INVESTIGATION OF EXTRACELLULAR ESTERASE ACTIVITIES FROM THERMOPHILIC FUNGI, AND PURIFICATION AND CHARACTERIZATION OF NOVEL SMALL ENZYMES

by

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ABSTRACT

The existence of naturally occurring, very small enzymes (<10 kDa, microenzymes) has been established with the findings that some enzyme activities are associated with proteins smaller than 10 kDa. This work investigated the extracellular esterase activities observed in some thermophilic fungi, and purified and characterised three novel thermostable microenzymes with esterase activities.

Among the nine thermophilic fungi studied, two esterase-positive organisms, *Emericella nidulans* and *Talaromyces emersonii,* exhibited the enzyme activities in less than 10 kDa fraction when grown in either malt extract medium or a synthetic medium.

Two small enzymes (E40 and E32) from *E. nidulans* and one (T40) from *T. emersonii* were identified, and purified to homogeneity by ultrafiltration, gel filtration and reverse-phase HPLC.

Nondenaturing gel filtration showed that MWs of E40 and T40 were 1.6 kDa, and E32 was 4.1 kDa, while electrospray and MALDI mass spectrometry indicated monomeric MWs of 510.3, 609.3 and 1424.7 Da for E40, T40 and E32 respectively. This was consistent with a trimer structure in solution. Sequence analysis revealed that all the three esterases had the $(Gly\text{-}Pro\text{-}Hyp)_n$ repeating unit, the characteristic of collagen. The esterase activities were associated with small diffusible factor(s). X-ray microanalysis indicated the esterases contained Zn and Al.

The esterases exhibited extremely high thermostability and unusual pH stability. They were more active against short chain-length fatty acids than long ones and hydrolysed glycerol esters with 1, 3 specificity.

An attempt at chemical synthesis of the enzymes showed that the synthetic peptide itself was inactive. Circular dichroism spectra showed that the native esterase possessed more triple-helical conformation than the synthetic peptide.

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LIST OF ABBREVIATIONS

A (Ala)	alanine
ACTH	adrenocorticotropic hormone
ATP	adenosine triphosphate
BF_3	boron trifluoride
Boc	t-butoxycarbonyl
C	concentration of crosslinking monomer relative to total monomer
C(Cys)	cysteine
CD	circular dichroism
D	aspartic acid
DIPEA	diisopropylethylamine
DMF	N, N-dimethylformamide
DNA	deoxyribonucleic acid
Dns (dansyl)	5-dimethylaminonaphthalene-1-sulfonyl
Dns-Cl	5-dimethylaminonaphthalene-1-sulfonyl chloride
E	glutamic acid
E32	microenzyme with minor esterase activity from *E. nidulans*
E40	microenzyme esterase from *E. nidulans*
EDTA	ethylenediaminetetraacetic acid
EG	ethylene glycol
EGF	epidermal growth factor
ESI	electrospray ionization

FAB	fast atom bombardment
Fmoc	9-fluorenylmethoxycarbonyl
G (Gly)	glycine
GGH	NH_2-glycyl-glycyl-histidine-COOH
GLC	gas liquid chromatography
H	histidine
HEEC	high molecular weight esterase from *E. chevalieri*
HFBA	heptafluorobutyric acid
HOBt	N-hydroxybenzotriazole
HPLC	high performance liquid chromatography
Hyp	hydroxyproline
I (Ile)	isoleucine
IgA	immunoglobulin A
IgG	immunoglobulin G
K_{cat}	turnover number of an enzyme
KDa	kilodalton
Lys	lysine
MALDI-TOF	matrix-assisted laser desorption ionizaton – time of flight
Min	minute
MRNA	messenger RNA
MS	mass spectrometry
MSR	macrophage scavenger receptor
MW	molecular weight

N	asparagine
NC	noncollagenous
NMR	nuclear magnetic resonance
NOEs	nuclear overhauser effects
P (Pro)	proline
PCR	polymerase chain reaction
PKa	the pH at which there is 50% dissociation of an acid
PLA_2	phosphatide 2-acylhydrolase
PON	paraoxonase
PTH	phenylthiohydantoin
PyBOP	benzo-triazol-1-yloxytris(pyrrolidino)phosphonium hexa-fluorophosphate
Q	glutamine
Rf	relative mobility
RNA	ribonucleic acid
RP-HPLC	reversed-phase HPLC
rpm	revolutions per minute
S	serine
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
SP	synthetic peptide
SPPS	solid phase peptide synthesis
T	total monomer concentration of acrylamide and bisacrylamide
T40	microenzyme esterase from *T. emersonii*
TBu	t-Butyl

TFA	trifluoroacetic acid
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
u/min	one fluorescence unit per minute
UV	ultraviolet
V_0	void volume
V_t	total available volume
Y	tyrosine

CHAPTER 1. INTRODUCTION

1.1 THERMOPHILIC MICROORGANISMS AND THERMOPHILIC ENZYMES

1.1.1 Thermophilic microorganisms

Microbiologists have long appreciated that temperature profoundly affects the activities and distribution of microorganisms in natural environments. The temperature range of growth has been used to classify groups of organisms. The commonly used divisions are psychrophiles (-5 to +20°C), mesophiles (15-40°C) and thermophiles (45-100°C or more) (Herbert, 1992).

It is with this latter group that the most exciting developments have occurred in recent years. Research in this area was originally stimulated by the isolation of the thermophilic eubacterium Thermus by Brock and Freeze (1969). Subsequent to this discovery, and following the pioneering work of Woese and Fox (1977), the traditional division of the biological world into Prokaryotes and Eukaryotes was re-structured to include a third primary kingdom, the Archaebacteria. In addition to the methanogens and extreme halophiles, this new kingdom includes some 19 genera of thermophilic, extremely thermophilic and hyperthermophilic bacteria. The hyperthermophiles (growth-temperature optimum ~100°C) have attracted the most attention following the unequivocal demonstration that they not only survive but also grow optimally at temperatures above the boiling point of water (Huber *et al.*, 1987; Fiala and Stetter,

1986).

Eukaryotic microorganisms are much more restricted in their distribution than prokaryotic microorganisms; the upper temperature limit for eukaryotes seems to be about 60°C (Tansey and Brock, 1972). Thus, above 60°C only prokaryotic organisms are found. It seems likely that structural characteristics of eukaryotes, perhaps in nuclear membrane systems, are incompatible with thermostability (Brock, 1985).

1.1.2 Biotechnological Application of Thermophilic microorganisms

Thermophilic microorganisms offer some major advantages for microbial technology, and a considerable amount of research on thermophiles is motivated by these potential applications. The most attractive attribute of thermophiles is that they produce enzymes capable of catalyzing biochemical reactions at temperatures markedly higher than those of conventional organisms. In addition, enzymes from thermophiles are more stable at conventional temperatures than are enzymes from mesophiles, prolonging the shelf life of commercial products.

1.1.2.1 Biocatalysts for biotechnology

Since many industrial enzymes are used at temperatures in excess of 50°C, there is considerable commercial pressure to develop thermostable forms as biocatalysts in modern biotechnology.

Thermophilic organisms have been shown to produce a variety of extracellular

enzymes, however, in varying amounts. Enzymes that are produced in large quantities primarily include proteases, amylases (including glucoamylase) and glucose isomerase. These are added to detergents, employed in the production of natural sweeteners and used in the manufacture of pharmaceuticals. More than 80% of thermostable enzymes are used in the detergent and starch industries and they have been identified as having the greatest growth potential. Data presented in Table 1 shows the applications of currently available thermostable enzymes (Herbert, 1992).

Table 1.1 Currently available thermostable enzymes and their applications.

Enzyme	Process Temperature (°C)	Application
Carbohydrates		
α-Amylase (bacterial)	90-100	Starch hydrolysis, brewing
α-Amylase (fungal)	50-60	Maltose
Glucoamylase	50-60	Maltodextrin hydrolysis
Pullulanase	50-60	High glucose syrups
Xylose isomerase	50-60	High fructose syrups
β-Galactosidase	30-50	Lactose hydrolysis
Cellulase	45-60	Cellulose hydrolysis
Pectinase	30-50	Clarification of fruit juices
Proteases		
Acid proteases	30-50	Food processing
Neutral proteases (fungal)	40-60	Baking, brewing
Alkaline proteases	40-60	Detergents
Lipases	30-70	Detergents, food processing

1.1.2.2 A place in PCR technology

The use of thermostable DNA polymerases, many of which are now obtained from hyperthermophilic microorganisms (Perler *et al.*, 1996), for the polymerase chain reaction (PCR) has been at the heart of the biotechnology revolution. The PCR technique has major applications in diagnostic medicine, taxonomy and molecular biology. The major advantage of using Taq polymerase in PCR reaction is its enhanced thermostability. At the denaturing temperature used (95°C), its half-life is 40 min and so the enzyme does not need to be added repeatedly as in the earlier procedures using the Klenow polymerase (Berquist and Morgan, 1992). This procedure has, in many instances, superseded cloning and has provided a much simpler method for the production of recombinant genes and plasmids which would have been much more tedious to be produced by conventional means.

1.1.2.3 Advantages of using thermophiles and their enzymes

Based on the first quantitative data on the kinetics of a number of thermophilic organisms, but especially because of the high temperature gradients and the expected acceleration of all reactions (according to the 'golden rule' that an increase of temperature by 10°C would double the reaction rate), it was assumed that the technical applications of thermophilic microorganisms could be highly advantageous, as compared with classic bioprocesses which require temperatures ranging between room temperature and/or about 37°C.

The following summary lists the advantages of using themophiles and their enzymes (Zeikus *et al.*, 1980; Amelunxen and Murdoch, 1978):

-- Productivity would be increased as the reaction rates (of organisms and enzymes) increase.

-- Mass cultivation of thermophiles would be cheaper than that of mesophilic microorgansims due to reduced investment required for heat exchange equipment.

-- Mass cultivation of thermophiles would be cheaper than that of mesophiles due to greatly reduced contamination problems.

-- Higher enzyme yields would result due to greater enzyme stability. This is of particular significance for enzyme recovery from the cultures and for enzyme purification.

-- Thermostable enzymes generally seem to be more resistant to the denaturing effects of detergents and organic solvents.

-- Volatilization of products might be valuable for two reasons: as a recovery or as a means for removing potentially inhibitory products.

Undoubtedly themophiles and their enzymes will play an important role in future biotechnological developments, particularly in processes which can benefit from the

use of more stable biocatalysts.

1.1.3 Molecular Basis of Thermophily

In conventional organisms macromolecules such as proteins and nucleic acids are inactivated irreversibly by heat, but in thermophiles these components are more stable (Langworthy *et al.*, 1979). Supramolecular structures such as ribosomes and membranes are also thermostable in thermophiles, although inactivated by heat in mesophiles. Further, the catalytic activity of thermophilic enzymes is low or absent at moderate temperatures at which conventional enzymes of similar function are optimally active. The temperature optima of thermophilic enzymes are frequently at or above the optima for the growth of the organisms.

Perutz and Raidt (1978) analyzed the factors involved in the thermostability of proteins. Comparisons were made of amino acid sequences and the three-dimensional structures of ferredoxins, hemoglobins and glyceraldehyde phosphate dehydrogenases exhibiting different degrees of thermostability. The results showed that the greater heat stability of the thermostable proteins was due to extra salt bridges between portions of the folded molecules.

But salt bridges may not fully explain protein thermostability. Yutani *et al.* (1977) studied the effect of single amino acid substitutions on the stability of the alpha subunit of the tryptophan synthetase of *Escherichia coli*. They found that a single amino acid change increased the stability of the molecule without a gross change in conformation.

The stability of the enzyme was greatly increased by an increase in hydrophobicity brought about by the substitution of a few suitable amino acid residues.

Matsumura *et al.* (1984) determined the nucleotide sequences of the gene encoded by the plasmid carrying a kanamycin-inactivation enzyme (kanamycin nucleotidyltransferase) in a thermophilic bacterium (*Bacillus stearothermophilus*) as compared with the same enzyme in a mesophilic bacterium (*Staphylococcus aureus*). Despite the fact that the plasmids coding these two enzymes had completely separate origins, the nucleotide sequences were identical except for only one base in the midst of the structural gene. This base change resulted in the substitution of a threonine residue with a lysine residue. The position of this lysine substituent was such that the protein surface could acquire increased electrostatic bridging without any significant change in three-dimensional structure, a situation consistent with the conclusion of Perutz and Raidt (1978).

Direct sequence comparisons show that thermophilic and mesophilic versions of the same enzyme typically have about 30-50% identity, but systematic analyses, even with small proteins, give no clue as to why one should be so much more stable than the other (Adams, 1993). Moreover, the first crystal structure for a thermophilic protein (which contained only 53 amino acids) showed that it was virtually superimposable on its mesophilic counterpart (Day *et al.*, 1992). To date, crystal structures have been reported for three enzymes and for one DNA-binding protein obtained from an organism that can grow at 100°C (Chan *et al.*, 1995; Russell *et al.*, 1997); although it is clear that protein thermostability is a result of relatively minor changes in protein

structure, there is as yet no consensus on the nature of these changes. Enhanced packing, additional salt bridges and hydrogen bonds, extended secondary structures, and abbreviated loops all appear to play a role in some (but not all) cases. The mechanisms involved appear to be unique to each enzyme type. It is apparent, however, that such stability is an intrinsic property of the protein. Thermophilic proteins are stabilized by the same types of intramolecular forces that stabilise mesophilic ones, although exactly how remains unclear.

Studies so far indicate two salient facts: first, amino acid sequence analyses do not indicate stabilising mechanisms, and so structural data are an absolute prerequisite; second, there does not appear to be a general strategy for converting, via site-directed-mutagenesis techniques, a mesophilic enzyme into a thermophilic one. Thus, for the foreseeable future, the only source of thermophilic enzymes will be the organisms themselves, or genes derived from them (Adam and Kelly, 1998).

1.2 MICROBIAL EXTRACELLULAR ESTERASES AND THEIR APPLICATIONS

1.2.1 Secretion and regulation of extracellular enzymes

The key feature of an extracellular enzyme is that it is transported across the cytoplasmic membrane. Proteins that traverse membranes typically possess an NH_2-terminal peptide extension of about 25 amino acid residues; the signal peptide. As the signal peptide emerges from the ribosome, it interacts with the inner surface of the

cytoplasmic membrane in bacteria or the endoplasmic reticulum in eukaryotes. As the polypeptide is elongated it passes through the membrane (cotranslational secretion) and a processing enzyme (signal peptidase) removes the signal peptide on the outer side. This allows the protein to assume its normal configuration on the outside of the membrane. Genetic studies have shown that there is a secretory machinery in the *E. coli* envelope that appears to transport the protein actively (Priest, 1984).

The secretion of proteins is remarkably similar in eukaryotic and prokaryotic cells to the extent that eukaryotic signal sequences are recognized and processed by prokaryotic cells and *vice versa*. However, the additional complexity of the eukaryotic cell requires that a series of membrane bounded vacuoles are needed to transport the enzyme from the endoplasmic reticulum to the outside of the cell. Cotranslational secretion is not universal, however, and some proteins are incorporated into, or transported through membranes, after they have been synthesised (post-translational secretion), again through the involvement of a signal peptide.

The synthesis of extracellular enzymes is regulated by the environment in the same way as cytoplasmic enzymes. If inducible, they are generally induced by a product from the substrate rather than the substrate itself since the latter is usually too large to enter the cell. Accumulation of the products generally leads to repression of the enzyme. Present evidence suggests that the control is exerted primarily at transcription and it seems probable that inducible extracellular enzymes are governed by repressor or activator proteins. Thus the operon model for gene regulation is applicable to these systems and has been confirmed in the case of penicillinase synthesis in *B. licheniformis*.

Extracellular enzyme synthesis is repressed by rapidly metabolized carbon sources (catabolic repression). Similarly, an ample supply of nitrogen as amino acids or ammonium represses the synthesis of proteases and related enzymes (Priest, 1984).

1.2.2 Microbial extracellular esterases

Esterases belong to the group of hydrolases (carboxylesterhydrolases; EC 3.1.1.1) which catalyse the formation or cleavage of ester bonds of water-soluble substrates. Esterases can be distinguished from lipases (EC 3.1.1.3) in that the catalytic activities of the former are generally restricted to short-chain fatty acid esters (Krisch, 1971).

The physiological functions of many esterases are not clear. Some of these enzymes are known to be involved in metabolic pathways that provide access to carbon sources; such enzymes include the acetyl- and cinnamoyl esterases that are involved in degradation of hemicellulose (Dalrymple *et al.*, 1996; Ferreira *et al.*, 1993). In some plant-pathogenic bacterial and fungal strains these cell-wall degrading esterase activities are believed to be pathogenic factors (McQueen and Schottel, 1987). Detoxification of biocides may be another important role. Insecticide resistance often results from amplification of genes for esterases that hydrolyze the insecticides (Blackman *et al.*, 1995). The fusidic acid resistance of *Streptomyces lividans* is due to a specific esterase which inactivates the antibiotic (Von den Haar *et al.*, 1997), and a *Bacillus subtilis* esterase that hydrolyzes the phytotoxin brefeldin A has been described (Wie *et al.*, 1996).

Esterases occur widely not only in animals and plants but also in microorganisms (Williamson, 1991; Higerd and Spizizen, 1973). They show a wide range of positional specificity, fatty acid specificity, thermostability, pH optimum, etc. This suggests that one could probably find a suitable esterase from nature that would be suitable for any desired application.

Esterases from mammalian origin have been extensively studied and characterised (Long *et al.*, 1988; Winkler *et al.*, 1990). More recently, a great deal of interest has been devoted to microbial esterases which could be a good alternative to the expensive mammalian enzymes. In addition, since microbial esterases are usually more thermostable than animal or plant esterases, they have received much attention for their potential use in industry and diagnostics. A large number of microbial esterases have been described, including esterases from *Escherichia* (Pacaud, 1982), *Bacillus* (Wood *et al.*, 1995), *Pseudomonas* (Nakagawa *et al.*, 1984), *Thermoanaerobacterium* (Shao and Wiegel, 1995), *Caldocellum* (Luthi *et al.*, 1990), *Aspergillus* (Okumura *et al.*, 1983), and *Sulfolobus* species (Sobek and Gorisch, 1988).

Most of the microbial esterases are extracellular, being excreted through the external membrane into the culture medium. Optimisation of fermentation conditions for these enzymes is of great importance, since culture conditions influence the properties of the enzyme producer as well as the ratio of extracellular to intracellular enzymes. The amount of enzyme produced is dependent on several environmental factors, such as cultivation temperature, pH, nitrogen composition, carbon and lipid sources,

concentration of inorganic salts and the availability of oxygen.

Many microbial esterases and lipases are only produced in the presence of an inducer. This may be a triglyceride, a fatty acid, or another lipid (Kroon *et al.*, 1996; Shimada *et al.*, 1992). Lipids or fatty acids do not appear to be required for the production of esterase (lipase) activity in a second group of microorganisms, but incorporation of these compounds increased the level of enzyme activity produced (Yoshida *et al.*, 1968; Chen *et al.*, 1992). In a third group of microorganisms, the enzymes are only produced constitutively (Chander and Klostermeyer, 1983).

1.2.3 Applications of esterases

Both esterases and lipases are very diverse in their enzymatic properties and substrate specificities, which makes them attractive for industrial applications (Gandhi, 1997; Boland *et al.*, 1991). In the application of biocatalysts, esterases, lipases and proteases account for more than half of all reported biotransformations.

A large number of lipases and esterases have been screened for application as food additives (flavour-modifying enzymes), industrial reagents (glyceride-hydrolysing enzymes) and stain removers (detergent additives), as well as for medical applications (digestive drugs; diagnostic enzymes).

Furthermore, in the presence of suitable organic solvents, esterases and lipases may also be used to effect stereoselective esterification, interesterifications and

transesterification of substrates (Cambou and Klibanov, 1984; Margolin, 1993).

The fact that different enantiomers interact differently in an organism and may even have hazardous effects, has led to a growing demand for enantiomerically pure compounds (Schoffiers *et al.*, 1996). Lipases and esterases have been used successfully in organic synthesis of optically pure substances. For instance, an esterase from *Arthrobacter globiformis* was used in the resolution of ethyl chrysanthemate derivatives (Nishizawa *et al.*, 1995), which are key compounds during the synthesis of pyrethrin insecticides. A heroin-specific esterase can selectively convert heroin into morphine; this is followed by further degradation to morphinone by a morphine dehydrogenase (Rathbone *et al.*, 1997). A Bacillus carboxyl esterase has been used for stereospecific resolution of R, S-naproxen esters to S-naproxen (Quax and Broekhuizen, 1994), which is an important anti-inflammatory drug, and a p-nitrobenzyl esterase was genetically engineered in order to synthesize cephalosporin-derived antibiotics (Moore and Arnold, 1996).

Apart from their industrial importance, the medical and therapeutic applications of this class of enzymes are obvious. The manipulation of lipolytic activities plays a part in the methods for treating malfunctions of fat metabolism and thus control cardiovascular diseases. Pancreatic lipase is necessary for the absorption of fat, cholesterol esterases for the absorption of cholesterol esters and perhaps of cholesterol, hormone-sensitive lipase for the mobilization of fat from adipose tissue, and pancreatic and intracellular phospholipases for the absorption and metabolism of polyunsaturated fatty acids.

The lipase level in blood serum is a diagnostic indicator for conditions such as acute pancreatitis and pancreatic injury (Lott and Lu, 1991). Lipase activity determination is also important in the diagnosis of heart ailments (Schnatz *et al.*, 1963) and blood cholesterol determinations (Imamura *et al.*, 1989). Lipid-storage diseases have been connected with cholesterol esterase and sphingomyelinase.

Lipase, being an activator of the tumour necrosis factor, can be also used in the treatment of malignant tumours (Kato *et al.*, 1989).

1.2.4 Molecular weight of esterases

A lot of esterases have been isolated, purified and sequenced with different molecular weights. Their molecular weight is usually in the range of 20-160 kDa. The mammalian esterases reported by Krisch (1971) was about 160 kDa, the esterase from beef liver isolated by Bauminger and Levine (1971) was 54 kDa, the molecular weight of the esterase from *B. stearothermophilus* was estimated to be 42-47 kDa (Matsunaga *et al.*, 1974) and the gene of a 44 kDa esterase from *Pseudomonas fluorescens* has been sequenced and characterized (Khalameyzer *et al.*, 1999).

Some esterases and lipases with molecular weight less than 20 kDa have also been reported. An intracellular esterase of 16.7 kDa was isolated from *Arthrobacter viscosus* NRRL B-1973 and purified to homogeneity (Cui *et al.*, 1999). The 16 kDa lipase (BTL-1) was purified directly from the culture broth of a thermophile *Bacillus thermocatenulatus* and characterized. As expected for a thermophilic enzyme, the BTL-1 showed maximum activity at elevated temperatures of 60-70°C, a good

temperature stability (50% residual activity after 30 min at 60°C) and a good stability toward different organic solvents and detergents (Schmidt-Dannert *et al.*, 1997). The animal PLA_2 (phosphatide 2-acylhydrolase, EC 3.1.1.4) are a diverse family of well-studied enzymes in which several distinct groups belong to a class of small (13-18 kDa), secretory, and extremely heat-stable enzymes having between five and eight disulfide bonds and requiring millimolar concentrations of Ca^{2+} for maximum activity (Dennis, 1994); a plant phospholipase A_2 with MW 13.9 kDa was purified and characterized from developing seeds of elm (Ståhl *et al.*, 1998). The purified enzyme had an alkaline pH optimum and required Ca^{2+} for activity. It was stable both in organic solvents and at extreme pH conditions, it was not denatured under the condition of 40-55% of acetonitrile with 0.1% TFA gradient elution during the reverse phase HPLC purification, and was also extremely heat stable: incubations with the purified enzyme at 100°C for 5 min did not affect activity, whereas a 30-min incubation at that temperature caused only a 40% decrease in activity.

Apart from these low molecular weight ester hydrolases, a very few even smaller esterases with MW less than 10 kDa have also been reported. An esterase of 5.7 kDa from *Candida lipolytica* was isolated and characterized by Adoga and Mattey (1979, 1985). The enzyme had 56 amino acid residues, with a high percentage of proline (11 residues) and the activity of the enzyme was related to the presence of Fe^{3+}. A small lipase of 7.0 kDa was also isolated by Chadan and Shahani (1963). And recently, another extremely thermostable esterase was isolated from *Bacillus stearothermophilus* by Simões *et al.* (1997, 1995), the MW of which is only 1566Da

by MALDI-TOF spectrometry, and it retains more than 90% activity after incubation at 90°C for 2 hr.

1.3 MICROENZYMES, THEIR PURIFICATION AND CHARACTERIZATION

1.3.1 Microenzymes

Microenzymes are defined as enzymes, including monomer or subunit forms, with molecular weights below 10 kDa (Schenk and Bjorksten, 1973). The study of microenzymes was initiated at Bjorksten Research Laboratory in U. S. A. from the early 1970's as a result of aging research which indicated a possible therapeutic utility for such enzymes (Bjorksten, 1968).

In the aging process, amorphous 'hyalin' materials accumulate in and around cells and gradually impede the functionality of the cells and consequently of the body. These hyalin materials are mostly proteinaceous, and appear to be highly cross-linked, dense, insoluble, and resistant to dissolution by the body's enzymes. The insolubility and high density of the materials suggest that inability to penetrate the hyalin matrix may explain why normal body enzymes do not eliminate this material. Since these materials are broken down naturally after death, enzymes for achieving such dissolution clearly do exist. If the high density of these hyalin materials prevents their dissolution by the body's enzymes, then a very low molecular weight proteolytic enzyme - a microenzyme - seems called for. The search for such enzymes in that laboratory had centred around spore-forming microorganisms whose spores also have extremely dense

walls, which they obviously must at least partially dissolve in order to germinate.

The results from Schenk and Bjorksten (Schenk and Bjorksten, 1973) showed that a single exogenous microenzyme with proteolytic activity was found to exist in equilibrium with oligomers in a strain of *Bacillus cereus* (BRL-70). The monomer of this enzyme, which still gave the enzyme activity, has a MW only 2.5 kDa, and calcium ions were found to stabilize and to activate the enzyme.

Despite this, it seems that the study of microenzymes has not received much attention since then as among the great number of enzymes found and studied over the past 30 years, only a very few have been reported with MW smaller than 10 kDa. In addition to the 5.7 kDa esterase from *Candida lipolitica* and the 1.57 kDa esterase from *Bacillus stearothermophilus* mentioned in Section 1. 2. 4, a microenzyme of which the amino acid composition has been investigated was a rennin of 9.7 kDa from a thermophilic actinomycete (Laxer *et al.*, 1981): again a high proline content was found. The enzyme was thermostable: 70°C for 90 min resulted in no loss of activity, and it required calcium ions for activity. Steele *et al.* (1992) isolated a novel spiral-shaped bacterium which produced a 8 kDa alkaline-active, thermostable protease with optimum activity at pH 11.0 and 60°C. A disulfide bond-forming enzyme from archaebacterium *Sulfolobus solfataricus* (Guagliardi *et al.*, 1992), with MW 6.2 kDa, was highly thermostable, heating to 90°C for 2 hr did not cause loss of activity. The extracellular amylase produced by *Bacillus caldolyticus* at 70°C was a Ca-dependent enzyme and had been shown to consist of subuits with a MW of less than 10 kDa, which were enzymatically active in the low temperature range (Heinen and Lauwers,

1976). It was also found that the subunits associate to a higher MW form when divalent Ca cation was bound to the subunits. The 10-13 kDa phytase described by Tambe *et al.* (1994) was shown to be the fragment of the native enzyme of 700 kDa. The two enzymes differed in their Km, pH optima, pI values and temperature sensitivity, and the small enzyme peptide retained a full complement of enzyme activity.

Apart from these microenzymes found in microorganisms, more recently a 30 amino acid survival-promoting peptide Y-P30 in medium conditioned by neural cell lines treated with hydrogen peroxide has been identified and found to possess phosphatase activity (Cunningham *et al.*, 1998).

The peptide Y-P30 supports neural cells and their processes *in vitro* and *in vivo*; and it was shown to inhibit neuronal atrophy and the response of microglia 7 days after direct application to cerebral cortex lesions. Its amino acid sequence has been determined as YDPEA ASAPG SGNPC HEASA AQCEN AGEDP. A very stable synthetic peptide made on the basis of sequences obtained during purification of this molecule is fully active when delivered to such lesions.

Y-P30 was found to be also able to catalyse the hydrolysis of para-nitrophenyl phosphate to nitrophenol in the presence of manganese ions. The reaction is inhibited by the phosphatase inhibitor sodium vanadate. These particular properties are similar to those displayed by much larger molecules belonging to the family of calcineurin-type phosphatases. The phosphatase activity of this peptide might be attributed to its amino

acid sequence where residues 12-17 of this peptide (Gly-Asp-Pro-Cys-His-) resemble a motif found in several phosphatases.

Interestingly, a cancer cachectic factor identified by Todorov *et al.* (1996) has the same sequence in N-terminal 20 amino acids as Y-P30 and contains the motif in the phosphatases as well. But if this peptide also exhibited phosphatase activity has not been investigated yet.

1.3.2 Importance of small enzyme studies

Of reported small enzymes, most are thermostable and require the presence of a metal (Ca, Fe or Mn) for optimum stability and activity. Such small enzymes offer a unique opportunity for the study of thermostability and are potentially useful in industry. Possible applications include use in organic synthesis reactions, and improved, more efficient immobilized enzyme systems.

Although speculation as to the mechanism of thermal stability is premature, decreased size may allow less structural and conformational options (i.e. less folding, hydrogen bonding, etc.) that would be labile to high temperatures (Steele *et al.*, 1992). In fact, most microenzymes reported have higher thermostability than their larger counterparts (Adoga, 1980; Guagliardi *et al.*, 1992; Simões *et al.*, 1997).

That decreasing molecular weight results in increasing thermostability has been indicated by some authors (Singleton and Ameluxen, 1973; Stellwagen and Wilgus, 1978). They studied the relationship between thermostability and protein molecular

weight. Their screening of a large number of globular proteins obtained from mesophilic organisms has revealed that a decrease in molecular weight was correlated with an increase in thermostability.

Another example is that an α-amylase was crystallized from *B. stearothermophilus* by Campbell and co-workers (Singleton and Ameluxen, 1973) and found to be heat stable. There was no loss of enzymatic activity after 24 h of incubation at 65 or 70 °C, and only a 29% loss of activity occurred after 20 h at 85°C. The enzyme was found to have a low molecular weight of 16 kDa and contain 15% proline, and to be nonspherical. It was proposed that this unique structure was solely responsible for its thermostability.

Decreasing protein size may be a strategy for increasing stability by increasing globularity due to an increase in intramolecular packing and deletion of surface loops (Hubbard and Argos, 1994; Jaenicke, 1991, 1991a). When a low molecular mass of protein is too small to provide the necessary size of a co-operative unit, the structure may be stabilised by covalent crosslinking or ligand binding which will improve the stability of the entire molecule.

Low molecular weight enzymes will occupy less space in an immobilised bioreactor system, which means that, in the same volume, the number of enzyme molecules will be larger than when compared with large enzymes, thus increasing the rate of reaction when other factors being equal (Gacesa and Hubble, 1987). Moreover, small enzymes are more easily made by chemical synthesis and could provide opportunities for the development of novel biocatalysts. In addition, enzymes with a lower MW may be

more readily cloned and expressed in heterologous systems and are, thus, of special interest (Adney *et al.*, 1995).

1.3.3 Other biologically important low MW proteins – bioactive peptides

Peptides play a number of important roles in organisms. These short polymers of amino acids can function as the basic recognition unit for receptors and other biological binding proteins, and are thus used for studying and affecting a very wide range of biochemical interactions. Some peptides themselves are biologically important, serving as hormones, neurotransmitters, growth factors, etc. Peptides are also important structural and functional elements of proteins, and the analysis of peptides from enzymatic digests of proteins is a critical tool in understanding protein structure.

Peptides are obtained from several quite distinct sources. Some peptides are separated from natural biological extracts such as serum, cell lysates, organ extracts, etc. Peptides with biological activities produced by several bacterial and fungal species belong to large and diverse family of natural products that includes antibiotics, enzyme inhibitors, plant or animal toxins and immunosuppressants. They are therefore of great benefit to medicine, agriculture, biological research and industry.

Microenzymes are the peptides with catalytic activities and have similarity to bioactive peptides with regard to their biosynthesis, purification and characterization.

1.3.4 Biosynthesis of peptides

There exist two biosynthesis pathways for peptide synthesis: ribosomal and non-ribosomal.

Some peptides are synthesized ribosomally, are gene-coded, but often undergo extensive post-transcriptional modification and proteolytic processing. The synthetic mechanisms for these small peptides are the same as those for larger proteins. Peptides with physiological activities are often derived from much larger precursor proteins by the action of specific proteinases.

Another group of bioactive peptides are synthesized non-ribosomally on a protein-template. These bioactive peptides are produced mainly by soil bacteria and filamentous fungi and are of linear, cyclic and branched-linear structures. They contain non-protein amino acids like D-amino acids or hydroxy acids and other amino acid constituents that can undergo extensive modifications, including N-methylation, acylation, glycosylation and covalent linkage to other unusual functional groups (Kleinkauf and Von Döhren, 1990; Lipmann, 1980).

Biosynthesis of peptides in non-ribosomal systems is catalysed by multifunctional enzymes that employ the thio-template mechanism (Lipmann, 1980). The constituents of the peptides, amino- and hydroxy acids, that have to be sequentially connected by the corresponding peptide synthetase are first activated as acyl adenylates where ATP serves as the energy source. This activation is similar to that catalysed by the aminoacyl-tRNA synthetases (Schimmel, 1987), however, no tRNA intermediates are formed, but instead peptide synthetases covalently link the activated amino acid as a

carboxy thioester (Katz and Demain, 1977; Kleinkauf and Von Döhren, 1990) (Figure 1.1).

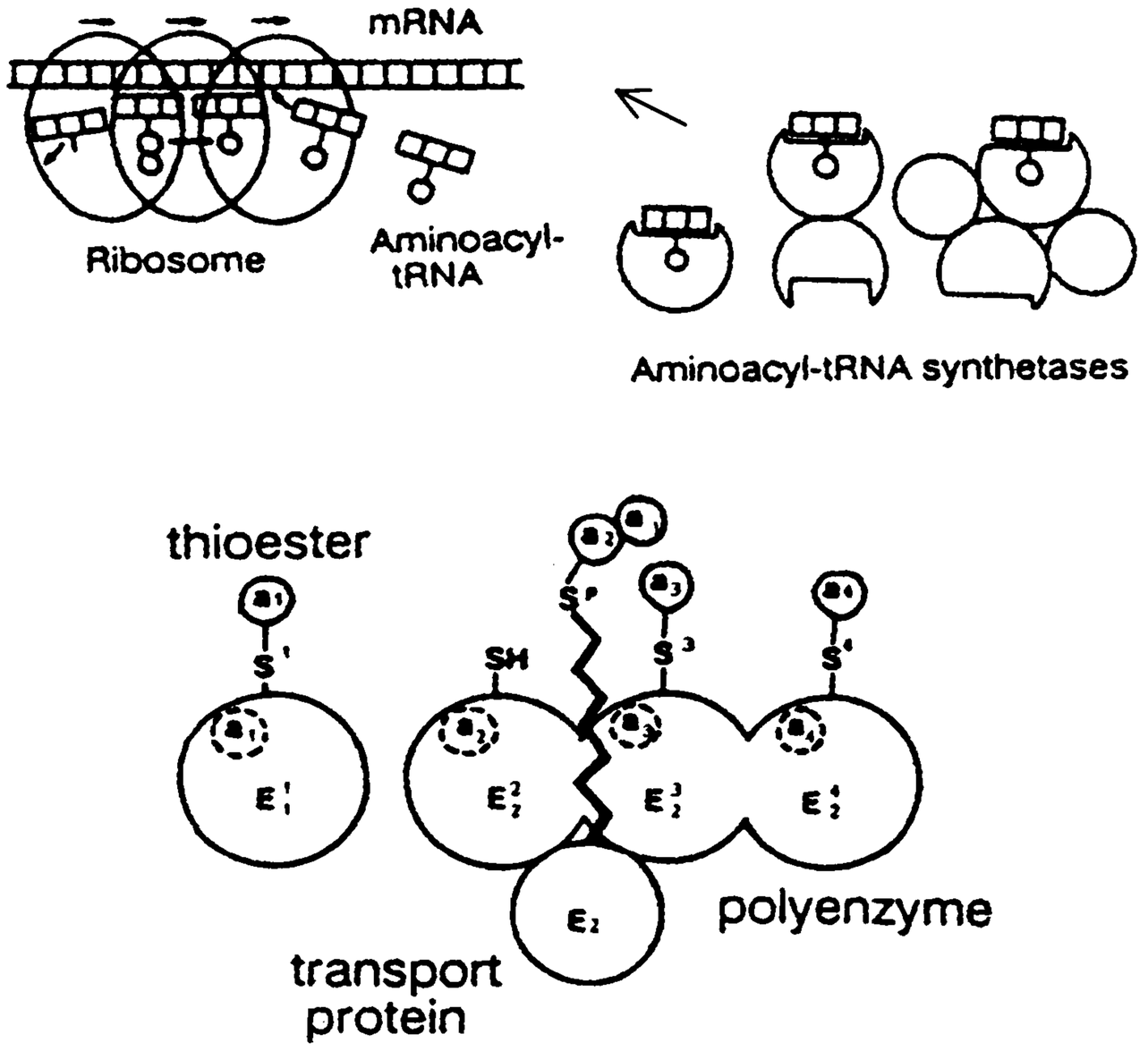


Figure 1.1 Scheme of ribosomal and enzymatic polypeptide biosynthesis.
In the ribosomal system amino acids are activated by aminoacyl-tRNA synthetases. These are symbolized here by their different types of subunit structures. The charged tRNA is then lined up at the ribosome according to the mRNA sequence. In the enzymatic system polyenzymes activate amino and hydroxy acids, and the template itself is aminoacylated, generally as a thioester. A transport system then assembles the lined-up residues, presumably by a sequential mechanism (Kleinkauf and Von Döhren, 1990).

The elongation reaction is catalysed by the enzyme bound cofactor 4'-phosphopantetheine (Kleinkauf *et al.*, 1971). The activated amino acid is then transferred to the thiol group of the cofactor which acts as an internal transport system. By repeated trans-peptidation and trans-thiolation reactions the peptide chain is completed and then released from the multi-enzyme either by cyclization or by the action of a specific thioesterase. The protein-thio-template pathway has been shown to direct the synthesis of many antibiotics (Kleinkauf and Von Döhren, 1990).

Recent studies on the analysis of the primary structure of several peptide synthetases have revealed that they are organized in highly conserved and repeated functional domains. The domains, which represent the functional building units of peptide synthetases, appear to act as independent enzymes whose specific linkage order forms the protein-template that defines the sequence of the incorporated amino acids (Stachelhaus and Marhiel, 1995).

1.3.5 Purification and characterization of small proteins

Naturally occurring peptides are often present at extremely low concentrations (often at the nanomolar level), in the presence of large amounts of various proteins, nucleic acids and other small molecules. The low concentrations, together with their low molecular weights, make purification and characterization of these natural peptides can be quite challenging.

1.3.5.1 Microseparation of small proteins

High-performance liquid chromatography (HPLC) and gel electrophoresis are the two techniques which have found extensive use in both the isolation and characterization processes of proteins present at very low concentrations.

The combination of sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) with electroblotting is one of the most versatile methods for the isolation of proteins at the microgram and submicrogram level for further protein chemical analysis (Eckerskorn, 1994). This strategy has several advantages compared to other protein isolation procedures. First, the separation method offers the high resolution potential of one and two dimensional (1D and 2D) PAGE. This is advantageous for complex protein mixtures, and especially for the separation of membrane proteins. Second, the sample handling steps are minimized due to direct transfer of the separated protein onto a membrane. Then the immobilised protein could be either directly sequenced (Eckerskorn *et al.*, 1988) or subjected to amino acid composition analysis (Nakagawa and Fukuda, 1989), mass spectrometry (Strupat *et al.*, 1994) and chemical or proteolytic cleavage (Jahnen *et al.*, 1990; Patterson *et al.*, 1992). Third, proteins purified by this technique can be prepared in a short time relatively free from contamination by other proteins, amino acids and salts. Fourth, very small amounts of proteins can be handled with high yields. Nowadays it is possible to separate most of the cellular proteins by 2D gel electrophoresis and to check their amount quantitatively.

