PURIFICATION, CRYSTALLIZATION AND CLONING OF TRIBUTYRIN ESTERASE FROM *LACTOCOCCUS*

by

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ABSTRACT

Tributyrin esterase is an enzyme that has been isolated and purified from lactococcal starter strain by research staff at the New Zealand Dairy Research Institute. It has been shown to play an important role in production and control of flavour development during cheese ripening, but little is known about its biochemical characteristics.

New studies on tributyrin esterase have been initiated, with the aim of carrying out a three dimensional structure determination to completely understand the molecular basis and the nature of its *in vivo* activity.

This thesis is divided into three main parts. In the first part, the purification of tributyrin esterase from a genetically modified strain *Lc. lactis* subsp. *cremoris* B1079 is described. The procedure investigated for optimization of the protocol and a partial study of factors affecting tributyrin esterase activity are described. In the second part, crystallization trials for tributyrin esterase are described. Several crystals were obtained, with the best ordered crystals being grown from 2.6M ammonium sulfate, these have been shown to diffract to 3.0 Å, and belong to the space group C222 with cell dimensions a=76Å, b=178Å c=179Å. In the third part, the lipase gene was ligated into 4.75kbp expression vector proEX, which contains a his-tag sequence upstream of the multiple cloning site. The ligation reaction mixture was transformed into competent *E.coli* DH5α cells. This should allow the expression of tributyrin esterase in *E.coli* and eventually provide a great yield of protein and make purification easier.

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ABBREVIATIONS

BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BTP	Bis-tris propane; 1,3-bis[tris-hydroxymethyl)-methylamino]propane
DNA	Deoxyribonucleic acid
DTT	D-Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid (di-sodium salt)
EPPS	N-[2-Hydroxyethyl]piperazine-N'[3-propanesulfonic acid]
FPLC	Fast protein liquid chromatography
HEPES	N-[2-Hydroxyethyl]piperazine-N'[2-ethanesulfonic acid]
HIC	Hydrophobic Interaction Chromatography
HPLC	High performance liquid chromatography
IEX	Ion Exchange Chromatography
MES	2-[N-Morpholino]ethanesulfonic acid
MOPS	3-[N-Morpholino]propanesufonic acid
NMWC	Nominal Molecular Weight Cut-off
NaAc	Sodium acetate buffer
$(NH_4)_2SO_4$	Ammonium sulfate
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEG-mme	Polyethylene glycol monomethyl ether
PIPES	1,4-Piperazinediethanesulfonic acid
Q-	Quaternary amino
SDS	Sodium dodecyl sulfate
SEC	Size Exclusion Chromatography
TE (*x*:*y*)	*x* mM Tris/HCl ph8.0, *y* mM EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TRIS	Tris(hydroxymethyl)aminomethane

ABBREVIATIONS FOR AMINO ACIDS

Amino Acid	Three Letter Symbol	One letter Symbol*
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter 1 INTRODUCTION AND LITERATURE REVIEW

1.1 Esterases and Lipases

Lipases and esterases constitute an important group of enzymes, which are associated with fat metabolism as well as with fat degration. They are widely distributed in nature, being present in numerous tissues and fluids of animals, plants and microbes. They are physiologically important since they hydrolyze fats and oils giving rise to free fatty acids and partial glycerides. These are essential for metabolic processes, such as fatty acid transport, oxidation, and the resynthesis of glycerides and phospholipids.

There is still much confusion concerning the exact meaning of the terms lipase and esterase, with these terms often being used interchangeably. According to the International Union of Biochemistry nomenclature (Florkin and Stotz, 1965), the term esterase is assigned to enzymes that catalyze the hydrolysis of carboxylic acid ester bonds. Esterases are therefore classified as hydrolyases and belong to the class 3.1.1. A lipase on the other hand is defined as an enzyme that hydrolyzes carboxylic esters of glycerol and belongs to class 3.1.1.3 hydrolyases. This means that the term esterase is more nonspecific and includes lipases. However, it should be noted that a typical lipase may also catalyze the hydrolysis of esters other than glycerides (Brocherhoff *et al.*1974).

Since Sarda and Desnuelle (1958) found that the lipolytic activity was significantly enhanced by the presence of micelles, this so called " interfacial activation" phenomenon has been regarded as the major distinction between lipases and esterases (Borgstrom, et al, 1984 and Tsujita *et al.*, 1990). More recently, however, Martinez, *et al.* (1992) and Lesuiss, (1993) discovered some lipolytic enzymes with clear sequence homology to lipases that are not activated by interfacial interaction. They therefore suggested that the classification based solely on the interfacial activatability is too simplistic. This idea was supported by Ransac *et al.*, (1996) and Brocherhoff *et al.*, (1974) who proposed to define lipases as carboxylesterases that are able to hydrolyze long chain acylglycerols (10 or more carbons). Nevertheless, the activity of the majority of the known lipases increases in the presence of a water-lipid interface. Thus, the molecular nature of this activation has become an important aspect of lipase research.

Apart from the obvious biological importance, there are other, more specific, reasons that make the study of lipolytic enzymes interesting and rewarding, such as medical and industrial applications. The manipulation of lipolytic activities will probably play a part in future methods for treating malfunctions of fat metabolism and thus control cardiovascular diseases. Of similar interest are the practical aspects of lipases in the food and other industries. In this current project, the lipase of interest was found to have the industrial importance in cheese making. The related background is thus reviewed extensively in following sections.

1.2 Cheese Making and Esterase

1.2.1 Cheese Making Technology

The manufacture of cheese is one of the oldest examples of biotechnology known to human beings. This process has long been regarded as a means of preserving the nutritional value of milk in a safe, palatable form. Traditionally, therefore, the long maturation time of some varieties of cheese was seen as an advantage. Modern cheese making technology, (see figure 1.1) which has evolved from these principles, can generally be regarded as a two stage process. In the first stage (manufacture stage), curds are made from milk, through a series of processes (standardization, pasteurization, curdling, cutting, cooking and draining). In the second stage (ripening stage), mature cheese is developed from curds. The details of this stage are discussed in next section.

