Studies on the AROM pentafunctional enzyme in *Saccharomyces cerevisiae* and *Aspergillus nidulans*

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For my Parents

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Abbreviations

The abbreviations used in this thesis are set out in the Biochemical Journal 'Instruction to Authors', except the following.

A	absorbance
AMM	*Aspergillus* minimal medium
ATP	adenosine triphosphate
AUFS	absorbance units full scale
BSA	bovine serum albumin
CAPS	3-[cyclohexylamino]-1-propanesulfonic acid
kDa	kilodaltons
DAHP	3-deoxy-D-arabino-heptulosonate 7-phosphate
DAHP synthase	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase
DHQase	dehydroquinase
DHQ synthase	3-dehydroquinate synthase
DTT	dithiothreitol
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra acetate
EPSP	5-enolpyruvyl-shikimate 3-phosphate
EPSP synthase	5-enolpyruvyl-shikimate 3-phosphate synthase
FPLC	fast protein liquid chromatography
HPLC	high performance liquid chromatography
HRP	horse raddish peroxidase
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
PAGE	polyacrylamide gel electrophoresis

PEP	phosphoenol pyruvate
PMSF	phenylmethanesulphonylfluoride
PVDF	polyvinylidene difluoride
shikimate DH	shikimate dehydrogenase
SDS	sodium dodecyl sulphate
TEMED	N,N,N'N'-tetramethylethylene diamine
TLCK	N-*p*-toluenesulphonyl-L-lysine chloromethylketone
TPCK	N-*p*-toluenesulphonyl-L-phenylalanine chloromethylketone
Tris	Tris (hydroxymethyl) aminomethane
U	units of enzyme activity
UV	ultra violet

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Summary

Aromatic amino acids are synthesised via the shikimate pathway. AROM is a pentafunctional enzyme which catalyses the five central steps of the shikimate pathway and is found in fungi and yeast. This thesis describes the purification and characterisation of AROM from overexpressing strains of *Saccharomyces cerevisiae* and *Aspergillus nidulans*.

S. cerevisiae AROM was purified 30-fold with a 10% yield from a yeast overexpression strain which exploited the ubiquitin-fusion system of yeast. The purified protein was found to possess all five enzyme activities in a similar ratio to that observed in crude extract and had a subunit molecular weight of 175kDa. The main *S. cerevisiae* protein was shown to have several minor, lower molecular weight contaminants following SDS PAGE and three of these were found to cross-react with anti-AROM antibodies raised against *Neurospora crassa* AROM. The peak AROM fraction eluted from gel filtration chromatography was found to be composed of two proteins which were separable by native PAGE and both of which were shown to have shikimate DH activity. The poor recovery and multiple protein bands suggested that during the AROM preparation limited proteolysis was occuring despite a number of anti-proteinase measures. No means of eliminating limited proteolysis during *S. cerevisiae* AROM isolation were found and purification studies were carried out on the AROM of *A. nidulans* in the hope that proteolysis might not be as problematic in this species.

A rapid procedure for the purification of *A. nidulans* AROM from the overexpresion strain *A. nidulans* 1314 has been developed which results in 13-fold purification and a 9% yield. The subunit molecular weight was estimated at 175kDa and the native molecular weight suggests that the protein is a dimer. The N-terminal DHQ synthase activity in the AROM protein was found to be severly deficient in both crude extract and the purified protein from *A. nidulans* 1314. This was independently attributed to the introduction of a missense mutation in this region of the polypeptide.

A preliminary limited proteolysis study was carried out on AROM purified from *A. nidulans* 1314 which suggested that the proteolysis pattern is complicated and which show that the DHQase and shikimate DH enzyme activities are least susceptible to limited proteolysis.

Chapter 1 Introduction

Chapter 1 Introduction

1.1 The shikimate pathway

1.1.1 General introduction

In plants, fungi, protozoa and bacteria, chorismate is the common metabolic precursor for the aromatic amino acids, phenylalanine, tryptophan and tyrosine, as well as for other important metabolites such as vitamin E and K, folic acid, ubiquinone, plastiquinone and enterochelin. Chorismate is the product of a seven step pathway known as the shikimate pathway (Haslam, 1974; Weiss & Edwards, 1980; Bentley, 1990; Haslam, 1993) (Figure 1.1). The oxidation of glucose via the glycolytic and pentose phosphate pathways yields phosphoenolpyruvate and erythrose 4-phosphate respectively which are the starting metabolites of the shikimate pathway.

Other organisms are unable to synthesise chorismate and rely on their dietary intake to provide aromatic amino acids and vitamins. Therefore, this pathway is a target for herbicides and antimicrobial agents since inhibition of enzymes in this pathway does not affect mammals (Coggins, 1986a). The commercially important herbicide glyphosate has been shown to act by inhibiting the sixth enzyme in the shikimate pathway, EPSP synthase (Steinrucken *et al.*, 1980; Boocock & Coggins, 1983). Recently there has also been interest in the shikimate pathway as a target for antimicrobials against the pathogenic fungus *Pneumocystis carinii.* This fungus is responsible for a pneuomonia known to be the main cause of death in AIDS patients in Europe and the USA (Banerji *et al.*, 1993), and which also affects malnourished children (Pixley *et al.*, 1991).

In this thesis I have used abbreviations for some of the shikimate pathway enzymes: DAHP synthase, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; DHQ synthase, 3-dehydroquinate synthase; DHQase, 3-dehydroquinase; shikimate DH, shikimate dehydrogenase; EPSP synthase, 5-enolpyruvyl-shikimate 3-phosphate synthase. Shikimate kinase is written in its full form.

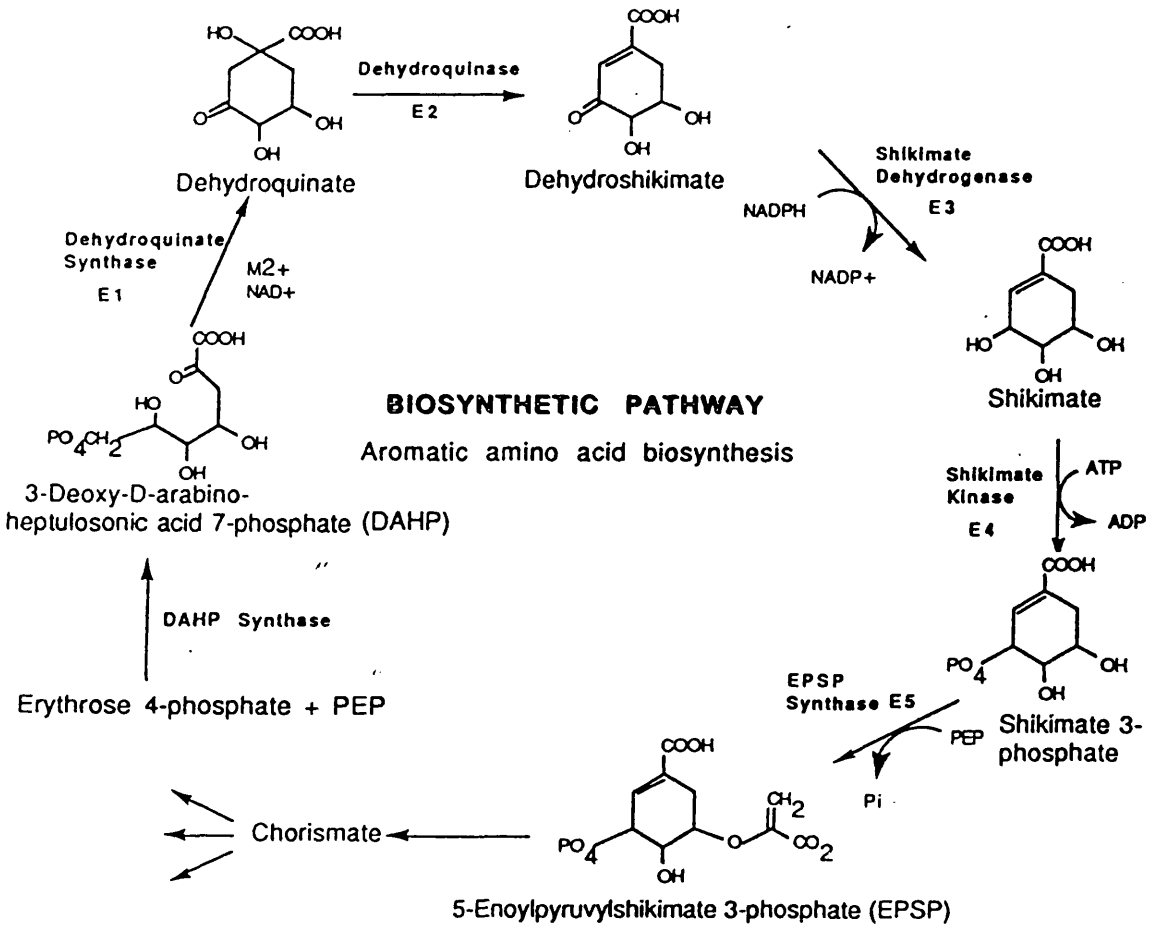


Figure 1.1 **The shikimate pathway**

1.1.2 Organisation of the shikimate pathway enzymes

Although chorismate is synthesised by the same, seven enzyme reactions in all organisms capable of making chorismate, the organisation of the enzymes involved varies between species (Figure 1.2). In *Eschericia coli* the seven enzymes of the shikimate pathway are all monofunctional enzymes (Berlyn & Giles, 1969) with genes scattered throughout the genome (Pittard & Wallace, 1966). In higher plants the enzymes DHQase and shikimate DH, which carry out two consecutive reactions in the pathway, are carried on a bifunctional polypeptide while the other five enzymes of the pathway are monofunctional (Polley, 1978; Koshiba, 1979; Mousdale *et al.*, 1987). In the fungi *Neurospora crassa* (Lumsden & Coggins, 1977, 1978; Gaertner & Cole, 1977) and *Aspergillus nidulans* (Ahmed & Giles, 1969; Charles *et al.*, 1986), the yeast *Saccharomyces cerevisiae* (Duncan *et al.*, 1988), and in *Euglena gracilis* (Patel & Giles, 1979) the enzymes for the five central steps of the shikimate pathway (steps 2-6, Figure 1.1) are contained on a pentafunctional polypeptide known as AROM. In this thesis I have used the accepted term 'AROM' to refer to the protein, where in the past *arom* has been used to define the *N. crassa* and *S. cerevisiae* proteins. The genes encoding *S. cerevisiae* and *A. nidulans* AROM are called *ARO1* and *aromA* respectiviely. The entire coding sequences of the AROM genes have been cloned and sequenced from *S. cerevisiae* (Larimer *et al.*, 1983; Duncan *et al.*, 1987), *A. nidulans* (Kinghorn & Hawkins, 1982; Charles *et al.*, 1985; Charles *et al.*, 1986), and recently from *P. carinii* (Banereji *et al.*, 1993). A comparison of the sequences with those of the individual *E. coli* monofunctional enzymes (Duncan *et al.*, 1984a, 1986; Millar *et al.*, 1986; Millar & Coggins, 1986; Anton & Coggins, 1988) show regions within the AROM polypeptide corresponding to the monofunctional enzymes (Duncan *et al.*, 1987; Hawkins, 1987; Banerji *et al.*, 1993). In the case of the *S. cerevisiae* gene the identity with the individual *E. coli* enzyme amino acid sequences varies between 21% and 38% (Duncan *et al.*, 1987). The regions of homology are illustrated in the schematic representation shown in

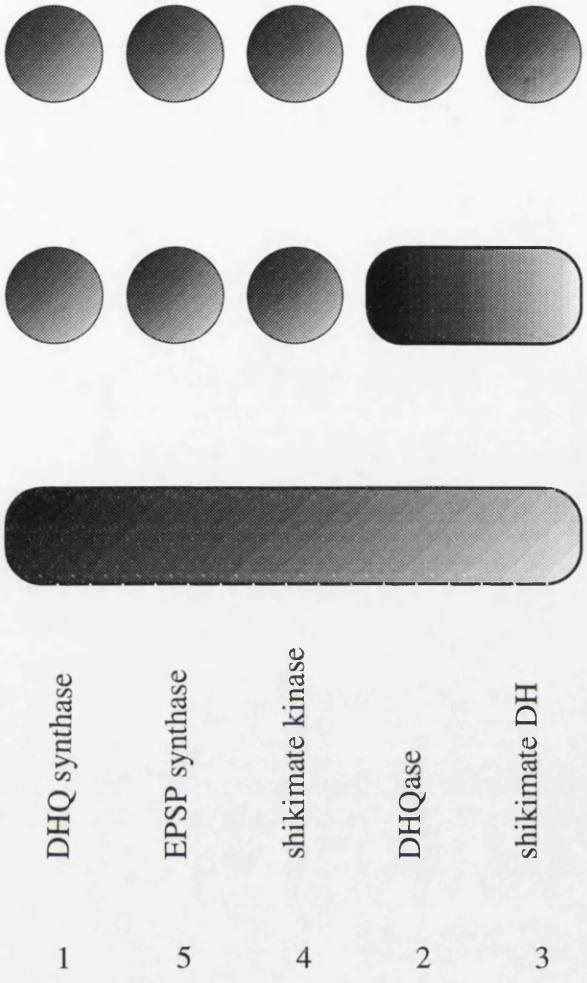


Figure 1.2 **Domain arrangement of *S. cerevisiae* and *A. nidulans* AROM with the corresponding shikimate pathway enzymes from *E. coli* and *P. sativum***

Figure 1.2. The deduced enzyme order along the polypeptide is the same for both *A. nidulans* and *S. cerevisiae* AROM, and agrees with the order deduced for *N. crassa* AROM from genetic and biochemical studies (Giles, 1978). The enzyme order along the polypeptide is not, however, the same as the reaction order. It has been proposed that the gene encoding AROM has evolved by fusion of ancestral monofunctional enzyme genes, such as those found in *E. coli* (Giles, 1978; Charles *et al.*, 1986; Duncan *et al.*, 1987; Hawkins,1987).

The differences in the arrangement of the shikimate pathway enzymes raise questions about the evolution of AROM and the bifunctional plant DHQase-shikimate DH enzymes. In relation to the AROM protein there are many questions to be answered as part of a larger research programme, such as:

1. What is the advantage, if any, of the fused arrangement of AROM over monofunctional *E. coli*-like enzymes?
2. Are the individual AROM enzymes able to fold to form functional protein when they are expressed independently and do they possess the same kinetic properties?
3. Is the order of the domains important?

Both *S. cerevisiae* and *A. nidulans* have well established genetic systems making them suitable for investigating AROM. This introduction will discuss each of the individual enzyme reactions catalysed by AROM, metabolic control of the pathway and its relationship with the quinate utilisation pathway found in some fungi. A brief account of multifunctional proteins is followed by a detailed description of the genetic, biochemical and molecular studies which have already been carried out on the AROM protein and the *arom* gene. Finally the aims of this project are described.

1.2 The individual enzyme reactions of AROM

1.2.1 DHQ synthase

DHQ synthase catalyses the second reaction of the shikimate pathway (Figure 1.1) which is the conversion of 3-deoxy-D-arabino-heptulosonic acid 7-

phosphate (DAHP) to 3-dehydroquinate (DHQ). DHQ is the first cyclic intermediate in the pathway and was identified by Weiss *et al.*(1953) in *E. coli*. The reaction was first demonstrated by Srinivasan *et al.* (1963) and both NAD^+ and a divalent metal cation are essential for DHQ synthase activity (Saijo & Kosuge, 1978; Yamamoto, 1980; Hasan & Nester, 1978; Lambert *et al*, 1985)

The monofunctional enzymes of *E. coli* (Frost *et al.*, 1984; Millar & Coggins, 1986) and *Pisum sativum* (Pompliano *et al.*, 1989) have been purified to homogeneity. In *Bacillus subtilis* the DHQ synthase is associated with chorismate synthase and flavin reductase in a multienzyme complex (Hasan & Nester, 1978). The DHQ synthase activity of the pentafunctional AROM of *N. crassa* was shown to be Zn^{2+} dependent (Lambert *et al.*, 1985).

1.2.2 DHQase

DHQase catalyses the conversion of 3-dehydroquinic acid to 3-dehydroshikimic acid (DHS) (step 3, Figure 1.1). The reaction is involved in two separate metabolic pathways: biosynthesis in the shikimate pathway, and catabolism in the quinate pathway. The quinate pathway is discussed in Section 1.4 and is found in some fungi. There are two classes of DHQase distinguished on the basis of their physical and catalytic properties (Kleanthous *et al.*, 1992). Type I DHQases' are heat labile and the enzymic reaction proceeds through the formation of a Schiff base with the substrate, DHQ (Walsh, 1979; Butler *et al.*, 1974). Type II DHQases are heat stable and do not act through a Schiff base mechanism (Kleanthous *et al.*, 1992). The type II enzymes form large aggregates from the monomeric subunits while the type I enzymes are dimers (Kleanthous *et al.*, 1992). The type I and type II DHQases are thought to have evolved independently.

The type I DHQases examined to date are exclusively involved in the biosynthetic shikimate pathway. They have been purified and characterised from *E. coli* (Chaudhuri *et al.*, 1986) and the *N. crassa* AROM protein, and the Schiff base intermediate of the reaction has been shown to be formed at a conserved lysine

residue (Lumsden & Coggins, 1977; Smith & Coggins, 1983; Lambert *et al.*, 1985; Chaudhuri *et al*, 1991). Other type I enzymes studied from *S. cerevisiae* (Duncan *et al.*, 1987) and *A. nidulans* (Charles *et al.*, 1985; Hawkins *et al.*, 1993a) as part of AROM, and from *Salmonella typhi* (Servos *et al.*, 1991) have similar characteristics to the *E. coli* and *N. crassa* enzymes. They are heat labile and all contain the conserved active site lysine residue thought to be involved in Schiff base formation (Chaudhuri *et al.*, 1991). The bifunctional DHQase-shikimate DH of *Pisum sativum* has also been shown to be a type I DHQase (Deka *et al*, 1994).

Type II DHQases which are quinate inducible and involved in quinate catabolism (see 1.4) have been isolated from *A. nidulans* (Hawkins *et al.*, 1982a; Da Silva et al., 1986) and *N. crassa* (Hawkins *et al.*, 1982b) . Type II enzymes have also been isolated from *Streptomyces coelicolor* (White *et al.*, 1990) and *Mycobacterium tuberculosis* (Garbe *et al.*, 1991) which are involved in the shikimate pathway. More recently, a dual function, quinate inducible type II DHQase has been identified from *Amycolatropsis methanolica*, which is involved in both the catabolism of quinate and the biosynthesis of aromatic amino acids (Euverink *et al.*, 1992).

The reactions catalysed by type I and type II DHQases have been shown not only to be mechanistically distinct but also to have different stereochemistries (Harris *et al.*, 1993). The type I *E. coli* DHQase has been shown to catalyse a *syn* elimination, while the type II DHQase from *A. nidulans* catalyses an *anti* reaction.

1.2.3 Shikimate DH

Shikimate DH catalyses the fourth step of the shikimate pathway (Figure 1.1) which is the reduction of 3-dehydroquinic acid to shikimic acid utilising hydride transfer from NADPH (Yaniv & Gilvarg, 1955; Dansette & Azerad, 1974).

Monofunctional shikimate DH has been purified and characterised from *E. coli* (Chaudhuri & Coggins, 1985) and the shikimate DH of *N. crassa* AROM has also been characterised (Lambert *et al.*, 1985; Coggins *et al.*, 1987a). As mentioned

in Section 1.1.2 the shikimate DH activity in a variety of plants is part of a bifunctional polypeptide (see Mousdale & Coggins, 1992 for a recent review).

1.2.4 Shikimate kinase

Shikimate kinase catalyses the fifth step in the shikimate pathway (Figure 1.1) which is the phosphorylation of shikimate to shikimate 3-phosphate with ATP as co-substrate (Weiss & Mingioli, 1956).

E. coli and *Salmonella typhimurium* possess two shikimate kinase isozymes (Berlyn & Giles, 1969; Ely & Pittard, 1979): type I and type II. Sequence studies have shown the shikimate kinase II in *E. coli* K12 (Millar *et al.*, 1986; DeFeyter & Pittard, 1986) to have 34% homology with shikimate kinase type I in *E. coli* (Løbner-Olesen & Marinus, 1992). The shikimate kinases of AROM from *A. nidulans* (Charles *et al.*, 1986), *S. cerevisiae* (Duncan *et al.*, 1987) and *P. carinii* (Banerji *et al.*, 1993) and the shikimate kinase from tomato (Schmid *et al.*, 1992) show homology with the bacterial enzymes, and all shikimate kinases known contain an ATP binding region homologous with some other kinases and ATP-requiring enzymes (Walker *et al.*, 1982).

1.2.5 EPSP synthase

EPSP synthase (5-enolpyruvyl-shikimate 3-phosphate synthase) catalyses the transfer of phosphoenolpyruvate to shikimate 3-phosphate to give 5-enolpyruvyl-shikimate 3-phosphate (EPSP) and inorganic phosphate (Levin & Sprinson, 1964) (Step 6 in Figure 1.1).

EPSP synthase has been studied more exhaustively than any other activity in the pathway because it is the major target for inhibition by the broad spectrum herbicide, glyphosate (Steinrucken & Amrhein, 1980; Boocock & Coggins, 1983). It has been shown for the EPSP synthase component of *N. crassa* AROM that the inhibition of EPSP synthase by glyphosate is competitive with PEP (Boocock & Coggins, 1983). EPSP synthase from *E. coli* has been crystallised by the Monasanto

group and the backbone structure determined at 3-Å resolution (Stallings *et al.*, 1991). Glyphosate tolerance is also of great commercial interest and cell cultures of a number of plant species, selected for herbicide tolerance, have been shown to have an accompanying increase in EPSP synthase levels (Nazfiger *et al.*, 1984; Smart *et al.*, 1985; Steinrucken *et al.*, 1986; Dyer *et al.*, 1988).

EPSP synthase from *E. coli* has been purified, sequenced and overexpressed (Lewendon & Coggins, 1983; Duncan *et al.*, 1984a; 1984b) and is a monomer. It has also been purified from a range of bacteria and plants, including *K. pneumoniae* (Steinrucken & Amrhein, 1984) and pea seedlings (Mousdale & Coggins, 1984). Sequence data have been obtained for EPSP synthase from *Bordetella pertussis* (Maskell *et al.*, 1988), petunia and tomato (Gasser *et al.*, 1988), pea (Granger, 1989), *Arabidopsis thaliana* (Klee *et al.*, 1987), tobacco (Wang *et al.*, 1991), and as part of AROM in *S. cerevisiae* (Duncan *et al.*, 1987) and *A. nidulans* (Charles *et al.*, 1986). There is considerable homology between the plant, fungal and bacterial sequences.

1.3 Metabolic control of the shikimate pathway

There is control of the flux of metabolites through the shikimate pathway by feedback inhibition at the first step in the pathway. Both *E. coli* (Doy & Brown, 1965) and *N. crassa* (Hoffmann *et al.*, 1972) have three DAHP synthase enzymes, each one regulated by one of the three aromatic amino acids. *S. cerevisiae* has only two DAHP synthase isozymes - one inhibited by tyrosine, and the other by phenylalanine (Teshiba *et al.*, 1986; Paravicini *et al.*, 1988; Paravicini *et al.*, 1989; Künzler *et al.*, 1992).

The *ARO1* gene encoding AROM in *S. cerevisiae* has been shown to have the 5' flanking sequence which places amino acid biosynthetic enzymes under the general amino acid control system found in yeast (Duncan *et al.*, 1988). The general control system regulates the levels of many different amino acid biosynthetic pathways at the transcription level and the starvation of one amino acid involved in

general control leads to the derepression of all the enzymes involved in the response (Jones & Fink, 1982; Fink, 1986; Braus, 1991). The level of derepression is not the same for all the enzymes. The *GCN4* gene product is a trans-acting regulatory factor which binds to the 5' flanking region of regulated genes under conditions of amino acid starvation. Duncan *et al.* (1988) showed that the levels of AROM transcription increased on derepression, and the DHQase and shikimate DH specific activities increased 2-3-fold. Interestingly, the *P. carinii arom* locus has a putative GCN4 binding site which may place it under general amino acid control (Banerji *et al.*, 1993).

1.4 The quinate utilisation pathway

The metabolite quinate makes up between 2% and 10% of the dry weight of the leaves of higher plants (Bentley, 1990) and is, therefore, a good energy source for many soil microorganisms which catabolise it via the quinate pathway (Giles *et al.*, 1985).

The quinate pathway is illustrated in Figure 1.3 and is described here because it shares two intermediates with the shikimate pathway: dehydroquinate and dehydroshikimate. Dehydroshikimate is converted to protocatechuate by dehydroshikimate dehydrase. Species such as the fungi *A. nidulans* and *N. crassa* which grow as saprophytes on decaying plant material, have been shown to have two DHQase enzymes as described already in Section 1.2.2: one involved in the quinate utilisation pathway and the other in the shikimate pathway. The type II DHQase is involved in the catalysis of dehydroquinate to dehydroshikimate in the quinate pathway and is encoded by the *qa-2* gene in *N. crassa* (Vapnek *et al.*, 1977; Giles *et al.*, 1985) and by the *QUT E* gene in *A. nidulans* (Hawkins *et al.*, 1985; Da Silva *et al.*, 1986). The same reaction is catalysed via a different mechanism (see 1.1.2) by the DHQase region of AROM in *A. nidulans* and *N. crassa* during biosynthesis of chorismate through the shikimate pathway (Lumsden & Coggins, 1977; Charles *et al.*, 1985). It has been suggested that the intermediates common to both pathways are

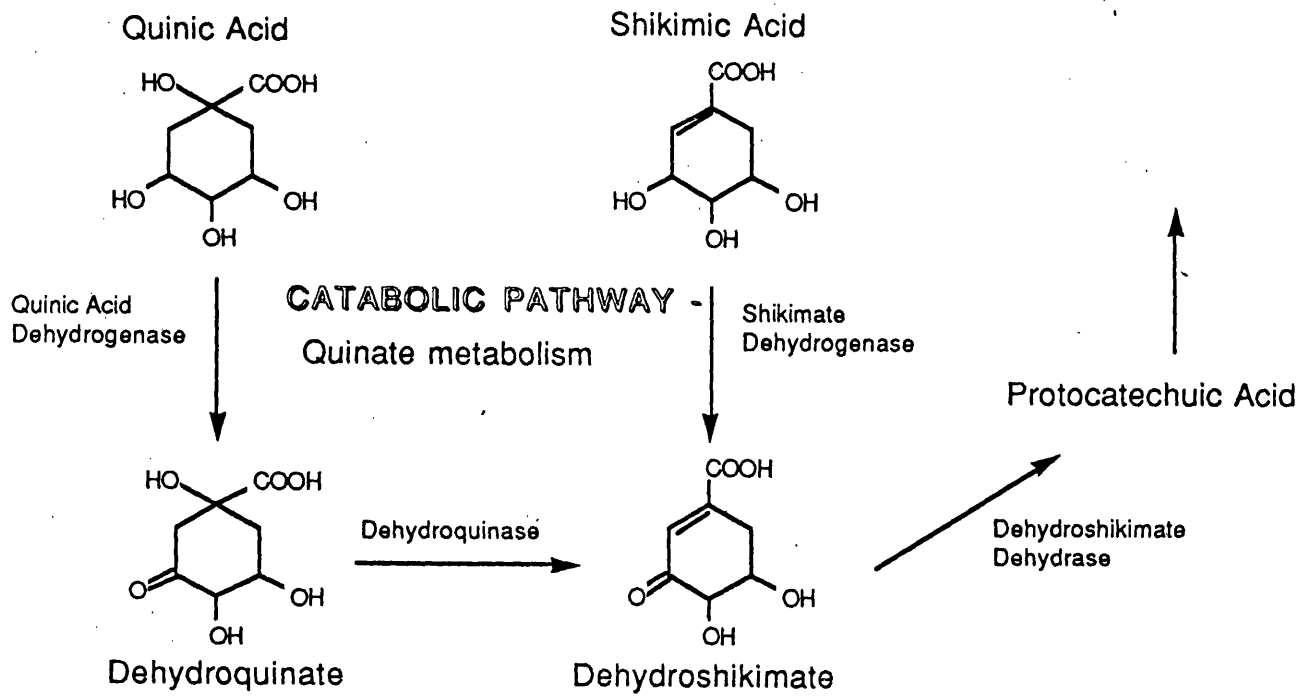


Figure 1.3 **The quinate pathway**

segregated once the catabolic pathway has been induced, by means of channelling of the intermediates of aromatic amino acid biosynthesis by the AROM protein (Giles, 1978). It has been proposed that this has been the evolutionary selection pressure for the formation of a multifunctional AROM protein. Substrate channelling in *N. crassa* AROM was reported by Welch & Gaertner (1975), however, their AROM preparation was deficient in both DHQ synthase and EPSP synthase activity, and there is no direct evidence for channelling from measurements of the steady state levels of the intermediates from an intact AROM preparation (Coggins & Boocock, 1986). It has been shown that the pathway intermediates, DHQ and DHS, are able to leak from the AROM protein and can be used by both pathways (Lamb *et al.*, 1991), although Lamb *et al.* (1991) have suggested that AROM might have a low level channelling function under conditions of nutrient limitation.

The enzymes of the quinate pathway are quinate-inducible and subject to carbon catabolite repression (Giles *et al.*, 1985; Hawkins *et al.*, 1993d). The genes are part of a tightly-linked cluster of seven genes in the case of *N. crassa* (Giles *et al.*, 1985) and eight genes in the case of *A. nidulans* (Hawkins *et al.*, 1993d), which comprise the quinic acid utilisation gene cluster in these species (Giles *et al.*, 1985; Grant *et al.*, 1988). Two of the genes in each system encode repressor and activator proteins which control transcription of the quinate cluster genes. It has been shown that the repressor protein of both organisms are homologous with the three most C-terminal domains of the AROM polypeptide (Anton *et al.*,1987; Hawkins *et al.*, 1992). More recently, the activator protein of both species has been shown to be homologous with the two most N-terminal domains of the AROM polypeptide (Hawkins *et al.*, 1993c).which raise interesting questions about their evolution.

1.5 Multifunctional proteins

Multifunctional proteins are defined as those proteins which have more than one distinct biochemical function on a single polypeptide (Kirschner & Bisswanger, 1976). They are found in both prokaryotes and eukaryotes, and are

particularly prevalent in amino acid biosynthetic pathways. Multifunctional enzymes often catalyse consecutive reactions in a biosynthetic pathway although this is not universal, and multifunctional enzymes occuring in one species may be present as monofunctional enzymes in another species.

It is now recognised that proteins are constructed from structural domains which fuse to make more complex structures (Doolittle, 1989; Coggins, 1991). A domain is defined as a spatially separate, compact, globular structure made up from a continuous stretch of polypeptide which is stable as an independent entity (Rossmann & Argos, 1981), and domains of a protein are readily identified by x-ray crystallography. Kirschner & Bisswanger (1976) proposed a model which suggests that the individual functions of a multifunctional protein are located in discrete domains within the polypeptide chain. This is known as the mosaic model and it is now generally thought that multifunctional proteins evolved from ancestral monofunctional enzymes by gene fusion. This model is supported by much of the protein sequence data for multifunctional proteins which are often found to be composed of regions which are homologous to monofunctional proteins which carry out the same reaction in another species. Proteolysis studies on multifunctional proteins also show that functions of the multifunctional polypeptide can be assigned to discrete regions of the polypeptide. An account of the use of these techniques in studying a multifunctional protein is provided in Sections 1.5 and 1.6 which describe the work carried out on the AROM multifunctional protein.

The best studied multifunctional proteins are tryptophan synthase, fatty acid synthase and the CAD multifunctional protein. The bifunctional tryptophan synthase of *N. crassa* and *S. cerevisiae* has been compared to the individual monofunctional polypeptides of *E. coli* and *Salmonella typhimurium* which form a multienzyme complex (Burns & Yanofsky, 1989; Zalkin & Yanofsky, 1982). The three-dimensional structure of the tryptophan synthase multienzyme complex of *S. typhimurium* has been solved by x-ray crystallography and shows a tunnel which is thought to allow diffusion of indole, the product of one subunit, to the active site of

the other subunit for which indole is the substrate (Hyde *et al.*, 1988). In the case of tryptophan synthase the order of gene fusion of the two monofunctional genes encoding polypeptides such as those found in *E. coli* , has been shown to be important in constructing a bifunctional polypeptide from the *E. coli* monofunctional enzyme coding sequences (Burns *et al.*, 1990).

The multifunctional mammalian fatty acid synthase is also thought to have arisen by gene fusion (Hardie & McCarthy, 1986; Amy *et al.*, 1992) although the sequence homology of the multifunctional proteins with monofunctional counterparts is limited to areas around substrate binding sites (Schweizer *et al.*, 1989). The CAD multifunctional protein in animals catalyses the first three steps in pyrimidine biosynthesis (reviewed by Davidson *et al.*, 1993) All three of these well-studied multifunctional proteins represent examples where fusion of monofunctional components is thought to have led to formation of the multifunctional protein.

The reason for the formation of multifunctional proteins is the focus of much speculation. The main proposals are catalytic facilitation, substrate channelling, co-ordinate regulation of enzyme activity, coordinate expression, and protection of unstable intermediates. There is often no obvious reason why the multifunctional protein has arisen. In the case of the AROM protein a low level channeling of pathway intermediates has been suggested by Lamb *et al.* (1991) in *A. nidulans* in conditions of nutritional stress (see 1.4). Duncan *et al.* (1988) have suggested that the ability to express the individual AROM enzyme activities stoichiometrically avoids the problems of co-ordinating the expression of individual proteins. This idea is supported in the case of the *S. cerevisiae* AROM which has been shown to be under transcriptional control of the general control system found in yeast which responds to amino acid starvation (see 1.3).

The next section describes the initial work carried out on the the multifunctional AROM protein.

1.6 Early studies on the AROM multifunctional enzyme

1.6.1 Genetic studies of the *arom* locus

Mutants requiring all three aromatic amino acids for growth, termed *arom*, were first isolated from *N. crassa* by Gross and Fein (1960). In *Neurospora* complementation experiments are carried out in hybrid heterokaryons which express two different mutations. The *arom* mutants were placed in groups according to their ability to complement one another in this trans configuration. Less detailed studies were also made of *arom* mutants of the fission yeast, *Schizosaccharomyces pombe* (Strauss, 1979) and *Aspergillus nidulans* (Roberts, 1969) which suggested similar complementation patterns.

The *N. crassa* mutants fell into five distinct complementation groups which were shown to map very close together in five non-overlapping regions on linkage group II (Giles *et al.*, 1967). Mutations in each complementation group were found to be associated with the loss of one enzyme activity of the five central steps of the shikimate pathway, and this region was named the '*arom* cluster' (Gross & Feinn, 1960; Giles *et al.*, 1967). Detailed complementation studies in *N. crassa* revealed two categories of mutants in the '*arom* cluster'. The first category consists of mutants in which a single enzyme activity is lost, and the second category contains pleiotropic mutants where two or more activities are lost (Gross & Feinn, 1960; Giles *et al.*,1967). The five activities were shown to co-sediment in sucrose density gradients (Giles *et al.*, 1967).

The single activity mutants generally have normal wild-type activity levels of the other four enzymes (Giles *et al*, 1967; Rines *et al.*, 1969). These single enzyme mutations map to distinct non-overlapping subregions of the '*arom* cluster' in fine structure studies. They also have the normal wild-type native molecular weight, estimated to be 200-230kDa by sucrose density gradient centrifugation studies (Giles *et al.*, 1967; Rines *et al..*, 1969; Case *et al.*, 1971). This suggests that a mutation in a single subregion does not affect the overall quaternary stucture of the protein. The mutations were interpreted as missense mutations resulting from a single amino acid

substitution (Rines *et al.*, 1969; Strauss, 1979) which can lead to incorrect polypeptide folding in the region of the mutation (Crick & Orgel, 1964). There is little evidence that the mutants of one enzyme activity affects the activity of a separate AROM enzyme. Therefore, the missense mutations in the '*arom* cluster' provided good evidence that the cluster encodes five functionally independent enzymes. At the time of the study these data were interpreted as evidence that the '*arom* cluster' is composed of five genes which produce five polypeptides forming a multienzyme aggregate, since all the activities have the same sedimentation coefficient on sucrose density centrifugation (Giles *et al.*, 1967).

The second class of '*arom* cluster' mutants are polarity mutants which lack two or more activities. This indicates that the *arom* 'cluster' is transcribed as a single mRNA and this was thought to be subsequently translated as five separate polypeptides which form the AROM 'aggregate' (Giles *et al.*, 1967; Burgoyne *et al.*, 1967).

The complementation map allowed regions of the *arom* 'cluster' to be assigned to individual AROM enzyme activities and the genetic map is summarised in Figure 1.4.. The mRNA 5' to 3' transcription order is: DHQ synthase - EPSP synthase - shikimate kinase - DHQase - shikimate DH (Rines *et al.*, 1969).

One group of pleiotropic mutants, class E, are unable to complement with any other *arom* mutants and lack all five enzyme activities. The mutation is located at the 5'-DHQ synthase subregion of the *arom* 'cluster'. Some mutants of this class have been shown to be nonsense mutants (Case *et al.*, 1968) which result in the production of truncated protein. The phenotype is suppressed in *N. crassa* strains believed to carry a nonsense suppressor tRNA gene and AROM of wild-type molecular weight with all five enzyme activities results (Case *et al.*, 1968). The remaining polar mutations have phenotypes which can also be interpreted as due to premature polypeptide termination (Case & Giles, 1971).

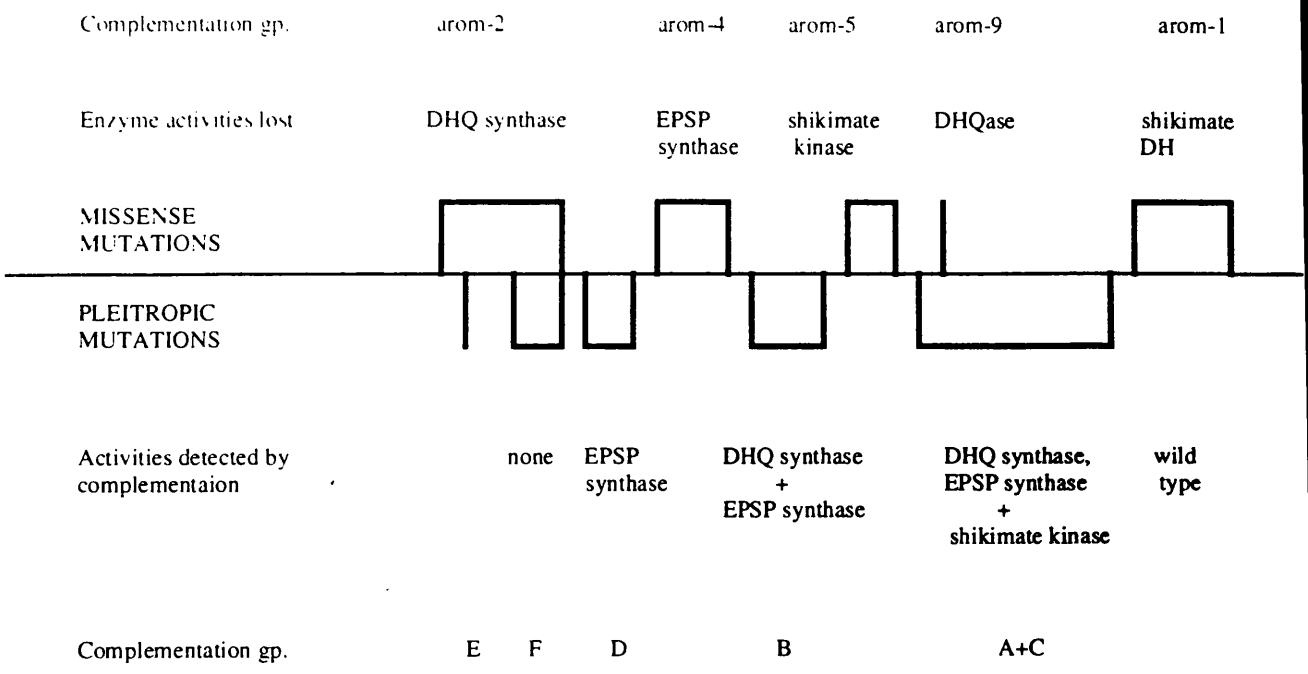


Figure 1.4 **Genetic map of the *arom* gene of *N. crassa***

The genetic map is based on the data of Giles *et al.* (1967); Rines *et al.*, 1969; Case & Giles, 1971).

1.6.2 Purification of *N. crassa* AROM protein

Early AROM protein preparations from *N. crassa* suggested that the protein is able to dissociate into a number of low molecular weight species (Burgoyne *et al.*, 1969; Gaertner, 1972; Jacobson *et al.*, 1972) which were thought to be 'subunits' of an AROM multienzyme complex. Low molecular weight subunits were later shown to result from proteolysis of a single AROM polypeptide by endogenous *N. crassa* proteinases and it was suggested that AROM is, in fact, a pentafunctional polypeptide (Gaertner & Cole, 1976; Lumsden & Coggins, 1977; Giles, 1978; Boocock, 1983). Lumsden & Coggins (1977) used an antiproteinase strategy and purified AROM of 165kDa when analysed by SDS PAGE. Centrifugation on glycerol density gradients showed that native AROM had a molecular weight of 270kDa suggesting that it was a dimer (Lumsden & Coggins, 1977). This was confirmed by crosslinking studies with dimethyl suberimidate (Lumsden & Coggins, 1977). Peptide mapping showed that the two 'subunits' were chemically very similar (Lumsden & Coggins, 1978). This is consistent with the genetic studies which also suggested that AROM is a dimer (Case & Giles, 1971).

It was not until each of the individual AROM enzyme activities was followed throughout the purification that it was realised that the purified preparations were severely deficient in EPSP synthase and DHQ synthase activities (Boocock, 1983). The purification procedure was modified to prevent the loss of zinc ions from the DHQ synthase domain which was found to be a metallo-enzyme, and to prevent the oxidation of the EPSP synthase domain. In the resulting AROM preparation the individual AROM activities co-purified in constant activity ratio (Lambert *et al.*, 1985; Coggins *et al.*, 1987a). The genetic data were re-evaluated with the knowledge that AROM is a pentafunctional polypeptide in order to provide information about the structure of the protein.

1.7 Structure of the AROM protein

1.7.1 Genetic data

The studies of AROM mutants in *N. crassa* provide some information about the structure of the AROM protein. Interallelic complementation is considered to involve the interaction in a multimer of two differentially defective polypeptides with the restoration of enzyme activity (Crick & Orgel, 1964). Within the AROM enzymes, interallelic complemetation is only observed between missense mutants of the N-terminal subregion encoding DHQ synthase, and between missense mutants of the C-terminal shikimate DH subregion (Giles *et al.*, 1967; Rines *et al.*, 1969; Case *et al.*, 1969; Case & Giles, 1971). Interallelic complementation occurs in a small subset of these mutants and the shikimate DH domain has been implicated in dimer formation by such studies (Case & Giles, 1971). There is no evidence for contacts between any of the other AROM subregions from interallelic complementation studies.

A detailed analysis of the AROM pleiotropic mutants of *N. crassa* by Case & Giles (1971) showed that several mutants have a smaller native molecular weight than the wild-type enzyme as determined by sucrose density gradient centrifugation. The mutants have not been characterised as nonsense mutations but are probably best interpreted as premature termination mutants (Case & Giles, 1971). These partial enzyme aggregates are able to complement DHQ synthase activity, while the EPSP synthase and shikimate kinase activities can be assayed in extracts. Therefore, EPSP synthase and shikimate kinase subregions of the AROM polypeptide can fold as part of the N-terminus to produce functional enzymes, albeit at a reduced level, without the C-terminal DHQase and shikimate DH regions. The reason why DHQ synthase can only be detected by complementation and cannot be detected in assays, suggests that the picture is more complicated. The molecular weight data suggests the proteins are monomers since they are approximately one quarter of the intact AROM molecular weight. As with the missense mutants, this is consistent with the model of AROM where the shikimate DH region is required for dimerisation. The lack of

DHQ synthase activity in assays would suggest in such a model, that interaction between the homologous DHQ synthase regions of the polypeptide are required for enzyme activity.

The genetic studies of AROM have provided a wealth of information on the *arom* gene and the AROM protein, which complements the biochemical studies which are described in the remainder of this Section.

1.7.2 Biochemical evidence for the mosaic model of AROM

The mosaic model of a multifunctional protein proposes that each of the functions of the protein are located on discrete regions of the polypeptide. This section describes the biochemical evidence for such a model of AROM. Biochemical studies on AROM purified from *N. crassa* suggest that each of the five enzyme active sites act independently of one another. The evidence is described below for each AROM activity in turn:-

DHQ synthase: The zinc-dependent DHQ synthase activity can be inactivated with the chelating agent EDTA in the absence of substrate (DAHP) without affecting the other four activities (Lambert *et al.*, 1985).

DHQase: The type I DHQase of AROM works through an imine intermediate which is formed between the amino group of a lysine at the enzyme active site and the keto group of the substrate, DHQ (Butler *et al.*, 1974). DHQase is specifically inactivated with sodium borohydride in the presence of DHQ by reducing the Schiff base formed between the active site lysine and DHQ thus trapping the substrate (Smith & Coggins, 1983; Coggins & Boocock, 1986; Chaudhuri *et al.*, 1986). Under these conditions the other four AROM enzymes are active.

Shikimate DH: Sodium borohydride, inactivates shikimate DH in the presence of formaldehyde (Smith, 1980). The lysine-specific reagent, methylacetimidate also inactivates shikimate DH (Coggins & Boocock, 1986). Shikimate protects shikimate DH against inactivation by both reagents (Coggins & Boocock, 1986; Coggins *et al.*, 1987). The DHQase of AROM can be specifically inactivated with sodium

borohydride in the presence of DHQ, and the intact shikimate DH can then be inactivated by methylacetimidate. The other enzymes are unaffected by this treatment.

Shikimate kinase: Limited proteolysis of AROM with trypsin or subtilisin specifically inactivates the shikimate kinase activity leaving the other four activities unaffected (Coggins *et al.*, 1985; Coggins & Boocock, 1986). This is described more fully in Section 1.6.

EPSP synthase: Limited proteolysis of AROM with trypsin and chymotrypsin simultaneously, produces a fragment with only EPSP synthase activity which can be isolated chromatographically (Coggins *et al.*, 1985; Coggins & Boocock, 1986). This is described more fully in the next section (1.6.3).

These results indicate that each AROM enzyme has distinct active sites and supports the mosaic model of AROM in which the five enzymes form five autonomous structural domains each of which has one of the component enzyme activities. This also supports the genetic data.

1.7.3 Limited proteolysis studies on *N. crassa* AROM

Limited proteolysis of native protein is used as a tool to provide conformational information. It has been particularly useful in studies of multifunctional proteins, which often consist of tight globular domains separated by 'linker' polypeptide, and provides evidence for a mosaic structure. The globular domains are less accessible to proteinases than the inter domain regions which may lack secondary structure. Therefore, limited proteolysis provided an excellent technique for conformational studies on AROM (Price & Johnson, 1989).

Smith & Coggins (1983) isolated an active AROM fragment after limited proteolysis with trypsin or subtilisin. Analysis by SDS PAGE showed that the AROM was proteolysed to two stable fragments of 110kDa and 68kDa with the loss of shikimate kinase activity. Analysis by native PAGE showed that the proteolysed AROM is identical to the intact protein which suggests that the proteolysed AROM is

held together, at least in part, by the same kind of non-covalent interactions responsible for the quaternary structure. Smith & Coggins (1983) showed that the DHQase active site is located on the 68kDa fragment by labelling the DHQase active site with tritiated sodium borohydride in the presence of substrate, DHQ. This traps the substrate and the active site of DHQase was specifically labelled. After denaturation with 8M urea, the 68kDa fragment was able to renature and showed shikimate DH activity in an activity stain. This indicates that DHQase and shikimate DH active sites are able to function without the other AROM activities and furthermore the information for refolding to produce functional shikimate DH is within the proteolytic fragment.

Prolonged proteolysis with trypsin and chymotrypsin simultaneously (Boocock, 1983; Coggins *et al.*, 1985; Coggins & Boocock, 1986) resulted in the loss of DHQ synthase activity. Native AROM of '530kDa' (this was thought to be erroneously high due to the shape of the protein) was no longer observed on gel filtration HPLC and species of 200kDa and 130kDa were present. The 200kDa and 130kDa species were shown to be 74kDa and 63kDa polypeptides, respectively, when they were analysed by SDS PAGE suggesting that both species are dimers under native conditions. The 63kDa fragment was found to be derived from the 68kDa fragment and was shown to have both DHQase and shikimate DH activity. The 74kDa fragment was derived from the 110kDa initial fragment and was separated from the other fragment by chromatography on DEAE cellulose and phosphocellulose. The EPSP synthase activity was shown to be associated with the 200kDa species. The DHQ synthase activity was assumed to be located on the 110kDa fragment and was lost when this was proteolysed further to 74kDa. The proteolysis of AROM is summarised in Figure 1.5.