However, electrophoresis has a molecular size limit below which smaller proteins and peptides are difficult to separate. For small molecules (e.g., small proteins, peptides

or amino acids) chromatography has the best separation power, which is reflected in almost exclusive use of HPLC for peptide fractionations, peptide maps or amino acid separation.

Reversed-phase HPLC (RP-HPLC) is one of the most powerful separation techniques for either purifying or analysing peptides and small proteins. The high resolution offered by the technique is essential for separating the many very closely related species present in a typical peptide mixture, through the use of proper ion pairing agents (such as TFA), reversed-phase can be made to resolve peptides based on charge as well as hydrophobic characteristics. Both the eluent (e.g., acetonitrile) and ion pairing agents (TFA) can be volatile, simplifying sample clean-up after purification, thus making the separated products suitable for direct amino acid sequence analysis, mass spectrometry and other protein structure analysis. RP-HPLC on micro-columns provides purified fractions in small volumes suitable for microsequencing.

RP-HPLC is generally used in the final purification step following ion-exchange and/or gel filtration chromatographic procedures.

There are many small proteins which have been purified by RP-HPLC. In the beginning of the 1980s Marqardt and Todaro (1982) reported the purification of two growth factors (multiplication stimulating activity and transforming growth factor, 7500 daltons) from conditioned media of tumor-derived cells (liver and melanoma cells, respectively), by using a combination of conventional gel chromatography and RP-HPLC. At the same time Petrides *et al.* (1981) applied RP-HPLC to the final

purification of murine epidermal growth factor (6000 daltons) obtained by conventional gel chromatography and described the presence of two EGF-related molecules which could not be resolved by conventional ion-exchange chromatography. Afterwards, RP-HPLC was utilized for the partial purification of other transforming growth factors with molecular weights between 6000 and 12,000 daltons (Robert *et al.*, 1982), identified in sarcoma virus transformed non-neoplastic cells and for the complete purification of transforming growth factors from physiological fluids.

Other biologically active peptides, such as peptide antibiotics, ACTH-like neuropeptides, hypothalamic releasing factors, intestinal peptides, have also been successfully separated and purified by RP-HPLC (Hancock, 1984).

1.3.5.2 Microcharacterization of small proteins

When the purified small proteins or peptides are obtained, then they are further characterized by three main methods: mass spectrometry, amino acid sequence analysis and amino acid composition analysis.

(a). Mass spectrometry of proteins

Estimating molecular weights has always been an important aspect of protein and peptide characterization. Molecular weight measurements have been used to prove homogeneity of the sample, establish identity, analyse quaternary structure (e.g. the presence of subunits) and to detect modifications such as glycosylation or proteolysis. Most molecular mass estimates are made using SDS-PAGE and size exclusion

chromatography, calibrated with known standards. These techniques are able to give useful indications of purity, relative molecular masses and approximate amounts of material present. However, the techniques are unable to provide the mass accuracy and resolution really needed to detect mass changes i.e. those due to post-translational modifications and amino acid exchanges. Two relatively recent developments in mass spectrometry, the "soft" ionisation processes electrospray ionisation (ESI) (Yamashita and Fenn, 1984) and matrix-assisted laser desorption/ionisation (MALDI) (Karas and Hillenkamp, 1988) extend the application of mass spectrometry to proteins and other biopolymers.

The desorption/ionisation techniques use different physical approaches for the conversion of the polar, nonvolatile molecules into intact, isolated ionised molecules in the gas phase; field desorption (Berdey, 1977) applies a high electric field to the sample; in fast atom bombardment the sample is bombarded by highly energetic ions or atoms; thermospray ionisation (Blakely *et al.*, 1980) and electrospray ionisation form ions directly from small, charged liquid droplets. Matrix-assisted laser desorption/ionisation make use of short, intense pulses of laser light to induce the formation of intact gaseous ions.

Electrospray ionisation, as well as matrix assisted laser desorption/ionisation have already demonstrated their capabilities for mass spectrometric analysis of proteins in the molecular mass range between hundreds and a few hundred thousand Daltons.

(b). Amino acid sequence analysis

Current methods of protein sequence analysis fall into two categories: Edman degradation (Edman, 1950), or mass spectrometry.

Edman degradation cleaves the N-terminal amino acid from a peptide or protein backbone and prepares the derivatized residue for its identification. In this way the amino acid sequence of a protein is determined by repetitive chemical reaction. Edman degradation comprises three individual steps: the coupling, the cleavage and the conversion. Finally, a derivatised amino acid, the PTH amino acid, is produced and identified. Edman degradation can be performed manually using phenyl isothiocyanate alone or with dimethylaminobenzene isothiocyanate (Chang, 1983) or fluorescein isothiocyanate (Mutamoto *et al.*, 1978). Quantities of protein or peptide down to below 1 nmole and 20 pmole respectively can be sequenced, with a realistic length of about 20 residues. Alternatively, the Edman degradation can be performed using automated methods where sensitivities of below 10 pmole are readily achieved and 50-80 residues can be obtained from 1 nmole of starting material. In the solid-phase sequencing approach (Findlay *et al.*, 1989), the peptide or protein is covalently linked to a solid support to minimize losses during the sequence reaction and to increase chemical cleanliness. 'Gas-phase' or liquid pulse sequencers (Hewick *et al.*, 1981) have largely superseded the liquid spinning cup sequencers due to their improved sensitivity.

Use of Fast Atom Bombardment (Morris, 1980) mass spectrometry for amino acid sequence analysis has some advantages over the Edman degradation. Peptides with blocked N-termini can be sequenced, and, since information can also be obtained on

the carbohydrate structure, glycoproteins can potentially be characterized in a single step. In addition, the sequences of mixtures of up to five peptides can be unambiguously determined. To analyse the sequence of an unknown protein it is more efficient to use tandem mass spectrometry (Weigt *et al.*, 1994). By utilizing FAB in combination with tandem mass spectrometry a great number of proteins have been sequenced (Biemann and Martin, 1987) and sites of post-translational modifications, e.g. phosphorylation sites, have been identified (Labdon *et al.*, 1992).

(c). Amino acid analysis

Amino acid analysis is used to estimate the amount and to determine the composition of proteins, peptides or free amino acids. It involves two stages: complete hydrolysis of proteins and peptides, followed by quantitation and identification of the released amino acids.

Amino acid residues are small and polar compounds which are difficult to handle for most separation techniques except for ion exchange chromatography. Furthermore, these molecules possess almost no UV or fluorescence activity that would allow their detection. The specific derivatization of amino acids causes a substantial change in their chromatographical behaviour as well as in their detectability. Throughout the 1970s, developments in chromatography and new derivatization chemistries provided alternative methods of amino acid analysis. HPLC gained wide popularity for the analysis of complex organic, synthetic and biological compounds. In particular, the

reversed-phase HPLC initiated work with new amino acid derivatives, and provided an increase in speed and sensitivity (Kellner *et al.*, 1994).

1.4 MICROENZYMES BY CHEMICAL SYNTHESIS

1.4.1 The de novo-designed peptides as biocatalysts

The design and synthesis of molecules having catalytic activity and substrate specificity resembling that of natural enzymes has long been a goal of chemists (Dugas, 1989). While the number of microenzymes from natural sources are rare, chemists interested in catalysis have made a large number of attempts to select aspects of enzyme behaviour capable of being mimicked in smaller systems and have succeeded in producing catalysts with a number of enzyme-like features.

Many relatively small peptides containing the amino acid residues corresponding to the active centres of enzymes have been synthesized and studied as "hydrolase models" (Fridkin and Goren, 1974) in comparison with α-chymotrypsin and imidazole. Peptides and copolypeptides with a weak esterase activity (Trudelle, 1982) and glycosidic activity (Chakravarty *et al.*, 1973) have been reported. A strong ribonuclease activity was found for a 70-residue synthetic polypeptide analogue of ribonuclease S-protein (Gutte, 1975). And a noticeable activity has been found with a 99-residue polypeptide of an aspartic protease (Veber *et al.*, 1989). Even very simple peptides can show such activities. For example, Lys-Trp-Lys is able to recognize and to cleave DNA strands at apuric or apurinic sites (Behmoaras *et al.*, 1981; Ducker and Hart, 1982).

These studies have yielded a deeper understanding of enzyme catalysis, however, in most cases the rate enhancements obtained were many orders of magnitude lower than those observed for the naturally occurring enzymes, probably owing to the inability of the molecule to realise a conformation similar to that of the active centre of a real enzyme. The de-novo design of artificial catalysts requires the generation of protein-like molecules having the general structural and functional properties found in natural enzymes. Thus, a productive strategy can be seen in the selection-based design of well defined secondary and tertiary structures, which maintain sufficient flexibility to allow the accessibility of the functionalities required for catalysis to occur.

The increasing number of de-novo designed polypeptides with well-defined tertiary structures have been reported recently (Hill and DeGrado, 1998; Bassil and Mayo, 1997). The successful introduction of metal (Walkup and Imperiali, 1996) and cofactor (Roy and Imperiali, 1996) binding sites and porphyrin binding pockets (Rabanal, 1996) and the development of peptides capable of forming ion channels (Lear *et al.*, 1997) are but a few examples of the diversity of applications that are being approached by the design of nonnatural amino acid sequences.

Designed polypeptide catalysts with widely different catalytic mechanisms have now been reported where the relationship between structure and function is at least partly understood and that show rate enhancements of approximately 3 orders of magnitude.

Johnsson *et al.* (1993) reported the catalytic activity of a 14-mer peptide, called oxaldie-1, for the oxaloacetate decarboxylation reaction. This short, self-associating,

Leu-Lys-rich peptide accelerated the rate of decarboxylation of oxaloacetate by a factor of 10 to 100, and catalyses decarboxylation by means of a Schiff's base intermediate between substrate and an amine. Such activity was postulated to be closely related to the ability of this peptide to adopt a partial α-helical conformation in aqueous solution.

A synthetic peptide ligase has been designed which catalyses ligation reactions with rate enhancements of 4×10^3 over that of the background reaction (Severin *et al.*, 1997). The catalyst is a 33-residue amphiphilic helix that binds the reacting peptide fragments to increase the effective concentrations of electrophile and nucleophile with dissociation constants in the micromolar range.

KO-42, a designed antiparallel helix-loop-helix hairpin dimer with 42 residues in the monomer sequence, catalyses acyl-transfer reactions of *p*-nitrophenyl esters. The reactive site contains six His residues (Broo *et al.*, 1997), and the reaction mechanism proceeds via rate-limiting formation of an acyl-imidazoyl intermediate followed by attack on the acyl intermediate by the appropriate nucleophile to form the reaction products.

1.4.2 Solid phase peptide synthesis

Solid phase peptide synthesis (SPPS) (Atherton and Sheppard, 1989) is based on sequential addition of α-amino and side-chain protected amino acid residues to an insoluble polymeric support (Fig. 1.2). The acid-labile Boc (t-Butoxycarbonyl, Me_3C-

OCO-) group or base-labile Fmoc (9-Fluorenylmethoxycarbonyl) group is used for N-α-protection. After removal of this protecting group, the next protected amino acid is added using a coupling reagent and pre-activated protected amino acid derivative. The resulting peptide is attached to the resin, via a linker, through its C-terminus and may be cleaved to yield a peptide acid or amide, depending on the linking agent used. Side-chain protecting groups are often chosen so as to be cleaved simultaneously with detachment of the peptide from the resin.

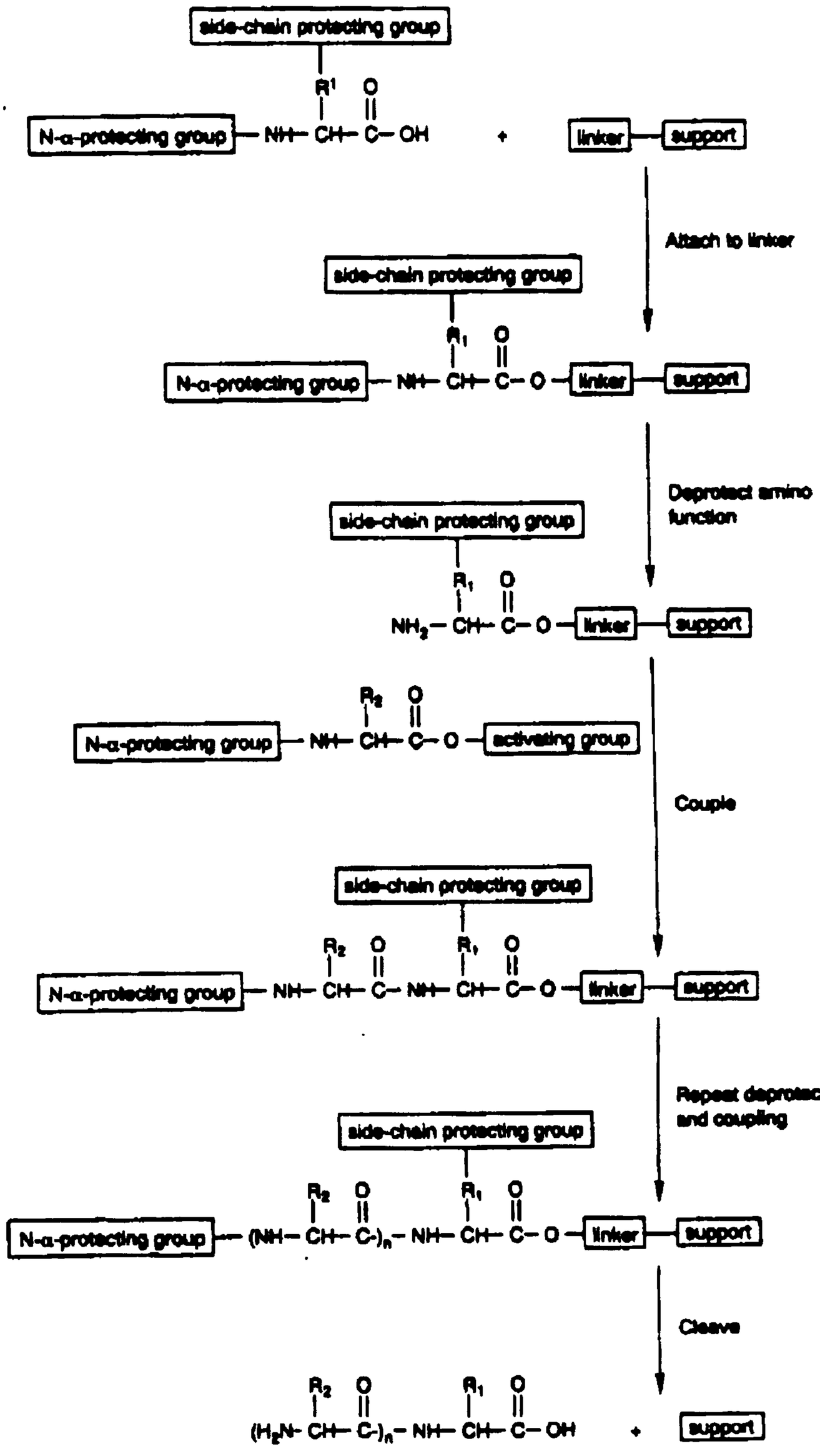


Figure 1.2 General scheme of SPPS.

(From *novabiochem Catalogue* 1998. Technical sections, Introduction and

background: 1. Solid phase peptide synthesis).

1.5 COLLAGEN PEPTIDES AND COLLAGEN RELATED PROTEINS

1.5.1 Collagens

Collagen is the major class of insoluble fibrous protein in the extracellular matrix and connective tissue. There are 19 different types of collagen known so far in vertebrates (Woessner, 1991). Collagen types I-III are the most abundant and form fibrils of similar structure (Van der Rest and Garrone, 1991). The main biomechanical function of fibrillar collagen types I, II, and III is to oppose tensile forces, due to the action of the spontaneously self-assembled fibrils and fibers in the extracellular space of connective tissues. Other collagens have specific functions in specialized areas, e.g., collagen type IV in basement membranes and type VII in anchoring fibrils (Kielty *et al.*, 1993). Collagens are also involved in biological processes such as interactions with diverse cell types (fibroblasts, chondrocytes, endothelial cells, platelets, etc.); these interactions appear to be very specific and important in cellular adhesion, migration, growth and differentiation, or even specific interactions with bacterial receptors (Visai *et al.*, 1995).

The collagen molecule is a triple-helical coiled-coil formed by the super coiling of three polypeptide chains (Figure 1.3). Each strand is in a left-handed, extended polyproline II helical conformation. Three such strands coil about a common axis in a right-handed helical fashion. Each strand is staggered by one residue from its adjacent chain and the

three strands are held together by inter-chain hydrogen bonding. The general amino acid sequence repeat of these polypeptide chains is of the form $(Gly\text{-}X\text{-}Y)_n$, in which X and Y can be any amino acid. The strict sequence constraint of Gly at every third position is required for the close packing of the three strands. The X and Y positions are frequently occupied by proline and hydroxyproline, respectively, comprising about 20% of all the residues in collagen. The high content of imino acids imposes a high degree of steric restriction which helps to stabilize the extended nature of the chains (Lodish and Darnell, 1995). Hydroxyproline results from the post-translational modification of proline by the enzyme prolyl hydroxylase, which adds a hydroxyl group to the C^{γ} atom of proline. Hydroxyproline plays a very important role in the stability of the triple helix, as compared with proline (Engel *et al.*, 1977). This can be clearly observed by melting studies on $(Gly\text{-}Pro\text{-}Pro)_{10}$ and $(Gly\text{-}Pro\text{-}Hyp)_{10}$ (Sakakibara *et al.*, 1973); while the Tm for $(Gly\text{-}Pro\text{-}Hyp)_{10}$ is about 58°C in aqueous 10% acetic acid solution, the Tm for $(Gly\text{-}Pro\text{-}Pro)_{10}$ is only 24°C. Collagen lacking hydroxyproline is unstable (Rosenbloom *et al.*, 1973), while the presence of Hyp at the Y-position has been found to enhance the stability of the assembly of collagen into microfibrils (Nemethy and Scheraga, 1986).

The unusual amino acid sequence of the collagens reflects their gene organization. For example, the gene encoding the human α1(I) chain contains 51 exons; 46 of these exons are quite small and encode portions of the triple helical segment of the collagen molecule. About half of these 46 exons consist of 54 base pairs and encode one so-called primordial unit containing six repeats of a Gly-X-Y seqeunce. Since three amino acids form one turn of the triple-stranded helix, each primordial unit corresponds to

six turns of the helix. It is currently believed that the ancestral collagen gene coded for one primordial unit and that the ancestral collagen protein was a 6 turn repeat of the Gly-X-Y sequence. It is therefore thought that fibrous collagen was evolved through multiple duplications of this primordial unit (Lodish and Darnell, 1995).

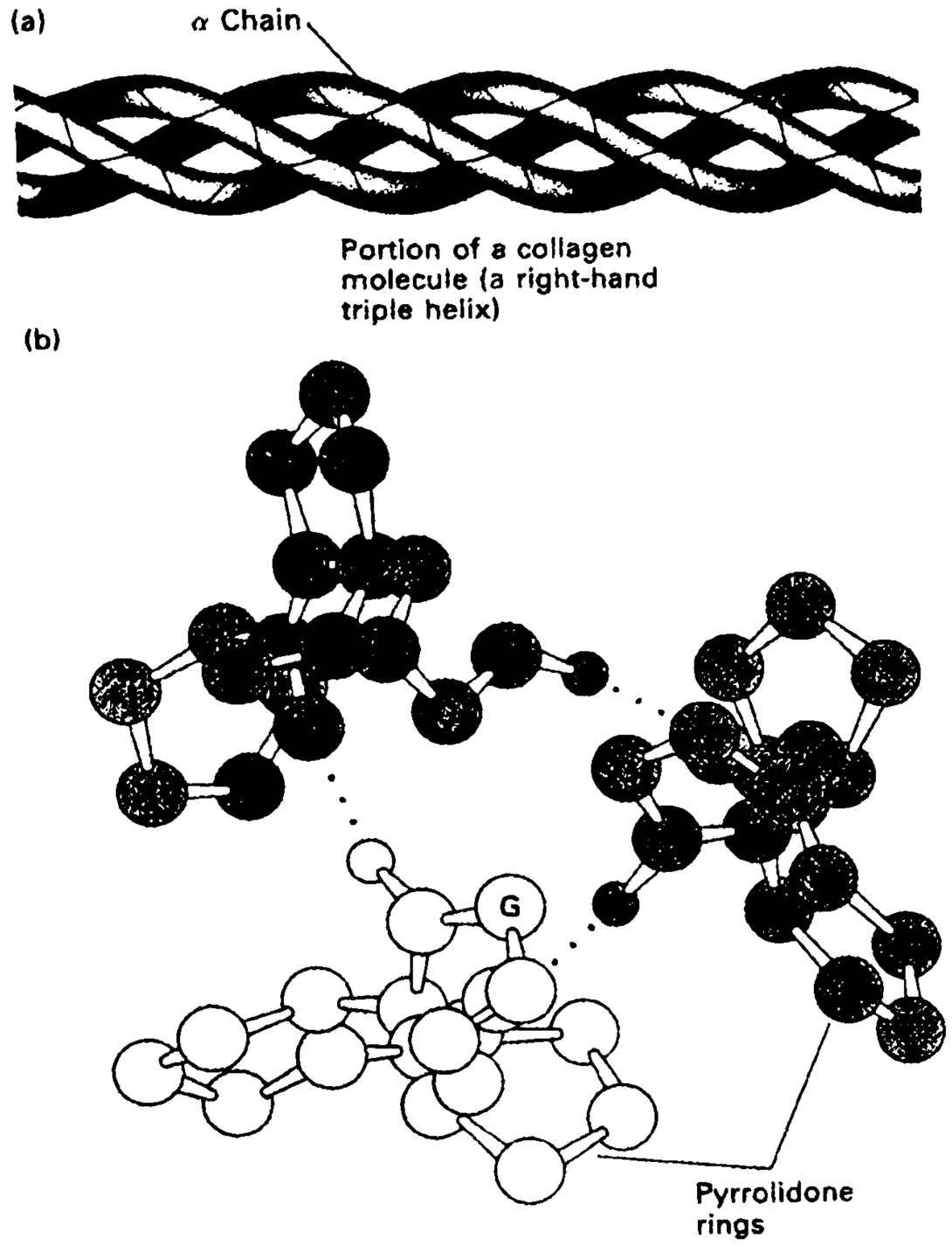


Figure 1.3 The structure of collagen.

(a) The basic structural unit is a triple-stranded helical molecule. Hydrogen bonds link residues in each chain to the other two chains, which makes the helix rigid. (b) Ball-and stick model of a collagen triple helix viewed down the helix axis. The α carbon of glycine is labled G; every third residue must be glycine because the space in the centre

of the helix will not accommodate a side-chain larger than H. The five-membered pyrrolidone rings from the proline residues are on the outside of the helix. The dots indicate the hydrogen bonds between the NH of a glycine residue on one chain with a peptide C=O residue on another (Lodish and Darnell, 1995).

1.5.2 Collagen peptides

There is renewed attention concerning studies on collagen peptides which bear either natural or model collagen-like sequences, especially those with imino acid-rich sequences such as (Pro-Pro-Gly)n or (Pro-Hyp-Gly)n. This is because of recent advances in studies on diseases due to mutations in the genes coding for collagen α chains and studies on collagen interactions with other extracellular matrix macromolecules and with cell receptors.

In the late sixties and the seventies studies were performed on collagen peptides mainly to elucidate their physicochemical properties. Examples are the studies on type I collagen CNBr peptides (Piez and Sherman, 1970; Saygin *et al.*, 1978), on imino acid-rich model peptides of differing length and composition (Engel *et al.*, 1977; Brown *et al.*, 1972). The development of solid phase peptide synthesis and the availability of powerful analytical techniques, such as high-resolution NMR, have enabled studies on model peptides which either incorporate natural sequences or bear modifications or interruptions of the repeating Gly-X-Y sequence (Fan *et al.*, 1993; Venugopal *et al.*, 1994). The crystal and molecular structure was recently determined for one of these peptides giving detailed information concerning the structural effect of a single glycine

substitution (Bella *et al.*, 1994).

At present, knowledge at the molecular level of interactions occurring between collagen and other connective tissue macromolecules and cells is insufficient. There are a few studies whereby the type I collagen binding domain for collagenase and fibronectin (Dzamba *et al.*, 1993) or cellular integrins (Staatz *et al.*, 1991) or proteoglycans like decorin (Scott and Glanville, 1993) has been investigated or postulated.

The structure of peptide species, whether single-stranded or triple-helical, is a relevant factor when considering their interactions with other extracellular matrix components or with cell receptors. In fact, when the inhibitory effect of a peptide on platelet adhesion to collagen was studied, it was found that inhibition was greater when the peptide is in a triple-helical conformation rather than when single-stranded (Santoro *et al.*, 1994). Peptide structure was also found to be an important factor in melanoma cell adhesion and spreading on a substratum of a synthetic peptide that incorporates a natural type IV collagen sequence (Fields *et al.*, 1993a).

Proper use of collagenous peptides will increasingly be of great value in collagen studies for solving the biological problems. For more than a century collagen has been known as a chemical and biological individuality, and after the seventies it became better known as a family of related individuals. However, it has still not revealed all its secrets.

1.5.3 Collagen related proteins

In addition to collagen, the triple helix is also found in a variety of other proteins like the C1q complement subunit, the collectins, the asymmetric form of acetylcholinesterase, ficolin and the macrophage scavenger receptors, all of which possess Gly-X-Y rich regions. The potential activities mediated by triple helices are quite varied. Collagenous domains of several proteins are found to bind to a wide variety of molecules. Examples include asymmetric acetylcholine esterase binding to proteoglycans (Brandan *et al.*, 1985), lung surfactant-associated apoproteins binding to lipids (Ross *et al.*, 1986), mannose-binding protein association with hydrophobic surfaces (Colley and Baenziger, 1987). Collagen can bind lipids (Frank and Fogelman, 1989) and many macromolecules (Takagi *et al.*, 1989), including fibronectin, thrombospondin, laminin, and von Willebrand factor.

It has been suggested that positive charges in the collagen domains of some molecules are involved in such interactions (Malhotra *et al.*, 1993; Krieger and Herz, 1994). The non-collagenous domains of the molecules play important roles in determining their interactions with other molecules or trimer assembly. This is true for the C1q complement subunit and the macrophage scavenger receptor.

Acetylcholinesterase inactivates acetylcholine as part of the process of cholinergic neuro-transmission (Massoulie and Bon, 1982). The asymmetric, insoluble form of acetylcholinesterase, which is concentrated at the neuromuscular junction of skeletal muscle, is composed of a tetrameric catalytic unit associated with a rod-shaped tail,

composed of three interwoven collagen-like chains (Hall, 1973; Duval *et al.*, 1992) (Figure 1.4a). The collagen-like domain of each chain is flanked by noncollagenous amino and carboxy termini containing cysteine residues thought to be important in stabilizing protein-protein interactions (Duval *et al.*, 1992). In particular, the cysteine residues in the amino terminus of the collagen-like chains of *Torpedo* acetylcholinesterase apparently play a role in its interactions with the catalytic portion of the enzyme (Duval *et al.*, 1992).

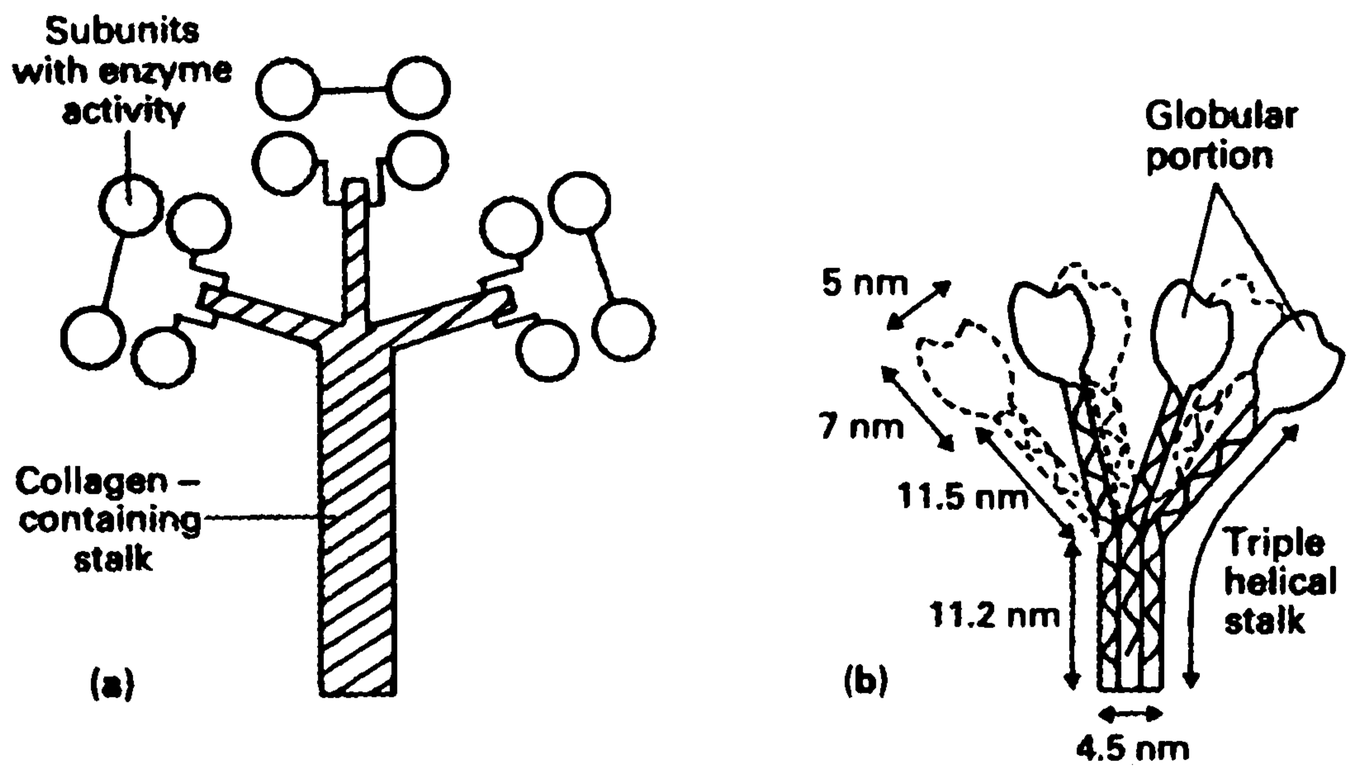


Figure 1.4 Schematic models of acetylcholinesterase and complement subcomponent C1q.

(a). Eel acetylcholinesterase. The enzyme contains a large number of catalytic subunits which are linked to a stalk built up of collagen-like structures. The circles are catalytic subunits. The cross-hatched region contains protein with a collagen-like triple helical conformation (Rosenberry and Richardson, 1977). (b). Human complement subcomponent C1q, based on electron microscopy and amino acid sequence studies of

the molecule. C1q comprises a group of six identical globular proteins each on a triple-helical collagen-like stalk (Reid and Porter, 1976).

The complement system is a multi-protein pathway involved in host defence. The classical pathway of complement is activated by the C1 enzyme which is composed of the C1r, C1s and C1q subunits. C1q consists of 18 polypeptides, six chains each of three distinct types (A, B, and C) (Reid and Porter, 1976). The amino terminus of each chain contains a collagen domain while the carboxy end forms a globular head structure (Sellar *et al.*, 1991) (Figure 1.4b). The collagen portions of one A, B and C chain of C1q form a triple helix which interacts with two C1r and C1s subunits. This binding is of importance in complement function since IgG and IgM binding to the globular head of the C1q induces a conformational change in the collagen tail. This then leads to activation of the C1s and C1r subunits (Reid and Porter, 1976).

1.5.4 Characterization of triple helical structures

The detection of triple-helical conformations of collagen, collagen model peptides or collagen related proteins has been carried out using a variety of experimental techniques. For example, x-ray diffraction and electron microscopy have been used to study the sizes and shapes of collagen fibrils as well as synthetic collagen-like polymers (Miller and Wray, 1971; Bruns and Gross, 1973). X-ray crystallography has also been employed to investigate the triple-helical packing of collagen segments and synthetic collagen analogues in the solid state (Bella *et al.*, 1994). In solution, equilibrium sedimentation and light scattering have been utilized to study molecular weight-

dependent properties, such as the formation and denaturation of triple helices (Venugopal *et al.*, 1994).

Circular dichroism spectroscopy is the most frequently used technique to study triple-helical conformations (Fields *et al.*, 1993, 1995). The natural collagen triple helix has a unique CD spectrum with a small positive peak around 220 nm, a crossover at 213 nm, and large trough around 197 nm. It has been found that polyproline II and polyproline II-like structures also exhibit CD spectra similar to that of triple helices.

Natural collagens undergo hyperchromatic changes in the uv absorbance under denaturation conditions. This effect can be observed in two regions around 190 and around 220 nm in the uv spectrum of collagen. Monitoring the uv absorbance in these two regions can lead to melting curves of collagen-like structures (Wetlaufer, 1962).

Recently, NMR spectroscopy has been used as a technique to detect triple-helical structures (Li *et al.*, 1993; Venugopal *et al.*, 1994). An NMR spectrum of a triple-helical array exhibits (a) a new set of resonances in one-dimensional NMR spectra absent in the nontriple-helical structure; (b) observable amide proton NH signals in D_2O, indicative of slow proton exchange arising on triple-helix formation; (c) interchain nuclear overhauser effects (NOEs) which are unique to the triple helix packing.

1.6 OBJECTIVES OF THE PRESENT STUDY

As indicated in Section 1.3.1, the existence of naturally occurring microenzymes has been established with the findings that some enzyme activities have been associated with proteins smaller than 10 kDa (Adoga and Mattey, 1979; Steele *et al.*, 1992; Cunningham *et al.*, 1998), but such small enzymes have not been extensively studied as among the great number of enzymes found and studied so far only a very few have been reported with MW smaller than 10 kDa. This may be because either this class of enzymes does not generally exist in organisms, or such small enzymes have been overlooked by investigators due to their properties, e. g., such microenzymes would not be observed by the classical methods for protein purification, they do not readily precipitate with ammonium sulphate, they pass through normal dialysis and ultrafiltration membranes and run together with the dye front in the routinely used SDS-PAGE.

Preliminary studies have shown esterase activities from several thermophilic microorganisms were also attributed to very small enzymes (Simões, 1997a). A 1.566 kDa thermostable extracellular esterase has been isolated and partially characterized from *Bacillus stearothermophilus* (Simoes *et al.*, 1997). The present study aimed at further investigating the extracellular esterase activities observed in some thermophilic fungi, screening and identifying the esterases smaller than 10 kDa, purifying and characterizing these microenzyme esterases, and then chemically synthesizing the esterase peptide and attempting to produce the active enzyme from the synthetic peptide.

The more specific objectives of this work were as follows:

- To investigate extracellular esterase activities in thermophilic fungi.

- To examine esterase activities in less than 10 kDa fraction.

- To screen thermophilic fungi producing microenzyme esterases.

- To identify and isolate the microenzymes by ultrafiltration and gel filtration.

- To optimise RP-HPLC procedures and purify the microenzymes to homogeneity.

- To examine the purities of the esterases by RP-HPLC, N-terminal amino acid analysis and SDS-PAGE.

- To characterize the microenzymes by structure analyses, including molecular weight determination by gel filtration, SDS-PAGE, and mass spectrometry; amino acid composition analysis by dansylation of hydrolysed amino acids; sequence analysis by Edman degradation; secondary structure determination by CD spectroscopy; qualitative element identification by X-ray microanalysis.

- To characterize the microenzymes by enzyme activity analyses, including catalytic activity analysis, thermostability, pH stability, optimum pH, specificity

determinations.

- To chemically synthesize the microenzyme peptide by solid-phase peptide synthesis and attempt to produce active esterase from the synthetic peptide.

CHAPTER 2. MATERIALS AND METHODS

2.1 MEDIA

2.1.1 Malt extract agar (Oxoid)

	g/l distilled water	
Malt extract	30.0	
Peptone	5.0	
Agar	15.0	pH 5.4

2.1.2 Czapek agar

	per litre distilled water
Sucrose	200.0 g
Yeast extract	5.0 g
KH_2PO_4	1.0 g
Cz concentrate	10.0 ml
Agar	15.0 g

(Cz concentrate: g/l distilled water

$NaNO_3$	300.0
KCl	50.0
$MgSO_4 \cdot 7\ H_2O$	50.0
$FeSO_4 \cdot 7\ H_2O$	1.0)

2.1.3 Malt extract broth

	g/l distilled water	
Malt extract	30.0	
Peptone	5.0	pH 5.5

2.1.4 Malt extract fermentation medium

	per litre distilled water	
Malt extract	30.0 g	
Peptone	5.0 g	
Tributyrin	4.0 ml	pH 5.5

2.1.5 Synthetic fermentation medium

	per litre distilled water
Sucrose	30.0 g
Urea	1.0 g
$NaNO_3$	3.0 g
K_2HPO_4	1.0 g
KCl	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$FeSO_4 \cdot 7H_2O$	0.01 g
Tributyrin	4.0 ml

2.1.6 Tributyrin agar medium

0.4% tributyrin was emulsified with the Malt extract agar or Czapek agar using a top-drive homogeniser at maximum speed for 5 min.

2.2 FUNGAL STRAINS

Stock cultures of nine thermophilic fungi obtained from the University of Strathclyde culture collection were grown at their optimum temperature on the appropriate agar medium slants (Table 2.1) and stored at 4°C.

Table 2.1 Thermophilic fungi tested and their growth conditions

Microorganisms	Optimum temperature (°C)	Medium
Absidia corymbifera	45	Malt extract
Chaetomium thermophilum	45	Malt extract
Emericella nidulans	45	Malt extract
Eurotium chevalieri	37	Czapek
Humicola lanuginose	37	Czapek
Rhizomucor pusillus	45	Malt extract
Talaromyces emersonii	45	Malt extract
Therandascos aurantiacus	45	Malt extract
Thermoascus crustaceus	45	Malt extract

2.3 CULTURE CONDITIONS

Stock cultures of the nine thermophilic fungi were plated out individually on tributyrin agar medium and incubated at their optimum temperature to screen for the production of extracellular esterase. Two days later, colonies exhibiting large clear zones were inoculated into 250 ml Erlenmeyer flasks containing 25 ml of malt extract broth and shaken at 100 rpm for 2 days at optimum temperature. For esterase production, a 5-ml sample of seed culture was used to inoculate 500 ml of malt extract fermentation medium which was grown at optimum temperature with aeration in a 1 l BIOTEC FE700 fermenter.

10 ml of culture was withdrawn daily. Fungal cells and excess tributyrin were removed from the medium by filtration through glass microfibre filter (Whatman GF/C). Growth was estimated as dry weight of mycelium by being dried to constant weight at 70 °C on the preweighed membrane filter, and 1 ml of cell free medium was used to determine the esterase activity.

At a later stage, to simplify the purification procedure, the synthetic medium was used instead of the malt extract fermentation medium, while other conditions were kept unchanged.

2.4 SCREENING OF ESTERASES WITH MOLECULAR WEIGHT LESS THAN 10 KDA

After removing fungal cells, the cell-free medium with esterase activity was then passed through a MICROSEP concentrator (Filtron Technology Corporation) containing an OMEGA^{TM} membrane with a nominal molecular weight cut-off 10 kDa.

Microsep is a device in which centrifugation provides the driving force for filtration (Figure 2.1). The device (cut-off 10 kDa) retains molecules bigger than 10 kDa while solvent and low molecular weight molecules pass through the membrane into the filtrate reservoir. Besides its wide application in concentrating molecules with molecular weight more than 10 kDa, MICROSEP microconcentrator can also be used in isolating low molecular weight compounds from fermentation broths for natural product screening.