1.2.2 Cheese Ripening

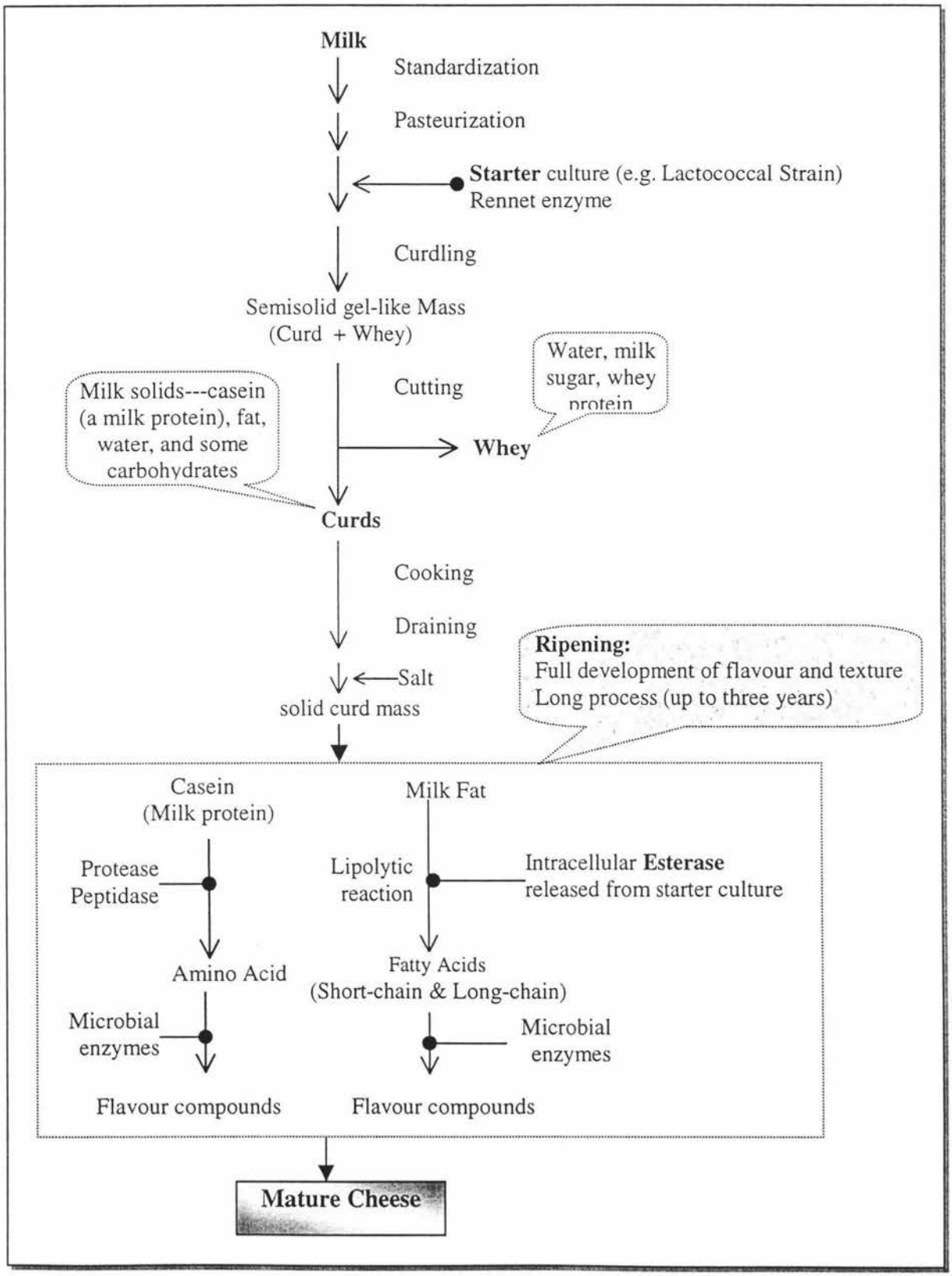
The maturation process involves the slow, controlled decomposition of the fats, proteins and carbohydrates of bland lactic curds. It is mediated by both the starter bacteria, which the cheese maker adds deliberately, and the secondary flora whose composition varies widely between different cheese types. Overall, the major objectives of a ripening system are:

1. To produce the typical flavour and texture of mature cheese;
2. To improve the reliability of mature flavour development;
3. To increase the range of products and flavours available to consumer;

In order to achieve these aims, the ripening process can take up to three years, depending on the type of cheese. In the commercial world, this prolonged period between making cheese and realising its value may be a disadvantage to a factory scale cheese maker who

needs a rapid turnover to cover the high costs of capital equipment and labour. Therefore, it is commercially advantageous to accelerate the ripening.

Figure 1.1: Scheme of Cheese Making Technology



1.2.3 Esterase in Cheese Ripening

Cheese ripening is essentially an enzymatic process involving curd breakdown by proteolysis, lipolysis and other enzyme-catalyzed reactions, resulting in cheese with typical flavours and texture. Some of these enzymes are present in milk whereas others are synthesised by starter lactic acid bacteria and liberated by the autolysis of these bacteria. While the role of proteinases in the cheese ripening process has been studied extensively (for reviews, see Fox, 1989; Olsen, 1990; Kok, 1993), the exact role of lipolytic enzymes is not yet completely understood. McNeil & Connolly (1989) suggested that after esterase was released from starter cells trapped in cheese curd, lipolysis occurred to release free fatty acids from triacylglycerols in milk fat. It has been known for some time that short-chain fatty acids such as *n*-butanoic and *n*-hexanoic acids are potent flavour compounds, at concentrations below 10 mg/kg (Brennand *et al.* 1989). Short chain fatty acids may also be esterified to compounds such as alcohols to give rise to a further range of potent flavour compounds (Bills *et al.* 1965). This evidence implies that the lipolytic activity in starter bacteria probably plays a significant role in flavor development (Crow *et al.*, 1994; Lawrence *et al.* 1976).

1.3 Esterases from *Lactococcus* and other starter bacteria

1.3.1 Lipolytic activity in starter bacteria

Lactococcus (formerly *Streptococcus*) species (and sometimes *Lactobacillus* species) are widely used as bacterial starters in cheese manufacture. Lipolytic activity due to these strains has been reported by a number of groups (Lawrence et al. 1967; Umemoto & Sato, 1975; Kamaly *et al.* 1989). The level of lipase activity in these strains is low however compared to lipolytic activity found in organisms such as *Pseudomonas* and other dairy spoilage microbes. While the lipases of different species of bacteria prefer to hydrolyze different substrates, most of the strains produce a lipase that is able to catalyze the hydrolysis of tributyrin. Umemoto *et al.* (1968) observed that when a tributyrin emulsion served as substrate, lipolysis in the presence of various dairy lactic acid bacteria was optimal between pH 6 and 8 and at 37°C. Oterholm *et al.* (1968) also tested a number of lactic acid bacteria, and found that all cell-free extracts possessed lipolytic activity against an emulsion of tributyrin, which suggested that the lipase associated with lactic acid bacteria is intracellular. In addition to lipolytic activity, extracellular esterase activity was also observed when cell free extracts of the various lactic acid bacteria were

incubated with an aqueous solution of triacetin (Lawrence, 1967a; Brockerhoff & Jensen 1974). Several esterases have been purified and partially characterised from several starter strains, and some of their features are summarised in Table1.1.

1.3.2 Esterase from *Lactococcus*

1.3.2.1 Tributyrin Esterase from *Lactococcus lactis* subsp *cremoris* E8

A tributyrin esterase from *Lactococcus lactis* subsp *cremoris* E8 (Holland & Coolbear, 1995) has been identified at the New Zealand Dairy Research Institute. The activity appeared to be located in the cytoplasm of the organism, although there was a limited degree of association with the cell membrane. The following purification protocol was used to isolate this enzyme from the bacteria. Salt fractionation (60% ammonium sulfate precipitation) was followed by hydrophobic interaction chromatography (HIC) on an alkyl Superose column. The active fractions from this step were subjected to gel filtration chromatography (SEC) using an Superose-6 column, and the final step involved ion exchange chromatography (IEX) on a Mono-Q anion exchange column. The enzyme was shown to have a monomeric molecular mass of 29 kDa by SDS-PAGE but to associate as a holoenzyme of molecular mass of approximately 109 kDa in solution, implying that it is a tetramer in its native configuration. It has optimum activity at neutral pH, on milk fat and liberates mainly C_4, C_{14}, and C_{16} fatty acids from milk fat.