The proteolysis studies of native AROM suggest that the DHQase and shikimate DH regions can function without the other activities, at least as part of a bifunctional polypeptide such as those seen in plant species (Polley, 1978; Koshiba, 1979; Mousdale *et al.*, 1987)). The EPSP synthase region can also function

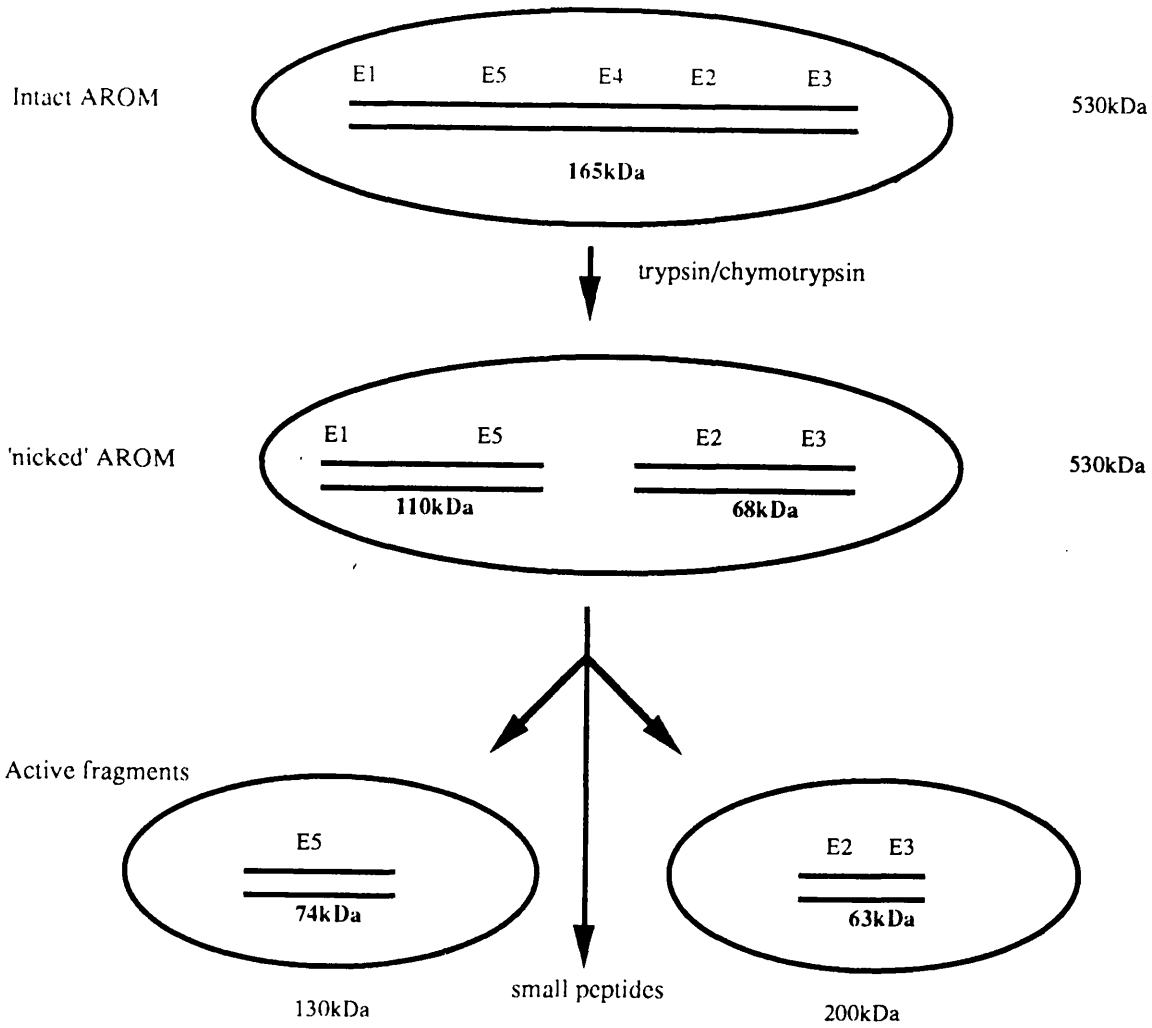


Figure 1.5 Summary of limited proteolysis studies on *N. crassa* AROM

The individual AROM enzyme domains have been abbreviated to their reaction order in the shikimate pathway: DHQ synthase, E1; DHQase, E2; shikimate DH, E3; shikimate kinase, E4; EPSP synthase, E5. The molecular weights directly under each protein are the subunit molecular weights estimated by SDS PAGE. The molecular weights indicated surrounding the entire protein, or fragments, show the native molecular weights determined under non-denaturing conditions by gel filtration HPLC. The diagram is taken from Boocock, 1983.

independently and this suggests that the EPSP synthase and DHQase-shikimate DH regions of AROM are compact globular structures which are not easily proteolysed. This agrees with the model suggesting that each AROM activity is spatially and functionally independent of the others. The evidence that the DHQase-shikimate DH fragment is a dimer supports the genetic evidence that shikimate DH allows dimerisation (Giles *et al.*, 1967; Rines *et al.*, 1969; Case *et al.*, 1969). It also provides evidence that the shikimate DH of one polypeptide is in contact with shikimate DH of the other polypeptide rather than DHQ synthase (see Figure 1.5). The dimeric association of the EPSP synthase subregion is rather more difficult to explain because the data on pleiotropic mutants which have the entire N-terminus (A and C mutants - see Figure 1.4 and Section 1.7.1) including DHQ synthase, EPSP synthase and shikimate kinase, is suggestive of a monomeric structure (Case & Giles, 1971). The reason for this discrepancy in the data has not been established.

1.7.4 Sequence alignment of AROM from *A. nidulans* and *S. cerevisiae* with the equivalent *E. coli* monofunctional enzymes

The genes encoding the entire AROM polypeptide have been sequenced from *A. nidulans* (Kinghorn & Hawkins, 1982; Charles *et al.*, 1986), *S. cerevisiae* (Larimer *et al.*, 1983; Duncan *et al.*, 1987) and *P. carinii* (Banerji *et al.* 1993). As described in Section 1.2, a comparison of the *S. cerevisiae* and *A. nidulans* sequences with those of the individual *E. coli* monofunctional enzymes (Duncan *et al.*, 1984a; 1986; Millar *et al.*, 1986; Millar & Coggins, 1986; Anton & Coggins, 1988) show functional regions, or domains, within the polypeptide corresponding to the monofunctional enzymes (Duncan *et al.* 1987; Hawkins, 1987). There were also regions with no homology with the *E. coli* enzymes which were located at two of the expected domain boundaries (Duncan *et al.*, 1987; Coggins *et al.*, 1987b; van den Hombergh *et al.* 1991) These interdomain, or linker regions are thought to be essential for the structural integrity of multifunctional proteins This data supports the model of AROM as a mosaic with semi-autonomous functional domains.

1.7.5 AROM domain expression studies

The hypothesis that the *arom* gene evolved by linking of ancestral *E. coli* type genes suggests as one possibility that there is an evolutionary advantage to the formation of this pentafunctional protein. The expression of fragments of the AROM protein will allow comparison with the intact polypeptide and perhaps provide an indication of why the protein is arranged pentafunctionally.

Fragments of the*S. cerevisiae* AROM protein have been shown to complement *E. coli* auxotrophic mutants of all the AROM activities (K. Duncan & R.M. Edwards, unpublished data) with the exception of shikimate kinase which has not been studied by complementation because there are two shikimate kinase isozymes in *E. coli* (Ely & Pittard, 1979). No DHQ synthase activity was found in *E. coli* constructs containing this domain. In *S. cerevisiae* where the entire AROM protein was successfully overexpressed using a ubiquitin fusion vector (see 3.4.1), attempts to express the DHQase and the DHQ synthase regions using the same system were unsuccessful.

In *A. nidulans* the domain expression studies have progressed further, although most of this work was carried out after starting the project described in this thesis. The domain expression studies are described below.

1. DHQase domain: An 80kDa fragment of AROM expressed in *E. coli* was found to have DHQase activity and extended into the shikimate DH domain (Charles *et al.*, 1985). The DHQase domain was later expressed in *E. coli* producing protein with activity levels 8-fold higher than in *A. nidulans*. More recently the DHQase domain has been overexpressed in *E.coli* as a glutathione S-transferase fusion protein which has been purified and shown to be a type I DHQase since it is inhibited by sodium borohydride in the presence of DHQ (Hawkins *et al.*, 1993a).

2. DHQase-shikimate DH: A DHQase-shikimate DH bifunctional domain in *A. nidulans* overexpressed both activities 50-fold over wild-type *A. nidulans*, but the same construct failed to be overproduced in *E. coli* (Moore & Hawkins, 1993).

3. DHQ synthase: A monofunctional DHQ synthase domain has been expressed in

E. coli (van den Hombergh *et al.*, 1992) and subsequently purified to homogeneity. This domain is currently undergoing kinetic characterisation (J. Moore, personal communication).

4. DHQ synthase-EPSP synthase: A bifunctional DHQ synthase-EPSP synthase has been overexpressed to about 10% of the total cell protein in *E. coli.*

5. EPSP synthase: EPSP synthase has so far not been successfully expressed in *E. coli* without DHQ synthase as part of the same polypeptide. EPSP synthase has been expressed as a bifunctional polypeptide in *E. coli* with a defective DHQ synthase with a point mutation at residue 144 (see Section 4.6.8), which was able to complement an *aro A*$^-$ mutant (lacks EPSP synthase activity). Expressing both active DHQ synthase and EPSP synthase independently in the same cell does not complement *aro A*$^-$ mutants, so EPSP synthase requires to be in the *cis* conformation with DHQ synthase and is not active when expressed in *trans* conformation. A glutathione S-transferase fusion protein expressed in *E. coli* produces a highly overexpressed protein which fails to complement an *aro A*$^-$ mutant. Switching of the domains, to form a reversed-order bifunctional EPSP synthase-DHQ synthase, followed by expression in *E. coli* and was found to relieve *aro A*$^-$ auxotrophy, but not *aro B*$^-$ auxotrophy (DHQ synthase mutation).

6. Shikimate DH: Shikimate DH has never complemented *aro E*$^-$ (lacking shikimate DH activity) mutants in *E. coli* even as part of the entire AROM protein (Hawkins & Smith, 1991; Moore & Hawkins, 1993). It now appears that there is a strong likelihood that there is a 39 nucleotide intron near the 3' end of the shikimate DH domain coding sequence, which was previously thought to be a $^{130a}_{\wedge}$ insertion when compared to the *S. cerevisiae* gene sequence. This might explain why the protein is inactive in *E. coli* but not *A. nidulans* since *E. coli* are unable to process the intron.

These studies have not looked at the shikimate kinase activity primarily because there is no complementation system available in *E. coli* ,as mentioned above. Therefore, the work has concentrated on the other domains. The study suggests that the mosaic model is somewhat oversimplified since the expression data indicate that

the domains which make up AROM do not act completely independently of one another.

From the expression studies, Hawkins has suggested a structure for AROM in which the AROM protein is in two halves: one half contains the N-terminal DHQ synthase and EPSP synthase activities, and the other has shikimate kinase, DHQase and shikimate DH activities. These two subregions can function independently, in this model, with each subregion stabilising the enzymes within that region and maximising individual enzyme activities (Hawkins & Smith, 1991; Hawkins *et al.*, 1993).

1.8 Aims of the project

The AROM protein has been shown to be a pentafunctional protein composed of five *E. coli*-like monofunctional domains catalysing five consecutive steps of the shikimate pathway. It is thought to have arisen by the fusion of ancestral monofunctional enzymes and domain expression studies are currently being used to investigate the structure of the pentafunctional protein and its component enzymes (see 1.7.4 and 1.7.5). In parallel with this study, to provide a comparison with those domains which have been expressed individually or fused with other domains, a detailed kinetic and biophysical analyses must be made of intact AROM protein. At the start of this project the only AROM protein which had been purified to homogeneity and characterised kinetically was *N. crassa* AROM. However, the *arom* gene has not been sequenced from this species. The gene encoding the AROM protein in *S. cerevisiae* was sequenced in our laboratory and this species was, therefore, used for domain expression studies. In order to compare the expressed domains with intact protein, it was essential to purify and characterise *S. cerevisiae* AROM. The original objective of this work was to purify and characterise *S. cerevisiae* AROM, and any independently expressed AROM domains, in order to assess any differences between the two which might provide an insight into the multifunctional arrangement of AROM. As it became clear that the problems

involved in the purification of AROM from *S. cerevisiae* would not be easily solved, and the domain expression studies were progressing in *A. nidulans*, it was decided that purification and characterisation of AROM from *A. nidulans* should be the new objective of the project.

Chapter 2 Materials and Methods

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and biochemicals

Triton X-100, formaldehyde, N,N,N',N'-tetramethylethylene diamine (TEMED), Tricine, PMSF, benzamidine, pepstatin A, leupeptin hemisulphate salt, nitroblue tetrazolium, chloronapthol, phenanzine methosulphate, Tween 20, shikimic acid and Coomassie Brilliant Blue G-250 were obtained from Sigma Chemical Co., Poole.

Bacto Yeast Extract, Bacto-Peptone, Bacto-Agar and BactoYeast Nitrogen Base without amino acids, were obtained from Difco, Detroit, USA.

Ammonium persulphate, enzyme grade ammonium sulphate (specially low in heavy metals), disodium hydrogen orthophosphate, sodium dihydrogen orthophoshate, ammonia, ß-mercaptoethanol and pH buffer tablets (pH4.0, pH7.0 and pH9.2) were obtained from BDH Chemicals, Poole, UK.

Tris, dithiothreitol, ATP, NAD^+, $NADP^+$, NADPH and phosphoenolpyruvate were obtained from Boehringer Mannheim, Lewes, UK.

Glutaric dialdehyde was obtained from Aldrich Chemical Co. Ltd., Gillingham, UK.

Silver nitrate was purchased from Johnson Mattey, Materials Technology UK, Royston, UK.

Glycine, acrylamide, NN' Methylenebisacrylamide, sodium chloride, D-glucose, diamino ethanetetra-acetic acid disodium salt, sodium hydroxide, sodium lauryl sulfate, di-potassium hydrogen orthophoshate, potassium di-hydrogen orthophoshate, hydrochloric acid, glacial acetic acid, hydrogen peroxide and orthophsphoric acid were obtained from Fisons, Loughborough, UK.

Sodium dihydrogen orthophoshate dihydrate was obtained from Formachem Ltd, Strathaven, UK.

The GOD-Perid glucose test kit was from Boehringer Mannheim GmbH Diagnostica, and kindly gifted by Dr. I. Hamilton.

Ammonium dehydroquinate was prepared following the procedure of Grewe and Haendler (1966). 3-Deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) was isolated from an *aro* B^- strain of *E. coli* (AB2847A) and prepared following the procedure of Lambert *et al.*, (1985). Both were gifts of Professor J.R. Coggins.

All other chemicals were of analytical reagent grade.

2.1.2 Enzymes and proteins

Dehydroquinase was purified from an overproducing *E. coli* strain (AB2848 pKD201) by Mr. J.Greene as described by Chaudhuri *et al.*(1987). AROM was purified by Dr H. Powell from *N. crassa* by the method described by Coggins *et al* (1987a).

Shikimate DH was purified from the *E. coli* overproducing strain AB2834/pIA321 as described by Anton & Coggins (1988).

Pyruvate kinase/lactate dehydrogenase in the form of a crystalline suspension and bovine serum albumin were obtained from Sigma Chemical Co., Poole, UK.

TPCK treated bovine trypsin, TLCK treated bovine α-chymotrypsin, Subtilisin Carlsberg Type III from *Bacillus subtilis*, and Thermolysin Type X from *Bacillus thermoproteolyticus* were supplied by Sigma Chemical Co. Proteinase K from *Tritirachium album* was supplied by Boehringer Mannheim GmbH, Germany. Lima bean proteinase inhibitor was the gift of Dr. M. Boocock

An SDS high molecular weight marker kit (MW-SDS-200) for protein molecular weight determination following SDS PAGE, contained the standards: rabbit muscle myosin, 205kDa; *E. coli* ß-galactosidase, 116kDa; rabbit muscle phosphorylase b, 97.4kDa; bovine serum albumin, 66kDa; egg albumin, 45kDa; carbonic anhydrase from bovine erythrocytes, 29kDa. The kit was obtained from Sigma Chemical Co., Poole, UK

A high molecular weight standard kit for native molecular weight determination of protein by gel filtration chromatography, was obtained from Pharmacia, Milton Keynes, UK. The kit contained the protein standards: rabbit muscle aldolase, 158kDa; bovine liver catalase, 232kDa; horse spleen ferritin, 440kDa; bovine thyroid thyroglobulin, 669kDa.

HRP donkey anti-rabbit IgG and normal donkey serum were obtained from the Scottish Antibody Production Unit, Carluke, UK.

Polyclonal anti-AROM antibodies were raised in rabbit against the *N. crassa* protein and were prepared by Dr. I. Hamilton.

2.1.3 Chromatography media

Hydroxyapatite was Bio-Gel HTP from BioRad Laboratories Ltd., Watford, UK. Pre-swollen DE52 was supplied by Whatman Biochemicals, Maidstone, UK. Q-Sepharose FF, CM-Sepharose FF, S200 and G-25 superfine gel filtration media were obtained from Pharmacia, Milton Keynes, UK. Cibacron Blue F-3GA on Sepharose 4B was gifted by ICI. Mimetic Yellow 1 A6XL was from Affinity Chromatography Ltd., Isle of Man, UK.

2.1.4 Pre-packed media

The PIKSI Mimetic dye screening kit was obtained from Affinity Chromatography Ltd., Isle of Man, UK. Pre-packed Mono Q, Superose 6 and Superose 12 columns were obtained from Pharmacia, Milton Keynes, UK, and were utilised on a Pharmacia FPLC system.

2.1.5 Pre-cast polyacrylamide gels

Polyacrylamide PhastGels, buffer strips and PhastGel Blue R Coomassie dye tablets were purchased from Pharmacia, Milton Keynes, UK, and were used for electrophoresis, and developed on the Pharmacia PhastSystem.

2.1.6 Blotting membranes

Cellulose nitrate was obtained from Schleicher & Schuell, Dassel, Germany. Polyvinylidene difluoride (PVDF) membrane (Problott) was obtained from Applied Biosystems, Warrington, UK.

2.1.7 Miscellaneous materials

Cell scrapers used for *A. nidulans* spore collection were from Corstar, Cambridge, USA. Sterile plates were from Sterilin, Stone, UK. Sterile universal containers were supplied by Greiner Labortechnik,

2.1.8 Plasmids, *S. cerevisiae* and *A. nidulans* strains

Plasmid Yep42a-Gsaa was the kind gift of Dr. Tauseef Butt (Smith Kline & French, Pennsylvania). *S. cerevisiae* strains ABYS106 and BJ1991 were gifted by Dr. M. Stark, Dundee University, UK. *A. nidulans* strains 1314 and R153 were the gift of Dr. A. Hawkins, Newcastle University, UK. The *S. cerevisiae* and *A. nidulans* strains used during this study are shown in Table 2.1.

2.2 General methods

2.2.1 pH measurement

pH measurements were made with a Corning pH meter 220 calibrated at room temperature.

2.2.2 Conductivity measurement

Conductivity was measured at 4°C with a Radiometer Model CDM2e conductivity meter.

2.2.3 Protein determination

Protein was estimated by the method of Bradford (1976), with bovine serum albumin as standard.

	Genotype	Phenotype
***S. cerevisiae* strains**		
S288C	haploid wild-type strain	Wild-type
ABYS106	*pra1-2 prb1-1 prc1-1 cps1-3 his3 ura3*	Lacks proteinase yscA, proteinase yscB, carboxypeptidase yscY and carboxypeptidase yscS.
BJ1991	*α leu2 trp1 ura3-52 prb1-112 pep4-3 gal2*	Lacks proteinase yscB. Reduced levels of proteinase yscA, carboxypeptidase yscY and aminopeptidase yscI.
***A. nidulans* strains**		
R153	*wA3 pyroA4*	White spore, requires pyridoxine.
1314	*aromA qutB*	AROM and quinate dehydrogenase mutant strain transformed with pNUFC2 (*aromA* fused to the *qutE* promoter).

Table 2.1 ***S. cerevisiae* and *A. nidulans* strains used during the study**

2.2.4 Phosphate buffer

1M solutions of both disodium hydrogen orthophosphate and sodium dihydrogen orthophosphate were made up. These were mixed until the pH required was reached, resulting in a 1M phosphate buffer.

2.2.5 Dialysis membranes

Dialysis membranes were boiled for 15 min at 100°C, in 1 litre of solution containing 2g sodium hydrogen carbonate and 1.2g EDTA. The dialysis tubing was rinsed with distilled water and stored in 25% (v/v) ethanol at 4°C.

2.3 Regeneration of dye chomatography media

2.3.1 Regeneration of Cibacron blue F-3GA on Sepharose 4B

The regeneration of Cibacron blue dye matrix following chromatography to remove bound proteins, was carried out using the following regime:

1. 5 column volumes of 50mM-Tris/HCl pH7.5 containing 50mM-EDTA and 6M-guanidinium hydrochloride.
2. 20 column volumes of 1.9M-KCl in 100mM-Tris/HCl pH7.5, containing 5mM-EDTA.
3. 30 column volumes of 30mM-KCl in 50mM-Tris/HCl pH7.5, containing 0.1% (w/v) sodium azide for storage.

2.3.2 Regeneration of Mimetic dyes

The regeneration of mimetic dyes following chromatography, to remove protein is described below:

1. 2 column volumes of 1M-NaOH.
2. 10 column volumes of 25:75 (v/v) of ethanol:0.1M-NaCl as preservative.

2.4 Polyacrylamide gel electrophoresis (PAGE)

2.4.1 SDS PAGE

Protein electrophoresis in polyacrylamide gels, in the presence of 0.1% SDS was carried out by the method of Laemmli (1970), with a 3% stacking gel and a 10% or 7% running gel. The ratio of acrylamide:bis-acrylamide ratio was 30 : 0.8 in all experiments and polymerisation was induced with 0.05% (w/v) ammonium persulphate and 0.07% (v/v) TEMED. SDS PAGE on Phastgels was carried out according to the manufacturers instructions using SDS buffer strips.

Protein samples were boiled for 3 min in SDS PAGE sample buffer providing a final concentration of 0.05% (v/v) ß-mercaptoethanol and 0.01% SDS.

2.4.2 SDS PAGE for subsequent protein renaturation

The protocol for protein renaturation following SDS PAGE requires several adaptations to the general method of SDS PAGE and has been described by Anton (1985). The polyacrylamide gel contained 1mM EDTA and was aged overnight prior to use. Protein samples were boiled in SDS PAGE sample buffer containing 1% (w/v) SDS and 2% (v/v) ß-mercaptoethanol, for exactly 2 min The running buffer contained 0.1mM-EDTA and 0.01% (v/v) ß-mercaptoethanol.

2.4.3 Native PAGE

Non-denaturing PAGE on 5% tube gels was carried out by the method of Davis (1964) at 4°C. Samples were incubated with 1-50mM DTT on ice for 1 h prior to loading and the gel was electrophoresed for 30 min prior to loading the sample.

Native PAGE on Phastgels was carried out in the same manner, with a 4-15% gradient slab gel using native buffer strips.

Following electrophoresis the gel was stained for protein with Coomassie Brilliant Blue G-250 (2.5.1) or silver stain (2.5.2), or for shikimate dehydrogenase activity using the nitroblue tetrazolium dye-linked method (2.6).

2.5 Protein stains

2.5.1 Coomassie stain

Proteins on polyacrylamide gels were stained with 0.1% (w/v) Coomassie Brilliant Blue G-250 in 50% (v/v) methanol and 10% (v/v) glacial acetic acid, for 2 h. The gel was destained with 10% (v/v) methanol and 10% (v/v) acetic acid for protein visualisation.

2.5.2 Silver stain

Protein visualisation by the silver stain technique was adapted from the method of Wray *et al.*(1981) and is described below.

1. The polyacrylamide gel was fixed overnight in 50% (v/v) methanol.
2. 21ml of 0.36%(w/v) sodium hydroxide was mixed with 1.4ml of ammonium hydroxide.
3. Silver stain was freshly prepared by adding 4ml of a 20% (w/v) silver nitrate solution, dropwise, to the sodium hydroxide/ammonium hydroxide solution (2). This was diluted to 100ml to give a final concentration of 0.8% (w/v) silver nitrate.
4. The gel was soaked for 15 min in freshly prepared silver stain
5. The gel was washed for 1 h in distilled water.
6. Developer (prepared freshly immediately prior to use) consisted of 120µl of 1M citric acid and 250µl of 38% (v/v) formaldehyde, made up to 500ml with distilled water. The gel was soaked in developer and the reaction was stopped with several washes in distilled water after the protein bands had developed.
7. The gel was stored in 10% (v/v) acetic acid, 45% (v/v) methanol to prevent further development of the stain.

Protein on Pharmacia Phastgels were stained with Coomassie Brilliant Blue or silver stain according to the manufacturers instruction manual.

2.6 Shikimate DH activity stain

2.6.1 Renaturation of protein after SDS PAGE

SDS PAGE for the subsequent renaturation of protein is described in Section 2.4.2. The gel was renatured according to the method of Anton (1985):

1. The gel was soaked at room temperature over a 7-12 h period in 3 x 330ml of 50mM-sodium phosphate buffer pH7.0, containing 1mM-DTT, 0.1mM-EDTA and 0.1% (v/v) Triton X-100.
2. The gel was incubated for a further 12 h with 3 x 330ml of 50mM-sodium phospate buffer pH7.0, containing 1mM-DTT and 0.1mM-EDTA (without Triton X-100).
3. The gel was soaked 2 x 250ml of 100mM Tris/HCl pH8.8 to remove DTT.
4. The gel was then stained for shikimate DH activity.

2.6.2 Shikimate DH activity stain

The shikimate DH activity of protein following SDS PAGE (2.4.2) and native PAGE (2.4.3) was visualised by the triazolium dye-linked method, adapted by Lumsden and Coggins (1977).

Native gels were washed in 100mM-Tris/HCl pH8.8 (100ml) for at least 1 h. prior to staining to remove DTT. Protein was renatured after SDS PAGE as described above in Section 2.6.1.

The gel was soaked in 250mM-Tris/HCl pH8.8 containing 0.5mM-$NADP^+$, 0.5mM-shikimic acid, 0.5mg/ml nitroblue tetrazolium and 5µg/ml phenanzine methosulphate (20ml total volume). This was incubated in the dark until black bands corresponding to shikimate DH activity developed. The reaction was stopped with several washes in distilled water.

2.7 Immunoblot

2.7.1 Electroblotting

The protein on polyacrylamide gels was electrophoretically transferred to nitrocellulose using a Bio-Rad Trans-blot cell by the method of Towbin *et al* (1979).

Blot transfer buffer:
- 42.8g glycine
- 9.1g Tris
- 0.8g SDS
- 600ml methanol, made up to 3 litres with distilled water.

The blotting sandwich was assembled in the following order from the anode, ensuring that no bubbles were present:- sponge : filter paper : nitrocellulose : membrane : gel : filter paper : sponge. The blotting sandwich was placed in the blotting apparatus in blot transfer buffer with the nitrocellulose membrane closest to the anode. Protein from the gel was transferred at 70 volts for 4 h onto nitrocellulose in this manner.

2.7.2 Immuno-stain

The cross-reaction of AROM with polyclonal antibodies raised against *N. crassa* AROM was carried out by the method of Batteiger *et al*. (1982) and is described below:

1. Following electroblotting of protein from the gel unoccupied binding sites on the nitrocellulose were blocked overnight by incubation with 0.5% (v/v) Tween 20 in Blot Incubation Buffer (BIB) made up of 0.24% (w/v) Tris with 0.88% (w/v) sodium chloride pH7.2.
2. The blot was incubated for 90 min while shaking in BIB with 0.5% (v/v) Tween containing 5% (v/v) normal donkey serum and 1% (v/v) anti-AROM.

3. The blot was washed for 4 x 12 min with BIB containing 0.5% (v/v) Tween, then transferred to BIB without Tween for a 12 min wash.

4. The blot was incubated in BIB containing 5% (v/v) normal donkey sera and 0.2% (v/v) donkey anti-rabbit peroxidase conjugate for 90 min.

5. It was then washed for 5 x 12 min with BIB.

6. The horse raddish peroxidase was detected by immersing the blot in 50ml of 10mM-Tris/HCl pH7.5, containing 30mg of chloronapthol in 10ml methanol, and 150µl of 4% (v/v) hydrogen peroxide. The reaction was stopped by flushing the blot with distilled water.

2.7.3 Amido black protein stain

Protein was stained directly on nitrocellulose membranes with 0.1% (w/v) amido black in 5% (v/v) acetic acid for 1 min. It was destained for protein visualisation with 45% (v/v) methanol and 5% (v/v) acetic acid.

2.8 Electroblotting of protein onto PVDF membrane after SDS PAGE for N-terminal sequence determination

2.8.1 Electrophoresis conditions

All the solutions used in this procedure were made with de-ionised, Millipore filtered water.

SDS PAGE was carried out by the method of Laemmli (1970) as described in Section (2.4.1) using a Biorad mini-gel system. The gel was aged at room temperature overnight and electrophoresis proceeded as described below:

1. Protein samples were mixed with SDS PAGE sample buffer and boiled for 2 min.

2. Glutathione was added to the upper tank running buffer to a final concentration of 50µM, and the gel was pre-electrophoresed for 60 min to remove any free radicals from the gel which might damage the protein.

3. The pre-run electrophoresis buffer was decanted and the reservoirs were filled with fresh buffer. 0.1mM final concentration of sodium thioglycollate (free radical scavenger) was added to the upper reservoir buffer.

4. The protein sample was loaded and the electrophoresis was carried out as normal.

2.8.2 Electroblotting

All the solutions used in this procedure were made with de-ionised, Millipore filtered water. The electroblotting buffer was 10mM-CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) in 10% methanol, titrated to pH11.0 with 2M-NaOH.

The PVDF membrane was cut to the size of the gel and soaked in 100% methanol for a few minutes, rinsed with 50% methanol, then transferred to electroblotting buffer. The gel was soaked in electroblotting buffer for 5 min to reduce theTris and glycine content of the gel.

The electroblotting was otherwise carried out as described in Section 2.7.1 at 250V, at room temperature, for 3 h 20 min.

The PVDF membrane was washed with de-ionised water prior to staining. The gel was stained with Coomassie Brilliant Blue G-250 (2.5.1) in order to estimate the protein transferred onto the membrane.

2.8.3 Amido black stain for protein visualisation prior to N-terminal sequence analysis

All the solutions used in this procedure were made with de-ionised, Millipore filtered water.

The PVDF membrane was stained with 0.1% (w/v) amido black, 1% (v/v) acetic acid and 40% (v/v) methanol. The amido black was dissolved in methanol for 60 min. Acetic acid was added and the solution was made up to volume with water.

After stirring for 60 min the solution was filtered and ready for use. The procedure for staining the PVDF membrane for protein visualisation is described below:

1. The washed PVDF membrane was saturated in 100% methanol for a few seconds.
2. It was then stained for 1 min with amido black stain.
3 The PVDF membrane was destained with several changes of de-ionised water.
4. It was hung to dry.and stored in a sealed container.

The PVDF membrane was sent for N-terminal sequence determination at the Aberdeen Amino Acid Sequencing Facility. The protein bands were cut from the PVDF membrane and analysed on a Pulsed-liquid sequencer.

2.9 Enzyme assays

2.9.1 General method

Enzyme assays were monitored at 25°C with either a Gilford/Unicam model 2600 or a Philips PU 8720 UV/VIS spectrophotometer. All the enzyme assays were carried out according to the method of Coggins *et al* (1987a). Enzyme rates were calculated subtracting any blank rate in the absence of substrate.

2.9.2 DHQ synthase assay

DHQ synthase was assayed in the forward direction through coupling with dehydroquinase to produce 3-dehydroshikimate. The enzyme was assayed continuously in 100mM-potassium phosphate buffer pH7.0, 40μM-NAD^+, 200μM-DAHP, 100μM-zinc sulphate and 0.6 U *E. coli* dehydroquinase. The formation of 3-dehydroshikimate was measured as an increase in absorbance at 234nm [E_{234nm}=1.2 x $10^4 M^{-1}cm^{-1}$].

2.9.3 DHQase assay

DHQase was assayed directly by following the formation of 3-dehydroshikimate. The activity was assayed continuously in 100mM-potassium phosphate buffer pH7.0, with 100μM-3-dehydroquinate and the increase in absorbance at 234nm was monitored [E_{234nm}=1.2 x $10^4M^{-1}cm^{-1}$].

2.9.4 Shikimate DH assay

Shikimate DH was assayed in the reverse direction at high pH measuring the formation of NADPH. The assay was carried out in 100mM-sodium carbonate/bicarbonate pH10.6 with 2mM-$NADP^+$ and 4mM-shikimic acid. The enzyme was assayed as an increase in absorbance at 340nm [E_{340nm}= 6.3 x $10^3M^{-1}cm^{-1}$].

2.9.5 Shikimate kinase assay

Shikimate kinase was assayed in the forward direction by coupling the production of ADP to pyruvate kinase and lactate dehydrogenase and following the resulting decrease of NADH. The enzyme was assayed in 50mM-triethanolamine pH7.0 containing 500mM-KCl, 25mM-$MgCl_2$, 200μM-NADH, 1mM-phosphoenolpyruvate, 2.5mM-ATP, 1mM-shikimic acid, with 3.5U of pyruvate kinase and 5U of lactate dehydrogenase. The enzyme activity was monitored by following the decrease in absorbance at 340nm [E_{340nm}=6.22 x $10^3M^{-1}cm^{-1}$].

2.9.6 EPSP synthase assay

EPSP synthase was assayed in the reverse direction by using pyruvate kinase and lactate dehydrogenase to measure the phosphoenolpyruvate produced. The enzyme was assayed in 100mM-potassium phosphate buffer pH7.0, containing 2.5mM-$MgCl_2$, 100μM-NADH, 2.5mM-ADP^+, 50μM-EPSP, with 3.5U of pyruvate

kinase and 5U of lactate dehydrogenase. The assay was measured as the decrease in absorbance at 340nm as NADH is oxidised [E_{340nm}=6.22 x $10^3M^{-1}cm^{-1}$].

2.10 Limited proteolysis of native protein

2.10.1 Proteinase stock solutions

The proteinase stock solutions used in the limited proteolysis study (Section 4.7) are described below and were taken from Flannery *et al.* (1989):

Proteinase	Stock solution
Trypsin	1mg/ml in 1mM-HCl with 20mM-$CaCl_2$, stored at -20°C.
Chymotrypsin	1mg/ml in 1mM-HCl, stored at -20°C.
Subtilisin	10mg/ml in distilled water.
Thermolysin	10mg/ml in 20mM-$CaCl_2$, stored at -20°C.
Proteinase K	10mg/ml in 50mM-Tris/HCl pH8.0, with 1mM-$CaCl_2$, stored at 4°C.

2.10.2 Conditions for proteolysis of native AROM

AROM was digested at a concentration of 200µg/ml, with proteinase at 25°C. Protein was removed during the digestion and the appropriate proteinase inhibitor was added to stop the proteolysis. This mixture was stored on ice while enzyme assays were conducted. The protein was denatured by boiling in SDS PAGE sample buffer and subsequently analysed by SDS PAGE. The buffers used for each individual digestion experiment are described in Section 4.7.

Specific requirements for the activation of individual proteinases, as well as the proteinase inhibitors used, are described below:

Trypsin:	Inhibited with a 3-fold molar excess of lima bean proteinase inhibitor.
Chymotrypsin:	Inhibited with a 3-fold molar excess of lima bean proteinase inhibitor.
Subtilisin:	Stock was diluted 10-fold in 2mM-Ca^{2+} to activate the proteinase. Inhibited with 1.15mM-PMSF.
Thermolysin:	Inhibited with 5mM-EDTA.
Proteinase K:	Stock was diluted 10-fold with digestion buffer containing 1mM-$CaCl_2$, to activate the proteinase.

2.11 Growth of *S. cerevisiae*

2.11.1 Minimal medium (GYNB)

The recipe for yeast minimal growth medium is described below, and the medium was sterilised by autoclaving at 5psi:

2% glucose

0.65% yeast nitrogen base (YNB)

2.11.2 Rich medium (YEPD)

The recipe for rich yeast growth medium is described below, and was sterilised by autoclaving at 5psi:

1% Bacto-yeast extract

2% Bacto-peptone

2% glucose

(2% Bacto-agar for plates)

2.11.3 SCGluc-trp plates

SCGluc-trp is a selective medium which was used to ensure the retention of plasmid Yep52g:ARO1 in *S. cerevisiae* strain BJ1991. (The plasmid contains TRP1). The protocol for making the medium is described below:

Supplements A

10ml of 1.2mg/ml adenine sulphate
5ml of 2.4mg/ml L-histidine HCl
5ml of 2.4mg/ml L-methionine
5ml of 3.6mg/ml L-leucine
5ml of 3.6mg/ml L-lysine-HCl
10ml of 6mg/ml L-glutamic acid
5ml of 45mg/ml L-serine
5ml of 2.4mg/ml uracil
5ml of 2.4mg/ml L-arginine-HCl
20ml of 0.9mg/ml L-tyrosine
5ml of 3.6mg/ml L-isoleucine
10ml of 3mg/ml L-phenylalanine
5ml of 18mg/ml L-valine

Supplements B

15ml of 4mg/ml L-asparagine
5ml of 24mg/ml L-threonine

Agar: 300ml of a 4% suspension of Oxoid No. 1 agar in water.

Broth: 60ml of 10x Yeast nitrogen base (filter sterile)
60ml 20% glucose (autoclaved)
65ml distilled H_2O
15ml total volume Supplements A
The pH was adjusted to 6.4.

The agar and broth solutions were autoclaved for 20 min, and when they had cooled somewhat supplements B were added. The broth and agar solutions were mixed and plates were poured in a laminar flow hood.

2.11.4 Yeast preservation mixture

The yeast preservation mixture described below was used for the long term storage of *S. cerevisiae*:

2X medium

6.3g K_2HPO_4

1.8g KH_2PO_4

0.45g trisodium citrate

0.09g $MgSO_4.7H_2O$

0.9g ammonium sulphate

200ml glycerol

This was made up to 500ml with distilled water and autoclaved. One volume of preservation mixture was added to an *S. cerevisiae* overnight culture in YEPD, and mixed. This was frozen with dry ice/ethanol, and stored at -80°C.

2.11.5 Growth conditions for *S. cerevisiae*

An 150ml overnight culture was innoculated from a single colony and grown with vigorous aeration on an orbital shaker at 30°C. This was used to innoculate 3 litres of medium in a 10 litre flask which was stirred vigorously and pumped with a forced draught of air at 30°C. The absorbance at 600nm was monitored and a growth curve constructed. Cells were harvested by centrifugation at 4, 500*g* at 4°C, washed with chilled distilled water, then re-pelleted by centrifugation. Harvested cells were stored frozen at -20°C or -80°C.

2.11.6 Growth conditions for *S. cerevisiae* BJ1991 containing the plasmid Yep52g:ARO1 for the overproduction of AROM

S. cerevisiae BJ1991 transformed with Yep52g:ARO1 were grown on rich medium (YEPD) to A_{600}=1.0 at 30°C. AROM induction was achieved by the addition of $CuSO_4$ to a final concentration of 20μM, and the cells were cultured for a further 3 h before harvesting.

2.12 Growth of *A. nidulans*

2.12.1 *Aspergillus* minimal medium (AMM)

The recipe for *Aspergillus* minimal medium is described below:

6g sodium nitrate

0.52g KCl

1.52g KH_2PO_4

This was made up to 1 litre after adjusting the pH to pH6.5 with sodium hydroxide. It was autoclaved at 15psi.

2.12.2 Medium for the growth of *Aspergillus nidulans* R153 and 1314

The recipe for the minimal growth medium used for the growth of *A. nidulans* R153 and 1314 is described below:

Trace element solution:

100mg $FeSO_4.7H_2O$

880mg $ZnSO_4.7H_2O$

40mg $CuSO_4.5H_2O$

15mg $MnSO_4.\ 4H_2O$

10mg $NaB_4O_7.10H_2O$

5mg $(NH_4)_6Mo7O_{24}.10H_2O$

The trace element solution was made up to 100ml with water and adjusted to pH2 to dissolve the salts. It was then filter sterilised.

Pyridoxine: A 50mg/ml stock solution was autoclaved.

$MgSO_4$: Filter sterilised 25% (w/v) stock solution.

Quinate A 20% (w/v) stock solution was adjusted to pH6.5 with sodium hydroxide and autoclaved at 15psi.

Medium: 400ml of AMM with 8ml of 20% (w/v) glucose (filter sterile), 0.8ml 25% (w/v) $MgSO_4$, 0.4ml pyridoxine stock solution and 0.4ml of trace element solution.

For agar plates 6g of agar was added to 400ml of AMM and autoclaved. 8ml of 20% (w/v) glucose, 0.8ml 25% (w/v) $MgSO_4$, 0.4ml pyridoxine stock solution and 0.4ml of trace element solution were added to partially cooled medium.

2.12.3 Spore Growth

The Tween-saline solution was used to liberate *A. nidulans* spores from the mycelia.

Tween-saline: 0.8% (w/v) NaCl
0.025% (v/v) Tween 20
This was autoclaved at 15psi.

A loop of Tween-saline solution was used to collect spores from a single plate colony of *A. nidulans*. The spores were streaked on plates of minimal medium with the appropriate supplements (2.12.2) and grown inverted at 37°C.

For the large scale production, spores from a single colony were collected on a loop of Tween-saline and transferred to an Eppendorf tube containing the same solution. The spores were pelleted using a microfuge and excess liquid was removed. The remaining spore suspension was spread evenly over an agar plate using a glass spreader. This was incubated upside down at 37°C for 3-4 days. The yield was approximately $3x10^8$ spores/plate

2.12.4 Harvesting spores

Tween-saline solution was added to the top of the agar plate covered in mycelia and the spores were loosened using a sterile cell scraper (normally used for animal cell culture). The spore suspension was collected in a sterile universal

container and centrifuged at 3,000 rpm on a Beckman model TJ-6 in order to concentrate the spores. The top layer of Tween-saline solution was replaced with 10mM-potassium phosphate buffer pH7.2. This process was repeated several times to replace all the Tween-saline solution with phosphate buffer. The spore solution is stable for 1 month at 4°C.

2.12.5 Estimation of spore numbers

The relationship between the number of spores and the absorbance at 500nm is linear, and the spore concentration was estimated knowing that $A_{500} = 0.4$ is equal to 3×10^6 spores/ml.

2.12.6 Growth conditions for *A. nidulans* 1314: Method 1

The method of growth for AROM induction in *A. nidulans* 1314 was also used for the growth of R153 (since it was only grown for comparative analysis).

Aspergillus minimal medium supplemented with 0.4% (w/v) glucose, 0.05% (w/v) $MgSO_4$, 0.1% (v/v) trace element solution and 0.05% (w/v) pyridoxine, was innoculated 1×10^6 spores/ml This was grown with vigorous aeration at 37°C for 17 h. Mycelia were harvested by filtration through muslin, washed with room temperature distilled water then transferred to minimal medium with 0.1% (w/v) quinate, 0.2% (v/v) glycerol, 0.1% (v/v) trace element solution, 0.05% (w/v) $MgSO_4$ and 0.05% (w/v) pyridoxine for a further 5 h growth at 37°C with aeration. Mycelia were harvested by filtration and washed with distilled water.

Small scale production was carried out in 2 litre baffled flasks on an orbital shaker, each with 500ml of medium. Harvested mycelia were stored at -80°C.

Large scale production of mycelia were carried out in 10 litre flasks, each with 2 litres of medium, stirred vigorously and pumped with a forced draught of air. Harvested mycelia were lyophilised then broken into a fine powder in a Waring blender and stored at -80°C.

2.12.7 Growth conditions for *A. nidulans* : Method 2

The above method of growth was used for AROM induction in *A. nidulans* strain 1314.

The second method of *A. nidulans* growth was adopted later in the study and involved the construction of a growth curve on medium supplemented with glucose:

Construction of an *A. nidulans* growth curve

Mycelia growth in minimal medium with glucose was monitored by following the depletion of glucose. The glucose concentration was determined by the GOD-Perid method in which the oxidation of glucose by glucose oxidase is used to elicit a measureable colour change. 200µl samples of appropriate dilution were added to 5ml of GOD-Perid solution, shaken, and incubated at room temperature in the dark for 25 min. A blank was prepared using 200µl of distilled water, and a standard with glucose solution of known concentration was similarly incubated with GOD-Perid solution. The absorbance was measured at 610nm and the glucose concentration calculated according to the following equation:

$$\frac{A_{sample}}{A_{standard}} \times 0.505 \times N \text{ (mM)},$$

where N is the dilution factor

In this way growth curves for *Aspergillus* were constructed.

Growth conditions

Minimal medium with 0.4% (w/v) glucose, 0.1% (v/v) trace element solution, 0.05% (w/v) $MgSO_4$ and 0.05% (w/v) pyridoxine was innoculated with 1×10^6 spores/ml. Mycelia were grown in 2 litre baffled flasks on an orbital shaker with 200ml of media per flask. Growth was monitored by the measurement of glucose concentration in the media by the GOD-Perid method described above. Log

phase cells were harvested by filtering through muslin, washed with distilled water and transferred to minimal medium with 0.2% (w/v) quinate, 0.2% glycerol, 0.1% (v/v) trace element solution, 0.05% (w/v) $MgSO_4$ and 0.05% (w/v) pyridoxine, for a further 7 h growth. Mycelia were harvested by filtration and washed with distilled water, then lyophilised until dry. The cells were broken into a fine powder in a Waring blender and stored at -80°C.

2.12.8 Long term storage of *Aspergillus nidulans*

A. nidulans strains were stored on silica for long term storage, as described below:

1. Sterile glass universal containers were half-filled with silica gel granules. These were heated in an oven overnight at 180°C with tinfoil covers. They were allowed to cool and covered with lids that had been autoclaved.
2. Reconstituted non-fat dried milk (Marvel) was autoclaved.
3. 1ml of milk was added to an agar slope of *A. nidulans*, and vortexed to create a spore suspension.
4. 0.5ml of the spore suspension was added to the universal container with the silica gel, pipetting it evenly over the silica granules. This was left on ice for 10 min to dissipate the heat from the reaction.
5. The *A. nidulans* viability was tested after 5-7 days storage at room temperature. The universal containers were taped up in an air tight container with humidity indicating silica gel and stored at 4°C.

Chapter 3 Purification of AROM from *Saccharomyces cerevisiae*

Chapter 3 Purification of AROM from *Saccharomyces cerevisiae*

3.1 Introduction

3.1.1 Aims

The original objective of the project was to purify and characterise AROM from *S. cerevisiae*. This was intended to complement the concurrent work of Dr. L. Graham in our laboratory who was studying AROM domain expression in *E. coli* and *S. cerevisiae* (see 1.7.5). This would allow a comparative study to be made between functional fragments of AROM and the intact protein.

The chapter describes the steps developed for the purification of AROM from *S. cerevisiae* and the anti-proteinase strategies adopted as it became apparent that endogenous proteinases were a major problem. During the development of the purification scheme, L. Graham successfully overexpressed AROM in *S. cerevisiae* using a ubiquitin fusion vector, and the purification and characterisation of AROM from this strain is described in Section 3.5.

3.1.2 General strategy for the purification of AROM from *S. cerevisiae*

Proteolysis was a very serious problem in the purification of AROM from *N. crassa* and it was some time before a protocol was developed which resulted in the purification of intact AROM (see 1.6.2). Proteolysis is also known to be a serious problem in protein purification from *S. cerevisiae* and has led to many proteolytic artefacts, such as 'isoenzymes', being isolated and reported (Pringle, 1975). Therefore, right from the start of AROM purification from *S. cerevisiae* the proteinase inhibitors EDTA, benzamidine, PMSF and leupeptin were included in the buffers wherever possible.

The intact AROM purified by Boocock (1983) from *N. crassa* was found to lose DHQ synthase and EPSP synthase activity. The purification scheme was modified, to optimise the co-purification of all five AROM activities, when it was discovered that DHQ synthase is a metallo-enzyme (Lambert *et al.*, 1985). The EPSP

synthase activity was also found to be very sensitive to oxidation (Boocock, 1983; Coggins *et al.*, 1987a). The buffers used in the purification were supplemented with zinc for the DHQ synthase activity and ß-mercaptoethanol was regularly added for the EPSP synthase activity. Using this approach the five AROM enzymes co-purified with a constant activity ratio. At this preliminary stage in the development of an AROM purification scheme for *S. cerevisiae* the buffers were supplemented with DTT to act as a reducing agent but not zinc because the initial emphasis was on the purification of unproteolysed AROM. It was hoped that a purification protocol would be developed producing intact AROM which could later be optimised for each of the individual AROM activities. Hence, only one AROM activity was followed during the purification.

The activity ratio of a multifunctional protein can be used to compare the individual component activities of the protein. It can give an indication of the integrity of a protein by comparing the activity ratio in crude extract with that of a purified sample, such as has been done for *N. crassa* AROM. As mentioned above for *N. crassa* AROM, all five AROM activities co-purified with a constant activity ratio (Boocock, 1983; Lambert *et al.*, 1985; Coggins *et al.*, 1987a). However, things are not always as straightforward. In some cases the activity ratio might alter during the purification of a protein because of the loss of an inhibitor of one of the activities. Thus, one must be careful not to read too much into activity ratio values. The DHQase activity of *N. crassa* AROM was found to be the most stable AROM activity and was given the arbitrary value of 100 (Boocock,1983; Lambert *et al.*, 1985; Boocock *et al.*, 1987a). Therefore, in keeping with this precedent, the *S. cerevisiae* AROM activities were also standardised against the DHQase activity. In Chapter 4, the activity ratio of *A. nidulans* AROM has been normalised against both the shikimate DH and shikimate kinase activities (see 4.2.4 and 4.6.5).

3.2 Exploratory steps for the purification of AROM from *S. cerevisiae*

3.2.1 Introduction

The AROM gene, *ARO1*, was originally cloned from the haploid, wild-type *S. cerevisae* strain S228C by Larimer *et al.* (1983). It was subsequently sequenced by Duncan *et al.*(1987), and the subunit molecular weight of the protein was estimated to be 175kDa. This clone was used by L. Graham for AROM domain expression studies. Therefore, the initial attempts to purify naturally expressed AROM were from strain S288C. This section describes the purification steps developed and the anti-proteinase strategies adopted.

3.2.2 Preliminary purification of AROM from *S. cerevisiae* S288C

During the purification of AROM from *S. cerevisiae*, the DHQase activity was monitored for several reasons. It is easy to assay and, at least in the case of *N. crassa* AROM, it is the most stable activity (Boocock, 1983). Yeast do not have a quinate utilisation pathway (Berlyn & Giles, 1972) such as that found in fungi (Rines *et al.*, 1969; Hawkins *et al.*,1982a; Hawkins *et al.*, 1982b) and therefore do not possess a catabolic DHQase activity which might complicate the assay. Later in this Section (see 3.2.8 and 3.3.3) I switched to assaying the shikimate DH activity for the routine monitoring of AROM during protein purification. Shikimate DH is also easily assayed and has the advantage over DHQase in *S. cerevisiae* that the assay is more sensitive due to a higher specific activity (see 3.3.2).

The proteinase inhibitor leupeptin is extremely expensive and, although this is not ideal, it was only used in the initial stages of purification and when the protein was in a concentrated state. It was not included in dialysis buffers.

All steps were carried out at 4°C unless otherwise stated. Protein samples were taken during the purification and stored at -20°C for protein determination and analysis by SDS PAGE. The buffers used in the purification are described in Figure 3.1.

Buffer A 100mM-Tris/HCl pH7.5, containing 0.4mM-DTT, 5mM-EDTA, 1.2mM-PMSF, 2mM-benzamidine and 10μM-leupeptin.

Buffer B 50mM-Tris/HCl pH7.5, containing 0.4mM-DTT, 5mM-EDTA and 2mM-benzamidine.

Buffer C 20mM-potassium phosphate buffer pH6.5, containing 0.4mM-DTT, 5mM-EDTA, 2mM-benzamidine and 1μM-pepstatin A.

Figure 3.1 **Buffers used in the purification of AROM from *S. cerevisiae***

3.2.3 Cell Growth

S. cerevisiae S288C was grown according to the method described in Section 2.11.6, on minimal medium (GYNB) at 30°C until the A_{600} reached 10. Harvested cells were stored at -20°C.

3.2.4 Extraction

Cells were suspended in 3 volumes of buffer A, and broken by 3 passages through a French pressure cell (6550kPa). The cell debris was removed by centrifugation at 100, 000g for 2 h.

