Tris-HCl buffer was used for the extraction. The extraction buffer selected for this study was Tris-HCl with some of the additives used in previous isolations from other plants [Hertel et al 1991, Poulsen et al 1993]. The most important stabilising factor was L-glutamine; glycerol, DTT, EDTA, PMSF and leupeptin were also important, but the enzyme stayed active for longer time in the presence of L-glutamine.

After the extraction, the next selected step was the precipitation with PEG. This procedure gave a better recovery than either Piksi agarose A6XL or ammonium sulphate precipitation. The results obtained with the agarose columns were not reproducible; on other hand, the precipitation with PEG removed more protein and the specific activity was higher than with the agarose columns. When columns were used after the precipitation, no advantages were found and therefore, PEG precipitation was chosen. In experiments using precipitation of protein with $(NH_4)_2SO_4$ the activity was always lower than with PEG and the

amount of protein removed with the organic polymer was higher.

Experimentation and analysis demonstrated that two precipitations were desirable: the first, with 10% (w/v) PEG, to remove protein and the second, with 18% PEG, to precipitate and concentrate the enzymes. During this step it always looked like there was considerable loss of total activity, but later with the next step it was possible to see that this was not so. Probably when the precipitation is performed the enzyme changes its structure and consequently loses its activity.

The next step in the process of purification was the Q-Sepharose column for which a discontinuous salt gradient was used to obtain the best performance with the column. There was an attempt to use an Orange A column after the Q-Sepharose column that proved to be a crucial step during the isolation of the enzyme from *Catharanthus roseus* [Poulsen et al 1993]. The loss of specific activity with this column was very high and, if this step had been included it would not be possible to continue with the enzyme purification due to the loss of enzyme.

A discontinuous salt gradient was also used with the Mono-Q column and two peaks with anthranilate synthase activity were detected. The two isoenzymes were not present always. From the many preparations that reached the last step (Mono-Q column), only in four of them were the two isoenzymes detected, and excepting for one preparation, AS-b was always the isoenzyme present. The fact that the isoenzymes were not always present, might be due to one of them being more unstable and decomposing throughout the isolation process.

During the isolation of AS enzymes, the discontinuous salt gradient in the Q-Sepharose and Mono-Q column were crucial for the separation of the chorismate mutase and anthranilate synthase enzymes. In the case of the isolation in *C. roseus*, the CM and AS were separable only with the use of an Orange A column.

COMPARISON OF *AILANTHUS ALTISSIMA* ANTHRANILATE SYNTHASE-B WITH OTHER ANTHRANILATE SYNTHASE ENZYMES

Optimum temperature and pH

Anthranilate synthase-b was found to operate optimally at an incubation time of 60 min., with a pH between 7.3 and 8, and a temperature of 35°C. Because temperatures above 35°C were deleterious to enzyme activity, 30°C was kept as the temperature to perform the assays testing AS activity. Also maintaining this temperature and a pH of 7.5, which was within the range of pH for maximum activity, the conditions of the assay coincided with the conditions used to measure AS activity in other cell suspension cultures [Poulsen et al 1991] and gave the opportunity to make comparisons with other plant cultures.

The pH optimal for AS activity (glutamine-dependent) in *Catharanthus roseus* was between 7.5 and 8.3 [Poulsen et al 1993], and the assay for the enzyme was the same as that used for *Ailanthus altissima* anthranilate synthase. The optimum temperature was not reported, but due to the assay being performed at 30°C, the optimum temperature must be near the value of the assay temperature. For *Ruta graveolens* anthranilate synthase, an optimum reaction temperature between 37 and 40°C was reported although the value for the optimum pH was not given [Hertel et al 1991].

In microorganisms, the pH optimal for the glutamine dependent AS reaction has been reported to be 7.6 in *Serratia marcescens* [Zalkin 1985] and 7.8 in *Pseudomonas putida* [Queener et al 1973]. The optimum temperatures of 37°C for *E. coli* [Ito and Yanofsky 1969] and *Pseudomonas putida* [Queener et al 1973], and 22°C for *Salmonella typhimurium* [Bauerle et al 1987] have been reported.