1.3.2.2 Esterase from *Lactococcus lactis* subsp. *lactis* NCDO 763

Chich *et al.*, (1997) purified an intracellular esterase from *L. lactis* subsp. *lactis* NCDO 763, using polyethylene glycol (PEG) fractionation, IEX (Mono Q at pH 8.0), HIC (phenyl Sepharose) and IEX (Mono-Q at pH 5.5). This enzyme was shown to be intracellular homotrimer with a monomeric molecular mass of 29 kDa, which is very similar to that of the tributyrin esterase (in 1.3.2.1). While the enzyme hydrolyses *para*-nitrophenyl esters from C2 to C12 and *ortho*-nitrophenyl butyrate, maximum activity is however, observed with *p*-nitrophenyl butyrate at pH 8.0 and 55°C. The enzyme was found to be strongly inhibited with pefabloc, diisopropyl fluoro-phosphate and 3,4-dichloroisocoumarin, demonstrating that it belongs to the group of B-esterases that have serine at their active site (Aldridge, 1993). While this esterase preferentially hydrolyzes *p*-nitrophenyl (pNP) butyrate, it is also able to hydrolyse pNP esters longer than C8. As this enzyme does not strictly correspond to the criteria for esterase defined by Tsujita *et*

al. (1990), the author proposed that this esterase should be classified as belonging to a new esterase/lipase family.

1.3.2.3 An Esterase from *Lactococcus lactis* subsp. *lactis* ACA-DC 127

Tsakalidou *et al.* (1992) purified an esterase from *Lactococcus Lactis* subsp. *lactis* ACA-DC 127 originating in Greek feta cheese. The purification steps involved were IEX (DEAE-cellulose) and SEC (Sephadex G100). The esterase had a molecular weight of 68,000 Da, optimum activity with 4-nitrophenyl butyrate at pH 8.0 and 45 °C and was strongly inactived by PMSF. Interestingly, 1,10-phenathroline and EDTA, which are metal chelators, had no effect on enzyme activity, showing that the enzyme is not dependent on the present of metal ions for activity. In fact the bivalent cations Hg^{2+} and Cu^{2+} were shown to inhibit the esterase activity.

1.3.2.4 The Genetically Modified Lactococcal Strain used in this study

The obvious benefits of controlling the production of desirable enzymes and proteins by lactic acid bacteria (LAB) used for fermented products (*e.g.* cheese) has led to the development of some genetic tools (for review, see Kleerebezen and Kupiers, 1997). De Ruyter (1996) developed some controllable expression systems based on the autoregulatory properties of nisin biosynthesis by *Lactococcus lactis*. Using the N-terminal sequence of the esterase purified from *Lactococcus lactis* subsp *cremoris* E8 by Holland & Coolbear (1995) to design probes, the gene of this esterase was isolated, cloned and overexpressed in a lactococcal strain using a controlled nisin promoter system (Holland, 1997). The resultant strain, *Lactococcus lactis* subsp. *cremoris* B1079 (est^+) has been utilised by the New Zealand Dairy Research Institute to overproduce tributyrin esterase in amounts 50-100 fold higher than in strain E8. The characteristics of the recombinant strain have been compared with that of original strain (Holland, 1997), and been show to have basically the same characteristics as those of the original esterase, except for one difference that shows up in the HIC purification step.

1.3.3 Esterase from other starter bacteria

Gobbetti, *et al.* (1997) purified an intracellular tributyrin esterase from *Lactobacillus plantarum* 2739, a strain isolated from good quality Cheddar cheese. This enzyme is a monomer with relative molecular mass of ca. 85 kDa that is able to hydrolyze β-naphthyl

Table 1.1: Summary of Comparison of Different Microbial Esterases

Microbial source	Molecular mass (kDa)	pI	Factors Affecting Activity[C]	Substrate	Purification steps	Reference
L. lactis ssp *cremoris* E8	109 with monomer 29 (25.5[a])	5.49[a]	Protease inhibitor	tributyrin, *p*-nitrophenyl butyrate	$(NH_4)_2SO_4$ fractionation, alkyl Superose, Superose 6, Mono Q	Holland,1995
L. lactis ssp. *Lactis* NCDO 763	91 with monomer 29	4.5	DFP +, pefabloc +, 3,4-DCI +, Hg^{2+} +,	C4 > C6, C8>C2 >C10 >C12	PEG fractionation, Mono Q (pH 8.0), Phenyl Sepharose, Mono Q (pH 5.5),	Chich,1997
L. lactis ssp. *Lactis* ACA-DC 127	68 monomer	ND	EDTA -, DFP -, PMSF +, Cu^{2+} +, Hg^{2+} +, Ca^{2+} -,	<C8	DEAE-cellulose, Sephadex G-100	Tsakalidou et al. (1992)
L. *plantarum* 2739	85 monomer	ND	Hg^{2+} +, Ag^{+} +,	C2-C10, prefer β-naphthyl	DEAE-cellulose, Sephacryl 200, CM-Cellulose, Mono Q	Gobbetti,1995
Lactobacillus fermentum DT41	67 monomer	ND	Hg^{2+} +, Ag^{+} +, PMSF +,	C2-C10 Limited in C12-C14	Q Sepharose, Sephacryl 200, phenyl Superose, Mono Q	Gobbetti et al. (1997)

(a) Predicticted from computer program
(b) Complete Protease Inhibitor (from Boehringer Mannhem)
(c) + : increase activity; -: decrease activity;

(β-NA) esters of fatty acids from C_2 to C_{10}, tributyrin, tricaprylin, and, to a lesser extent, milk fat. The same authors (Gobbetti *et al.*1997), recently purified a cell surface-associated esterase from *Lactobacillus fermentum* DT41, a starter used in the production of Parmesan cheese. This esterase was shown to be a monomer with a molecular mass of 67 kDa and has optimum activity at pH 7.0 between 30-35 °C.

1.4 Structure Studies on Lipase

As mentioned in previous sections, lipases are widely used in a number of practical applications. Studies on the applied technology of these enzymes are hindered by the lack of detailed knowledge about the molecular basis for their activity. The ability to manipulate lipase activity and to construct designed enzymes is dependent on such knowledge, allowing rational site directed mutagenesis to be carried out in order to bring about changes in activity. Fundamental studies of lipolytic enzymes have been driven by these demands. In this section reported structural studies on esterases from starter bacteria and other lipases are discussed extensively.

1.4.1 Primary Amino Acid Sequences of Lipases

1.4.1.1 Consensus Sequence

The first complete amino acid sequence of a lipase was reported by De Caro (1981), and a review by Antonian (1988) listed 13 sequences; today their number probably exceeds 60. While there is little sequence similarity between the microbial lipases, they all contain the G-X-S-X-G motif, in which the Ser has been identified as being essential for catalysis. In many cases this has been confirmed either by chemical labelling with inhibitors or site-directed mutagenesis, or both (Cygler, *et al.*1997). Another common feature in the sequences of microbial lipases is a HG (His-Gly) dipeptide located about 70 residues N-terminal to the catalytic serine at the margins of the lipid-binding pocket (Langin, *et al.*, 1993).