3.2.5 Ammonium sulphate fractionation

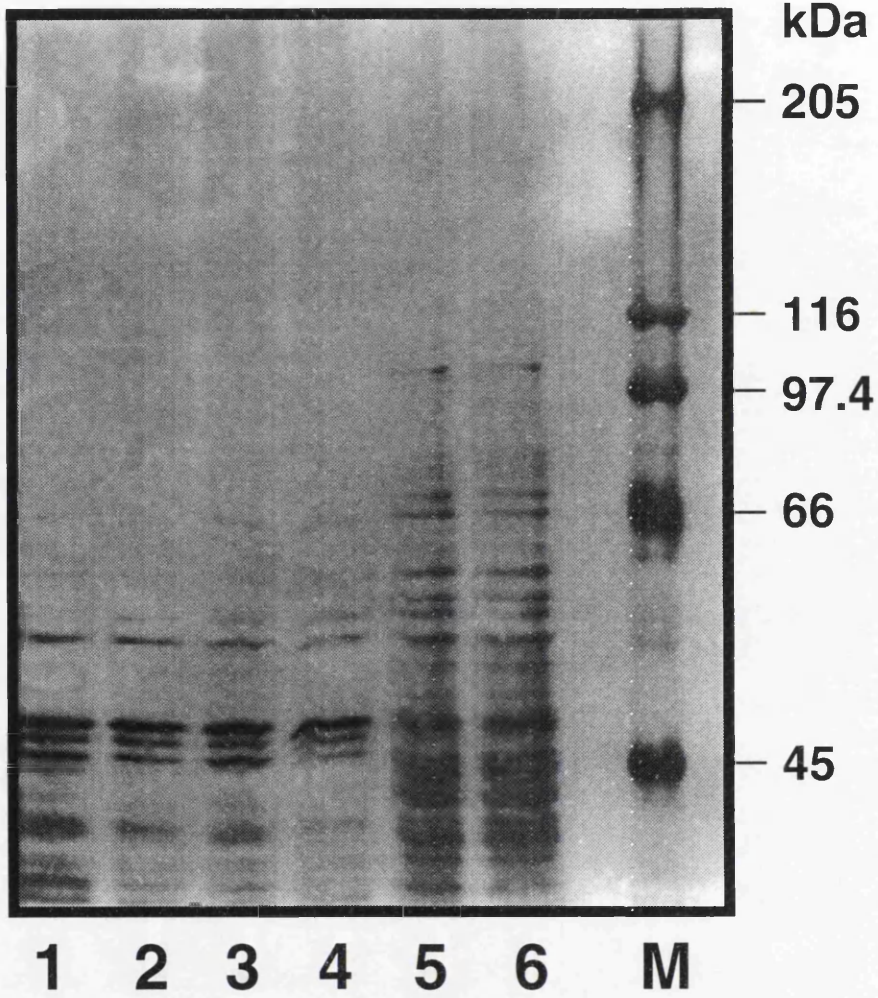
The initial ammonium sulphate cut was 0-30% saturation. AROM was found to precipitate in the 30-50% saturation range. Pelleted protein was resuspended in buffer B containing 10μM-leupeptin and 1.2mM-PMSF and dialysed overnight into buffer B containing 1.2mM-PMSF. The ammonium sul-phate fractionation yield varied between 56% and 81% in three separate experiments with between 2- and 12-fold purification. As much as 65% of this activity was lost during dialysis.

Figure 3.2 shows the ammonium sulphate fractionation of AROM from *S. cerevisiae* S288C analysed by SDS PAGE and stained with silver for protein visualisation. The gel shows the protein present in each of the ammonium sulphate fractions. There are no high molecular weight proteins in the sample lanes and no protein of the expected subunit molecular weight of AROM (175kDa). The gel also required an apparently massive protein loading for protein visualisation. Clearly extensive proteolysis has occurred since no high molecular weight species remain in the samples. Some of the proteolysis may have occurred during the preparation of protein samples for SDS PAGE. It is known that some proteinases are activated in SDS, and a gradual increase in heat as the protein samples are boiled in SDS PAGE sample buffer facilitates rapid protein degradation. This phenomenon produces

Figure 3.2 **SDS PAGE analysis of ammonium sulphate fractionation of AROM from *S.cerevisiae* S288C**

Protein samples were taken containing during ammonium sulphate fractionation of AROM from *S. cerevisiae* S288C and stored at -20°C. The protein concentration was determined by the method of Bradford (1976). Protein samples were analysed by SDS PAGE on a 7% gel and stained by silver for protein visualisation.

M	molecular weight markers: 205kDa, 116kDa, 97.4kDa, 66kDa and 45kDa.
Lane 1.	clarified extract, 26μg protein.
Lane 2.	0-30% saturation $(NH_4)_2SO_4$ supernatant, 28μg protein.
Lane 3.	0-30% saturation $(NH_4)_2SO_4$ pellet, 24μg protein.
Lane 4.	30-50% saturation $(NH_4)_2SO_4$ supernatant, 47μg protein.
Lane 5.	30-50% saturation $(NH_4)_2SO_4$ pellet, 50μg protein.
Lane 6.	dialysed 30-50% saturation $(NH_4)_2SO_4$ pellet, 73μg protein.


kDa
205
116
97.4
66
45
1 2 3 4 5 6 M

proteolytic artefacts with *S. cerevisiae* proteins and results in a similar gel to that observed in Figure 3.2 (Pringle, 1976; North, 1989). So, protein samples taken during the purification of AROM were mixed immediately with SDS PAGE sample buffer and plunged into a boiling water bath for 5 min. This should immediately inactivate the proteinases and prevent proteolytic artefacts being produced during the denaturation process. The boiled protein samples were stored at -80°C along with separate samples taken for protein determinations.

3.2.6 Chromatography of AROM on mono Q

A mono Q FPLC anion exchange chromatography column (HR5/5 or 10/10) was equilibrated at room temperature in buffer B. The buffer did not contain PMSF because it has a short half-life at room temperature. There was also the worry that it would precipitate in the buffer during the chromatography and block the column. AROM was loaded at 1ml/min and the protein concentration in the eluent was allowed to fall to zero. AROM was eluted with a continuous linear gradient of 0-350mM-NaCl in buffer B (Figure 3.3). AROM eluted between 185mM and 215mM-NaCl in buffer B with the peak fraction eluting at 200mM-NaCl in buffer B. The yield for this step ranged, in four separate experiments, between 51% and 68% with a 6- to 18-fold purification. AROM was dialysed into buffer B containing 1.2mM-PMSF, however, as much as 66% of the activity was lost on dialysis.

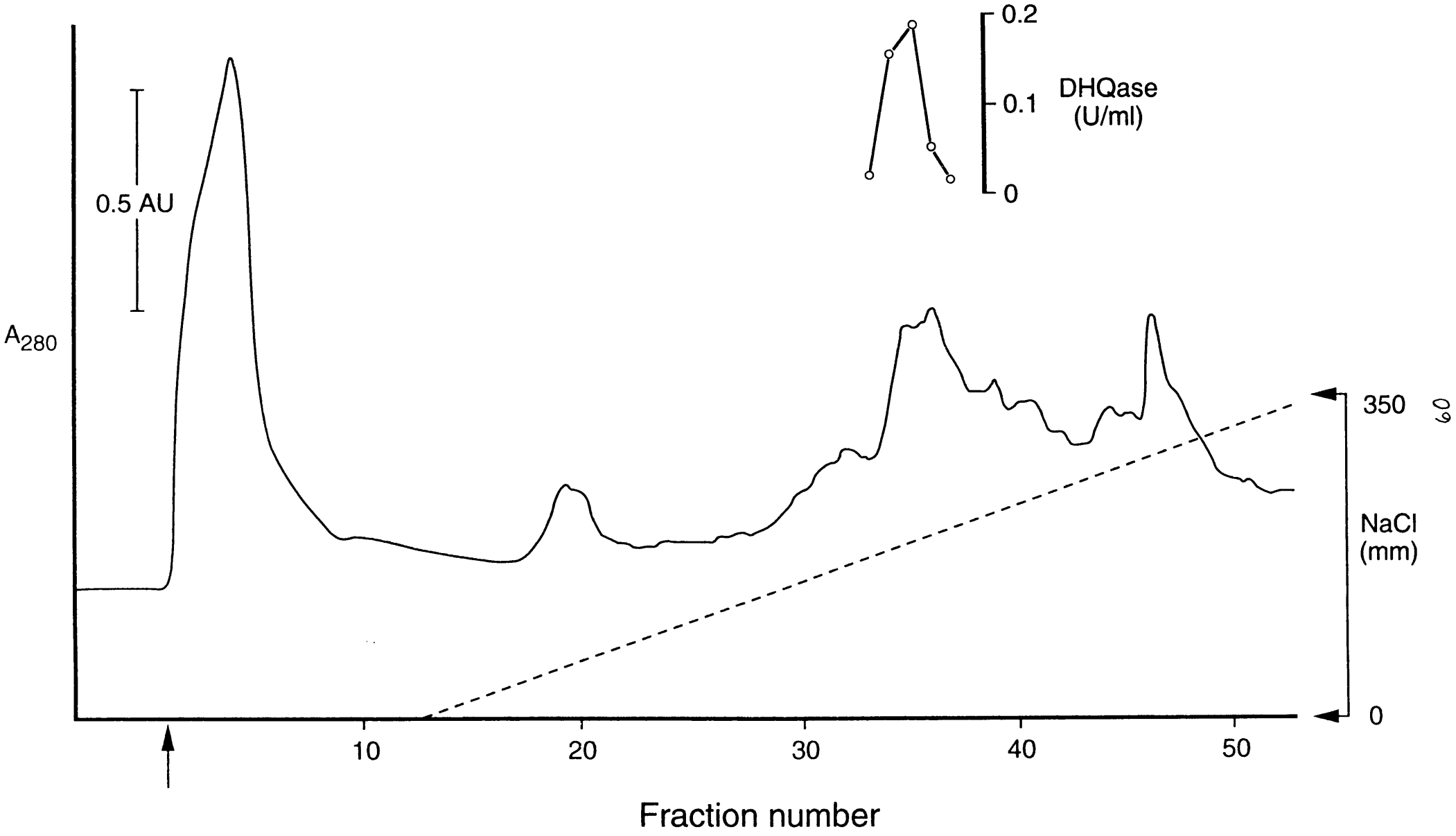
3.2.7 Superose gel filtration chromatography of AROM

A Superose 6 FPLC gel filtration column was equilibrated in buffer B at room temperature. AROM was loaded at 0.3ml/min and 0.9ml fractions were collected. No DHQase activity was detected in the column eluent. Purified *N. crassa* AROM was applied to both Superose 6 and Superose 12 FPLC chromatography columns under identical conditions, however, no DHQase activity was detected in the eluent. In later attempts, *S. cerevisiae* AROM again failed to elute from Superose gel filtration columns. Superose gel filtration was

Figure 3.3 Chromatography of AROM on mono Q

A mono Q (HR5/5) FPLC anion exchange column was equilibrated in buffer B, at room temperature. Filtered sample was loaded, then washed with buffer B at 1ml/min until the A_{280} had fallen to zero. AROM was eluted with a continuous linear gradient of 0-350mM-NaCl in buffer B and 0.5ml fractions were collected. The remaining protein was eluted with a steeper continuous linear gradient of 350mM-1M-NaCl in buffer B.

The protein concentration was monitored continuously at 280nm during the chromatography and the DHQase activity of the individual fractions was assayed. The figure also shows the salt gradient.


0.2
0.1
0
DHQase
(U/ml)
0.5 AU
A_{280}
350
0
NaCl
(mm)
10
20
30
40
50
Fraction number

therefore abandoned as a purification step for *S. cerevisiae* AROM. It was later discovered that other groups within the department routinely added 200mM-KCl to their buffers to prevent protein binding to the column. *A. nidulans* AROM was successfully eluted from Superose gel filtration media when 200mM-KCl was added to the buffer (see 4.3.8 and 4.6.2).

3.2.8 Chromatography of AROM on Cibacron Blue F-3GA coupled to Sepharose 4B

The dye binding column, Blue Dextran Sepharose was used as the final step in the purification of AROM from *N. crassa* and the resulting AROM preparation was purified 680-fold compared to the starting material (Boocock, 1983). The Blue Dextran Sepharose used for the purification of *N. crassa* AROM was Cibacron Blue F-3GA coupled to Sepharose 4B by the method of Ryan & Vestling (1974). It was thought likely that *S. cerevisiae* AROM would also be successfully purified on this dye column. For the purification of *S. cerevisiae* AROM Cibacron Blue F-3GA coupled directly to Sepharose 4B (without Dextran)was used.

The AROM pool from mono Q anion exchange chromatography was dialysed into 30mM-KCl in buffer B containing 1.2mM-PMSF and 1μM-pepstatin A. The pepstatin A was added in later purification schemes as a proteinase inhibitor and is described in Section 3.3.1. The Cibacron Blue F-3GA column was equilibrated with 30mM-KCl in buffer B containing 1.2mM-PMSF and 1μM-pepstatin A and AROM was loaded at 5ml/h. Figure 3.4 shows the elution profile for AROM. The column was washed with a salt step of 0.5M-KCl in buffer B containing 1.2mM-PMSF and 1μM-pepstatin A. There was no further protein elution with 1.0M-KCl in buffer B. AROM started to elute from the column with a salt step of 1.5M-KCl in buffer B containg 1.2mM-PMSF and 1μM-pepstatin A and the remaining AROM was desorbed with 2.0M-KCl in buffer B. The protein concentration of the pooled protein was too low to be determined. The AROM eluting from the blue dye column when wild-type *S. cerevisiae* was used for the purification, was only visualised after SDS

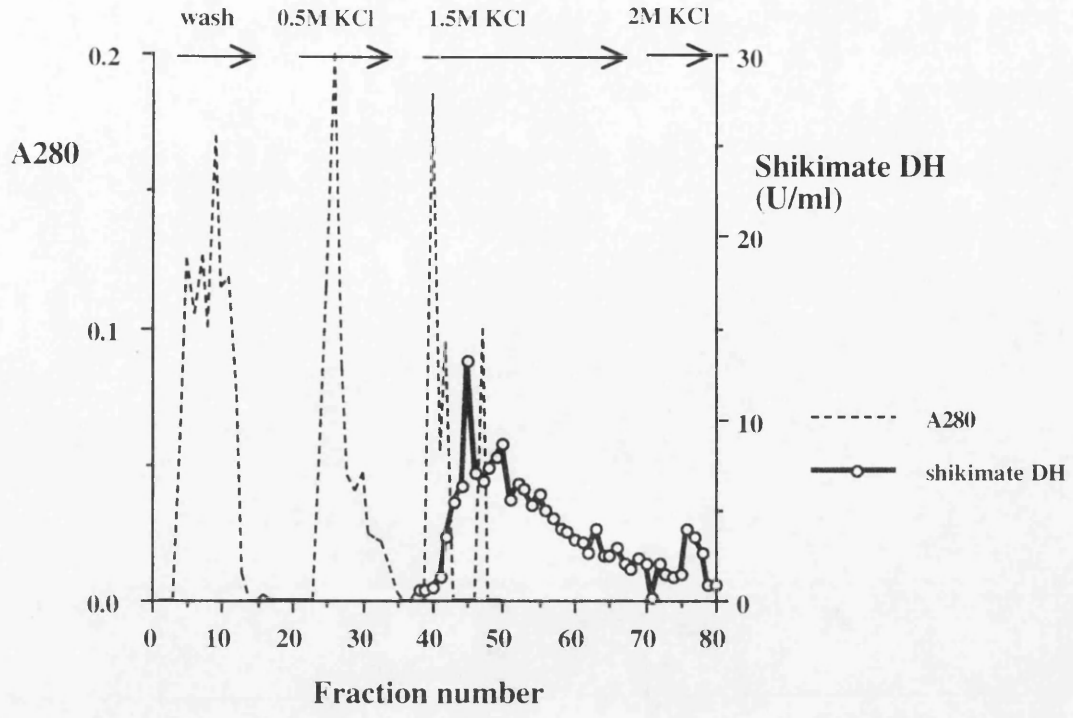


Figure 3.4 **Chromatography of AROM on Cibacron Blue F-3GA**

A 2ml column of Cibacron Blue F-3GA on Sepharose 4B, in a 2ml disposable syringe, was equilibrated with 30mM-KCl in buffer B containing 1.2mM-PMSF and 1μM-pepstatin A. Sample was loaded at 5ml/h and washed with equilibration buffer until the A_{280} had dropped to zero. The column was washed with 0.5M-KCl in buffer B containing 1.2mM-PMSF and 1μM-pepstatin A until the A_{280} had fallen. It was then washed with 1.0M-KCl in buffer B containing 1.2mM-PMSF and 1μM-pepstatin A but no further protein was eluted. AROM was eluted with 1.5M-KCl in buffer B containing 1.2mM-PMSF and 1μM-pepstatin A. 0.6ml fractions were collected and the protein concentration was measured at 280nm. The shikimate DH activity of individual fractions was assayed.

PAGE when the protein was lyophilised to concentrate it. A typical AROM preparation after chromatography on Cibacron Blue analysed by SDS PAGE is shown later in Figure 3.6. The AROM yield in this case was 60% and analysis of the elution profile shows that it had been purified significantly. On dialysis 70% of the shikimate DH activity was lost and only 6% of the protein eluted from the column was recovered after vacuum dialysis to concentrate the protein. The massive activity loss on dialysis did not necessarily result from proteolysis but may have been due to the very dilute protein 'sticking' to the dialysis tubing. This can be a severe problem when dealing with such small quantities of protein. It can be overcome by coating the dialysis tubing, and other vessels that the protein is in contact with, in BSA. However, this contaminates the protein and must be removed later if pure protein is required.

The DHQase activity could not always be detected on elution from the Blue dye column because of the low levels of AROM remaining at this stage in the purification. The shikimate DH activity of *S. cerevisiae* AROM has a higher specific activity than DHQase (see 3.3.2) and can be detected at very low levels when the DHQase activity cannot. Therefore, the shikimate DH rather than DHQase activity was monitored during subsequent AROM purifications.

3.3 Anti-proteinase strategies adopted for the purification of AROM from *S. cerevisiae*

3.3.1 General measures

The massive activity losses, thought to be due to endogenous proteinases, which occured during the purification of AROM from *S. cerevisiae*, made progress very slow. Several general measures were introduced in an attempt to reduce the effects of proteinases in the extracts and also to reduce the proteinase levels themselves.

The proteinase inhibitor pepstatin A inhibits some aspartic proteinases such as the vacuolar proteinase yscA of *S. cerevisiae*. The inhibitor was added to all the buffers used in the purification, at a concentration of 1μM.

Breaking yeast cells with a French pressure cell was found to be very time consuming and it was difficult to maintain a low temperature in the pressure cell. The extraction was made faster by reducing the volume of extraction buffer to one volume rather than three volumes. The extract was also centrifuged for 1 h rather than 2 h to remove cell debris to reduce the processing time of the crude extract. As I have already described in Section 3.2.5, protein samples were taken during AROM purification for SDS PAGE analysis, and boiled immediately in SDS PAGE sample buffer. This ensured that the fresh proteinase inhibitors in the sample were still active in the samples during protein denaturation.

Attempts were made to minimise the levels of proteinase production in *S. cerevisiae* by several means. The levels of proteinase production in *S. cerevisiae* are lower in cells grown on rich medium rather than minimal medium (Pringle, 1975; Jones, 1991). Also, with the exception of carboxypeptidase yscY, the levels of vacuolar proteinases increase as the cells reach the stationary phase of growth (Jones, 1991). Section 3.3.2 examines the levels of AROM production in *S. cerevisiae* grown on minimal and rich media. A growth curve was constructed and cells in the log phase of growth were harvested in order to reduce proteinase levels. The purification of AROM from an *S. cerevisiae* strain which lacks four vacuolar proteinases is described in Section 3.3.3. Section 3.3.4 describes the use of a 'negative' anion exchange chromatography column which it was hoped might remove some proteinases. This later strategy had proved useful during the purification of AROM from *N. crassa* (Lumsden & Coggins, 1977).

3.3.2 Comparison of AROM activity levels in *S. cerevisiae* S288C grown on rich and minimal medium

The levels of AROM production were examined at different stages of growth on both minimal and rich (YEPD) medium.

Figure 3.5(a) shows that the optical density of stationary phase *S. cerevisiae* grown in rich medium is more than double that of cells grown in minimal medium. At point (1) on the growth curve, after 8.5 h growth, the optical density of the cells grown on the rich medium was 3.6-fold higher than those grown on minimal medium. Therefore the yield of *S. cerevisiae* is, as expected, is greater when the cells are grown on rich medium. The figure also shows that the cells previously grown on minimal medium to an A_{600} of 10 had already reached the stationary phase of growth. Figure 3.5(b) shows the shikimate DH and DHQase activity levels of AROM from *S. cerevisiae* S288C during both the log phase and the stationary phase of growth on both rich and minimal medium. The figure shows that the shikimate DH activity is much higher than the DHQase activity. This is very different from the levels found in *N. crassa* and *A. nidulans* AROM which show a DHQase:shikimate DH activity ratio of 100:266 in the former (Boocock, 1983) and 100:117 in the latter (Table 4.4). The activity ratio in crude extract in this experiment varies between 100:560 to 100:2200. The variability is probably due to error because of the very low activity levels in crude extract. It is quite clear, however, that the shikimate DH activity is substantially higher compared to DHQase than in *N. crassa* and *A. nidulans*. This is discussed further in Chapter 5.

Figure 3.5(b) also shows that the specific activities of the AROM enzymes from *S. cerevisiae* S288C grown on minimal medium, to the mid-log phase of growth, are higher than those from cells harvested at the stationary phase of growth and those from cells grown on rich medium. For cells grown on minimal medium the shikimate DH activity decreases 4-fold between the log phase of growth and approaching stationary phase. For cells grown on rich medium, the shikimate DH activity apparently increases in the stationary phase of growth compared to the log

Figure 3.5 **Comparison of the AROM activity levels in *S. cerevisiae* S288C grown on minimal and rich medium**

(a) 'Growth curve'

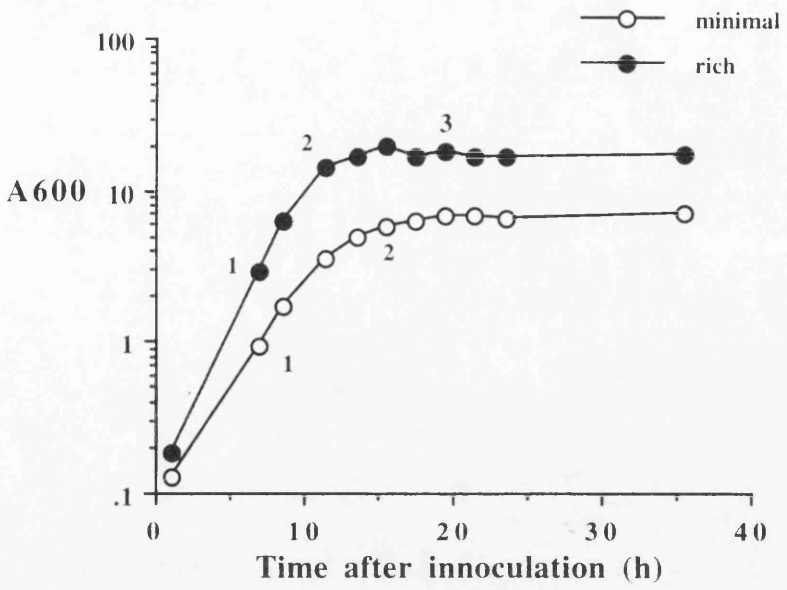
Overnight cultures of *S. cerevisiae* S288C were used to innoculate one flask containing minimal medium and another containing rich (YEPD) medium (see 2.11.1 and 2.11.2). The yeast were grown at 30°C with vigorous stirring and pumped air. The absorbance at 600nm was measured at regular intervals during the growth and the figure shows the A_{600} plotted on a logarithmic scale versus the time after innoculation. 50ml aliquots were taken at intervals during the growth and centrifuged at 35,000g for 10 min at 4°C, washed with distilled water and re-pelleted. The cells were stored at -20°C. The points during the growth of yeast where these samples were taken are numbered and are shown on Figure (a).

(b) 'DHQase and shikimate DH activity levels'

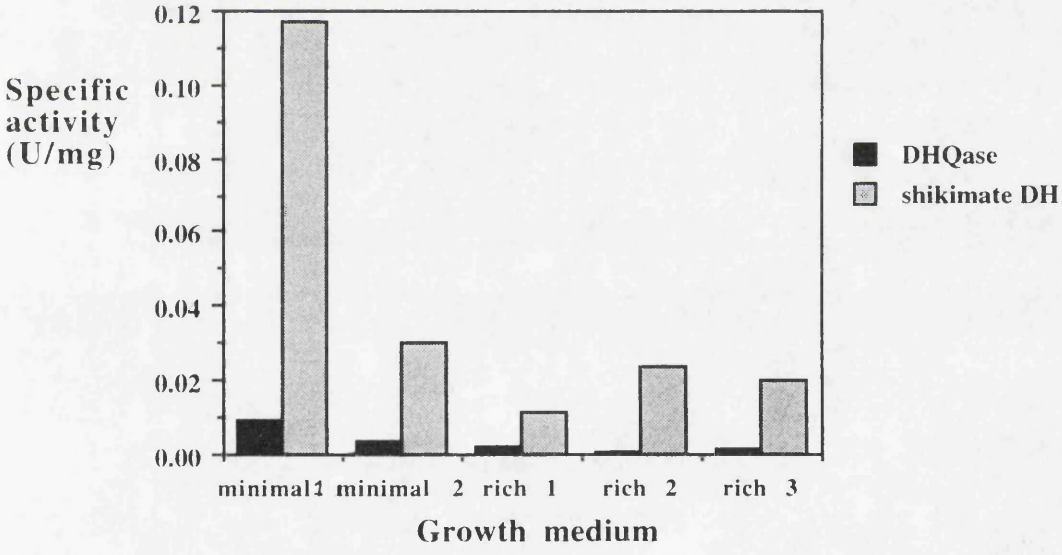
Yeast samples were taken during the exponential phase of growth described in part (a) above (labelled 1 in Figure (a)) and on entering the stationary phase of growth (labelled 2 and 3 in Figure (a)). The cells were resuspended in a total volume of 1.5ml of buffer A and broken by 3 passages through a mini French pressure cell (6550kPa). The extracts were centrifuged for 30 min at 4°C using a microfuge, and the supernatants were assayed for both DHQase and shikimate DH activity. The protein concentration was determined by the method of Bradford (1976).

The figure shows the shikimate DH and DHQase activity levels in extracts from *S. cerevisiae* S288C grown on minimal and rich medium.

(a) 'Growth curve'



(b) 'DHQase and shikimate DH activity levels'



phase, although the effect is not as pronounced, and the DHQase apparently decreased although the DHQase activity was difficult to detect in crude extracts.

The *ARO1* gene encoding AROM in *S. cerevisiae* has been shown to be under the general amino acid control system found in yeast (Duncan *et al.*, 1988). The general control system is described in Section 1.3. Duncan *et al.* (1988) showed that the levels of AROM transcription increased on derepression, and the DHQase and shikimate DH specific activities increased 2 to 3-fold. There is a mistake in the printed DHQase specific activities reported by Duncan *et al.*, (1988) which are 1000-fold too high.

The rich medium used for *S. cerevisiae* growth contains peptone and amino acids and these repress the levels of transcription of the enzymes under general control. Hence, the levels of AROM production in cells grown on rich medium are lower than the levels in cells grown on minimal medium during the logarithmic phase of growth. The levels of AROM production, from the specific AROM activities, had fallen by the time the cells grown on minimal medium were approaching the stationary phase of growth. The shikimate DH and DHQase specific activities fell 3.9-fold and 2.8-fold respectively between the logarithmic phase of growth on minimal medium and the stationary phase. The apparent difference between the two activities is probably due to the low DHQase activities in the crude extracts.

Although the levels of AROM production are lower in cells grown on rich growth medium, the cell yield is higher than on minimal medium. For the rest of this study *S. cerevisae* was grown on rich growth medium to the mid-log phase of growth to minimise proteinase levels in the cells.

The purification of AROM from *S. cerevisiae* S288C grown in this new way made no noticeable difference to the stability of AROM during the purification. The new growth regime was kept but it was clear that other anti-proteinase strategies had to be adopted in order purify AROM from *S. cerevisiae*. One approach was to purify AROM from a strain of *S. cerevisiae* which lacks several vacuolar proteinases.

3.3.3 Purification of AROM from *S. cerevisiae* strain ABYS106 lacking several vacuolar proteinases

S. cerevisiae strain ABYS106 was the gift of Dr. M. Stark (University of Dundee) and lacks four vacuolar proteinases: proteinase yscA, proteinase yscB, carboxypeptidase yscY and carboxypeptidase yscS. A growth curve was constructed with cells grown on rich medium and after large scale growth, ABYS106 cells were harvested at the mid-log phase. The cells were stored at -20°C. No pepstatin A was used at this stage in the development of the purification scheme.

19.6g wet weight of cells were broken and AROM was purified by ammonium sulphate fractionation, Mono Q anion exchange chromatography and Cibacron blue chromatography as described in Sections 3.2.4, 3.2.5, 3.2.6 and 3.2.8 respectively. Only a tiny amount of AROM eluted from the Blue dye column and this was lyophilised for visualisation by silver staining of a gel after SDS PAGE

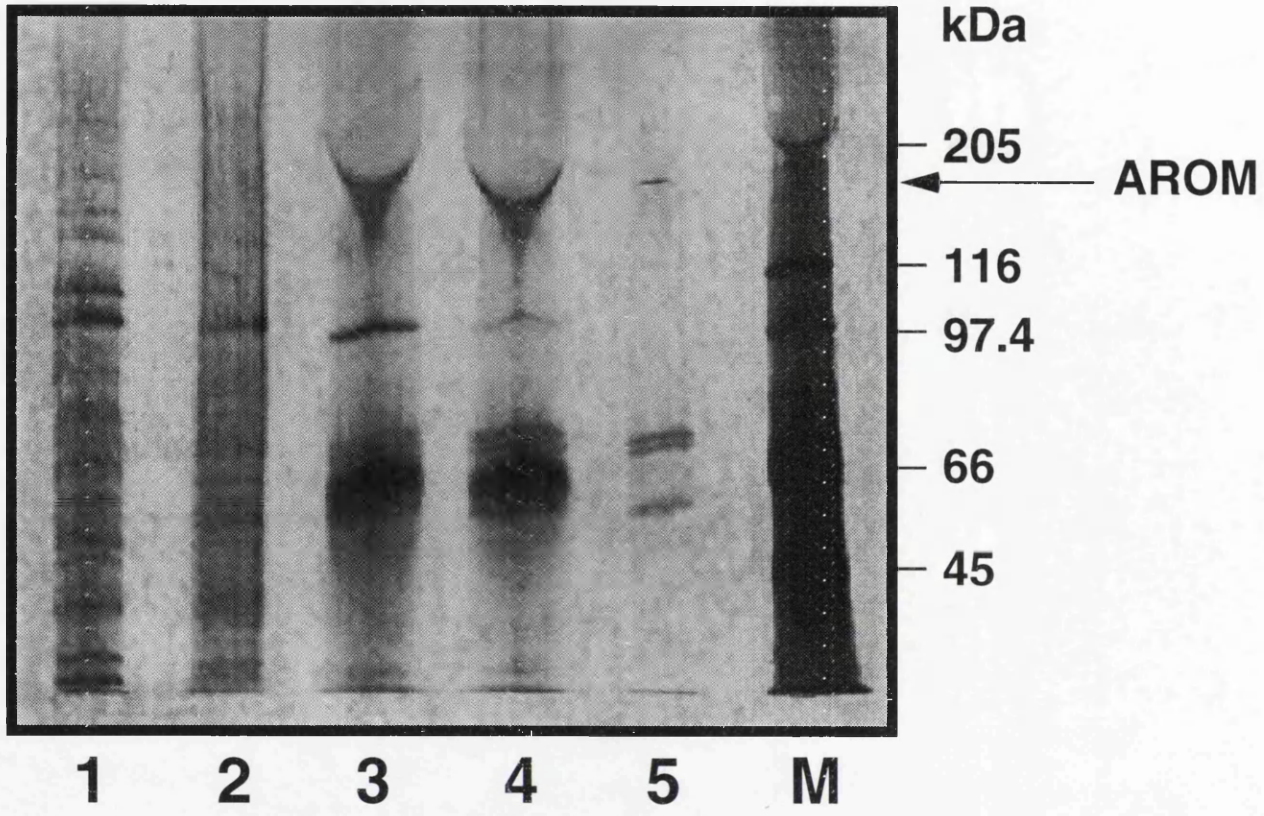
The DHQase activity apparently increased to 133% of the starting activity on ammonium sulphate fractionation. The reason for this is unclear, although it is possible that an inhibitor of the DHQase activity had been removed during the fractionation. There was a large activity loss when the ammonium sulphate precipitated protein was dialysed and only 39% of the starting activity remained. This was reduced to 6% of the starting activity after chromatography on Mono Q. No DHQase activity was found in fractions eluted from the Cibacron Blue dye column, but two fractions which were eluted with 1.5M-KCl in buffer B were found to have shikimate DH activity. This is consistent with the observation, discussed above, that the shikimate DH specific activity is much higher in *S. cerevisiae* than the DHQase specific activity. The specific activity (shikimate DH) of the protein apparently fell during the purification.

The analysis of the AROM fractions from Mono Q anion exchange chromatography and Cibacron Blue chromatography by SDS PAGE is shown in Figure 3.6. The major high molecular weight protein observed on the gel in the Cibacron Blue fractions runs in the correct position for AROM (175kDa). The gel

Figure 3.6 **SDS PAGE analysis of AROM purified from *S. cerevisiae* ABYS106**

Samples of protein were taken during the purification of AROM from *S. cerevisiae* ABYS106 and boiled immediately in SDS PAGE sample buffer, then stored at -80°C. Protein from the Cibacron Blue dye column was very dilute and was lyophilised to concentrate the protein. There was only sufficient lyophilised protein to analyse by SDS PAGE and not enough for a protein determination. The protein concentration in the mono Q eluent fractions were estimated from the trace of A_{280} which was measured continuously during chromatography on mono Q. Re-boiled samples were analysed by SDS PAGE on a 7.5% Phastgel and the protein was visualised by the silver stain method.

M	Molecular weight markers: 205kDa, 116kDa, 97.4kDa, 66kDa and 45kDa, 0.3μg protein.
Lane 1.	mono Q side fraction, 0.3μg protein.
Lane 2.	mono Q pool, 0.2μg protein.
Lane 3.	Cibacron Blue fraction.
Lane 4.	Cibacron Blue fraction.
Lane 5.	*N. crassa* AROM, 0.03μg protein.


kDa
205
AROM
116
97.4
66
45
1
2
3
4
5
M

apparently shows that AROM has been greatly purified by Cibacron Blue chromatography. There are several lower molecular weight proteins also present, the most prominent at 105kDa and 63kDa.

Using the proteinase deficient strain did not lead to a better recovery of AROM activity. The massive DHQase activity losses were similar to those observed when AROM was purified from the wild-type strain S288C. It is possible that some other proteinases present in *S. cerevisiae* have been overproduced to compensate for the loss of the four vacuolar proteinases. Many of the *S. cerevisae* proteinases were not discovered until proteinase deficient strains were isolated (Suarez Rendueles & Wolf, 1988). Since there was no apparent improvement in AROM stability during the purification from the proteinase deficient strain further studies were resumed on the wild-type strain, S288C.

3.3.4 'Negative' anion exchange chromatography of *S. cerevisiae* AROM on DE52

1. Chromatography of AROM on DE52

The major breakthrough in the purification of intact AROM from *N. crassa* was the use of 'negative' chromatography on DE52: AROM does not bind to the column while four endogenous proteinases do bind (Lumsden & Coggins, 1977). It seemed possible that such a chromatography step might also remove *S. cerevisiae* proteinases from the extract. Because all other methods had failed to alleviate the proteinase problem the approach was tried with *S. cerevisiae*.

The conditions for binding of *S. cerevisiae* AROM to DE52 anion exchange medium were determined. A clarified *S. cerevisiae* S288C cell extract was applied to a DE52 anion exchange column equilibrated in buffer B containing 1.2mM-PMSF and 1μM-pepstatin A. AROM was eluted with a continuous linear gradient of 0-300mM-KCl in buffer B containing 1.2mM-PMSF and 1μM-pepstatin A. The elution profile is shown in Figure 3.7. AROM eluted between 55mM and 120mM-KCl with the peak fraction at 65mM-KCl in buffer B. For 'negative'

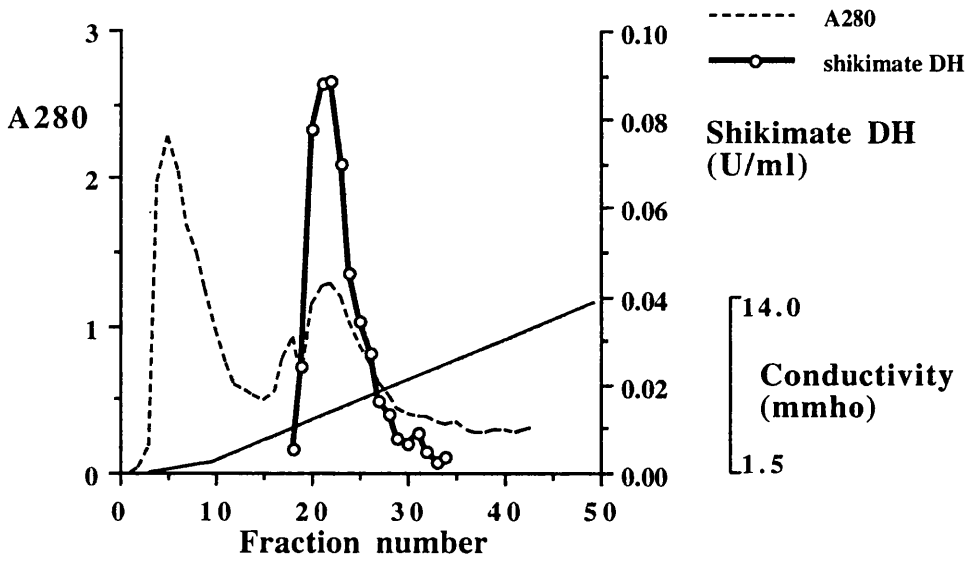


Figure 3.7 **Chromatography of AROM on DE52**

S. cerevisiae S288C was extracted into 2 volumes of buffer A containing 1μM-pepstatin A by 3 passages through a French pressure cell (6550kPa) and centrifuged at 100,000g for 60 min. The supernatant was loaded onto a 10ml DE52 anion exchange column (22mm diameter) which had been equilibrated in buffer B containing 1.2mM-PMSF and 1μM-pepstatin A at 4°C. The column was run at 1ml/min and 1.5ml fractions were collected. The column was washed in buffer B containing 1.2mM-PMSF and 1μM-pepstatin A until the A_{280} had fallen. AROM was eluted with a continuous linear gradient of 0-300mM-KCl in buffer B containing 1.2mM-PMSF and 1μM-pepstatin A and the individual fractions were assayed for shikimate DH activity. The protein concentration was measured at 280nm and the conductivity of fractions was also monitored.

chromatography of AROM on DE52, 75mM-KCl in buffer B was selected. Some AROM binds to the column under these conditions, however, in this preliminary experiment it was decided to sacrifice yield in the hope of achieving stability.

2. AROM purification, from *S. cerevisiae* S288C, incorporating negative anion exchange chromatography on DE52

66g of *S. cerevisiae* S288C (which had been grown on rich medium to mid-log phase) were suspended in 1 volume of buffer A containing 1μM-pepstatin A. Cells were broken by 3 passages through a French pressure cell (6550kPa) and the broken cells were centrifuged at 100,000g for 2 h.

A 200ml (7.5cm diameter) DE52 anion exchange column was equilibrated with 75mM-KCl in buffer B containing 1μM-pepstatin A. The column was flushed with one column volume of 75mM-KCl in buffer B containing 1μM-pepstatin A and fresh PMSF immediately prior to loading the sample. The conductivity of the clarified yeast extract was adjusted to that of 75mM-KCl in buffer B, which had been used to equilibrate the DE52 column, by the addition of 3M-KCl. The extract was then loaded onto the column and 14ml fractions were collected. Fractions containing shikimate DH activity were pooled.

The AROM pool was precipitated by treatment with 30-50% saturation ammonium sulphate. Dialysed AROM was further purified by chromatography on mono Q as described in Section 3.2.6.

There was a 64% recovery from the 'negative' column with 1.3-fold purification. The yield had fallen to 33% after ammonium sulphate fractionation with a 2.6-fold purification. After chromatography on mono Q the yield was 12% with a 9.6-fold purification which was further reduced to 7% after dialysis. The protein eluted from Cibacron Blue was too dilute for the protein concentration to be determined and the yield was reduced to 5%.

AROM was loaded onto Cibacron Blue as described in Section 3.2.8. A salt wash of 1.0M-KCl in buffer B containing 1μM-pepstatin A and 1.2mM-PMSF

was used without a wash of 0.5M-KCl in buffer B. AROM eluted from the column even although it had remained bound in a previous experiment at this salt concentration (see 3.2.8). The salt concentration was increased to 2M-KCl in buffer B to remove the remaining AROM, resulting in 81% recovery from this column. The Cibacron Blue chromatography was later discarded from the purification scheme of AROM because of irreproducible results in several experiments (see 3.5.1). Such irreproducible results were also observed in the purification of AROM from *A. nidulans* 1314 (see 4.3.6).

The 'negative' chromatography on DE52 did not appear to improve the stability of AROM in the preparation and the massive activity losses were probably due to the combination of the delay caused by the extra step early in the purification, and to the large activity loss on the 'negative' column itself which greatly reduced the amount of AROM for the subsequent purification steps. The 'negative' chromatography was abandoned as an anti-proteinase measure.

3.3.5 Summary

The purification of AROM from wild-type *S.cerevisiaae* was severely hampered by great activity losses at every stage in the purification. The initial activity losses, at least, are thought to be due to endogenous *S. cerevisiae* proteinases. Later in the purification, when the protein concentrations were extremely low, the activity losses may also be accounted for by adsorption of the protein to dialysis tubing and any vessels which the protein was in contact with. Several measures were taken to try and reduce the proteinase effects and these are summarised below:-

1. Use of proteinase inhibitors: EDTA, PMSF,benzamidine, leupeptin and pepstatin A.
2. *S. cerevisiae* cells used in the purification of AROM were grown to the mid-log phase of growth on rich medium to minimise proteinase levels.
3. Use of *S. cerevisiae* ABYS106, which lacks several vacuolar proteinases, for the purification of AROM.

4. Use of a 'negative' chromatography step in an attempt to remove proteinases from crude extract.

Application of the first two measures slightly improved the recovery of AROM activity during the purification from wild-type *S. cerevisiae* cells. The last two measures did not improve recovery and the problem of activity loss was clearly not solved by these anti-proteinase strategies.

By this time Dr L. Graham in our group had developed a ubiquitin fusion vector capable of overexpressing AROM in *S. cerevisiae*. The next section describes the purification of AROM from this overproducing strain.

3.4 Overexpression of *S. cerevisiae* AROM

3.4.1 *S.cerevisiae* AROM overproducer strain

The overexpression of yeast AROM as a ubiquitin fusion protein is described in Graham *et al.* (1993). The plasmid Yep52g:ARO1 expresses ubiquitin fused to the amino-terminus of AROM. An endogenous yeast proteinase cleaves the ubiquitin rapidly from such fusion proteins*in vivo* to yield active protein in its native form except when proline is the first residue of the guest protein (Bachmair *et al.*, 1986; Butt *et al.*, 1988; Sabin *et al.*, 1989). The fusion protein is placed under the control of the *CUP1* promoter which is the copper-inducible promoter of yeast metallothionein (Butt *et al.*, 1988). This allows the controlled expression of the fusion protein when copper is added to the yeast growth medium. The overexpression of several proteins which are not native to yeast has been shown to have been enhanced by expression as a ubiquitin fusion protein in *S. cerevisiae* (Ecker *et al.*, 1989). The plasmid containing a copper inducible fusion protein was the kind gift of Dr. Tauseef Butt (Smith Kline and French, Pennsylvania).

The expression vector Yep52g:ARO1 was transformed into *S. cerevisiae* strain BJ1991 which lacks proteinase yscB and has reduced levels of proteinase yscA, carboxypeptidase yscY and aminopeptidase yscI. Although the use

of *S. cerevisiae* ABYS106, which lacks several vacuolar proteinases, had failed to improve the purification of AROM (see above) it was decided that this new low proteinase strain was worth investigating.

The AROM produced as a result of the cleavage of ubiquitin differs from wild-type AROM in that it contains an amino-terminal methionine which is normally removed co-translationally from yeast proteins that have valine as their second residue (Tsunasawa *et al.*, 1985; Huang *et al.*, 1987). AROM expressed as a ubiquitin fusion protein would be expected to retain methionine at the amino-terminus.

3.4.2 Level of AROM overexpression in *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1

In order to determine the level of AROM overexpression in *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1 growth curves were constructed for strain BJ1991 with and without the expression vector. This allowed determination of when copper should be added to the growth medium for the 3 h induction of AROM. It was important to ensure that harvested cells would still be in the logarithmic phase of growth. These experiments led to AROM transcription being induced with 100μM copper sulphate, when the A_{600} reached between 1 and 2. The cells were grown for a further 3 h prior to harvesting.

Table 3.1 shows the specific activities for each of the five individual AROM activities in strain BJ1991, with and without, the expression vector. It also shows the level of overexpression achieved in the overexpression strain for each individual activity. The level of AROM overexpression ranges from 12-fold for the DHQ synthase activity to 78-fold for EPSP synthase. The variable levels of overexpression observed between each of the activities are probably attributable to the low levels of activity in the untransformed strain, and the high background rates observed. The most reliable indicators of the level of overexpression are the DHQase and shikimate DH activities which show 27- and 30-fold overexpression respectively. Dr Graham obtained comparable overexpression values of 22-fold and 34-fold for

Table 3.1 **Comparison of AROM activities in *S. cerevisiae* BJ1991 with and without plasmid Yep52g:ARO1**

S. cerevisiae BJ1991, and BJ1991 transformed with Yep52g:ARO1 were grown at 30°C in YEPD growth medium on an orbital shaker and grown to A_{600}=1.4. $CuSO_4$ was added to each flask to a final concentration of 100µM, and the cells were grown for a further 3 h. Cells were harvested at 4°C by centrifugation, washed with cold distilled water, and repelleted. The *S. cerevisiae* were stored at -80°C.

0.5g of BJ1991 cells, with and without Yep52g:ARO1, were resuspended in 100mM-Tris/HCl pH7.5, containing 5mM-EDTA, 1.2mM-PMSF, 2mM-benzamidine, 10µM-leupeptin, 1µM-pepstatin A and 1.4mM-ß-mercaptoethanol. An equal volume of glass beads was added, and cells were broken by vortexing for 3 x 30 s. Each extract was clarified by a 15 min spin in a cold room microfuge. Extracts were assayed in quadruplicate for each of the five individual AROM activities as described in Section 2.9 (zinc sulphate was added to the DHQ synthase assay buffer to a final concentration of 100µM). The protein concentrations were determined by the method of Bradford (1976).

The table shows the five AROM activities for *S. cerevisiae* BJ1991 with and without the plasmid Yep52g:ARO1 and the activity ratio for each standardised against the DHQase activity. The table also shows the level of AROM overexpression in strain BJ1991 with the plasmid, compared to the strain without.

	BJ1991		BJ1991 with Yep52g:ARO1		over-expression (fold)
	specific activity (U/mg)	DHQase=100	specific activity (U/mg)	DHQase=100	
DHQ synthase[f]	0.00365	120	0.0437	53	12
DHQase[f]	0.00303	100	0.0819	100	27
shikimate DH[r]	0.0275	907	0.812	1860	30
shikimate kinase[f]	nd	nd	0.0956	117	nd
EPSP synthase[r]	0.00097	32	0.0761	93	78

nd = not determinable
[f]assayed in the forward direction
[r]assayed in the reverse direction

DHQ synthase and DHQase respectively, with a final concentration of copper sulphate of 20μM for AROM induction (Graham *et al.*, 1993). Over a period of time, results suggested that the concentration of copper ions in our distilled water was often sufficient for substantial (and in some cases, full) induction of the metallothionein promoter. The induction of AROM was, therefore, subsequently achieved by the addition of copper sulphate to a final added concentration of 20μM.

Table 3.1 also shows the activity ratios for both strains standardised against the DHQase activity. The activity ratio varies between the transformed, and untransformed yeast, and this is probably due to the inaccuracy in measuring wild-type activity levels already mentioned in this section. It is also possible that in the case of DHQ synthase, the lower activity in the overexpression strain might be due to the additional methionine at the amino-terminus of AROM not found in wild-type AROM (see 3.4.1). This might affect the DHQ synthase domain in such a way as to reduce its specific activity.

Dr. Graham observed a similar increase in DHQ synthase and DHQase activities when AROM was expressed as a fusion protein with ubiquitin, in a manner such that ubiquitin is not readily cleaved from AROM. This shows that a construct which has ubiquitin fused to the N-terminus does not have a major effect on the AROM activity (Graham *et al.*, 1993). Dr Graham showed that AROM expressed from vector Yep52g:ARO1 was slightly smaller than AROM produced from the vector in which ubiquitin is not readily cleaved when analysed by SDS PAGE. This provided evidence for ubiquitin cleavage.

3.5 Purification of AROM from BJ1991 transformed with Yep52g:ARO1

3.5.1 Introduction

A scheme was developed for the purification of AROM from the overexpression strain BJ1991 containing Yep52g:ARO1. Q-Sepharose was used rather than mono Q, in order to scale up the preparation. Since the AROM activity

was degrading rapidly despite all the measures previously introduced (see 3.3.5), it was decided to explore the use of other chromatographic steps which might alleviate the problem. Hydroxyapatite chromatography proved a useful purification step and appeared to stabilise AROM. Gel filtration on Sephacryl S-200 was introduced following hydroxyapatite chromatography and Cibacron Blue chromatography (3.3.4) was removed from the purification scheme because it gave variable results. Although it was a good purification step the yields were often low due to AROM leaching from the column during the washes, and variability in the salt concentration at which AROM eluted. The development of the new purification scheme meant that the Cibacron Blue column became redundant.

3.5.2 Protocol for the purification of AROM from *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1

All steps were carried out at 4°C. The purification was followed by assaying the shikimate DH activity throughout the purification. Samples were taken during the purification for protein determination and stored at -80°C. Protein samples for analysis by SDS PAGE were boiled immediately in SDS PAGE sample buffer, then stored at -80°C.

Cell growth

S. cerevisiae BJ1991 transformed with Yep52g:ARO1 was grown on YEPD medium and AROM expression was induced for 3 h with 20μM-copper sulphate, as described in 2.11.6. Washed cells were stored at -80°C.

Step 1 Extraction

52.3g wet weight of cells were resuspended in 1 volume of buffer A containing 1μM-pepstatin A, and broken by 3 passages through a French pressure cell (6550kPa). The cell debris was removed by centrifugation at 100,000g for 1 h.

Step 2 Ammonium sulphate fractionation

The initial ammonium sulphate cut was 0-30% saturation. AROM was precipitated with 30-50% saturation. Pelleted protein was resuspended into buffer B containing 10μM-leupeptin, 1μM-pepstatin A and 1.2mM-PMSF. The protein was dialysed into 50mM-NaCl in buffer B containing 1μM-pepstatin A and 1.2mM-PMSF.

Step 3 Q-Sepharose

The dialysed AROM sample was loaded onto a Q-Sepharose anion exchange column equilibrated in 50mM-NaCl in buffer B containing 1μM-pepstatin A. Figure 3.8 shows the elution of AROM with a continuous linear gradient of 50-500mM-NaCl in buffer B containing 1μM-pepstatin A. AROM eluted in a double peak with the major peak eluting in 100mM-NaCl and the minor peak at 150mM-NaCl in buffer B. Due to the massive activity losses observed in the purification of *S. cerevisiae* AROM in the past it was decided to maximise the AROM yield, at this stage, and AROM was pooled across both peaks (fractions 42-47). The pooled AROM sample was dialysed into buffer C containing 1.2mM-PMSF.