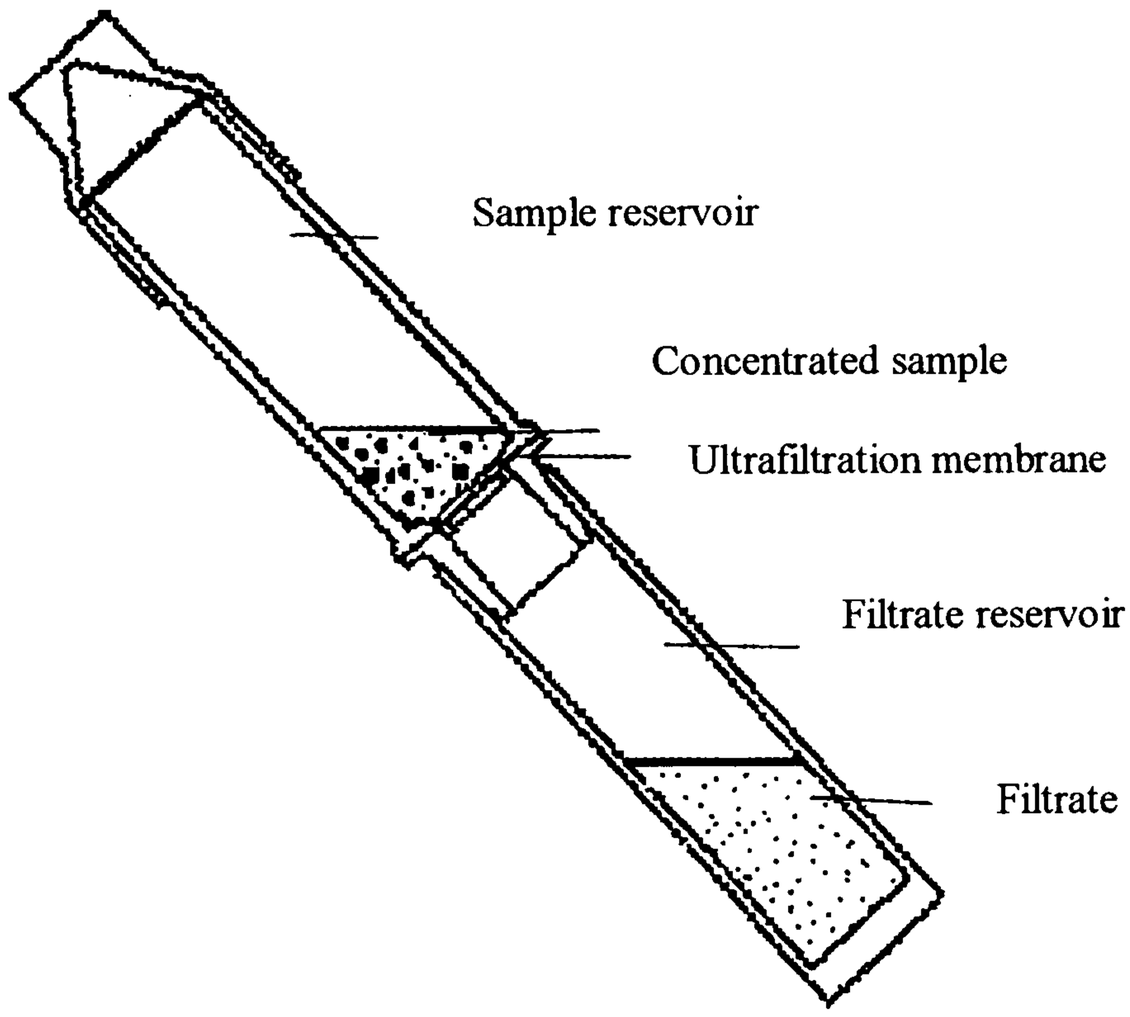


Figure 2.1 Diagram of the Microsep centrifugal concentrator.

3 ml of medium from each organism was filtered at 6000 rpm until 2 ml of filtrate was obtained, then 1 ml of filtered and retained fractions were taken to measure the esterase activity in the <10 kDa and > 10 kDa fractions respectively.

2.5 POLYACRYLAMIDE GEL ELECTROPHORESIS

2.5.1 Native-PAGE

Native PAGE was carried out to observe whether a protein was associated with the esterase activity. Samples were desalted by the MICROSEP microconcentrators (MW cut-off 1 kDa) and loaded two identical gels which were run simultaneously in the absence of SDS. Electrophoresis was carried out on a vertical slab gel apparatus (Mini-PROTEIN II Cell, Bio-Rad Laboratories Ltd.). After electrophoresis, one of the gels was developed for esterase activity using α-naphthyl acetate as substrate (Nachlas and Seligman, 1949), while the other gel was stained for protein by Coomassie Blue or Silver Staining. Results were analysed by comparing the relative mobility (Rf) of the bands (Segel, 1976) observed in both gels, where:

$$Rf = \frac{\text{Distance migrated by protein}}{\text{Distance migrated by dye}}$$

The buffer system of Laemmli (1970) with a 12% separating gel was initially used and the protein bands were stained with Coomassie Blue by placing the gel in fixer (50% v/v methanol, 10% v/v acetic acid, 40% v/v water) for 30 min, transferring to the

staining solution (0.025% w/v Coomassie Blue G250 in 10% v/v acetic acid) for 1 h and then destaining in 10% v/v acetic acid as quickly as possible.

In order to separate small proteins with MWs <10 kDa, Schägger and Von Jagow (1987) discontinuous Tricine-PAGE system with a 16.5% T (total monomer concentration of acrylamide and bisacrylamide), 6% C (concentration of crosslinking monomer relative to the total monomer) separating gel was used. The desalted samples were freeze-dried to concentrate, re-suspended in 50 µl 50 mM tris buffer (pH 6.8) and loaded onto the gels. Proteins were visualized with the Sigma Silver Stain Kit (AG-5) for Polyacrylamide Gels, following the procedures provided in the Sigma Technical Bulletin No. P 3040.

For the esterase activity stain, the staining solution was prepared by dissolving 0.2 g Fast Blue RR salt in 2 ml 50% acetone containing 20 mg α-naphthyl acetate and mixing with 100 ml 0.2 M Tris-acid maleate (pH 6.4). The gel was incubated in the stain for 15-30 min at room temperature on a water bath with gentle shaking and then washed with distilled water. Brownish to red bands developed where esterase activity was present.

2.5.2 SDS-PAGE

The desalted and concentrated samples were incubated at 100 °C for 3 min in 50 mM Tris-HCl buffer (pH 6.8) with 4% SDS and 2% (v/v) 2-mercaptoethanol, and the electrophoresis was run in the presence of SDS. The molecular masses of the esterases

were estimated by the buffer system of Schägger and Von Jagow (1987) discontinuous Tricine-SDS-PAGE with 16.5% T and 6% C acrylamide slab gel. The peptide markers (BDH Calibration Kit) contained myoglobin (16.9 kDa), myoglobin I+II (14.4 kDa), myoglobin I+III (10.7 kDa), myoglobin I (8.2 kDa), myoglobin II (6.2 kDa), myoglobin III (2.5 kDa). Proteins were revealed by the silver stain.

2.6 ENZYME ASSAY

A fluorescent assay (Guilbault and Kramer 1964, Kramer and Guilbault 1963) was used to measure esterase activity. Fluorescein dibutyrate was dissolved in 2-methoxyethanol at a concentration of 5×10^{-4} M. 30 µl of this substrate solution was added to 3 ml of 0.1 M Tris-HCl buffer (pH 8.0) giving a final concentration of 5×10^{-6} M. The measurement was done at room temperature in a Perkin-Elmer 3000 Fluorescence Spectrometer set to 470 nm and 520 nm for excitation and emission wavelengths respectively. Controls not containing enzyme were used to measure the non-catalysed rate. One unit of enzyme activity was defined as the amount of the enzyme which produced a change of one fluorescence unit per minute (u/min) after subtracting the non-catalysed rate.

2.7 DETERMINATION OF PROTEIN CONCENTRATION

The protein concentration was determined by the method of Bradford (1976), using lysozyme as standard.

The fractions from gel filtration were monitored by measuring UV absorption at 280 nm using a Shimadzu uv-visible recording spectrophotometer UV-160.

The protein concentrations of the purified esterases and the synthetic peptide were estimated by comparing eluted peak (from reverse-phase HPLC) areas monitored at 214 nm with peak areas of standard protein insulin (Buck *et al.*, 1989; Ståhl *et al.*, 1998).

2.8 PURIFICATION OF THE ESTERASE MICROENZYMES

2.8.1 Separation by a tangential flow filter

After removing fungal cells and excess tributyrin, large-scale esterase preparation was performed by filtration of the fermentation medium through a tangential flow filter (Mini-ultrasette, Filtron Technology Corporation) with a MW cut-off 10 kDa for 20 minutes at a flow rate of 220 ml/min and at a pressure of 0.7 bar (Simões 1997a). The filtrate was collected, freeze-dried to concentrate and stored at –20 °C for further purification.

2.8.2 Purification by gel filtration

The freeze-dried esterase was dissolved in 0.025 M Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl and 1 ml of the sample was loaded onto a Bio-Gel P-10 (Bio-Rad Laboratories Ltd., fractionation range of 1.5-20 kDa) column (1.1 x 91 cm) for gel filtration. The column was pre-equilibrated with 0.025 M Tris-HCl buffer (pH

8.0) containing 0.2 M NaCl, and eluted with the same buffer at a flow rate of 0.125 ml/min at room temperature. 1.1 ml fractions were collected and all the fractions were analysed for esterase activity and protein content.

Before the purification, the column was calibrated using blue dextran (2,000 kDa) for the void volume (V_0) and potassium ferricyanide (0.3 kDa) for total available volume (V_t) determination.

For native MW determination, the column was calibrated with reference proteins, which were cytochrome C (12.4 kDa); aprotinin (6.5 kDa); vitamin B12 (1.35 kDa).

2.8.3 Purification by reverse-phase HPLC

The HPLC equipment used was a Gilson HPLC system which contained a controller pump (305), a slave pump (306) and a 119 UV/VIS detector. The results were analysed by a Gilson 715 controller software, version 2.1.

Fractions which showed esterase activity from Bio-Gel P-10 were pooled, freeze-dried and further purified by HPLC using a Nucleosil C-18 reverse-phase column (10 μ, 10 Å, 250 x 4.6 mm, Phenomenex) equilibrated with 0.1% TFA (trifluoroacetic acid) in water (solvent A), and eluted with 0.1% TFA in acetonitrile (solvent B). The column was developed at 1 ml/min and peaks were monitored at 214 nm. Esterase activity was examined after evaporating the collected fractions in a Savant Speed-Vac concentrator (SC210A) to remove volatile acetonitrile and TFA, which interfered with the

fluorescent assay.

The purified esterases from HPLC were collected, pooled, concentrated either by freeze drying or by evaporation in the Savant Speed-Vac concentrator and stored at –20 °C for further characterization.

2.9 CHARACTERIZATION OF THE ESTERASE MICROENZYMES

2.9.1 Electrospray and MALDI mass spectrometry analysis

The molecular weights of the esterase were confirmed by electrospray and MALDI mass spectrometry at the Michael Barber Centre in University of Manchester Institute of Science and Technology. The concentrations of samples for mass spectrometry analysis were: E40, 15 μM; T40, 3 μM; E32, 4 μM.

A Micromass Quattro triple quadrupole mass spectrometer was used for electrospray mass spectrometry. Samples in 15 μl H_2O were dissolved in 20 μl 80% (v/v) aqueous acetonitrile with 0.1% (v/v) formic acid and were infused to the source at 5 μl/min. 8 scans were averaged to produce the final spectra.

For MALDI mass spectrometry, mass spectra were obtained using a Voyager Elite mass spectrometer (PerSeptive Biosystems) equipped with an N_2 laser (337 nm), and positive-ion mass spectra were acquired using reflectron mode. The matrix used was α-cyano-4-hydroxy cinnamic acid (Aldrich) and the accelerating voltage was 24 kV.

100 to 200 scans were averaged to produce the final spectra.

2.9.2 Amino acid composition analysis

The amino acid composition analysis was performed by total acid hydrolysis, followed by dansylation of the amino acids and separation and identification of dansylated amino acids by reverse phase HPLC.

(a). Hydrolysis

Freeze-dried esterase samples (E40 and E32) were re-suspended in 0.5 ml of 6 N HCl (constant boiling) containing 0.1% phenol and transferred to dried Pyrex glass test tubes, which were then flushed gently with N_2 until no further frothing and the tubes were sealed quickly. Hydrolysis was carried out for 16 hours at 105 °C using a heating block.

After hydrolysis, the samples were dried down thoroughly *in vacuo* on a Savant Speed-Vac concentrator to remove HCl and 0.5 ml of distilled water was added and evaporation repeated. It is important to remove residual acid as dansylation will be inefficient if the pH is below 7.5.

(b). Dansylation

The Dansyl derivatization procedures were based on the methods of Gray (1972).

Before the dansylation, the efficiency and reliability of dansylation conditions were examined by derivatizing standard amino acids (125 nmol amino acid in 0.25 ml 0.2 M $NaHCO_3$ and 0.25 ml Dns-Cl reagent) and separating and identifying the dansylated amino acids (by comparing with standard Dansyl amino acids) by HPLC.

0.3 ml of 0.2 M $NaHCO_3$ was added to the hydrolysate of the sample, and the pH was examined. When the pH was > 8.5, dansylation was performed (if pH <8.5, samples were dried down again and then dissolved in 0.3 ml of distilled water). 1 vol. of dansyl chloride reagent (5 mg/ml in acetone, stored in the dark in a refrigerator) and 1 vol. of water were mixed to give the dansyl chloride working solution. 0.3 ml of dansyl chloride working solution was then added into the hydrolysate. The tubes were then sealed with Parafilm and incubated at 37 °C for 1 hour. During this time, most of the excess reagent was hydrolysed to DNS-OH, and the solution became colorless or pale yellow. The dansylated samples were then analysed directly by reverse-phase HPLC.

(c). Separation of the dansyl derivatives

The Dns-derivatized total hydrolysates were separated on a Nucliosil C18 (10 μ, 100 Å, 250 x 4.6 mm, Phenomenex) reverse phase HPLC column. The chromatographic conditions were: solvent A, 50 mM sodium acetate, pH 6.5; solvent B, 80% methanol-20% 50 mM sodium acetate, pH 6.5; linear gradient from 10% B to 100% B for 50 min was developed; flow rate, 1 ml/min; detection, 254 nm; injection volume, 20 μl.

These conditions were set up by testing the separation of standard Dns-amino acid mixture and identifying individual dansyl amino acid before analysing esterase samples.

2.9.3 N-terminal amino acid analysis

N-terminal amino acids of esterase samples were analysed by the method of Gray for peptides (Gray, 1972).

200 µl of 0.2 M $NaHCO_3$ was added into dried peptides and 200 µl of dansyl chloride reagent (2.5 mg/ml in acetone) was then added. The tubes were sealed with Parafilm and were incubated for 1 h at 37 °C. After being dried down under vacuum, the dansylated materials were hydrolysed with 0.3 ml of 6 M HCl in sealed tubes at 105 °C for 16 hours. After the removal of acid *in vacuo,* the residues were dissolved in 200 µl of solvent A (50 mM sodium acetate, pH 6.5) for HPLC injection. The chromatographic conditions were similar to those of amino acid composition analysis in Section 2.9.2 except a 55-min linear gradients from 10-100% B over 50 min followed by 100-10% B for 5 min.

2.9.4 Sequence analysis

Amino acid sequence analysis was performed at the Protein Facility in University of Aberdeen using the Edman degradation method.

Samples for sequencing (E40, 15 µM; T40, 3 µM; E32, 4 µM) were spotted onto a glass-fibre filter which had been treated with 1.5 mg polybrene sample carrier and

precycled. Automated sequencing was carried out on one of two Applied Biosystems model 477A sequencing instruments, each equipped with on-line PTH amino acid analysis monitoring at 269 nm. Sequencing programs used were specifically designed in-house for use with either blotted samples or solution-applied samples, incorporating fast cycle times (< 27 minutes), pulsed-liquid extraction and pressurized cleavage.

2.9.5 Circular dichroism analysis

CD spectra were recorded on a JASCO J-600 spectropolarimeter at the Scottish Circular Dichroism Facility in University of Stirling.

The samples (E40, 3.6 μg; T40, 5.1 μg; E32, 5.07 μg; synthetic peptide, 105 μg) were dissolved in 100 μl ethylene glycol/H_2O (EG/H_2O, v/v, 2:1) and the solutions were left to equilibrate at 4 °C overnight before CD analysis. Spectra were obtained at 20 °C using a 0.05 cm path length cell by signal-averaging 3 scans from 190 to 260 nm at a scan speed of 10 nm/min.

For the CD analysis of the effects of metal ions on conformation of the synthetic peptide, the synthetic peptide in EG/H_2O was mixed with 1 equivalent of $ZnCl_2$, $AlK(SO_4)_2 \cdot 12\ H_2O$, or $FeCl_3$ solutions (EG/H_2O). The mixtures were then equilibrated and analysed as mentioned above.

2.9.6 X-ray microanalysis

Qualitative element identification of the esterases was performed by JSM-840A

Scanning Microscope (JEOL) with Tracor X-ray microanalysis at Department of Bioengineering Unit in University of Strathclyde.

5 µl of the samples (E40, 15 µM; T40, 3 µM; E32, 4 µM) was applied to the analysis.

2.9.7 Effect of substrate concentration

The dependence of the hydrolysis rate of the esterase on the concentration of fluorescein dibutyrate was carried out at pH 8.0 and at room temperature. The activity of the esterase solution (0.5 ml, 15 µg/ml) was determined against increasing concentrations of the substrate (0 to 7 x 10^{-5} M). Values of Km and Vmax were determined using a Lineweaver-Burk plot (Segel, 1976).

The plot of fluorescence units against concentration of fluorescein was obtained by preparing a standard solution of fluorescein (15 µg/ml) and determining fluorescence units against increasing volume of the fluorescein solution. One unit change in fluorescence related to fluorescein produced was then calculated from the linear correlated plot.

2.9.8 Determination of thermostability

The thermostability of the esterases was examined by incubating each 1 ml enzyme (15 µg) solution in a sealed glass tube at a range of temperatures between 30 °C and 140 °C in a heating block for 2 hours. Three tubes were incubated at each temperature.

After the incubation, the tubes were removed, cooled down at room temperature

and kept at –20 °C. At the end of the incubation period, samples were assayed for the residual enzyme activity by using fluorometric assay under standard conditions. Activity values were expressed as a percentage of the maximum activity (100%) at 110 °C.

2.9.9 Determination of pH stability

The pH stability of the enzyme activity was examined by incubating the enzymes (15 μg) in 1 ml of buffers of different pH values at room temperature for 2 hours. The buffers utilized were 0.1 M citric acid-Na_2HPO_4 (pH 2.6-7.6), 0.1 M tris-HCl (pH 8.0-9.0) and 0.1 M Na_2HPO_4-NaOH (pH 11.0-12.0). Triplicate aliquots of 0.2 ml at each pH was taken and assayed for enzyme activity using the fluoremetric assay under standard conditions. Activity values were expressed as a percentage of the maximum activity (100%) at the corresponding pH.

2.9.10 Determination of optimum pH

The optimum pH of the enzyme activity was examined by mixing the enzyme (15 μg) with 3 ml different pH buffers, which were same as the buffers used in Section 2.9.9, and equilibrating the solutions for 2 hours at room temperature. Then, the fluorimetric assay analysis was performed immediately after adding 30 μl of fluorescein dibutyrate solution (prepared as described in Section 2.6). The experiment was carried out in triplicate. Blanks which contained different pH buffers without enzyme were used to measure the non-catalysed rate. Activity values were expressed as percentage of the

maximum activity (100%) at the corresponding pH.

2.9.11 Positional specificity

The positional specificity of the esterases was determined using mixed substituted triglyceride 1,3-dipalmitoyl-2-oleoylglycerol (C16:0/C18:1, cis-9/C16:0) as substrate. The released fatty acids were derivatized into their methyl esters which were subsequently analysed by gas liquid chromatography (GLC).

(a) Digestion of triacylglycerol substrate by esterases

10 mg substrate was added to 4 ml 0.25 M Tris-HCl buffer (pH 8.2) containing 1% gum arabic and 0.25 ml of 0.1 M $CaCl_2$, the aqueous mixture was warmed until the substrate melted and then was emulsified with a Branson Sonifier. 2 ml of the substrate and 2 ml of 0.25 M Tris-HCl buffer (pH 8.2) were added to the freeze-dried enzyme (15 μg) and the digestion was carried out at 37 °C for 2 h in a shaking water bath. The reaction was terminated by the addition of 5 ml diethyl ether.

A commercial lipase (from *Candida rugosa*, Sigma) was used as a control following the same procedures as the esterase microenzyme.

(b) Extraction and derivatization of the fatty acids

The digestion products were extracted three times with 5 ml diethyl ether by repeated centrifugation and phase separation. The extracts were combined and evaporated to

dryness in a small tube in a stream of nitrogen. The residue was then dissolved in 2.5 ml ether and 0.25 g anhydrous sodium sulphate was added to dry the solution. The solution was shaken well and allowed to stand for a few minutes. The supernatant was decanted into a screw-capped tube and evaporated to dryness by warming in a stream of nitrogen.

0.5 ml BF_3-MeOH (boron trifluoride-methanol) reagent (Sigma) was added to the residue and the tube was capped and placed in a boiling water bath for 30 min. At the end of this time, the tube was cooled, 0.5 ml water and 0.5 ml hexane were then added, shaken, centrifuged. The upper phases which contained the methyl esters of the fatty acids were collected and the volume of the methyl ester solution was reduced by evaporation in a stream of nitrogen.

(c) GLC analysis of the methyl esters of the fatty acids

Fatty acid methyl esters were analysed by GLC (CARLO ERBA Strumentazione 4200) using a 30 m x 0.25 mm (df = 0.25 μm) fused silica CP-Wax 52 CB column (CHROMPACK) and a flame ionisation detector. The carrier gas was helium, at a constant flow of 1 ml/min. The column was programmed from 190 °C (5 min) to 240 °C at 8 °C/min, and then held there for 3 min.

1 μl of the prepared sample solutions was introduced onto the column and peaks were identified by compared with standard methyl esters. Palmitic acid methyl ester and

oleic acid methyl ester (Sigma) at a stock solution of 10 mg/ml in hexane were used as the standards.

2.9.12 Substrate specificity on different carbon chain length

The chain length specificity of the esterase was determined (in triplicate) by the fluorescent assay to measure the enzyme activity against different fluorescein ester substrates at a concentration of 5×10^{-4}. The substrates used were fluorescein dipropionate, fluorescein dibutyrate, fluorescein dicaproate, fluorescein dicaprylate, fluorescein dilaurate. Activity values were expressed as the percentage of the maximum activity (100%) at the corresponding substrate.

2.9.13 Determination of the effect of dialysis on enzyme activity

The effect of dialysis on enzyme activity was determined using a Spectra/Por 7 molecular porous dialysis membrane of 1 kDa cut off (Medicell International Ltd.). 1 ml of the esterase solution was dialyzed against 400 ml of distilled water, 0.025 mM EDTA or a 2% suspension of iminodiacetic acid chelating resin (Chelex 100, Sigma) for 24 h at 4 °C. The dialyzed esterase solution was then freeze-dried and re-dissolved in 1 ml distilled water. The residual activity was determined by the fluorescent assay. 1 ml of the un-dialyzed enzyme was used as a control. Activity values were expressed as the percentage of the control (100%).

In addition, the water that surrounded the dialysis tube was freeze dried and mixed with the enzyme after dialysis to observe if the activity could be restored.

To test the possibility that the loss of activity on dialysis was due to the removal of dialyzable metal ions from the enzyme, metal ions (Zn^{2+}, Al^{3+}, Fe^{3+}, Fe^{2+}, Ca^{2+}, Mn^{2+}) at a final concentration of 3.75 mM were added to the dialyzed enzyme and equilibrated at room temperature for 1 h. The restoration of enzyme activity was then measured by the fluorometric assay.

2.10 SOLID-PHASE SYNTHESIS OF THE ESTERASE PEPTIDE

The esterase peptide was synthesized using standard solid phase peptide synthesis procedures (Figure 1.2) by a NovaSyn Crystal solid phase peptide synthesizer at Department of Pure and Applied Chemistry in University of Strathclyde.

The synthesis was performed on a NovaSyn PA 500 resin (0.37 mmol/g loading) using Fmoc protecting group strategies (Figure 2.2) and benzo-triazol-1-yloxytris(pyrrolidino)phosphonium hexa-fluorophosphate (PyBOP) coupling protocols.

0.5 g Fmoc-Gly-NovaSyn PA 500 resin was swelled in DMF (N, N-Dimethylformamide) for one hour and loaded into the column. The required peptide sequence was entered and the following parameters were set:

Coupling: Each amino acid was double coupled, Fmoc-Gly-OH and Fmoc-Pro-OH in a 5 fold excess while Fmoc-Hyp(tBu)-OH in a 2.5 fold excess to the resin. PyBOP (with 1 equivalent to the relative amino acid) and HOBt (N-Hydroxybenzotriazole) were used as coupling reagents and DIPEA (diisopropylethylamine) as the base;

Deprotection: 20% piperidine in DMF;

Column temperature: 30 °C;

Recirculation time: 40 min;

Deprotection time: 15 min.

After the synthesis was completed, the resin was removed from the reactor column and sequentially washed with DMF, t-amyl alcohol, acetic acid, t-amyl alcohol, dichloromethane and ether. The Fmoc resin cleavage and deprotection was carried out with 95% TFA (Figure 2.3). The solution was then completely evaporated and ether was introduced to form a precipitate. The product was collected by centrifugation and dried under high vacuum for 3 hours.

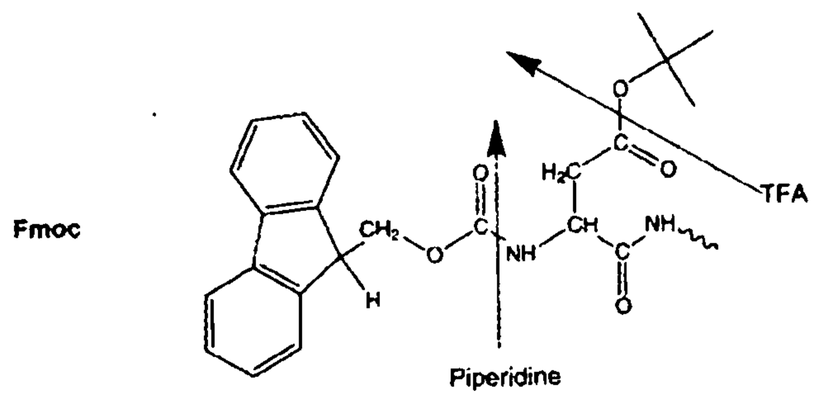


Figure 2.2 Fmoc protecting group strategies in SPPS.

(From *novabiochem Catalogue* 2000. Technical sections, Introduction and background: 1. Solid phase peptide synthesis).

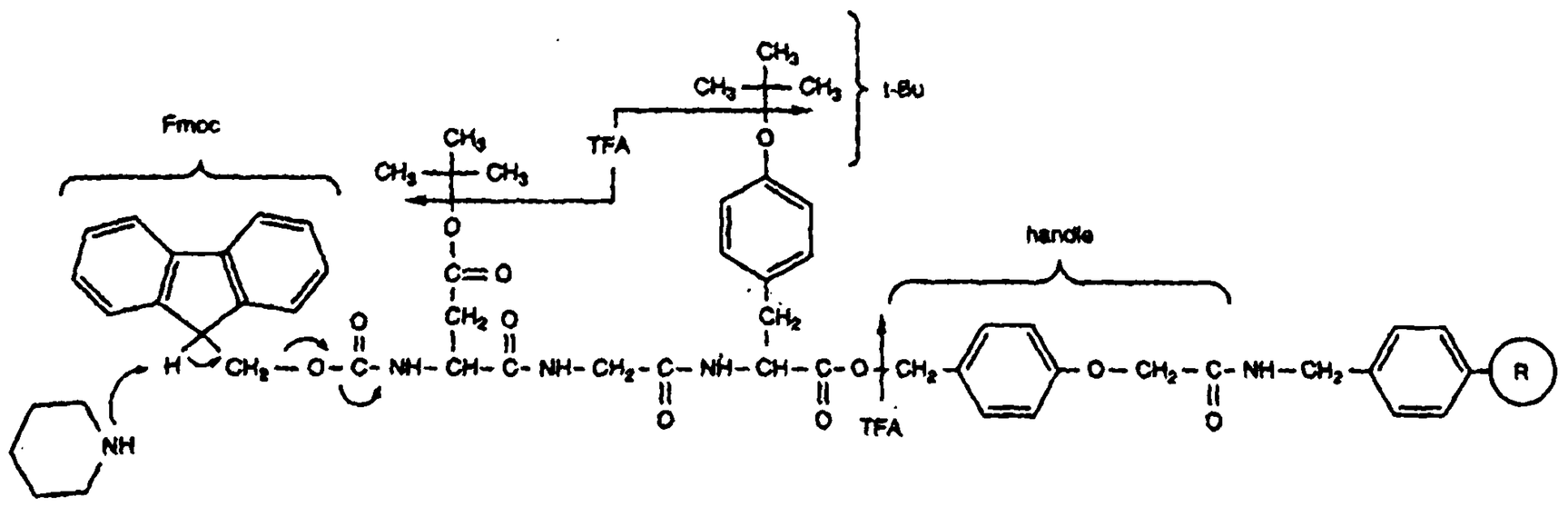


Figure 2.3 General protecting group and cleavage strategy for Fmoc chemistry. (From *novabiochem Catalogue* 1998. Technical sections, Introduction and background: 1. Solid phase peptide synthesis).

2.11 DETERMINATION OF ESTERASE ACTIVITY OF THE SYNTHETIC PEPTIDE

The esterase activity of the synthetic peptide was examined by preparing a stock solution of 340 μg/ml synthetic peptide in distilled water and determining the activity over a concentration range of 34~340 μg/ml using the fluorescent assay.

To investigate the effects of metal ions on the restoration of the enzyme activity of the synthetic peptide, 500 μl of 0.5mM synthetic peptide was incubated respectively with 500, 50 and 5 μl of 5 mM $ZnCl_2$, $AlK(SO_4)_2 \cdot 12H_2O$ and $FeCl_3$ aqueous solution for

2 h at room temperature. To reach equilibrium for triple helix formation, the mixtures were incubated for a longer time (68 h) at 4 °C. Activity was then measured by the fluorescent assay. Each concentration of different metal ions not containing the synthetic peptide was used as respective blank for the non-catalysed rate. Activity values were expressed as percentage of the native enzyme E40 (100%).

CHAPTER 3. RESULTS

3.1 SCREENING AND IDENTIFICATION OF MICROENZYMES WITH ESTERASE ACTIVITIES FROM THERMOPHILIC FUNGI

3.1.1 Extracellular esterase activities in thermophilic fungi

Nine thermophilic fungi were preliminarily examined for their extracellular esterase production by observing the formation of clear zones on the tributyrin agar plates. Six of them showed large clear zones: *A. corymbifera, E. nidulans, E. chevalieri, H. lanuginosa, R. pusillus, T. emersonii.* (Table 3.1).

The six esterase-producing organisms were selected and grown in malt extract broth with 0.4% tributyrin as an inducer: all the six strains exhibited extracellular esterase activities and the activities were growth related. Figure 3.1 and Figure 3.2 shows the cell growth and esterase production curves of these thermophilic fungi. It can be seen that

1). The esterase activities in five strains (*R. pusillus, E. nidulans, H. lanuginosa, A. corymbifera,* and *E. chevalieri)* were low during the early stage of growth and attained a maximum at the stationary phase, at different times between three and six days of incubation, except in *T. emersonii* where the highest activity was in the exponential growth phase and then decreased with fast growth of the organism;

2). *E.chevalieri* showed highest esterase production and high biomass;

3). *T. emersonii* gave highest biomass and most rapid growth, which might be related to the low esterase activity observed in this organism.

The fermentation media of six organisms were collected when the esterase activities reached their maximum. After removing fungal cells, the media were separated into two fractions with the Microsep microconcentrators. The Microsep filter is designed to retain materials of molecular weight greater than 10 kDa, and retained over 99% of an aqueous solution of cytochrome C (MW 12.4 kDa) under the conditions used.

Esterase activities were then examined in both retained (>10 kDa) and filtrate (<10 kDa) fractions. It was found that in the retained fraction, only *E. chevalieri* showed high esterase activity. The esterase activities of other five strains were found almost entirely in less than 10 kDa fractions (Figure 3.3). Esterase activities in less than 10 kDa were different among six organisms: *E. nidulans* exhibited the highest and *E.chevalieri* gave the lowest. It is obvious that the high extracellular esterase activity in *E.chevalieri* mainly came from a high molecular weight esterase (MW >10 kDa).

Table 3.1 Formation of a clear halo on tributyrin agar plates

Microorganisms	Clear halo formation
Absidia corymbifera	positive
Chaetomium thermophilum	negative
Emericella nidulans	positive
Eurotium chevalieri	positive
Humicola lanuginose	positive
Rhizomucor pusillus	positive
Talaromyces emersonii	positive
Therandascos aurantiacus	negative
Thermoascus crustaceus	negative

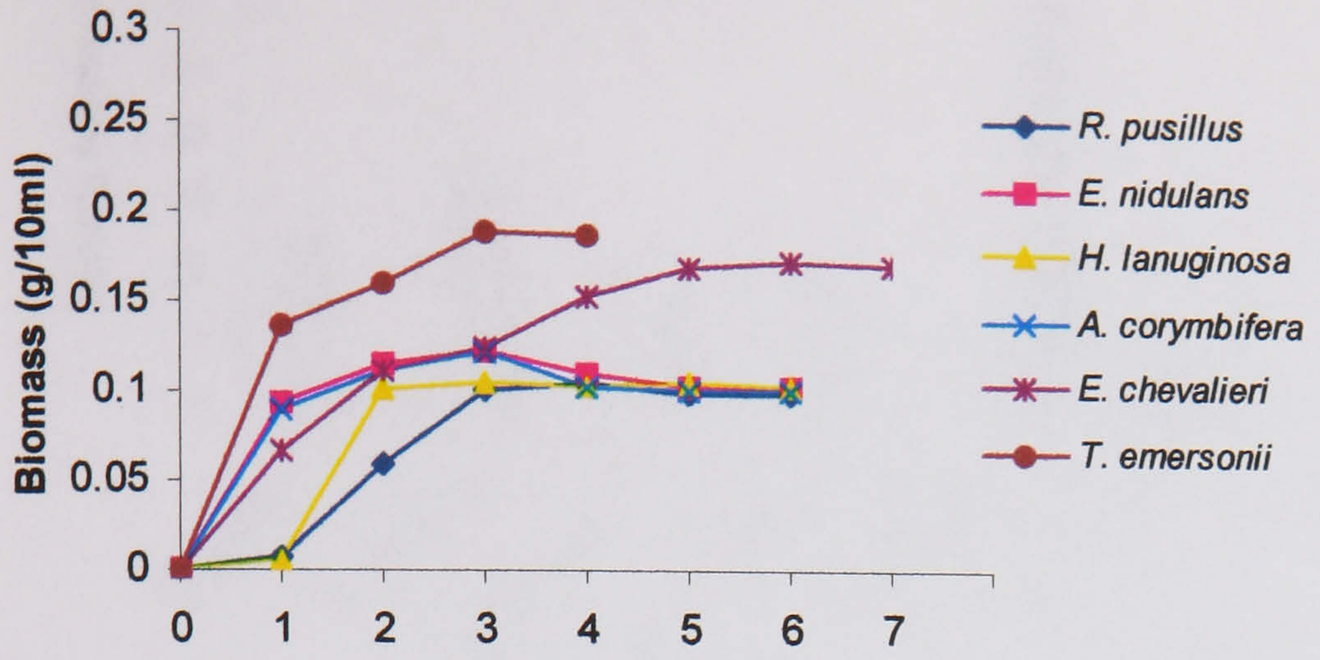


Figure 3.1 The time course of cell growth of thermophilic fungi.

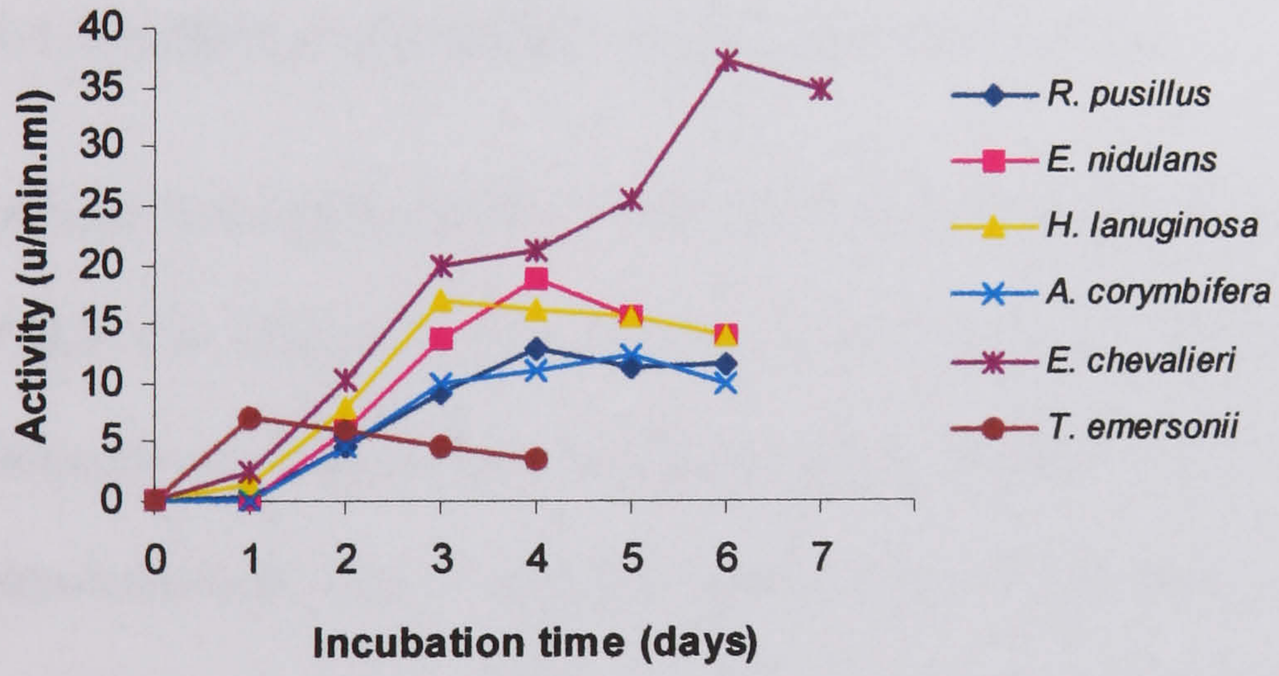


Figure 3.2 The time course of esterase production by thermophilic fungi

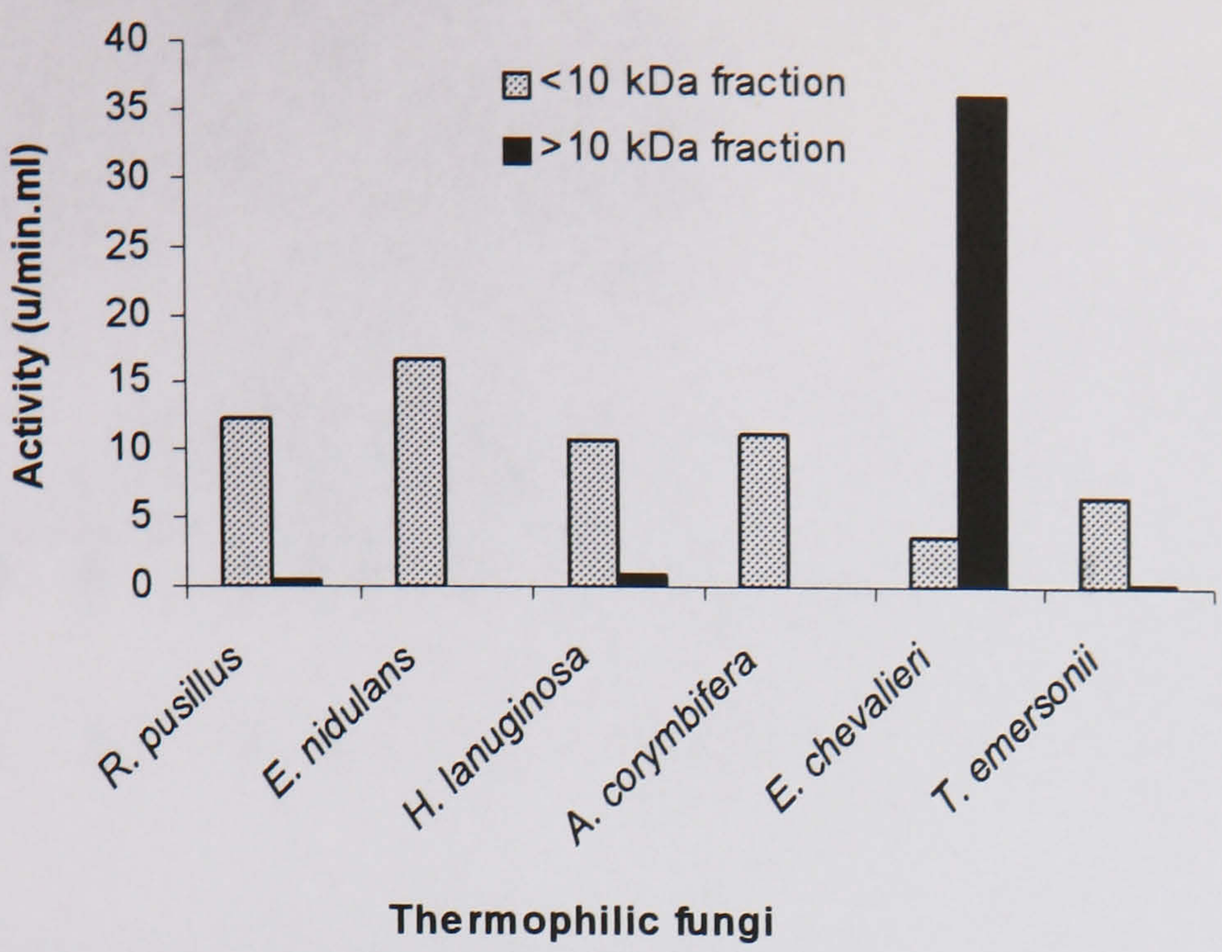
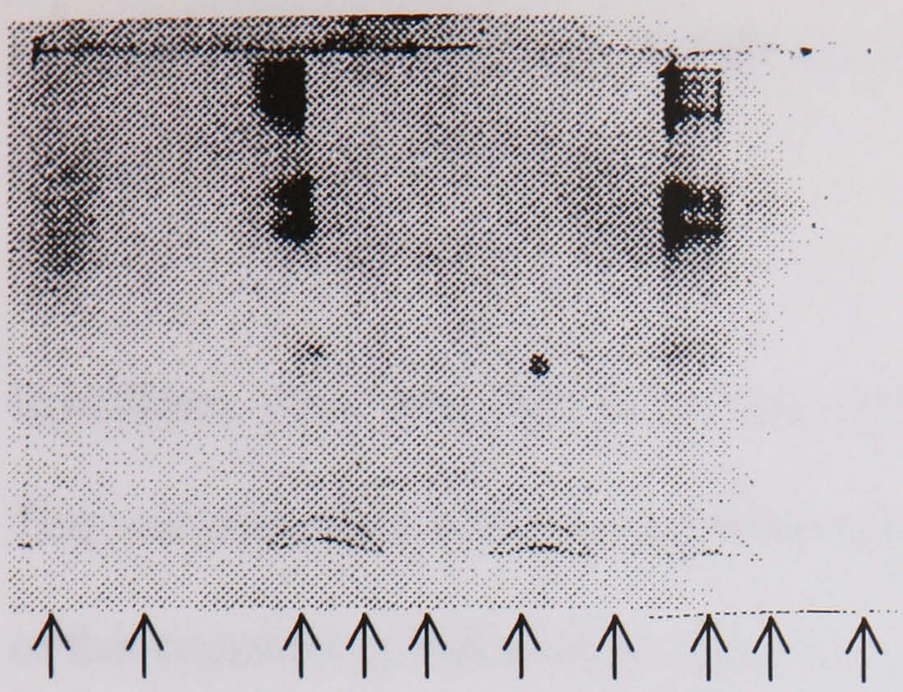


Figure 3.3 Esterase activities observed in >10 kDa and <10 kDa fractions after passing through Microsep centrifugal filters.