1.4.1.2 Primary Amino Acid Sequence of Tributyrin Esterase

Comparison of all the known N-terminal sequences of esterases from starter bacteria (Table1.2) reveals a striking similarity between the esterase from *Lactococcus lactis* sp. *lactis* NCDO 763 (see 1.3.2.2) and that from *L. lactis* sp. *cremoris* E8 (1.3.2.1). In

addition, they also have same monomeric molecular weight, suggesting these two lipases might be the same protein or very closely related in their structure and kinetics.

Table 1.2: Comparison of N-Terminal sequences of lipases from *Lactococcus* species

Microbial source	N-terminal sequence	Reference
L. lactis ssp *cremoris* E8	AVINIEYYSEVLGMNRKVNVIYPESSK	Holland,1995
L. lactis ssp. *lactis* NCDO 763	AVINIEYYSEVLGMNXKVNVIYPEXRFE	Chich,1997
L. plantarum 2739	SNEHTQEVLNQTVAD	Gobbetti,1995

* X= unidentified residue

A search of the GCG database revealed that there are some sequence similarities between tributyrin esterase (EC3.1.-.-) from *L. lactis* ssp *cremoris E8*, and two xylanases, acetylxylan esterase (EC 3.1.-.-) from *Caldocellum saccharolyticum* (coded by the *Xyn C* gene) (~45% overall similarity and ~34% identity) and xylanase Z (EC 3.2.1.8) from *Clostridium thermocellum* (~38% overall similarity and ~30% identity). The comparison is shown in Figure1.2.

```
TEL    1  MAVINIEYYSEVLGMNRKVNVIY....PESSKVEDFTQTDIPVLYLLHGMSGNE  50
XynC   1  MAIMQINFYSKMLKKNTTILAILPVDKPDKKFQKDVDSENLKTLYLLHGYAGNY  54
XynZ  51    VVNISYFSTATNSTRPARVYL....PPGYSKD....KKYSVLYLLHGIGGSE  94

TEL   51  NSWIIRSGIERLIRHTNLAIVMPSTDLGFYVNT.TYGMNYF.DAIAHELPKVI  101
XynC  55  MDWLCGARIVELSMRYNVAVFLPSGENSFYLDD.EEKEEYFGEFVGNEIIEFT  106
XynZ  95  NDWFEGGGRANVIADNLIAEGKIKPLIIVTPNTNAAGPGIA.DGYENFTKDLL  146

TEL   102 NNFFPNLSTKRE...KREKNFIAGLSMGGYGAYR..LAL.GTDYFSYAASLS   147
XynC  107 RSVFP....IPQ...KREKTFIGGLSMGGYGALRNGLKY.NKNFVGIIALSS   150
XynZ  111 NSLIPYIESNYSVYTDREHRAIAGLSMGGGQSFN..IGLTNLDKFAYIGPIS   196

TEL   143 GVL..TFDGMEENFKENPA...YWGGIFGNWETFKGSDNEILSLADRKQENK   188
XynC  147 ALIIHKIAGIPKDYRNAYASYNYYRRVFGDLNSLIGSDKDINALVTKLKQEK

TEL   189 ...PKLYAWCGKQDFLFPGNEYATAELKKLG    222
XynC  197 GSIPKIYMACGRDDFLVQENRDLFNFLKNEG    233
```

Figure 1.2 Comparison of amino acid sequence

TEL (tributyrin esterase from *L. lactis* ssp. *cremoris E8*), Xyn C (acetylxylan esterase from *Caldocellum saccharolyticum* coded by Xyn C gene) and Xyn Z (Xylanase Z from *Clostridium thermocellum*)

In general, proteins with common functions sometimes have similar sequences limited to short stretches around essential catalytic residues (Drables, *et al.*, 1997). From the comparison of the above proteins, it is clearly shown that the sequence similarity among

these proteins is around the LYLLHGXXG (40~50) and GLMSGG region (114~128). Hence, Ser (124) can be postulated as the catalytic centre. However, the three-dimensional structure of acetylxylan esterase from *Caldocellum saccharolyticum*, which has similar molecular size, is still unknown. The three-dimensional structure of the catalytic domain of xylanase Z from *Clostridium thermocellum* has been determined at 2.6Å (Dominguez, *et al.*, 1995). The catalytic domain, residues 531 to 789 of Xyn Z is located in the C-terminal region of this enzyme (Souchon, *et al.* 1994 and Grepinet, *et al.* 1988), which does not contain the sequence similarity with tributyrin esterase. This comparison, however, indicates that these enzymes may have a common evolutionary origin. Furthermore, it has been shown that homologous proteins usually have the same main-chain folding and possibly related functions (Topham, *et al.*, 1990). Comparison and analysis of known three-dimensional protein structures has also clearly demonstrated that proteins with ~30% sequence identity or more, are likely to adopt similar tertiary folds (Schrag, *et al.*, 1997). It will not be unexpected therefor, if the tertiary structures of the tributyrin esterase and the non catalytic domain of xylanase Z, are shown to be similar.

1.4.2 Three-Dimensional Structures of Lipases

In 1990, the first crystal structures of two unrelated lipases were reported: one of an enzyme purified from a fungus *Rhizomucor miehei* (RmL) at 1.9 Å resolution (Brady, *et al.*, 1990) and the other of the human digestive enzyme, pancreatic lipase (hPL) at 2.3Å resolution (Winkler, 1990). To date the 3-dimensional structures of more than 10 lipases from mammalian and bacterial sources have been determined by X-ray crystallography and work on more is near completion. Several common structural features have been identified in these proteins.

1.4.2.1 Catalytic Triad in the Active Site

In all the lipase structures solved to date, the serine in G-X-S-X-G consensus is located in a sharp turn between a β-strand and a buried α-helix. The side chain of the catalytic serine is hydrogen bonded to the side chains of two other residues, usually His and Asp (or Glu), which serves to make the serine hydroxyl group strongly nucleophilic. (Derewenda, *et al.*, 1991)

1.4.2.2 Lipase Fold

Analysis of known protein structures has shown that similar folds are sometimes adopted by proteins with little sequence similarity (Bowie, *et al.*,1991 & Alon, 1995). The α/β hydrolase fold is one example of a tertiary fold that is adopted by a number of proteins displaying no sequence similarity (Schrag, *et al.*, 1997). Apart from the pancreatic lipases, all the lipases with known three-dimensional structures contain only one domain with an α,β doubly wound protein fold and are formed from a parallel β sheet and a number of helices that flank the sheet on both sides. The minimal fragment of this fold common to all lipases is a subset of the α/β-hydrolase fold as described by Ollis *et al.* (1992) that contains a five-stranded β sheet and two α helices.

1.4.2.3 Two Classes of Conformers: Closed and Open

One of the important features of an enzyme is the shape of the substrate-binding site and its accessibility to the substrate. Structures of the majority of the enzymes show that their active sites are on the surface of the molecule that are accessible to solvent. This is not always the case for lipases. The various lipase structures determined to date can be divided into two categories: those with the active site accessible to the solvent (the open form) and those with an inaccessible active site, where a "lid" closes over the active site (the closed form). For some lipases both forms have been experimentally observed (Cygler, *et al.*,1997).

1.4.2.4 Oxyanion Hole

Previous crystallographic and biochemical studies of lipases have also shown that the mechanism of hydrolysis by lipases is similar to that of the serine proteases. In both cases, an oxyanion created during hydrolysis, is located in the so-called "oxyanion hole" and is stabilised through non-covalent interaction with some electrophiles (Martinez, *et al.*, 1992).