Step 4 Hydroxyapatite chromatography

The dialysed AROM preparation was loaded onto an hydroxyapatite column equilibrated in buffer C. AROM was eluted with a continuous linear gradient of 20-500mM-potassium phosphate buffer pH6.5, containing 0.4mM-DTT, 5mM-EDTA, 2mM-benzamidine and 1μM-pepstatin A. The elution profile (Figure 3.9) shows a double AROM activity peak. The major and minor activity peaks eluted in 150mM- and 230mM-potassium phosphate buffer pH6.5, respectively. During the protein loading and subsequent column wash, no protein eluted in four column volumes and the gradient was started. However, several fractions at the end of the wash, immediately prior to starting the gradient, were found to contain protein. This protein probably bound to the column with a low affinity and took some time to elute

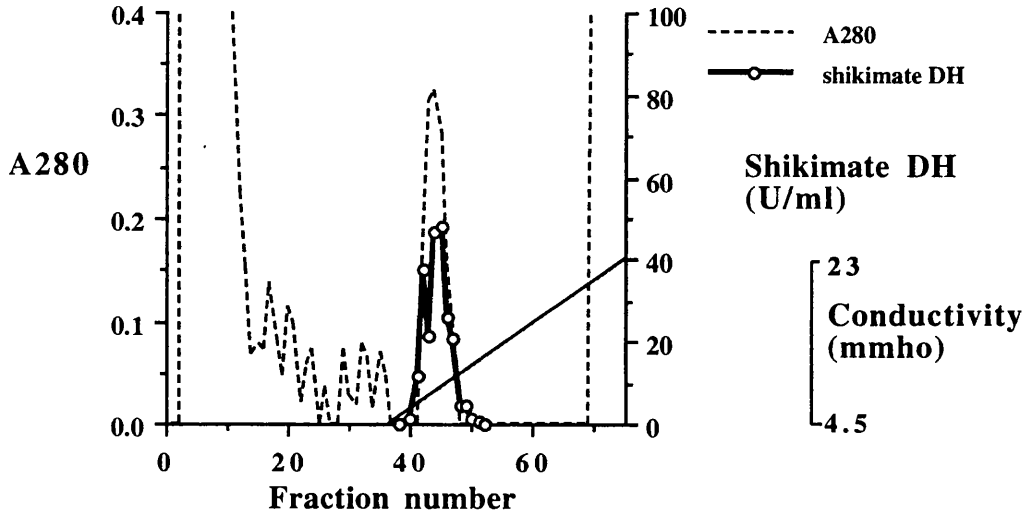


Figure 3.8 **Chromatography of AROM on Q-Sepharose**

A 50ml column of Q-Sepharose (22mm diameter) was equilibrated with 50mM-NaCl in buffer B containing 1μM-pepstatin A. AROM sample was loaded at 5ml/min and the column washed with equilibration buffer until the A_{280} had fallen below 0.1. AROM was eluted with a continuous linear gradient of 50-500mM-NaCl in buffer B containing 1μM-pepstatin A. 14ml fractions were collected and the shikimate DH activity of the individual fractions was assayed. The protein concentration was measured at 280nm and the conductivity was also monitored.

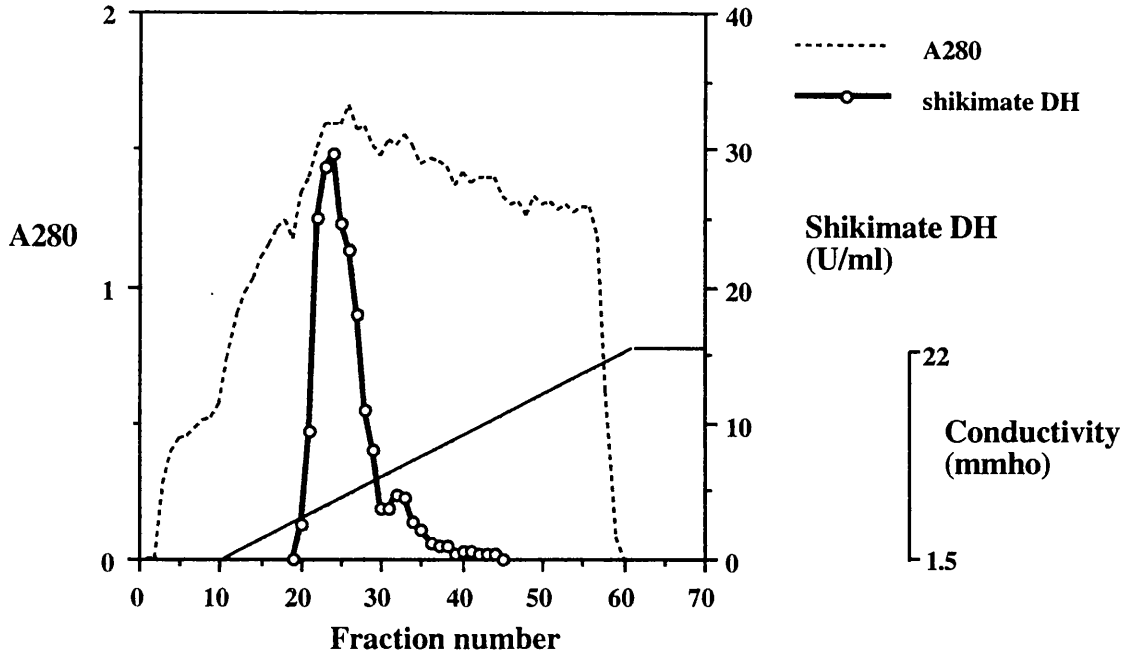


Figure 3.9 **Chromatography of AROM on hydroxyapatite**

A 24ml column of hydroxyapatite (22mm diameter) was equilibrated with buffer C. The AROM sample was eluted with a 250ml gradient of 20mM (buffer C)-500mM-potassium phosphate buffer pH6.5. Fractions were collected of 4.8ml and the shikimate DH activity of individual fractions was assayed. The protein concentration was measured at 280nm.

from the column during the wash period, so it would most likely have eluted from the column as soon as the salt gradient was started.

Protein from the AROM peaks was analysed by SDS PAGE and the fractions across the major peak were pooled and dialysed into 100mM-Tris/HCl pH7.5, containing 0.4mM-DTT and 5mM-EDTA. The reason for the double peak is unclear, and the AROM appears the same after SDS PAGE except for the contaminants present (data not shown). It is possible that the minor peak represents the chromosomally encoded AROM which differs from the overexpressed AROM since it no longer has an amino-terminal methionine residue (see 3.4.1).

PMSF was not included in this purification step because it is extremely insoluble in these buffering conditions and blocks the column and tubing.

Step 5 Sephacryl S-200 chromatography

The dialysed AROM preparation was concentrated using an Amicon centriprep 30 and loaded onto a Sephacryl S-200 gel filtration column equilibrated in 100mM-Tris/HCl pH7.5, containing 0.4mM-DTT, 5mM-EDTA and 2mM-benzamidine. The elution profile (Figure 3.10(a)) shows a double peak of AROM activity. The individual fractions were analysed by SDS PAGE (Figure 3.10(b)) on which the protein was overloaded to allow visualisation of the minor polypeptides also present in the preparation. The peak fraction shows minor contaminants with molecular weights of 150kDa, 125kDa, 110kDa, 69kDa and 52kDa. As the elution of AROM proceeded across the peaks there is an increase in 103kDa and 74kDa species. The reason for the double peak is unclear as both fractions contain high molecular weight species. The double AROM peak eluted over the same volume range as Blue Dextran on the same column showing it eluted in the void volume. The more detailed analysis of the individual AROM fractions eluted from Sephacryl S-200 chromatography are described in Sections 3.5.4 - 3.5.7.

Figure 3.10 Chromatography of AROM on Sephacryl S-200

(a) 'chromatography'

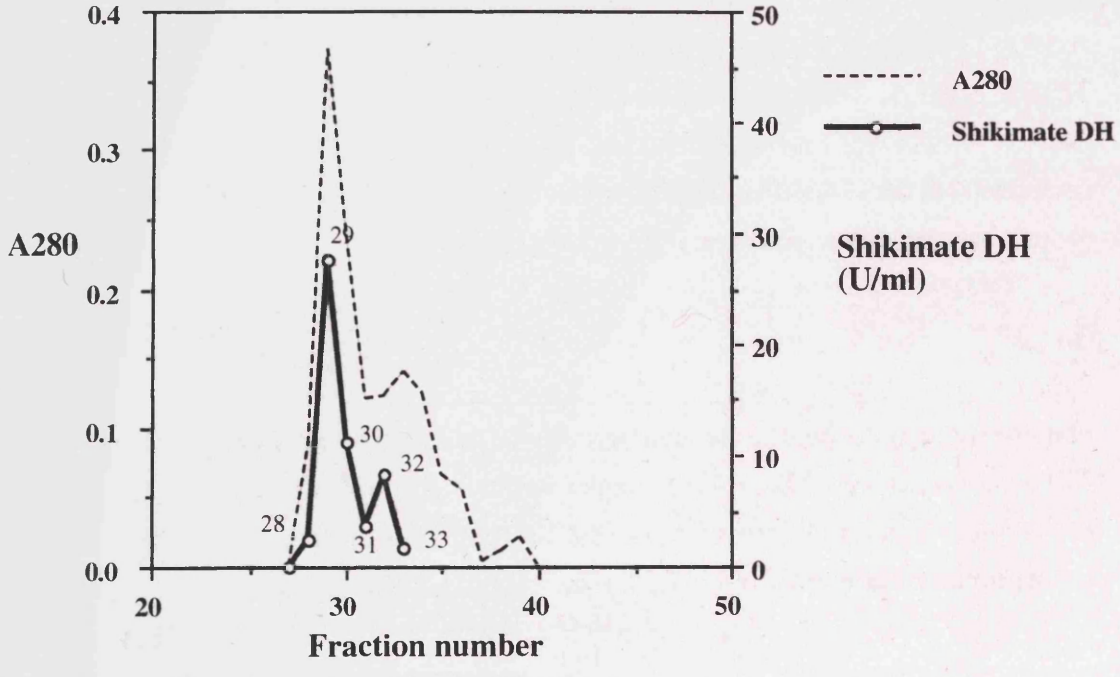
A 500ml column of Sephacryl S-200 (3cm diameter) was equilibrated in 100mM-Tris/HCl pH7.5, containing 0.4mM-DTT, 5mM-EDTA and 2mM-benzamidine. AROM sample was loaded and the column was run at 6ml/h. 9.3ml fractions were collected and the individual fractions were assayed for shikimate DH activity. The protein concentration was measured at 280nm.

(b) 'gel'

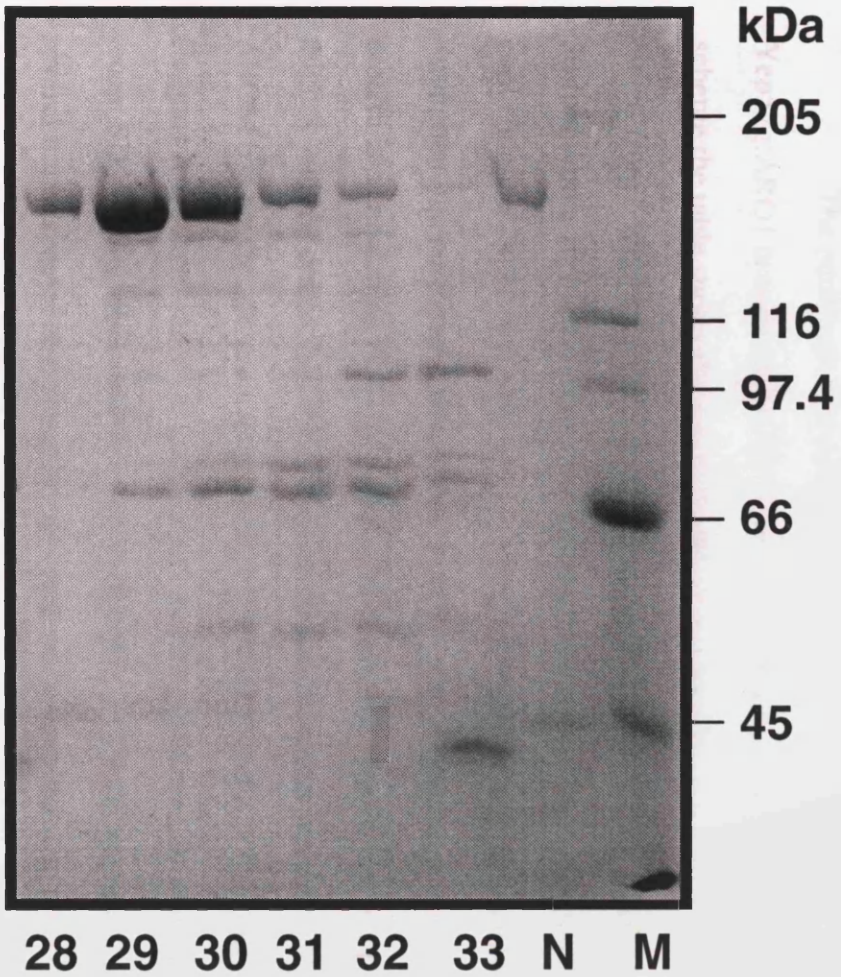
Fractions eluted during the chromatography of *S. cerevisiae* AROM on Sephacryl S-200 were boiled with SDS PAGE sample buffer and subjected to SDS PAGE on a 7% gel. Protein was stained with Coomassie Brilliant Blue G-250. The protein concentrations were determined by the method of Bradford (1976).

M.	molecular weight markers: 205kDa, 116kDa, 97.4kDa, 66kDa and 45kDa.
N.	*N. crassa* AROM, 2µg protein.
28.	S-200 fraction 28, 5µg protein.
29.	S-200 fraction 29, 14µg protein.
30.	S-200 fraction 30, 13µg protein.
31.	S-200 fraction 31, 7µg protein.
32.	S-200 fraction 32, 6µg protein.
33	S-200 fraction 33, 7µg protein.

(a) 'chromatography'



(b) 'gel'


kDa
205
116
97.4
66
45
28
29
30
31
32
33
N
M

Step 6 Storage

Each of the individual AROM fractions eluted from Sephacryl S-200 chromatography were dialysed into 50% (v/v) glycerol in buffer B containing 10μM-leupeptin, 1μM-pepstatin A, 1.2mM-PMSF and 40μM-zinc sulphate. These fractions were stored at -20°C.

3.5.3 Analysis of the purification of AROM from *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1

The purification of AROM from *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1 is summarised in Table 3.2. In order to evaluate the purification scheme the table shows the purified AROM resulting from a pool of the major peak eluted from Sephacryl S-200 chromatography (fractions 28-31). AROM was purified 30-fold with a 10% yield using this purification scheme. The same Sephacryl S-200 pool (fraction 28-31) was also analysed by SDS PAGE, along with samples taken throughout the purification (Figure 3.11). The subunit molecular weight of the protein is estimated as 175kDa by SDS PAGE and the protein was found to co-migrate with *N. crassa* AROM. The polypeptide molecular weight of *N. crassa* AROM has previously been estimated as 165kDa (Lumsden & Coggins, 1977; Coggins & Boocock, 1986; Coggins *et al.*, 1987a). However, it is now believed from recent SDS PAGE results that *N. crassa* AROM is a 175kDa species. There are several minor species also present in the purified preparation which have been described already (see 3.5.2). The most prominent low molecular weight species are 74kDa and 69kDa. Both the purification table and the gel show that Q-Sepharose proved a good purification step for AROM. However, there was a massive activity loss on dialysis of the Q-Sepharose pool. Analysis of the Q-Sepharose pool, before and after dialysis, by SDS PAGE (lanes 3 and 4 respectively, of Figure 3.11) did not reveal any apparent degradation products and the reason for the loss of activity during dialysis is not known.

Step	Volume (ml)	Shikimate DH Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Purifi-cation (fold)	Yield (%)
crude extract	72	3500	1.2	4162	1	100
30-50% $(NH_4)_2SO_4$ precipitate	54	1650	2.1	3420	1.7	82.2
Q-Sepharose	83	120	23.2	2870	19.5	66.8
Dialysed Q-Sepharose	91	108	4.3	467	3.6	11.2
Hydroxyapatite	43	35	23.4	813*	19.7	19.5
Sephacryl S-200	37	11.6	35.9	416	30.2	10.0

*the increase in activity may be due to the removal of an inhibitor by the hydroxyapatite step, or may result from the refolding of reversibly denatured AROM.

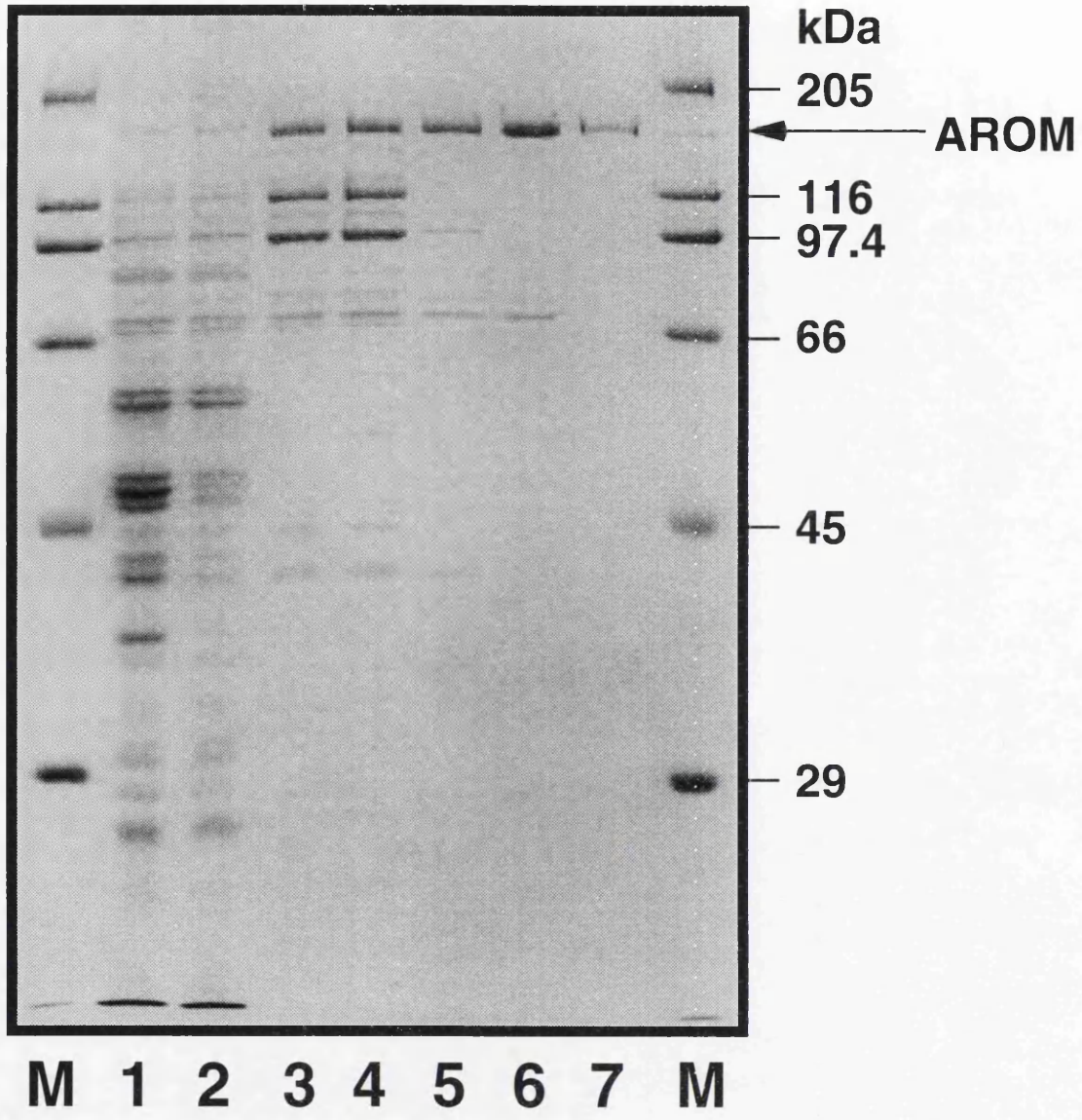
Table 3.2 **Scheme for the purification of AROM from *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1**

The purification of AROM from the *S. cerevisiae* overexpression strain was followed by assaying for shikimate DH activity. Samples were assayed immediately for enzyme activity and stored at -80°C prior to protein determination. Protein concentrations were determined by the method of Bradford (1976).

Figure 3.11 **SDS PAGE analysis of the purification of AROM from *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1**

Samples were taken at each stage of the purification of AROM from *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1. The samples were boiled immediately in SDS PAGE sample buffer and stored at -80°C. The protein samples were subjected to SDS PAGE on a 10% gel. Protein was stained with Coomassie Brilliant Blue G-250 and protein concentrations were determined by the method of Bradford (1976).

M.	molecular weight markers: 205kDa, 116kDa, 97.4kDa, 66kDa, 45kDa and 29kDa.
Lane 1.	clarified extract, 30μg protein.
Lane 2.	dialysed 30-50% $(NH_4)_2SO_4$ pellet, 30μg protein.
Lane 3.	Q-Sepharose pool, 10μg protein.
Lane 4.	dialysed Q-Sepharose pool, 10μg protein.
Lane 5.	dialysed hydroxyapatite pool, 5μg protein.
Lane 6.	Sephacryl S-200 pool, 5μg protein.
Lane 7.	*N. crassa* AROM, 2μg protein.


kDa
205
AROM
116
97.4
66
45
29
M 1 2 3 4 5 6 7 M

Chromatography on hydroxyapatite also proved to be a good purification step, and was the only chromatographic step in any *S. cerevisiae* AROM purification scheme in which the AROM activity was stabilised. Following chromatography on hydroxyapatite the AROM preparation remained stable. AROM was later purified with hydroxyapatite as the first step in the purification scheme, in an attempt to stabilise the protein as early as possible in the purification, however, this failed to stabilise the AROM activity. When cells are broken open, at least in the case of proteinase yscA, ysc B, and carboxypeptidase yscY, the proteinases are known to complex with endogenous proteinase inhibitors (Jones, 1991). During protein purification the proteinase inhibitors may become dissociated and the proteinases activated (Jones, 1991; Pringle, 1975). It seems likely that during the purification of AROM on Q-Sepharose, proteinases dissociate from their inhibitors giving rise to active proteinases. During the subsequent dialysis AROM is proteolysed. The proteinases are probably separated from AROM during chromatography on hydroxyapatite. This would explain why bringing the hydroxyapatite step forward in the purification scheme failed to remove the proteolytic activity affecting AROM. One way to cut down the massive activity losses would be to reduce the dialysis time following Q-Sepharose chromatography. The large pool volume makes it difficult to desalt the protein quickly, although the volume could be reduced by concentrating the sample and then desalting it on an FPLC desalting column. Leupeptin was added to the Q-Sepharose pool but it was not used during chromatography. It is possible that the addition of leupeptin to the buffers used during Q-Sepharose chromatography and to the dialysis buffer may help to prevent proteolysis at this stage in the purification. Another possible solution to the problem is to try hydrophobic interaction chromatography, immediately following Q-Sepharose chromatography as a means of stabilising the AROM activity since this would not require the removal of salt from the AROM sample.

There was an apparent rise in shikimate DH activity resulting from chromatography on hydroxyapatite. This phenomenon has also been observed for the

shikimate DH activity of *A. nidulans* AROM (Section 4.2.6). A possible explanation is that an inhibitor of the shikimate DH activity has been removed during chromatography on hydroxyapatite. Another explanation is that this C-terminal region of AROM is reversibly denatured during the early purification steps and has renatured during hydroxyapatite chromatography.

Chromatography on Sephacryl S-200 removed several low molecular weight contaminants from the AROM preparation and was, therefore, a useful step in the purification of AROM.

To allow a more thorough evaluation of the purification of *S. cerevisiae* AROM, each of the individual AROM activities would ideally be assayed throughout the purification. This is very time consuming and in a preparation where the activity is rapidly lost (such as this one) it is only feasible to follow a single activity in order to minimise the purification time.

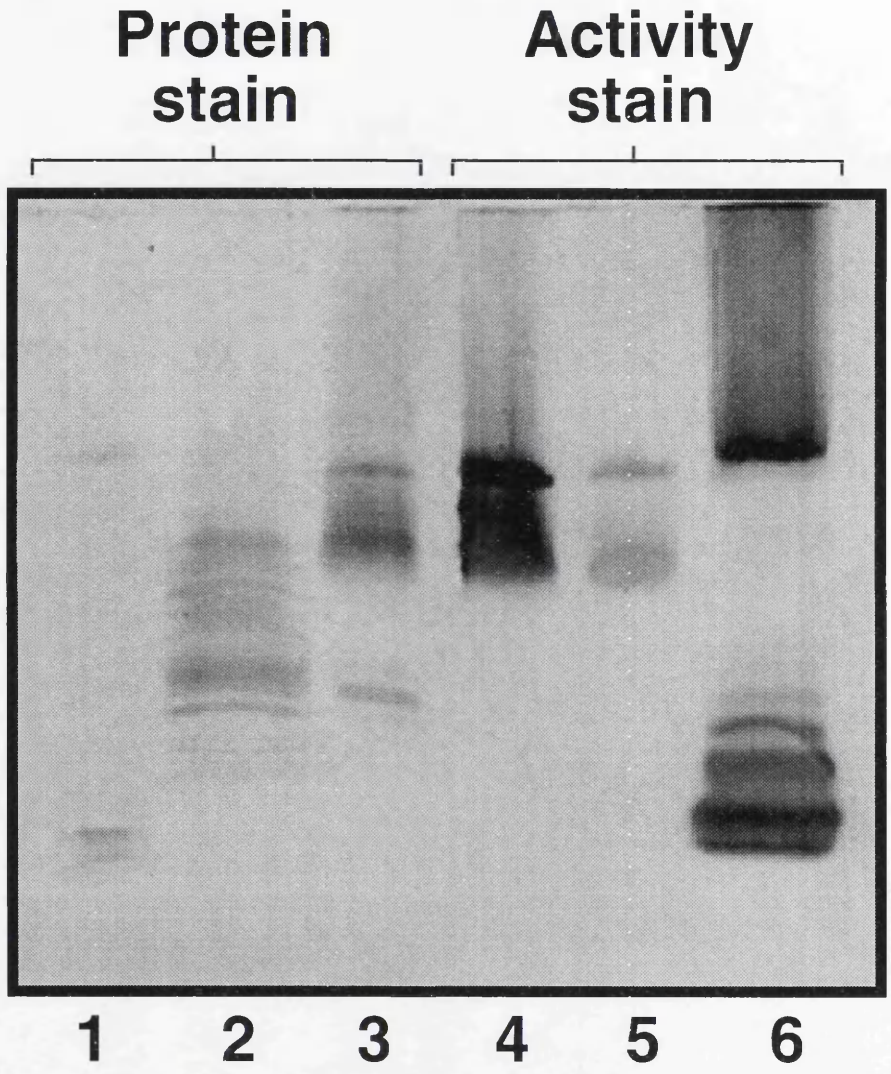
3.5.4 Native PAGE analysis of AROM purified from *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1

The major and minor peak Sephacryl S-200 fractions collected during the purification of AROM from *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1 (3.5.2) were analysed by native PAGE on a 4-15% gradient Phastgel. The major peak (fraction 29) and the minor peak (fraction 32) were run alongside *N. crassa* AROM and the samples were loaded in duplicate. Half the gel was stained for protein with Coomassie Brilliant Blue, while the other half was stained for shikimate DH activity (Figure 3.12). The *N. crassa* AROM preparation shows a low mobility protein species (lane 1), and many other high mobility polypeptides are shown to have shikimate DH activity (Lane 6). This suggests that the *N. crassa* AROM preparation, which had been stored in 50% (v/v) glycerol at -20°C and which was over a year old, had suffered from degradation. Analysis of the major *S. cerevisiae* AROM peak fraction eluted from Sephacryl S-200 chromatography showed a double protein band under native conditions with a less prominent higher

Figure 3.12 **Analysis of *S. cerevisiae* AROM by native PAGE**

Individual Sephacryl S-200 fractions were collected during the purification of AROM from *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1, and dialysed into buffer B containing 10μM-leupeptin, 1μM-pepstatin A, 1.2mM-PMSF, 40μM-zinc sulphate and 50% (v/v) glycerol, and stored at -20°C. Samples were loaded in duplicate onto a 4-15% gradient Phastgel and subjected to native PAGE. Following electrophoresis, half of the gel was stained for protein with Coomassie Brilliant Blue and the other half was stained for shikimate DH activity (see 2.6). Protein concentrations were determined by the method of Bradford (1976).

Protein stain	Lane 1.	*N. crassa* AROM, 0.27μg protein.
	Lane 2.	Sephacryl S-200 fraction 32, 1.9μg protein.
	Lane 3.	Sephacryl S-200 fraction 29, 4.7μg protein.
Shikimate DH activity stain	Lane 4.	Sephacryl S-200 fraction 29, 4.7μg protein.
	Lane 5.	Sephacryl S-200 fraction 32, 1.9μg protein.
	Lane 6.	*N. crassa* AROM, 0.27μg protein.


Protein stain
Activity stain
1
2
3
4
5
6

mobility species also present (Lane 3). After staining for shikimate DH activity the two low mobility species can be seen to have shikimate DH activity and the lowest mobility protein appears to have the highest specific activity according to stain intensity. This high activity, low mobility protein migrates at a similar position to the intact *N. crassa* AROM suggesting that is intact yeast AROM. It is possible that the higher mobility species with shikimate DH activity are proteolysed AROM which retain shikimate DH activity under native conditions (*i.e.* 'nicked ' AROM protein). It is very likely that 'nicked' AROM would occur as a complex under non-denaturing conditions; this complex would 'fall apart' when denatured. This explanation would account for some of the low molecular weight species observed when the AROM sample is analysed by SDS PAGE (Figure 3.10b). 'Nicked' species have been observed in the case of *N. crassa* AROM (Gaertner & Cole, 1977; Smith & Coggins, 1983; Coggins *et al.*, 1985). It is also possible that the two species could represent ubiquitinated and de-ubiquitinated protein. However, this is unlikely because these species would be resolved on SDS PAGE and there is only one 175kDa species on the SDS PAGE gel (Figure 3.10b).

The second peak of *S. cerevisiae* AROM eluted from Sephacryl S-200 in a larger elution volume (fraction 32; see 3.5.2). It also shows the double shikimate DH activity band found in the major AROM peak (Lane 5). The protein stained sample shows many high mobility species, which do not have shikimate DH activity (Lane 5), suggesting that this protein is degraded more than the major AROM peak fraction or contains several contaminants. Chromatography of these AROM samples on gel filtration media with a large enough pore size for good separation in the AROM molecular weight range would allow resolution of the different AROM species present. However, the results might not be straightforward to interpret because both *N. crassa* and *A. nidulans* AROM elute with higher apparent molecular weights than expected. *N. crassa* AROM has an anomalously high molecular weight of 530kDa in at least some HPLC gel filtration experiments (Coggins *et al.*, 1985; Coggins &

Boocock, 1986). AROM purified from *A. nidulans* 1314 eluted from an FPLC gel filtration column with an apparent native molecular weight of 410kDa (Section 4.6.6).

These experiments indicate that the purified AROM is proteolytically damaged. If the AROM protein is 'nicked', forming several lower molecular weight species during SDS PAGE, any polypeptides which contain the shikimate DH domain might renature following SDS PAGE as has been shown for *N. crassa* AROM (Coggins *et al.*, 1985).The renaturation experiment is described in the next section.

3.5.5 Renaturation of shikimate DH activity following SDS PAGE of AROM purified from *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1

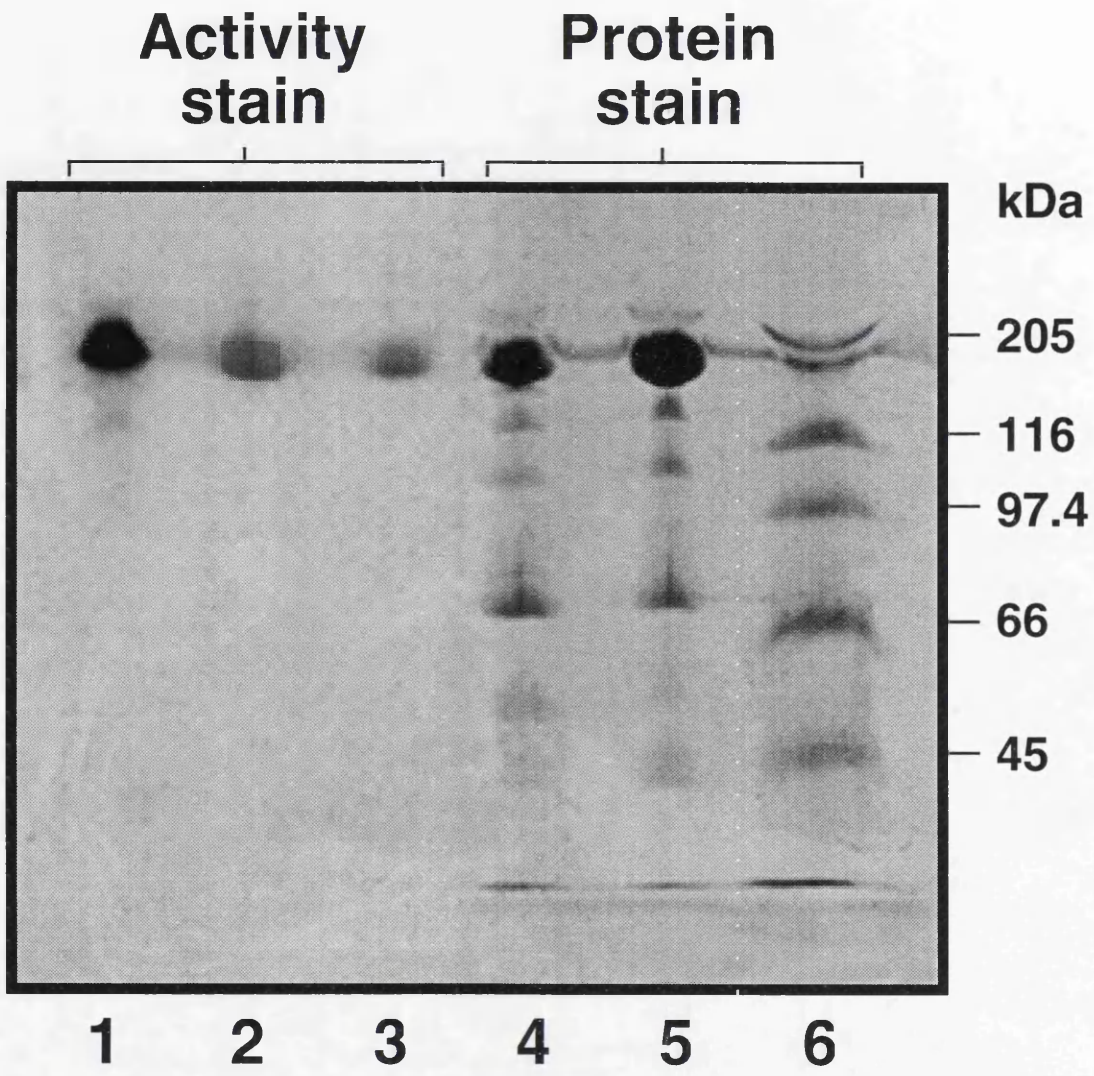
AROM collected during the purification described in Section 3.5.2 was subjected to SDS PAGE in duplicate. The protein was renatured and half of the gel was stained for protein while the other half was stained for shikimate DH activity. Figure 3.13 shows that the high molecular weight species at 175kDa is capable of renaturing to form active shikimate DH and is AROM. The relative activity stain intensities indicate that, as with AROM purified from *A. nidulans* 1314 (data not shown), the *S. cerevisiae* AROM is not able to renature to form active shikimate DH as well as the *N. crassa* AROM. Figure 3.13 also shows that none of the lower molecular weight species present in the *S. cerevisiae* AROM preparation are able to renature to form active shikimate DH. There are several possible reasons for this. Possibilities, are that none of the lower molecular weight species contain the shikimate DH region of AROM, polypeptides containing the shikimate DH region are not able to renature, or that none of the lower molecular weight species are proteolysed AROM fragments.

Another way in which it is possible to show that polypeptides are proteolytic fragments of a protein is to show that they cross-react with antibodies raised against the intact protein and this is described in the next section.

Figure 3.13 **Renaturation of shikimate DH activity of *S. cerevisiae* AROM following SDS PAGE**

AROM from *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1, eluted during Sephacryl S-200 chromatography was subjected to SDS PAGE on a 7.5% Phastgel. Half of the gel was stained for protein with Coomassie Brilliant Blue, and the other half was renatured and stained for shikimate DH activity, as described in Section 2.6. The protein concentrations were determined bu the method of Bradford (1976)

Shikimate DH activity stain	Lane 1.	*N. crassa* AROM, 0.27μg protein.
	Lane 2.	Sephacryl S-200 fraction 29, 5.8μg protein.
	Lane 3.	Sephacryl S-200 fraction 30, 5.4μg protein.
Protein stain	Lane 4.	Sephacryl S-200 fraction 30, 5.4μg protein.
	Lane 5.	Sephacryl S-200 fraction 29, 5.8μg protein.
	Lane 6.	Molecular weight markers: 205kDa, 116kDa, 97.4kDa, 66kDa and 45kDa.


Activity stain
Protein stain
kDa
205
116
97.4
66
45
1
2
3
4
5
6

3.5.6 Immunoblot of AROM purified from *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1 with anti-AROM antibodies

AROM collected during the purification described in Section 3.5.2 was immunoblotted with polyclonal antibodies raised against *N. crassa* AROM in rabbit. The major (fraction 29) and minor (fraction 32) peak AROM fractions eluted from Sephacryl S-200 gel filtration chromatography (Figure 3.10) and the hydroxyapatite pool (Figure 3.9), were loaded onto the gel in duplicate alongside *N. crassa* AROM.

The immunoblot (Figure 3.14) shows that at least some of the antibodies cross-react with *S. cerevisiae* AROM as has been shown previously by Likidlilid (1989). They also cross-react with three low molecular weight polypeptides in the AROM samples of molecular weights 110kDa, 103kDa and 69kDa. The 110kDa and 69kDa species were also observed when the Sephacryl S-200 fractions were analysed by SDS PAGE (Figure 3.10b). The banding pattern is possibly the result of proteolysis and is also seen in the hydroxyapatite pool suggesting proteolysed material was present at this stage in the purification.

3.5.7 Individual enzyme activities of AROM purified from *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1

AROM fractions eluted from Sephacryl S-200 chromatography during the purification described in Section 3.5.2 were stored in 50% (v/v) glycerol at -20°C. The 3 month old samples were assayed for each of the five individual AROM activities (Table 3.3). Part (a) shows the specific activities for each fraction. The specific activity is highest in the peak fraction (29) and a general deterioration is seen across the peak for all the activities. The decrease in specific activity of all the fractions compared to the peak fraction is similar for each of the five individual enzymes. The final AROM fraction (33) shows a 17-fold decrease in specific activity for each activity except for shikimate kinase which fell 30-fold.

A comparison of the shikimate DH specific activities with those in freshly purified material prior to dialysis into storage buffer, show that the three month old

Figure 3.14 **Immunoblot of AROM from *S. cerevisiae* with anti-AROM antibodies.**

Samples taken during the purification of AROM from *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1were subjected to SDS PAGE on a 7% gel. Protein was electroblotted onto nitrocellulose and lanes 1-4 were stained with amido black for protein visualisation. Lanes 5-8 were probed with an antiserum raised against *N. crassa* AROM in rabbit. Bound antibodies were visualised using HRP-anti-rabbit conjugate. The colour was developed with chloronapthol and hydrogen peroxide as described in Section 2.7. The protein concentration was determined by the method of Bradford (1976).

Protein stain	Lane 1.	*N. crassa* AROM, 2.5μg protein.
	Lane 2.	Sephacryl S-200 fraction 32, 22μg protein.
	Lane 3.	Sephacryl S-200 fraction 29, 44μg protein.
	Lane 4.	hydroxyapatite pool, 30μg protein.
Immunoblot	Lane 5.	*N. crassa* AROM, 0.25μg protein.
	Lane 6.	Sephacryl S-200 fraction 32, 22μg protein.
	Lane 7.	Sephacryl S-200 fraction 29, 44μg protein.
	Lane 8.	hydroxyapatite pool, 30μg protein.

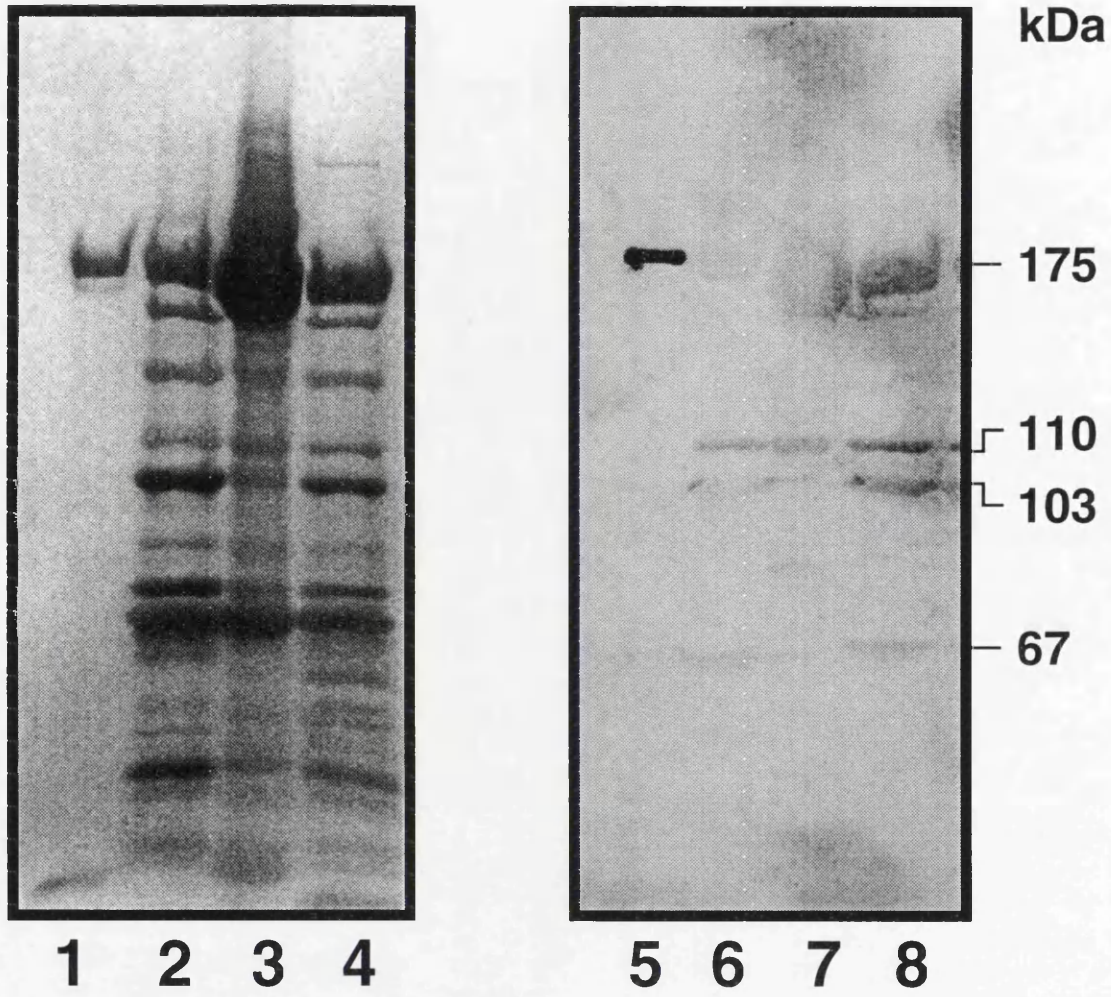

kDa
175
110
103
67
1 2 3 4
5 6 7 8

Table 3.3 **Individual AROM activities of purified *S. cerevisiae* AROM fractions eluted from Sephacryl S-200 chromatography**

(a)'Specific activities'

AROM purified from *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1 was eluted from Sephacryl S-200 chromatography (Figure 3.10). The specific activities for each of the individual AROM enzymes was assayed in quadruplicate, as described in Section 2.9. The DHQ synthase, DHQase and shikimate kinase activities were assayed in the forward direction. The DHQ synthase assay buffer was supplemented with 100μM zinc sulphate. Shikimate DH and EPSP synthase activities were assayed in the reverse direction. The protein concentrations were determined by the method of Bradford (1976)

(b) 'Activity ratios'

The activity ratio for each of the individual AROM fractions eluted from Sephacryl S-200 chromatography shown in part (a) above, were standardised against the DHQase activity for each sample. The DHQase activity was given the arbitrary value of 100.

(a) 'Specific activities'

	Specific activity (U/mg) Fraction number					
	28	**29**	**30**	**31**	**32**	**33**
DHQ synthase[f]	1.6	1.94	1.38	0.869	0.497	0.112
DHQase[f]	1.3	3.19	2.55	1.62	0.565	0.187
shikimate DH[r]	16	52.8	39.4	19.2	8.75	1.77
shikimate kinase[f]	3.46	5.64	4.05	2.10	1.74	0.422
EPSP synthase[r]	3.30	5.54	3.01	2.85	1.3	0.338

(b) 'Activity ratios'

	Activity ratio (DHQase=100) Fraction number						
	crude extract*	**28**	**29**	**30**	**31**	**32**	**33**
DHQ synthase[f]	53	123	61	54	54	62	60
DHQase[f]	100	100	100	100	100	100	100
shikimate DH[r]	1860	1230	1655	1549	1188	1087	946
shikimate kinase[f]	117	266	177	159	130	216	226
EPSP synthase[r]	93	254	174	118	146	162	181

[f]assayed in the forward direction

[r]assayed in the reverse direction

*values taken from Table 3.1.

preparation has retained between 22% and 183% of its original value. Fractions 28 and 29 have 72% and 76% of their original activity, respectively, showing some activity loss on storage. In the case of fractions 30 and 31 the shikimate DH activity has risen on storage to 183% of its original value in the former, and 122% for the latter. It is possible that the C-terminal shikimate DH region of AROM was reversibly denatured and has renatured as discussed in Section 3.5.3, during storage. Fractions 32 and 33 have fallen to 22% and 32% of their original values respectively showing that the shikimate DH activity of the minor AROM peak eluted on Sephacryl S-200 chromatography is less stable. However, these results do not tell us if this activity loss is selective for the shikimate DH activity alone , or whether it is a more general degradation of the whole protein.

The activity ratio for each of the fractions has been normalised against the DHQase activity and is shown alongside the activity ratio for AROM in crude extract (Table 3.3b). The activity ratios for the fractions are similar to one another and to the activity ratio in crude extract which is also shown. One might have expected to have seen a fall in the DHQ synthase activity since the buffers used in the purification were not supplemented with zinc. In the case of the purification of AROM from *N. crassa* it was not until the buffers were supplemented with zinc that AROM was isolated with full DHQ synthase activity (Lambert *et al.*, 1985; Coggins *et al.*, 1987). The DHQ synthase activity is still rather low compared to the activity found in crude extract of untransformed *S. cerevisiae* and a possible reason for this has been discussed in Section 3.4.2. The only Sephacryl S-200 fraction with an obviously different activity ratio is fraction 28 which is the AROM first eluted from the column. The activity ratio shows a higher DHQ synthase, shikimate kinase and EPSP synthase activities than the other fractions. The shikimate DH activity appears to have dropped in all the fractions and this may be due to proteolytic trimming of this carboxy-terminal domain by an exopeptidase which is not detectable by SDS PAGE. There was an apparent increase in shikimate DH activity during the purification (Table 3.2) so the decrease in shikimate DH activity may be an underestimate. The shikimate

kinase and EPSP synthase activities appear to be slightly higher than those found in crude extract but it is possible that inhibitors of these activities have been removed during purification. The assays are also more accurate in purified protein due to the removal of background rates which interfere with the assay. Another possible reason for the increase in activity ratio of these enzymes is that the DHQase activity has been selectively inactivated, thus resulting in an apparent increase in other activities. The EPSP synthase activity of *N. crassa* AROM was found to be sensitive to oxidation (Boocock, 1983) but there was no apparent activity loss in this AROM preparation from *S. cerevisiae.*

Therefore, the activity ratio of the purified AROM is very similar to the activity ratio in crude extract and the activity is stable in 50% (v/v) glycerol at -20°C over this time period.

3.5.8 Summary

AROM has been purified from the overexpression strain *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1. The protein has been purified 30-fold with a 10% yield (Table 3.2). It runs as a 175kDa polypeptide on SDS PAGE with several lower molecular weight species also present (Figure 3.11). Two protein species were observed following native PAGE both of which had shikimate DH activity (Figure 3.12), although only the major AROM polypeptide was able to recover shikimate DH activity following SDS PAGE (Figure 3.13). Three of the low molecular weight polypeptides in the preparation were shown to immunoblot with anti-AROM antibodies raised against *N. crassa* AROM (Figure 3.14). The second AROM peak which eluted from Sephacryl S-200 chromatography during the purification of AROM (see Figure 3.10) is thought to contain less intact protein, and the shikimate DH activity was lost more readily on storage at -20°C. This suggests that there was still a low level of proteinase present in the preparation. The purified protein was found to have all five AROM activities with an activity ratio similar to that observed in crude extract, suggesting that the enzyme activities have co-purified (Table 3.3).

Attempts to reduce proteolysis of AROM during the purification by changing the column order were unsuccessful (3.5.3) and the only time that AROM activity was stabilised in any *S. cerevisiae* AROM preparation, was when it was purified according to the protocol described in Section 3.5.2. This AROM preparation could have been examined in more detail and the nature of the multiple species in the preparation studied more closely. The two species shown to have shikimate DH activity following native PAGE (Figure 3.12) should be separable by gel filtration or ion exchange chromatography. The purification could also be carried out more rapidly to avoid a long dialysis following Q-Sepharose chromatography. This should reduce the massive activity losses seen at this stage in the purification (see Table 3.2). The nature of the double activity peaks seen during chromatography of AROM on Q-Sepharose (Figure 3.8) and hydroxyapatite (Figure 3.9) could also be investigated. However, the general conclusion from this work is that even the purification of overproduced AROM from *S. cerevisiae* is very difficult. The material obtained was almost certainly partially proteolysed and detailed characterisation was not considered worthwhile.

3.6 Discussion

This chapter describes the steps investigated for the purification of AROM from *S. cerevisiae.* The problems encountered with rapid proteolysis of AROM in yeast extracts due to the presence of endogenous proteinases throughout the purification are discussed. Measures introduced in an attempt to minimise the effects of proteolysis are also described, however, it was not until AROM was purified by hydroxyapatite chromatography following chromatography on Q-Sepharose, that stabilised protein was produced. AROM was purified from *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1 which overexpresses AROM as a ubiquitin fusion protein.

The DHQ synthase domain has been implicated at the dimer interface in interallelic complementation studies of *N. crassa* AROM (see 1.7.1). If *S. cerevisiae*

AROM proves to be a dimer like the *N. crassa* and *A. nidulans* proteins it would be interesting to see if a ubiquitin-AROM fusion protein with an altered ubiquitin sequence that is not cleaved, is able to dimerise since it is attached to the DHQ synthase region of AROM.