Kinetic properties

The K_m values for chorismate, L-glutamine and Mg^{2+} for *A. altissima* anthranilate synthase are 72 μM, 0,050 mM and 0.18 mM, respectively. The purified AS from *C. roseus* showed a K_m value of 67μM for chorismate which is very similar to the one obtained for *A. altissima*. The K_m value for L-glutamine, 0.37mM, and for Mg^{2+}, 0.26mM, were higher than the values obtained for *A. altissima* anthranilate synthase.

The K_m values for the substrate chorismate and L-glutamine, and for the cofactor Mg^{2+}, were determined for the partially purified AS enzymes of two cell lines of *Ruta graveolens*. The obtained values were 0.22 and 0.28mM for chorismate, 0.25 and 0.18mM for glutamine, and 0.6 and 0.55mM for Mg^{2+} [Hertel et al 1991]. In crude extracts of *Daucus carota*, the same parameters were obtained for normal and resistant cells to growth inhibition by DL-5-methyltryptophan: 46 and 26μM for chorismate, 0.249mM and 0.237mM for L-glutamine and 0.101 and 0.026mM for Mg^{2+} [Widholm 1972b].

In the case of microorganisms, most of the K_m values for chorismate in purified enzymes are between 2.3 and 18μM except for *Clostridium butyricum* which crude extract has a K_m of 76μM [Baskerville and Twarog 1972]. For L-glutamine the values vary from 0.5mM in *Serratia marcescens* [Zalkin and Hwang 1971] to 10mM in *Claviceps sp.* [Lara and Mills 1973]. The K_m values for Mg^{2+} are less frequent reported in the literature and one of the few examples is the K_m for *Sacharomyces cereviciae* which is 0.57mM.

Molecular weight

The molecular weight of the enzyme from *A. altissima* using gel filtration was 77±3kDa. From the plants where AS has been studied, the values for the Mr vary from 95.3kDa in *Pisum sativum* [Hankins et al 1976] to 220kDa in *Ruta graveolens* [Hertel et al 1991] (Table 3.3, Chapter 3). In *Arabidopsis thaliana*

the isolated genes encode proteins with molecular mass of 66kDa for α-subunit and 30kDa for β-subunit [Niyogi and Fink 1992, Niyogi et al 1993].

The Mr values for microorganisms vary from 65kDa for *Staphylococcus aureus* [Procter and Kloos 1973] to 400kDa for *Claviceps sp.* SD 58 [Lara and Mills 1973]. Therefore, there are microbial AS enzymes with similar molecular weight to AS-b. For example species from the genera *Bacillus, B. subtilis* and *B. pumilis* with Mr of 84 and 86kDa; *Chromabacterium violaceum* with 86kDa, *P. aeruginosa* with 79kDa, *Acinobacter calcoaceticus* with 86kDa, *Euglena gracilis* with 80kDa, and *Clostridium butyricum* with 84kDa. In the cases where the structures of these enzymes were determined, all of them were found to be dimers with the exception of *Euglena gracilis* where the two subunits, α and β, are fused, and *Clostridium butyricum*, where the enzyme is a tetramer.

SDS-PAGE electrophoresis of fractions from the Mono-Q column in *A. altissima*, showed a main single protein band corresponding to a molecular weight of 60±1kDa, but also there was at least another band at 37±1kDa, which was not very clear. There were several attempts to detect these bands in the active fractions from the Superose column, but due to low quantity, it was not possible to detect them. There were also problems to detect them from the Mono-Q column.

Based in the results obtained with gel filtration and SDS-PAGE electrophoresis, it is possible that the enzyme was not quite purified by the Mono-Q column, because if the values obtained from the two bands from gel electrophoresis are added, the obtained Mr value, 97kDa, is much larger than the 77±3kDa obtained from gel filtration. In spite of this, there have been occasions where the values for Mr from gel filtration and gel electrophoresis do not match very well. One case is AS from *C. roseus* for which the molecular weight by gel filtration was 143±5kDa, but by electrophoresis was 184kDa. In this case the explanation given was that the native AS could be a very compact molecule giving a smaller Stokes radius than that which would be expected from the sum

of the subunits [Poulsen et al 1993]. Because of these possibilities it is necessary to run the gel electrophoresis from the active fractions from Superose column to be sure about the structure of the enzyme. In the case when one band was observed in the gel electrophoresis, then it would indicate that the enzyme is a single polymer where the two subunits are fused, but if two bands were presented, the enzyme could be a dimer. There has been only one case where AS enzyme has the two subunits fused and it is *Euglena gracilis* [Mann and Floss 1977].