1.5 Needs and aims of the current project

As reviewed in above section, little is known about the molecular basis for trybutyrin esterase activity in starter culture bacteria. We know from studies undertaken by Holland (1995) that tributyrin esterase hydrolyses tributyrin. But we cannot be sure however, that tributyrin is the preferred substrate *in vivo*. The primary structural homology to the non

catalytic domain of xylanase Z from *Clostridium thermocellum* is intriguing, as it is not-known whether this domain has any specific functions. However, the amino acid sequence homology has raised the possibility that the enzyme, which contains a postulated active site similar to many other hydrolases, may be able to hydrolyse other biomolecules. Many families of enzymes that are classified through function have been found to have similar or conserved structures or contain conserved structural motifs (Schrag, *et al.*, 1997). Often the structural homology is more striking than sequence homology. Therefore the determination of the three dimensional structure of tributyrin esterase and the subsequent analyses and comparison with other three dimensional structures in the protein data base should unequivocally show whether or not the enzyme is a true esterase, or whether it belongs to another enzyme class.

The three-dimensional structure determination of a protein by X-ray crystallographic techniques requires the production of large single crystals. As reviewed in the following section, the difficulties of screen searching for crystal growth conditions may require milligrams of protein for the initial crystallisation trials. Once crystals suitable for X-ray analysis can be reproducibly grown, additional protein will be needed to improve their quality and size and to prepare heavy-atom derivatives. For all these reasons it is essential to develop a suitable purification procedure in order to produce enough fresh protein of reproducible quality.

The addition of poly-His terminal extensions on target proteins using genetic engineering has for a long time been regarded as a powerful tool for use in the isolation of target proteins due to the ease of the purification procedures which can be applies (see review in section 1.6). In this technique, the gene of a target protein is cloned into a vector that contains the codons for six histidine residues and a fusion protein is produced in a high expression system. A convenient and abundant source of pure protein can thus be relatively easily obtained.

The aim of the current project is to provide a foundation for the subsequent three dimensional structural determination of tributyrin esterase. For all the reasons described above, the immediate goals addressed are first, to purify the tributyrin esterase from the genetically modified lactococcal strain (section 1.3.2.4), secondly, to crystallize this enzyme, and thirdly, to develop an expression system in *E. coli* suitable for over-

expressing the tributyrin esterase. As a longer-term goal, the development of a tributyrin esterase overexpression system in *E.coli* would not only facilitate the preparation of the tributyrin esterase necessary for the structural determination, but would also provide a foundation for site directed mutagenesis studies of the enzyme.

Having addressed the above goals of the current project, in the following sections, a brief overview of facilitating protein purification by histidine tagging will be given. The topic of protein crystallization will also be reviewed.

1.6 Facilitating Protein Purification by Histidine Tagging

In biochemistry and molecular biology, the expression of histidine-tagged fusion proteins is a well-established technique for the identification, and purification of gene products (Hochui *et al.*, 1988; Porath *et al.*, 1976). This technique is based on the affinity of five to six consecutive histidine residues for metal chelating lidands, such as N-nitrilotri-acetic acid (NTA) or imidodiacetic acid (IDA). Association and dissociation of the His-tagged protein can be triggered by competitors such as histidine and imidazole, or by changing the pH of the buffer solution.

Recently, Ledent *et al.*, (1997) reported that the addition of a His-tag to the C-terminal end of β-lactamase had an unexpected influence on both the structure and the thermal unfolding of the protein. Lee *et al.*, (1997) also proved that the direct addition of a His-tag to both the C-terminal and the N-terminal end of an aryl-esterase resulted in a significant change in the enzyme activity. These results served as a warning that His-tag addition may not always be as 'neutral' as has been assumed. Therefore, careful comparison of wild-type and His-tagged proteins is necessary.

1.7 Protein Crystallization

1.7.1 Principles of Protein Crystallization

While protein crystallization has been mainly a trial-and-error procedure in which the protein is slowly precipitated from solution, recent reports have identified the basic processes that occur when crystals form. (Ries-kautt, *et al.*, 1990 and Drenth, 1994) The crystallization of a macromolecule is directly related to its solubility characteristics, which can be represented by a phase diagram (Fig.1.3). Of the many variables affecting

solubility, two of the most important are the concentration of the protein and the concentration and type of precipitant used. In the region under the solubility curve, both nucleation and crystal growth are impossible and any crystal formed previously will dissolve. Above the solubility curve, the concentration of protein is higher and the protein is supersaturated. This region may itself be subdivided into three zones: (i) The precipitate phase, where excess protein immediately separates from the solution in an amorphous state; (ii) The labile phase (nucleation zone), where excess protein separates from the solutions in a crystalline form. Crystallization may occur both as a shower of microcrystals and an amorphous precipitate. (iii) The metastable zone , in which the supersaturated solution may not be nucleated for a long period of time, unless the solution is mechanically shocked or a seed crystal introduced. In this zone, crystals are able to grow and the nucleation of new crystals is minimal.

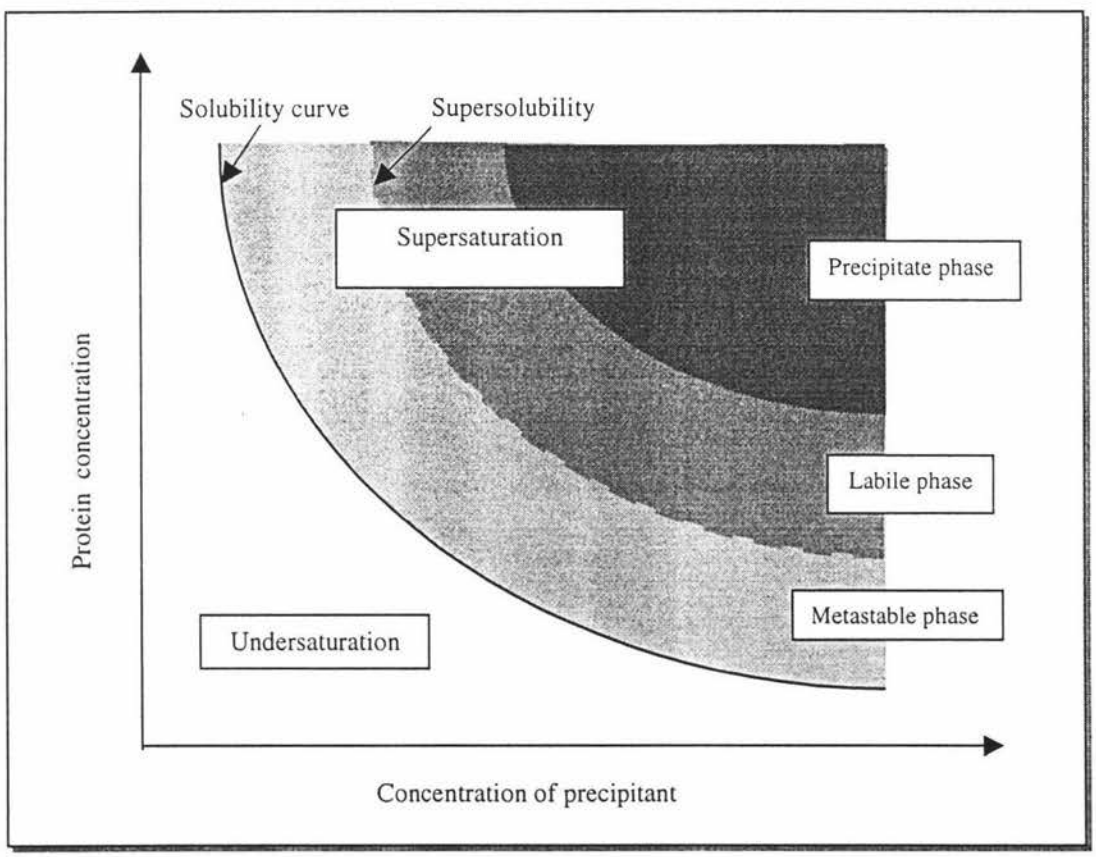


Figure 1.3 Phase diagram.