AROM purified from the *S. cerevisiae* overexpression strain was thought to be proteolysed during the purification summarised in Table 3.2, despite the introduction of several anti-proteinase measures. The very nature of AROM as a large protein makes it susceptible to proteolysis. The purified AROM could be characterised further, as mentioned in Section 3.5.8 and the nature of the multiple active species identified. However, the objective of this project was to purify intact AROM in order that it might be characterised for comparison with individually expressed AROM domains. Therefore, time was spent trying to solve the proteolysis problem and purifying intact AROM. All other purification attempts led to AROM preparations which rapidly lost activity. It was felt that the problems of proteolysis of AROM purified from *S. cerevisiae* BJ1991 transformed with Yep52g:ARO1 described in this chapter were not easily solved. *S. cerevisiae* AROM domain expression studies were also unsuccessful (Graham *et al.*,1993). However, the domain expression studies on *A. nidulans* AROM in the laboratory of Dr. A. Hawkins were progressing well, and J. Moore in his group had overexpressed AROM in *A. nidulans*. Due to the time constraints of the project, it was decided to purify AROM from *A. nidulans* in the hope that the proteolytic problems in that species were less severe.

Chapter 4 Purification and characterisation of AROM from *Aspergillus nidulans* strain 1314

Chapter 4 Purification and characterisation of AROM from *Aspergillus nidulans* strain 1314

4.1 Introduction

4.1.1 Background

Because of the problems encountered with the purification of AROM from *S. cerevisiae* described in Chapter 3 and the finite time available for this project, it was decided to purify AROM from *Aspergillus nidulans*. This work was carried out in collaboration Dr. Alastair Hawkins, University of Newcastle upon Tyne.

The *aromA* gene of *A. nidulans* has been extensively studied by Hawkins' group: the gene has been cloned and sequenced (Charles *et al.*, 1985; Charles *et al.*, 1986), and overexpressed in *A. nidulans* (Lamb *et al.*, 1991; Moore *et al.*, 1992). The expression of domains of the *Aspergillus* AROM has been more successful than the expression of the yeast protein (see 1.7.5). Fragments of the *A. nidulans* AROM protein have been expressed both in *E. coli* (Hawkins & Smith, 1991; van den Hombergh *et al.*., 1992; Moore & Hawkins, 1993) and in *A. nidulans* (Moore & Hawkins, 1993). The AROM protein had previously only been partially purified by ammonium sulphate fractionation from *A. nidulans* (Ahmed & Giles, 1969).

This chapter describes the exploratory purification steps leading to the development of a rapid purification of AROM from the *A. nidulans* overexpression strain 1314. AROM purified by this procedure was characterised and a preliminary proteolysis study of the native protein was undertaken.

4.1.2 *A. nidulans* AROM overproducer strain 1314

E. coli was an unsuccessful host for the expression of full length *A. nidulans* AROM (Hawkins & Smith, 1991) as it had been for *S. cerevisiae* AROM (L. Graham, unpublished results). The *Aspergillus* protein has been constitutively overexpressed in *A. nidulans* resulting in a 12-fold increase in enzyme activity (Lamb *et al.*, 1991). Inducible overexpression was achieved with plasmid pNUFC2 (Lamb *et*

al., 1991) using the promoter of the quinate-inducible *qutE* (catabolic DHQase) gene (see Section 1.4). One *aromA*$^-$ mutant strain transformed with this plasmid has a 12-fold increase over wild-type in enzyme activity after induction by quinate. The level of AROM production was later greatly improved by transforming the *A. nidulans* *aromA*$^-$*qutB*$^-$ double mutant with pNUFC2 (Moore *et al.*, 1992). This mutant host is unable to catabolise quinate (the inducer) since it lacks quinate dehydrogenase activity although there is evidence that shikimate DH of AROM is able to carry out the reaction (van den Hombergh *et al.*, 1992) - see Section 4.5.3. This transformant strain is called 1314 and was found to have an 120-fold increase in enzyme activity over an *A. nidulans* strain with wild-type levels of AROM. Moore reported that this 175kDa protein made up approximately 6% of the soluble protein in cell extracts.

A. nidulans strain 1314 was used as the source of material for the purification of the AROM protein in this study.

4.2 AROM overexpression in *A. nidulans* 1314 relative to AROM wild-type strain R153

4.2.1 Background

The extent of AROM overproduction in *A. nidulans* 1314 was determined by comparison with strain R153 which has wild-type levels of AROM. This strain was used because it is the strain that AROM was originally cloned from (Charles *et al.*, 1986). Each strain was grown under identical conditions and then induced with quinate. Extracts of each strain were assayed for the five individual AROM activities and analysed by SDS PAGE. Moore *et al.* (1992) had previously measured DHQase and shikimate DH levels in each strain but had not examined the other activities.

4.2.2 *A. nidulans* growth conditions for AROM induction

A. nidulans conidia were produced, and the mycelia grown at 37°C for

17 h as described in Section 2.12.6. The mycelia were induced on minimal medium with 0.1% (w/v) quinate and 0.2% (v/v) glycerol for 5 h at 37°C. Strain 1314 is unable to grow on quinate as sole carbon source because it lacks quinate dehydrogenase activity. Therefore, quinate is present to induce AROM production. Glycerol is used as the carbon source because it does not show pronounced catabolite repression of the quinate utilisation gene cluster (Lamb *et al.*, 1991; Moore *et al.*, 1992) and will therefore not repress the *aromA* gene under the control of the *qutE* promoter.

4.2.3 DHQ synthase activity of *A. nidulans* 1314

No DHQ synthase activity was detected in extracts of *A. nidulans* 1314. This was rather alarming. The chromosomal copy of the *aromA* gene in this strain is unable to complement any missing AROM activity in heterokaryons (Roberts *et al.*, 1969; J. Moore, personal communication). Therefore, the ability of strain 1314 to grow without aromatic amino acid supplements suggested that it has at least a low level of DHQ synthase activity. Ahmed & Giles (1969) found that, in ammonium sulphate fractions of *A. nidulans* (Yale Culture Collection no. A1), the activity of DHQ synthase was only 2-5% of the shikimate DH activity. The assays were carried out under similar conditions to those used in this study except the higher temperature of 37°C was used. Their samples were lacking in proteinase inhibitors and the reported DHQ synthase activity was very low compared to values for the closely related *N. crassa* AROM (Boocock, 1983; Lambert *et al.*, 1985) and *S. cerevisiae* AROM shown in Table 3.1. At this stage it was thought that the lack of DHQ synthase might be due to the assay conditions.

The DHQ synthase of *N. crassa* AROM is Zn^{2+}-dependent (Lambert *et al.*, 1985) while Ahmed & Giles (1969) included Co^{2+} in the assay buffer. The DHQ synthase activity in crude extracts was measured in assay cocktail supplemented with particular divalent metal cations- Co^{2+}, Mn^{2+}, Ni^{2+}, Ca^{2+}, Cu^{2+} and Mg^{2+}, in case

the *A. nidulans* enzyme had a different metal preference from the enzyme in other species. No activity was detected using this method.

On increasing the DAHP concentration of the standard assay supplemented with Zn^{2+} some DHQ synthase activity was finally observed. By extrapolation of the double reciprocal plot obtained with increasing DAHP, a value for V_{max} was determined. This gave an activity for DHQ synthase of 0.00244 U/mg compared to 0.453 U/mg for shikimate DH, an activity ratio of 0.54:100.

It was still unclear why the DHQ synthase activity was so low but several explanations seemed possible. Firstly, crude extracts might contain some inhibitory substance: if so the activity should be easier to assay in material that was at least partially purified. Secondly, the DHQ synthase activity might be readily proteolysed in extracts with accompanying loss of activity. A third possibility was that during the construction of the overproducing strain there might have been some accidental change to the coding sequence in the DHQ synthase domain which adversely affected activity.

4.2.4 AROM activity levels in *A. nidulans* 1314 and R153

The individual AROM activities in *Aspergillus* extracts are shown in Table 4.1 except for DHQ synthase (see Section 4.2.3). The level of AROM overexpression in strain 1314 has been determined for each activity by comparison with strain R153 which has wild-type levels of AROM and was grown under identical conditions. All four activities are being overexpressed at high level. However, the variation in the level of overexpression (from 65 to 194-fold) requires comment.

The shikimate kinase and EPSP synthase activities were difficult to determine accurately in crude extracts of the wild-type strain due to a high background rate of NADH oxidation. This may explain the higher values for overexpression observed for these activities. The DHQase levels in the table were due to both the biosynthetic type I activity (bDHQase) associated with AROM and the catabolic type II activity (cDHQase) due to the catabolic enzyme (see below).

	R153 wild-type AROM		1314 overproducer			over expression (fold)
	U/mg	shikimate DH=100	U/mg	shikimate DH=100	shikimate kinase=100	
DHQase*	0.0064	nd	0.42	111	76	nd
shikimate DH	0.0034	100	0.38	100	69	110
shikimate kinase	0.0036	104	0.55	146	100	152
EPSP synthase	0.0021	60	0.40	107	73	194

*DHQase includes both biosynthetic and catabolic DHQase activity (see text, Section 4.3.3)

nd, not determinable

Table 4.1 **Comparison of AROM activities in *A. nidulans* R153 and 1314 crude extracts**

A. nidulans was grown according to method 1 described in Section 2.12.6.

Cells were broken with a pestle and mortar in liquid nitrogen and extracted into 15 volumes of extraction buffer and shaken gently on ice for 1 h. The extraction buffer was 100-mM-Tris/HCl pH7.2, containing 1.4mM-ß-mercaptoethanol, 5-mM-EDTA, 2mM-benzamidine, 1.2-mM-PMSF and 1µM-pepstatin A. The extracts were spun for 30 min using a cold room microfuge then assayed for each of the individual AROM activities. The protein concentration of each extract was determined by the method of Bradford (1976). Enzyme activities were assayed in quadruplicate.

The table shows specific activities for each extract alongside the activity ratio normalised against the shikimate DH activity which was given the arbitrary value of 100. The same has also been done for each activity normalised against the shikimate kinase activity. The DHQase activity includes both the biosynthetic and catabolic DHQase activities. The extreme right-hand column shows the extent of overexpression for the activities in *A. nidulans* 1314 compared to R153 which has wild-type levels of AROM.

The most reliable measurement of the level of overexpression between the wild-type R153 and 1314 is therefore obtained by comparing the shikimate DH activity which shows 110-fold overexpression. The overexpression of AROM was previously measured by Moore *et al.* (1992) who compared the shikimate DH and bDHQase activities of strain 1314 to wild-type strain R153 grown under non-inducing conditions. The specific enzyme activities are not directly comparable to my own because the assays were carried out at a different temperature.

In *A. nidulans,* unlike in yeast, a complication arises from the presence in crude extracts of both the biosynthetic (AROM) DHQase (bDHQase) and the inducible catabolic DHQase (cDHQase): see Sections 1.2.2 and 1.4. A rough estimate can be made of the contributions of these two activities to the total DHQase activity by exploiting the heat lability of the bDHQase and the heat stability (10 min at 70°C) of the cDHQase. Therefore, bDHQase = total DHQase - heat stable DHQase. This would have allowed the bDHQase and cDHQase components of the extract to be measured. It does not however, provide accurate specific activities due to the presence of heat-labile inhibitors of the cDHQase, which cause the cDHQase activity to rise by approximately 20% after the heat step (A. Hawkins, personal communication). In bDHQase assays the cDHQase activity is only partially seen for two reasons. The first is that cDHQase is inhibited by the potassium phosphate buffer (100mM) used for the bDHQase assay: the K_i for phosphate is 10mM (R. Deka, 1993). The second is that the cDHQase has a much higher K_m for the substrate DHQ than bDHQase. In the absence of inhibitors the cDHQase of *A. nidulans* has a K_m for DHQ of 150μM (Kleanthous *et al.*, 1992) compared to the bDHQase of *N. crassa* AROM which has a K_m of 5μM (Coggins & Boocock, 1986). Therefore cDHQase is routinely assayed with a ten fold higher DHQ concentration than bDHQase. In strain R153, which has wild-type AROM, cDHQase constitutes as much as 80% of the total DHQase when it is induced by quinate. In the AROM overexpression strain 1314 the cDHQase is approximately 8% of the total DHQase in assays after induction by

quinate (J. Moore, personal communication). Therefore, the presence of cDHQase does not lead to large errors in estimating the level of AROM in strain 1314.

Table 4.1 shows the activity ratios standardised against shikimate DH for both strains. This allows a comparison between strains R153 and 1314. The activity ratio is also shown for strain 1314 standardised against shikimate kinase although not for strain R153 since shikimate kinase is difficult to determine accurately in crude extract. The reason for listing the activity ratio for strain 1314 standardised against shikimate kinase is that the shikimate DH activity can increase during AROM purification (see 4.3.4 and 4.3.9). The shikimate kinase activity appears to be fairly stable and does not have the complications associated with the presence of two forms of DHQase. Therefore, shikimate kinase has been used to standardise the AROM activities to allow comparison with the AROM activities in purified protein (see 4.6.5).

4.2.5 SDS PAGE analysis of *A. nidulans* 1314 and R153

Extracts of *A. nidulans* R153 and 1314 were analysed by SDS PAGE. Figure 4.1 shows a 175kDa protein from strain 1314 which is not visible in extract of strain R153 that has wild-type AROM. The overexpressed protein has the expected subunit molecular weight of AROM, in agreement with Moore *et al.* (1992). An estimation of band intensity by laser densitometry suggested that the AROM protein comprises 6% of the total soluble protein in the overproducing strain, the same value estimated by Moore *et al.*(1992).

4.2.6 AROM stability in crude extract of strain 1314

An extract of the overproducing strain 1314 containing proteinase inhibitors was assayed for the individual AROM activities immediately after extraction, and 48 h later after incubation at 4°C. The specific activity after the period of incubation is shown as a percentage of the original activity in Figure 4.2.

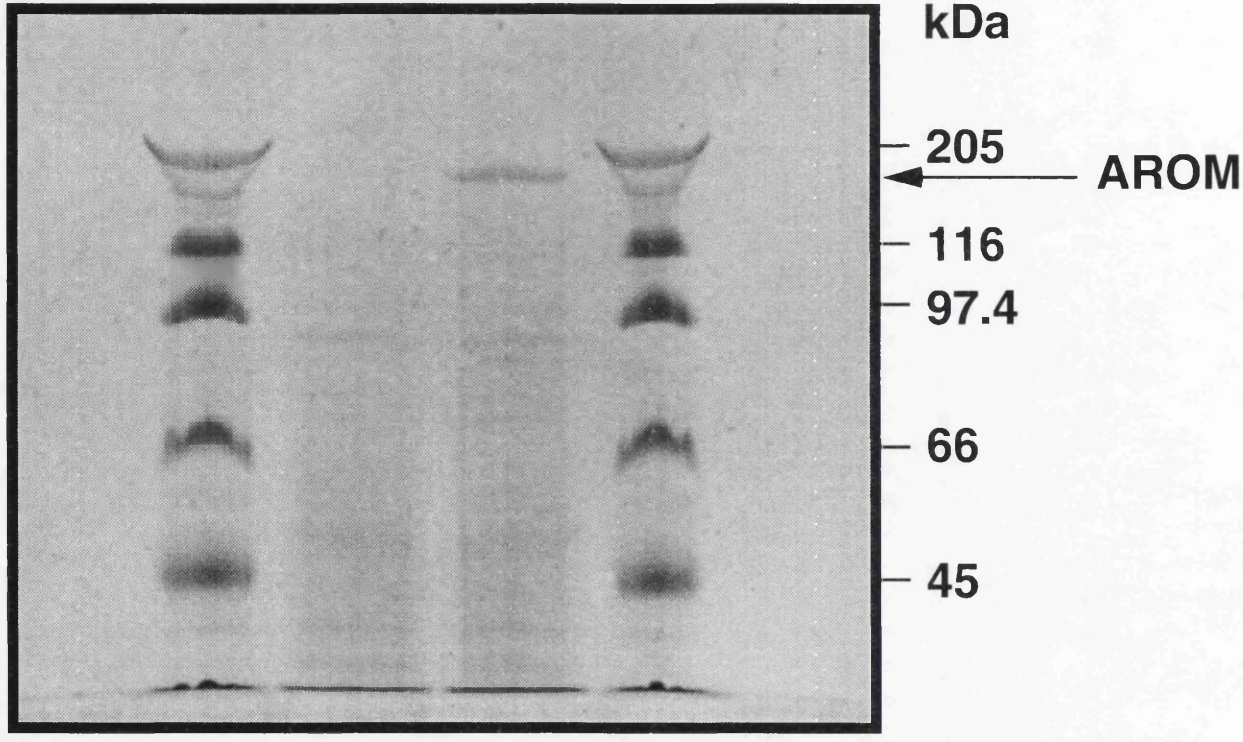
Figure 4.1 **SDS PAGE analysis of AROM production in *A. nidulans* R153 and 1314**

Mycelia were grown according to method 1 described in Section 2.12.6.

Cells were broken with a pestle a mortar in liquid nitrogen, extracted into 15 volumes of extraction buffer and shaken gently on ice for 1 h. The extraction buffer was 100mM-Tris/HCl pH7.2, containing 1.4-mM-ß-mercaptoethanol, 5mM-EDTA, 2mM-benzamidine, 1.2mM-PMSF and 1µM-pepstatin A. Extract was spun for 30 min with a cold room microfuge.

The photograph shows R153 and 1314 clarified supernatants separated on a 7.5% Phastgel and stained for protein with Coomassie Brilliant Blue G-250 (2.5.1). 3.6µg of protein was loaded on each of the central lanes and 2µg of molecular weight markers were run on the outer tracks.

R153 1314



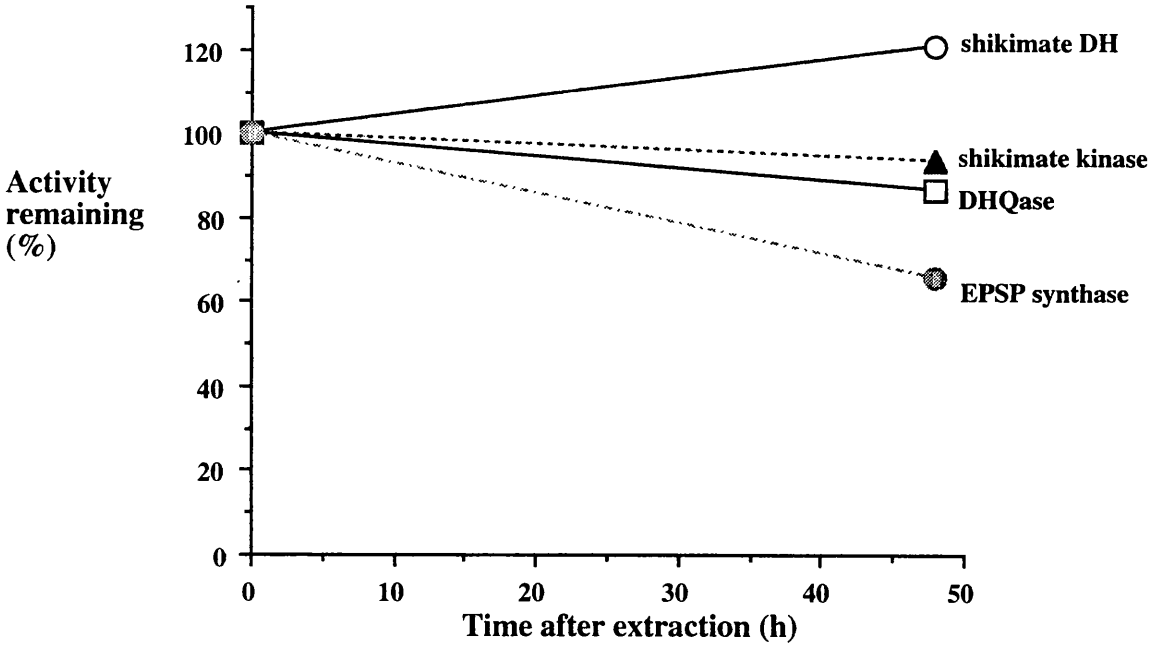


Figure 4.2 **Stability of AROM from *A. nidulans* 1314 in crude extracts**

Mycelia were grown according to method 1 described in Section 2.12.6.

Cells were broken with a mortar and pestle in liquid nitrogen, extracted into 15 volumes of extraction buffer and shaken gently on ice for 1 h. The extraction buffer was 100mM-Tris/HCl, pH7.2, 1.4mM-ß-mercaptoethanol, 5mM-EDTA, 2mM-benzamidine, 1.2mM-PMSF and 1µM-pepstatin A. Extract was spun for 30 min in a cold room microfuge.

Extracts were assayed for all the AROM activities with the exception of DHQ synthase. Assays were performed at 25°C as described in Section 2.9. The extract was stored for 48 h at 4°C then reassayed.

The graph shows the activities after 2 days as a percentage of the activity on initial extraction.

The graph shows an apparent rise in the shikimate DH activity. This phenomenon was observed in subsequent purification steps (see 4.3.4 and 4.3.9) and has also been seen in the purification of *S. cerevisiae* AROM (Table 3.2) and occassionally in the purification of *E. coli* shikimate DH in this laboratory (S. Chackrewarthy, personal communication). The reason for this is unclear, although it is conceivable that not all the protein is properly folded and that only some of it has reached an active conformation, bearing in mind that shikimate DH is at the carboxy-terminus of the polypeptide. Another possibility is that a labile inhibitor of shikimate DH is lost during incubation.

EPSP synthase activity was found to be the most vulnerable, falling to 66% of its original value. Boocock (1983) found that EPSP synthase was sensitive to an endogenous proteinase in *N. crassa* and the activity occasionally vanished during purification. This activity loss was associated with the appearance of a peptide of 125kDa on SDS PAGE, thought to be derived from the 165kDa intact protein. EPSP synthase of *N. crassa* AROM is also sensitive to oxidation and it is thought to have an active site thiol group (Boocock,1983). Padgette *et al.*(1988) have identified a cysteine residue which is sensitive to cysteine-directed reagents in the EPSP synthase of *E. coli*. It is not essential for the enzyme activity and modification is thought to cause inactivation through steric hindrance. For this reason DTT is used in buffers throughout EPSP synthase purifications.

The DHQase activity fell to 87% of its original value after 48 h. This value includes both the catabolic and biosynthetic enzyme activities. The cDHQase constitutes approximately 8% of the total activity under these assay conditions in crude extract (J. Moore, personal communication). Since the cDHQase activity is generally more stable than the bDHQase it seems likely that most of the activity loss is bDHQase activity.

Clearly not all AROM activities are equally stable in crude extracts of *A. nidulans*, despite the presence of various proteinase inhibitors. The susceptibility of *N. crassa* AROM to proteolysis has been well documented (Gaertner & Cole,

1976; Gaertner & Cole, 1977; Lumsden & Coggins, 1977; Coggins & Boocock, 1986). Similarly early attempts to purify the cDHQase of *A. nidulans* (Hawkins *et al.*, 1982; Da Silva *et al.*,1986) showed that proteolysis could be an obstacle in at least some cases. The *N. crassa* cDHQase was also found to be sensitive to proteolysis during purification (Hawkins *et al.*, 1982b). This emphasised the need to adopt a purification strategy which might stabilise AROM.

4.3 Preliminary purification

4.3.1 Strategy

A. nidulans and *N. crassa* both belong to the Euascomycetes sub-class of Ascomycetes. This led me to believe that the chromatographic steps used successfully to purify AROM from *N. crassa* might also prove useful for *A. nidulans* AROM. In particular, the initial 'negative' step of anion exchange chromatography was adopted since, for *N. crassa*, this had proved effective in removing the most troublesome proteinases (Lumsden & Coggins,1977). Thus the purification strategy was developed from the starting point of the latest generation of the *N. crassa* AROM purification procedure (Coggins *et al.*,1987a).

As with the yeast AROM preparations, it was advantageous to include proteinase inhibitors in the buffers used to prepare crude extracts and for the early chromatographic steps. The zinc requirement of DHQ synthase precludes the addition of EDTA to the buffers. These contradictory requirements were balanced: EDTA was used in the buffers only in the early stages of the purification, and zinc was added to the chromatography buffers with the exception of the Cibacron Blue step since its capacity for AROM is reduced in the presence of zinc (Boocock, 1983). In this way I hoped that the resulting AROM purification would result in intact protein which would have DHQ synthase activity.

The following section describes the exploratory phase of AROM purification from *A. nidulans* 1314. It describes the steps which would be retained in

the final purification protocol, and others which were abandoned. Each, is described more fully in the individual sub-sections.

During the preliminary purification DHQase activity was monitored for two reasons. Firstly, it is easily assayed, and secondly, it is one of two activities which may be susceptible to degradation (4.3.5). After the rapid loss of shikimate DH and DHQase activity observed during *S. cerevisiae* AROM preparations it was felt important to monitor any similar losses in the case of the *Aspergillus* protein. The observation that shikimate DH activity increased after extraction (4.3.5) might have confused the issue if only this easily assayed activity had been followed in the first experiments. In later experiments only shikimate DH activity was monitored. This was a compromise - it saved time on assays and allowed the purification to be carried out more rapidly. It also avoided complications due to varying levels of cDHQase.

All the yields and purification factors given for the stages of the preliminary purification are in comparison with the values for the previous step. Samples were taken at each stage in the purification and stored at -80°C for later protein determination. Protein samples were also taken at each stage which were boiled immediately in SDS PAGE sample buffer and stored at -80°C for analysis by PAGE.

All the steps were carried out at 4°C unless otherwise stated. The buffers used throughout are described in Table 4.2.

4.3.2 Extraction

Powdered mycelia grown by method 1 described in Section 2.12.6, were suspended in 15 volumes of bufferA and stirred gently for 60 min. This extract was centrifuged at 35,000g for 30 min and the resulting supernatant filtered through two layers of muslin. Analysis of clarified supernatant by SDS PAGE is included in Figure 4.7.

Buffer A:	100mM-Tris/HCl pH7.5, 0.4mM-DTT, 5mM-EDTA, 2mM-benzamidine, 1.2mM-PMSF, 1μM-pepstatin A and 10μM-leupeptin.
Buffer B:	50mM-Tris/HCl pH7.5, 2mM-benzamidine, 1.2mM-PMSF and 1μM-pepstatin.
Buffer C:	20mM-Tris/HCl pH7.5, 2mM-benzamidine, 1.2mM-PMSF and 1μM-pepstatin A.
Buffer D:	10mM-potassium phosphate buffer pH6.5, 0.4mM-DTT, 20μM-zinc acetate, 2mM-benzamidine and 1μM-pepstatin A.
Buffer E:	200mM-KCl in 50mM-Tris/HCl pH7.5, 0.4mM-DTT, 20μM-zinc acetate, 2mM-benzamidine and 1μM-pepstatin A.
Buffer F:	50mM-sodium phosphate buffer pH7.5, 0.4mM-DTT, 5mM-EDTA, 2mM-benzamidine and 1μM-pepstatin A.

Table 4.2 **Buffers used in the purification of AROM from *A. nidulans* 1314**

4.3.3 'Negative' anion exchange chromatography

In the purification of AROM from *N. crassa*, anion exchange chromatography on DE52 was performed 'negatively', so that AROM did not adsorb to the column while four endogenous proteinases were bound (Lumsden & Coggins, 1977).

The conditions for the elution of *A. nidulans* AROM from DE52 were determined on a small scale extract. A clarified *A. nidulans* 1314 extract in buffer A was loaded onto a DE52 column equilibrated with buffer B containing 0.4mM-DTT and eluted with a continuous linear gradient of 0-370mM-KCl in buffer B containing 0.4mM-DTT. The elution profile in Figure 4.3 shows that the DHQase activity starts to elute from the column immediately the gradient is started. The DHQase activity appeared to elute over several peaks rather than the two expected from AROM and the cDHQase. Later chromatography on Q-Sepharose anion exchange failed to resolve these peaks (see Figure 4.4), and mono Q anion exchange resolved the activity into a double peak (data not shown). The reason for this multiple activity peak is not clear. The cDHQase is known to elute from DE52 in potassium phosphate buffer pH7.2, during a 0-350mMKCl gradient (Hawkins *et al,* 1982). Here, one of the minor peaks is likely to be cDHQase since it is the minor activity. The major peak is most likely to be AROM because it has the greatest DHQase activity.

From this elution profile the conditions for 'negative' chromatography used in the purification of AROM were selected as 100mM-KCl in buffer B. The conductivity of clarified *Aspergillus* extracts was subsequently adjusted with 3M-KCl to that of the DE52 equilibration buffer. The yield on three separate extracts, where the shikimate DH activity was monitored, varied between 70% and 75% with between 1.9- and 2.3-fold purification.

4.3.4 Ammonium sulphate fractionation

Following the 'negative' chromatography step described in Section 4.3.3 the eluate was subjected to salt fractionation. The initial ammonium sulphate cut was

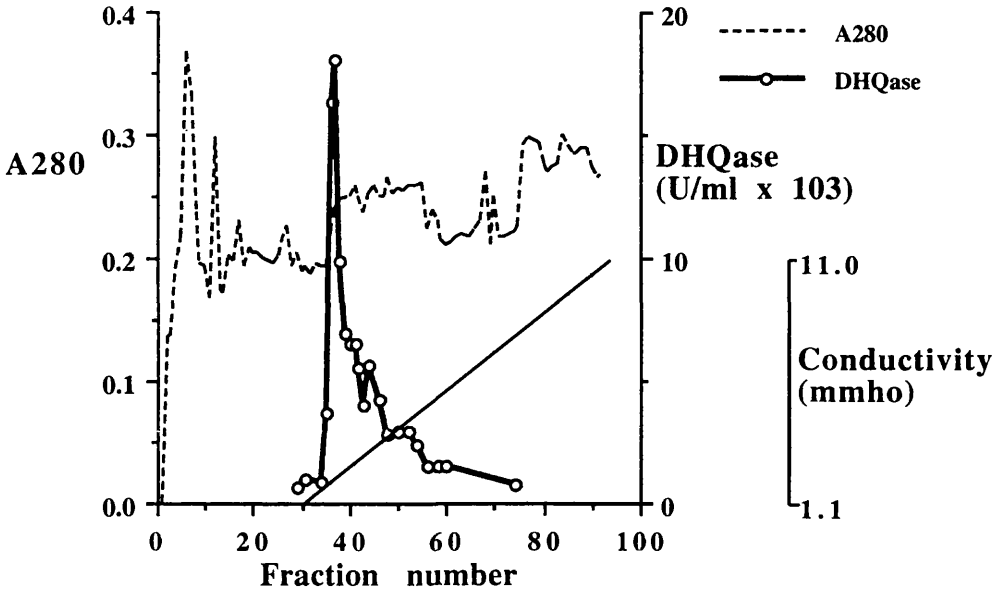


Figure 4.3 **Chromatography of AROM on DE52**

A 30ml DE52 column of 22mm diameter was equilibrated with buffer B containing 1.4mM-ß-mercaptoethanol. Clarified extract of *A. nidulans* 1314 in Buffer A was applied to the column at 1ml/min, and washed with 5 column volumes of extraction buffer. The column was developed with a 300ml continuous linear gradient of 0-370mM-KCl in buffer B containing 1.4mM-ß-mercaptoethanol, run at 1ml/min. 5.4ml fractions were collected and assayed for DHQase activity. The protein concentration was measured as the absorbance at 280nm.

0-30% saturation. Ammonium sulphate was added to the resulting supernatant to 60% saturation and much of the AROM was found in the precipitate. The pellet was resuspended in buffer B containing 0.4mM-DTT and 10μM-leupeptin. AROM fractionated in this manner was analysed by SDS PAGE (Figure 4.7, lane 4).

The ammonium sulphate cut was later tightened, and AROM shikimate DH activity was found to precipitate in the 30-50% saturation range. This gave yields of between 77% and 120% with between 1.4- and 3.2-fold purification on three separate experments. The increase in shikimate DH activity has been discussed in Section 4.2.6.

4.3.5 Chromatography of AROM on Q-Sepharose

After negative DE52 chromatography and ammonium sulphate fractionation, the AROM preparation was dialysed into buffer C containing 0.4mM-DTT and 20μM-zinc acetate. This material was loaded onto a Q-Sepharose anion exchange column equilibrated in buffer C containing 0.4mM-DTT and 20μM-zinc acetate. The loading buffer was 20mM rather than the 50mM used for chromatography on DE52 (see 4.3.3) to allow tighter binding of AROM to the medium. AROM was eluted with a 0-420mM-NaCl gradient in buffer C containing 0.4mM-DTT and 20μM-zinc acetate as shown in Figure 4.4. AROM elutes in a single peak and the cDHQase, if it is still present, is apparently not separated from AROM under these conditions. The peak fractions were pooled and dialysed into buffer B. The degree of purification could probably be improved slightly by using a shallower gradient. The pool from this step was not all used immediately. It was found to lose 30% of its DHQase activity per day which is probably due to proteolysis and the absence of EDTA in the buffer possibly accentuated the problem. The instability coupled with the poor purification factor from this step, led me to believe that a powerful step was required early on, which would hopefully stabilise the AROM activity.

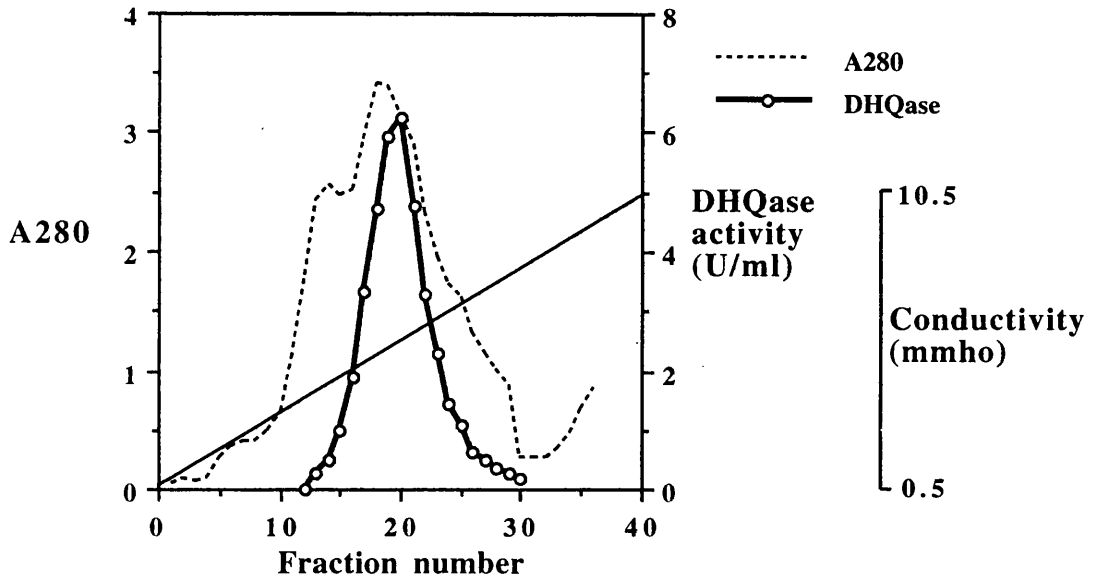


Figure 4.4 **Chromatography of AROM on Q-Sepharose**

A 50ml column of 22mm diameter was equilibrated in buffer C containing 0.4mM-DTT and 20μM-zinc acetate. AROM was applied to the column and washed with equilibration buffer until the absorbance at 280nm had dropped below 0.1. The column was developed at 5ml/min with a 500ml continuous linear gradient of 0-420mM-NaCl in buffer C containing 0.4mM-DTT and 20μM-zinc acetate. The DHQase activity was monitored and the protein concentration measured as the absorbance at 280nm. 14ml fractions were collected.

Blue Dextran Sepharose was a useful step in the purification of *N. crassa* AROM, producing highly purified AROM (680-fold purification) with reliably large yields of 50% (Smith & Coggins, 1983; Boocock, 1983). Therefore, chromatography on Cibacron Blue was thought likely to be a useful step in the purification of *A. nidulans* AROM.

4.3.6 Chromatography of AROM on Cibacron Blue F-3GA

A pool of AROM from the Q-Sepharose chromatography step (Section 4.3.5) was dialysed into buffer C containing 1.4mM-ß-mercaptoethanol, and loaded onto a Cibacron Blue F-3GA column equilibrated in buffer C containing ß-mercaptoethanol. Figure 4.5 shows AROM eluting after a 0.5M-KCl step with a smaller amount eluting after a second step of 1.5M-KCl. 40% and 12% yields were achieved for the medium and high salt washes respectively, with 5-fold purification over the major activity peak. The material from the major peak (0.5M-KCl) was analysed by SDS PAGE (see Figure 4.7, lane 6). Many of the contaminants have been lost. The protein eluted with 1.5M-KCl also showed a major band of 175 kDa, corresponding to AROM, on SDS PAGE (data not shown).

In a preliminary experiment, the *Aspergillus* AROM remained adsorbed to Cibacron Blue in 0.5M-KCl, and was only eluted by 1.5M-KCl. This variability in AROM elution was reminiscent of the attempts to use this chromatography procedure for *S. cerevisiae* AROM purification, which also gave variable results (see 3.5.1). However, given the potentially useful 5-fold purification factor from the Cibacron Blue step, it was thought worthwhile to see whether another dye column might give more reproducible results. This approach will be returned to in Section 4.3.9.

4.3.7 Chromatography of AROM on CM-Sepharose FF

Following Q-Sepharose chromatography, part of the pooled fractions was dialysed into buffer D and loaded onto a CM-Sepharose Fast Flow (FF) cation exchange column equilibrated in buffer D. The AROM bound to the matrix was

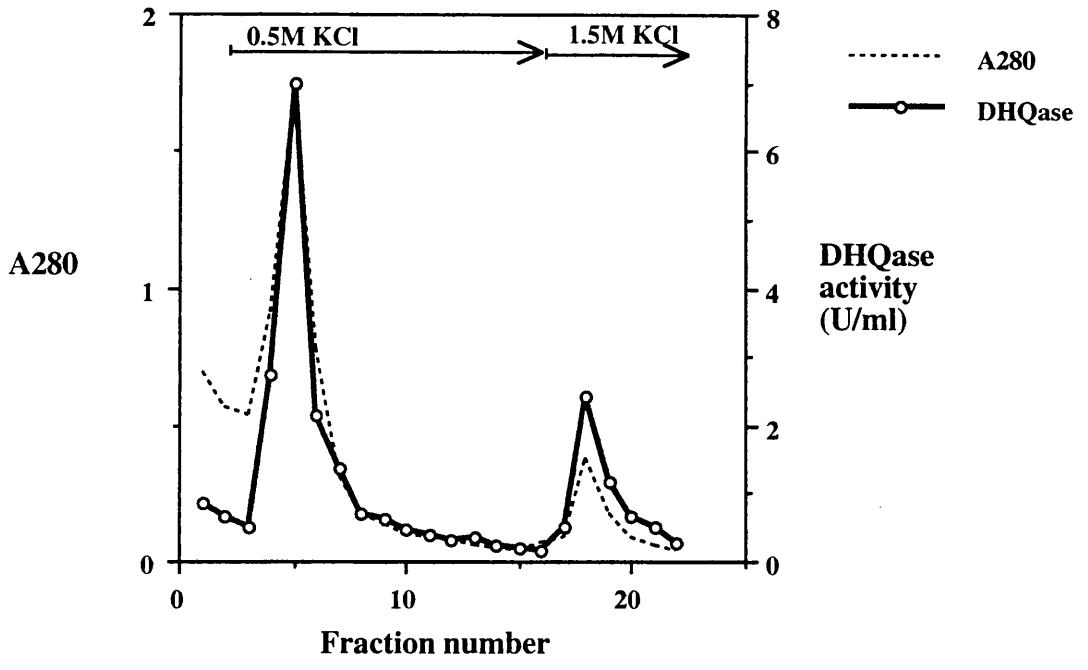


Figure 4.5 **Chromatography of AROM on Cibacron blue F-3GA**

A 2ml plastic syringe packed with 2ml of Cibacron blue F-3GA on Sepharose 4B was equilibrated in buffer C containing 1.4mM-ß-mercaptoethanol. AROM from Q-Sepharose chromatography was loaded at 10ml/h and the column was then washed with loading buffer until the absorbance at 280nm had fallen to 0.5 (data not shown). The column was washed with a salt step of 0.5M-KCl in buffer C containing 1.4mM-ß-mercaptoethanol until the A_{280} dropped below 0.1. The remaining AROM was eluted with a second salt step of 1.5M-KCl in buffer C containing 1.4mM-ß-mercaptoethanol and 20µM-zinc acetate. The DHQase activity was monitored and the protein concentration meaasured as the absorbance at 280nm. 4ml fractions were collected.

eluted with a continuous linear gradient of 10-400mM-potassium phosphate buffer pH6.5. There was no PMSF added to the buffer because it precipitates in this buffer, clogging the matrix and the tubing.

Some activity did not bind to the column under these conditions (8%). Also, the elution profile shown in Figure 4.6(a) displays a double DHQase activity peak with a shoulder prior to the second, major activity peak. The peak fraction eluted in 100mM-potassium phosphate buffer pH6.5. Samples of fractions collected over these peaks were analysed by 7% SDS PAGE (Figure 4.6(b)): this indicates as before that AROM elutes with the second peak and is the major protein present. The first peak (fractions 47 and 48) is possibly cDHQase which runs at the dye-front on 7% SDS PAGE because the quantity of AROM is comparatively low in these fractions. The peak AROM fraction (54) separated on 10% SDS PAGE is shown in Figure 4.7 (lane 8) and appears considerably different to the same sample separated on 7% SDS PAGE shown in Figure 4.6 (b). AROM is not such a prominent band in Figure 4.7, however the samples were exactly the same and the reason for the discrepancy is not known.

The recovery of activity over the gradient was only 20% indicating substantial loss of AROM activity under these conditions. Although AROM was stable during dialysis into buffer D at pH6.5, the Donnan effect may result in a lower pH in the cation exchange matrix during chromatography. It is possible that AROM precipitated in the column at this lower pH if it is at, or near the proteins isoelectric point. Alternatively, the enzyme may have denatured. Interestingly, on overnight storage in the elution buffer the activity of the fractions over the peak increased between 21% and 61% without dialysis, suggesting that the protein had renatured or redissolved in the buffer.

On the basis of these results CM-Sepharose does not provide a useful step for AROM purification.

Figure 4.6 Chromatography of AROM on CM-Sepharose FF

(a) 'Profile'

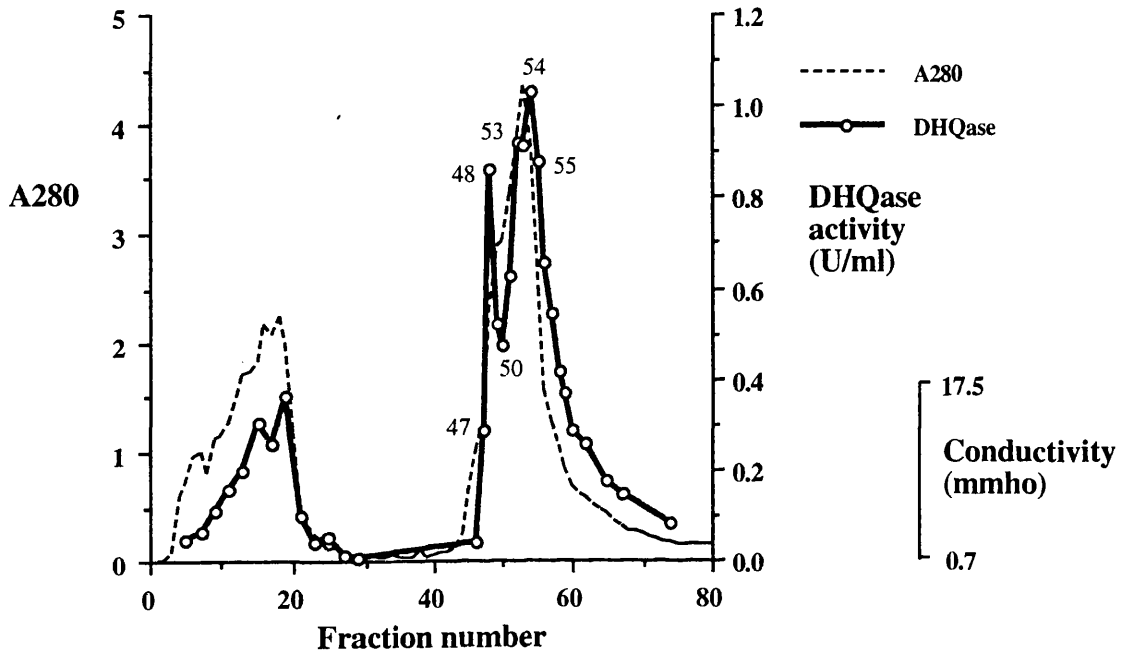
A 22mm diameter column containing 20ml bed volume of CM-Sepharose FF was equilibrated in buffer D. Protein was loaded at 2ml/min. and the column washed washed with buffer D until the absorbance at 280nm had fallen below 0.05. The column was developed with a 200ml continuous linear gradient of 10mM-400mM-potassium phosphate buffer pH6.5, containing 0.4mM-DTT, 5mM-EDTA, 2mM-benzamidine, and 1μM-pepstatin A. 4.4ml fractions were collected and assayed for DHQase activity. The protein concentration was monitored as the absorbance at 280nm.

(b) 'Gel'

Peak fractions were individually desalted and fresh PMSF was added immediately prior to boiling in SDS PAGE sample buffer. The proteins were separated on a 7% SDS-Phastgel which was then stained for protein with Coomassie Brilliant Blue G-250.

Lane 47.	fraction 47, 0.3μg protein
Lane 48.	fraction 48, 0.5μg protein
Lane 50.	fraction 50, 0.3μg protein
Lane 53.	fraction 53, 0.5μg protein
Lane 54.	fraction 54, 0.7μg protein
Lane 55.	fraction 55, 0.8μg protein
M.	Molecular weight markers from top to bottom: 205kDa, 116kDa, 97.4kDa, 66kDa and 45kDa, 0.5μg protein

(a) 'Profile'



(b)'Gel'

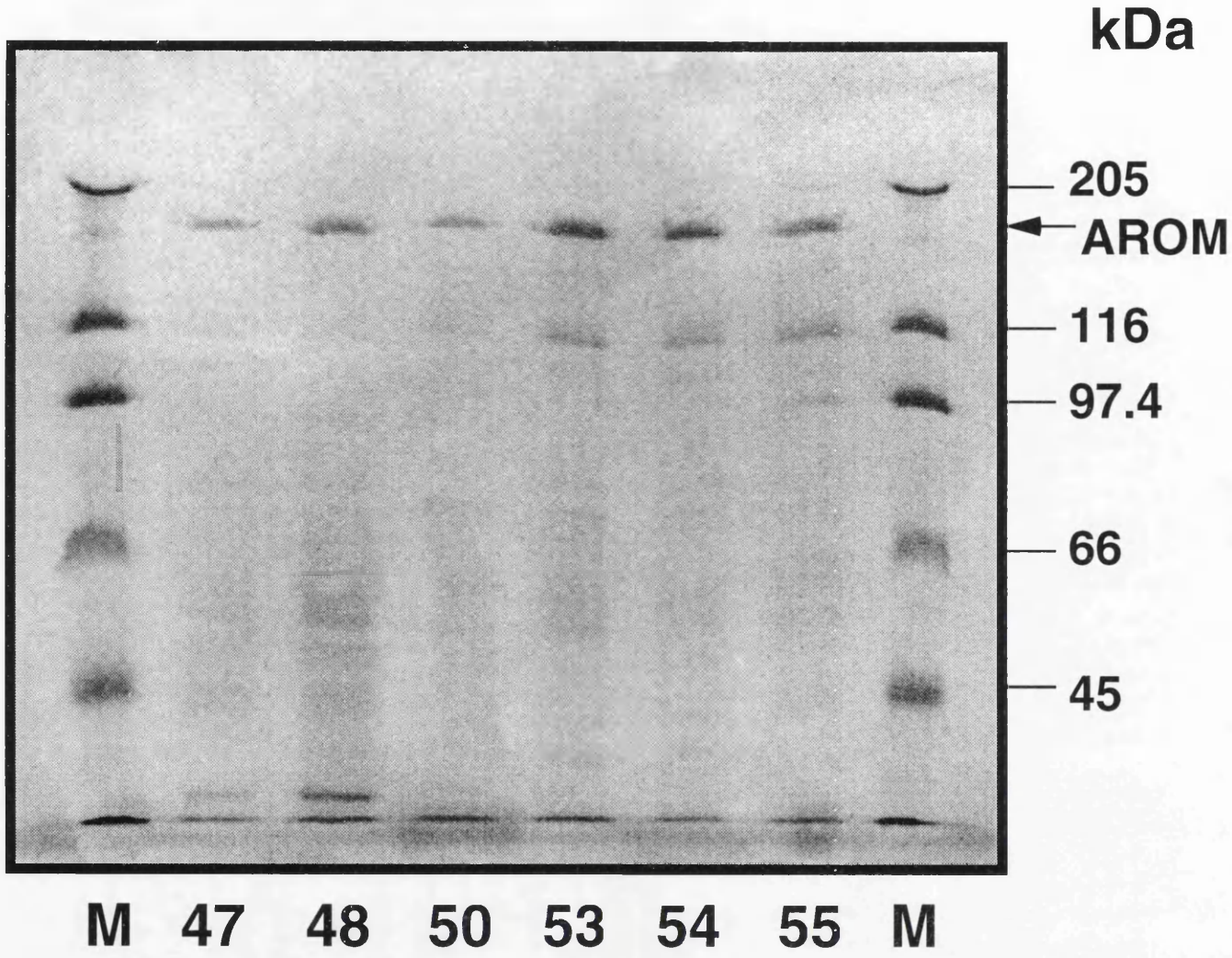
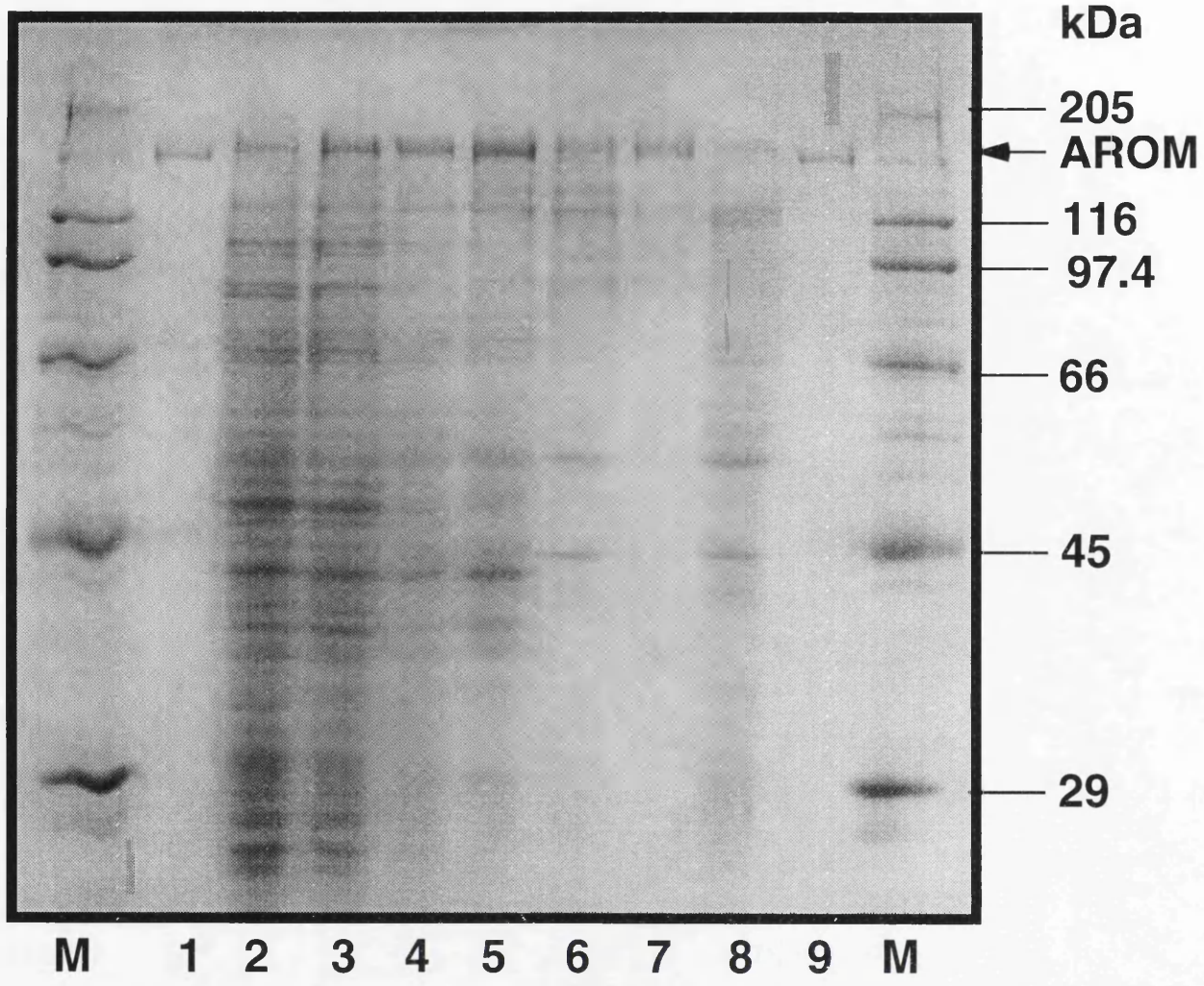

kDa
205
AROM
116
97.4
66
45
M
47
48
50
53
54
55
M

Figure 4.7 **SDS PAGE of samples from exploratory steps for the purification of *A. nidulans* AROM**

Samples were taken from each step and boiled immediately in SDS PAGE sample buffer and stored at -80°C. Samples were reboiled prior to loading on the polyacrylamide gel. Protein concentrations were determined by the method of Bradford (1976). Samples were subjected to SDS PAGE on a 10% gel which was then stained for protein with Coomassie Brilliant Blue.