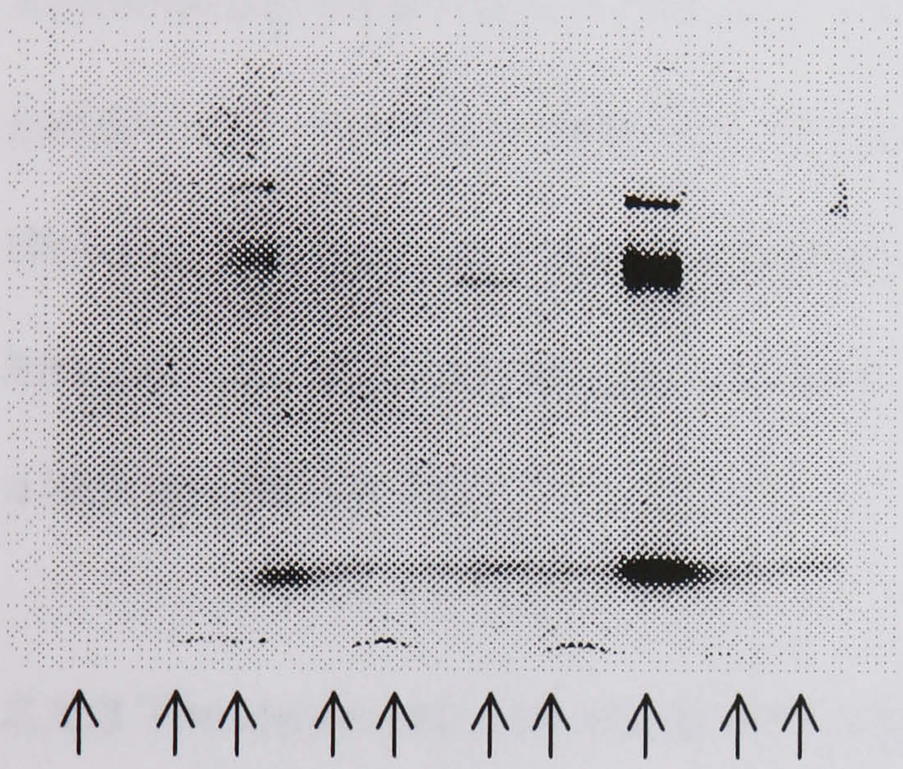
3.1.2 Native polyacrylamide gel electrophoresis

In order to confirm that the esterase activities observed were related to proteins, native PAGE was carried out. The desalted crude extract samples were loaded onto two 12% discontinuous slab polyacrylamide gels using Laemmli's buffer system and run simultaneously. One of the gels was stained for protein with coomassie brilliant blue, while the other gel was stained for esterase activity using α-naphthyl acetate as substrate (Figure 3.4).



(A)

1 2 3 4 5 6 7 8 9 10



(B)

1 2 3 4 5 6 7 8 9 10

Figure 3.4 Native PAGE of the esterase.

(A), the protein stain gel; (B), the esterase stain gel. 1. medium; 2. blank; 3. *E. chevalier*; 8. *E. chevalier* (concentrated); 4. *A. corymbifera*; 5, 7. *E. nidulans*; 6. *H. lanuginosa*; 9. *T. emersionii*; 10. *R. pusillus*

It can be seen that all the six organisms exhibited bands of esterase active stain and protein stain in the position of the dye front, where proteins with molecular weights

less than 10 kDa normally co-migrate in 12% separating gel of Laemmli's buffer system.

In addition, there were high molecular weight esterase stain bands in *E. chevalieri*. This was consistent with the significant esterase activity observed in >10 kDa fraction of this organism as indicated in Figure 3.3. The presence of two esterase stain bands may be due to two forms of one esterase or two different esterases.

An interesting observation is that there was an intense esterase stain band at high MW position in *H. lanuginosa* while very low activity was detected in > 10 kDa fraction as shown in Figure 3.3. In addition, compared with the same position of the esterase stain band, the protein stain band was very faint, which implies that this esterase might have a high specific activity.

3.1.3 The esterase activities in *E. nidulans*

It can be seen from Section 3.1.1 and 3.1.2 that the extracellular esterase activities with low molecular weights were present in the crude extracts of the six thermophilic fungi and these esterases catalysed both fluorescein dibutyrate and α-naphthyl acetate. In order to investigate the nature of these small enzymes, further work was focused on *E.nidulans,* which exhibited the highest esterase activity in the less than 10 kDa fraction.

As shown in Figure 3.4, small proteins (<10 kDa) could not be resolved by 12% separating gel in Laemmli buffer system so that they co-migrated with the dye front.

In order to resolve the low molecular weight proteins, Schagger and Von Jagow's Tricine-PAGE system was tested using the peptide standards (MW 2.5 ~ 16.9 kDa) and found to exhibit a good separation, and was thus utilized to resolve the microenzymes.

The <10 kDa fraction of the crude extract from *E. nidulans* was desalted and concentrated and loaded onto two identical 16.5%T, 6%C separating gels, and run under native conditions. After electrophoresis, one of the gels was stained for proteins by a silver stain and another stained for esterase activity. The result showed the presence of multiple protein bands in the protein gel and the esterase active stain gel exhibited a very broad band at the position between 2.5 kDa and 6.2 kDa bands of the standard peptides, which was associated with the corresponding protein bands.

The separation of the esterase was carried out by gel filtration. The concentrated esterase sample from <10 kDa fraction was loaded onto a Bio-gel P-10 column. The result is shown in Figure 3.5. It is clearly seen that the esterase activity presented as one major peak between fraction 39 and 43 (E40) and one minor peak between fraction 30 and 33 (E32).

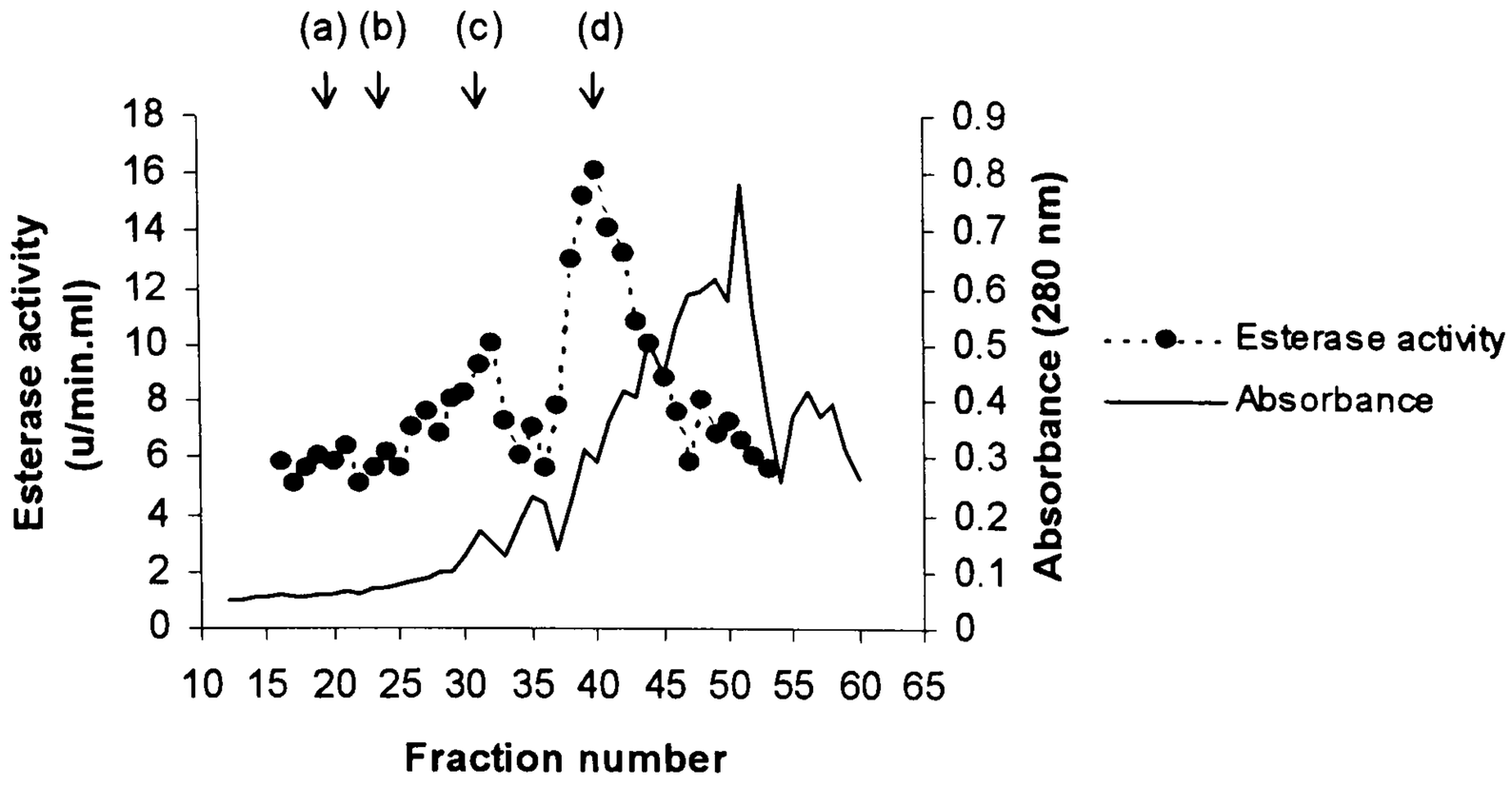


Figure 3.5 Elution profile of protein and esterase activity of *E. nidulans* from Bio-gel P-10 column chromatography.

Arrows indicate position of Blue Dextran MW 2000 kDa (a); Cytochrome C MW 12.4 kDa (b); Aprotinin MW 6.5 kDa (c); Vitamin B-12 MW 1.35 kDa (d) from a calibration run.

Figure 3.5 and the subsequent reverse-phase C18 HPLC showed the presence of multiple peptide peaks in the active fractions pooled from gel filtration. In addition, preliminary observation showed that low esterase activities were present in the complex medium, such as corn steep liquor, yeast extract, peptone, malt extract, etc., which are routinely used for the culture of the microorganisms.

In order to get rid of the interference from the complex medium, a synthetic medium was developed to substitute the malt extract fermentation medium.

3.1.4 Extracellular esterase activities of thermophilic fungi in synthetic medium

Different synthetic media for fungi growth and esterase activity production reported in the literature were tested. It was found that only the improved Czapek medium described in Section 2.1.5 using urea as the N source, sucrose as the C source and tributyrin as an inducer gave both cell growth and esterase activities in some of the thermophilic fungi used. Two of the six organisms, *E. nidulans* and *T. emersonii,* exhibited good cell growth and obvious esterase activities in crude extract (Figure 3.6). No significant activities were detectable in *E. chevalieri* and *H. lanuginosa,* which means that these two organisms produced neither high molecular weight nor low molecular weight extracellular esterases as they did in the malt extract medium.

E. nidulans showed slower growth and lower esterase activity in the synthetic medium than in the malt extract medium: growth reached a maximum after 8 days' incubation and the highest esterase activity occurred on the 10^{th} day, compared with maximum cell growth and activity on the 3^{rd} and 4^{th} day of fermentation in malt extract medium (Figure 3.7).

The esterase activities in *E. nidulans* and *T. emersonii* were further examined and they were all found again in less than 10 kDa fraction after passing through the microconcentrators (Figure 3.8).

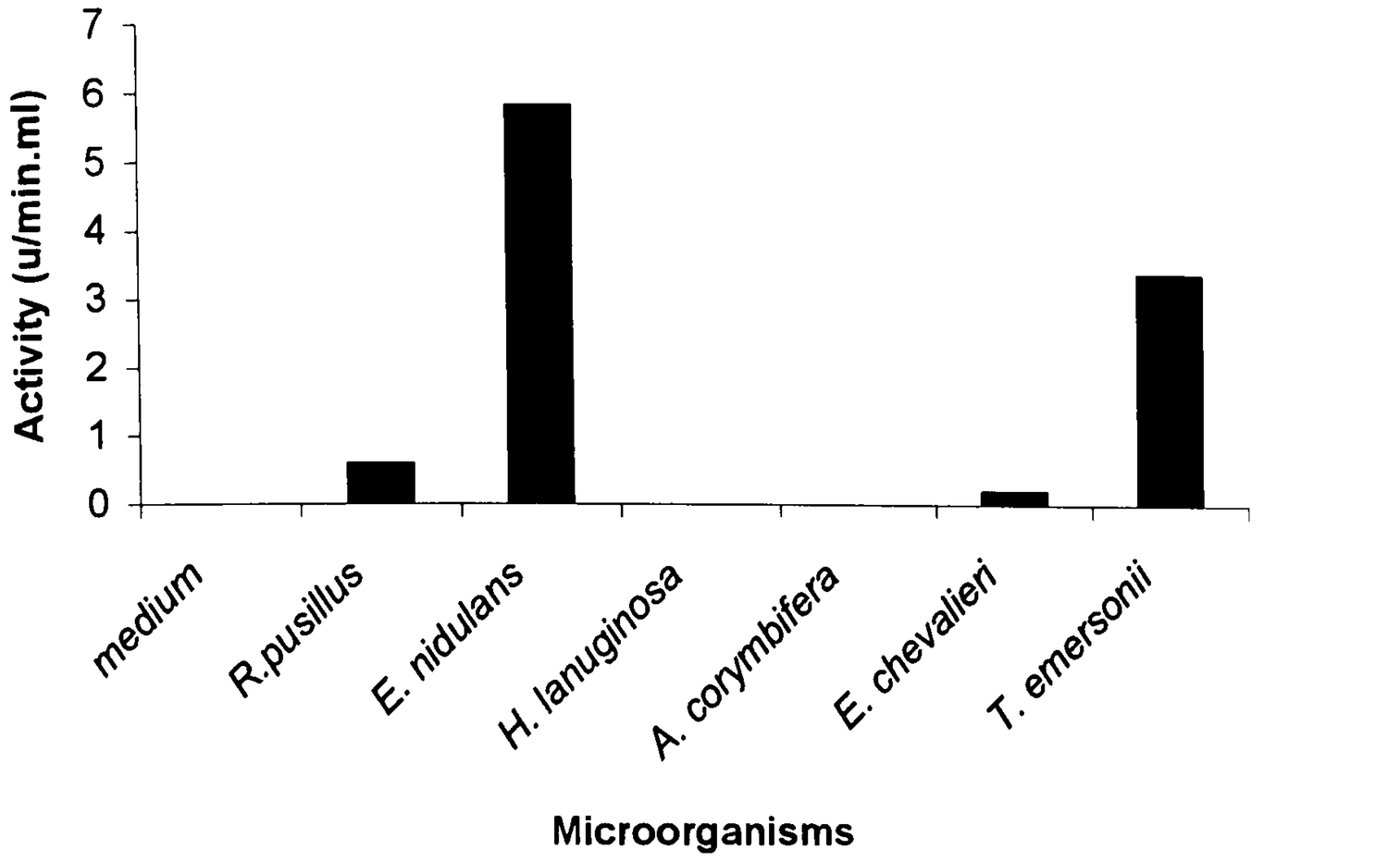


Figure 3.6 Extracellular esterase activity of thermophilic fungi in synthetic medium.

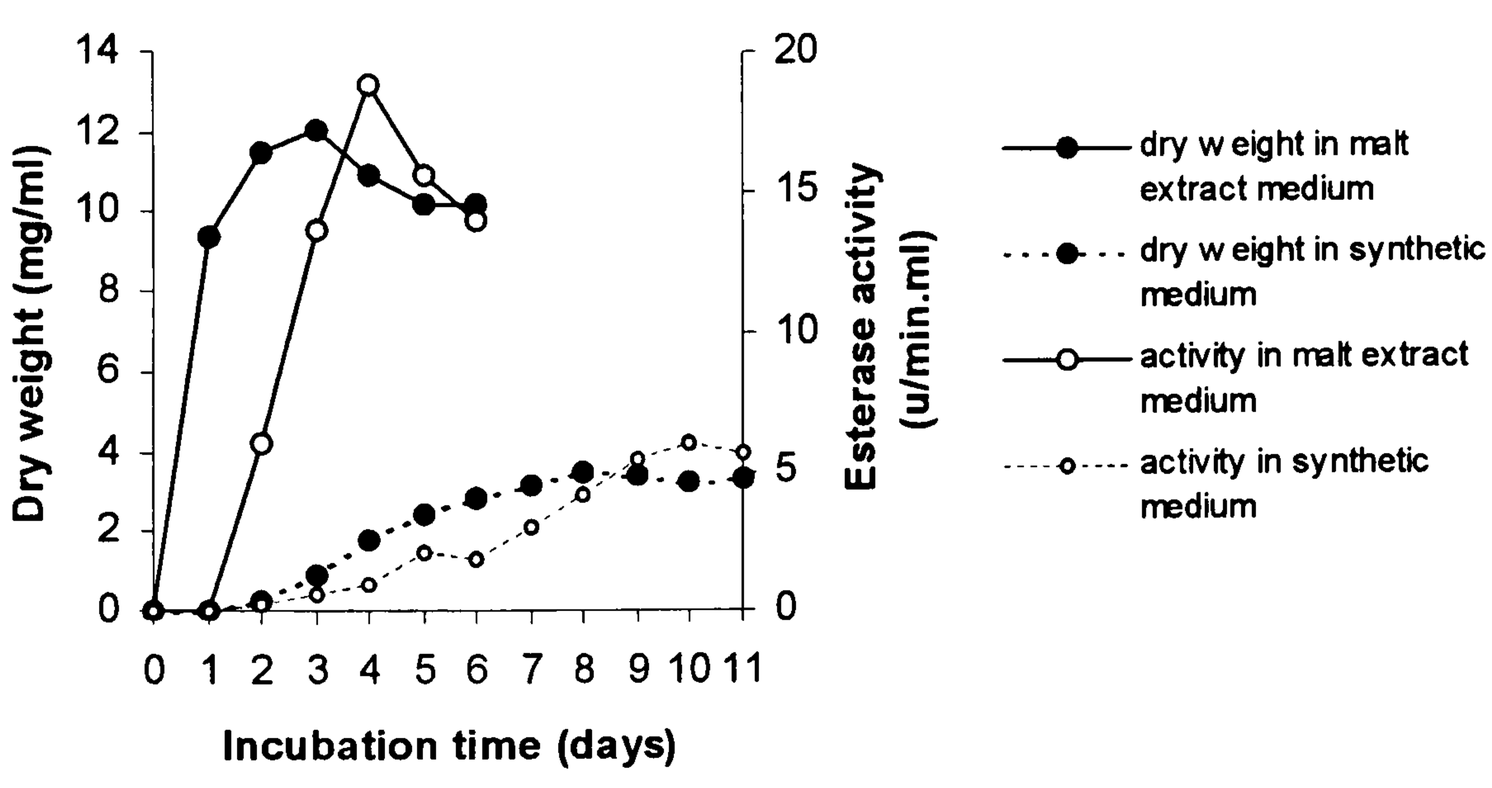


Figure 3.7 The time course of growth and esterase production by *E. nidulans* in malt extract medium and synthetic medium.

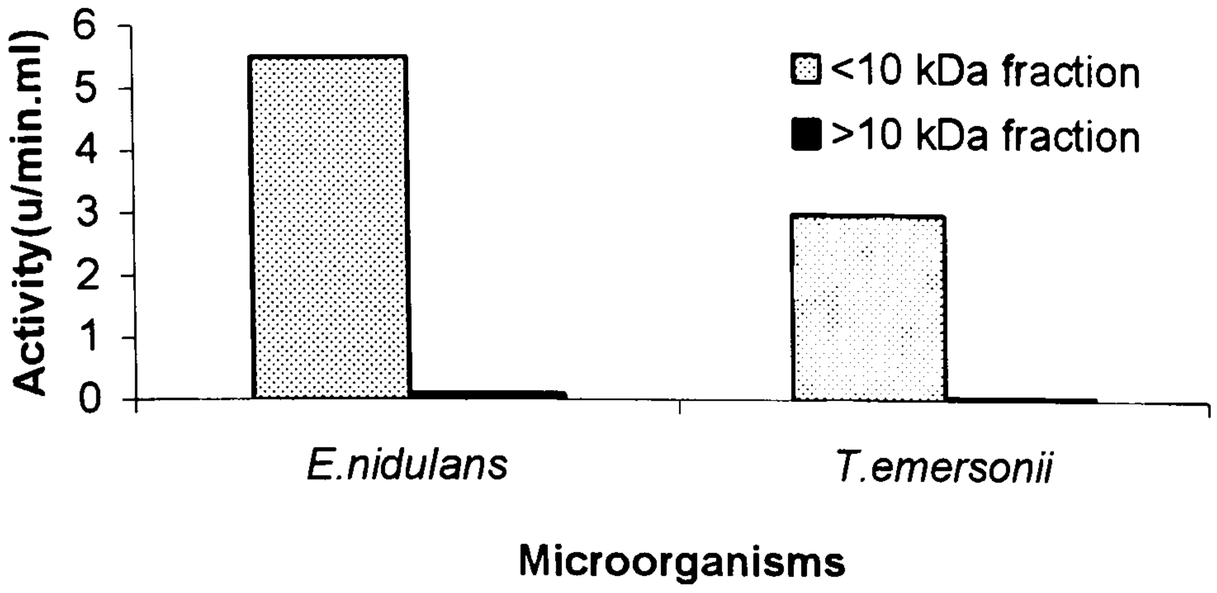


Figure 3.8 Esterase activities of *E. nidulans* and *T. emersonii* in synthetic medium after passing through microconcentrators with MW cut off 10 kDa.

3.2 PURIFICATION OF THE ESTERASE MICROENZYMES

3.2.1 Purification of the esterases from *E. nidulans*

After demonstrating that the esterase activity in *E. nidulans* was due to a small enzyme with molecular weight less than 10 kDa, large-scale enzyme preparation was carried out by filtering the 410 ml cell free fermentation broth using a tangential flow filter (MW cut-off: 10 kDa). The less than 10 kDa fraction (filtrate) was concentrated by freeze drying. Then, this crude extract from the synthetic medium was purified by Bio-gel P-10 gel filtration under the same conditions as described in Section 3.1.3. The result (Figure 3.9) showed that the esterase activity peaks appeared in the same range of fractions as those from the malt extract medium: most of the activity was between fraction 39-42 (E40) and a low esterase activity was observed between fraction 31-33

(E32).

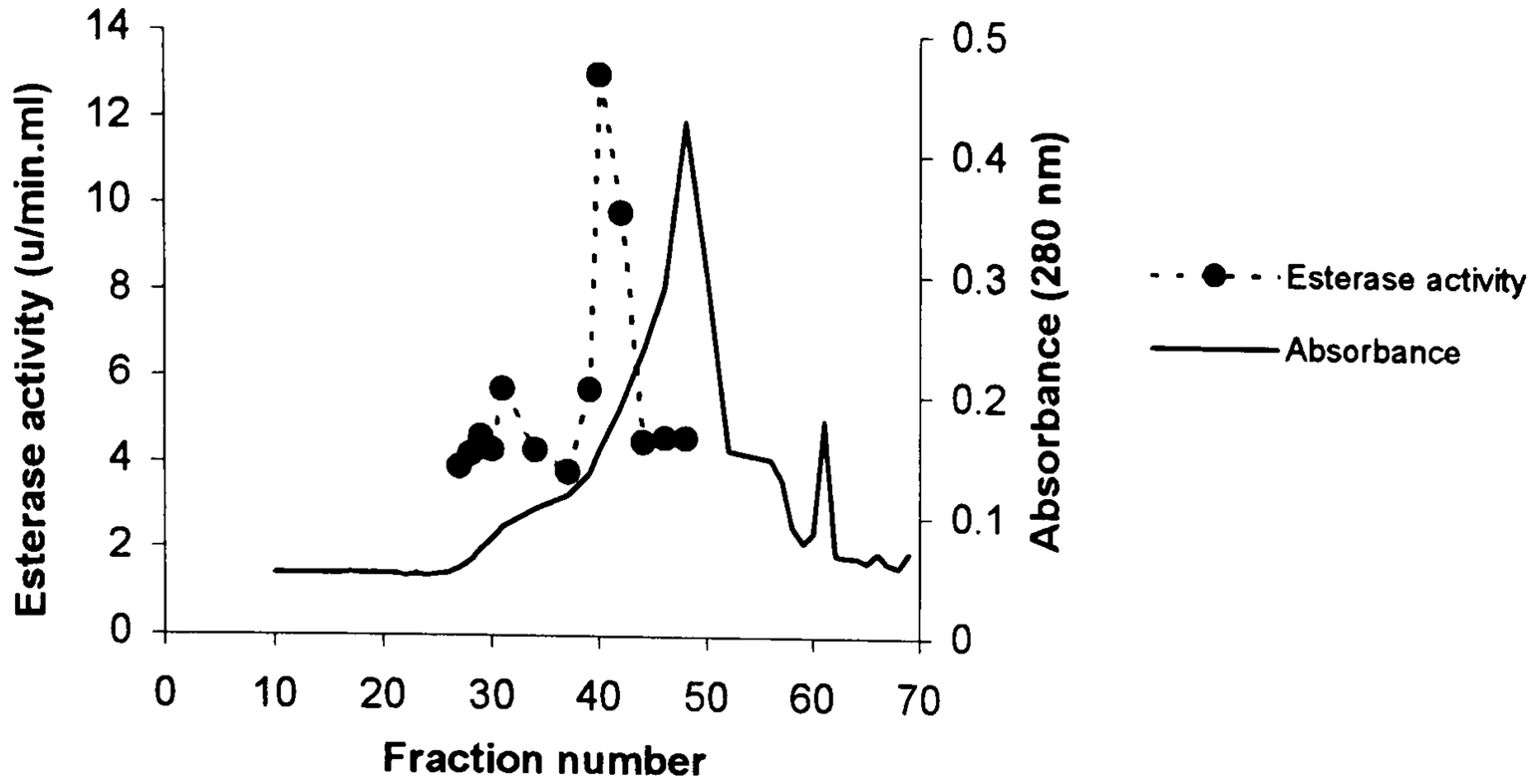


Figure 3.9 Elution profile of Bio-gel P-10 gel filtration in synthetic medium from *E. nidulans*.

The active fractions from E40 and E32 were pooled separately, freeze-dried to concentrate and further purified by reverse phase HPLC.

The conditions for the separation of the enzymes by reverse phase HPLC were determined using a high resolution, small particle Nucleosil C-18 column with TFA as the ion-pairing agent and acetonitrile as the elution solvent.

The chromatography using reverse phase HPLC with 0.1% TFA and elution with an acetonitrile gradient is harsh condition that denatures most enzymes but was suitable for the purification of microenzyme esterases because of the extreme stability of these enzymes in organic solvents at a low pH.

The central task in developing a reversed-phase procedure is to determine the starting and ending solvent concentrations of the gradient which give the optimal separation. The first attempt was to use 0-60% acetonitrile gradient developed in 30 min. It was found that enzyme activity of E40 and E32 and most of the uv absorbance peaks were eluted below 30% acetonitrile. Thus, 0-30% acetonitrile gradient was set for the separation of the enzymes.

The method (method A) with 20-min linear gradients from 0-30% B over 10 min, 30% B over 5 min followed by 30-0% B for 5 min was initially used. Figure 3.10 represents the elution profile of E40 under this method. It shows a major uv-absorbing peak with elution time of 7.9 min (23.7% acetonitrile), which coincided with the esterase activity. It was also noted that a number of minor peaks were very close to the major peak which means the major peak separated at this stage was not pure and it may contain more than one peptide. So the separation by this method was not satisfactory.

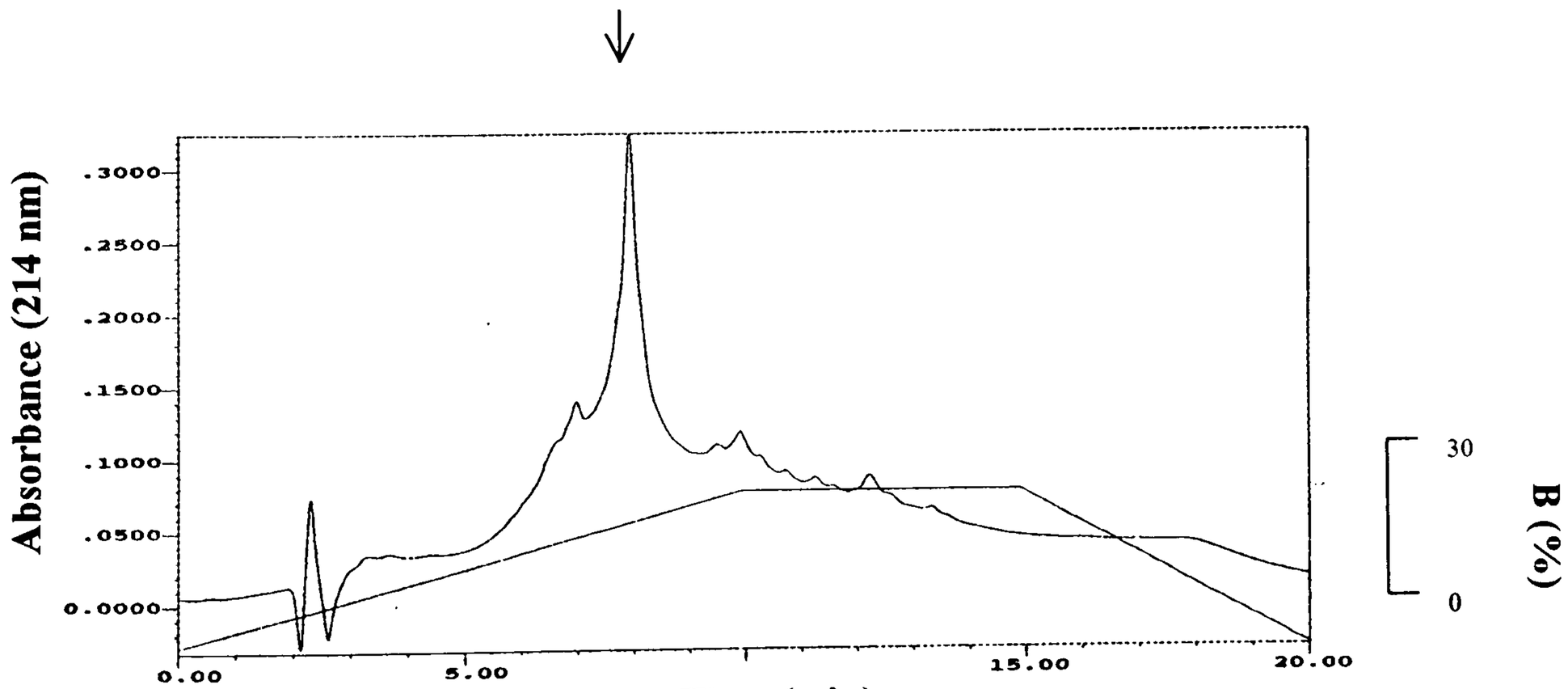


Figure 3.10 HPLC elution profile of E40 by method A.

Column: Nucleosil C18 (10μ, 100Å, 250 x 4.6 mm, Phenomenex). Flow rate: 1 ml/min. Mobile phase: solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile. 20-min linear gradients from 0-30% B over 10 min, 30% B over 5 min followed by 30-0% B for 5 min. Arrow indicates the position of enzyme activity.

In order to optimize the separation, a series of methods were attempted, which included:

1) Change the column to use the polymer-based reverse phase column Hamilton PRP-3 C18 (10μ, 300Å, 150 x 4.1 mm, Phenomenex) which allows the separation to be conducted at pH>7, so that basic pH condition can be tested;

2) Use different ion pairing agent heptafluorobutyric acid (HFBA) instead of TFA as HFBA is more hydrophobic than TFA;

3) Change flow rates (from 0.25 to 2 ml/min);

4) Change gradient to decrease the gradient slope and thus increase the gradient duration based on method A while the starting and ending solvent concentrations were kept unchanged.

Of the above methods tested, it was found that only the fourth method (method 4) using a shallow gradient of longer duration with TFA as the ion pairing agent at a flow

rate of 1 ml/min on the silica-based Nucleosil C18 column gave the best separation results. The gradient developed used 45-min linear gradients from 0-30% B over 40 min, 30% B over 3 min followed by 30-0% B for 2 min. Figure 3.11 shows the elution profile of E40 by this method.

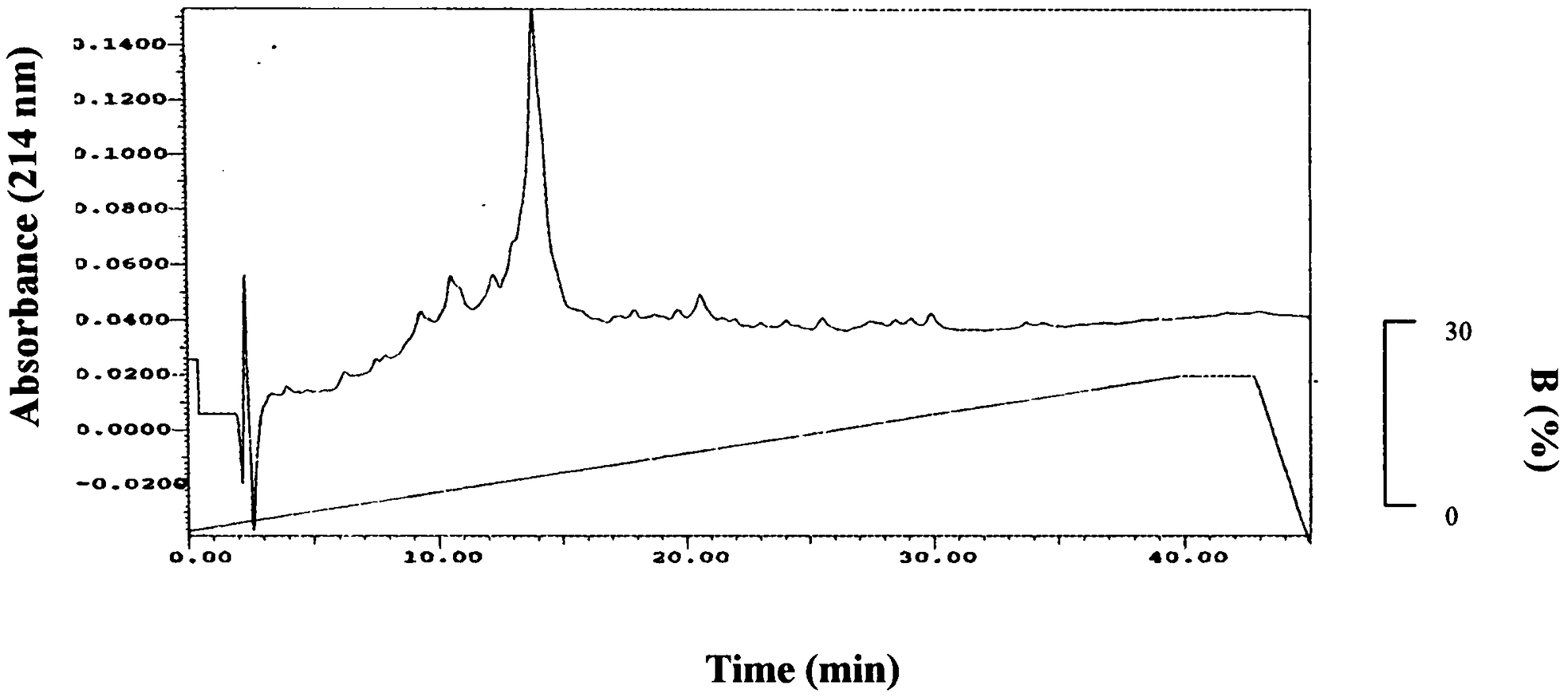


Figure 3.11 HPLC elution profile of E40 by method 4.

45-min linear gradients from 0-30% B over 40 min, 30% B over 3 min followed by 30-0% B for 2 min. Other conditions were same as those in Figure 3.10.

It is evident that decreasing gradient slope caused peaks to elute further apart, thus increasing resolution, and the peaks were eluted at lower eluent concentration. The major peak (enzyme active peak) appeared at 13.8 min (10.1% acetonitrile) and improved resolution of the minor peaks nearby was attained. The major peak was collected and rechromatography of the major peak (Figure 3.12) showed a single peak in the preparation which implied the separated peptide was pure. Subsequently, successive runs were made to collect enough protein for further analysis. The collected peak fractions from separate runs were pooled and concentrated either by freeze

drying or by evaporation in a Savant Speed-Vac concentrator as both acetonitrile and TFA are volatile, so that the sample obtained at this stage can be directly applied to mass spectrometry and protein sequencing analysis etc.