Overall, there are three major steps in crystallizing a protein:

1. The protein is dissolved in a suitable solvent from which it must be separated in crystalline form. It is important that there should be no aggregation of protein molecules in this solvent.
2. The solution is slowly brought to supersaturation, as spontaneous formation of nuclei is best achieved at a high supersatuation.
3. Once nuclei have formed, actual crystal growth can begin.

1.7.2 Screening for Crystallization Conditions

A large number of parameters influence the crystallization process: protein purity and concentration, buffer type, pH, temperature, ionic strength, and the presence of minute amounts of detergent or organic or inorganic molecules. As a general rule, the more pure the protein, the better the chances of growing crystals. In order to successfully grow crystals suitable for X-ray diffraction, proteins not only have to be pure in terms of lack of other compounds, they have also to have the same surface properties, especially the same charge distribution on their surface (Drenth, 1994).

Finding the best combination among many crystallization parameters is a multivariable optimisation problem. During the past decade, the development of incomplete factorial screening protocols and optimisation strategies have provided investigators with an extended portfolio of effective crystallization tools (Carter, 1990; Jancarik & Kim, 1991; Kingston, 1996).

The process of crystallizing a protein can be divided into three discreet stages. These are (1) screening for useful crystallization conditions or leads; (2) optimisation of one or more initial conditions to produce single crystals suitable for X-ray diffraction analysis; and (3) reproducible production of single crystals for X-ray data collection.

1.7.3 Methods of Crystallization

1.7.3.1 Vapor Diffusion Methods

One of the most commonly employed techniques for growing protein crystals is the hanging drop method. A droplet containing the protein, with buffer, crystallization agent, and additives, is equilibrated against a reservoir containing a solution of crystallizing agent at a higher concentration than in the droplet. Thus, concentrations within the

protein-containing droplet are manipulated remotely by diffusion through air. Equilibration proceeds by diffusion of the volatile species (usually H_2O) until the vapour pressure of the components in the droplet equals those of the reservoir.

1.7.3.2 Dialysis methods

The protein is separated from a large volume of solvent by a semi-permeable membrane which gives small molecules (ions, additives, buffer, and so on) free passage through the membrane, but prevents free passage of the protein. Thus, the dialysis method is uniquely suited to crystallization at low ionic strength and in the presence of volatile reagents such as alcohols. The kinetics of equilibrium depend on the ratio of the concentration of precipitant inside and outside the membrane, the temperature, and the geometry of the cell.

CHAPTER 2 PREPARATION OF TRIBUTYRIN ESTERASE FROM *Lactococcus Lactis* strain B1079

The aim of this section of work was to produce, extract and purify the tributyrin esterase enzyme from a genetically modified strain of *Lactococcus lactis*, strain B1079, for use in crystallization trials. As discussed in the introduction, the purity of the protein is very important in crystallization (see section 1.6). In general, the likelihood of achieving diffraction quality crystals is increased if a completely homogenous protein solution is used to carry out crystallization growth trials. This requires absolute purity of the protein involved, as judged by SDS-PAGE, electrospray mass spectrometry and other methods where applicable. It also requires that there is little aggregation of the protein molecules at the concentrations and under the conditions (pH, temperature, etc,) used for the trials. Preliminary studies were therefore undertaken to optimize the purification methods used and the factors affecting enzyme activity.

2.1 Materials

2.1.1. Bacterial strain

The organism used was a genetically modified lactococcal strain---*Lactococcus lactis* strain B1079 (est^+), which was obtained from the New Zealand Dairy Research Institute (NZDRI) Palmerston North (see section1.3.2.4).

2.1.2 Microbiological Media Constituents

M17 media (Difco Laboratories Ltd., Surrey, UK) containing 0.5% glucose and 10 $\mu g \cdot mL^{-1}$ antibiotic chloramphenicol was used to grow all cultures. (The chloramphenicol was added following autoclaving)

2.1.3 Chemicals

-BDH Laboratory Supplies, Poole, England

Tris(hydroxymethylamine; glycine; glycerol; acetonitrile; sodium dodecyl sulphate (SDS); glucose; ammonium sulfate; sodium phosphate;

-Bio-Rad, Laboratories, Hercules, Carlifornia, USA

Acrylamide/Bis 37.5:1(2.6%C); ammonium persulphate (APS); TEMED (N,N,N',N'-Tetrmethylethylenediamine) ; bromophenol blue; Coomassie Brilliant Blue R-250; Protein assay reagent;

-Hampton Research

Polyethleneglycol (PEG) 6000;

-Sigma Chemical Co, St. Louis, Missouri

DTT (DL-Dithiothreitol); HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]); *p*-nitrophenyl butyrate; BTP (Bis-tris propane)(1,3-bis[tris9Hydroxymethyl)-methylamino]propane); EPPS (N-[2-Hydroxyethyl]piperazine-N'[3-propanesulfonic acid]) ; RNase; DNase; BCA (Bicinchoninic acid) reagent;

-Pharmacia Biotech,

Q-Sepharose fast-flow gel, Sephadex G-75 and G-150; Mono Q HR 5/5 prepacked column; Alkyl Superose HR10/10 prepacked column; Phenyl Sepharose HR 5/5 prepacked column; Butyl sepharose 4 fast flow and Octyl sepharose 4 fast flow HiTrap HIC Test Cartridge; Superose 12 prepacked column;

2.1.4 PAGE Protein Standards

Rainbow protein molecular mass markers (whole molecular mass range) were obtained from Amersham Life Science, Little Chafont HP79NA, UK. Low-Mr protein standards were obtained form Bio-Rad, Laboratories, Hercules, Carlifornia, USA.

2.1.5 Deionized water

The water used in all steps of the purification was purified through a Milli-Q filtration system (Millipore Corp., Bedford, Massachusetts, USA) containing ion exchange, solvent exchange, organic and inorganic removal cartridge.

2.2 Methods in Microorganism Growth

2.2.1 Microbiological Media Sterilization

All culture media were sterilized by autoclaving at 121°C and 20 psi for 15 min. Culture media (5 L) was sterilized in 1 L aliquots.

2.2.2 Stock Culture preparation

To prepare stocks, strain *Lc. lactis* subsp. *cremoris* B1079 (est^+) was cultured in 10 mL of medium for 16 hours at 30°C. Five mL of this culture were transferred to 100 mL of medium and grown at 30°C. After 8 hours, 2 mL aliquots were taken and stored at -90 °C as stock inocula. These frozen cultures were the stock used for all subsequent work. One of

these cultures was checked for homogeneity by streaking on agar plates (M17 agar containing 5% lactose and 5% glucose).