Lanes 1 and 9.	*N. crassa* AROM, 2μg total protein
Lane 2.	cleared extract, 40μg total protein
Lane 3.	negative anion-exchange pool, 40μg protein
Lane 4.	dialysed 30-60% ammonium sulphate pellet, 20μg protein
Lane 5.	dialysed Q-Sepharose pool, 20μg protein
Lane 6.	desalted peak fraction (54) of Cibacron Blue 6μg protein
Lane 7.	Superose 12 peak fraction, 4μg protein
Lane 8.	CM-Sepharose peak fraction, 25μg protein
M.	Molecular weight markers from top to bottom: 205kDa, 116kDa, 97.4kDa, 66kD, 45kDa and 29kDa, 10μg total protein.


kDa
205
AROM
116
97.4
66
45
29
M
1
2
3
4
5
6
7
8
9
M

4.3.8 Chromatography of AROM on Superose 12

AROM purified by Cibacron Blue chromatography was concentrated using an Amicon Centricon 30 microconcentrator. It was then run at room temperature on an FPLC Superose 12 gel filtration column equilibrated in buffer E. The column was run at 0.5ml/min and AROM eluted as expected in the void volume. Analysis of the peak fraction by SDS PAGE (Figure 4.7, lane 7) indicated that Superose gel filtration was a useful purification step for AROM since many of the lower molecular weight proteins were removed. The cDHQase should have been separated from AROM by Superose 12 gel filtration chromatography and it is not observed on SDS PAGE (data not shown).

This exploratory purification is summarised in diagram A of Figure 4.9.

4.3.9 Chromatography of AROM on the 'PIKSI' Mimetic dye test kit

The promising AROM purification achieved on a Cibacron Blue column (Section 4.3.6) led me to test AROM binding to other dyes. The binding of AROM to Cibacron Blue may be attributable to the multiple nucleotide binding sites on the protein: the NAD^+ site of the DHQ synthase domain, the NADPH site of the shikimate DH domain, and the ATP binding site of the shikimate kinase domain. It seemed likely that AROM would bind to other similar dyes.

The 'PIKSI' test kit from Affinity Chromatography Ltd. contains ten different Mimetic ligands (Red-2 and -3, Orange-1,-2 and -3, Yellow-1 and -2, Green-1 and Blue-1 and -2) coupled through a spacer arm to 6% cross-linked agarose (A6XL). These adsorbents have the advantage of surviving harsh regeneration treatments (1-2M-NaOH) and can therefore be used repeatedly to fractionate fairly crude protein extracts. The adsorbents are in the form of 1ml columns (in a screening kit) which are run under gravity.

Initial binding studies were performed in 25mM-sodium phosphate buffer pH6.0, however, AROM lost 30% of its shikimate DH activity during dialysis,

possibly due to precipitation or denaturation as discussed in Section 4.3.7. AROM was able to bind most of the dye ligand adsorbents at higher pH.

From these initial studies, five of the Mimetic dyes were selected on the basis of AROM purity of the eluent on analysis by SDS PAGE (data not shown). The dyes selected for further trials were - Mimetic Red-2 and -3, Orange-1, and Yellow-1 and -2. AROM purified by negative DE52 chromatography and ammonium sulphate fractionation (30-50% saturation) was dialysed into buffer F. Aliquots of this AROM preparation were loaded onto each of the five columns, which were then washed with 0.5M-KCl in buffer F followed by a 1.5M-KCl step. The results are shown in Figure 4.8. The elution pattern is roughly similar in each case and the eluates look alike by SDS PAGE (data not shown). Specific activities could not be measured in every eluate because the protein concentration was too low in some. Under these conditions Mimetic Yellow-1 appears better than the other dyes at separating AROM from most of the other proteins originally present. The AROM eluted in the 0.5M-KCl wash from Mimetic Yellow-1 showed a 7-fold purification of specific activity. This estimate is not accurate because the shikimate DH yield was between 116% and 142% on the Mimetic ligands. This increase in shikimate DH activity has been discussed prevoiusly in Section 4.2.6.

The proteinase inhibitor, PMSF, is very insoluble in sodium phosphate buffer and the buffer requires filtration prior to use to remove solid PMSF. The chromatography was repeated in 25mM-Tris/HCl pH7.5, in which PMSF is more soluble, however, analysis of the eluate on SDS PAGE suggested that the purification factor was greatly reduced using this buffer. An alternative might have been HEPES buffer, however, I proceeded using sodium phosphate buffer.

Mimetic Yellow-1 chromatography provides a quick and effective purification step. It was hoped that its use early in the protocol would stabilise AROM.

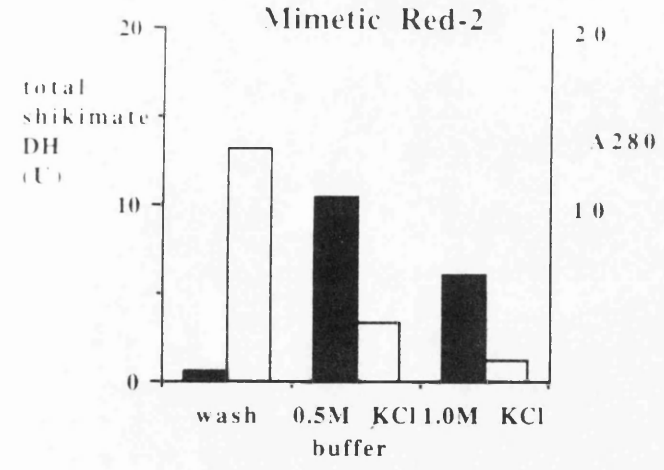
This exploratory purification is summarised in diagram B of Figure 4.9.

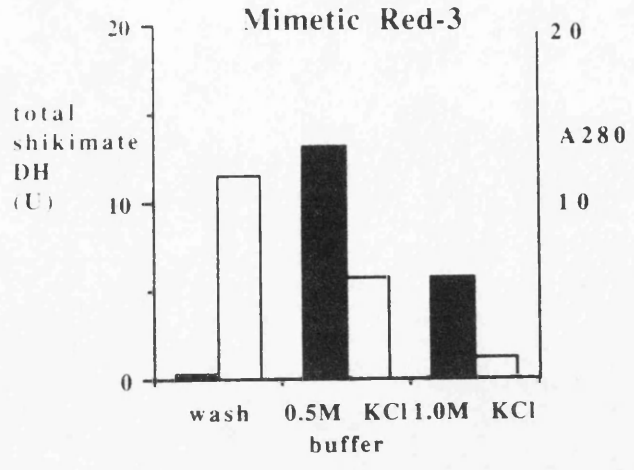
Figure 4.8 AROM binding to Mimetic dye columns

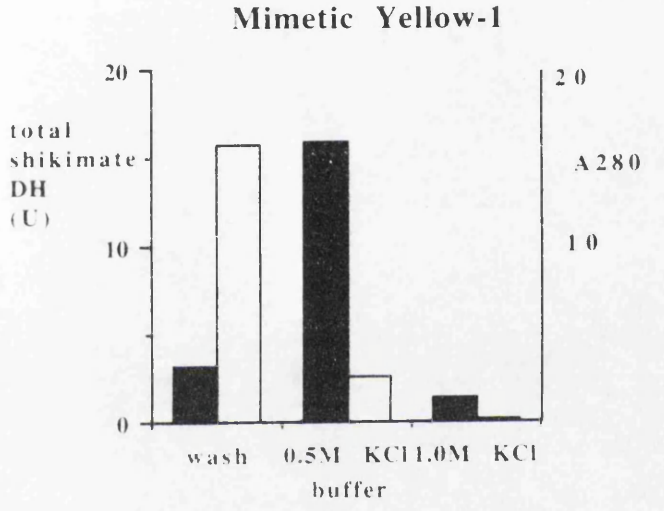
5g dry weight of *A. nidulans* 1314 was extracted in buffer A. This was stirred for 60 min then centrifuged at 35, 000g for 60 min. Supernatant was run through a 'negative' DE52 column and a 30-50% saturation ammonium sulphate cut was then taken. The resuspended precipitate was dialysed overnight into buffer F containing 1.2mM-PMSF

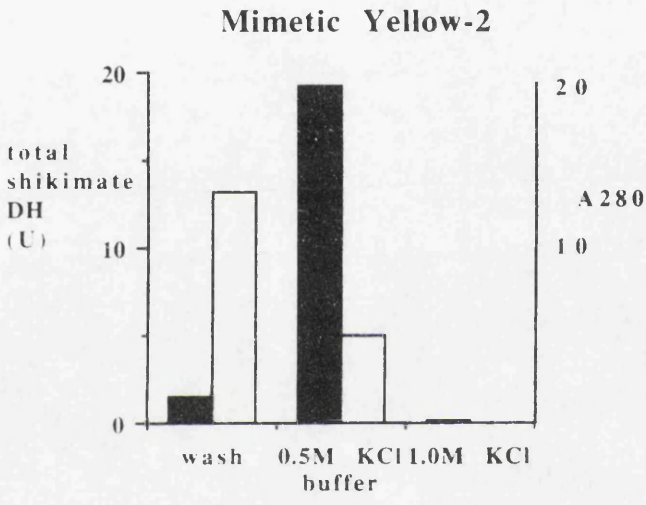
The 1ml Mimetic dye columns in the PIKSI kit were equilibrated in 10ml of buffer F. The AROM preparation was centrifuged at 35,000g for 20 min to remove precipitated protein. 14mg of protein (15 U of shikimate DH activity) was loaded onto each column in a volume of 0.8ml. Each column was washed with 4ml of loading buffer, and the eluate monitored for both shikimate DH activity and for protein concentration by measuring the absorbance at 280nm. Each column was washed with a 4ml step of 0.5M-KCl in buffer F, then 4ml of 1.0M-KCl in buffer F. Eluate was pooled from each salt step and assayed for shikimate DH activity and the protein concentration was monitored as the absorbance at 280nm.

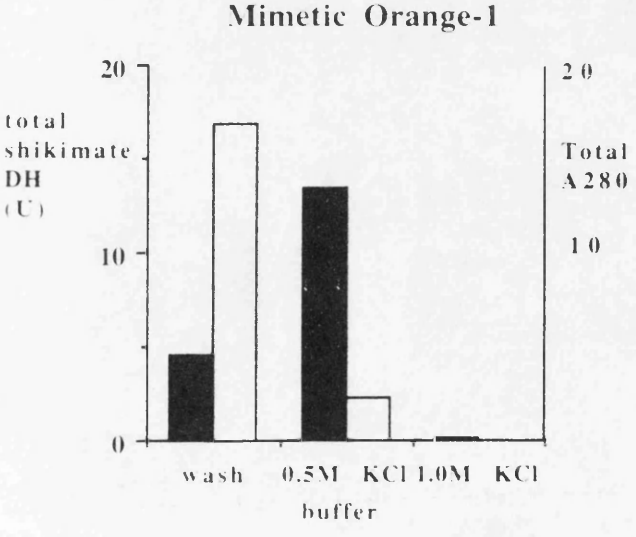
The figure shows both shikimate DH and protein elution from the Mimetic dye columns Red-2 and -3, Yellow-1 and -2, and Orange-1. The A_{280} shown is the absorbance at 280nm for 1ml multiplied by the volume, which is 4ml, to give an estimate of the total protein.

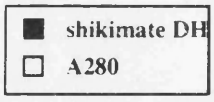

Mimetic Red-2
total shikimate DH (U)
A280
20
10
0
wash
0.5M KCl
1.0M KCl
buffer


Mimetic Red-3
total shikimate DH (U)
A280
20
10
0
wash
0.5M KCl
1.0M KCl
buffer

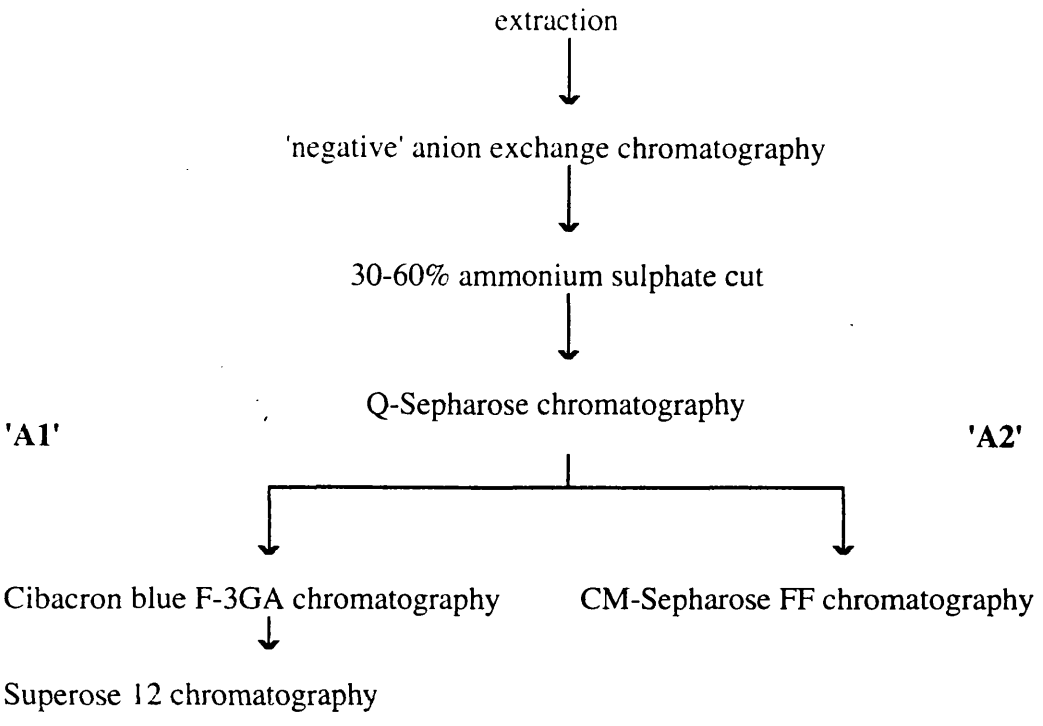

Mimetic Yellow-1
total shikimate DH (U)
A280
20
10
0
wash
0.5M KCl
1.0M KCl
buffer


Mimetic Yellow-2
total shikimate DH (U)
A280
20
10
0
wash
0.5M KCl
1.0M KCl
buffer


Mimetic Orange-1
total shikimate DH (U)
Total A280
20
10
0
wash
0.5M KCl
1.0M KCl
buffer


shikimate DH
A280

A.



B.

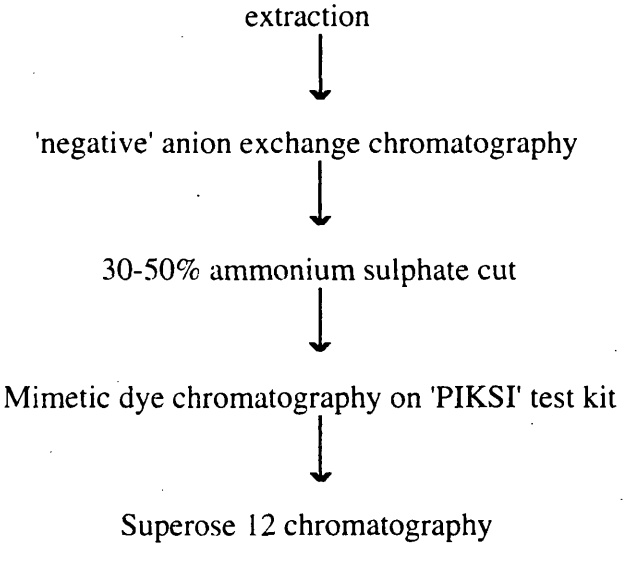


Figure 4.9 **Summary of the exploratory steps used in the purification of AROM from *A. nidulans* 1314**

4.3.10 Summary

Figure 4.9 summarises the steps tried in developing the protocol for the purification of AROM from *A. nidulans* 1314. Part A is described in Sections 4.3.2 to 4.3.8, while route B outlines Section 4.3.9.

The purification steps in route B form the basis of the preparative purification finally used (see Section 4.6).

4.4 Evaluation of the preliminary purification of AROM from *A. nidulans* 1314.

4.4.1 Individual enzyme activities of AROM partially purified from *A.nidulans* 1314

AROM which had been purified from, *A. nidulans* 1314 by route A1 (see Figure 4.9) was assayed for the five individual AROM activities. The AROM lacked DHQ synthase and EPSP synthase activities which are the activities associated with the two N-terminal domains of AROM. It had been hoped that the DHQ synthase, which was at very low levels in crude extract (see 4.2.3), might be easier to assay in purified preparations of AROM in which the buffers used for the preparation had been supplemented with zinc. At this stage, it was believed that the absence of the DHQ synthase and EPSP synthase activities might be due to proteolysis of AROM during purification or the loss of zinc from the N-terminal DHQ synthase domain. The EPSP synthase activity of *N. crassa* AROM is very sensitive to both proteolysis during purification and oxidation as mentioned in Section 4.2.6. The use of DTT in buffers should have prevented inactivation caused by oxidation.

The activity ratio of the three remaining AROM activities was standardised against the shikimate kinase activity. The ratio of DHQase:shikimate DH:shikimate kinase was 78:164:100 compared to 76:69:100 in crude extract (Table 4.1). Although this preparation retains three of the AROM activities, both the DHQ synthase and EPSP synthase activities have been lost in the purification of AROM by route A1.

4.4.2 Immunoblot of AROM from *A. nidulans* 1314 with anti-AROM raised against *N. crassa* AROM

A pool of AROM purified by mimetic dye chromatography, during the binding studies described in Section 4.3.9, was further purified by Superose 12 chromatography (route B in Figure 4.9). Following separation by SDS PAGE protein was immunoblotted with polyclonal antibodies raised against *N. crassa* AROM (Figure 4.10).

The immunoblot shows that at least some of the antibodies cross-react with *A. nidulans* AROM. They also cross-react with several low molecular weight polypeptides in the AROM sample. In particular, species of 130kDa, 115kDa, 88kDa, 59kDa and 40kDa cross-react quite strongly.

The banding pattern is probably the result of proteolysis of AROM as has been seen in the yeast AROM preparation (Sections 3.5.3 and 3.5.4). These fragments are likely to occur as complexes which resemble native AROM under non-denaturing conditions but which 'fall apart' when denatured.

The cross-reactivity of low molecular weight species in the AROM preparation with anti-AROM antibodies suggests that the AROM has been cleaved by endogenous proteinases despite the use of proteinase inhibitors. Procedures to minimise endogenous proteinases in *A. nidulans* are described in the next section

4.5 Optimisation of the growth conditions for *A. nidulans* 1314

4.5.1 Outline of the stages adopted to minimise endogenous proteinase production in *A. nidulans* 1314

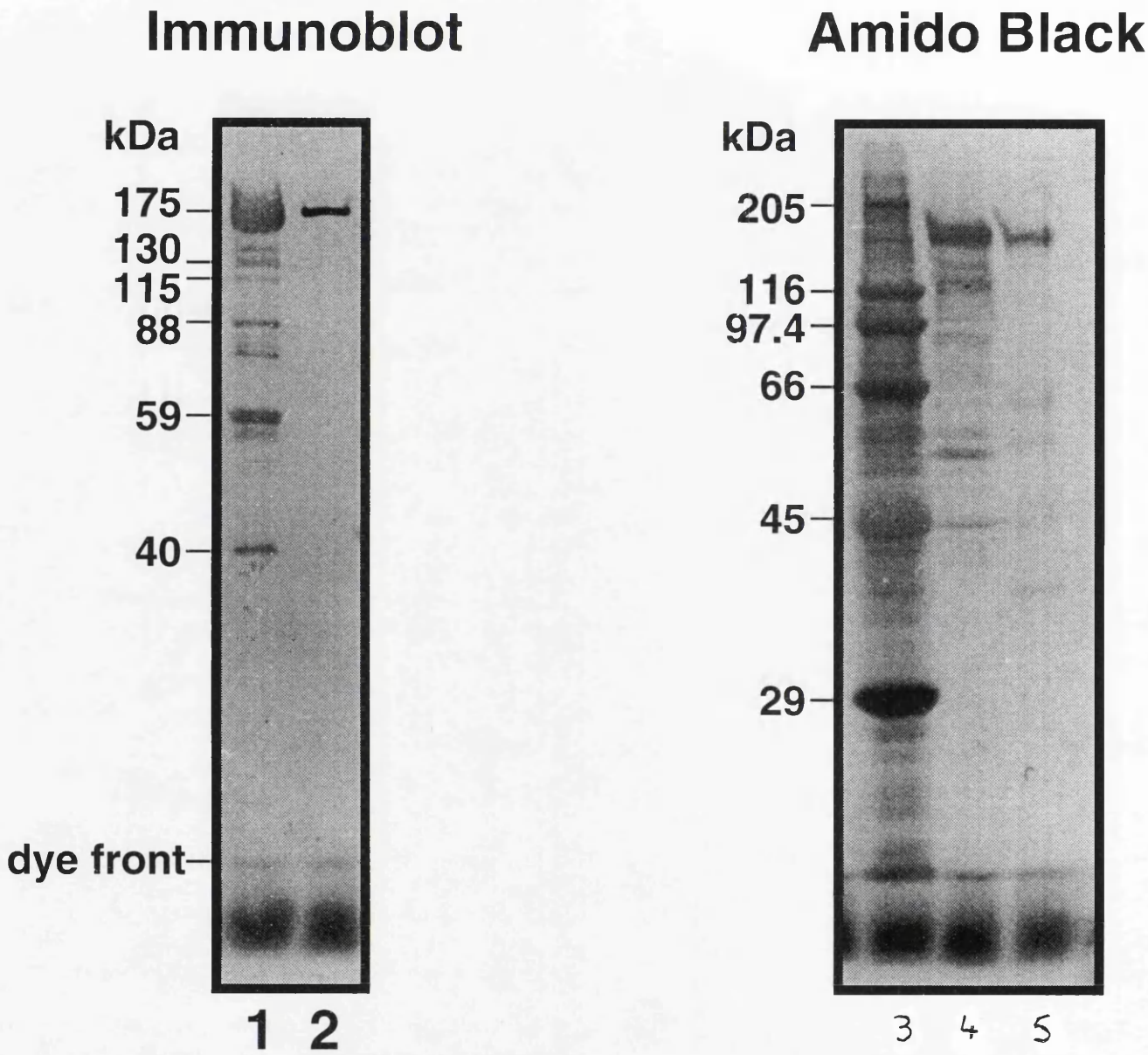
Evidence for proteolysis of *A. nidulans* AROM comes both from enzyme activity losses and immunoblotting studies. This led me to change the method of mycelial growth in an attempt to minimise endogenous proteinase production.

The final method of mycelial growth is described fully in Section 2.12.7 (Method 2). The reasons for the changes made to the growth conditions are outlined below:

Figure 4.10 **Immunoblot of AROM from *A. nidulans* 1314 with anti-AROM antibodies**

AROM purified from *A. nidulans* 1314 by route B (Figure 4.9) was subjected to 10% SDS PAGE. Protein was electroblotted onto nitrocellulose and lanes 3-5 were stained for protein with amido black. Lanes 1 and 2 were probed with an antiserum raised against *N. crassa* AROM in rabbit. Bound antibodies were visualised using HRP-anti-rabbit conjugate. The colour was developed with chloronapthol and hydrogen peroxide as described in Section 2.7.2. The protein concentration was determined by the method of Bradford.

Immunoblot	Lane 1.	*A. nidulans* AROM, 14μg protein
	Lane 2.	*N. crassa* AROM, 0.4μg protein
Protein stain	Lane 3.	molecular weight markers, 10μg protein
	Lane 4.	*A. nidulans* AROM, 10μg protein
	Lane 5.	*N. crassa* AROM, 2μg protein


Immunoblot
kDa
175
130
115
88
59
40
dye front
1 2
Amido Black
kDa
205
116
97.4
66
45
29
3 4 5

1. The native environment for *A. nidulans* is a woodland environment and the fungus lives off rotting plant matter (Hawkins *et al.*,1982(b)). The former mycelial growth temperature of 37°C was considered to be rather high , therefore, the growth temperature was changed to 30°C.

2. It was a concern that the large 10 litre flasks used for *Aspergillus* growth did not allow adequate aeration even with air forced onto the medium as it was stirred vigouously. Mycelia were subsequently grown in 2 litre baffled flasks on an orbital shaker.

3. Proteinase production is known to be maximal in *N. crassa* (Yu *et al.*, 1973) and in yeast (Pringle, 1975) when cells reach the stationary phase of growth. Therefore, growth on the pre-induction medium was monitored and the mycelia were harvested for transfer to inducing medium while still in the log phase of growth. This is explained more fully in Section 4.5.2.

4. The induction of AROM was followed during mycelial growth on quinate and is described in Section 4.5.3. Mycelia were subsequently harvested when the cells were in the log phase of growth as determined by an increasing AROM production on quinate.

4.5.2 Construction of a growth curve for *A. nidulans* grown on glucose

The medium used for the initial bulk mycelial growth, prior to AROM induction, contains glucose. The growth rate of mycelia can be followed indirectly by monitoring the fall of glucose concentration.

The samples of medium taken during growth were centrifuged to pellet the mycelia. The glucose concentration of the supernatant was determined using the GOD-Perid method (see Section 2.12.7). In this way the depletion of glucose was monitored and mycelia were transferred to the inducing media while the rate of

glucose consumption was still growing logarithmically. It was hoped that this would avoid the possibility of a high level of proteinases seen when the cells of other fungi enter stationary phase. Obviously the change in carbon source, from glucose to glycerol may affect the range of the proteins produced by the cell, and it will possibly target those involved in glucose metabolism for degradation. However, such proteolysis of proteins will hopefully be directed at a specific subset of the cellular proteins rather than resembling a more general breakdown of proteins in stationary phase cells.

4.5.3 Measurement of AROM levels in *A. nidulans* 1314 during induction on quinate

The effect of reducing the growth temperature from 37°C to 30°C on the rate of AROM induction on quinate was investigated. Although quinate DH is absent from strain 1314, there is some evidence that the quinate used to induce AROM production is catabolised by the shikimate DH activity of the overexpressed AROM protein (Moore *et al.*, 1992). The rate of quinate catabolism presumably increases with the degree of overexpression of AROM. Van den Hombergh *et al.* (1992) have shown that *A. nidulans* quinate DH can complement an *E. coli* mutant strain lacking shikimate DH. Shikimate DH of AROM and quinate DH of *A. nidulans* show extensive homology at the protein sequence level (Hawkins *et al.*, 1988). The quinate concentration of the inducing medium was doubled from 0.1% to 0.2% (w/v) and the AROM induction was monitored.

A. nidulans 1314 was grown on minimal medium with 0.4% glucose at 30°C and the glucose concentration was monitored. At this stage, and at hourly intervals during induction on quinate, *Aspergillus* samples were collected and extracts made. These were assayed for shikimate DH activity and analysed by SDS PAGE. Figure 4.11(a) shows a steady increase in shikimate DH specific activity over the induction period. After six hours the specific activity had reached that seen previously when the mycelia were induced for five hours at 37°C (Table 4.1). These

Figure 4.11 **Increase in AROM production in *A. nidulans* 1314 during induction on quinate.**

A. nidulans 1314 was grown on AMM supplemented with 0.4% glucose at 30°C on an orbital shaker. Glucose utilisation was monitored by the GOD-Perid method (see Section 2.12.7). Log phase cells were filtered through muslin, washed with distilled water and transferred to fresh minimal medium containing 0.2% (w/v) quinate and 0.2% (v/v) glycerol. 20ml samples were taken immediately prior to induction, and at regular intervals during induction on quinate. These samples were filtered under vacuum and the mycelia were stored at -80°C.

Mycelia were broken with a pestle and mortar in liquid nitrogen and extracted into buffer A. Extract was shaken gently at 4°C for 60 min then centrifuged for 15 min using a microfuge to remove cell debris. Protein concentration was determined by the method of Bradford (1976).

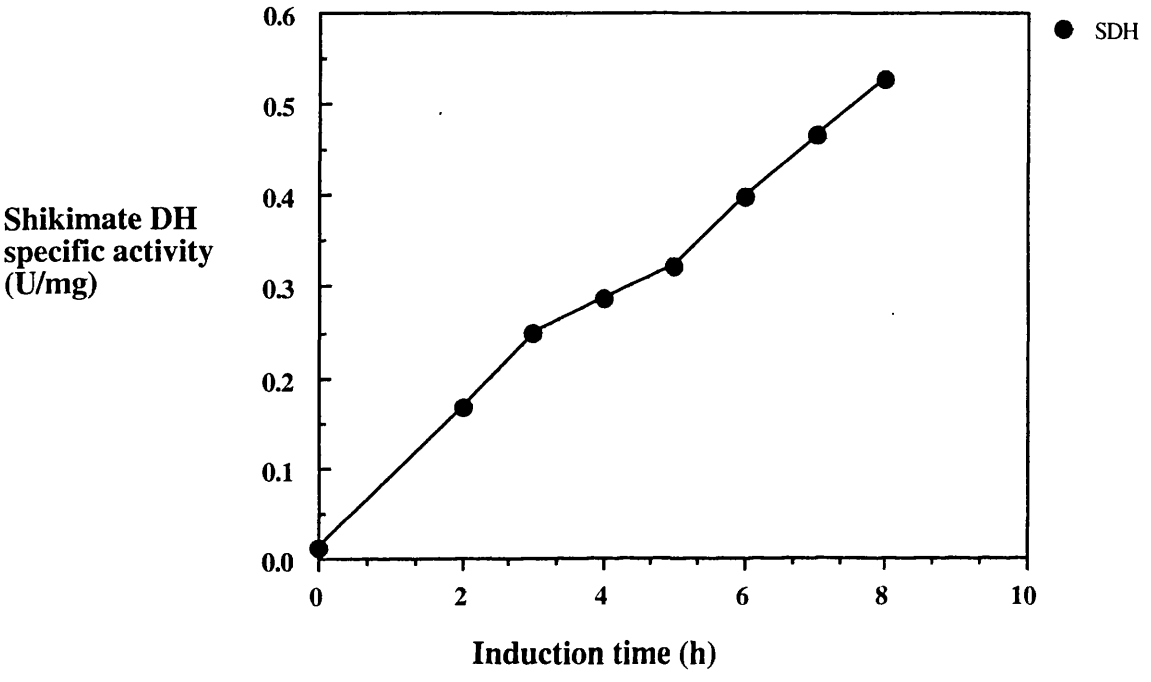
(a) 'Activity levels'

Supernatant from the crude extract samples were assayed in duplicate for shikimate DH activity. The graph shows an increase in the shikimate DH specific activity with induction on quinate.

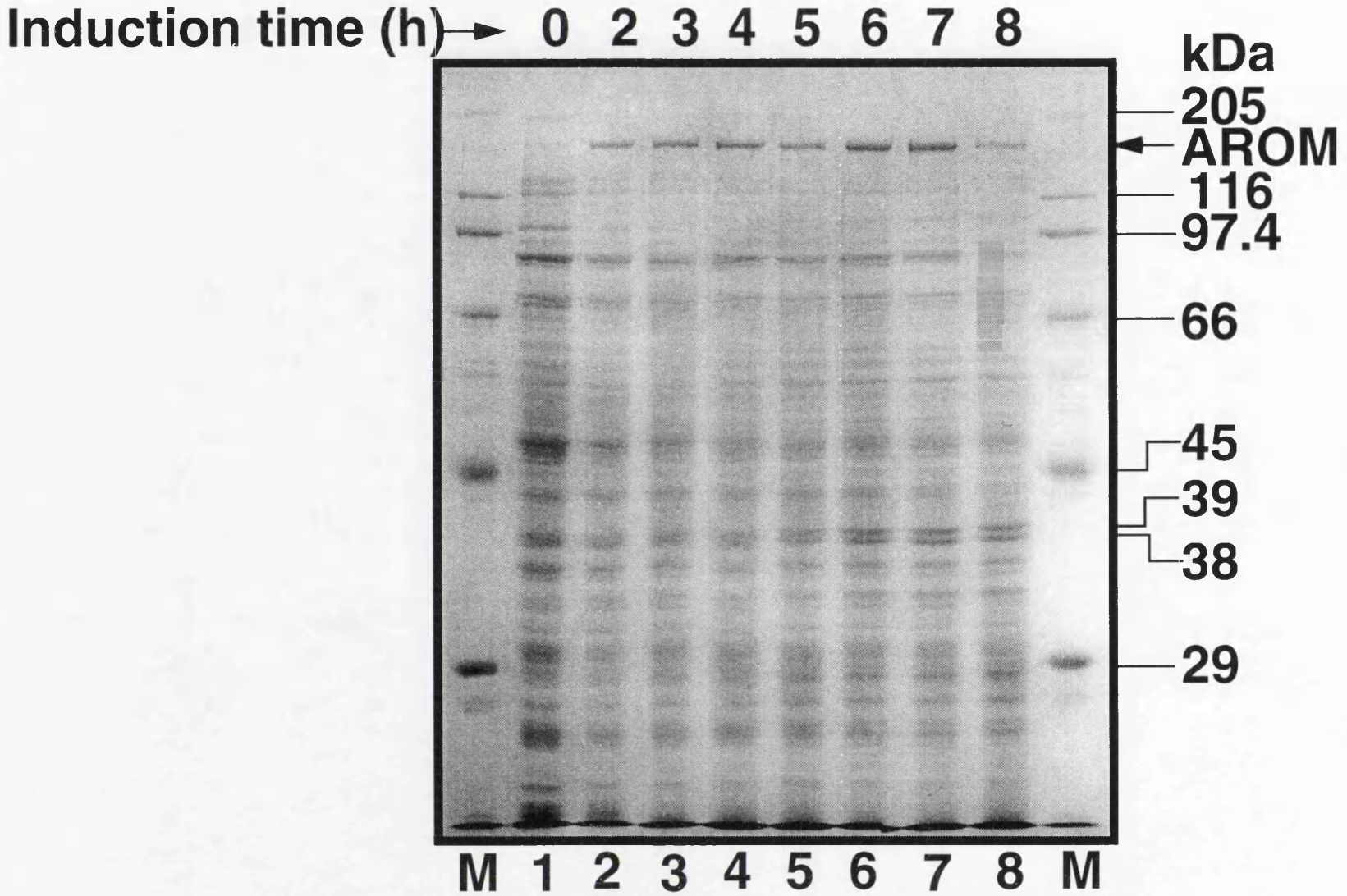
(b) 'Gel'

The figure shows the induction of AROM in *A. nidulans* 1314 in which the protein in cleared supernatant has been separated by SDS PAGE in a 10% gel. 30µg of protein was loaded in each lane. The gel was stained with Coomassie Brilliant Blue G-250. Lanes 1 to 8 are pre-induction, and after 2h, 3h, 4h, 5h, 6h, 7h and 8h induction on quinate respectively. Lane M, molecular weight markers from the top: 205kDa, 116kDa, 97.4kDa, 66kDa, 45kDa and 29kDa. Markers were loaded at 10µg of protein per lane.

(a) 'Activity levels'



(b)'Gel'


Induction time (h)
0 2 3 4 5 6 7 8
kDa
205
AROM
116
97.4
66
45
39
38
29
M 1 2 3 4 5 6 7 8 M

data suggest that quinate is not limiting up to 8 hours growth. Analysis of the samples, taken during growth, by SDS PAGE is shown in part (b) of Figure 4.11. This shows AROM levels increasing over the first seven hours with an apparent decrease at eight hours although the shikimate DH activity was still increasing. A possible explanation for this is that the AROM is becoming proteolytically 'nicked' while still retaining activity. The gel besides showing increasing levels of AROM up to 7 hours also shows an increase in the level of several other polypeptides during the induction period: most noticeable is a 38/39kDa doublet.

From these results I subsequently induced AROM synthesis for seven hours on quinate to maximise production of AROM and minimise proteinase production.

4.5.4 Summary

The revised protocol for mycelial growth is described in Section 2.12.7 (method 2). Briefly, it uses growth at 30°C with maximal aeration. Mycelia are transferred to inducing medium while they are still in the log phase of growth, and induced on quinate for seven hours prior to harvesting.

4.6 Preparative scale AROM purification from *A. nidulans* 1314

4.6.1 Introduction

The preparative method for the purification of AROM from *A. nidulans* 1314 is decribed in this section. It uses the successful purification steps developed in Section 4.3. The aim of the initial purification was to get intact protein. Optimisation of the conditions for particular activities would come later with refinement of the protocol. Proteinase inhibitors were used extensively, including EDTA. The time required for the entire purification was minimised by the use of desalting FPLC instead of dialysis prior to loading onto the Mimetic Yellow-1 dye column. The anti-proteinase effect of the negative anion exchange chromatography was questionable, but the column was retained in case it did reduce proteolysis.

Shikimate DH activity was monitored throughout the procedure.

4.6.2 Protocol for the purification of AROM from *A. nidulans* 1314

Unless otherwise stated, all steps were carried out at 4°C. Samples were taken at each stage of the purification and stored at -80°C for later estimation of the protein content by the method of Bradford (1976). Samples for analysis by SDS PAGE were boiled immediately in SDS PAGE sample buffer and stored at -80°C.

Step1 Extraction

6.09g dry weight of *A. nidulans* 1314, grown by method 2, Section 2.12.7, was suspended in 15 volumes of buffer A and stirred on ice for 60 min. The cell debris was removed by centrifugation at 35,000g for 60 min, and the cleared supernatant was filtered through two layers of muslin.

Step 2 'Negative' chromatography

The conductivity of the supernatant was adjusted with 3M-KCl until it was identical to the conductivity of 0.1M-KCl in buffer B containing 5mM-EDTA. An 80ml (22mm diameter) DE52 column equilibrated with 0.1M-KCl in buffer B with 5mM-EDTA was flushed with one column volume of the buffer containing fresh PMSF immediately prior to loading the crude extract. The extract was run on the DE52 column, which was then washed with equilibration buffer at 60ml/h. Fractions containing shikimate DH activity were pooled.

Step 3 Ammonium sulphate fractionation

The 'negative' chromatography pool was fractionated with ammonium sulphate. A 30-50% saturation ammonium sulphate cut contained the AROM. The precipitate was resuspended in buffer A with freshly added proteinase inhibitors.

Step 4 Desalting

The resuspended ammonium sulphate precipitate was desalted at room temperature on an FPLC G-25 superfine gel filtration column (HR 16/10). The column was run at 4ml/min in buffer F with 4ml of the AROM preparation per run. Fractions containing shikimate DH activity were pooled, and fresh PMSF and leupeptin were added.

Step 5 Chromatography on Mimetic Yellow-1

A 24ml Mimetic Yellow-1 dye column (22mm diameter) was equilibrated in buffer F. PMSF was added to buffer F, then the solution was filtered to remove any precipitated PMSF. The column was immediately flushed with one column volume of this buffer. Desalted AROM was filtered to remove precipitated PMSF (to avoid the column and tubing becoming clogged). The AROM preparation was loaded at 60ml/h and washed with four column volumes of buffer F. AROM was eluted overnight with a salt step of 0.5M-KCl in buffer F containing PMSF (filtered). The elution profile is shown in Figure 4.12. 1.0M-KCl in buffer F failed to elute any more AROM.

Peak fractions 36, 37 and 38 were pooled and dialysed into buffer B containing 5mM-EDTA.

Step 6 Superose 6 gel filtration chromatography

The dialysed AROM preparation was concentrated using an Amicon centriprep 30 and loaded onto an FPLC Superose 6 gel filtration column (HR 10/30) in 3 separate runs. The elution profile (Figure 4.13) shows a single AROM peak. Protein from the peak fractions from the three runs were analysed by SDS PAGE and the AROM containing fractions with fewest contaminants were pooled.

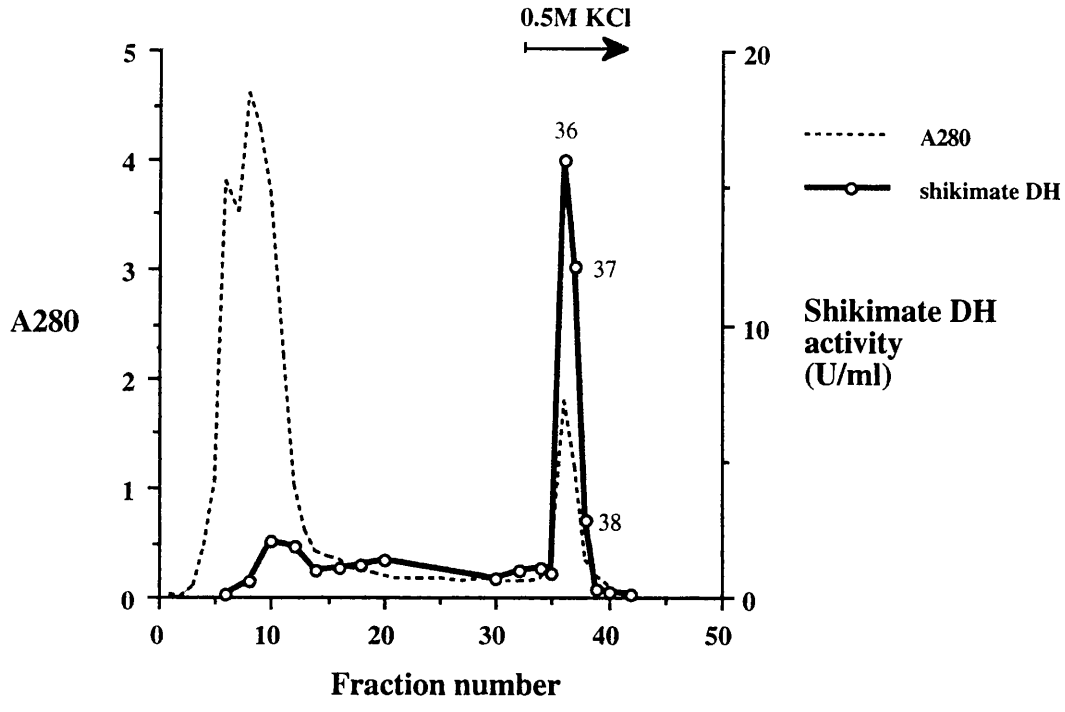


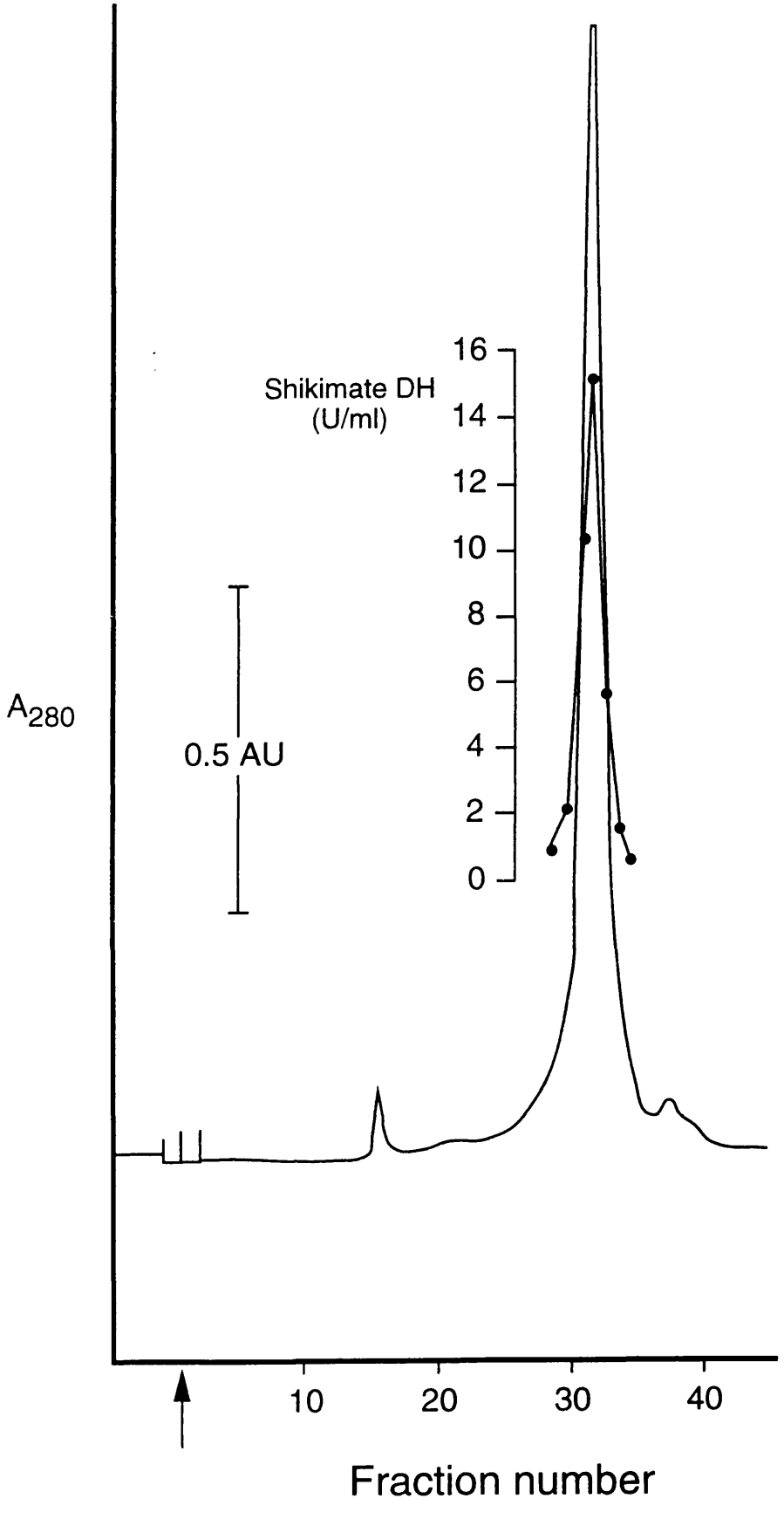
Figure 4.12 **Chromatography of AROM on Mimetic Yellow-1 A6XL**

A 24ml column (22mm diameter) of Mimetic Yellow-1 was equilibrated in buffer F. Filtered sample was loaded at 60ml/h. The column was washed with 4 column volumes of loading buffer until the absorbance at 280nm had dropped below 0.2. AROM was eluted with 0.5M KCl in buffer F. Fractions of 4.5ml were collected during loading and the wash, and 4.0ml fractions during the salt wash. Fractions were assayed for shikimate dehydrogenase activity and the protein concentration monitored at 280nm.

Figure 4.13 **Chromatography of AROM on a Superose 6 gel filtration column**

A Superose 6 (HR 10/30) FPLC column was equilibrated at room temperature in buffer E and subsequently run at 0.25ml/min. The AROM preparation was concentrated using an Amicon centriprep 30, and three separate 200µl aliquots were loaded and run. The A_{280} was continuously monitored and the shikimate DH activity was assayed in the individual 0.5ml fractions collected.

The profile shows one column run. AROM eluted in a single peak, and the fractions were pooled on the basis of the appearance of the protein analysed by SDS PAGE (data not shown).


Shikimate DH
(U/ml)
16
14
12
10
8
6
4
2
0
A_{280}
0.5 AU
10
20
30
40
Fraction number

Step 7 Storage

The AROM pooled from Superose 6 chromatography was dialysed into 50% (v/v) glycerol in 50mM-Tris/HCl pH7.5 containing 0.4mM-DTT, 20μM-zinc acetate, 2mM-benzamidine, 1.2mM-PMSF, 1μM-pepstatin A and 10μM-leupeptin. Some was stored at -20°C, and the remainder at -80°C.

4.6.3 Analysis of the preparative purification of AROM from *A. nidulans* 1314

The purification of AROM from *A. nidulans* 1314 is summarised in Table 4.3. AROM was purified 13-fold with 9% yield. The subunit molecular weight was estimated to be 175kDa by SDS PAGE (Figure 4.14), the size predicted from the sequence. It is likely that the actual yield is slightly lower due to the activation of shikimate DH activity during the preparation shown in Table 4.3. The purified AROM has a specific shikimate DH activity of 4.1U/mg. At a higher protein loading minor contaminating polypeptides of 130kDa and 120kDa can be seen when the protein is analysed by SDS PAGE (Figure 4.14). Although not seen in the photograph there are several other trace contaminants ranging from 45kDa to 130kDa.

The cDHQase has been removed from the AROM preparation by Mimetic Yellow chromatography as determined by SDS PAGE which shows no protein running at the dye front (Figure 4.14). The possible reason for the increase in shikimate DH activity after negative chromatography on DE52, ammonium sulphate fractionation and very slightly after dialysis following chromatography on Mimetic Yellow-1 has been discussed in Section 4.2.6. The latter activity increase may result from the loss of an inhibitor of shikimate DH during dialysis.