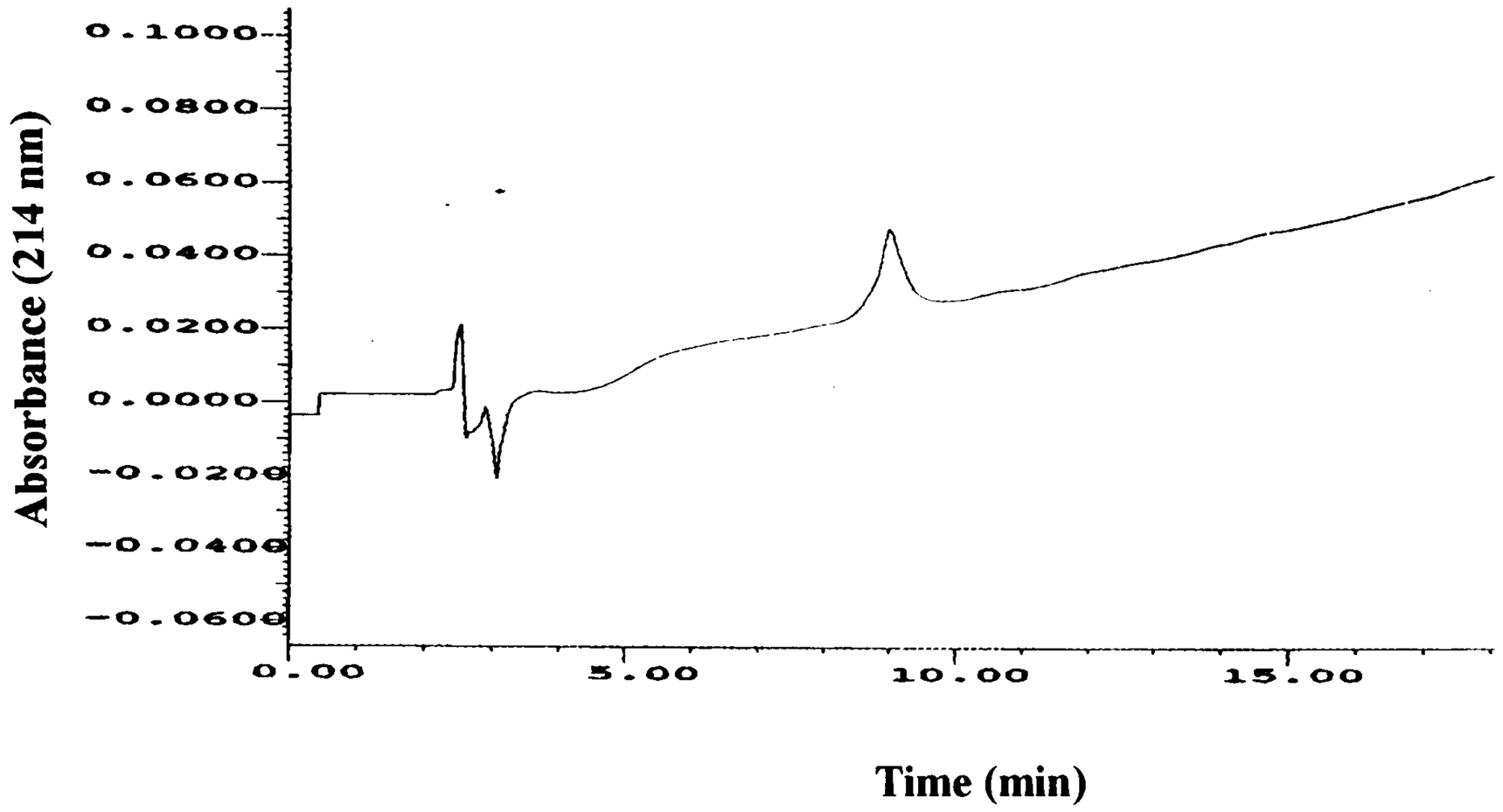


Figure 3.12 Rechromatography of the major peak of E40.

30-min linear gradients from 0-30% B over 20 min, 30% B over 5 min followed by 30-0% B for 5 min. Other conditions were same as those in Figure 3.10.

A summary of the purification procedure for E40 is shown in Table 3.2; about 28-fold purification was achieved, with a final yield of 35.3%.

Table 3.2 Purification of E40 from *E. nidulans*

Purification step	Total activity ($u\ min^{-1}$)	Total protein (mg)	Specific activity ($u\ min^{-1}\ mg^{-1}$)	Yield (%)
Culture broth	2320	90	26	100
<10 kDa fraction	2114	62.1	34	91.1
P-10 gel filtration	1153	3.48	331	49.7
HPLC elution	820	1.12	732	35.3

Likewise, E32 was further purified by method 4 and the esterase activity coincided with the major protein peak eluted at an acetonitrile concentration of 18.6% (25 min). Therefore, the peak was collected and reloaded. Figure 3.13 represents rechromatography of the purified E32.

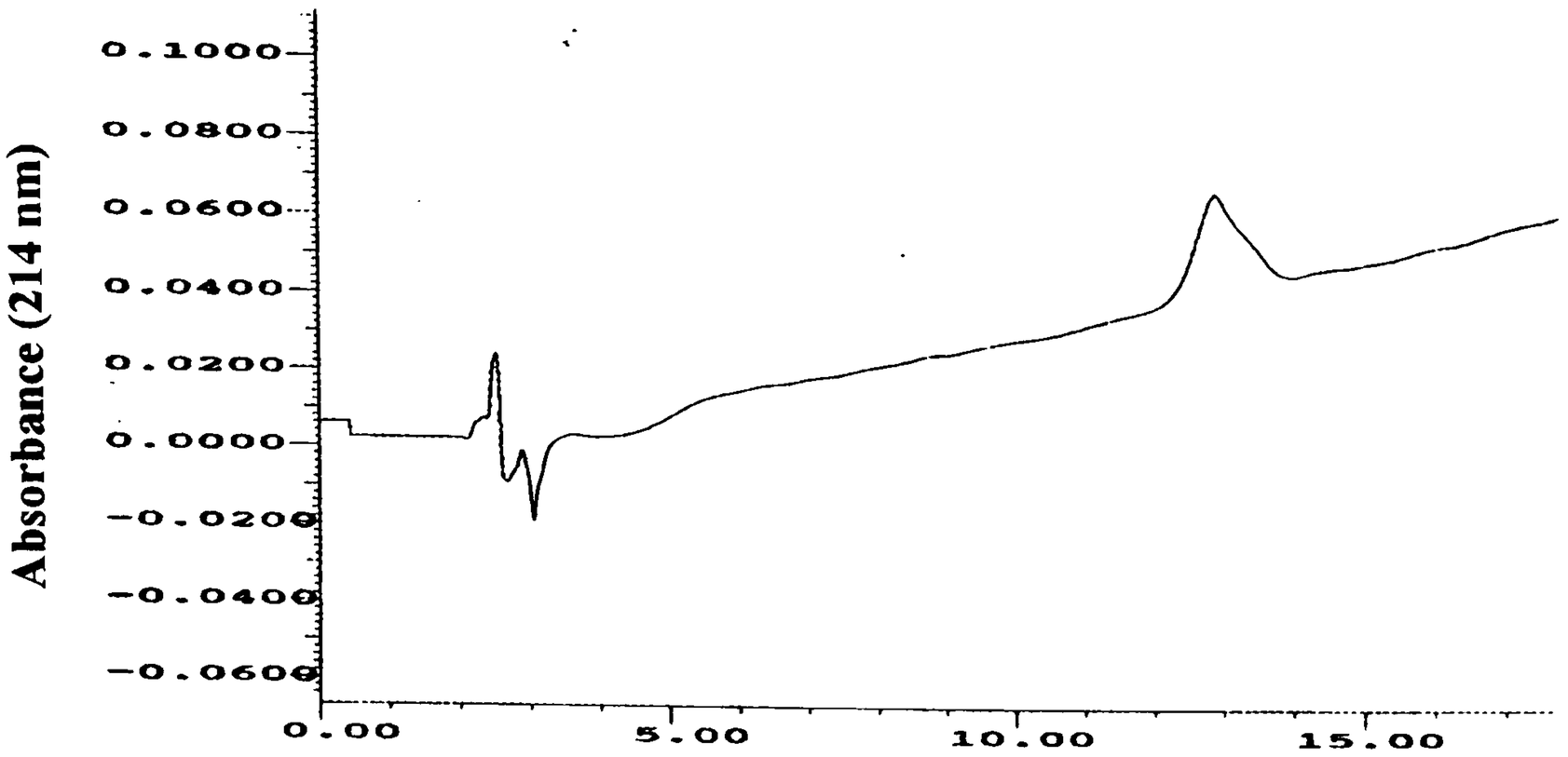


Figure 3.13 Rechromatography of the purified E32.

The conditions were same as those in Figure 3.12.

3.2.2 Purification of the esterase from *T. emersonii*

The purification of the esterase from *T. emersonii* was conducted in the same way as from *E. nidulans*. The concentrated sample from <10 kDa fraction was loaded onto the Bio-gel P-10 column and the result of gel filtration showed one active peak between fraction 39 and 43 (T40), which was at the same position as the major peak (E40) of *E. nidulans*. But unlike *E. nidulans*, no esterase activity was detected in other fractions in this organism.

The subsequent HPLC using method 4 indicated that the esterase active peak of T40 was eluted at 15% acetonitrile concentration (20 min) and the rechromatography of the collected peak is shown in Figure 3.14.

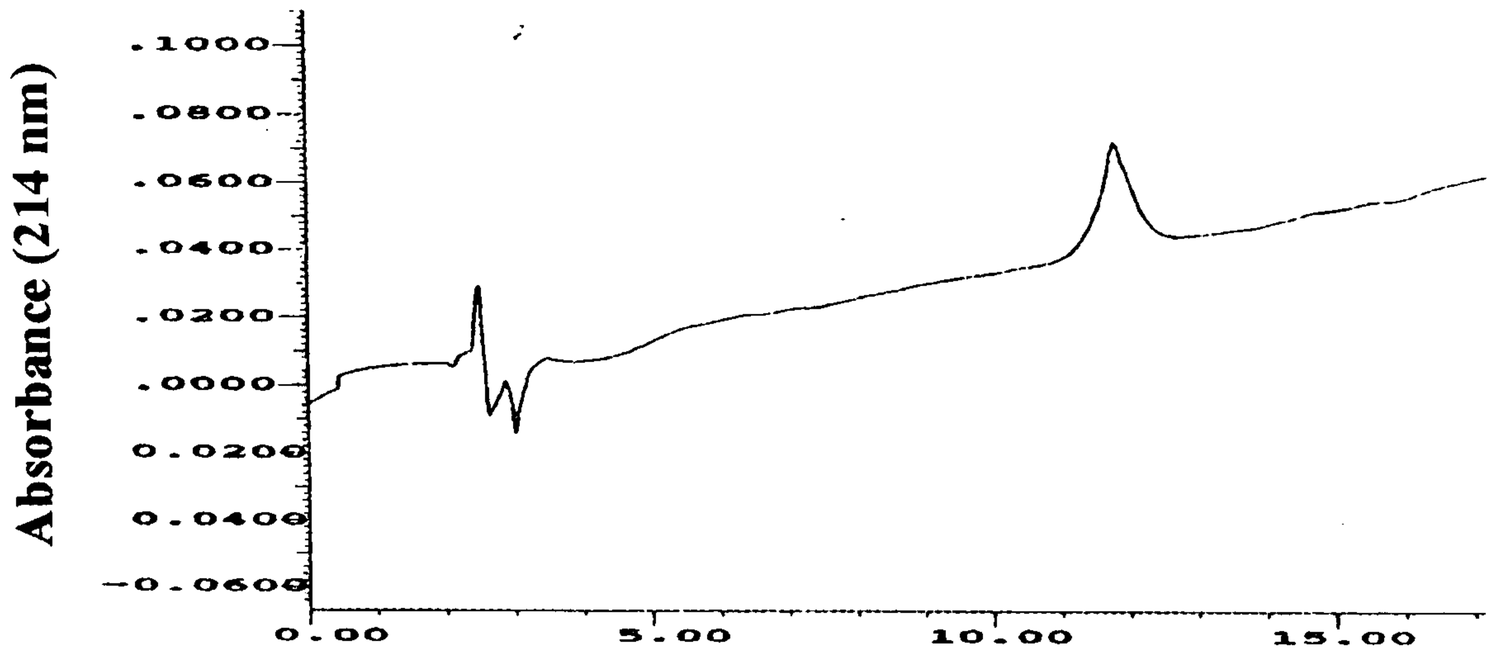


Figure 3.14 Rechromatography of the purified T40.

The conditions were the same as those in Figure 3.12.

3.3 CHARACTERIZATION OF THE ESTERASE MICROENZYMES

3.3.1 Molecular weight determination

The microenzymes were first characterized by examining their molecular weights. The molecular weights were determined by three methods: gel filtration, SDS-PAGE and mass spectrometry.

3.3.1.1 Molecular weight determination using gel filtration

The native molecular weights of the three esterases were estimated by Bio-Gel P-10. The calibration curve is shown in Figure 3.15. The molecular weights of E40 and T40 were 1.6 kDa, while molecular weight of E32 was 4.1 kDa by gel filtration.

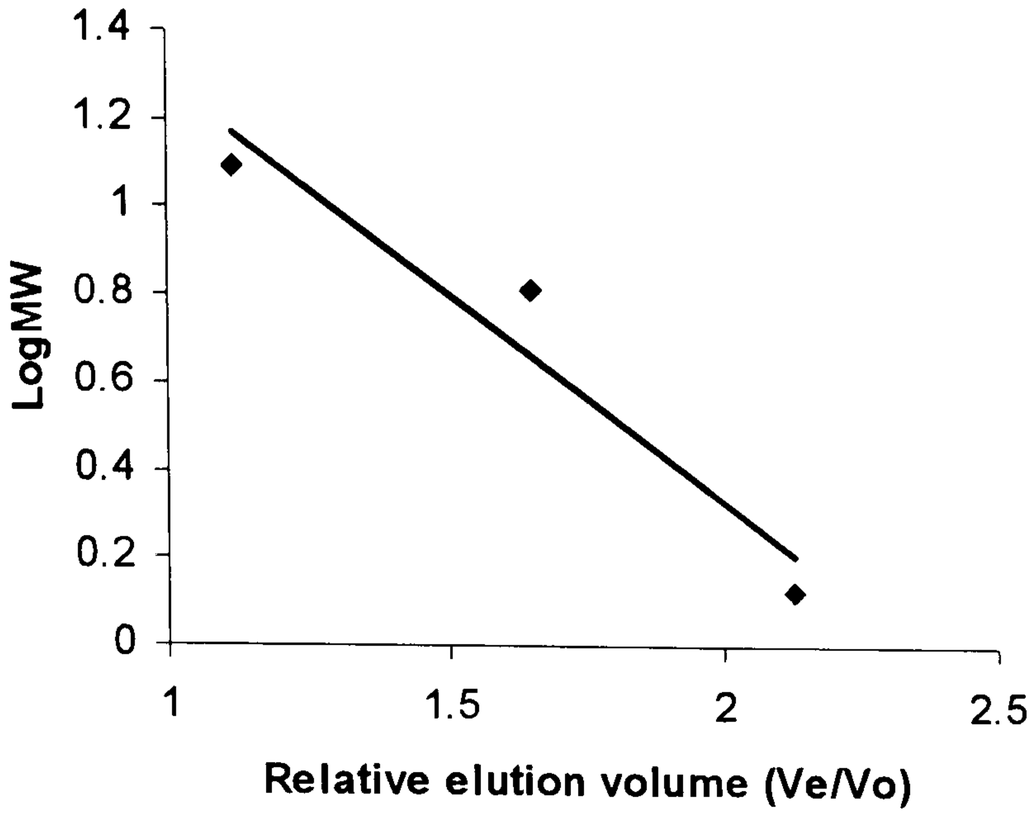


Figure 3.15 Calibration of the Bio-gel P-10 gel filtration column.

The standards were Cytochrome C (12.4 kDa), Aprotinin (6.5 kDa) and Vitamin B-12 (1.35 kDa).

3.3.1.2 Molecular weight determination using SDS-PAGE

The purified E40, E32 and T40 all exhibited a single band at the MW between 2.5 kDa and 6.2 kDa of the myoglobin MW markers when subjected to the Schägger and Von Jagow Tricine-SDS-PAGE system with a 16.5%T, 6%C separating gel and revealed by silver stain (Figure 3.16). E40 and T40 migrated at the same position and E32 ran slightly slower.

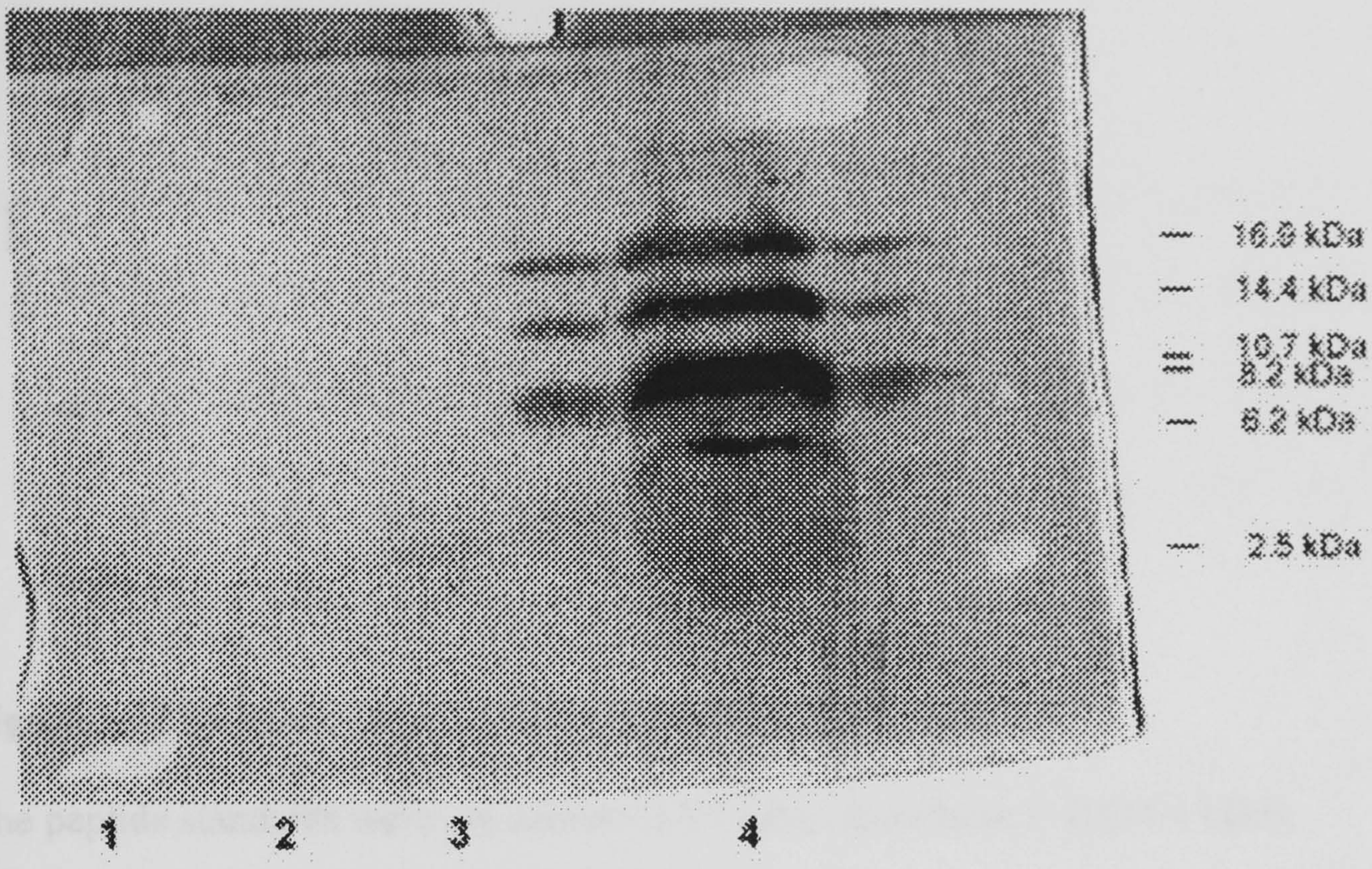


Figure 3.16 Tricine-SDS-PAGE of purified esterases.

Lane 1, T40; lane 2, E40; lane 3, E32; lane 4, molecular size markers.

The estimation of molecular weights of the esterases was also performed by Tricine-SDS-PAGE. The standard curve was obtained from Figure 3.16 and is shown in Figure 3.17. The molecular weights of E40 and T40 were 3.1 kDa while the molecular weight of E32 was 3.9 kDa by the method of gel electrophoresis.

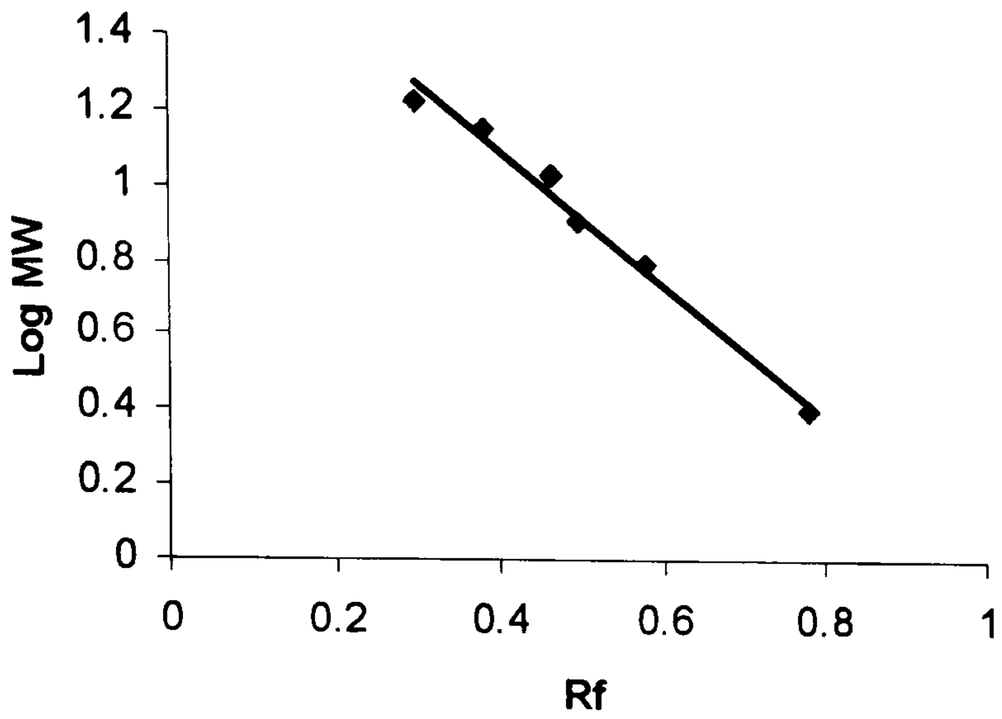


Figure 3.17 Standard curve for Tricine-SDS-PAGE.

The peptide standards were: myoglobin (16.9 kDa), myoglobin I+II (14.4 kDa), myoglobin I+III (10.7 kDa), myoglobin I (8.2 kDa), myoglobin II (6.2 kDa), myoglobin III (2.5 kDa).

It was noticed that the molecular weight estimation of E40 and T40 was inconsistent by gel filtration and SDS-PAGE while that of E32 seems consistent. The small size of these esterases may have unique structural features which have an important effect on electrophoretic mobility of these microenzymes.

In order to determine the accurate molecular weights of these esterases, electrospray and MALDI-TOF mass spectrometry were performed.

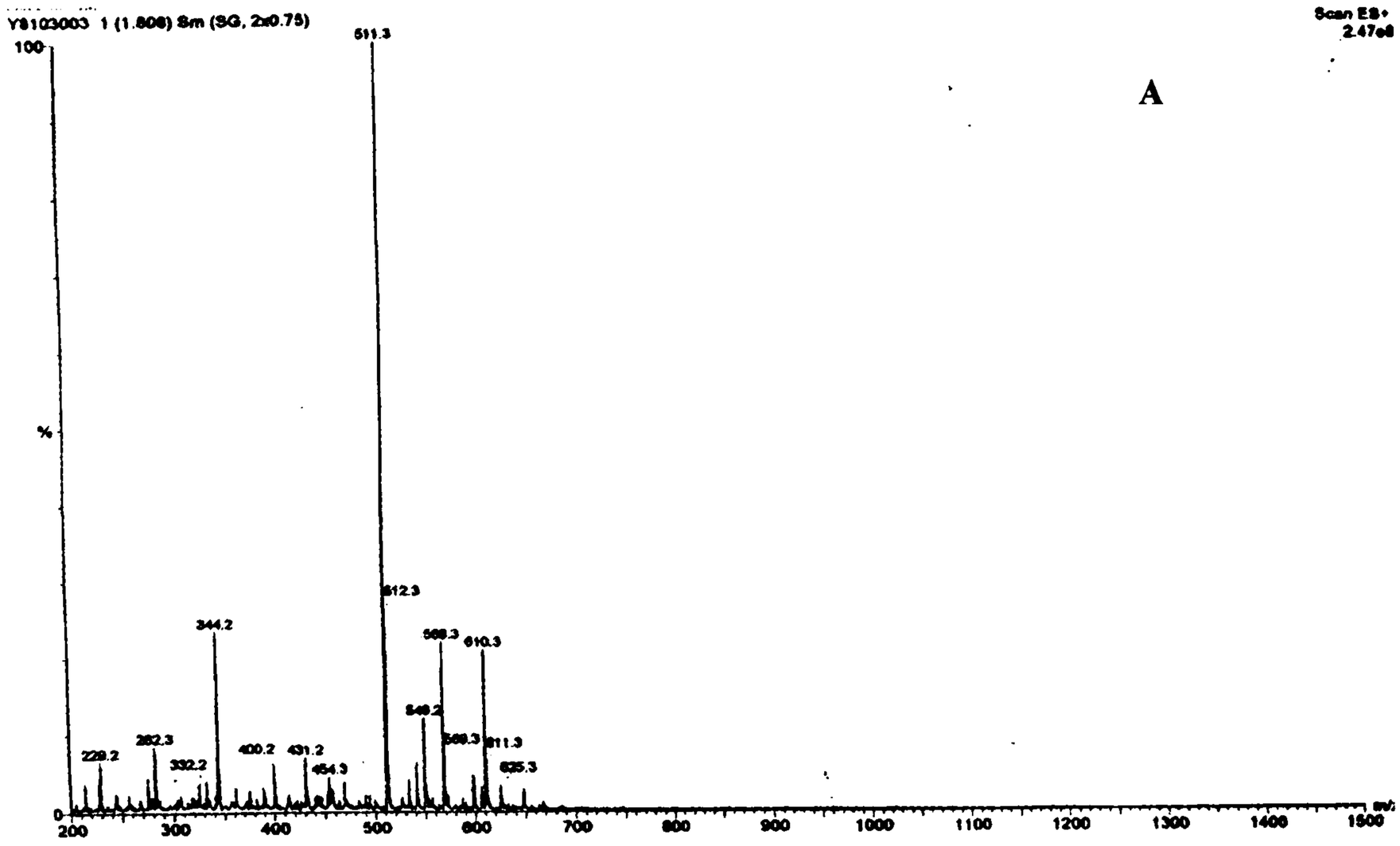
3.3.1.3 Molecular weight determination using electrospray and MALDI mass spectrometry

The ion electrospray mass spectra analysis revealed that a singly-charged species of high relative abundance is observed at m/z 511.3 in E40 (Figure 3.18A) and a singly-charged species of high relative abundance is observed at m/z 610.3 in T40 (Figure 3.18B). Therefore, the molecular weight of E40 is 510.3 and molecular weight of T40 is 609.3.

The MALDI mass spectrum of E32 (Figure 3.18C) shows a cluster of significant ions at m/z 1425.7, 1447.6 and 1463.6 which correspond to $[M_x + H]^+$, $[M_x + Na]^+$ and $[M_x + K]^+$ species respectively (M_x is a non-target molecular weight component of the sample). Thus, the molecular weight of E32 is 1424.7.

It was obvious that the molecular weights determined by mass spectrometry of the three esterases were much smaller than estimated by either gel filtration or gel electrophoresis, however it was also noticed that the molecular weights obtained by nondenatured gel filtration were just about three times that determined by mass spectrometry for all three esterases.

So it is especially of interest to explore further the structure of these esterases.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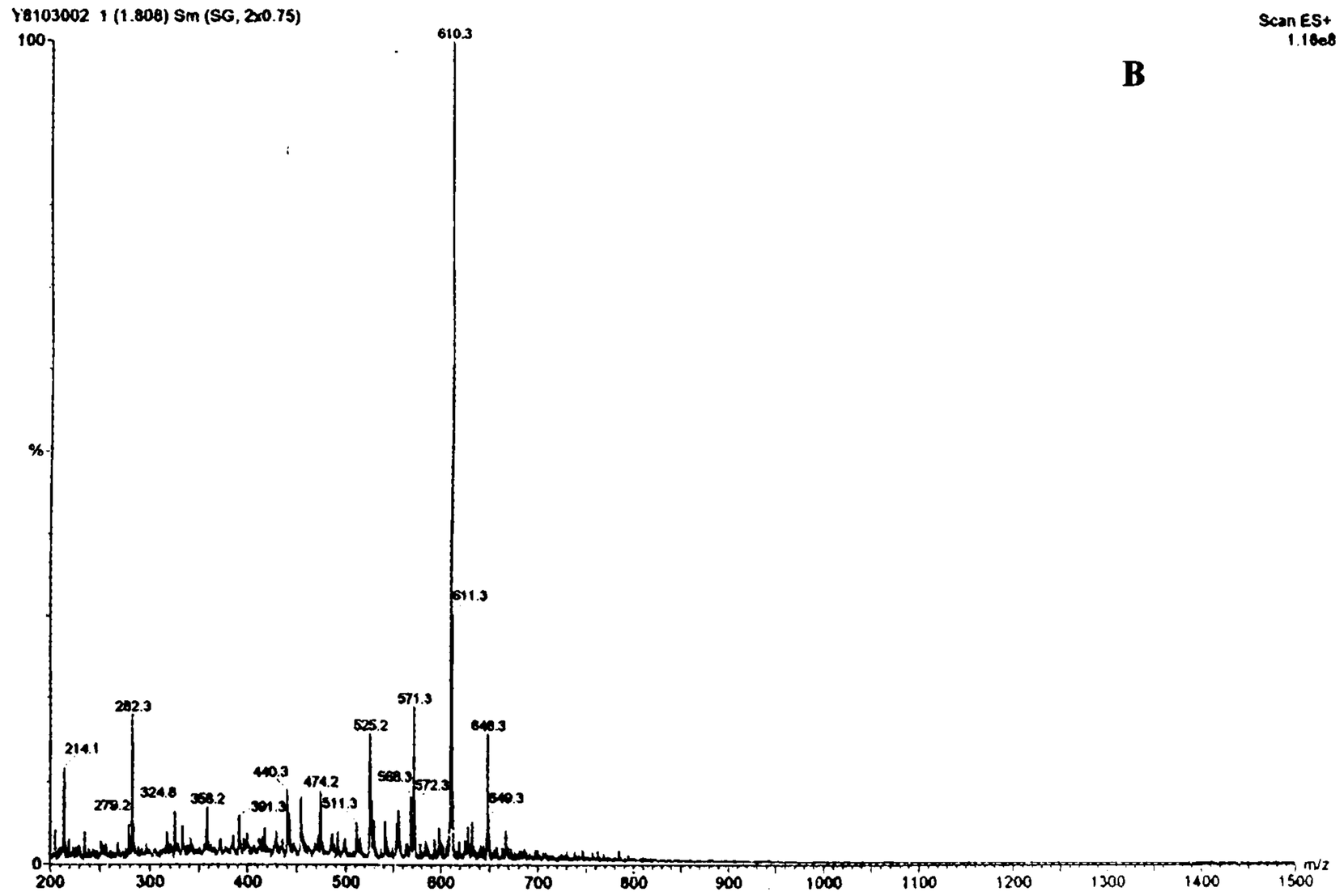
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1.16e8

B

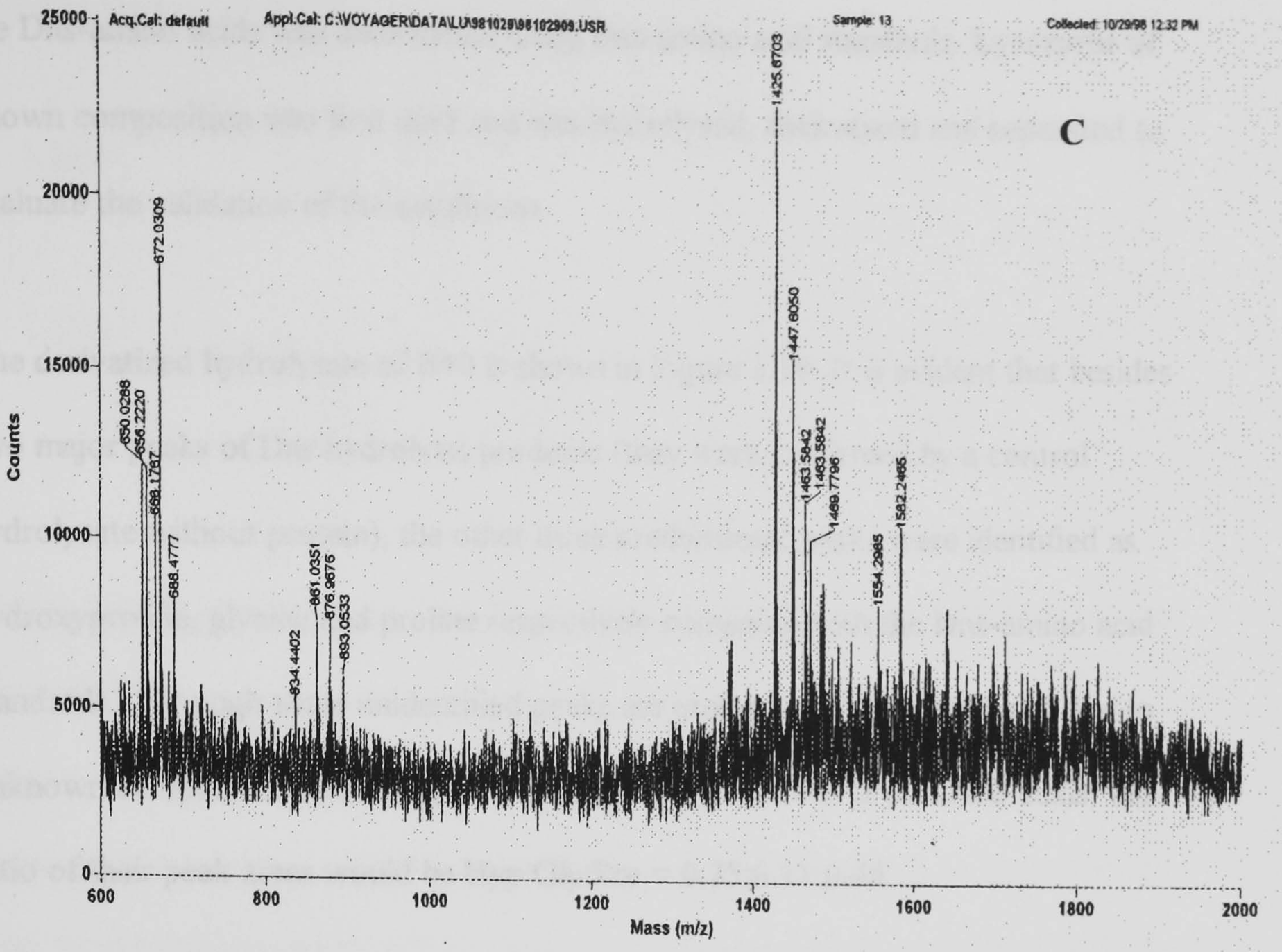


Figure 3.18 Mass spectrometry of the esterases.

A, ion electrospray mass spectrum of E40; B, ion electrospray mass spectrum of T40; C, MALDI mass spectrum of E32.

3.3.2 Amino acid composition analysis & N-terminal amino acid analysis

In order to confirm that the purified UV absorption peaks with esterase activities contained peptides, amino acid composition analysis was performed.

The conditions for dansylation of the amino acids of the microenzymes were determined using amino acid standards and the conditions for RP-HPLC separation of

the Dns-amino acids was determined using Dns-amino acid standards. Lysozyme of known composition was first used and was hydrolysed, derivatized and separated to evaluate the validation of the conditions.

The derivatized hydrolysate of E40 is shown in Figure 3.19. It is evident that besides two major peaks of Dns hydrolysis products (they were confirmed by a control hydrolysate without protein), the other three predominant peaks were identified as hydroxyproline, glycine and proline respectively compared with the Dns-amino acid standards. Although some unidentified peaks are present, they are minor and due to unknown compounds. Assuming E40 consisted of only these three amino acids, the ratio of their peak areas would be Hyp:Gly:Pro = 0.25:0.31:0.44.

The derivatized hydrolysate of E32 (Figure 3.20) exhibited the similar pattern as that of E40, which implied that E32 had a very similar amino acid composition to E40 and mainly consisted of the three amino acids hydroxyproline, glycine and proline. Another peak at 42.5 min was also apparent in E32 and was identified as di-Dns-Lys. Assuming that E32 consisted of only these four amino acids, the ratio of their peak areas would be Hyp:Gly:Pro:Lys = 0.16:0.42:0.36:0.06.

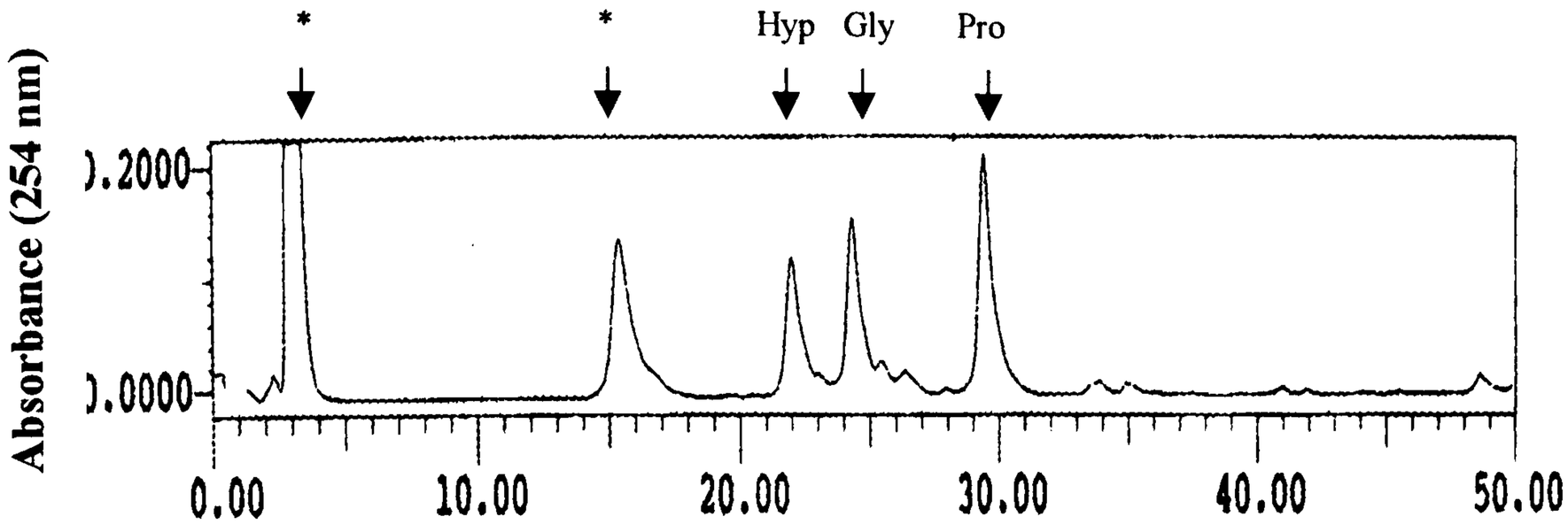


Figure 3.19 Separation of the Dns-derivatized total hydrolysate of E40 using reverse phase HPLC.

Column, Nucleosil C18 (10μ, 100Å, 250 x 4.6 mm, Phenomenex); flow rate, 1 ml/min; mobile phase: solvent A, 50 mM sodium acetate, pH 6.5; solvent B, 80% methanol-20% 50mM sodium acetate, pH 6.5; linear gradient from 10% B to 100% B for 50 min was developed. The peaks marked with an asterisk are Dns hydrolysis products. Unmarked peaks are due to unknown compounds.