2.2.3 Fermentation culture conditions

Stock cultures were revived by inoculating a thawed aliquot of 0.5 mL into 10 mL of growth medium and incubating anaerobically at 30 °C for 16 hours, or until an optical density of 1.8 at 600 nm was reached. This culture (1 mL) was then transferred to 100 mL of growth medium, and incubated at 30 °C for approximately 8 hours. A portion of this culture was then used to inoculate the main 5 L fermentation [1 % (v/v) inoculum], which was then incubated at 30 °C for 16 hours.

2.2.4 Tributyrin Esterase Induction

Purified nisin stock ~ 4mg/mL, from Netherlands Institute of Dairy Research (NIZO) was diluted 1:1000 with 0.05% (w/v) acetic acid, to give 1 mL of working solution with a nominal nisin concentration of $4\mu g \cdot mL^{-1}$. The exact nisin concentration in the working solution was determined by measuring the absorbance of the solution at 220nm using 0.05% (w/v) acetic acid as a blank; the concentration, in $mg \cdot mL^{-1}$, equals $A_{220}/18$. When the growing cultures of *Lc. lactis* subsp. *cremoris* B1079 (est^+) had reached a cell density equivalent to an A_{600} of 0.5. The est^+ gene was induced by the addition of freshly prepared nisin working solution to give a final concentration of $3\mu g \cdot L^{-1}$ nisin.

2.3 Analytical Methods

2.3.1 Growth Studies

The growth curve of a 500 mL culture of *Lc. lactis* subsp. *cremoris* B1079 (est^+) was measured by removing 5 mL aliquots from the culture every hour and measuring the absorbance at 600nm using a Hitachi 4625 UV/VIS spectrophotometer (Hitachi Analytical, Japan). The sample was diluted with water to ensure that the absorbance stayed within the instrument range. The pH was measured using a pH M-82 Standard pH meter (Radiometer, Copenhagen, Denmark) as another indication of growth.

2.3.2 Cell Dry Weight Determinations

In order to calculate the dry weights of the cell in each culture, duplicate 200 µL samples were dispensed onto preweighed 0.4 µm cellulose acetate filters (Whatman). The cell

were washed with 25 mL of distilled water. The liquid was removed by vacuum filtration, and the filters were dried in a vacuum oven (Squariod, Lab-line) at 80°C. They were re-weighed after cooling in a desiccator.

2.3.3 Determination of Protein Concentration

Five different assay methods were tested to determine protein concentration. These were the Biuret, Lowry, Bicinchoninic acid, Bradford, and UV methods. In all of these methods, standard curves were constructed using bovine serum albumin at a concentration of 0~0.5 mg/mL. The Bradford method was found to be the most suitable method experimentally and was thus used in all purification steps to determine the protein concentration. The UV method was, however, used to determine protein concentration of the final concentration (protein used for crystallization) as it is a non-destructive method. The details of methods used are listed as follows. (All absorbance was measured in either HP 8452 or Hitachi 4625 UV-visible spectrophotometer.)

(A) The Lowry Method:

The Lowry method for protein determination was tested as described by Lowry *et.al.*, (1951). 0.75 mL Lowry reagent was added to 0.15mL sample at concentration 0 ~ 8mg/mL, and incubated at room temperature for 30 min, A_{540} was read.

(B) Bicinchoninic Acid Method

1 ml of 4% $CuSO_4$ was added to 50 ml of premixed BCA solution to form the reaction mixture. 0.1 ml of the standard or sample protein was then added to 1.9 ml of reaction reagent, and incubated at room temperature for 30 min, before reading the absorbance of 562 nm.

(C) Bradford Method

In all enzyme purification steps, protein was estimated by the method of Bradford (1976) using a Bio-Rad kit. Diluted dye reagent (5 mL) was added to 100 μL of sample or standard. The reaction mixture was incubated at room temperature for at least 5 minutes (no more than 1 hour), and the absorbance at 595 nm was then measured. While this method normally gives lower than average values for protein concentrations, in this case the buffers had the least effect on the determination of the protein concentration

(D) UV Method

The first experiment to estimate protein concentration by UV absorption was set up as described by Wetlaufer (1962). The absorbances at 280 nm and 260 nm were measured and protein concentration was estimated using the following equation:

$$\text{Protein concentration (mg/mL)} = F \times 1/d \times A_{280}$$

Where d is the cuvette width in cm, and the F, a factor related to the ratio of A_{280}/A_{260}. The value obtained from this method was then correlated with the value obtained using the Bradford method. The simple 280 nm reading was then used to determine the protein concentration. Each measurement was carried out by using 10 μL of protein sample diluted with 0.99 mL of BTP buffer (pH 7.0) in a 1 ml quartz cuvette, using BTP buffer as a blank. While this method is not very accurate, it has the advantage of being non denaturing, and is a useful comparison with the Bradford method.

2.3.4 Esterase Activity Assay

Esterase activity was estimated spectrophotometrically using *p*-nitrophenyl butyrate as substrate (Lee & Lee, 1989). 10 μL of diluted enzyme was added into a 2.0 mL reaction mixture containing 100 μL 50 mM methanolic *p*-nitrophenol butyrate in 50 mM sodium phosphate buffer, pH 7.5 at 30 °C. The change in absorbance due to esterase activity was measured at 410 nm, 1.0 unit of esterase being the amount of enzyme required to release 1.0 μmole of *p*-nitrophenol per minute. To monitor the elution of esterase from FPLC columns, 50 μL of the fractions to be tested were added to 200 μL of esterase reaction mix in a 96-well plate. The development of yellow colour indicated the fractions containing esterase. The formula used to calculate the activity is:

$$\text{Activity} = \frac{1}{12.2} \times \frac{2}{1} \times \frac{1000}{x} \times \frac{\Delta Abs}{min}$$

(1.0 unit = $\mu mol \cdot min^{-1} \cdot mL^{-1}$ enzyme)

Where: 12.2 is the millimolar extinction coefficient of *p*-nitrophenol butyrate ;

x is amount of sample added (μL);

2.0 is total eaction mixture in mL ;

Factors affecting enzyme activity were tested as follows.

(A) Protease inhibitor

One tablet of "Complete" protease inhibitor (from Boehringer Mannhem) was dissolved in 2 mL 100 mM phosphate buffer, pH 7.0 to make up stock solution. 1 μL of this stock solution was added to 50 μL of pure tributyrin esterase. The reaction

solution was incubated at room temperature. Enzyme activity was measured after one hour and after 16 hours.

(B) DTT

10 μL of 100mM DTT stock solution was added to 100 μL of pure tributyrin esterase to take the final concentration of DTT to 10mM. The reaction mixture was incubated at room temperature for 30 minutes. The enzyme activity was then measured.

(C) Buffer Type

The tributyrin esterase buffer was exchanged by ultrafiltration. 500 μL of protein solution (in BTP buffer) was placed in a Centricon-3 unit with a 3,000 MW cut-off and spun to ca. 50 μL. After adding three times of 450 μL of the buffer to be tested, Enzyme activity was then measured by using the same method described above.