The protein concentration of the clarified extract loaded onto the DE52 column was rather high and in future the dry *A. nidulans* should be suspended in 20 rather than 15 volumes of buffer A. In this case the step did result in an apparent 2-fold purification of AROM. Table 4.3 also shows that the recovery of AROM from the Mimetic Yellow-1 dye column was rather low. Only 26% of the activity loaded onto the column eluted with the 0.5M-KCl salt step. The large drop in yield (Table

	Shikimate DH					
Step	**Volume (ml)**	**Total protein (mg)**	**Specific activity (U/mg)**	**Total activity (U)**	**Purification (fold)**	**Yield (%)**
crude extract	85	1088	0.32	348	1	100
'Negative' DE52 anion exchange	84	514	0.694	357	2.17	103
30-50% $(NH_4)_2SO_4$ pellet	19.5	273	1.57	429	4.91	123
FPLC Fast desalting column	24	185	2.23	413	6.97	119
Mimetic Yellow-1	12	23.6	4.57	108	14.3	31
Dialysed Mimetic Yellow-1	14	22.0	5.07	111	15.8	32
Centriprep concentrated	0.7			68		19.5
Superose 6	7.5			47		13.5
Superose 6 dialysed	5.3	7.31	4.14	30	12.9	8.6

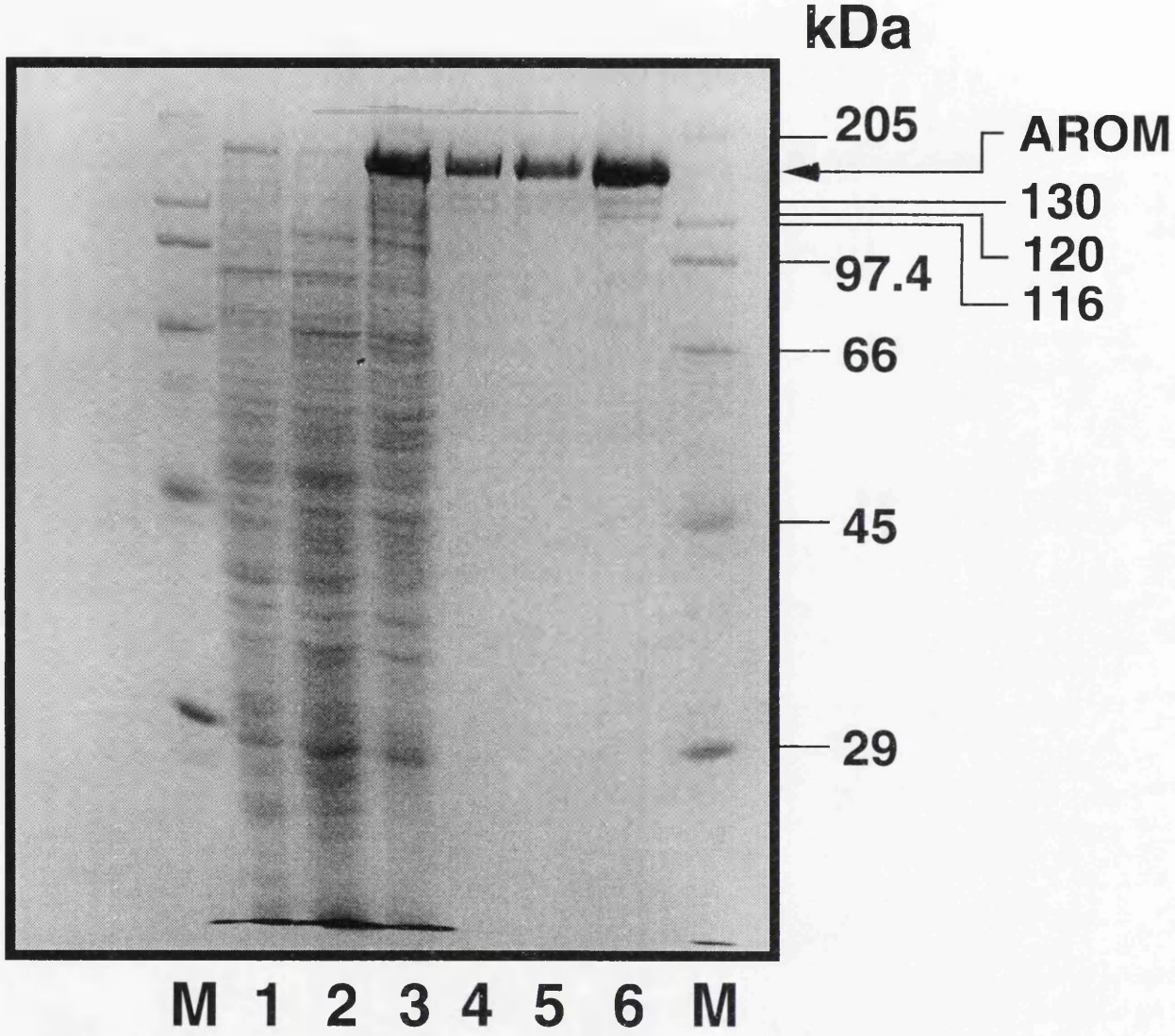
Table 4.3 Purification scheme for AROM from *A. nidulans* 1314

The purification of AROM from *A. nidulans* 1314 was followed by assaying for shikimate DH activity. Samples were assayed immediately for enzyme activity and stored at -80°C prior to protein determination. Protein concentrations were determined by the method of Bradford (1976). The reason for yields shown as greater than 100% is discussed in Section 4.6.3.

Figure 4.14 **SDS PAGE of preparative scale AROM purification from *A. nidulans* 1314**

Samples were taken at each stage of the purification of AROM from *A. nidulans* 1314 and boiled immediately with SDS PAGE sample buffer, then stored at -80°C. The samples were reboiled prior to separation on 10% SDS PAGE. The gel was stained with Coomassie Brilliant Blue G-250.

Lane 1.	cleared extract, 30μg protein
Lane 2.	negative DE52 anion exchange pool, 30μg protein
Lane 3.	desalted 30-50% $(NH_4)_2SO_4$ pellet, 20μg protein
Lane 4.	dialysed mimetic yellow dye pool, 2μg protein
Lane 5.	dialysed Superose 6 pool, 2μg protein
Lane 6.	dialysed Superose 6 pool, 5μg protein
M.	Molecular weight markers from top to bottom: 205kDa, 116kDa, 97.4kDa, 66kDa, 45kDa and 29kDa


kDa
205
AROM
130
120
116
97.4
66
45
29
M 1 2 3 4 5 6 M

4.3) was partly due to AROM 'leaching' off during the loading and subsequent washing (32% of the AROM was lost in this way). No further AROM could be eluted with 1.0M-KCl. The difference in binding of AROM to the Mimetic Yellow-1 here, and that in the 'PIKSI' dye test kit (Section 4.3.9) is most likely due to the batch variation of the dyes. The variation in protein binding from batch to batch of dyes used as affinity ligands in chromatography is notorious. The other 42 % of AROM not recovered might be irreversibly 'stuck' on the column.

Nearly 40% of the AROM was lost on concentrating the protein with an Amicon centriprep 30 prior to gel filtration chromatography on Superose 6 (Table 4.3). The loss is probably due to AROM 'sticking' to the membrane of the centriprep concentrator. In future concentrating the protein shoud be avoided if possible. Indeed, the apparent reduced purification of AROM after chromatography on Superose 6 (Table 4.3) and the failure to remove any contaminants as analysed by SDS PAGE (Figure 4.14), suggest that this step in the purification protocol might be omitted for future AROM purification.

In summary, AROM has been purified from *A. nidulans* 1314. On very heavy protein loading, several lower molecular weight polypeptides are observed by SDS PAGE. These might be due to proteolytically damaged AROM which co-purifies with the native protein. To test this hypothesis the AROM preparation was analysed by non-denaturing PAGE.

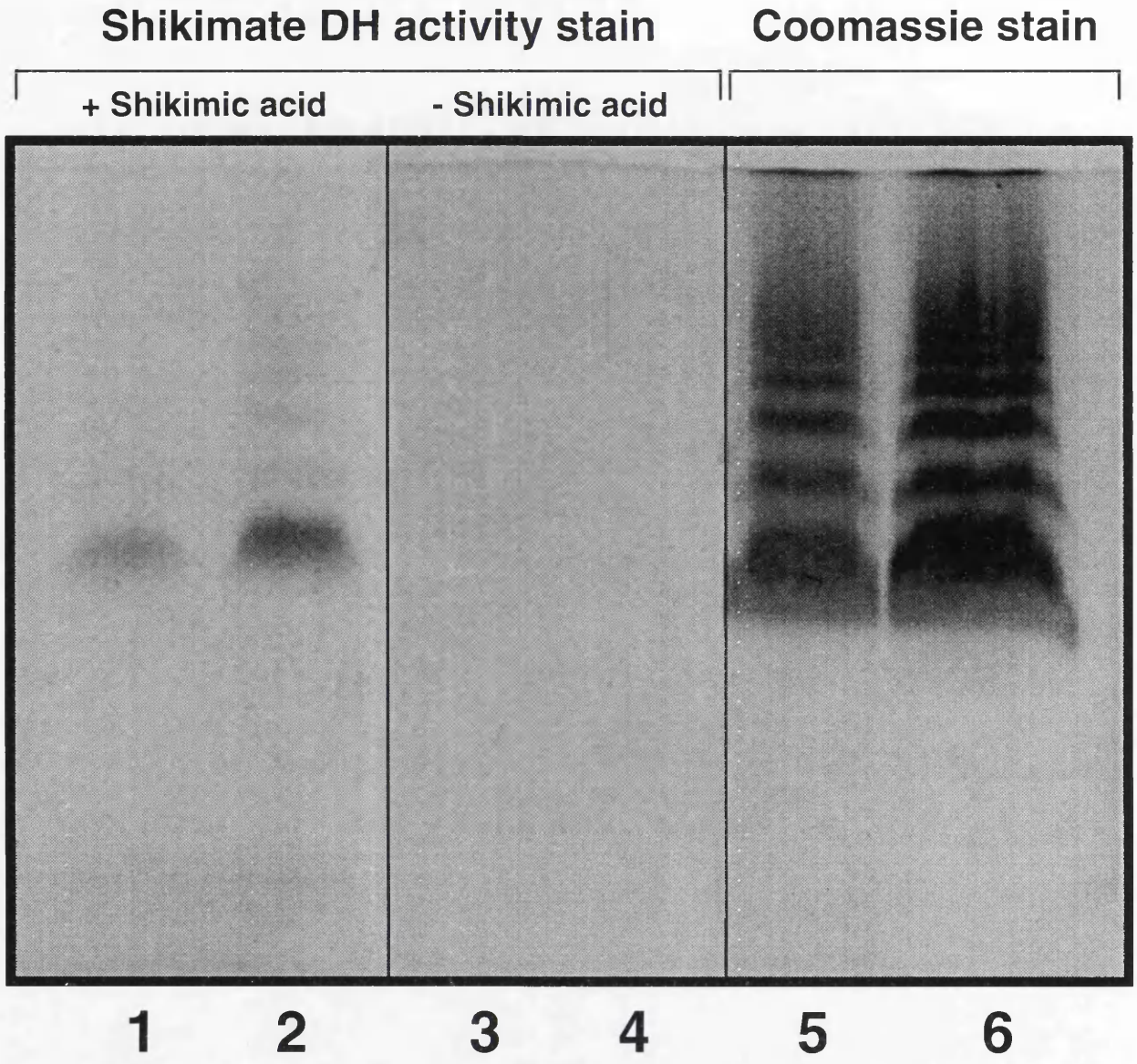
4.6.4 Analysis of AROM purified from *A. nidulans* 1314 by native PAGE

The AROM purified according to Table 4.3 was analysed by native PAGE on a 4-15% gradient gel (Figure 4.15). One major protein band was observed after staining with Coomassie Brilliant Blue G-250. There are also several other protein bands of lower mobility. Staining for shikimate DH activity showed every protein band had shikimate DH activity. (No staining was seen in the absence of the specific substrate, shikimate). The protein bands get closer together towards the top of the separating gel, and this 'laddering' effect can be explained by oxidative cross-linking

Figure 4.15 **Analysis of AROM purified from *A. nidulans* 1314 by native PAGE**

AROM purified from the overexpression strain of *A. nidulans* was incubated with 50mM-DTT at 4°C for 1 h prior to mixing with native running buffer. Samples containing 0.2μg and 0.5μg were electrophoresed in triplicate on a 4-15% gradient PhastGel using the native buffering system. The gel was sliced into 3 identical sections and one section was stained for protein with Coomassie Brilliant Blue G-250. The remaining sections were stained for shikimate DH activity using the nitroblue tetrazolium dye-linked method in the presence or absence of the substrate, shikimate (see Section 2.6.2).

Lane 1. 0.2μg AROM }	Stained for protein with
Lane 2. 0.5μg AROM }	Coomassie Blue
Lane 3. 0.2μg AROM }	Stained for shikimate DH activity
Lane 4. 0.5μg AROM }	in the absence of shikimate
Lane 5. 0.2μg AROM }	Stained for shikimate DH activity
Lane 6. 0.5μg AROM }	in the presence of shikimate


Shikimate DH activity stain
Coomassie stain
+ Shikimic acid
- Shikimic acid
1
2
3
4
5
6

of AROM, despite pre-incubation of the samples with a high concentration of DTT prior to loading the gel. No such multimerisation was observed on gel filtration chromatography (Figure 4.4 and Section 4.6.6).

On examination of the native gel (Figure 4.15) no other bands that might account for the contaminants seen on SDS PAGE can be seen with Coomassie stain. This might be because they are hidden by the other AROM protein bands, because insufficient protein was present to see these minor contaminants, or because they do in fact arise on SDS PAGE from 'nicked' AROM that is not separated from intact AROM under native conditions.

4.6.5 Individual enzyme activities of AROM purified from *A. nidulans* 1314

The five individual AROM activities of AROM purified from *A. nidulans* 1314 were assayed on a ten day old preparation which had been kept at -20°C in storage buffer (see 4.6.2). The specific activities, and activity ratios standardised against shikimate kinase, are shown in Table 4.4. The specific shikimate DH activity had not decreased since the AROM was purified (see Table 4.3). Comparisons made with the activity ratio in crude extract were with mycelia grown under different conditions although there is no reason to suppose that the activity ratio is any different for the individual enzymes.

Most notable in this AROM preparation is the low DHQ synthase activity assayed in 2mM-DAHP. As a precaution against DHQ synthase activity loss zinc was added to the buffers until the gel filtration step. Also the purification was carried out as quickly as possible. The activity ratio is similar to that observed in crude extract (Section 4.2.3). An explanation for the low DHQ synthase activity of AROM from *A. nidulans* 1314 was discovered later and this is discussed in Section 4.6.8.

The EPSP synthase activity shown in Table 4.4 is more encouraging since this activity was completely extinct in AROM purified by route A1 of the preliminary purification (Section 4.4.1). In the material from the large-scale purification the EPSP synthase activity is a little lower than the activity ratio observed in crude

AROM activity	Specific activity (U/mg)	Activity ratio shikimate kinase=100
DHQ synthase[f]	0.071	1.1
DHQase[f]	4.1	63
Shikimate DH[r]	4.8	72
Shikimate kinase[f]	6.6	100
EPSP synthase[r]	3.5	54

[f]assayed in the forward direction

[r]assayed in the reverse direction

Table 4.4 **Individual enzyme activities of AROM purified from *A. nidulans* 1314**

Ten day old AROM was diluted 10-fold in 50mM-Tris/HCl pH7.5 containing 1.4mM-ß-mercaptoethanol and 2mM-benzamidine. Each enzyme was assayed in quadruplicate and the mean value for each is shown. The protein concentration was determined by the method of Bradford (1976). DHQ synthase activity was assayed by coupling DHQ release to the DHQase reaction (see 2.9.2) with 2mM-DAHP and 100µM-zinc sulphate in the assay buffer.

extract of 100:75 for shikimate kinase :EPSP synthase (Table 4.1). Therefore some EPSP synthase activity has almost certainly been lost during purification.

The activity ratio of DHQase is 17% less than the value in crude extract. At least some of this is presumably due to the loss of cDHQase which was in the crude extract but not in the purified AROM. The shikimate DH activity ratio is approximately the same as in crude extract. I expected this to increase because the total shikimate DH activity increased at several stages during the purification (see Table 4.3).

The above analysis assumes that the shikimate kinase activity is stable throughout purification which may not be the case. There are no increases in the other AROM activities compared to shikimate kinase, so there is no evidence that shikimate kinase is unstable during purification. Ideally the specific activity at each stage of the purification should be assayed for each enzyme. However, this would be very time consuming and would slow the purification down considerably unless done with assistance. Also it did not seem sensible to follow all five activities throughout the purification until the problem of the very low DHQ synthase activity had been resolved.

4.6.6 Determination of the native molecular weight of AROM purified from *A. nidulans* 1314

N. crassa AROM is known to be a dimer of identical pentafunctional polypeptides, each estimated by SDS PAGE to be 165kDa (Lumsden & Coggins, 1977). The native molecular weight was estimated to be 270kDa on glycerol density centrifugation (Lumsden & Coggins, 1977). It shows an anomalously high molecular weight of 530kDa in at least some HPLC gel filtration experiments (Coggins *et al.*, 1985; Coggins and Boocock, 1986). The native molecular weight of *A. nidulans* AROM was determined by Ahmed and Giles (1969) to be 217kDa on sucrose density gradient centrifugation which is rather low for a dimer of two identical subunits of 175kDa.

Figure 4.16 shows the estimation of the native molecular weight of AROM purified from *A. nidulans* 1314 by gel filtration chromatography. From the plot, the native molecular weight is 410kDa. This suggests AROM is a dimer. The fact that it runs as a slightly larger protein on gel filtration chromatography than the 350kDa predicted for the dimer from the nucleotide sequence of the gene suggests that the protein is asymmetric.

4.6.7 Attempted N-terminal sequencing of the AROM polypeptide

DHQ synthase is located at the N-terminus of AROM. It was therefore of interest to see if the AROM purified from *A. nidulans* 1314, which was seriously defective in DHQ synthase, would show a mutation or truncation on sequencing the N-terminus which might explain the low activity.

200pmol of AROM purified from *A. nidulans* 1314 was separated by SDS PAGE and subsequently electroblotted onto Problott membrane for 3.5 h by the method described in Section 2.8. The protein on Problott was stained with Amido Black. An 80% transfer of AROM was estimated. The band of protein was cut out and analysed on a Pulsed-liquid sequencer at the Aberdeen University Amino Acid Sequencing Facility.

The data obtained from the N-terminal sequencing showed 4.6pmol of threonine in the first cycle and 2.64pmol of proline in the second round. The values are much lower than expected for the amount of protein present and the trace had a high background of peaks which made the determination of any further sequence impossible. There is a threonine-proline in the *A. nidulans* AROM sequence at residues 52 and 53. It seems likely that the major protein present is chemically blocked at the N-terminus, possibly by formylation or acetylation. The trace from sequencing is most likely to be from a contaminating protein - perhaps a truncated form of the AROM polypeptide. Thus no information was gained on the DHQ synthase domain of AROM from N-terminal sequencing.

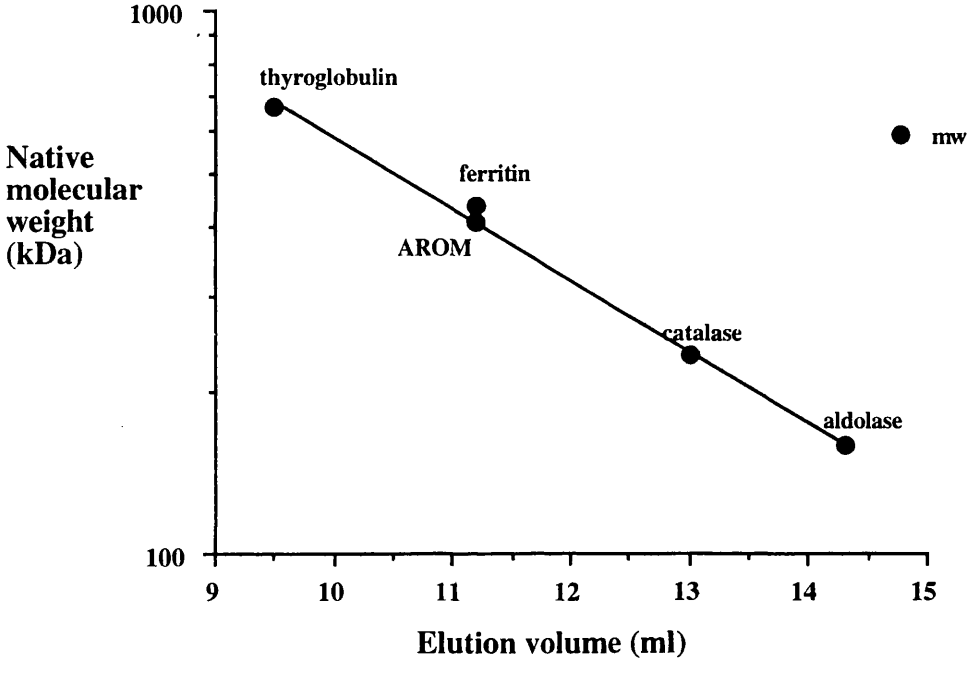
Figure 4.16 **Determination of the native molecular weight of AROM purified from *A. nidulans* overexpresser determined from calibrated gel filtration chromatography.**

Superose 6 HR 10/30 FPLC gel filtration column was equilibrated with buffer E. The column was calibrated with high molecular weight standards from the Pharmacia gel filtration calibration kit for proteins:-

Standard	Source	Native molecular weight
aldolase	rabbit muscle	158kDa
catalase	bovine liver	232kDa
ferritin	horse spleen	440kDa
thyroglobulin	bovine thyroid	669kDa

A mixture of the standards containing 40µg aldolase, 100µg catalase, 10µg ferritin and 100µg thyroglobulin were separated at 0.1ml/min and full scale deflection of 0.1 monitoring at A_{280} and the elution volumes measured. 20µg of AROM purified from *A. nidulans* 1314 was mixed with aldolase and thyroglobulin and separated on Superose 6 chromatography and the elution volumes measured.

The plot shows the native molecular weight of the standard proteins on a logarithmic scale versus the elution volume for the protein. From the measured elution volume of AROM, the native molecular weight was estimated as 410kDa from the calibration curve.


1000
100
Native molecular weight (kDa)
thyroglobulin
ferritin
AROM
catalase
aldolase
mw
9
10
11
12
13
14
15
Elution volume (ml)

The large number of 'background' peaks observed in the sequencer is similar to the picture observed more usually after 30 cycles of the Edman degradation when the polypeptide start to 'fall apart' under the intermittently acid conditions. It is conceivable that this AROM is hypersensitive to the acid conditions, and quickly 'falls apart' on sequencing.

4.6.8 The DHQ synthase activity of AROM from *A. nidulans* 1314

The DHQ synthase deficiency of AROM from *A. nidulans* 1314, both in crude extract (see 4.2.3) and in a purified form (see 4.6.5), gave cause for concern. It seemed possible that this activity had somehow been lost by proteolytic or other damage. Since this domain is N-terminal, one could envisage proteolytic inactiv-ation which would be undetectable by SDS PAGE. While this work on AROM was proceeding, the DHQ synthase domain of AROM was expressed in *E. coli* by van den Hombergh in Hawkins' group (van den Hombergh *et al.*, 1992). This DHQ synthase domain is able to complement an *aro B*$^-$ mutant (lacking DHQ synthase) *E. coli* strain . The domain was expressed at high level, comprising 30% of the total cell protein. J. Moore from Hawkins' group tested this stable domain for DHQ synthase activity in our laboratory and found it to be highly active (J. Moore, 1993).

An attempt by Moore to express a bifunctional DHQ synthase-EPSP synthase domain in *E. coli* resulted in the complementation of *aro A*$^-$ mutants (which lack EPSP synthase) but not *aro B*$^-$ mutants. The first 730 nucleotides of this DHQ synthase were replaced with the first 730 nucleotides of the DHQ synthase domain which had been successfully used by van den Hombergh to complement an *aro B*$^-$ mutant. The resulting DHQ synthase was capable of complementing *aro B*$^-$ mutants (J. Moore, 1993). This suggested that the two supposedly identical DHQ synthase regions are actually different, with one able to produce active protein, and the other not. Subsequent sequencing of the 730 nucleotides at the N-terminus of the 'faulty' DHQ synthase showed that a missense mutation is present which has altered residue

143 from alanine to proline (Moore & Hawkins, 1993). It transpired that this mutation had been accidentally introduced in an oligonucleotide which had been used to remove a restriction site at nucleotide 428 when plasmid pNUFC2 was constructed (Lamb *et al.*, 1991; Moore *et al.*, 1992). (A description of the mutation by Moore & Hawkins(1993) has an error in the text and states that the mutation is at residue 144, rather than 143). All the AROM constructs derived from pNUFC2 contain the missense mutation including that used to make the *A. nidulans* strain 1314 studied here.

In constructing plasmid pNUFC2, the second residue from the N-terminus of AROM was changed from serine to alanine in order to introduce a restriction site. The DHQ synthase domain expressed as a functional protein in *E. coli* by van den Hombergh *et al.* (1992) also has this missense mutation. Therefore, this second mutation is not responsible for the inactivation of DHQ synthase and the activity loss can be attributed to the missense mutation at residue 143.

Plasmid pNUFC2, which contains the entire AROM gene, including the 'faulty' DHQ synthase, is unable to complement *aro* B^- mutants of *E. coli* which lack DHQ synthase activity (J. Moore, personal communication). The chromosomal copy of AROM in *A. nidu-lans* 1314 is derived from the *arom* A^- mutant strain 1103 which is unable to complement any AROM activity (Roberts, 1969; J. Moore, personal communication) and is likely to have a chain termination mutation in the DHQ synthase region of the gene. This raises the question of how the low level of DHQ synthase actually observed in *A. nidulans* 1314 (see 4.2.3 and Table 4.4) has arisen, since neither the original chromosomal nor the integrated plasmid borne copies of AROM DHQ synthase have the ability to grow on medium without amino acid supplements. There are three possible explanations;-

(1) **Gene conversion**: plasmids are unstable in *A. nidulans* and integrate into the chromosomal DNA. There are approximately twelve copies of pNUFC2 containing the AROM coding region in strain 1314 (J. Moore, personal communication). If a single integrated copy undergoes gene conversion with the

'correct' sequence at residue 143 of the original chromosomal DHQ synthase, then one copy of AROM might be expressed in *A. nidulans* 1314.

(2) **Recombination**: It is possible that a recombination event may have occurred between the chromosomal DNA and a copy of integrated pNUFC2, resulting in a single copy of AROM which has the wild-type sequence. The recombination could also have occured during the integration of the plasmid into the chromosomal AROM coding region.

(3) **Interallelic complementation**: the third possibility is that interallelic complementation occurs. In this scenario the mutation on one polypeptide is able to complement the mutation on the other polypeptide resulting in active protein. This is dependent on both polypeptides being in contact with one another in the region of the mutations (Crick & Orgel, 1964). Interallelic complementation has been observed for DHQ synthase of *N. crassa* AROM, both *in vitro* and *in vivo*. Several *arom-2* (DHQ synthase deficient) *N. crassa* strains were able to complement one another in hybrid heterokaryons (Giles *et al.*, 1967). Complementation was also seen *in vitro* when partially purified AROM from two complementary *arom-2* strains were incubated together under alkaline conditions. Substantial DHQ synthase activity was reconstituted (Case *et al.*, 1969). This provided evidence that AROM is a dimer which is thought to interact at the dimer interface through DHQ synthase-DHQ synthase and shikimate DH-shikimate DH contacts (Case *et al.*, 1969).

In the first two cases the low level of DHQ synthase activity compared to the other four AROM enzymes can be explained by only one of the AROM copies reverting to wild-type (and being driven by the wild-type promoter) since the wild-type enzyme levels are about 100-fold less than strain 1314 (see Table 4.1). In the case of interallelic complementation no more than the wild-type levels can be expected because interallelic complementation requires both mutant polypeptides to interact.

The predicted protein sequences for AROM from *S.cerevisiae* (Duncan *et al.*, 1987) and *A. nidulans* (Charles *et al.*, 1986; Hawkins, 1987) have been aligned

with the protein sequence deduced from the *E. coli aro* B gene which encodes DHQ synthase (Duncan et al., 1987). The alignment suggests that the DHQ synthase domains may form two subdomains separated by a linker region of variable length: 27 amino acids in the *S. cerevisiae* sequence and 13 amino acids in *A. nidulans* (Figure 4.17). Within each subdomain are highly conserved regions. The N-terminal subdomain has the conserved nucleotide binding motif Gly-X-Gly-X-X-Gly within a ßαß nucleotide binding fold (Duncan *et al.*, 1987; Hawkins, 1987). In both *A. nidulans* and *S. cerevisiae* the motif sequence is Gly-Gly-Gly-Val-Ile-Gly starting at residue 113 in the *A. nidulans* sequence. Therefore, this region of DHQ synthase is thought to bind the catalytic NAD^+. The mutation at residue 143 from alanine to proline is in a very highly conserved region immediately adjacent to the putative nucleotide binding site. Proline residues lack an NH-group and this frequently disrupts the secondary structure of proteins (Milner-White *et al.*, 1992). In this case the alanine to proline mutation may disrupt a crucial structural feature near the nucleotide binding domain. This could explain the absence of DHQ synthase activity.

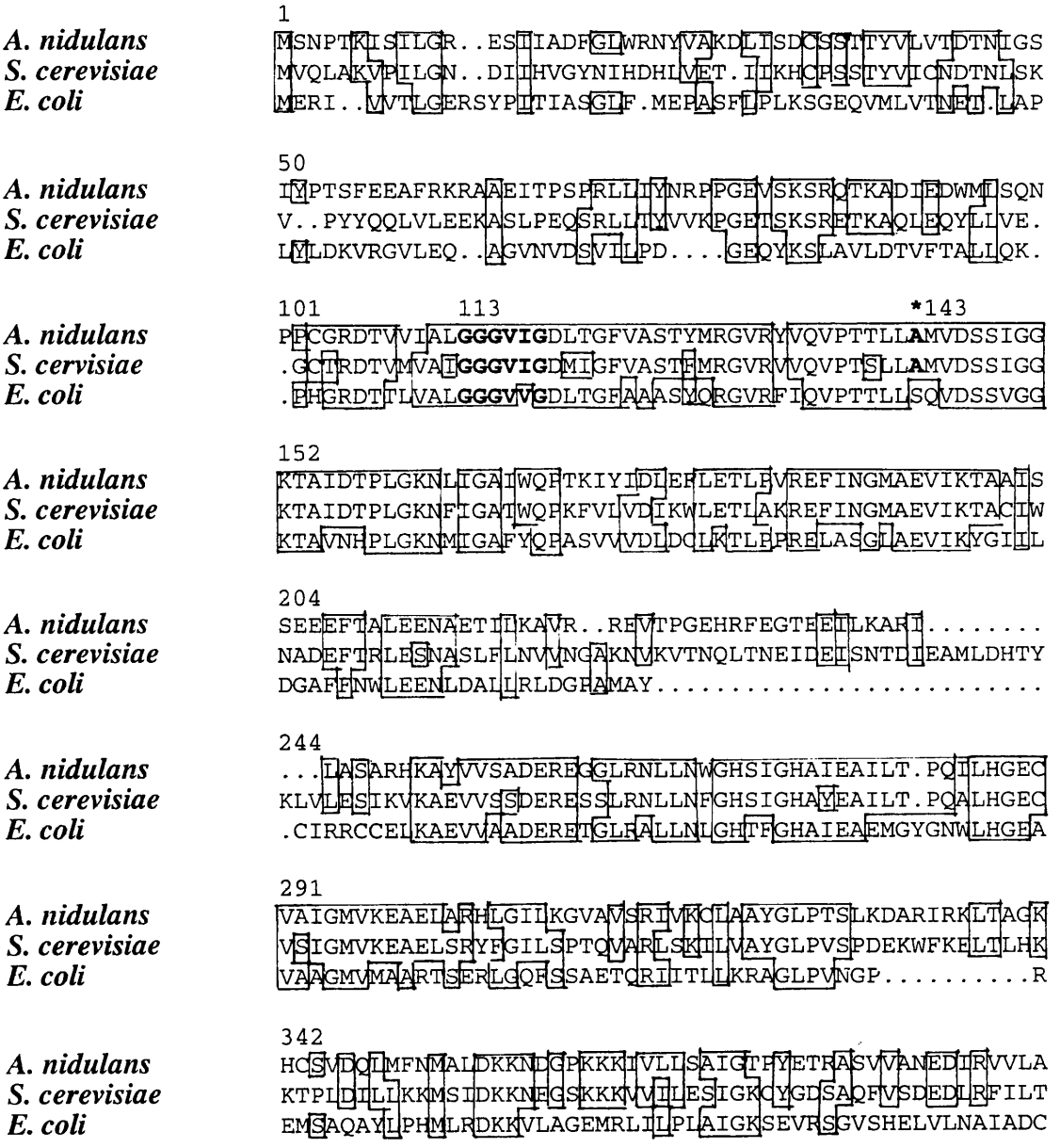
A. nidulans 1314, therefore, contains several copies of the AROM gene with a mutation which may disrupt the NAD^+ binding site of DHQ synthase and render the enzyme inactive. The low level of DHQ synthase activity observed in strain 1314 can be explained by genetic recombination, gene conversion or interallelic complementation.

4.6.9 Summary

A rapid procedure has been developed for purifying AROM from *A. nidulans* 1314. The purified AROM is a dimer and has only a low level of DHQ synthase activity. The polypeptide population is thought to be mixed, with the majority of the protein completely lacking DHQ synthase activity because of a missense mutation at residue 143. The other AROM activities of the purified protein have similar activity ratios to those found in crude extract (Tables 4.1. and 4.4).

Figure 4.17 **Amino acid identity between the *E. coli* DHQ synthase and the DHQ synthase regions of *A. nidulans* and *S. cerevisiae* AROM, showing the location of the missense mutation in AROM from *A. nidulans* 1314**

The figure shows the location of the missense mutation found in AROM from *A. nidulans* 1314. The mutation at residue 143 has a proline residue (P) rather than alanine (A) and is in bold type face. The nucleotide binding motif is also bold. The *A. nidulans* sequence has been numbered.



At the time that AROM was purified from *A. nidulans* 1314, it was not known that the deficiency of DHQ synthase activity was caused by a mutation. Therefore, I did not proceed with kinetic studies of the protein because it still remained unclear whether the activity had somehow been lost, possibly by proteolysis. If this is the case, kinetic data can be misleading. Now that the cause of inactivity is clear, kinetic studies of AROM from *A. nidulans* 1314 would provide interesting data. This is discussed in Section 4.8.

The AROM purified from *A. nidulans* 1314 was used in the limited proteolysis study described in the next section (4.7) since the mutation does not appear to have a major effect on the other AROM activities. The protein is also dimeric suggesting that the quaternary structure is not greatly affected.

4.7 Limited proteolysis studies of native AROM from *A. nidulans* 1314

4.7.1 Introduction

Limited proteolysis of native proteins can provide information about the domain structure. The method has proved particularly useful in studies of multifunctional proteins which are arranged as compact, globular domains separated by interdomain 'linker' regions which are more vulnerable to proteinases. Cleavage of interdomain sequences may produce separate domains of the protein with intact activity.

Two functional dimeric polypeptides were isolated from *N. crassa* AROM by proteolysis (Smith & Coggins, 1983; Coggins *et al.*, 1985; Coggins & Boocock, 1986). The first had a polypeptide fragment of molecular weight of 74kDa determined by SDS PAGE and its native form showed EPSP synthase activity. The other polypeptide fragment had a molecular weight of 63kDa, as determined by SDS PAGE and it was also isolated in a dimeric form which had both DHQase and shikimate DH activity. This study of *N. crassa* AROM has been described more fully in Section 1.7.3. The work showed that the isolated polypeptides are functionally independent of the other AROM enzymes. The isolated 63kDa polypeptide (with

shikimate DH and DHQase activity) was able to renature to produce active shikimate DH. This shows that the information for correct protein folding of shikimate DH is carried on the 63kDa polypeptide. The data support other biochemical and genetic evidence which suggest a 'mosaic model' for AROM. These studies were carried out prior to sequencing of the gene coding AROM and alignment of the deduced amino acid sequence with the sequences of the individual *E. coli* enzymes (see 1.7.4 and Figure 1.2).

Now that the *A. nidulans arom A* gene has been sequenced, a proteolysis study will provide a much better picture of AROM because the exact positions of proteolytic cleavage can be determined by sequencing of the N-termini of the polypeptide fragments produced. One of the long term aims of the project is to compare intact AROM with domains expressed independently (see 1.7.5 and 1.8). Attempts in this laboratory to express stable, functional domains of *S. cerevisiae* AROM in *E. coli* and in *S. cerevisiae* were unsuccessful (Graham *et al.*, in press; L.D. Graham, personal communication). Hawkins and Smith (1991) examined whether expression of *A. nidulans* AROM domains could complement *aro*$^-$ mutant *E. coli* strains which lack the individual shikimate pathway enzymes. For complementation to occur, only a minute quantity of protein need be expressed and the domains produced in the study were not stable (J. Moore, 1993). The study was carried out before the group had the facilities to use the polymerase chain reaction and they relied on restriction sites to clone the domains. This meant that the domains did not always begin and end in the correct places, and some constructs resulted in C-terminal extensions of non-AROM sequence due to read-through into the plasmid (Moore & Hawkins, 1993). These extensions could target the protein produced for proteolysis within the cell. Similarly, domains which are short of the entire domain sequence, may not fold into a compact globular structure and may again be unstable. This would explain why complementation occurs but no protein is observed.

The polypeptide domain boundaries were identified by sequence alignment with the individual monofunctional *E. coli* enzymes. The polymerase

chain reaction has allowed precise translational initiation and termination at a variety of possible domain boundary sites to see which produce stable protein. An example where this strategy was adopted is the study by van den Hombergh *et al.* (1991) in Hawkins' laboratory. He made two constructs encoding DHQ synthase with an identical N-terminus but with different C-termini (residues 393-434). Both plasmids were able to complement *aro* B^- *E. coli* mutants (lacking DHQ synthase) but only the strain containing the DHQ synthase terminating at residue 393 showed an inducible production of a stable 43kDa protein. In the same study the presumptive position of the C-terminus of the DHQ synthase domain was located more precisely by producing several constructs with different C-termini. The specific activity of DHQase in transformed strains varied 20-fold. Constructs with both shorter and longer polypeptides resulted in a lower specific activity in crude extracts.

Clearly, then, the position of the domain boundaries is very important in domain expression studies. At the time of this study of *A. nidulans* AROM only the DHQ synthase and DHQase domains had been successfully expressed (Kinghorn & Hawkins, 1982; van den Hombergh *et al.*, 1991). Therefore, the proteolysis study of AROM from *A. nidulans* 1314 - to be described here - was undertaken to produce stable, active domains. As already described, the interdomain linkers are generally most accessible to proteinases so any stable domains remaining may, once defined, provide a good starting point for domain expression studies. The N-terminus of the proteolysed fragment can be sequenced to this end. Obviously though, the same fragment will not neccessarily produce functional, stable protein in expression studies because it may be unable to fold into the correct conformation independently.

This section describes the preliminary proteolysis study that I made of AROM isolated from *A. nidulans* 1314.

4.7.2 Predicted domain sizes for *A. nidulans* AROM

The domain sizes of AROM from *A. nidulans* have been predicted both from the sequence alignment of *A. nidulans* and *S. cerevisiae* AROM with the

individual *E. coli* enzymes (Charles *et al.*,1986; Duncan *et al.*, 1987; Hawkins, 1987), and from domain expression studies (van den Hombergh *et al.*,1991). There are believed to be interdomain linker polypeptides between DHQ synthase and EPSP synthase, and between EPSP synthase and shikimate kinase (Duncan *et al.*, 1987). Secondary structure predictions for the *S. cerevisiae* AROM linker polypeptides suggest that the linkers may be devoid of secondary structure. The DHQ synthase and EPSP synthase domains may have 'internal' linker polypeptides dividing each domain into subdomains (Duncan *et al.*, 1987; Coggins *et al.*, 1987b). A domain expression study has suggested that there is no interdomain linker between DHQase and shikimate DH (van den Hombergh *et al*, 1991). There is also no obvious linker between the shikimate kinase and DHQase domains (Coggins *et al.*, 1987b; Duncan *et al.*, 1987).

Figure 4.18 shows the predicted molecular weights of the functional domains of *A. nidulans* AROM. In the preliminary study that follows, I looked at the loss of individual AROM enzyme activities during proteolysis and examined the polypeptides that resulted. The shikimate DH activity was renatured after SDS PAGE, to identify polypeptides with this functional domain of AROM.

4.7.3 Preliminary proteolysis of native AROM from *A. nidulans* 1314

In the first instance, I used trypsin, chymotrypsin and subtilisin for proteolysis since they had been used to isolate active domains of *N. crassa* AROM (Smith & Coggins, 1983; Coggins *et al.*, 1985; Coggins & Boocock, 1986). Thermolysin and proteinase K were also used. Initially the same conditions as Boocock (1983) were employed with digestion in potassium phosphate buffer pH7.0. The precipitate formed between potassium ions and SDS (in the SDS PAGE sample buffer) was problematic and so Tris/HCl pH7.0 was used instead: the results are described in this section. However, Tris/HCl is not the most suitable choice of pH7.0 buffer as it has a low buffering capacity. Also, the phosphate buffer might have had a stabilising effect on the conformation of AROM. For these reasons the proteolysis

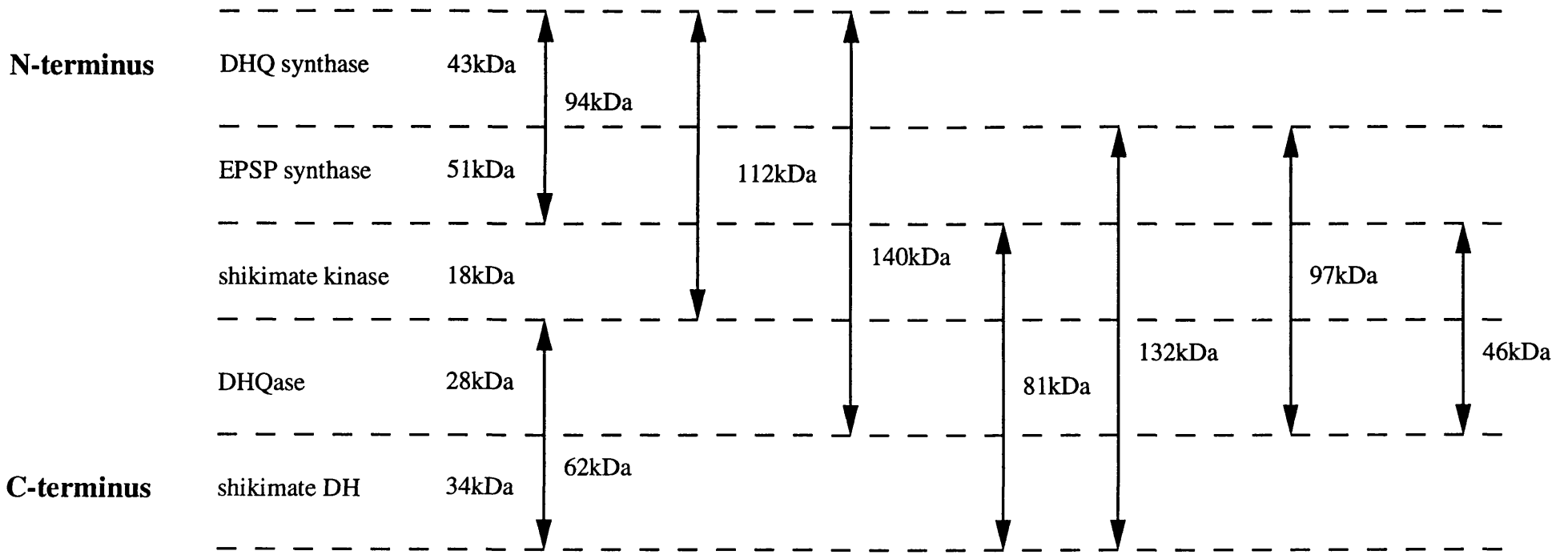


Figure 4.18 **Predicted domain sizes of *A. nidulans* AROM based on the alignment of the *A. nidulans* AROM sequence with the sequences of the corresponding *E. coli* monofunctional enzymes and the *S. cerevisiae* AROM sequence**

was subsequently repeated in sodium phosphate buffer pH7.0 and the fragmentation pattern observed by SDS PAGE was examined. Several polypeptides produced with each proteinase were common to each buffering system and it is believed that any differences were merely due to termination of proteolysis at different stages in the cleavage resulting in some different transient polypeptides.

Because of the very low DHQ synthase activity of this AROM preparation (see above) DHQ synthase was not assayed in the proteolysis study. For each proteolysis, native AROM was incubated with proteinase at 25°C and samples were removed at the stated time intervals. A specific proteinase inhibitor was added to the sample and the mixture stored on ice while enzyme assays were carried out. The protein remaining after assays was boiled in SDS PAGE sample buffer and analysed by SDS PAGE. The control samples - AROM alone and proteinase alone - were incubated for the time of the longest digestion and proteinase inhibitor was subsequntly added. The AROM control was assayed for the individual AROM activities and these values were taken as 100% activity. The enzyme activities of proteolysed AROM are shown as a percentage of this value. The proteolysis results are later discussed in the light of the experiment which examined shikimate DH renaturation after proteolysis.

1. Digestion of native AROM with trypsin

AROM was incubated with TPCK-treated trypsin in a 2:1 ratio. The digestion was terminated by the addition of a 3-fold mass excess of lima bean proteinase inhibitor. Figure 4.19 shows the individual AROM activities over 60 min digestion. After 5 min DHQase and shikimate DH remain fully active and only fall to 80% and 58% respectively of their original values after 60 min. EPSP synthase activity is most sensitive to proteolysis and is completely lost after 60 min digestion.

The proteolysed AROM fragments were separated by SDS PAGE (Figure 4.21) and show a complex cleavage pattern over the incubation period. After 60 min the major digestion species is a stable 41kDa polypeptide; There is also a minor

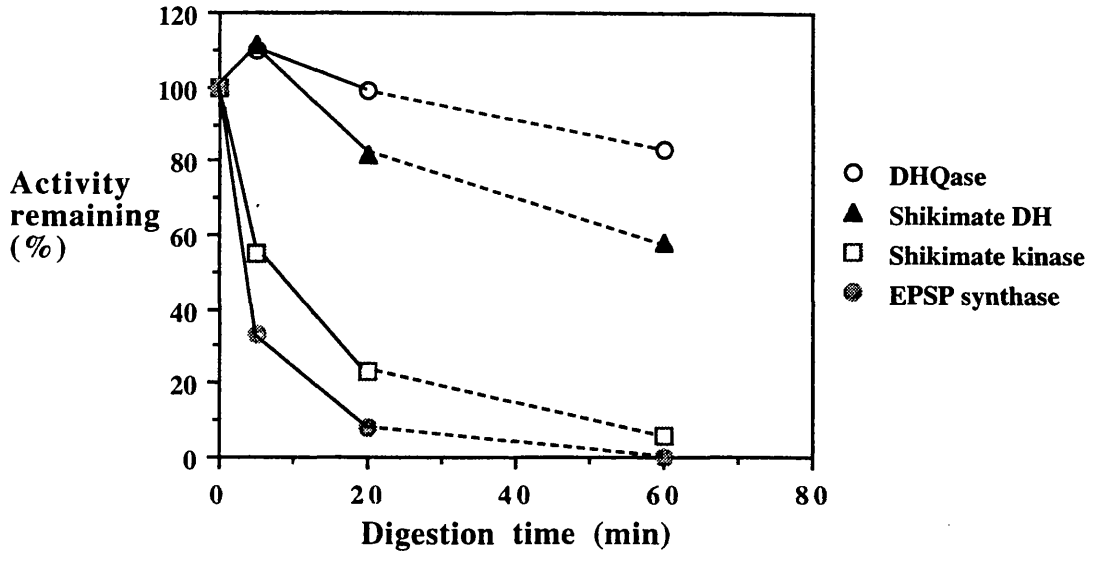


Figure 4.19 **Loss of AROM activities during digestion with trypsin**

AROM purified from *A. nidulans* 1314 was incubated at 200µg/ml with 100µg/ml TPCK-treated trypsin in 50mM-Tris/HCl pH7.0, containing 1.4mM-ß-mercaptoethanol at 25°C. Digestion was terminated after 5 and 20 min by the addition of 300µg/ml lima bean proteinase inhibitor and the samples stored on ice. A 60 min time point is also shown from a separate, identical experiment. Enzyme assays were carried out in duplicate.

The graph follows the loss of individual AROM activities with time during proteolysis with trypsin. The enzyme activities are given as a percentage of the zero time point which was taken as the activity found in an AROM sample incubated for 60 min at 25°C, and subsequently treated with lima bean proteinase inhibitor.

70kDa polypeptide. Both these species were observed when the proteolysis was carried out in sodium phosphate buffer. From Figure 4.18 the DHQase-shikimate DH bifunctional polypeptide has an estimated molecular weight of 63kDa. It is therefore possible that the 70kDa polypeptide has both these activities. The 40kDa polypeptide might contain the DHQase region of AROM or the shikimate DH region or be a mixture of both since both these activities survive to a significant degree over the time course of the digestion.

2. Digestion of native AROM with chymotrypsin

AROM was incubated with TLCK-treated chymotrysin in a ratio of 5:1. Figure 4.20 shows that all four enzyme activities are lost after 60 min digestion and no polypeptide fragments can be seen by SDS PAGE (Figure 4.21). Transient polypeptides of 82kDa, 60kDa, 48kDa and 38kDa are produced within 5 min digestion and at this stage 60% of the DHQase and shikimate DH activities remain. The 60kDa polypeptide is possibly a bifunctional DHQase-shikimate DH fragment, and the 82kDa polypeptide is the correct size for a trifunctional shikimate kinase-DHQase-shikimate DH frament. Both polypeptides are, however, degraded quite quickly and only a little of the 60kDa polypeptide remains after 20 min digestion. There are apparently no stable proteolytic fragments produced by digestion with chymotrypsin.

3. Digestion of native AROM with subtilisin

AROM was digested with Subtilisin Carlsberg in a 20:1 ratio. After 60 min incubation none of the enzyme activities remain and no fragments of AROM can be seen by SDS PAGE (Figure 4.22). Both shikimate kinase and EPSP synthase activities are lost very quickly. After 5 min digestion the DHQase and shikimate DH activities have fallen to 65% of their original values and several polypeptides are seen on SDS PAGE (72kDa, 59kDa, 52kDa, 51kDa and 33kDa). The major species appear as a 52kDa and51kDa doublet although this is too small to carry both the

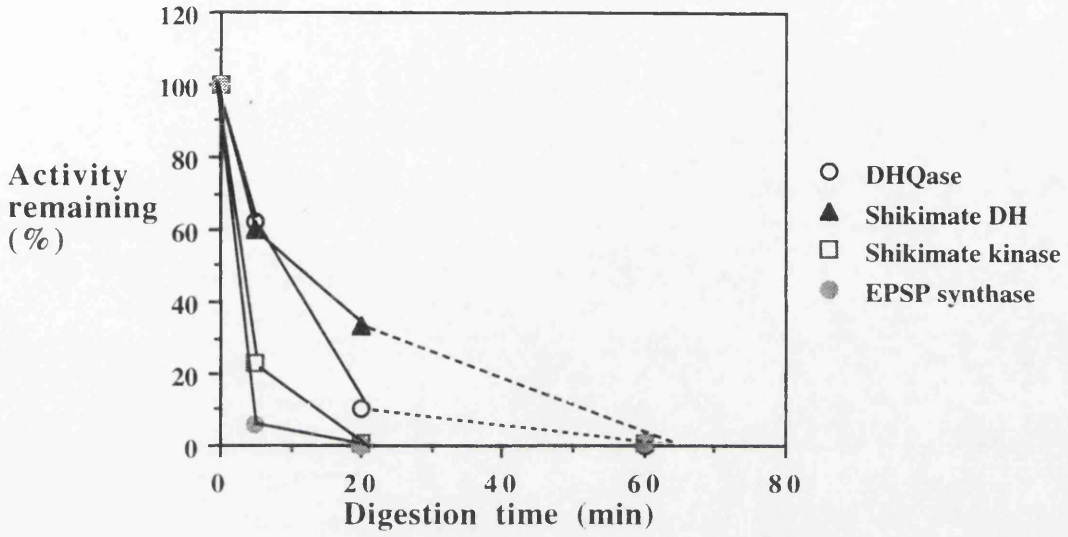


Figure 4.20 **Loss of AROM activities during digestion with chymotrypsin**

AROM purified from *A. nidulans* 1314 was incubated at a concentration of 200µg/ml with 40µg/ml of TLCK-treated chymotrypsin in 50mM-Tris/HCl pH7.0, containing 1.4mM-ß-mercaptoethanol at 25°C. Digestion was terminated at 5 and 20 min. by the addition of 120µg/ml lima bean protease inhibitor. The 60 min digestion data were obtained from a separate, identical experiment. The enzyme assays were carried out in duplicate.