Inhibitor studies

The tryptophan biosynthetic pathway was elucidated with microorganisms and all the information indicates that tryptophan is produced from chorismate in plants by the same sequence.

The control of tryptophan biosynthesis can occur in two ways, by feedback inhibition of AS by tryptophan and by repression of enzyme synthesis involve in the biosynthesis of tryptophan [Ito and Crawford 1965].

In all plants and microorganisms, with the exception of *Pseudomonas aeruginosa*, the enzyme was found to be feedback inhibited by tryptophan. Besides tryptophan, the enzyme can be inhibited by other tryptophan analogs such as 5-methyltryptophan [Widholm 1972a] and 5-hydroxytryptamine for example [Grosse 1977].

The two anthranilate synthase enzymes detected in *A. altissima* were inhibited by tryptophan, but at different levels: with 20mM, 66% inhibition was obtained for AS-a and 97% inhibition for AS-b. Anthranilate synthase-b was also inhibited by tryptamine and D-tryptophan and was not affected by L-phenylalanine, L-tyrosine and indole acetic acid. Other enzymes from plants have shown the inhibition effects with these compounds. E.g. AS from *C. roseus* [Poulsen et al 1993].

PRESENCE OF TWO CM AND TWO AS ISOENZYMES

As described in **Results**, two peaks of chorismate mutase activity were detected when enzyme extract was eluted from a Q-Sepharose column. Unfortunately it was not possible to determine whether they showed a different inhibition by L-phenylalanine and L-tyrosine.

Different chorismate mutase isoenzymes can be found in microorganisms, and some of them are bifunctional, but in plants, the CM isoenzymes are monofunctional (Chapter 4). In plants it is possible to find from one to three CM isoenzymes, and all of them show different regulation by L-tyrosine and L-phenylalanine. For example, when two isoenzymes have been detected in the same plant, one of them is regulated by tyrosine and phenylalanine, and the other one is not regulated by the aminoacids [Singh et al 1986].

The two AS enzyme detected in this study showed different level in regulation by tryptophan. The presence of two isoenzymes have been detected in other plants. Two AS isoforms, one feedback-sensitive and other feedback-resistant to tryptophan were separated in *Solanum tuberosum* cell suspension cultures which were resistant to growth inhibition by D,L-5-methyltryptophan [Carlson and Widholm 1978]. Also in *Nicotiana tabacum*, two AS isoenzymes were detected: the tryptophan-sensitive was found in the organelle fraction and the tryptophan-resistant in the cytosol [Brotherton and Widholm 1985, Brotherton et al 1986].

In microorganisms, two AS isoenzymes have been found in *Pseudomonas aeruginosa*: one participates in tryptophan biosynthesis and is strongly inhibited by tryptophan, and the other, which is not inhibited by tryptophan, participates in the biosynthesis of pyocyanin [Essar et al 1990c].

There is no doubt about the presence of a complete pathway in plastids. Isolated chloroplasts synthesized the three aromatic aminoacid from $NaH^{13}CO_3$,

^{14}C-3-phosphoglycerate and ^{14}C-phosphoenolpyruvate [Bagge and Larsson 1986, Schultze-Siebert and Schultz 1989, Homeyer and Schultz 1988], but in early results it was found that the isolated chloroplasts could not account for the total production of aromatic amino acids, therefore suggesting that the balance of production comes from the cytoplasm [Buchholz et al 1979].