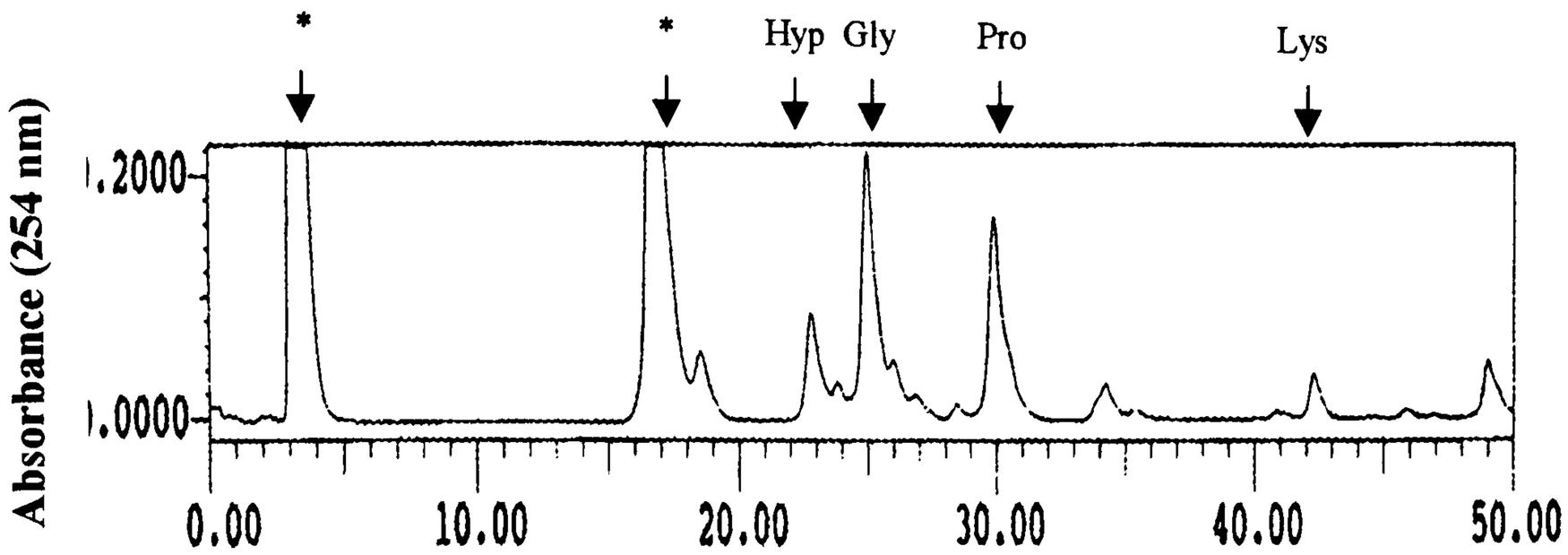


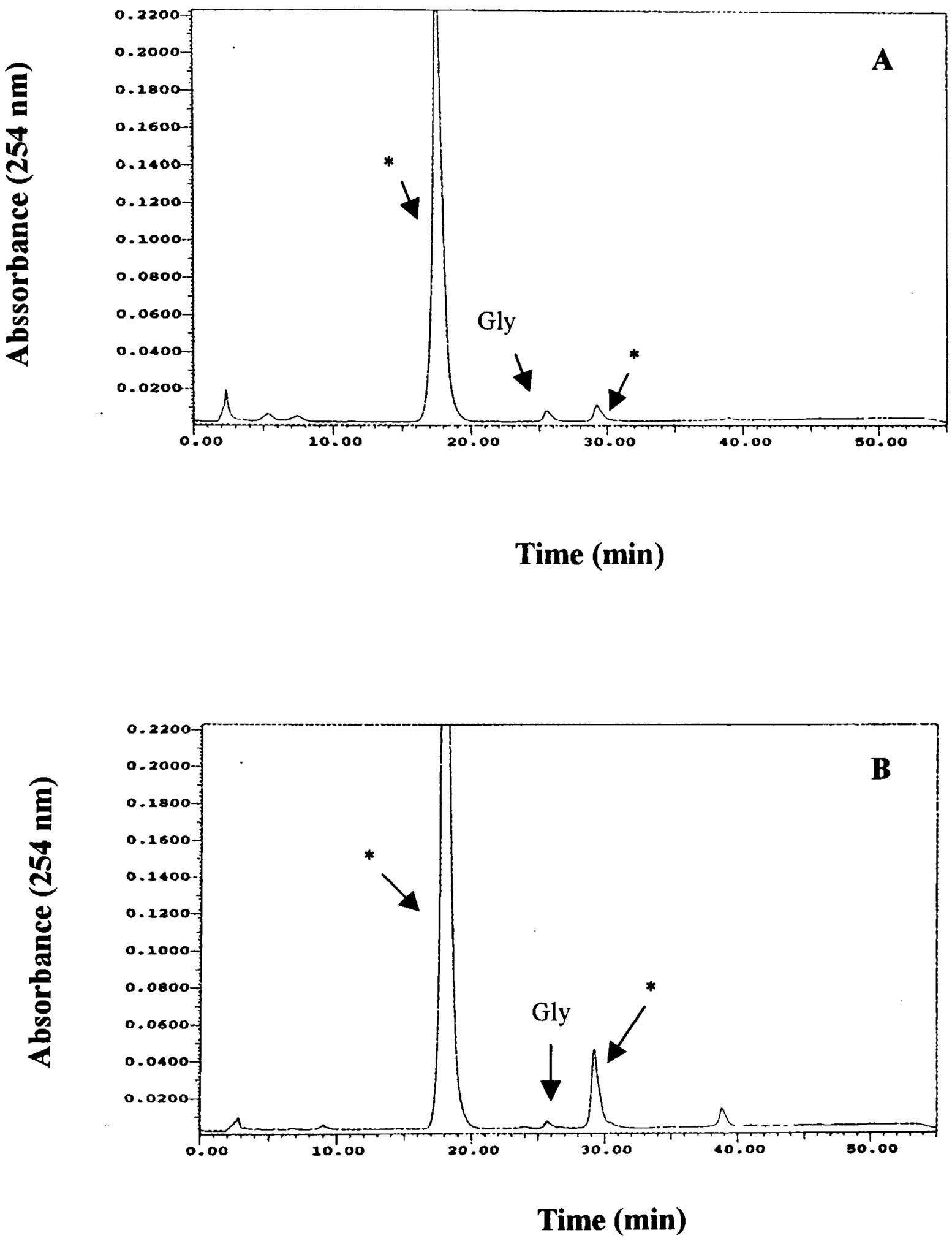
Figure 3.20 Separation of the Dns-derivatized total hydrolysate of E32 using reverse phase HPLC.

The conditions are the same as those shown in Figure 3.19. The peaks marked with an asterisk are Dns hydrolysis products. Unmarked peaks are due to unknown compounds.

After finding that the purified peaks from reverse phase HPLC were due to peptides, the N-terminal analysis of the peptides was performed so as to assess the purity as well as to identify the N-terminal amino acid.

E40, E32 and T40 as well as a control without protein were first Dns derivatized, then hydrolysed and analysed by reverse phase HPLC under the similar conditions as those used for the amino acid composition analysis. The results are shown in Figure 3.21. It can be seen that the three peptides exhibited very similar chromatographic profiles. Compared with the control, two major peaks at 17.5 min and 29.1 min can be clearly identified as by-products derived from the reagent. Another peak at 38.9 min which was also apparently present in all three samples were due to an unknown

compound which had different retention time from any known Dns-amino acids, as were the peaks eluted during the first 10 min. Thus, the peak at 25.6 min was the only one eluted exactly in the position of one of the standard Dns-amino acids (Dns-Gly) and was present in all three peptides. Therefore, glycine was identified as the N-terminal amino acid of all three esterases of E40, E32 and T40.



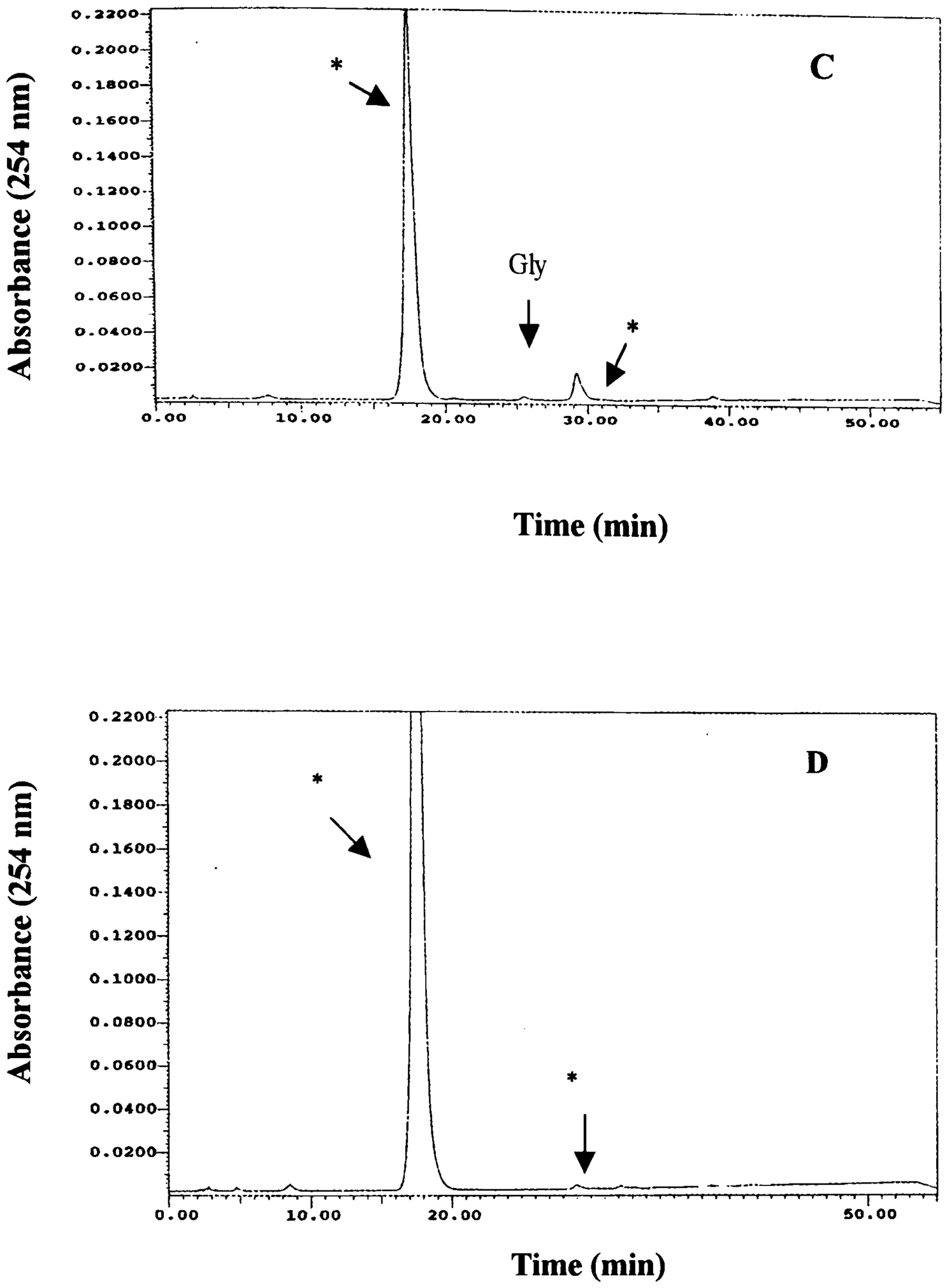


Figure 3.21 Chromatographic profiles for the hydrolysates of the Dns-derivatized product and the identification of the N-terminal residue.

(A), E40; (B), E32; (C), T40; (D), control. The chromatographic conditions are as described in Figure 3.19 except that a 55-min linear gradient from 10-100% B over 50 min was followed by 100-10 % B for 5 min. The peaks marked with an asterisk are

Dns hydrolysis products. Unmarked peaks are due to unknown compounds which have different retention times from any known Dns-amino acids..

3.3.3 Sequence analysis

The amino acid sequences of the esterases were determined by automatic Edman degradation and the results are shown in Table 3.3. It was found that all three esterases consisted of (Gly-Pro-Hyp)n, which is the characteristic amino acid sequence of collagen.

E40 contained 6 amino acids but there was inconsistency between MW calculated from sequence determination (552 Da) and mass spectrometry (510 Da).

The sequence analysis of T40 and E32 did not get to the end of the peptides before the signal faded as the yield of the C-terminal residue is normally low, and it was inferred that there is one more amino acid which is glycine in T40 and there are two more amino acids which are lysine and glycine in E32. The possibility of a lysine involved in E32 is based on 1) there is a di-Dns-Lys peak in Figure 3.20 in amino acid composition analysis of E32 and 2) ESI-MS analysis of E32 (result not shown) indicated that a typical doubly-charged ion was observed in the peptide which suggests there could be a lysine, arginine or histidine in the sequence (Section 4.3.1).

Based on this assumption, the calculated MWs of T40 and E32 from sequence analysis fit exactly with the MWs determined by mass spectrometry.

Table 3.3 Sequence analysis of the esterases

Esterase		Amino acid residue identification																MW (Da)	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	calculated	corrected
E40	Amino acid	Gly	Pro	Hyp	Gly	Pro	Hyp ?											552	?
	Yield (pmoles)	695	437	ND	490	200	ND												
T40	Amino acid	Gly	Pro	Hyp	Gly	Pro	Ile	Gly?										552	609
	Yield (pmoles)	330	228	ND	350	150	100	--											
E32	Amino acid	Gly	Pro	Hyp	Gly	Pro	Hyp	Gly	Pro	Hyp	Gly	Pro	Hyp	Gly	Pro	-- Lys?	-- Gly?	1240	1425
	Yield (pmoles)	500	210	ND	335	180	ND	190	120	ND	130	70	ND	100	60	--	--		

3.3.4 Secondary structure determination by CD spectroscopy

From the sequence data, we knew the esterases were collagen-related peptides. In order to investigate the secondary structures of these peptides, circular dichroism analysis was performed.

The esterase samples (E40, 3.6 μg; T40, 5.1 μg; E32, 5.07 μg) were dissolved in 100 μl ethylene glycol/H_2O (EG/H_2O, v/v, 2:1) solvent and the CD spectrum is shown in Figure 3.22. The three peptides exhibited similar CD spectral shapes and peak positions for the minimum, crossover, and maximum, with a big negative peak at about 200 nm which is similar to that of a collagen-like triple-helical conformation. The small positive peak was also clearly present but at 242 nm, which was red-shifted by about 20 nm compared with a typical collagen-like structure reported (215-227 nm). The peak intensities of E32 were bigger than those of E40 and T40, which was attributed to the longer chain thus with higher triple-helix content in E32.

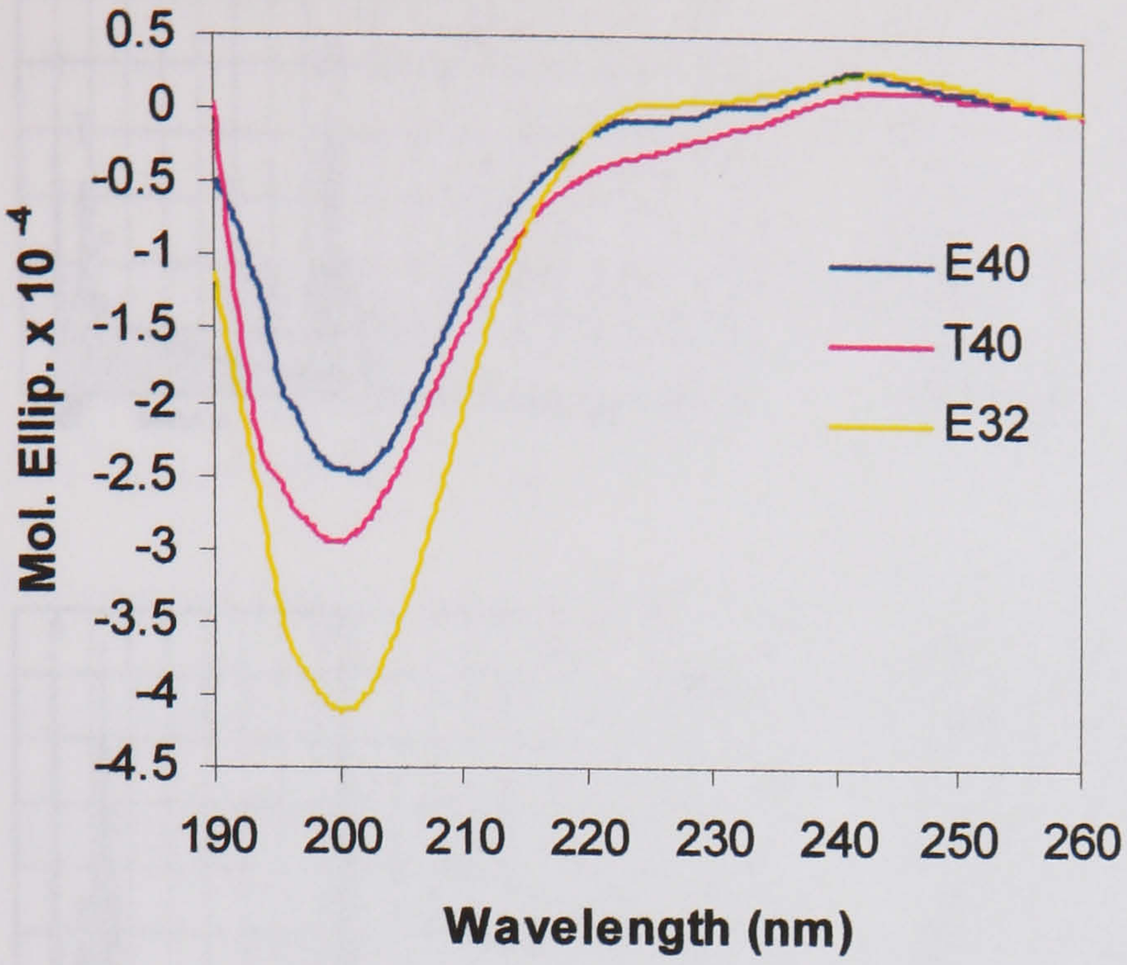
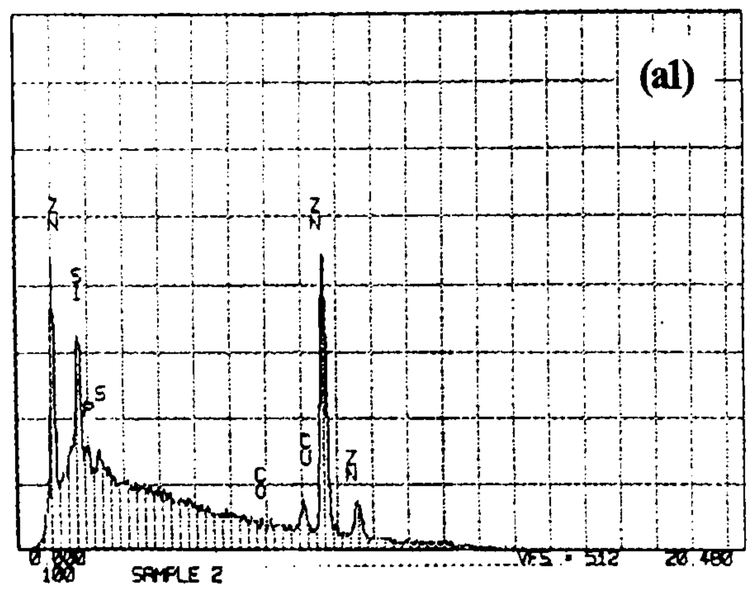
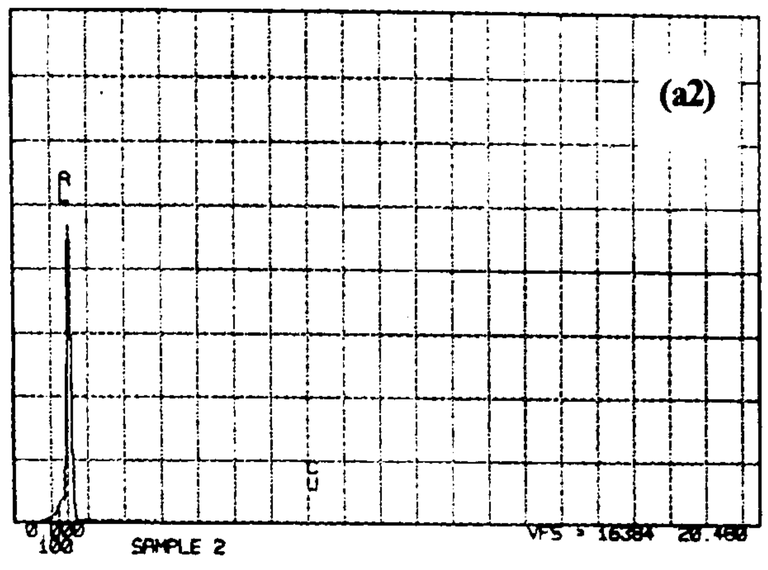


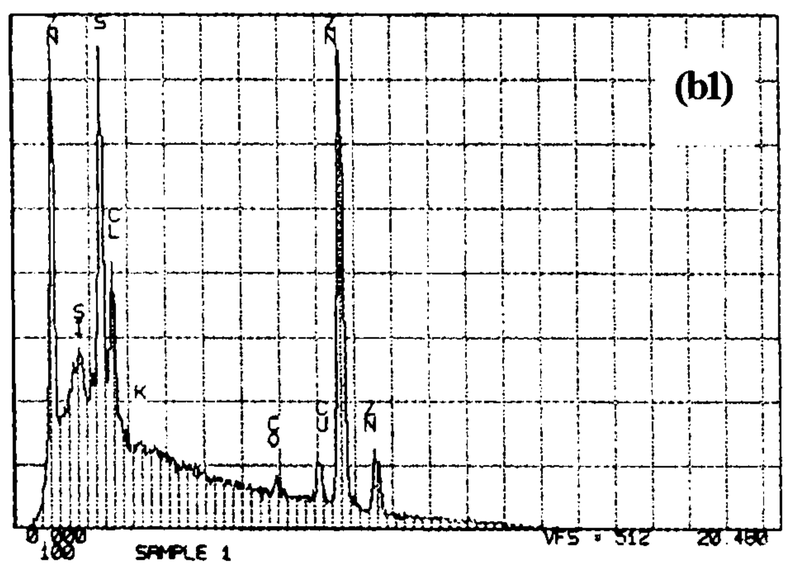
Figure 3.22 CD spectrum of the esterases.

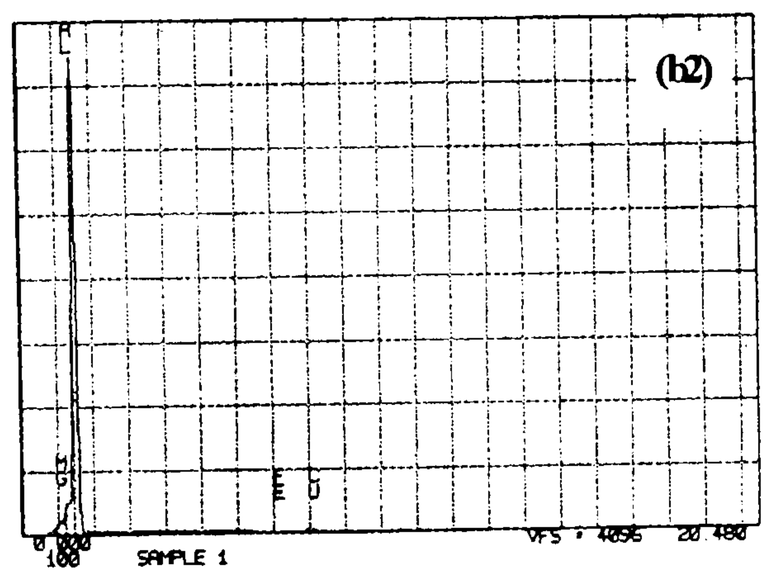
3.3.5 X-ray microanalysis

Qualitative element identification by X-ray microanalysis (Figure 3.23) indicated that all the three esterases contained substantial amounts of Zn, in addition, E40 and E32 were rich in Al as well, which suggests that Zn and Al ions may be bound and involved in the activity and stability of the esterases.









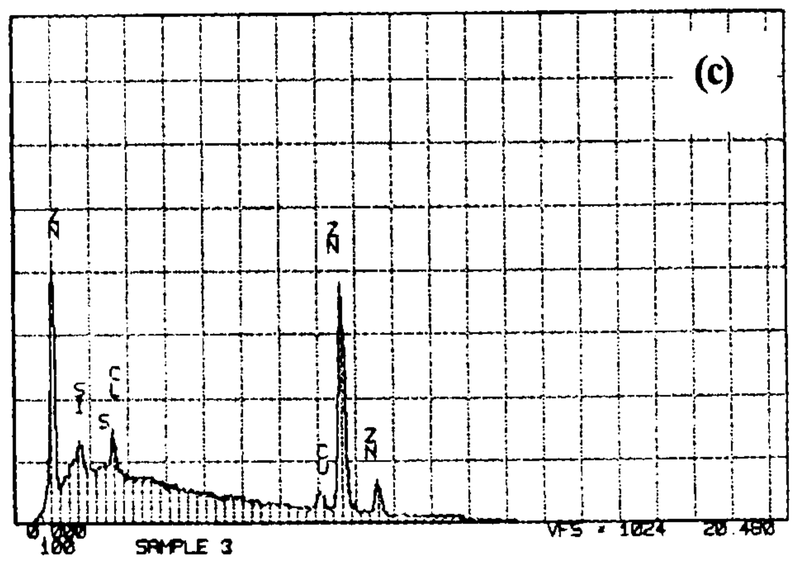


Figure 3.23 Qualitative element identification of the esterases by X-ray microanalysis. (a1) & (a2), different area of the sample in E40; (b1) & (b2), different area of the sample in E32; (c), T40.

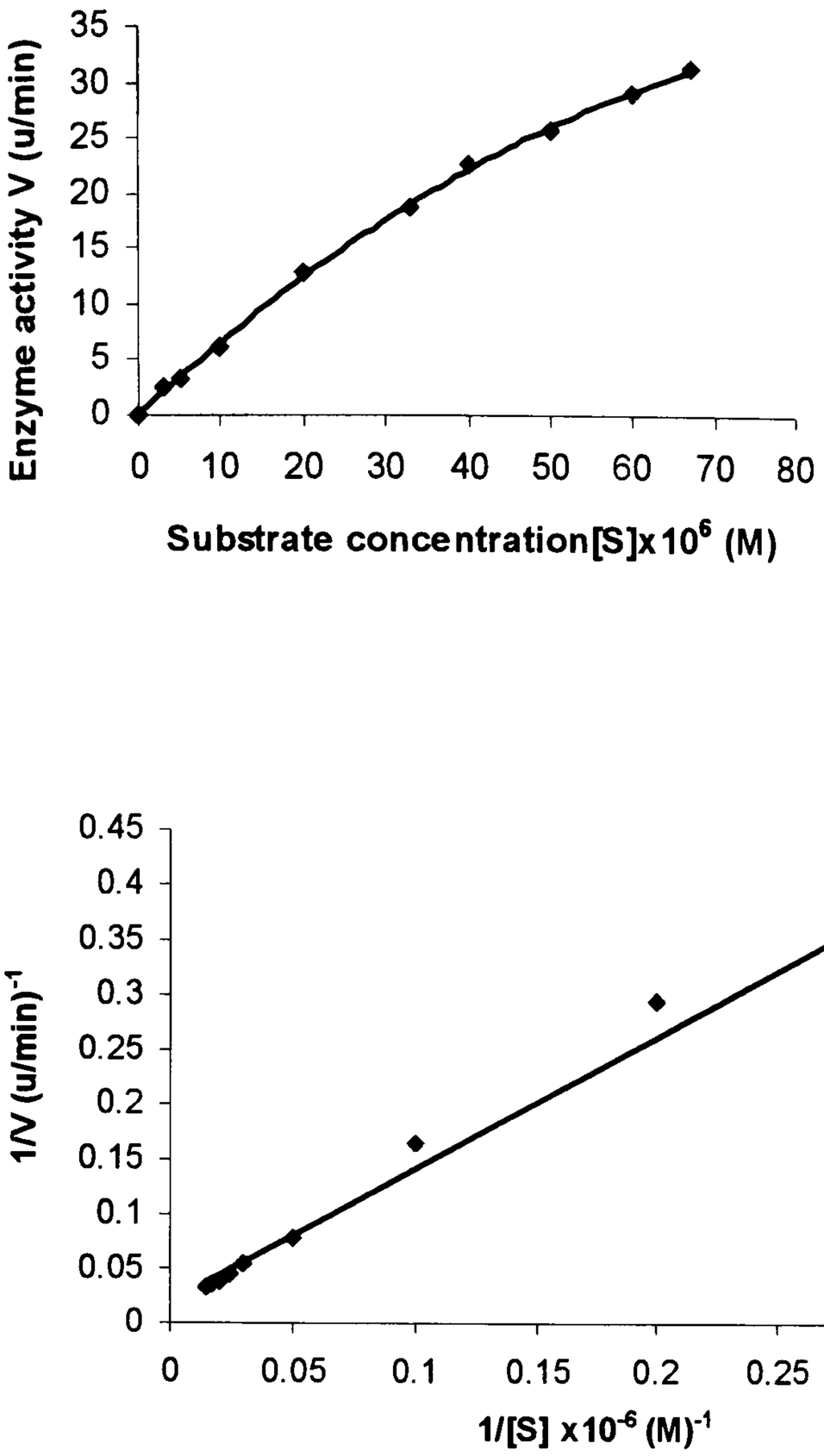
3.3.6 Characterization of esterase activity

E40 was used for all the studies on esterase activity.

3.3.6.1 Catalytic activity of the esterase

The dependence of the hydrolysis rate of the esterase on the concentration of fluorescein dibutyrate was shown in Figure 3.24A. Figure 3.24B is the reciprocal plot of the values obtained. K_m and V_{max} values were 5.87×10^{-5} M and 6.5×10^{3} u/min.mg respectively.

From the linear correlated plot of fluorescence units against concentration of fluorescein, it was calculated that one unit change in fluorescence was corresponded to 1.8×10^{-4} µmol produced. Thus, estimated V_{max} and K_{cat} were 1.17 µmol/min.mg and 2.98×10^{-2} s^{-1} respectively.



igure 3.24 Catalytic activity of the esterase.

A, effect of substrate concentration on the activity of the esterase; B, Lineweaver-Burk reciprocal plot of esterase activity and fluorescein dibutyrate concentration.

3.3.6.2 Thermostability of the esterase

The esterase was extremely heat stable. After incubation for 2 hours at different temperatures, the esterase showed no activity loss up to 110 °C, whereas significant

inactivation began to happen at 120 °C with more than 20% activity decrease (Figure 3.25A).

As a contrast, Figure 3.25B shows thermostability of the high MW esterase from *E.chevalieri* (HEEC) as mentioned in Section 3.1.1. It was obvious that thermostability of HEEC was not high as the esterase microenzyme, but HEEC was also a relatively thermostable esterase, retaining more than 60% activity at 65°C.

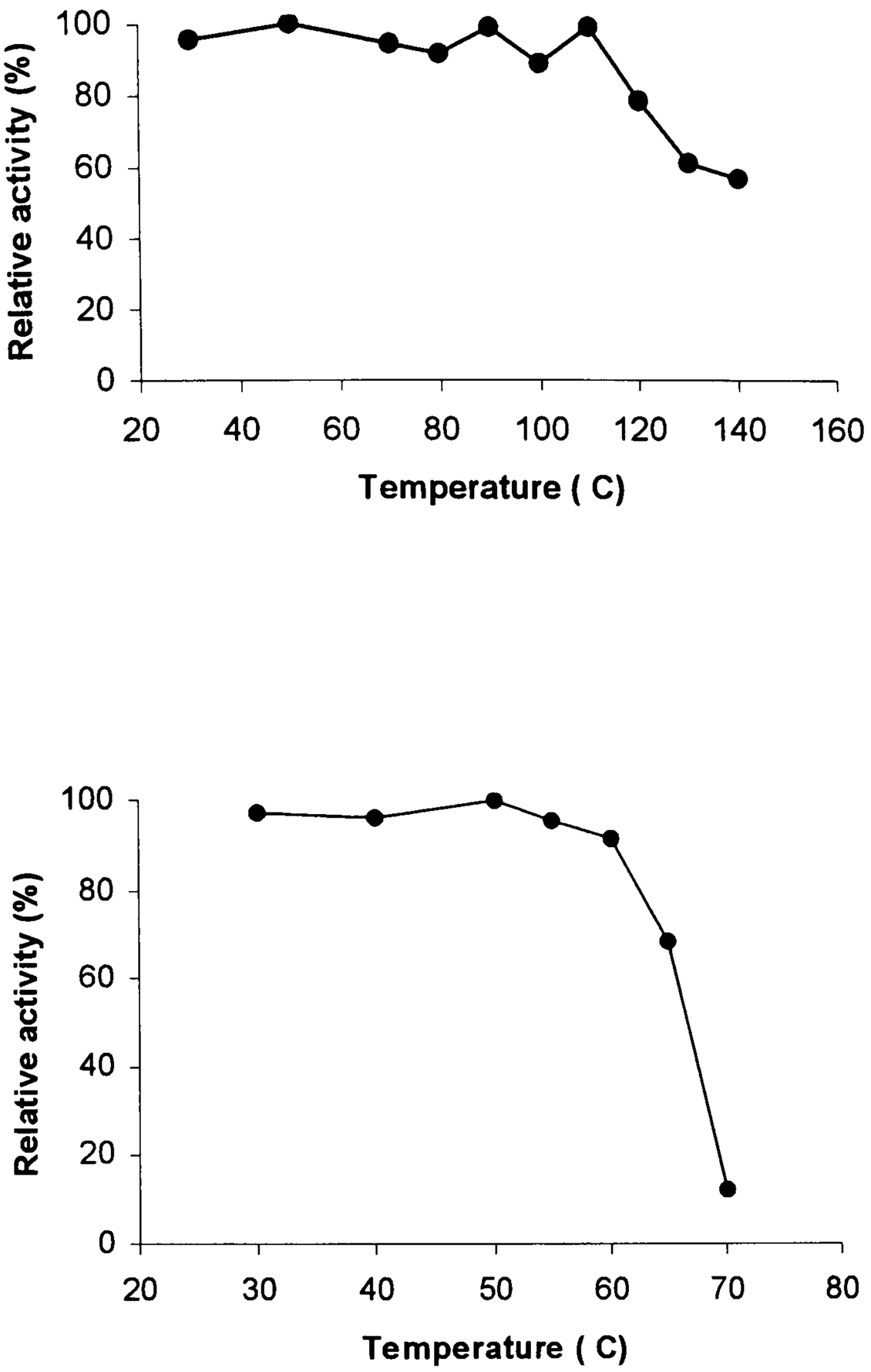


Figure 3.25 Thermostability of the esterase.

A, microenzyme; B, HEEC.

3.3.6.3 Effect of pH on the esterase

The esterase was stable over a wide range of pH after incubation for 2 hours in buffers at different pH values (Figure 3.26A), with no activity loss either in acid or neutral pH, which was consistent with the high stability of enzyme activity observed under the extreme acid pH elution condition (0.1% TFA, pH 2) during RP-HPLC purification. It seems the esterase gradually began to lose the activity after pH 9.0, but the inactivation was not great with only 22% decrease in activity at pH 11. Figure 3.26B shows the pH stability of HEEC as a contrast. HEEC was only stable at pH between 7 and 9 and lost activity at pH<4 and pH>10.

The pH profile of activity (Figure 3.27A) indicated that the esterase was remarkably active at alkaline pH, showing optimum activity at pH 11 and with no activity at pH<6. The curve is characteristic for one ionising group involved in catalysis; it can be inferred from the graph that the group has a pK of 8.5. Figure 3.27B is the pH activity profile of HEEC, with optimum activity at pH 6.5.

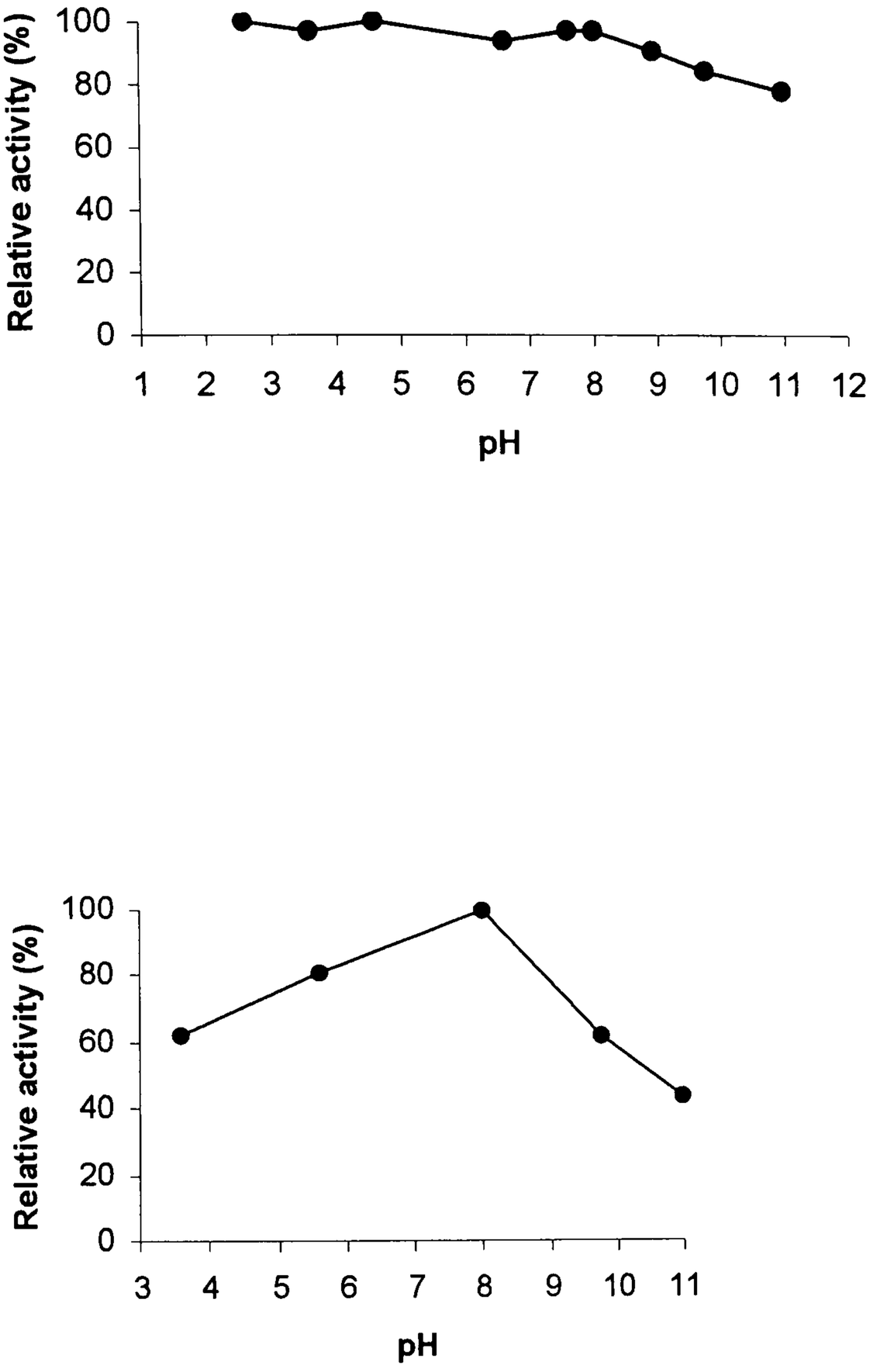


Figure 3.26 The pH stability of the esterase.

A, microenzyme; B, HEEC.