The graph shows the loss of individual AROM activities with chymotrypsin digestion under native conditions. Activities are shown as a percentage of the zero time point. The zero time sample was taken as the activity of an AROM sample incubated for 60min at 25°C without proteinase, and subsequently treated with lima bean protease inhibitor.

Figure 4.21 **Analysis by SDS PAGE of native AROM proteolysed by trypsin and chymotrypsin**

AROM purified from *A. nidulans* 1314 was incubated at 200µg/ml under native conditions with 100µg/ml TPCK-treated trypsin or 40µg/ml TLCK-treated chymotrypsin in 50mM-Tris/HCl pH7.0, containing 1.4mM-ß-mercaptoethanol. Proteolysis was terminated after 5 and 20 min with the addition of a 3-fold excess of lima bean proteinase inhibitor. In separate individual experiments AROM was digested in an identical manner for 60 min with each proteinase. Samples were boiled for 5 min in SDS PAGE sample buffer and analysed by SDS-PAGE on a 10% gel. The gel was silver stained for protein (see 2.5.2).

M	molecular weight markers: 205kDa, 116kDa, 97.4kDa, 66kDa, 45kDa, 29kDa, 3µg total protein .
Lane 1.	4.6µg AROM.
Lane 2.	4.6µg AROM incubated for 60 min, with subsequent addition of lima bean proteinase inhibitor.
Lanes 3	4.6µg AROM incubated for 5 min with trypsin.
Lane 4.	4.6µg AROM incubated for 20 min with trypsin.
Lane 5.	4.6µg AROM incubated for 60 min.with trypsin.
Lane 6.	2.3µg trypsin incubated for 60 min, with subsequent addition of lima bean proteinase inhibitor.
Lane 7.	4.6µg AROM incubated for 5 min with chymotrypsin.
Lane 8.	4.6µg AROM incubated for 20 min with chymotrypsin.
Lane 9.	4.6µg AROM incubated for 60 min with chymotrypsin.
Lane 10.	0.9µg Chymotrypsin incubated for 60 min, with subsequent addition of lima bean proteinase inhibitor.

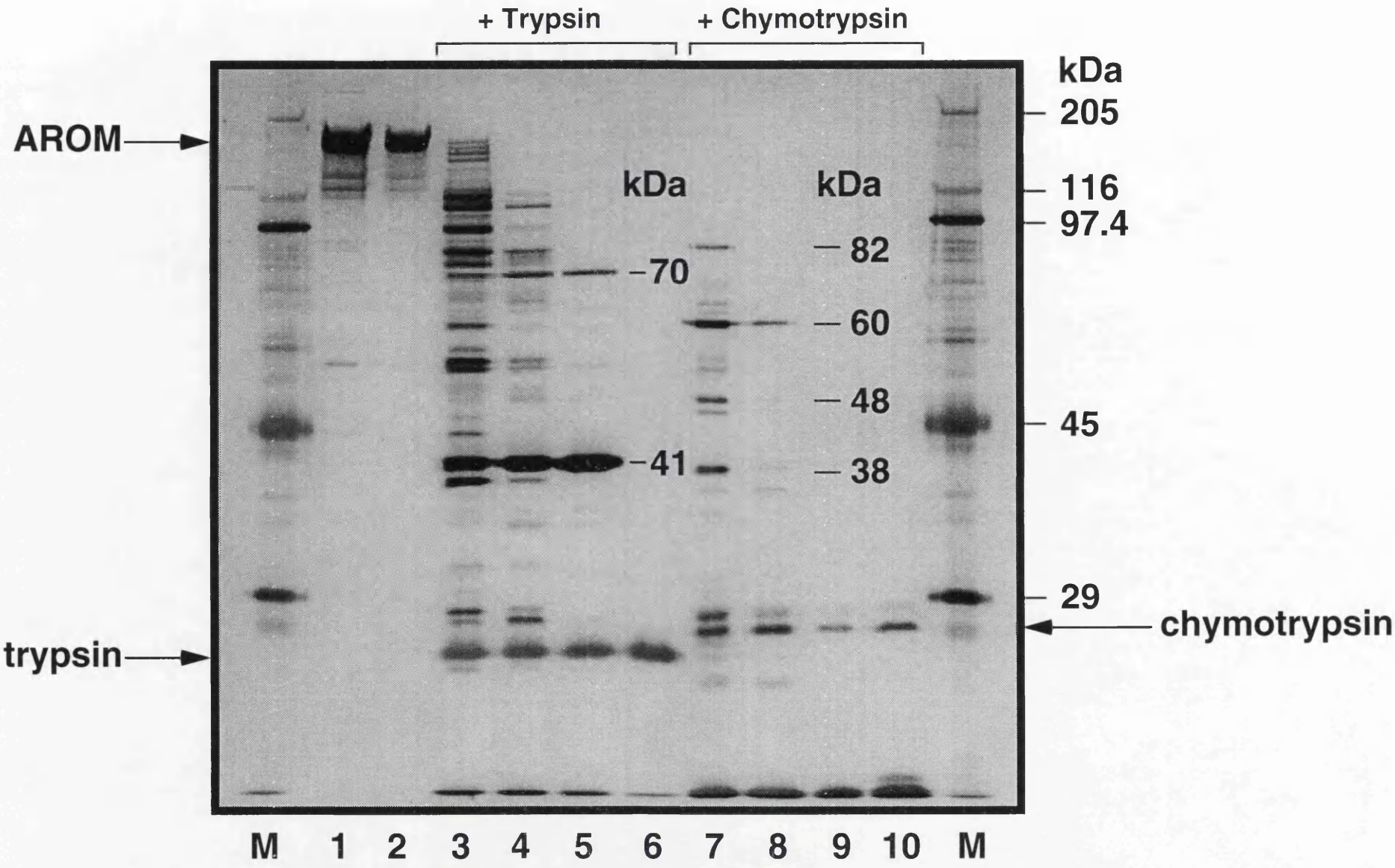

+ Trypsin
+ Chymotrypsin
AROM
trypsin
kDa
205
116
97.4
45
29
chymotrypsin
kDa
70
41
kDa
82
60
48
38
M 1 2 3 4 5 6 7 8 9 10 M

Figure 4.22 Digestion of native AROM with subtilisin

AROM purified from *A. nidulans* 1314 was incubated at 200µg/ml with 10µg/ml subtilisin Carlsberg at 25°C in 50mM-Tris/HCl pH7.0, containing 1.4mM-ß-mercaptoethanol. Digestion was stopped at 5 and 60 min with the addition of 1.15mM-PMSF and samples were stored on ice.

(a) 'Individual enzyme activities'

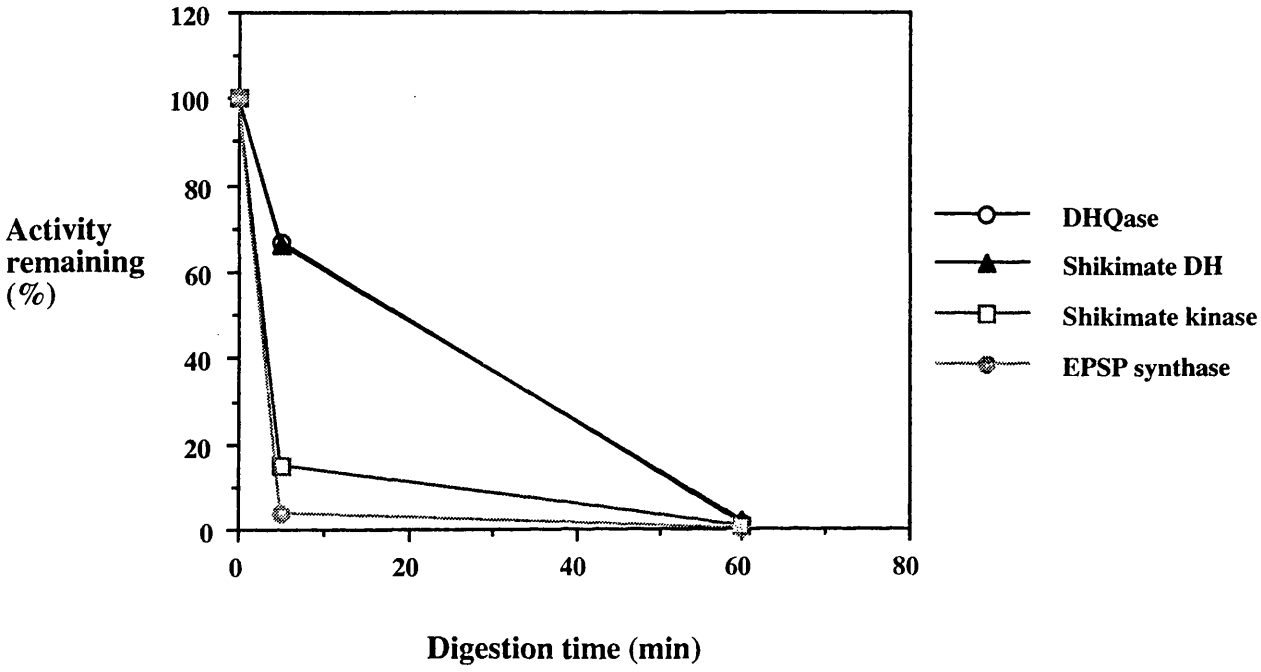
Enzyme assays were carried out in duplicate. The graph shows the loss of individual AROM activities with subtilisin proteolysis under native conditions. Activities are shown as a percentage of the zero time sample. The zero time point was taken as the activity in an AROM sample incubated at 25°C for 60 min without proteinase, and subsequently treated with PMSF.

(b) 'Gel'

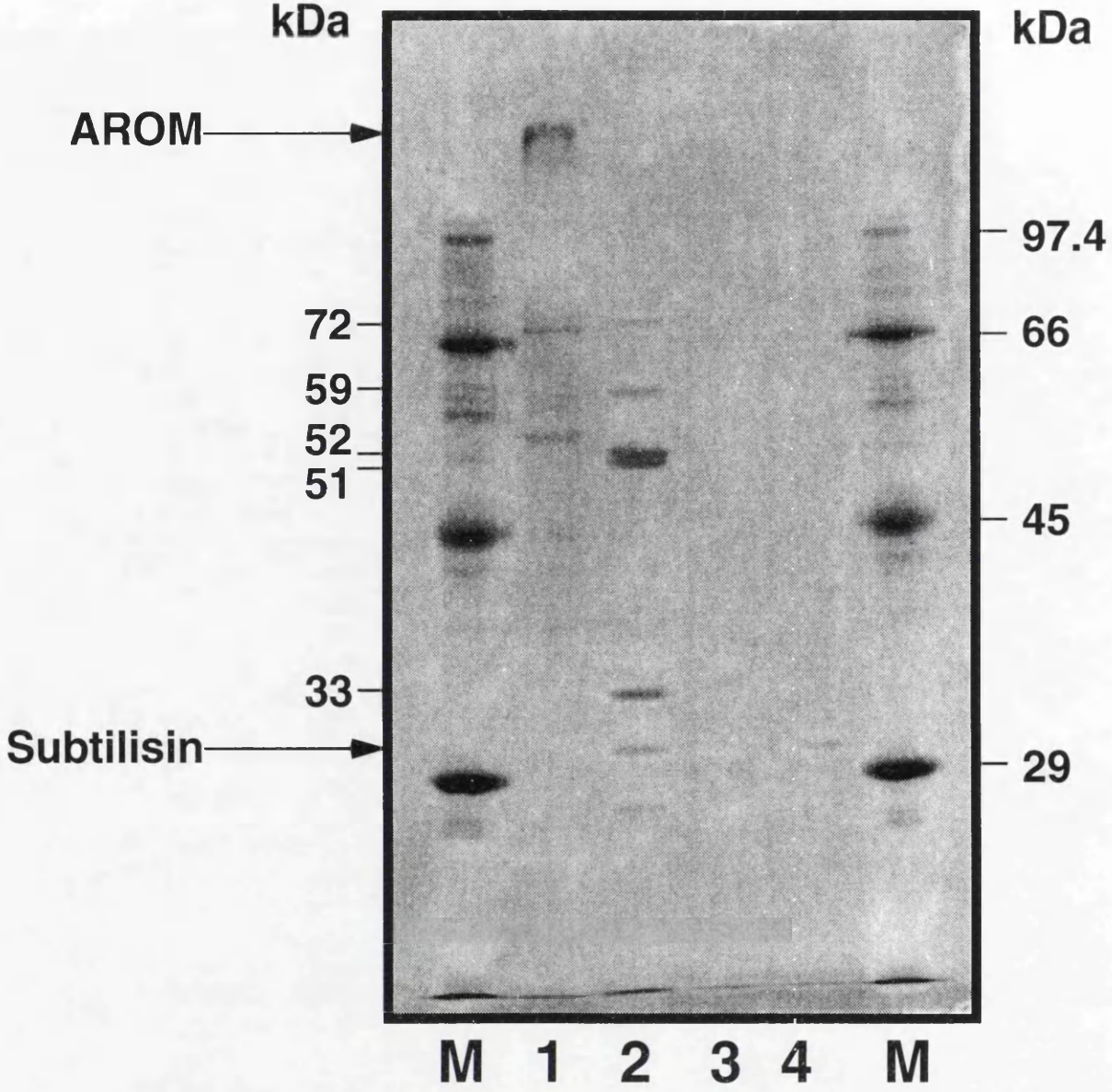
After completion of the individual enzyme assays described above (a), the protein was boiled for 5 min in SDS PAGE sample buffer and analysed by SDS PAGE on a 10% gel. The gel was silver stained for protein visualisation (see 2.5.2).

M	molecular weight markers: 205kDa, 116kDa, 97.4kDa, 66kDa, 45kDa and 29kDa.
Lane 1.	6µg AROM incubated 60 min, with subsequent addition of PMSF.
Lane 2.	6µg AROM incubated 5 min with subtilisin.
Lane 3.	6µg AROM incubated 60 min with subtilisin.
Lane 4.	0.3µg subtilisin incubated 60 min, with subsequent addition of PMSF.

(a) 'Individual enzyme activities'



(b)'Gel'


kDa
AROM
72
59
52
51
33
Subtilisin
kDa
97.4
66
45
29
M 1 2 3 4 M

shikimate DH and DHQase activities observed. The 33kDa polypeptide is conceivably the shikimate DH domain and such a fragment of 30kDa was observed after digestion of *N. crassa* AROM with a chymotrysin/trypsin mixture (Boocock, 1983). All of the polypeptides observed after 5 min digestion with subtilisin are rapidly digested.

4. Digestion of AROM with thermolysin

AROM was digested with thermolysin in a 5:1 ratio. Figure 4.23 shows that after 60 min of digestion there are two stable proteolysis products - a major 53kDa polypeptide and a minor polypeptide of 60kDa. At this stage all the DHQase activity remains and over 40% of the shikimate DH is active. The 60kDa polpeptide is possibly a DHQase-shikimate DH bifunctional fragment which has been slightly trimmed but is still active.

5. Digestion of native AROM with proteinase K

AROM was digested with proteinase K in a 5:1 ratio and the proteolytic fragments produced after 5 and 60 min were analysed by SDS PAGE (Figure 4.24). The protein was completely digested after 60 min but after 5 min several polypeptides were observed of 70kDa, 64kDa, 59kDa, 51.5kDa and 33.5kDa.. A 70kDa polypeptide was also seen with trypsin digestion and fragments of similar molecular weights to the other polypeptides were also observed with other proteinases. No enzyme activity studies were carried out after AROM digestion with proteinase K.

6. Shikimate DH activity of proteolysed AROM renatured after SDS PAGE

The digestion studies described above showed several fragments resulting from AROM proteolysis which might have shikimate DH activity. In an attempt to identify fragments with this activity, AROM was proteolysed with each of the proteinases: trypsin, chymotrypsin, subtilisin, thermolysin and proteinase K; the digested protein was separated by SDS PAGE and the gel renatured by the method

Figure 4.23 Digestion of native AROM with thermolysin

AROM purified from *A. nidulans* 1314 was incubated at 200µg/ml with 40µg/ml thermolysin in 50mM-Tris/HCl pH7.5, containing 1.4mM-ß-mercaptoethanol at 25°C. Digestion was terminated after 5, 20 and 60 min with the addition of 5mM-EDTA and samples were stored on ice.

(a) 'Individual enzyme activities'

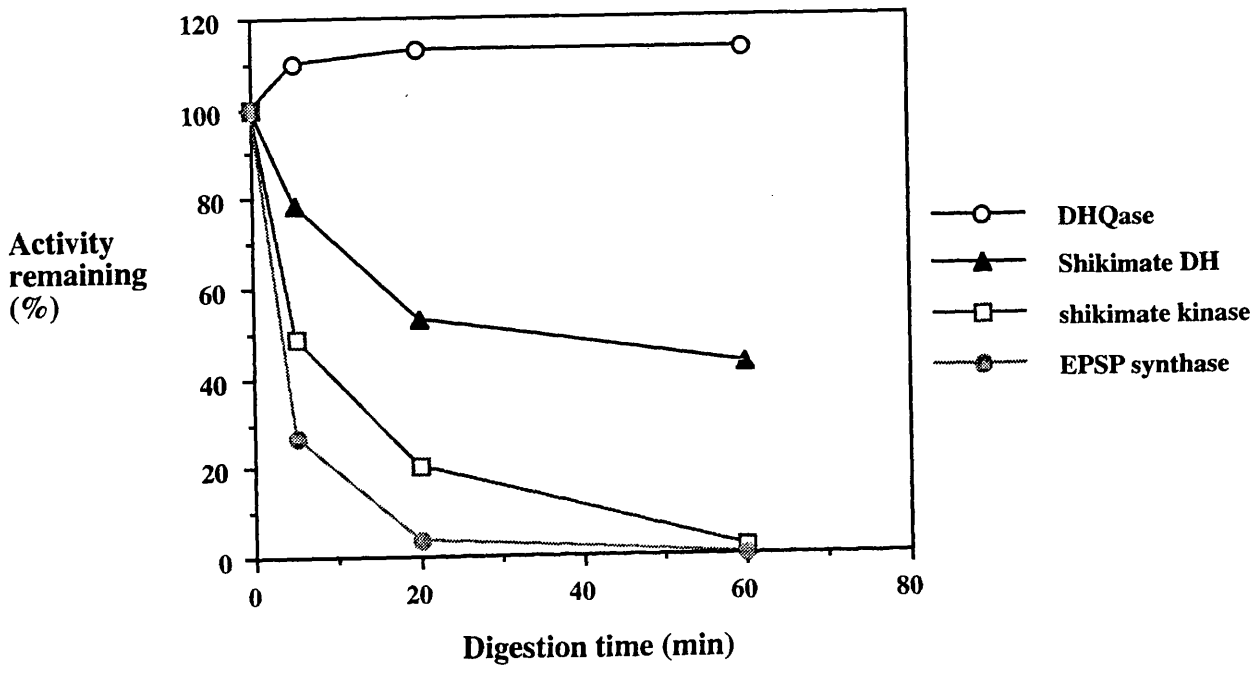
Enzyme assays were carried out in duplicate. The graph shows the loss of individual AROM activities with thermolysin digestion under native conditions. The zero time point was taken as the activity in an AROM sample incubated at 25°C for 60 min with the subsequent addition of EDTA. Activities are shown as a percentage of the zero time point.

(b) 'Gel'

After completion of the individual assays the proteolysed protein was boiled for 5 min in SDS PAGE sample buffer. Individual samples were analysed by SDS PAGE on a 10% gel using a Bio-Rad mini-gel apparatus. The gel was silver stained for protein visualisation (see 2.5.2).

M	molecular weight markers: 205kDa, 116kDa, 97.4kDa, 66kDa, 45kDa and 29kDa, 1µg protein.
Lane 1.	1.2µg AROM incubated 60 min, with subsequent addition of EDTA.
Lane 2.	1.2µg AROM incubated for 5 min with thermolysin.
Lane 3.	1.2µg AROM incubated for 20 min with thermolysin.
Lane 4.	1.2µg AROM incubated for 60 min with thermolysin.
Lane 5.	0.24µg thermolysin incubated for 60 min with subsequent addition of EDTA.

(a) 'Individual enzyme activities'



(b)'Gel'

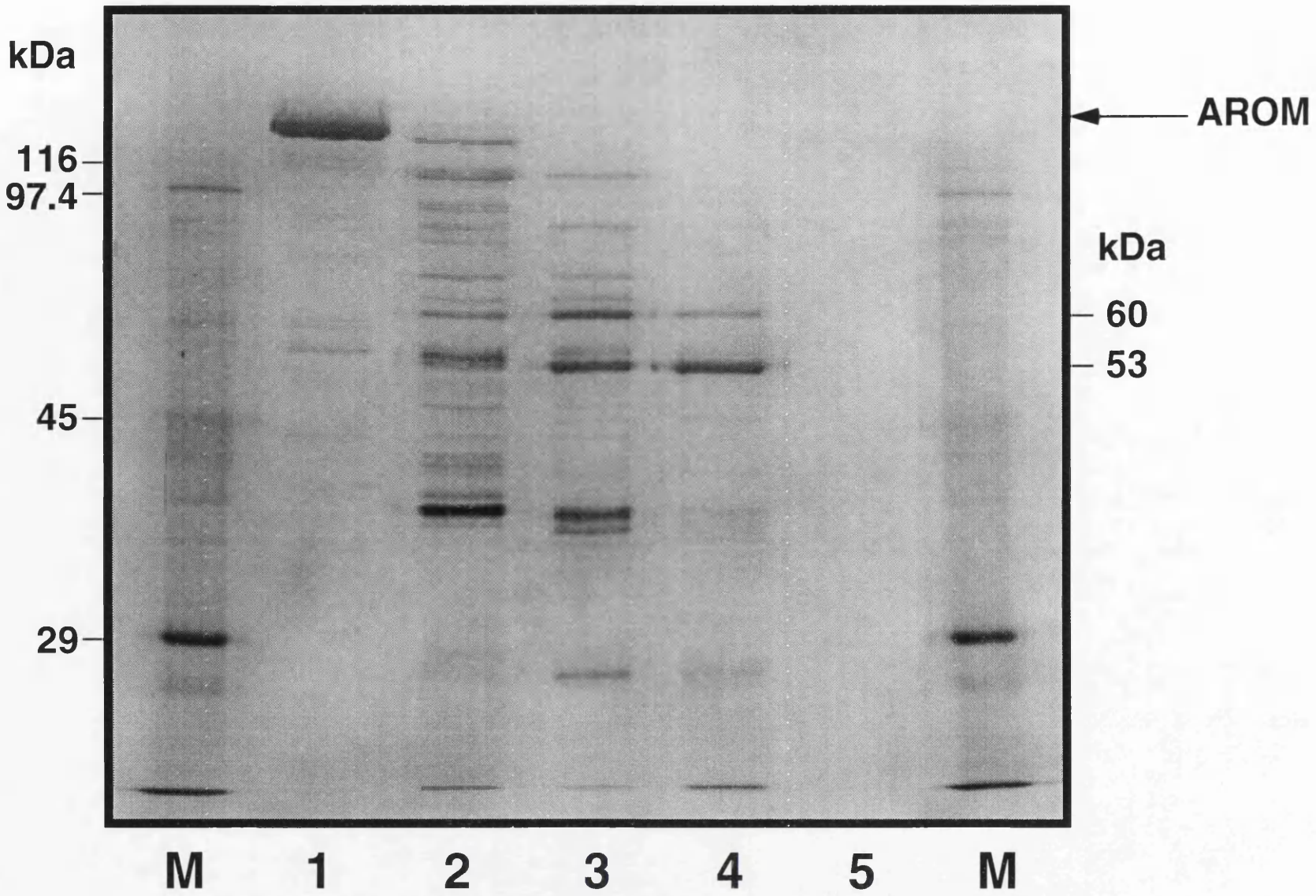
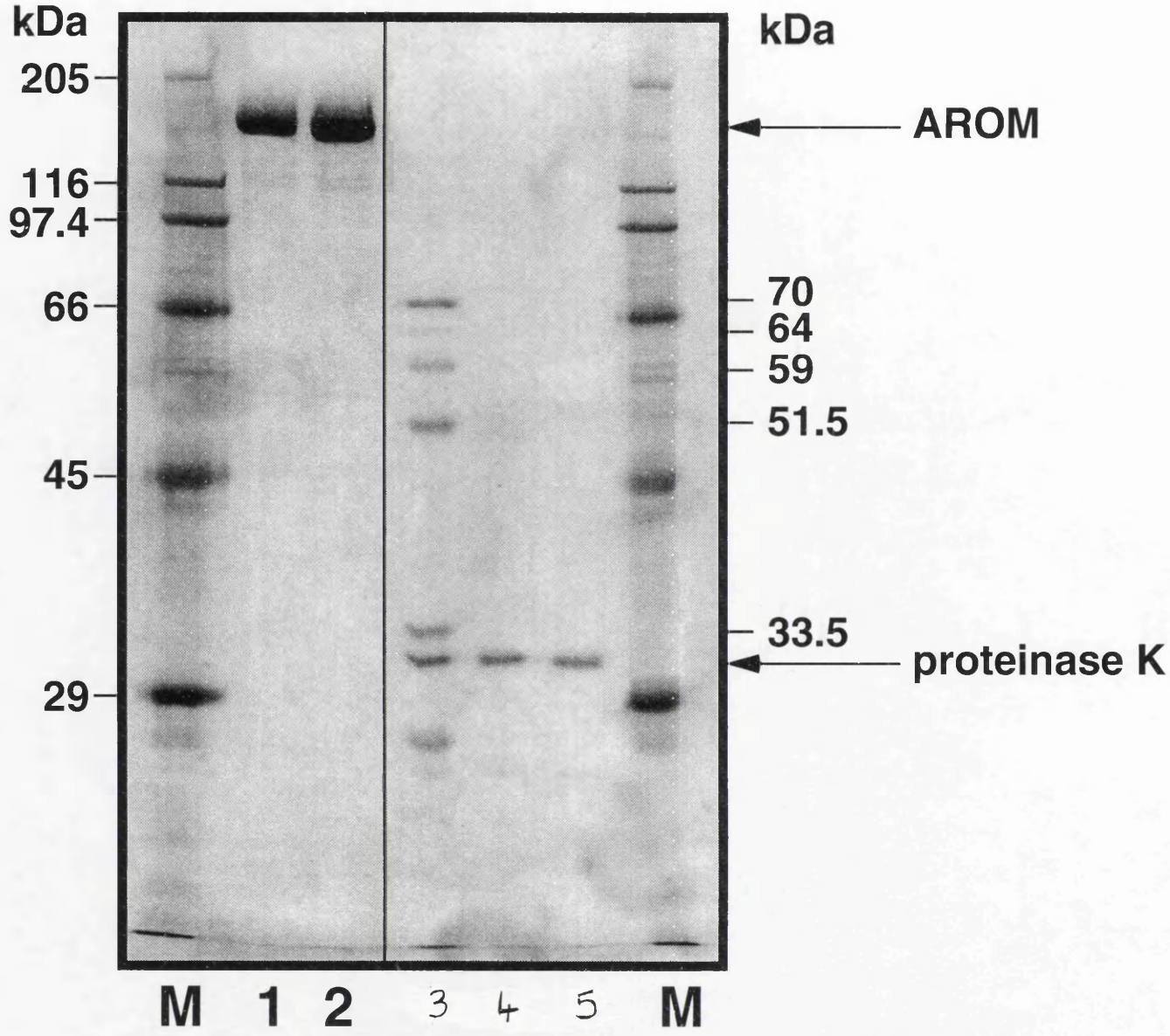

kDa
116
97.4
45
29
AROM
kDa
60
53
M
1
2
3
4
5
M

Figure 4.24 Digestion of native AROM with proteinase K

AROM purified from *A. nidulans* 1314 was incubated at a concentration of 200µg/ml with 40µg/ml proteinase K in 50mM-Tris/HCl pH7.5, containing 1.4mM-ß-mercaptoethanol at 25°C. Digestion was terminated after 5 and 60 min with the addition of 1.15mM-PMSF. The protein was boiled immediately in SDS PAGE sample buffer and analysed by SDS PAGE on a 10% gel. Protein was stained with Coomassie Brilliant Blue G-250.

M	molecular weight markers: 205kDa, 116kDa, 97.4kDa, 66kDa, 45kDa and 29kDa.
Lane 1.	6µg AROM.
Lane 2.	6µg AROM incubated at 25°C for 60 min with the subsequent addition of PMSF.
Lane 3.	6µg AROM incubated for 5 min with proteinase K.
Lane 4.	6µg AROM incubated for 60 min with proteinase K.
Lane 5.	1.2µg proteinase K incubated for 60 min with the subsequent addition of PMSF.


kDa
205
116
97.4
66
45
29
kDa
AROM
70
64
59
51.5
33.5
proteinase K
M 1 2 3 4 5 M

described in Section 2.6.1. The gel was then stained for shikimate DH activity (see 2.6.2).

Intact AROM used as a control was able to renature and form active shikimate DH (Figure 4.25). The technique was used to identify the shikimate DH active site of *N. crassa* AROM following proteolysis (Coggins *et al.*, 1985; Coggins & Boocock, 1986). It relies on the proteolytic fragment being able to renature independently. In an earlier experiment where intact AROM from *N. crassa* and *A. nidulans* were renatured after SDS PAGE on the same gel, the *N. crassa* AROM was able to recover more shikimate DH activity determined by the stain intensity (data not shown). This is similar to the findings in Section 3.5.5, where *S. cerevisiae* AROM is not as active after renaturation as *N. crassa* AROM, as shown by a shikimate DH activity stain.

Figure 4.25 shows the shikimate DH activity of fragments separated by SDS PAGE then renatured. The gel has a very dark background which is usually only seen when DTT is present in the gel. The DTT was washed out of this gel and has never caused a problem before. Despite this problem, shikimate DH activity is seen to be associated with intact AROM (lane 8) and shows as a dark band. Several other polypeptides with shikimate DH activity can also be seen in the gel. In lane 3 after digestion with proteinase K, both intact AROM and an 80kDa species have shikimate DH activity. This AROM has not digested as far as it did in the previous experiment with proteinase K (Figure 4.24) where no 80kDa polypeptide was observed. This indicates that the digestion of AROM is less complete in this renaturation experiment; the 80kDa polypeptide is presum-ably an unstable intermediate which is rapidly further proteolysed. It is possible that this 80kDa fragment is the C-terminal shikimate kinase-DHQase-shikimate DH trifunctional polypeptide.

Two polypeptides of lower activity were also observed after 5min digestion with chymotrypsin (lane 6) of 110kDa and 82kDa. The 110kDa polypeptide was not seen with the earlier chymotrypsin digestion (Figure 4.21) again

Figure 4.25 **Renaturation of shikimate DH activity following limited proteolysis of AROM from *A. nidulans* 1314**

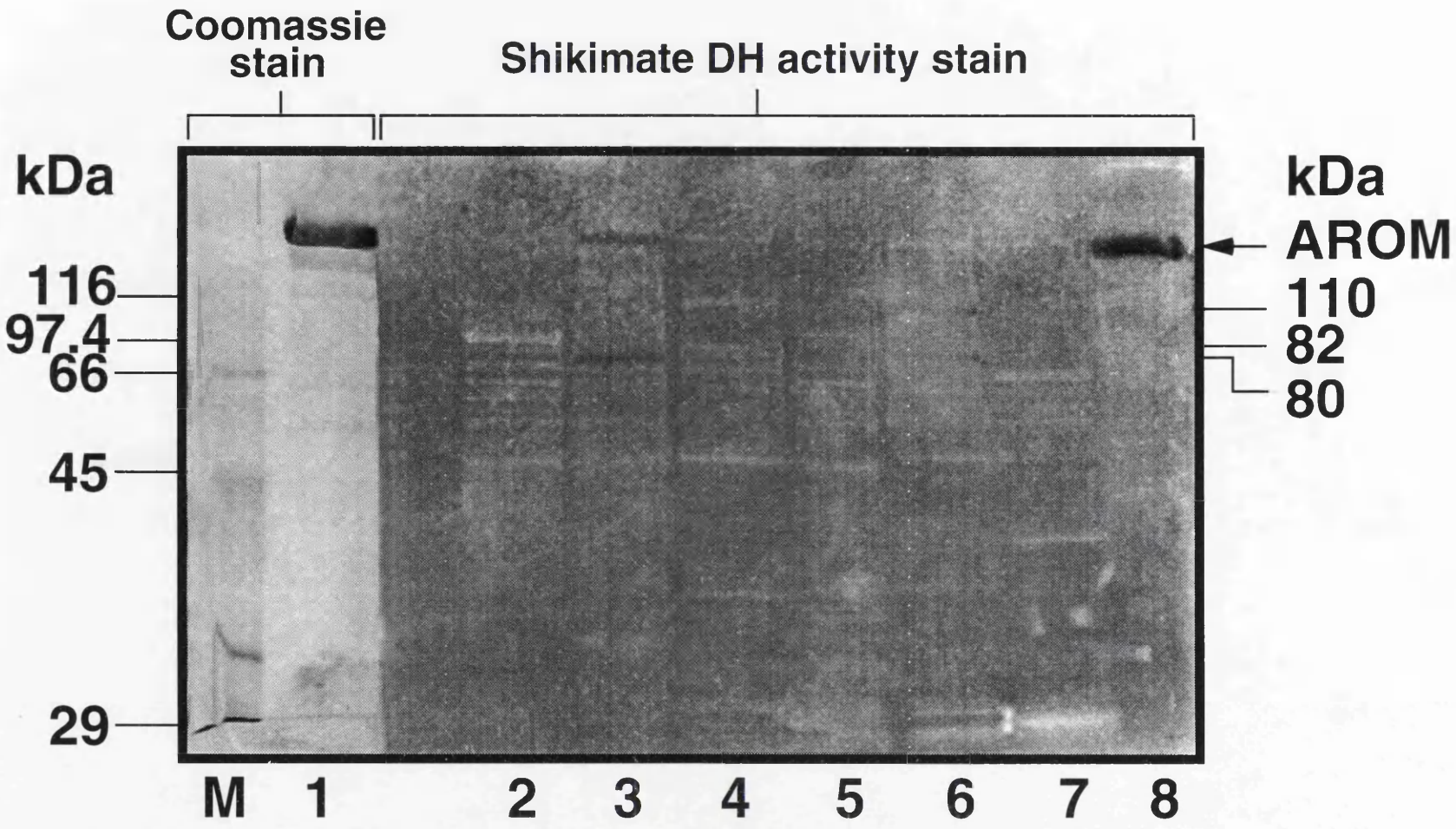
AROM purified from *A. nidulans* 1314 was proteolysed seperately with trypsin, subtilisin, proteinase K, thermolysin and thermolysin in the ratio of AROM:proteinase, and for the incubation time at 25°C described for each below. Digestion was terminated with the addition of an inhibitor specific to each proteinase, and samples were subsequently boiled for 2 min in SDS PAGE sample buffer. Each proteolysed AROM sample was separated by SDS PAGE on a 10% gel and two lanes with only intact AROM and molecular weight markers were stained for protein with Coomassie Brilliant Blue G-250. The remainder of the gel was renatured with Triton X-100, as described in Section 2.6.1, and stained for shikimate DH activity.
Note: The activity bands appear as black bands against the dark background. Light bands are protein bands.

Coomassie stain:

M	molecular weight markers: 205kDa, 116kDa, 97.4kDa, 66kDa, 45kDa and 29kDa.
Lane 1.	3µg AROM incubated for 60 min.

Shikimate DH activity stain:

Lane 2.	3µg AROM incubated for 5 min with subtilisin in the ratio 20:1.
Lane 3.	3µg AROM incubated for 5 min with proteinase K in the ratio 5:1.
Lane 4.	3µg AROM incubated for 60 min with thermolysin in the ratio 5:1.
Lane 5.	3µg AROM incubated for 5 min with subtilisin in the ratio 20:1.
Lane 6.	3µg AROM incubated for 5 min with chymotrypsin in the ratio 5:1.
Lane 7.	3µg AROM incubated for 60 min with trypsin in the ratio 2:1.
Lane 8.	3µg AROM incubated for 60 min, with the subsequent addition of PMSF.


Coomassie stain
Shikimate DH activity stain
kDa
116
97.4
66
45
29
kDa
AROM
110
82
80
M
1
2
3
4
5
6
7
8

indicating that the proteolysis is less complete in the renaturation experiment. The 110kDa polypeptide was observed in another digestion study with chymotrypsin (data not shown) and is an unstable proteolysis product. It is possibly due to the cleavage of AROM within the EPSP synthase domain. The 82 kDa polypeptide with shikimate DH activity was also seen in the chymotrypsin digestion shown in Figure 4.21 after 5 min digestion and like the active proteinase K fragment it is likely to represent a shikimate kinase-DHQase-shikimate DH trifunctional polypeptide from its size. However, it is only a minor component of the proteolysis products (Figure 4.20) and is rapidly degraded.

None of the stable fragments produced by digestion of AROM with thermolysin or trypsin, or with subtilisin showed any recoverable shikimate DH activity.

4.7.4 Summary

The preliminary proteolysis study of AROM from *A. nidulans* 1314 suggests that AROM is initially cleaved in many positions on the protein producing a complex fragmentation pattern. As digestion continues stable fragments are formed only with trypsin and thermolysin digestion (Figure 4.21 and 4.23). The data shows that for the proteinases tested the EPSP synthase activity of AROM is most sensitive to proteolysis and is lost very quickly (Figures 4.19, 4.20, 4.22 and 4.23).

In the case of *N. crassa* AROM, shikimate kinase was the activity found to be most sensitive to proteolysis with trypsin, subtilisin and chymotrypsin (Coggins & Smith, 1983; Coggins *et al.*, 1985; Coggins & Boocock, 1986). However EPSP synthase of *N. crassa* AROM was specifically proteolysed by an endogenous proteinase contaminant in some preparations. The data shown in this section confirms my own observation that of the four activities measured EPSP synthase of AROM is the activity most sensitive to inactivation in crude extracts (Figure 4.2). It is possible that EPSP synthase in this AROM preparation from *A. nidulans* 1314 is particularly sensitive to proteolysis because of the missense mutation in the adjacent

DHQ synthase domain (see 4.6.8). It will be interesting to see whether the proteolysis pattern is the same with wild-type *A. nidulans* AROM.

During proteolysis with trypsin, chymotrypsin and subtilisin equal DHQase and shikimate DH activities, expressed as a percentage of the level in intact AROM, are seen after 5 min digestion (Figures 4.19, 4.20 and 4.22). This suggests that AROM may be proteolysed to produce a bifunctional polypeptide which may have some of the shikimate kinase domain. There are four possible reasons why the polypeptides remaining after digestion fail to renature to produce active shikimate DH. The first is that they may not actually have the shikimate DH domain at all. The second is that the fragments are unable to refold although the ability of some polypeptides to renature makes this unlikely. Another possibility is that the proteolysed AROM is 'nicked' within the shikimate DH domain and retains activity under native conditions. However, when this proteolysed AROM is denatured for SDS PAGE the nicked protein 'falls apart'. The fourth possibility, which cannot be discounted is that AROM is further proteolysed when the protein is boiled in SDS PAGE sample buffer. The data are consistent with the hypothesis that the DHQase and shikimate DH regions of AROM form a compact globular structure. Evidence for this hypothesis also comes from the proteolysis study of *N. crassa* AROM in which a stable DHQase-shikimate DH bifunctional polypeptide was isolated (Smith & Coggins, 1983; Coggins *et al.*, 1985; Coggins & Boocock, 1986). Van den Hombergh *et al.* (1991) have provided evidence that there is no linker polypeptide between DHQase and shikimate DH in *A. nidulans* AROM. Interestingly in plants DHQase and shikimate DH are found to be produced as a bifunctional polypeptide (Polley, 1978; Koshiba, 1979; Mousdale *et al.*,1987).

The proteolysis study was not carried further due to the time constraints of this project. It should be possible to identify the DHQase active site of proteolysed AROM by labelling it with tritiated sodium borohydride in the same manner as Smith & Coggins (1983) identified the *N. crassa* AROM DHQase active site (see 1.7.3).

The unstable 80kDa polypeptide produced by digestion with proteinase K and the 82kDa polypeptide produced by digestion with chymotrypsin probably represent the trifunctional shikimate kinase-DHQase-shikimate DH C-terminus of AROM. N-Terminal sequence analysis would allow the identification of the domain boundary although the fragments are not ideal because they have only transient stability and are readily digested further. It may be possible to isolate the more stable AROM fragments chromatographically as was done for those from *N. crassa* AROM by Boocock (Coggins *et al.*, 1985; Coggins & Boocock, 1986) but it is possible that the mutation in the DHQ synthase domain of the AROM used in this study contributes to the instability of the protein fragments produced. Therefore, it would be more interesting to examine the proteolysis pattern produced by wild-type *A. nidulans* AROM before attempting the isolation and characterisation of fragments.

4.8 Discussion

This chapter has described the development of a rapid procedure for the purification of AROM from the *A. nidulans* overexpression strain 1314. The purified protein has been shown to have only a low level of DHQ synthase activity and it has emerged that the plasmid-borne copies of the AROM gene present in strain 1314 contain a missense mutation in the region encoding the DHQ synthase domain (Moore & Hawkins, 1993). The mutation does not appear to have a significant effect on the other four AROM activities (4.6.5) or on the dimerisation of AROM (see 4.6.6) even although the DHQ synthase domain has been implicated in contributing to the dimer interface by interallelic complentation studies of *N. crassa* AROM (see 1.7.1).

The protocol for the purification of AROM from *A. nidulans* 1314 described in this study may also prove useful in the purification of wild-type overexpressed AROM. However, it is possible that wild-type AROM may have a different elution profile on the Mimetic Yellow dye column because the mutant AROM inadvertantly used in this study has a mutation that may disrupt the nucleotide

binding site of DHQ synthase (see 4.6.7). The nucleotide binding site may well be important for dye binding and this may alter the elution pattern.

The purification of wild-type AROM from *A. nidulans* will allow a comparative study to be made with the missense mutant protein purified in this study. Such a comparative study would indicate whether the DHQ synthase activity has affected any of the other four AROM activities. Also, limited proteolysis studies of wild-type AROM may reveal differences in the domain packing of AROM which result from the mutation.

Since the work described here was completed, the domain expression studies of *A. nidulans* AROM by Hawkins' group have been very successful but it is obviously a high priority to purify and characterise wild-type AROM so that the integrity of the AROM 'fragments' can be established. Moore & Hawkins (1993) have expressed a stable DHQ synthase -EPSP synthase AROM fragment in *E. coli* and a DHQase-shikimate DH protein in *A. nidulans*. The same DHQase-shikimate DH sequence fails to complement the *aroE*$^-$ mutation in *E. coli* (see 1.7.5), and the failure of the shikimate DH region of AROM to complement *aroE*$^-$ mutants had previously been shown by Hawkins & Smith (1991). It is now thought that there is a thirty-nine nucleotide intron in the shikimate DH sequence. Since *E. coli* cannot excise the intron, it might explain the failure of this *A. nidulans* shikimate DH sequence to complement *E coli* mutants.

In conclusion, it remains a priority to purify and characterise wild-type AROM from *A. nidulans* and the purification scheme described in this study for the missense mutant of AROM is likely to form the basis of that purification.

CHAPTER 5 General discussion and future prospects

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The aim of the work presented in this thesis was to purify and characterise intact AROM so that the characteristics, for example kinetic properties, of genetically expressed domains of the protein might be compared with those of the native protein. The original species of choice was *S. cerevisiae* and AROM was purified from an overexpressing strain. However, the purified material was partially proteolytically degraded and no means of totally avoiding proteolysis was found. Thus the usefulness of the *S. cerevisiae* AROM preparation for an extensive kinetic analysis was limited since proteolytically altered proteins might well have different kinetic characteristics from the intact protein.

A rapid purification scheme was then developed for the isolation of AROM from the *A. nidulans* overexpressing strain 1314. The AROM enzyme was found to be defective in DHQ synthase activity due the accidental introduction of a missense mutation in the N-terminal domain of the polypeptide. It is expected that the purification scheme developed will provide the basis for the purification of intact AROM from *A. nidulans* and this has been discussed in Section 4.8.

Table 5.1 compares the activity ratio of AROM purified from the *S. cerevisiae* and *A. nidulans* overexpressing strains, as described in this thesis, with the activity ratio of purified *N. crassa* AROM. One obviously has to be careful not to read too much into these activity ratios because of the mutation in the DHQ synthase domain of the *A. nidulans* protein, and the partial proteolysis of the *S. cerevisiae* preparation (described above). The data shows that the activity ratios of AROM from all three species is broadly similar except for the lack of DHQ synthase activity in the mutated *A. nidulans* protein and for very high shikimate DH activity of the *S. cerevisiae* protein. The shikimate DH activity in the two fungal species, which are more closely related in evolutionary terms, is essentially similar. The high shikimate DH activity in *S. cerevisiae* is not thought to be an artefact since the activity is also high in crude extracts of both wild-type *S. cerevisiae* and the overexpressing strain (see Table 3.1).

	Activity ratio (DHQase=100)		
	N. crassa AROM	*S. cerevisiae* Yep52g:ARO1 AROM	*A. nidulans* 1314 AROM
DHQ synthase[f]	98	67	2
DHQase[f]	100	100	100
shikimate DH[r]	266	1472	117
shikimate kinase[f]	94	176	161
EPSP synthase[r]	69	170	85

[f]assayed in the forward direction

[r]assayed in the reverse direction

Table 5.1 Activity ratio of AROM purified from several species

The activity ratio of AROM purifed from *S.cerevisiae* BJ1991 transformed with Yep52g:ARO1 was taken as the activity ratio of the AROM eluted in the major AROM peak from Sephacryl S-200 chromatography (fractions 28-31) described in Section 3.5.2. The activity ratio for AROM purified from *A. nidulans* 1314 is taken from the values in Table 4.4 and the *N. crassa* AROM values are taken from Boocock (1983). The activity ratios were calculated by normalisation against the DHQase activity which was given the arbitrary value of 100.

It will be of interest to see whether the DHQ synthase activity of the *A. nidulans* AROM is similar to that found in *N. crassa* when intact, fully active AROM protein is eventually obtained from *A. nidulans*.

The purification of AROM from *S. cerevisiae* and *A. nidulans* highlights the problems which are still encountered with proteolysis during protein purification. This is despite the use of proteinase inhibitors and of strains of yeast which are deficient in several proteinases. The yeast system has become the focus for studies of eukaryotic protein degradation and a better understanding of this process might provide valuable information in preventing unwanted proteolysis during protein purification.

On the basis of a complementation study of fragments of the *A. nidulans* AROM gene in *E. coli*, Hawkins & Smith (1991) have proposed a model for the AROM protein in which the protein is separated into two functional units. In this model the N-terminal DHQ synthase and EPSP synthase domains make up one unit, and shikimate kinase, DHQase and shikimate DH domains the other. In this model is also suggested that the N-terminal DHQ synthase domain is in contact with the C-terminal shikimate DH domain. However, at the moment the genetic evidence from the *N. crassa* system, based on interallelic complementation, suggests that the C-terminal shikimate DH domains of the AROM polypeptide are closely associated with each other in the homodimer. Similar data suggest that the N-terminal DHQ synthase domains are also in contact *ie.* the polypeptides are arranged in a head to head arrangement in the AROM dimer (see 1.7.1). This was supported by the limited proteolysis study made by Boocock on purified *N. crassa* AROM (see 1.7.3 and Figure 1.5). The limited proteolysis study made of AROM purified from *A. nidulans* 1314 described in this Thesis concurs with a model in which the DHQase and shikimate DH of AROM form a compact globular domain (see 4.7.4). It might be possible to extend the proteolysis study on intact *A. nidulans* AROM to determine the domain interactions at the dimer interface.

Sequence homology has been observed between proteins involved in the shikimate pathway and the quinate pathway in fungi. The quinate dehydrogenase of the

quinate pathway is homologous to the prokaryotic shikimate DH enzymes and the shikimate DH regions of the fungal AROM enzymes (Hawkins *et al.*, 1988). The activator protein of the quinate pathway has recently been reported to be homologous to the two N-terminal domains of AROM and includes a zinc binding motif allowing DNA binding (Hawkins *et al.*, 1993c) . Also, the repressor protein of the quinate pathway had previously been shown to be homologous to the three C-terminal domains of AROM (Anton *et al.*, 1987; Hawkins *et al.*, 1992). This suggests the use of conserved functional modules in proteins of the shikimate and quinate pathways. A long term aim of work on the AROM protein is to determine the three dimensional structures of the proteins involved and to investigate these proposed structural similarities. To that end large quantities of the AROM protein must be purified.

Hawkins has proposed that the regulatory proteins involved in activating and repressing transcription of the quinate pathway have evolved by duplication and cleavage of the AROM gene into two distinct halves - one becoming the repressor protein and the other the activator protein (Hawkins *et al.*, 1993c; Hawkins *et al.*, 1993d). The repressor protein removes the effects of the activator protein and is thought to act by binding the activator protein. Hawkins has suggested that the homology of the repressor protein to the C-terminal half of the AROM protein (with homology to shikimate kinase, DHQase and shikimate DH) allows the repressor to bind the quinate pathway metabolites quinate, dehyroquinate and dehydroshikimate and enables regulation of the quinate pathway at the transcriptional level in this way (Hawkins *et al.*, 1993c; Hawkins *et al.*, 1993d). He has also suggested that the interaction of the repressor with the activator is similar to the non-covalent interactions found in the AROM protein between the homologous monomers which form the dimeric structure. Again, determination of the quaternary structure of the AROM protein will show these interactions. It is a long term aim of the project that AROM and the monofunctional prokaryotic enzymes of the shikimate and quinate pathways are crystallised and the three dimensional structures solved. This may confirm the recruitment of a small number of motifs to the quinate and shikimate pathways. It

might also allow the nature of the interactions between the activator and repressor protein of the quinate pathway to be established.

Hawkins research group has recently been very successful at expressing AROM domains and are now at the stage that the expressed fragments of the AROM protein are being characterised in more detail. In a recent publication the DHQase domain of *A. nidulans* AROM has been characterised and the kinetics have been compared to those of purified AROM from *N. crassa* (Hawkins *et al.*, 1993a). This has obviously got to be done with a degree of caution and it highlights the importance of the purification and characterisation of AROM from *A. nidulans*. It will also be of interest to compare the expressed *A. nidulans* AROM domains with those produced by limited proteolysis of the intact protein.

The procedure described in this Thesis for the purification of the *A. nidulans* AROM protein lacking DHQ synthase activity will almost certainly allow the purification and characterisation of intact AROM from *A. nidulans*. This will enable a detailed comparison to be made between AROM and independently expressed domains and provide an insight into the multifunctional character of the protein.

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