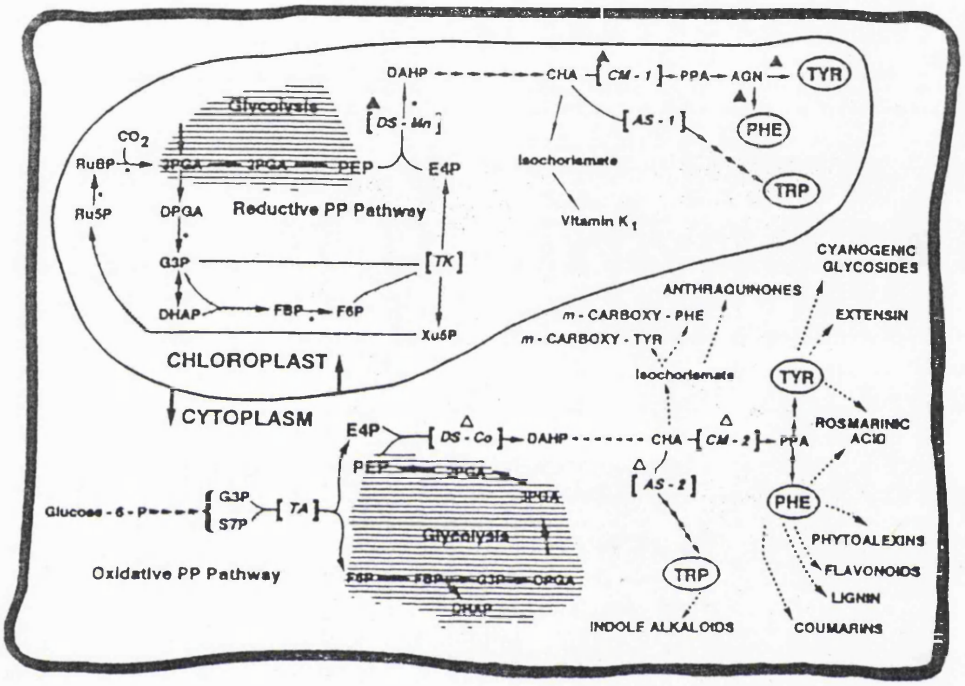
Further evidence for the cytosolic compartmentation comes with the finding of two isoforms of DAHP synthase and CM in several plants species. For example the regulated form of DAHP synthase was found in isolated chloroplasts from tobacco leaves [Ganson et al 1986] and the unregulated by aromatic amino acids was not present in the chloroplasts, being associated with cytosolic markers. Furthermore, in tobacco and sorghum, the regulated CM enzyme was located in chloroplasts, while the unregulated was cytosolic [d'Amato et al 1984, Singh and Conn 1986]. Besides this, evidence of extraplastidic isoforms of other shikimate pathway enzyme, like EPSP synthase and DHQ synthase for example, support the hypothesis that there are two complete aromatic amino acid biosynthetic pathways one in the plastids and one in the cytoplasm [Mousdale and Coggins 1985, Jensen 1986a, 1986b]. Furthermore this hypothesis, it has been proposed that each pathway, the plastidic and the cytosolic, has a specialized role [Goers and Jensen 1984, Ganson and Jensen 1987].

The rationale for the hypothesis that the chloroplastic pathway is for amino acid synthesis, and the cytosolic is for support of the chloroplastic pathway and for secondary products, is that the chloroplastic isoenzymes of DAHP and CM are regulated by the aromatic aminoacids, while their cytosolic forms are either unregulated or are inhibited by caffeic acid [Goers and Jensen 1984, Rubin and Jensen 1985]. Further support to the hypothesis is that the chloroplastic CM predominated in rapidly proliferating cultured tobacco cells, while the cytosolic isoenzyme predominated in older leaves [Goers and Jensen 1984]. Also during the tobacco cell cycle, the activity of the plastidic DAHP synthase was greater during early exponential growth, while the cytosolic peak was greater in late

exponential phase [Ganson and Jensen 1987]. Fig. 7.1 illustrates the hypothesis of the two specialised aromatic aminoacid biosynthesis.

The presence of two pairs of isoenzymes for anthranilate synthase and chorismate mutase in *Ailanthus altissima*, may support the hypothesis of the two aromatic aminoacid pathways, once the location of the enzymes is determined. The fact that the two AS showed different regulation by tryptophan give evidence of this possibility. Nevertheless, in some instances, no differences were found, between the components of isoenzymic systems and the existence of the two aminoacid pathway must be based in location of the enzymes.