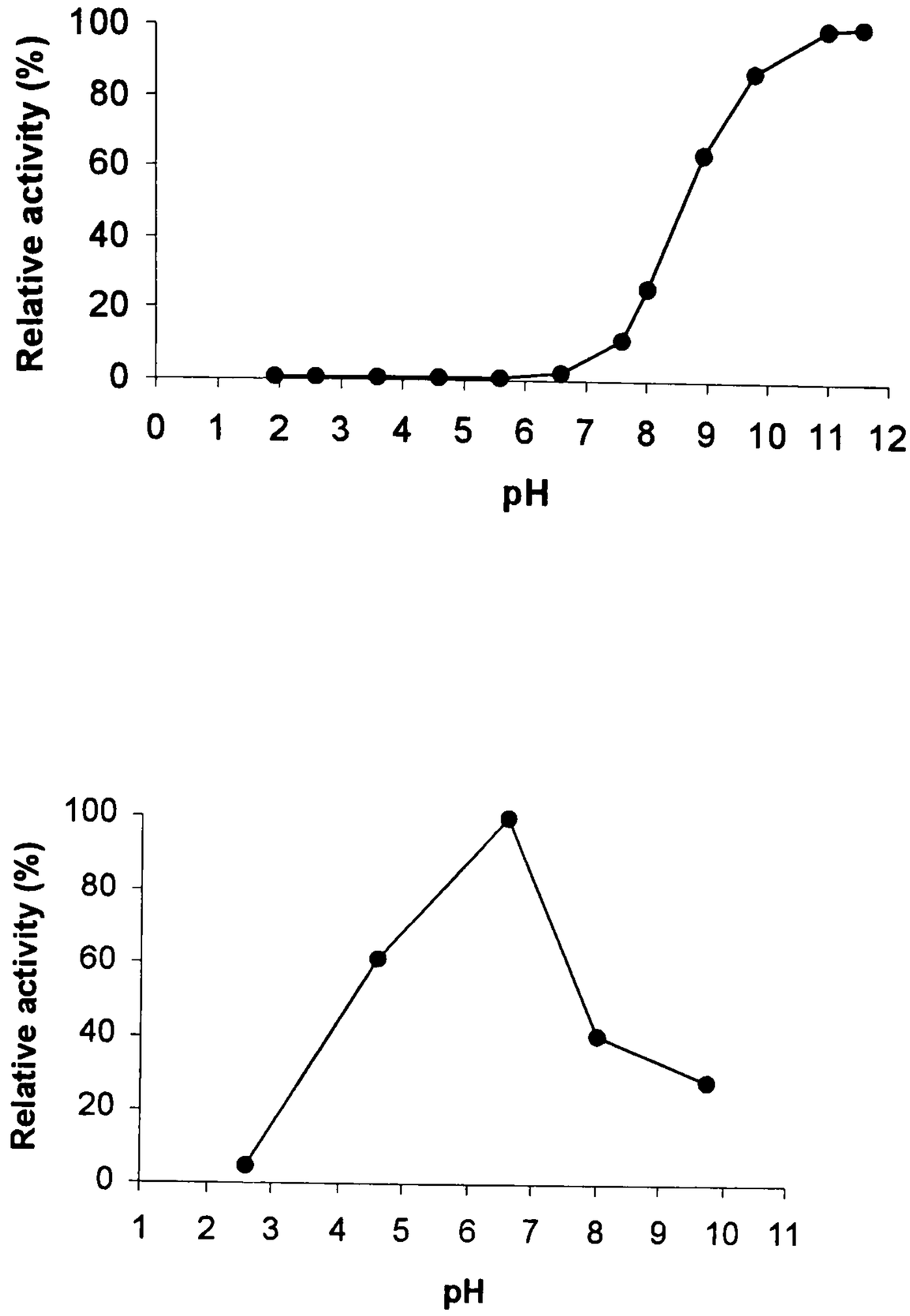


Figure 3.27 Effect of pH on the esterase activity.

A, microenzyme; B, HEEC

3.3.6.4 Positional specificity

The positional specificity of the esterase was determined using mixed substituted triglyceride as substrate. The products of hydrolysis were recovered and the fatty acids were identified and measured by GLC. Figure 3.28 shows the GLC elution of

esterified products of free fatty acids produced by the esterase. The standards used were palmitic acid methyl ester (C16) and oleic acid methyl ester (C18), with elution time at 7.88 and 11.02 min respectively (Figure 3.28A).

After 2 hr incubation with the substrate, the esterase released much larger quantities of C16 than C18 (Figure 3.28B). As a comparison, HEEC and a commercial lipase (Figure 3.28C & D) gave a similar profile, with much more C16 hydrolysed than C18. This suggests that the esterase microenzyme, like most of the esterases found, hydrolysed preferentially α-positions to β-positions in triglycerides.

Another possibility which can not be excluded is that these esterases or lipases released fatty acids of 16:0 more readily than those of 9-18:1 due to the enzymes specificity for saturated and unsaturated fatty acids or specificity for fatty acid chain length (C16 and C18).

Despite this possibility, this result reflected the fact that the esterase microenzyme was also able to hydrolyse triglycerides and with the same positional specificity which could be 1,3-specific as the high molecular weight esterase from *E. chevalieri* and a commercial lipase.

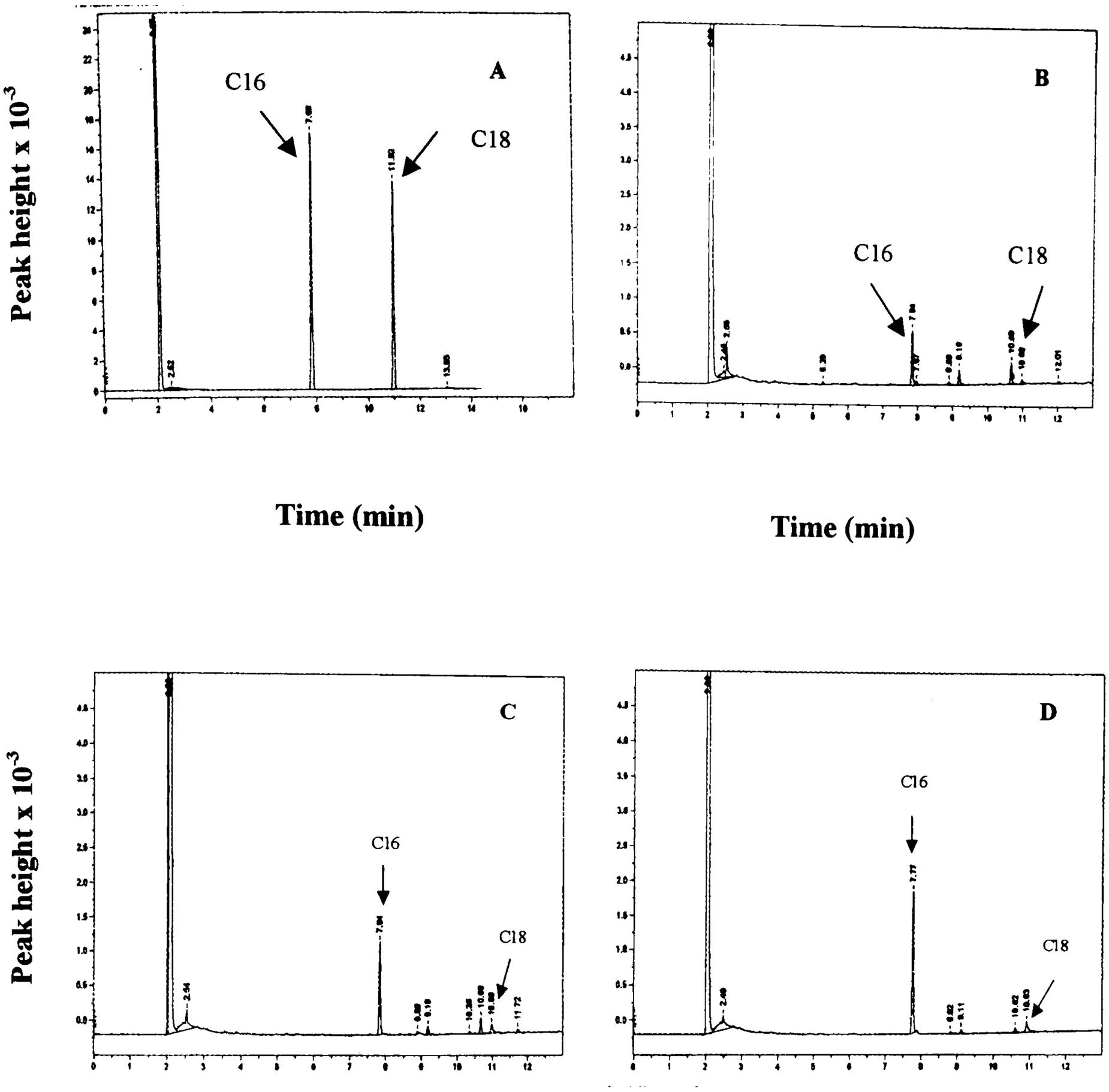


Figure 3.28 GLC analyses of the reaction products by esterase hydrolysis.

A, standards; B, esterase microenzyme; C, HEEC; D, commercial lipase.

3.3.6.5 Substrate specificity

The esterase was active toward fluorescein esters of various chain lengths. It prefers

short chain (C3, C4, C6) to long chain (C8, C12) fluorescein esters and the activity of C8 or C12 were about 35% that of C3 (Figure 3.29).

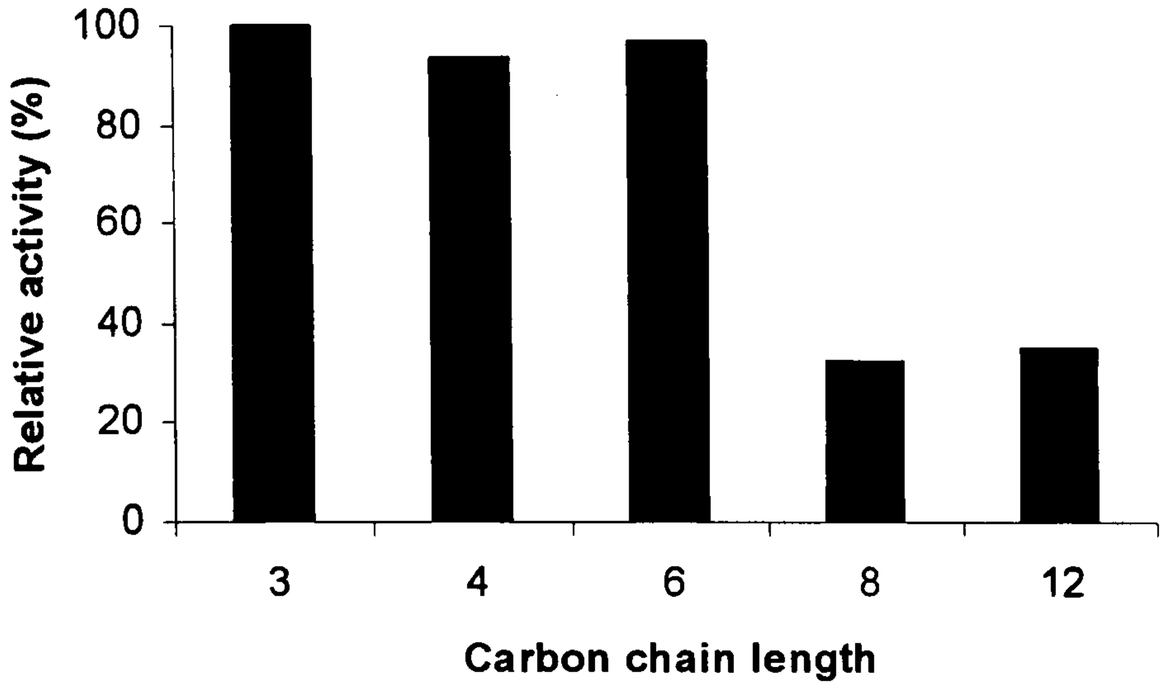


Figure 3.29 Esterase activity for several fluorescein esters of different chain length.

3.3.6.6 Effect of dialysis on enzyme activity

When the esterase was dialysed against distilled water, 0.025 mM EDTA or 2% Chelex 100, activity decreased markedly. Figure 3.30 shows the effect of 24 hours dialysis of 1 ml of the esterase solution against 400 ml of water, EDTA or Chelex 100. Although the data has not been corrected for manipulative losses, the higher loss of activity when dialysed against EDTA and Chelex 100 (87% and 83%) than against water (51%) suggests that the esterase contains a dialyzable component which might be a metal ion and this component is essential to maintaining enzyme activity.

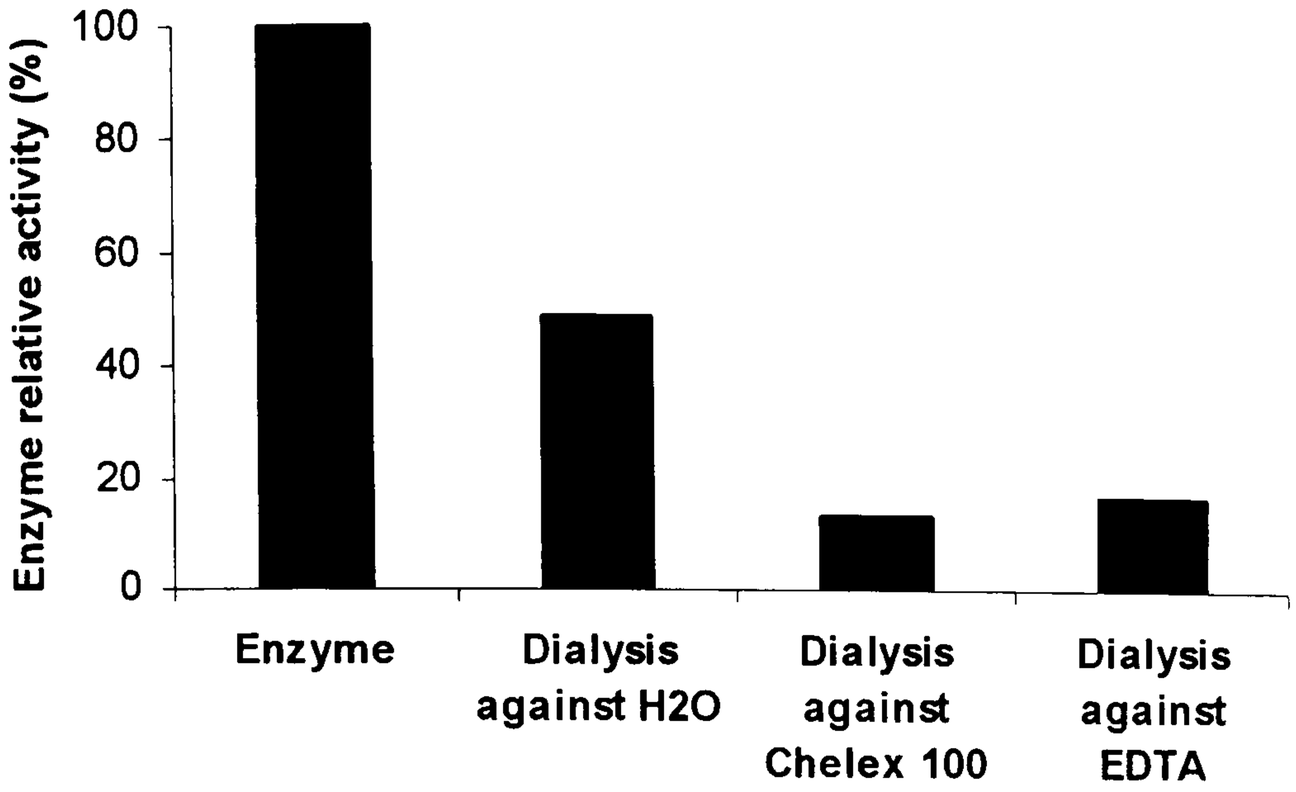


Figure 3.30 Effect of dialysis on enzyme activity.

3.4 CHEMICAL SYNTHESIS OF THE ESTERASE MICROENZYME

3.4.1 Solid-phase synthesis of the esterase peptide

After knowing the sequence and characteristics of the esterases, an attempt was made to chemically synthesize the esterase microenzyme by Solid Phase Peptide Synthesis. The peptide sequence synthesized was: NH_2-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-COOH (synthetic peptide, SP).

The C-teminus of the peptide was attached to a polymeric support and the synthesis was achieved by repetitive addition of N-protected amino acids by coupling and deprotection. The progress of each deprotection reaction was followed by monitoring the release of the cleaved Fmoc group at 300-320 nm and the peak area gave a crude measure of the coupling efficiency.

The reasonably consistent deprotection peak areas of each step with maximum peak area degradation of 7% (Table 3.4) indicated that there was no marked slowing of successive reactions and the couplings had proceeded satisfactorily.

Table 3.4 Successive deprotection peak areas in the synthesis of the SP

Amino acid	Area	Height	Peak area degradation (%)
Gly	121.18	--	
Hyp	121.43	2.043	0
Pro	113.72	2.031	7
Gly	114.30	2.309	0
Hyp	110.35	2.061	4
Pro	109.39	2.110	1
Gly	111.85	2.301	-2

3.4.2 HPLC and conformation analyses of the synthetic peptide

The efficiency of SPPS and the purity of the synthetic peptide were determined by reverse phase HPLC using the same condition as for the rechromatography of E40 in Figure 3.12 in Section 3.2.1. HPLC analysis (Figure 3.31) indicated the purity of synthetic peptide was >98% and the peptide synthesis was successful. The elution time of the synthetic peptide was 8.6 min, which was close to that of native esterase E40 (9.0 min).

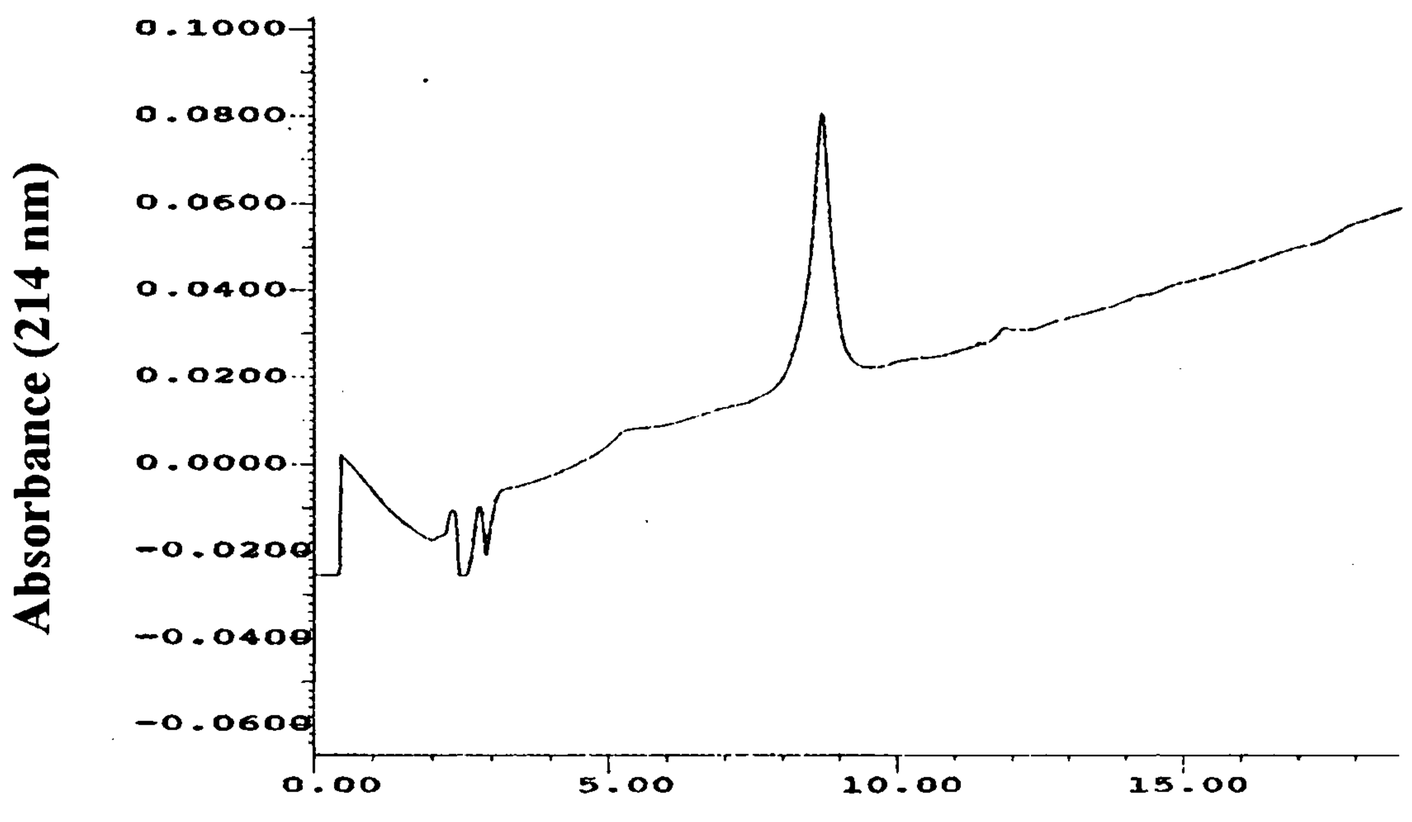
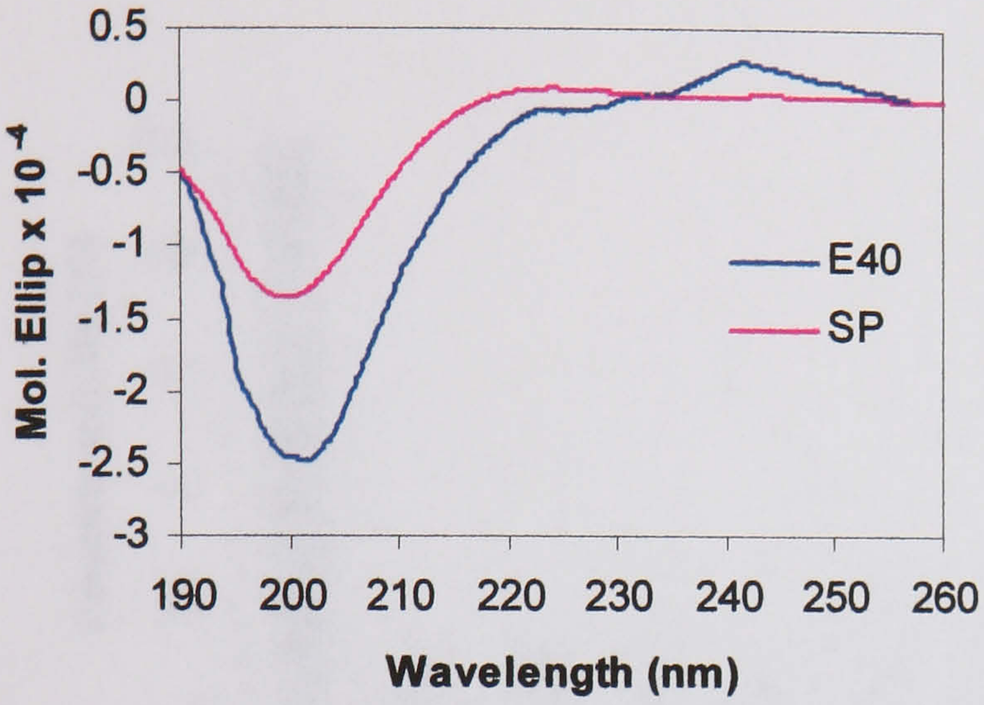


Figure 3.31 HPLC analysis of the synthetic peptide.

The conditions were same as those in Figure 3.12.

105 μg of the synthetic peptide was dissolved in 100 μl EG/H_2O (v/v, 2:1) and the CD analysis was performed under the same conditions as the native esterases. The CD spectrum of the synthetic peptide (Figure 3.32) exhibited a negative band but smaller than that of E40 at about 200 nm although the concentration of the synthetic peptide

was higher than that of E40, no positive band was seen in the region of 220 to 245 nm. This suggested that the synthetic peptide could be present in monomer form rather than in triple helical structure.



Fgure 3.32 CD spectrum of the synthetic peptide (SP) as compared with that of native esterase E40.

3.4.3 Esterase activity of the synthetic peptide

The synthetic peptide did not exhibit detectable activity over the concentrations tested (34~340 μg/ml).

In order to investigate if the metal ions can restore the activity, 2.5, 0.25 and 0.025 μmol Zn^{++}, Al^{+++} and Fe^{+++} were respectively mixed with 0.25 μmol synthetic peptide. However, no esterase activity was obtained.

The attempt for activity recovery by incubating the synthetic peptide and metal ions for

68 hours at 4°C to reach equilibrium for triple helix formation was also unsuccessful.

Figure 3.33 shows the results of these treatments.

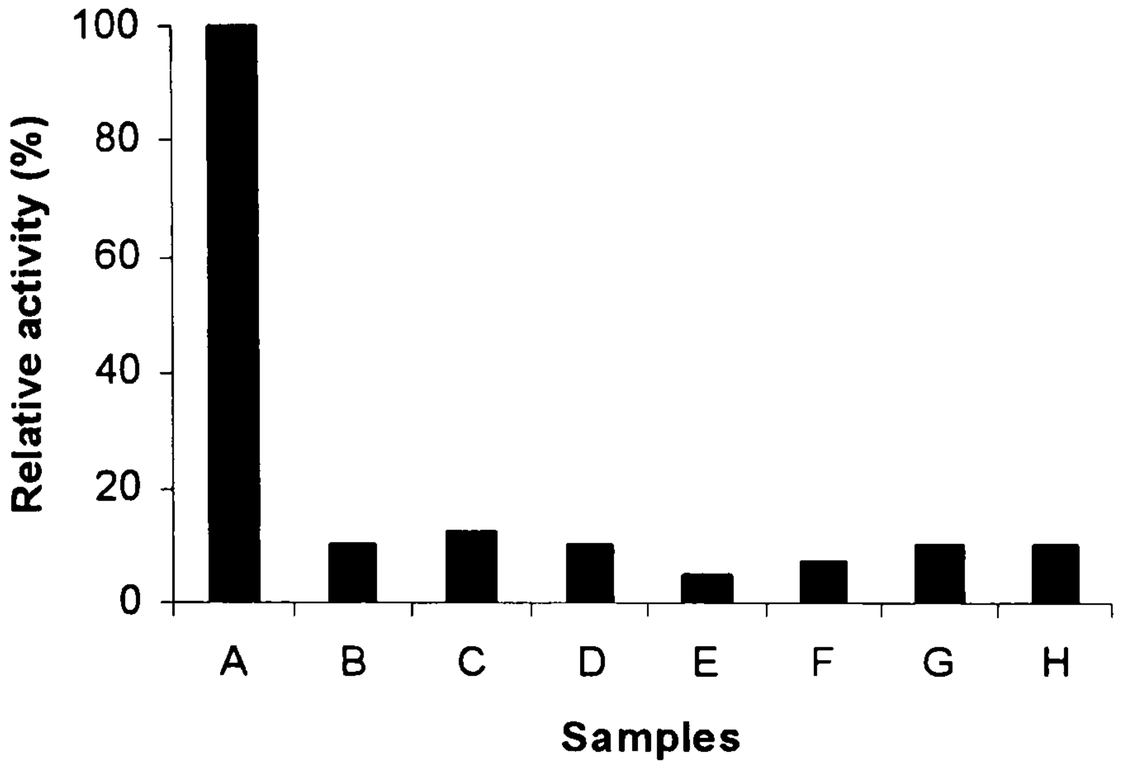


Figure 3.33 The esterase activity of synthetic peptide and the effects of metal ions on the activity of synthetic peptide*.

A, native enzyme; B, synthetic peptide (SP); C, SP + Zn^{++}; D, SP + Al^{+++}; E, SP + Fe^{+++}; F, SP + Zn^{++} + Al^{+++}; G, SP + Zn^{++} + Al^{+++} (after incubation at 4°C for 68 hours); H, control (buffer only).

*For the effects of metal ions, 0.25 μmol synthetic peptide was incubated with 0.25 μmol different metal ions.

Adding metal ions to the synthetic peptide gave the same CD spectrum as synthetic peptide (Figure 3.34). This suggested that the addition of the metal ions under the conditions used did not change the conformation of the synthetic peptide, which

was consistent with the result that adding metal ions had no effect on restoration of the activity of the synthetic peptide.

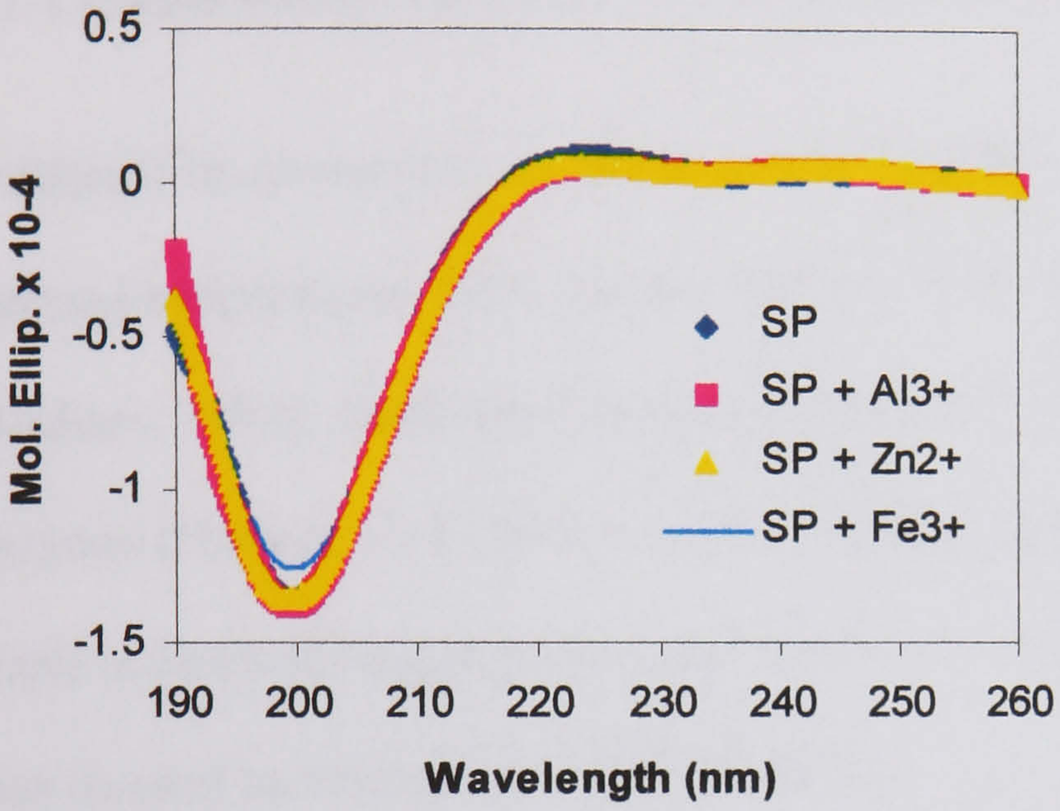


Figure 3.34 CD spectrum for the effects of metal ions on conformation of the synthetic peptide.

CHAPTER 4. DISCUSSION

4.1 SCREENING AND IDENTIFICATON OF MICROENZYMES WITH ESTERASE ACTIVITIES FROM THERMOPHILIC FUNGI

4.1.1 Extracellular esterase activities in thermophilic fungi

Proteins from thermophiles generally exhibit high thermodynamic stability, both at elevated temperatures and in organic solvents (Jaenicke and Závsdosky, 1990; Mozhaev, 1993), which are important properties for the industrial application of enzymes (Herbert, 1992; Brock, 1985). Although many esterases from mesophiles are stable at elevated temperatures (Sugihara *et al.*, 1991; Gilbert *et al.*, 1991), our work was focused on esterases from thermophiles.

Among the nine thermophilic fungi screened, the six which showed large clear zones on tributyrin agar plates also exhibited esterase activities in malt extract fermentation medium. This indicated that the esterase activities observed in the six fungi were all extracellular.

The extracellular esterase activities in these thermophilic fungi were growth-related. The maximum secretion of the esterase by five organisms at the stationary phase of growth is in accordance with the findings of several other authors (Jacobsen *et al.*, 1989), while the behaviour of *T. emersonii* in having a low esterase production may be explained as 1). This organism could produce some inhibitor which interfered with the

enzyme activity; or 2). The microenzyme produced at the early growth stage was then reused by the organism for its rapid growth.

The Microsep filter with molecular weight cut-off 10 kDa was proved a convenient and reliable tool to screen the microenzymes when proper attention was given to the speed and time of centrifugation.

Among the six esterase-producing fungi, only *E. chevalieri* produced high MW esterase active towards fluorescein dibutyrate. The esterase activities observed in the other five fungi were all due to a low MW enzyme with MW less than 10 kDa. Apart from the high MW esterase with relatively high activity, *E. chevalieri* also produced a microenzyme esterase but with low activity compared with microenzymes from other organisms.

An interesting observation was that the microenzyme esterase activities were lower in *E. chevalieri* and *T. emersonii,* which had higher biomass, than in the other four fungi. Possible explanations are:

1). The fermentation conditions used which greatly stimulated cell growth in these two organisms were unfavourable to the microenzyme esterase production, but could be favourable to the high MW esterase production in *E. chevalieri;*

2). Microenzyme esterase production required the nutrients which had been utilized for rapid cell growth;

3). The large amount of metabolites produced by these two organisms with their rapid growth may contain some inhibitor(s) which repressed the activities of the small esterases; or

4). These inhibitor(s) might interfere with the fluorescent assay used for enzyme activity determination.

4.1.2 Native gel electrophoresis

Native PAGE indicated that the six esterase-positive fungi all exhibited bands that were active to an esterase stain as well as staining for protein at the dye front in 12% separating gel in Laemmli's buffer system, which suggested that these organisms could all produce a low MW enzyme with MW less than 10 kDa which hydrolysed α-naphthyl acetate.

It was also noticed that no high MW esterase band was seen in *A. corymbifera; E. nidulans; T. emersionii* and *R. pusillus*. Thus, the esterase activities observed in these four organisms were entirely attributed to the low MW microenzymes.

In addition, *E. chevalieri* and *H. lanuginosa* also produced high MW esterases in addition to their microenzyme esterases. *E. chevalieri* secreted many high MW extracellular proteins and two (or one) of them were esterases. The high MW esterase(s) in *E. chevalieri* can hydrolyse both fluorescein dibutyrate and α-naphthyl acetate. However, the high MW esterase from *H. lanuginosa* was only active towards

α-naphthyl acetate, probably due to substrate specificity, or the inhibition effects of some metabolites on the fluorescent assay.

From native PAGE, together with the results in Figure 3.3, preliminary conclusion may be drawn that very low MW esterases were present in the six thermophilic fungi screened although the activities were different to some extent, and these esterases can catalyse the hydrolysis of both fluorescein dibutyrate and α-naphthyl acetate.

4.1.3 Esterase activities in *E. nidulans*

Although *E. nidulans* is a genetically well characterized fungus, few studies on esterases have been reported in this organism. Kawasaki *et al.* (1995) reported an extracellular lipase produced by *E. nidulans* when grown in solid or liquid cultures containing lipids as carbon source. This lipase is glucose-repressed and has a MW of 35 kDa. The study done by García-Lepe *et al.* (1997) indicated that *Aspergillus nidulans* 2544 exhibited a significant lipase activity in its autolysed culture.

The extracellular esterase activity observed in *E. nidulans* used in this work was due to a small esterase with MW less than 10 kDa.

The excellent electrophoresis system of Laemmli (1970) has an insufficient resolving power below 10 kDa. Several SDS-PAGE systems capable of separating peptides of MW below 10 kDa have been reported, which include using separation gels with urea (Swank and Mundres, 1971; Anderson *et al.*, 1983), highly concentrated and crosslinked polyacrylamide (West *et al.*, 1984), or steep polyacrylamide gradients

(Fling and Gregerson, 1986) in order to achieve successful resolution of the peptides. More recently, Schägger and Von Jagow (1987) published a non-gradient Tricine-SDS-PAGE system without urea, exhibiting high-resolving power especially in the MW range of 1-20 kDa. In this system free amino acids like glycine are omitted and only moderate acrylamide concentrations are needed. This method has been proved efficient and successful in resolving small proteins (Schägger and Von Jagow, 1987, Ploug *et al.*, 1989, Schägger, 1994), and has been widely used by researchers.

When the Schägger and Von Jagow Tricine-SDS-system was used in this study, it has also been found to exhibit a good separation for the less than 10 kDa fraction of crude extract from *E. nidulans*.

Native-Tricine-PAGE revealed a broad band when stained for esterase activity, which corresponded to protein bands in protein stain gel. This suggested the esterase activity in *E. nidulans* was very probably related to one of the proteins. The reason for the broad band might be due to the small size or specific structure features of the microenzyme, a similar behaviour was found in an extremely basic pH-tolerant extracellular lipase from *Bacillus subtilis* 168 (Lesuisse *et al.*, 1993). Actually, as discussed in Section 4.3.4.2 and Section 4.3.4.3, the microenzyme possessed some other properties similar to this enzyme, such as basic pH optimum and stability, as well as positional specificity.

Tricine-SDS-PAGE was performed at this stage to estimate the MW range of the esterase. It was found that protein bands exhibited very similar profile in SDS gel

and in native gel, which suggested that SDS had no effects on the proteins. And actually it was noticed that the esterase retained its activity in the Tricine-SDS-PAGE system. A similar phenomenon was observed by Kuo and La Du (1995) when a rabbit serum paraoxonase (PON) was compared with human PON. It was found that the rabbit monomeric species retains some enzymatic activity after SDS-PAGE, but a dimer is the smallest unit to retain activity with the human PON, after non-denaturing acrylamide gel electrophoresis. Rabbit PON seems more resistant to SDS-denaturation than the human esterase.

Thus, the MW of the esterase was estimated to be between 2.5 kDa and 6.2 kDa by gel electrophoresis at this stage.

Bio-gel P-10 gel filtration revealed that two esterases may be present in the less than 10 kDa fraction of *E. nidulans,* of which the smaller one (1.6 kDa) accounted for most of the esterase activity observed in this organism while the bigger one (4.1 kDa) has with weaker activity.

From these results, we knew that the esterases produced by *E. nidulans* were microenzymes with MW less than 6.2 kDa. Accurate MW could not be obtained until further purification.

Multiple peptide peaks were present in the active fractions from gel filtration suggesting that the esterase, with its extremely low MW, was co-eluting with peptides from the malt extract medium.

The low esterase activities found in the complex medium were not further investigated, but a similar observation and study have been done by Bartolome *et al.* (1996), who found the esterase activity present in malt extracts, and partially purified a ferulic acid esterase. The esterase was active upon the cinnamic acid methyl esters as well as acetyl esters.

4.1.4 Extracellular esterase activities of thermophilic fungi in synthetic medium

The defined media described by either Valero *et al.* (1988) or Jacobsen *et al.* (1989) did not give satisfactory results either in cell growth or extracellular esterase production with the thermophilic fungi. Improving the Czapek medium (Atlas, 1993) by substituting yeast extract with urea (Valero *et al.*, 1988, Jacobsen *et al.*, 1989) and using tributyrin as an inducer allowed two organisms *E. nidulans* and *T. emersonii* to grow satisfactorily and to produce extracellular esterases. Little extracellular esterase activities were observed in the other four thermophilic fungi, perhaps because this synthetic medium was not optimal for esterase production in these organisms.

That the extracellular esterase activities of *E. nidulans* and *T. emersonii* in synthetic medium all came from less than 10 kDa fraction was consistent with the observation in malt extract medium. This further confirmed that the microenzyme esterases were present at least in *E. nidulans* and *T. emersonii*.

Among the nine thermophilic fungi screened, two organisms *E. nidulans* and *T. emersonii* exhibited microenzyme esterase activities when grown in either malt

extract medium or synthetic medium. This finding, together with the previous studies on microenzymes (Section1.3.1), implies that such small enzymes may not be uncommon and might be produced by many microorganisms; other low-molecular-weight enzymes may have been overlooked by investigators due to the properties of such microenzymes that either they would not be observed by the routinely used methods for protein purification due to their small size, or the methods for detection of enzymatic activity are not sufficiently sensitive for the microenzymes.

4.2 PURIFICATION OF THE ESTERASE MICROENZYMES

The tangential flow filter Mini-ultrasette (MW cut off 10 kDa) has been proved to be an efficient procedure for microenzyme separation from large sample volumes at the early purification stage when proper attention is given to the pressure and time of filtration (Simões, 1997a). The conditions of filtration used in this work were based on those developed by Simões (1997a).

That esterase activity peaks from *E. nidulans* grown in synthetic medium were eluted by gel filtration at the same fractions as those in malt extract medium further supported the concept that the esterase activity observed was from the organism itself rather than from some substances in the medium.