Fig 7.1 Biochemical juxtaposition of carbohydrate metabolism, aromatic biosynthesis, and secondary metabolims in the chloroplast and cytosol microenvironments of higher-plant cells (Hrazdina and Jensen 1992)



Light-activated enzymes are denoted with an asterisk. The three pairs of isozymes participating in aromatic biosynthesis and known to be compartmented as diagrammed are shown within heavy brackets. Solid and open triagles denote enzymes sensitive and resistant to feddback inhibition, repectively. **Metabolite abbreviations:** PP: pentose phosphate; RuBP: ribulose 1,5-bisphosphate; Ru5P: ribulose 5-phosphate; Xu5P: xyulose 5-phosphate; 3PGA: 3-phosphoglycerate; 2PGA: 2-phosphoglycerate; DPGA: 1,3-diphosphoglycerate; G3P: glyceraldehyde 3-phosphate; DHAP: dihydroxyacetone phosphate; FBP: fructose 1,6-bisphosphate; F6P: fructose 6-phosphate; E4P: erythrose 4-phosphate; S7P: sedoheptulose 7-phosphate; DAHP: 3-deoxy-D-arabino-heptulosonate 7-phosphate; CHA: chorismate; PPA: prephenate; AGN: L-arogenate. **Enzyme acronyms:** [TK]: transketolase; [TA]: transaldolase; [DS-Mn] and [DS-Co]: isozymes of DAHP synthase; [CM-1] and [CM-2]: isozymes of chorismate; [AS-1] and [AS-2]: isozymes of anthranilate synthase.

CONCLUSIONS

1. During the growth cycle of *Ailanthus altissima* cell suspension cultures, isochorismate synthase activity was not detected. The absence of this enzyme activity may be correlated with the absence of anthraquinones in either the cell cultures or the parent plant, and it is not known if phylloquinone is a vitamin in cell suspension cultures. It is also possible that the activity of ISC is below the detection limit of the assay.

2. Anthranilate synthase and chorismate mutase activities were detected throughout the growth cycle, but the peaks of maximum activity occured at different stages. AS had a maximum activity between days 8 and 17, and CM activity increased after day 20. The activity of CM was 150x the activity of AS. This result agrees with reports of other plants in the literature, where CM was always more active than AS.

3. Two AS and two CM isoenzymes were detected. The chorismate mutases could be separated on a Q-Sepharose column, but the AS isoenzymes were separable only after a Mono-Q column. The two anthranilate synthases showed different level in regulation by tryptophan. The presence of two isoenzymes from AS and CM may support the hypothesis of there are two complete aromatic amino acid biosynthetic pathways one in the plastids and one in the cytoplasm. Once the location of the isoenzymes is found, this supposition could be confirmed.

4. The two AS were only detected four times, and except for one preparation, AS-b was the isoenzyme always present. This may be due to instability of the other isoenzyme.

5. One isoenzyme from anthranilate synthase was isolated and characterised. The enzyme exhibited optimum activity at a temperature of 35°C, a pH of 7.3-8 and an incubation time of 60 min. The molecular weight of the enzyme

determined by gel filtration was 77±3kDa.

6. The SDS-PAGE electrophoresis from the Mono-Q column active fractions showed two bands, one at 60±1kDa and one at 37±1kDa. The band at 37±1kDa could be an impurity, and since the total of the two, 97 kDa, is higher than the value obtained by gel filtration (77 kDa), no conclusion can be drawn concerning the structure of the enzyme.

FUTURE WORK

The results of this investigation have raised a number of questions that could be answered with further investigations. It will be necesary to determine the molecular structure of anthranilate synthase-b to complete the characterisation of the enzyme. The location of the enzymes, anthranilate synthase and chorismate mutase should be determined to solve the possibility of two aromatic aminoacids pathways.

Genes encoding for anthranilate synthase enzyme have been isolated in *Arabidopsis thaliana*, this information can be used to obtain the genes from *A. altissima* and compare them.

In the case of chorismate mutase, the gene enconding this enzyme has also been isolated also in *Arabidopsis*, hence it would be interesting to compare it with that from *A. altissima* and see if, in plants, it is the same as in microorganisms, where the chorismate mutase enzymes are very different and no similarities are found between them.

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