The esterase microenzymes were stable in organic solvents and low pH, as they retained the activities when purified using reverse phase HPLC with 0.1% TFA and elution with an acetonitrile gradient. Similar stability was found in a low MW (13.9

kDa) phospholipase A_2 from developing elm seeds (Ståhl *et al.*, 1998) and several low MW (13 to 15 kDa) secretory phospholipase A_2s from animal tissues (Forst *et al.*, 1986; Kramer *et al.*, 1989).

Optimisation of the method for reverse-phase HPLC separation of microenzyme was performed by 1) changing pH of the mobile phase; 2) using different ion pairing agent; 3) changing flow rate; and 4) changing the elution gradient.

The pH changes the charge distribution in the sample molecules. Charge functional groups are generally hydrophilic, but when a charge is titrated out, the same functional group can exhibit significant hydrophobic character. The pH of the mobile phase can thus have a significant effect on the selectivity of reverse-phase during elution. As the silica-based column is unusable above pH 8, high pH elution was performed on the stable, polymeric Hamilton PRP-3 column. No evident improvement was attained in separation of E40 when the pH was changed from acid to basic conditions, which implied that no acidic or basic amino acid residue existed in this microenzyme. Subsequent sequence analysis proved this to be so.

Ion pairing agents, which are molecules with both charge and hydrophobic functionality, can modify the effect of charge groups on sample molecules. Ion pairing agents enhance the interaction of charge groups with the surface, by binding hydrophobically to the bonded phase surface and ionically to the charge groups of the sample molecules. Different ion pairing agents can provide very different selectivity in reverse-phase chromatography. TFA is the most common ion pairing agent used

with silica reverse-phase packing. It is a good ion pairing agent with amine functionalities and is such a strong acid that it suppresses the ionisation of silanols and helps to solubilise difficult peptides and proteins. TFA was also proved to be better than the relatively hydrophobic ion pairing agent HFBA in the separation of microenzymes.

When changing flow rate from 0.25 to 2 ml/min, it was found that a flow rate of 1 ml/min exhibited the best separation. Lower or higher flow rates caused either increased band spreading and longer elution time or inefficient peak resolution.

Reversed-phase separations of peptides show the best resolution with a very shallow gradient. There is a very narrow range in solvent concentration over which a given molecule goes from no elution at all to no binding at all. Very slight shifts in solvent concentration can greatly affect retention, so a shallow gradient is more efficient than steep or isocratic elution. As the gradient slope is decreased, molecules tend to elute at a lower solvent strength.

After a 0-30% acetonitrile gradient was set for the separation of E40, the decrease of the gradient slope required the increase of the duration. Increased duration resulted in increased resolution. Therefore, it was obvious that peaks showed better resolution in 0-30% B over 40 min (Figure 3.11) than over 10 min (Figure 3.10).

By using method 4 (Section 3.2.1), the three microenzymes were eluted at different time and acetonitrile concentrations with E40 at 13.8 min (10.1% acetonitrile), T40 at

20 min (15% acetonitrile) and E32 at 25 min (18.6% acetonitrile). This indicated that the three microenzymes separated were different, although E40 and T40 were eluted at the same position by gel filtration. The hydrophobicity was: E40 < T40 < E32.

During the purification of the esterase from *T. emersonii* by gel filtration, the fact that only one esterase active peak (T40) with the same MW as the major peak (E40) from *E. nidulans* was obtained implied that these smaller enzymes may account for most of esterase activities observed in the thermophilic fungi.

4.3 CHARACTERIZATION OF THE ESTERASE MICROENZYMES

4.3.1 Molecular weights of the microenzymes

Gel filtration revealed that the MWs of the microenzymes in their native form were: E40 and T40, 1.6 kDa; E32, 4.1 kDa.

Tricine-SDS-PAGE indicated that MWs of the microenzymes in their "denatured" form were: E40 and T40, 3.1 kDa; E32, 3.9 kDa.

It has been noted by some researchers (Kratzin *et al.*, 1989, Ploug *et al.*, 1989) that when using Schägger and Von Jagow (1987) or Swank and Munkers (1971) electrophoresis system to determine the molecular weights of less than 10 kDa proteins, substantial discrepancies could happen with polypeptides smaller than 6 kDa where unique structural features have an important effect on electrophoretic mobilities. For example, the B-chain of insulin (3.5 kDa) was found to migrate like a 7 kDa

protein and bradykinin could only be detected as a very broad band at much higher apparent molecular mass. The discrepancy was also observed in this case as the esterase molecular weights of E40 and T40 measured by gel filtration were 1.6 kDa but were 3.1 kDa indicated by gel electrophoresis, while the molecular weight of E32 seems consistent by the two methods.

Although there existed discrepancy in MW determination between gel filtration and SDS-PAGE, it was still known at this stage that 1) all the three microenzymes have MWs at least less than 6 kDa; 2) E40 and T40 have the similar MW while E32 has a higher MW.

It was mass spectrometry that revealed the accurate MWs of the microenzymes. The ion electrospray mass spectra indicated that the monomeric MW of E40 was 510.3 Da and the monomeric MW of T40 was 609.3 Da. The singly charged peak of E40 and T40 suggests there was no lysine, arginine or histidine in these esterases while a doubly-charged ion peak was observed in E32 implying that such an amino acid existed in this esterase, which was later identified and inferred as lysine by amino acid composition and sequence analysis.

The multiplicity of organic functional groups in a biological molecule allows for the presence of multiple charges, because these functional groups can attract protons. This characteristic allows ESI-mass spectra to contain more than one mass spectral peak for a biological analyte. In most cases, multiple peaks of the same biological molecule will be evident in a mass spectrum, and these peaks will differ only by the number of

protons attached to the biological species. For peptides and proteins in the positive-ion mode, the terminal amine (NH_2), the amine functionality in the side chains of lysine (Lys) and arginine (Arg), and the nitrogen atoms in the histidine amino acid (His) residue are the preferred sites of protonation because of their relatively high proton affinities. N-terminal amine, Lys, Arg, and His locations on a peptide or protein determine the degree of presence of the $(M + nH)^{n+}$ mass spectral peaks and maximum charge state of the molecules (Snyder, 2000, Mirza *et al.*, 1993).

The MALDI mass spectrum of E32 unambiguously shows that the its monomeric MW is 1424.7 Da.

The inconsistency of MW determination by three methods was caused by the unique structural features of these peptides. Subsequently sequence analysis revealed these peptides purified were actually collagen-like peptides, which exhibited triple-helical conformation so that the MW of native esterase should be three times of their monomeric form. Thus, from the mass spectrometry data, the homotrimeric MWs of native esterases would be: E40, 1530 Da; T40, 1827 Da; and E32, 4275 Da. This is consistent with the MWs measured by nondenatured gel filtration.

E40 and T40 migrated on SDS-PAGE with an apparent mass of 3.1 kDa, which is much higher their monomer form (510.3 Da and 609.3 Da), even higher than their trimer form. One possible explanation is that the collagen peptides aggregated to form a multimer complex under the condition of SDS gel electrophoresis due to some other component present in the esterase preparations, with a noncovalent bond which is

resistant to SDS treatment. This also may explain why the esterases retained the activity in the Tricine-SDS-PAGE system.

Different collagens exhibit different stability. A study done by Rossi *et al.* (1996) indicated that only weak intermolecular noncovalent bonds are involved in trimer formation and their aggregation in CNBr peptides from type I collagen, so that SDS-PAGE analysis of the peptides showed only one band corresponding to single-stranded species. In contrast, one of the striking features exhibited by type X collagen and their isolated NC-1 domains (noncollagenous domains) is the unusual stability of the trimeric molecules which migrate as trimers in SDS-PAGE even after denaturation under reducing conditions (Schmid and Linsenmayer, 1984; Chan *et al.*, 1996).

Whether the behaviour of the microenzymes in SDS-PAGE has some similarity to that of type X collagen is unknown.

4.3.2 Structure of the microenzymes

4.3.2.1 Amino acid composition analysis & N-terminal amino acid analysis

5-dimethylaminonaphthalene-1-sulfonyl chloride (Dns-Cl) has been proved to be a very convenient derivatizing reagent for the trace detection of amino acids (Weiner and Tishbee, 1981). It is commonly used for qualitative amino acid analyses, the determination of the amino acid terminus of peptides, and for manual microsequence determination (Grego and Hearn, 1983). It reacts with both primary and secondary amino acids. The highly fluorescent derivatives are resistant to acid and alkaline

hydrolysis and can be separated by RP-HPLC. These derivatives provide high sensitivity and are stable over relatively long periods. The high sensitivity, together with the fact that proline and hydroxyproline can be determined, provide certain advantages over the more commonly used post-column fluorescent derivatization methods using reagents such as o-phthalaldehyde or fluorescamine.

Amino acid composition analyses of microenzymes by dansylation following hydrolysis confirmed that the UV absorption peaks purified with esterase activities were peptides, where E40 contained amino acids glycine, proline and hydroxyproline while E32 contained amino acids glycine, proline, hydroxyproline and lysine. That the lysine in E32 was detected in di-Dns-Lys form is because the peptide was first hydrolysed completely and then both α-NH_2 and ε-NH_2 of the free lysine reacted with dansyl chloride.

Hydroxyproline is found only rarely in other proteins but it comprises about 10% of all amino acids in collagen while a further 10% are unconverted proline (Woodhead-Galloway, 1980). The hydroxyproline in collagen is not a naturally occurring free amino acid but is formed from proline, the chemical change taking place after the protein is synthesized. In addition to that in mammals, the hydroxylation of free L-proline has also been reported in microorganisms, and the enzyme that hydroxylates free L-proline has recently been purified from *Streptomyces griseoviridis* (Lawrence *et al.*, 1996).

As the aim of this experiment was just qualitative analysis of the composition of the microenzymes, although peak areas were determined for E40 and E32, quantitative analysis was not done. The formation of Dns-amino acids involves a series of reactions and product yield appears to be dependent on the relative amounts of Dns-Cl and amino acid and on conditions used during incubation (Neadle and Pollitt, 1965). The problems of dansylating some amino acids have been discussed by some authors, and it has been shown by Neadle and Pollit (1965) that it is not possible to obtain quantitative dansylation of free amino acids although it has been reported by some people that it would be possible to obtain semi-quantitative information on peptide composition.

N-terminal amino acid analysis by dansylation followed by acid hydrolysis revealed that glycine was the N-terminal amino acid for all three peptides. The small peak eluting at 38.9 min, which appeared in all peptide hydrolysate runs, did not correspond to any known amino acid, and probably came from some unknown compound in the samples with NH_2- or OH- which could react with Dns-Cl.

The unique Dns-amino acid produced in N-terminal amino acid analysis indicated the peptides were pure.

4.3.2.2 Amino acid sequence analysis

From the amino acid composition analysis, it has been clearly shown that the typical collagen amino acids glycine and imino acids, proline and its derivative hydroxyproline, were present in the hydrolysate of the microenzymes, which means the peptides

purified were actually collagen-like peptides.

Sequence analysis further confirmed the fact that the purified peptides were all collagen-related peptides with Gly-Pro-Hyp repeat sequence, although the molecular weights of the three peptides were different.

By assuming that the last amino acid is glycine in T40 and the last two amino acids are lysine and glycine in E32, the MWs calculated from sequence analysis exactly fit with the MWs determined by mass spectrometry for these two esterases. For E40, the inconsistent MW obtained from sequence analysis and mass spectrometry is hard to explain. One of the possibilities is that the mistaken determination of the last amino acid. If it were Ala instead of Hyp, the MW from the sequence analysis would fit that from the mass spectrometry.

As elucidated in Section 1.5, the collagen stalk motif Gly-X-Y has been found in addition to collagens in other secretory mammalian proteins such as complement C1q, pulmonary surfactant apoprotein, asymmetric acetylcholinesterase, and serum mannose-binding protein (Reid *et al.*, 1982; Benson *et al.*, 1985; Drickamer *et al.*, 1986), as well as in the nonsecretory protein macrophage scavenger receptor (Brown and Goldstein, 1990). More recently, the occurrence of collagen-like sequences in phages and bacteria has also been reported (Engel and Baechinger, 1999; Smith *et al.*, 1998).

Sequences with glycine in every third position have been detected in DNA-derived

sequences of proteins in phages and bacteria and it was suggested that these regions trimerize to collagenous structures. Related sequences were found in proteins of molluscs and slime mold (Engel and Baechinger, 1999).

Several examples of the characteristic collagen-like repeat $(Gly\text{-}X\text{-}Y)_n$ in bacteriophage genomes encoding structural components of bacteriophage virions have been identified (Smith *et al.*, 1998). These examples include lactococcal phage BK5-T, with 64 repeats distributed in 11 short stretches within one gene (Boyce *et al.*, 1995); *E. coli* phage PRD1, with six repeats (Bamford and Bamford, 1990); and actinophage fC31, with five and 10 repeats separated by four amino acids (GenBank accession number Z99661). The bacteriophage collagen sequences have the bias toward proline at the second and third positions of the motif that has long been known in animal collagens.

It has been postulated that there would be four possible evolutionary explanations for the occurrence of collagen in such phylogenetically diverse locations as animals and bacteriophages: (i) collagen already existed in a common ancestor of animals and bacteriophages (or their bacterial hosts), (ii) collagen arose independently in animals and phages, (iii) collagen arose in animals and was passed horizontally to phages, or (iv) collagen arose in phages and passed horizontally to animals. However, there is no evidence to support any of these scenarios at present (Smith *et al.*, 1998).

In this work, the collagen-like peptides associated with esterase activities were found in the crude extracts of two thermophilic fungi, *E. nidulans and T. emersonii*. Further

work on the gene structural studies of these organisms would reveal the nature of these collagen-like peptides.

When comparing the microenzymes in this work with two other microenzymes which have been characterized to the extent of amino acid composition, it is interestingly found that they all have a large content of proline residues. The 5.7 kDa esterase from *Candida lipolytica* had 56 anino acid residues of which 11 were proline (Adoga and Mattey, 1979), and the 9.7 kDa rennin also possessed a high proline content (Laxer *et al.*, 1981). In addition, as mentioned in Section 1.3.2, the low MW (16 kDa) thermostable α-amylase contained 15% proline and with a nonspherical structure; it was proposed that this unique structure was solely responsible for its thermostability (Singleton and Ameluxen, 1973). Further investigations to determine if these proline residues are directly involved in the activity, or to explore the relationship between the proline residues and the enzyme active site in these low MW enzymes would also be interesting areas.

4.3.2.3 Secondary structure determination

Peptides with Gly as every third residue and a high content of the imino acids Pro and Hyp adopt a triple-helical conformation (Sakikabara *et al.*, 1973; Long *et al.*, 1993). Collagen-like triple-helical structure exhibits CD spectra in solution which are identified by a large negative peak around 200 nm and a positive peak around 215-227 nm (Sreerama *et al.*, 1994). These features have been used as a basis to provide information about the presence of a collagen-like triple-helical structure in solution for

natural and synthetic peptides (Inouye *et al.*, 1982; Toumadje and Johnson, 1995).

All three esterase samples exhibited CD spectra similar to that of collagen-like triple-helical conformation, with a minimum, negative molar ellipticity at 198-200 nm, but a maximum, positive molar ellipticity at 242 nm which was red-shifted by 20 nm compared with a typical collagen-like structure.

The reason for the red-shifted positive peak is not clear. One possibility postulated is due to presence of some other compound in the samples, for example, nucleic acids, which exhibit maximum peaks around 240 nm region in the CD spectrum.

Triple-helix domains in proteins bind to a variety of molecules as part of their biological function (Brodsky and Shah, 1995; Hoppe and Reid, 1994). These include the binding of the collagen triple helix to integrins, collagenase, fibronectin, and heparin, as well receptor binding by the collagen-like domain of C1q and ligand binding by the collagen-like domain of MSR (macrophage scavenger receptor).

Collagen related protein MSR binds a variety of polyanionic ligands (Resnick *et al.*, 1993, Pearson *et al.*, 1993), and in particular shows selectivity for tetraplex forms of nucleic acids. The ligand binding region has been shown to lie in the triple-helical collagen-like domain of MSR. Studies done by Mielewczyk *et al.* (1996) showed a complex was formed between a triple-helical peptide and a tetraplex nucleic acid, which was accompanied by the changes in the intensity of nucleic acid CD spectrum in the 240-300 nm region. Electrostatic interactions likely form the basis for binding of

the positively charged triple helix to negatively charged ligands.

But for the microenzmes studied, there has not been direct evidence for the presence of the similar compound or for the presence of the binding between collagen-related peptide and such compound (if any).

That ethylene glycol/H_2O were used as the solvents for CD determination is because ethylene glycol is known to stabilize helical structures and therefore can be very useful to amplify and detect very weak triple-helical propensities (Choma *et al.* 1994).

4.3.3 Catalysis of the microenzymes

4.3.3.1 Catalysis caused by peptide catalysts

Peptides, which exhibit catalytic activities, may include three groups: (a). Naturally occurring peptides; (b). De novo-designed peptides; and (c). Metal-peptide complexes.

Apart from the naturally occurring peptides and de novo-designed peptides catalysts which were described in Section 1.3.1 and Section 1.4.1, many studies have showed that complexes of transition metals with histidine and histidine-containing peptides exhibit catalytic activities for some substrates. Typically, Ni(II) and Cu(II) peptides have been shown to have DNA- and protein-cleavage as well as autoxidation of sulfite capabilities (Chiou, 1983; Merkler *et al.*, 1995; Muller *et al.*, 1997).

Nickel has been shown to bind to the Cys-Ala-Ile-His sequence of histone H3, and the

resulting complex promotes oxidative damage to DNA in the presence of H_2O_2 (Bal *et al.*, 1996). The complex formed by the tripeptide NH_2–glycyl-glycyl-histidine-COOH (GGH) and Ni(II) salts is known to mediate the oxidative cleavage of both DNA (Mack and Dervan, 1992) and proteins (Cuenoud *et al.*, 1992) in the presence of peracids. This complex was also shown to cause efficient covalent crosslinking of certain proteins in solution when incubated with oxidants such as monoperoxyphthalic acid (Kathlynn *et al.*, 1995). And an identical reaction is catalysed by the Ni^{2+} complex of poly-histidyl sequences, which had been incorporated into recombinant proteins to facilitate their purification by immobilised metal affinity chromatography (Fancy *et al.*, 1996).

It was also demonstrated that the complex formed by GGH and Cu(II) salts had DNA- and protein-cleavage capabilities (Chiou, 1983) and possessed antitumor activity in the presence of ascorbate (Kimoto *et al.*, 1983). This reactivity of the motif is due to the strong oxidizing potential of Cu(II) (Shriver *et al.*, 1994).

4.3.3.2 Proof for metal ion(s) involved as cofactor(s) in microenzymes

As discussed in Section 1.3.1, 1.4.1, and Section 4.3.3.1, it was clearly demonstrated that it is possible for a peptide, either naturally occurring or designed, and with even only a few amino acids, to be able to exhibit some kind of catalytic activity. But at this stage, a question was obviously raised about whether collagen peptides can produce the observed esterase activity.

Actually, the microenzymes isolated are not the only enzymes known which contain a collagen type structure, but they are the only ones known where the collagen type structure is part of the active unit of the enzymes. For example, eel acetylcholinesterase has a collagen-like triple helical stalk but its active subunits with enzymatic activity are separate.

As stated in Section 1.5.3, the triple helix is found in a variety of proteins in addition to collagen, such as macrophage scavenger receptors types I and II, complement component C1q, pulmonary surfactant apoprotein, acetylcholinesterase, and mannose binding protein. The potential activities mediated by triple helices are quite varied. However, no report has shown any esterase activity related to the peptide structure only with Gly-Pro-Hyp repeat sequence. And actually, subsequent study (Section 3.4) on synthetic peptides with only Gly-Pro-Hyp repeat sequence showed no comparable esterase activity.

Based on this fact, together with the observation that most of the reported, naturally occurring microenzymes require the presence of a metal (Ca, Fe or Mn) for optimum stability and activity (Cunningham *et al.*, 1998; Schenk and Bjorksten, 1973; Adoga and Mattey 1979; Laxer *et al.*, 1981), it was postulated that there might be something else (probably a metal ion) in the microenzymes which interacted with the collagen peptides and caused the esterase activity.

The presence of metal ions in the microenzymes was supported by 1) X-ray microanalysis; and 2) dialysis experiment.

X-ray microanalysis clearly showed substantial amounts of Zn were present in all the three esterase preparations while Al was also enriched in E40 and E32. Thus, it is postulated these two metal ions, most probably Zn^{++}, were involved in the activity and stability of the esterases, and the microenzymes obtained their activities not from the structural backbone but from metals attached to them.

Collagen-related peptides have been shown to possess the properties of binding Zn^{++}. The studies done by Vettakkorumakankav and Ananthanarayanan (1999) indicated that the collagen-related peptide GPQGIAGQ was able to bind Zn^{++} and Ca^{++} to form a stable peptide:Ca^{++}:Zn^{++} ternary complex when both cations were present together in excess. And the binding resulted in a quite different CD spectrum from that of the free peptide, which suggested a different conformation between the free peptide and the metal-peptide complex.

Zinc is an important metal in biological systems. In proteins, zinc ions play valuable roles both in enzyme catalysis and in maintaining structure.

The catalytic role of zinc involves its electrophilic character. The Zn^{2+} ion can stabilize the negative charges in the reaction intermediate, as in both carboxypeptidase (Coleman, 1992) and alcohol dehydrogenase (Cho *et al.*, 1997). Catalytic zinc ions can also ionise bound solvent to nucleophilic hydroxyl, as in carbonic anhydrase (Lilsjas *et al.*, 1994). In catalytic sites, the zinc ion is usually exposed and bound to a solvent molecule.

Zinc also plays structural roles in proteins. In "Zn-finger" DNA binding domains, the zinc ion stabilizes the folded conformation of the protein required for interactions with the nucleic acid (Vallee and Auld, 1990). Zinc ions are also required for the assembly of polymeric species, for example, the hexameric insulins (Derewenda *et al.*, 1989) or can be involved in stabilizing the active site structure in enzymes, such as superoxide dismutase (Lippard *et al.*, 1977).

Zinc-containing enzymes have been extensively studied, and examples of enzymes that contain zinc ions can be found in each one of the six classes of enzymes (Vallee and Auld, 1990). The roles that zinc ions play in catalysis are varied; however, a single essential zinc ion can usually be categorized as either structural or catalytic. Loss of either a catalytic or a structural zinc ion may result in similar activity losses upon Zn^{2+} removal. This activity loss is due to either impaired catalytic function or to loss of structural integrity of the protein. In the majority of studies where an enzyme requires a single zinc ion for hydrolysis of an amide bond, such as with the carboxypeptidases, neutral endopeptidases, and angiotensin converting enzyme, the role of that zinc ion is catalytic (Christianson and Lipscomb, 1989). Some amide-bond-cleaving enzymes, such as *Aeromonas* aminopeptidase and collagenase, contain a second zinc ion, in addition to the catalytic Zn^{2+}, that plays a structural role in enzyme activity (Lovejoy *et al.* 1994).

There are several properties of Zn^{2+} that make it particularly useful for catalysis (Williams, 1995). Zinc is inert to oxidoreduction, so it can be used to catalyse hydrolytic reactions while avoiding the formation of potentially damaging radical

species. Zinc is a strong Lewis acid, yet it retains flexibility in ligand geometry, as it is able to adopt 4-, 5-, or 6-coordinate geometries readily. This characteristic allows a zinc-containing enzyme to take advantage of the stability afforded by three protein residues coordinating the active Zn^{2+} while still leaving open one or two sites on the coordination sphere for binding of water molecules or substrates during the reaction.

The esterase microenzyme rapidly lost activity on dialysis against suspension of the chelating resin Chelex-100 and EDTA and also when dialysed against distilled water. The higher rate of loss of activity when dialysis against Chelex-100 and EDTA suggests that a non-protein dialyzable cofactor, probably metal-ion, was being removed. The ease of the removal of the cofactor suggests a weak interaction between it and the protein component.

Enzymes requiring metal ion cofactors are divided into two general groups (Vallee, 1955). In the one group, metal ions form an intrinsic component of the enzymes and are not easily removed from the molecule since they are firmly bound. Such enzymes are called metalloenzymes to differentiate them from the other group, the metal-enzyme complexes. In these complexes, the metal ions are loosely associated with the protein, generally loss of ions occur during purification and activity is regained by the addition of the metal ions (Vallee, 1955). The esterase in this study may therefore be regarded as a metal-enzyme complex.

Attempts to identify the cofactor by adding different cations (Zn^{2+}, Al^{3+}, Fe^{3+}, Fe^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+}) to the dialysed enzyme did not restore significant

esterase activity. But when the dialysate was concentrated by freeze-drying and then added back to the dialysed preparation, a significant increase in enzyme activity was observed (data not shown). This may be due to 1). The concentration of metal ions added was not optimal to the restoration of esterase activity; 2). Both Zn^{2+} and Al^{3+}, in a certain ratio, were required for the restoration of esterase activity; 3). In addition to Zn^{2+} or/and Al^{3+}, another unknown dialyzable component was also essential for enzyme activity or stability.

4.3.3.3 Catalytic activity of the microenzymes

Although the microenzyme seems to catalyse the hydrolysis of fluorescein dibutyrate with Michaelis Menten saturation kinetics, the catalytic activity is very low with Kcat only $2.98 \times 10^{-2}\ s^{-1}$ and the specific activity 1.17 μmole/min.mg when compared with other naturally occurring esterases reported in the literatures where catalytic activities range from 28 to 6800 μmole/min.mg (Matsunaga *et al.* 1974; Adoga, 1980; Castanares *et al.*, 1992). Even the 1.57 kDa microenzyme investigated by Simões *et al.* (1997a) has higher esterase activity (15.5 μmole/min.mg) than the microenzyme studied here.

The low esterase activity exhibited by the microenzyme may be due to:

1). Metal ions involved in the enzyme activity and stability were partially lost during purification, so the purified enzyme had a lower activity than the esterase activity observed on the tributyrin agar;

2). The peptide concentration determination by the method used was not accurate due to the nature of enzyme size and structure so that the specific activity calculated was lower than it actually was;

3). Structural instability of the mcroenzyme due to its unusually small size, as similar levels of enzyme activities have been observed in most *de novo*-designed peptide biocatalysts.

Although the rate enhancements obtained of some *de novo*-designed small peptides are several fold over that of the background reaction, most of them are still many orders of magnitude lower than those observed for the naturally occurring enzymes. For example, Kcat for decarboxylation of oxaloacetate catalysed by two14-residue peptides, oxaldie1 and oxaldie2, is 7×10^{-3} and 8×10^{-3} respectively (Johnsson *et al.*, 1993); Kcat for the 33-residue synthetic peptide ligase is 1×10^{-2} (Severin *et al.*, 1997). This has been attributed to structural instability or the lack of fixed conformations with peptides of such small size (Corey and Corey, 1996).

For the microenzyme studied, although metal ions were present in the esterase and may play some kind of role in maintaining its secondary or tertiary structure for esterase activity, but due to the small size of the enzyme or the possibility of partial loss of the metal ions during purification, the structure formed might be unstable.

4.3.4 Other biochemical properties of the microenzymes

4.3.4.1 Thermostability

The microenzyme exhibited extraordinary thermostability, with no activity loss up to 110 °C after 2 hours incubation, which was similar to the microenzyme isolated by Simões *et al.* (1997). It exceeds most of the thermophilic enzymes from thermophiles, although an extremely thermostable esterase from hyperthermophilic archaeon *Pyrococcus furiosus,* with activity optimum temperature at 100°C and thermostability half-life of 50 min at 126°C, has recently been reported (Ikeda, 1998).

As elucidated in Section1.1.3, to date, the intrinsic and extrinsic bases of thermostabilization and thermoactivation of thermophilic enzymes are not completely clear. But recent studies have indicated that mechanisms of enhanced thermal stability involve improved packing density, additional salt bridges and hydrogen bonds, extended secondary structures, abbreviated loops, as well as specific local interaction (Jaenicke, 1991; Adams and Kelly, 1998). Amino acid sequence analyses do not indicate stabilizing mechanisms: instead, the three-dimensional structures are an absolute prerequisite.

The stability of proteins from thermophiles used to be attributed to only intrinsic factors (Jaenicke, 1981), i.e., that is specified by the amino acid sequence and thus resides in the secondary and tertiary structure. But more recently extrinsic factors (or cellular components) have been found to be involved.

In searching for specific cellular responses to a variety of stress conditions, low-molecular-mass compounds and, in increasing number, stress proteins ('chaperons') have been uncovered. The latter assist in the stabilization of (nascent) proteins or in

the salvage of denatured polypeptides. The low-molecular-mass compounds refer to a wide spectrum of ions, metabolites, coenzymes or specific protectants, some of which have been known as structure-stabilizing factors for a long-time. The occurrence of polyphosphates as specific ligands which enable non-thermostable proteins to gain thermal stability has been uncovered (Huber *et al.*, 1989). Another example is caldolysin, a protein from an extreme thermophile, which depends on six calcium ions for its very high stability (Khoo *et al.*, 1984).

The extremely high thermostability of the microenzyme in this study may have a similar explanation. As discussed above, the microenzymes contain not only collagen-like peptides, but metal ions were also involved in the esterase stability and activity. Thus, the high thermostability could be attributed to participation of such small molecules, which assist to maintain the three-dimensional structure of the esterases by providing noncovalent interaction with the collagen peptides.

The three major terms accounting for the absolute heat capacity of a protein (Gomez *et al.*, 1995) are the following:

i) one term that depends only on the primary or covalent structure of a protein and contains contributions from vibrational frequencies arising from stretching and bending modes of each valence bond and internal rotation,

ii) a term that contains the contributions of noncovalent interactions arising from secondary and tertiary structures, and

iii) a term that contains the contribution of hydration.

When considering thermophilic enzymes at temperatures beyond 120 °C, hydrothermal degradation of amino acids becomes significant (Jaenicke, 1991), whereas, up to 110°C, most covalent bonds in the proteins are stable (Cowan, 1995). Thus, up to 110°C, the thermodynamic stability of a protein exhibits a balance between large stabilising forces (derived from non-covalent intramolecular interactions) and large destabilising forces, such as primarily chain conformational entropy.

That the microenzyme kept its activity at 110°C and lost its activity rapidly at 120 °C also suggested that the integrity of the collagen peptide might be essential to maintain the microenzyme's three-dimensional structure for esterase activity.

In addition, the extremely high thermostability of the microenzyme is consistent with the stability of the microenzyme in SDS-PAGE and HPLC, as many thermostable enzymes generally seem to be more resistant to the denaturing effects of detergents and organic solvents (Ljungdahl, 1979; Ståhl *et al.*, 1998).

4.3.4.2 Effects of pH

The remarkably activity at alkaline pH, together with the extreme stability over a wide range of pH, was another unusual feature exhibited by the microenzyme esterase.

An esterase with similar behaviour has recently described by Kademi *et al.* (2000). The thermostable esterase isolated from a moderate thermophile *Bacillus circulans*

showed high activity at alkaline pH (around 11) and the activity was completely stable for 1 hr at 37 °C at all pH tested. And the 8 kDa thermostable protease described by Steele *et al.* (1992) also possessed optimum activity at pH 11. Some other extremely acidic and basic pH-tolerant enzymes have also been reported, for example, an extracellular lipase purified and characterized from *Bacillus subtilis* 168 showed maximum stability at pH 12 and maximum activity at pH 10 (Lesuisse *et al.*, 1993); the low-molecular-weight phospholipase A_2 isolated from developing seeds of elm was unusually stable at extreme acid pH conditions and had an alkaline pH optimum, it was also extremely heat stable and resistant to organic solvents (Ståhl *et al.*, 1998); and the 5.7 kDa esterase produced by *Candida lipolytica* exhibited the optimal pH at 1 (Adoga, 1980).

Usually, enzymes are stable over only a limited range of pH. Outside this range, changes in the charges on ionisable amino acid residues result in modifications of the tertiary structure of the protein and eventually lead to denaturation. But within the range at which an enzyme is stable, the catalytic activities depend on pH. The pH dependence reflects ionisable residues that constitute the active site on the enzyme or are essential for maintaining the structure of the active site. The active sites of enzymes generally contain important acidic or basic groups. It is to be expected that if only one protonic form of the acid or base is catalytically active, the catalysis will somehow depend on the concentration of the active form.

But in the case of the microenzymes studied, both amino acid composition amino acid composition analysis and sequence analysis indicated neither acidic nor basic

groups were present in two most active esterases E40 and T40 although E32 contained a lysine residue. In addition, unlike most of the esterases or lipases reported, which are serine containing enzymes where serine hydroxyl (or sometimes the imidazole of histidine) functioned as nucleophilic reagent in catalysis, the microenzymes contained neither serine nor histidine.

Based on these facts and the behaviour of the microenzyme in regard to its pH effect, together with other features such as thermostability and the low catalytic activity exhibited by the microenzyme, it is postulated that the active site of the microenzyme catalysis would be more related to metal ions, in complex with the collagen peptides, but the details about the catalysis mechanism are unknown. This would only be possible to become clear after the esterase structure is completely revealed, as from the data available so far, it is only known that the microenzyme composed of the collagen-related peptides, Zn^{2+} and probably Al^{3+}, but how these components bound each other to construct the microenzyme and if there was any other component involved are not clear.

Compared to the microenzyme, the high MW esterase from *E. chevalieri* (HEEC) represents a typical thermostable enzyme as those isolated from thermophilic microorganisms in regard to its thermostablity, pH stability and pH optimum, as well as positional specificity.

4.3.4.3 Positional and chain length specificity

Although the microenzyme exhibited some unusual features as discussed above, it resembled most of the esterases reported with respect to its positional and chain length specificity.

The microenzyme was able to hydrolyse triglycerides and showed the specificity for the α-positions as the controls of high MW esterase from *E. chevalieri* and a commercial lipase exhibited the same positional specificity. Actually, most of esterases (or lipases) studied are 1,3-specific, such as the lipases from *Pseudomonas fragi* (Mencher and Alford, 1967) and *Pseudomonas aeruginosa* (Nadkarni, 1970) as well as the extremely basic pH-tolerant extracellular lipase from *Bacillus subtilis* 168 (Lesuisse *et al.*, 1993). The microenzyme esterase reported by Adoga (1980) was also 1,3-specific while the microenzyme esterase from *Bacillus stearothermophilus* seems to prefer the 2-positional ester-bond (Simões, 1997a).

The microenzyme showed higher activity towards short chain fluorescein esters than long chain ones, which was the same as the esterase from *Candida lipolytica* (Adoga, 1980). Several other esterases described in the literature also exhibited the similar substrate specificity (Kanaya *et al.*, 1998; Schmidt-Dannert *et al.*, 1997).

4.4 CHEMICAL SYNTHESIS AND ANALYSIS OF THE ESTERASE PEPTIDE

The successful chemical synthesis of the esterase peptide by Fmoc chemistry was clearly indicated both by successive deprotection peak areas in the synthesis and by subsequent HPLC analysis results.

Reverse-phase HPLC analysis of the synthetic peptide showed that the elution time of the synthetic peptide (8.6 min) was close to that of native esterase E40 (9.0 min). This suggested that the native E40 had the similar hydrophobic characteristic with the synthetic peptide, although the amino acid sequence may be different.

That an increase in peptide concentration enhances the triple helicity of single chain collagen analogues has been demonstrated by some researchers (Feng 1996). And peak intensity is related to triple-helix content: the more triple-helix content there is in peptides, the higher the amplitude of the peak. The CD spectrum of the synthetic peptide exhibited apparently lower peak amplitude than that of the native esterase E40 although the concentration of the synthetic peptide (105 μg) was much higher than that of E40 (3.6 μg). This suggested that E40, if not completely triple helical structure, at least possessed a higher percentage of triple-helical conformations in solution than the synthetic peptide.

The analyses of esterase activity of the synthetic peptide indicated that the synthesized collagen peptide did not exhibit esterase activity, and addition of metal ions (Zn^{++}, Al^{+++}, and Fe^{+++}) at different concentrations and under conditions used could not restore its activity.

In addition, the CD spectrum showed that the conformation of the synthetic peptide was not changed after addition of metal ions, which was different from the observation that the complex formed by collagen-related peptide GPQGIAGQ with saturating amounts of Zn^{2+} and Ca^{2+} exhibited a relatively stronger negative CD band at 200 nm in trifluoroethanol when compared with the CD of the free peptide (Vettakkorumakankav and Ananthanarayanan, 1999). In contrast, the CD spectrum exhibited by the native microenzyme seems similar to this metal-peptide complex.

The folding of the peptide into its secondary or tertiary structure is the fundamental requirement to induce the proper biological response or activity. That the active esterase E40 had more triple-helix content than the inactive synthetic peptide implied that the esterase activity might be related to the triple helix conformation. For synthetic collagen peptides with only 7 amino acids, formation of triple helix structure would require other component's binding (i.e., metal ions) to provide some kind of non-covalent bond.

That addition of metal ions could not produce activity or the conformation of the native enzyme may be due to the following reasons:

1). For the formation of the active microenzyme, it was required that the collagen peptide bound Zn^{2+}, and probably Al^{3+}, with definite stoichiometries;

2). Specific conditions were required for the formation of the active metal-peptide complex;

3). In addition to Zn^{2+} and Al^{3+}, another unknown component was also required for the enzyme activity; or

4). Failure to remove the protecting group from the hydroxyproline residues during chemical synthesis.

CONCLUSIONS AND FURTHER STUDIES

The investigations of the extracellular esterase activities from the nine thermophilic fungi showed the existence of very small esterases in *E. nidulans* and *T. emersonii.*

Of the three extracellular microenzymes found, E40 and T40 respectively accounted for most of the esterase activities observed in *E. nidulans* and all the activities in *T. emersonii,* and were similar in their molecular weights. These two esterases were also similar to the microenzyme esterase described by Simões *et al.* (1997, 1995) in their molecular weights and thermostability. These observations implied that the low molecular weight esterase activities observed in these thermophilic microorganisms could be attributed to the same class of microenzymes.

The microenzymes were active towards fluorescein dibutyrate and α-naphthyl acetate as well as triglycerides, but the K_{cat} was relatively low. The esterases exhibited extremely high thermostability and unusual pH stability at either alkaline or acid pH, with the maximum activity at alkaline pH. The esterases were more active against short chain length fatty acids than long ones and hydrolysed glycerol esters with 1, 3 specificity.

The purified small enzymes exhibited trimer structure in solution and the molecular weights in their native form were postulated to be 1.5 kDa, 1.8 kDa and 4.2 kDa for E40, T40 and E32 respectively. The small enzymes were found to be metal-enzyme complexes, which were composed of collagen-related peptides, Zn^{2+} and/or Al^{3+}.

The metal ion(s) may play an important role in maintaining enzyme activity and structural stability.

The chemically synthesized peptide with only Gly-Pro-Hyp repeating unit was shown to be esterase-inactive and esterase activity to the level of the native enzyme could not be produced by adding Zn, or/and Al under the conditions tested. To convert this peptide into active esterase, further experiments should be designed to investigate other conditions to see if the activities can be restored, such as testing other metal ions (i.e. Ca) in addition to Zn and Al, using two metal ions at different stoichiometries or increasing incubation time.

It is also necessary to measure the molecular weight of the synthetic peptide by mass spectrometry to confirm its structure.

It may also be interesting to study the relationship between the microenzyme and collagen, and to observe if any enzyme activity can be produced by hydrolysing collagen and attempting to bind metals to it.

The observation that the native esterase had more triple-helical conformation than the synthetic peptide implied that the esterase activity might also be related to triple-helical conformation. But this postulation has yet to be verified by further elucidation of the microenzyme structure.

Further investigation on the microenzyme structure, such as how the metal ions and

collagen-related peptides bound each other to construct the microenzyme and if there was any other component in the microenzyme in addition to Zn/Al and collagen peptides, would also be important to revealing the detailed catalysis mechanism for such small enzymes and to explaining some unusual features exhibited by the microenzymes (such as extremely high thermostability, pH stability and remarkable activity at alkaline pH, etc.), as well as perhaps allowing the synthesis of the active esterase from the inactive, synthetic collagen peptides. In addition, as discussed in Section 4.3.2.2, the investigation of the role of proline residues in such small enzymes would also be helpful to the studies of catalysis mechanism as well as thermostability for these esterases.

Another area of interest would be to investigate the gene structure of these thermophilic fungi, and to see if it has any relationship to collagen genes. This would eventually reveal the nature of these collagen-like peptides.

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