(D) Salt Concentration

A pure protein solution of trybutyrin esterase was first changed to BTP buffer without any salt by ultrafiltration as described above. NaCl solutions were made up at the following concentrations: 10, 20, 40, 100, 200, 300, 400mM respectively. 25 μL of pure trybutyrin esterase and 25 μL of the different NaCl solutions were mixed together to give final NaCl concentration of 5, 10, 20, 50, 100, 150, 200mM respectively. Enzyme activity was then measured using the same method described above.

2.3.5 Polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE and native PAGE were performed using the Mini Protean II Dual Slab Cell system (Bio-Rad Laboratories, Richmond, CA, USA) and the method described by Creamer (1991). A standard procedure using the following method was followed:

2.3.5.1 SDS-PAGE

Stock solutions of Acrylamide/Bis 37.5:1(2.6%C), SDS-PAGE resolving gel buffer (1.5M Tris-HCl, pH 8.8), SDS-PAGE stacking gel buffer (0.5 M Tris-HCl, pH 6.8), APS (10% w/v in mili-Q water) and SDS (10% w/v in Milli-Q water) were made. Resolving gels (12.5%) and stacking gels (5%) were made as described in Appendix A and gels were polymerised overnight.

2.3.5.2 Native-PAGE

Stock solutions of Acrylamide/Bis 37.5:1(2.6%C), native-PAGE resolving gel buffer (1.5M Tris-HCl, pH 8.8), native-PAGE stacking gel buffer (0.5 M Tris-HCl, pH 6.8) APS (10% w/v in mili-Q water) were made. Resolving gel (12.5%) and stacking gel (5%) were made as described in Appendix B. Prior to use Polymerised gels were always left for 24 hours in sealed plastic bags at 4 °C to ensure complete polymerisation had occurred.

2.3.5.3 Sample preparartion

SDS-PAGE sample buffer was prepared by mixing 1.25 mL SDS-PAGE stacking gel buffer, 0.25 ml Bromophenol blue (0.1% w/v), 0.5 mL β-mercaptoethanol (or 100mM DTT), 1.0 ml glycerol, 10% SDS and 5.0 mL Milli-Q water, final pH 6.8. For SDS-PAGE analysis, protein samples were diluted with equal volume of sample buffer and heated at 100°C for 2 minutes, before being loaded onto the gel (generally 10 -20 µL samples, dependent on the protein concentration). A constant voltage of 150 V (current ≤50 mA) was applied across the electrodes, until the Bromphenol Blue dye reached the bottom of the gel (typically 1 hour). Either a Bio-Rad Bio-Rad 1000/500 or a 200 power supply was used in these experiments. The running buffer consisted of 25 mM Tris-HCl, 0.2 M Glycine and 0.1% SDS, pH8.3.). For native gels, the same procedure was followed, except samples were not heated and SDS was not added to the loading and running buffers.

2.3.5.4 Stain and Destain

Gels were fixed and stained with a solution of 1 g Coomassie brilliant blue R-250 in one liter of methanol-acetic acid - water (40:1:50, by volume) and destained with methanol/ acetic acid / water (40:1:50).

2.4 Enzyme Purification

2.4.1 Overview of the purification of Tributyrin Esterase

Two main preparations were carried out, in an attempt to obtain enough product of pure tributyrin esterase. The purification strategy was similar to that described by Holland (personal communication). Cells from two 5 litre cultures were used for preparation one, and from a 10 litre culture for preparation two. All purification steps used in the two preparations are summarised in Figure 2.1. All steps except those involving IEX and HIC

chromatography were carried out at 4 °C. Details of the methods used in each step are described in following sections. Method tested in an attempt to improve the protocol are also described.

2.4.2 Harvesting of Cells and Extraction of Protein

Cultures (5 liters) were harvested at the beginning of the stationary phase by centrifugation at 11700 g for 10 min at 4°C. The cells were washed with 20 mM Bis-Tris propane buffer (BTP), pH7.0 then resuspended in 90 mL of the same buffer before being disrupted by two passes through a French Pressure cell (Aminco) at 3,800 kPa. DNase and RNase (0.5 mg each) were added to the homogenate, which was centrifuged at 39,000 g for 30 min. Cell free supernatants were obtained by further centrifugation of the supernatant at 39,000 g for 30 min at 4°C.

2.4.3 Purification by Precipitation

2.4.3.1 Ammonium Sulfate Salt Fractionation

Precipitation by increasing the ionic strength (salting-out) exploits the hydrophobic properties of the protein (Scopes, 1994) and was one of the major steps used to reduce the total amount of protein. Finely powdered $(NH_4)_2SO_4$ was added to the cell free supernatant slowly with stirring at 4°C, until the solution reached 60% saturation. After equilibration with stirring for 2 hours at 4°C, the precipitated material was removed by centrifugation at 39,000 g for 30 min. To investigate the efficiency of this step, fractionation was also carried out at 20%, 40% and 60% saturation, and the protein fractionated in both pellet and supernatant was tested for protein concentration and activity in order to determine the salt concentration required for optimum separation conditions.

2.4.3.2 Polyethylene glycol (PEG) Fractionation

PEG is also used to precipitate proteins, and has the advantage of not interfering with ion exchange chromatography. The procedure exploits the polar nature of the protein, rather than the hydrophobic properties, and thus may have advantages compared to ammonium sulfate. In order to investigate whether PEG precipitation could be used as a purification step, trials were carried out on 10 mL samples of the cell free supernatant. Solid polyethylene glycol (PEG) 6000 was added to the protein solutions to 4%, 4.5%, and 5% saturation. After gentle stirring for 1 h, the suspension was centrifuged, and both pellet and supernatant were analyzed for protein concentration and enzyme activity.

2.4.3.3 Precipitation by alteration of the pH

At the isoelectric point, the negative and positive charges on the surface of a protein molecule cancel one another out. Electrostatic repulsion between individual molecules no longer occurs, and hydrophobic interaction between molecules may occur, resulting in the formation of a precipitate. As the isoelectric point of tributyrin esterase is predicted to be 5.49 from sequence data using Expassy Tools, (1994). The pH of the protein solution was brought to 5.5 by the slow addition of 0.1 M acetic acid at 4°C.

2.4.4 Chromatographic Methods

Most of the chromatographic steps were carried out by using medium pressure chromatography. Low-pressure chromatography was also used to test the separation on Phenyl Sepharose column and to run the final size exclusion column. Medium pressure chromatography uses resins with a smaller particle size than is used in low pressure system and therefore results in better resolution (Scopes, 1994). Medium pressure chromatography purification steps were carried out using either a FPLC (Pharmacia, Biotechnology) or a Waters 650 System. Molecular weight determination using SEC were carried out using a SMART system (Pharmacia, Biotechnology). Low-pressure chromatography was carried out on an Econo system (Bio-Rad). All solutions for FPLC were filtered through a 0.22 μm filter, before use.

2.4.4.1 Ion Exchange Chromatography (IEX)

Ion exchange chromatography separates proteins on the basis of the overall charge on the molecule (positive or negative). The matrices can be either cationic or anionic carrying ligands that retain their charge over a narrow pH range (weak ion exchange) or a wide pH range (strong ion exchanger). The functional ligands on the resin (stationary phase) interact with proteins through electrostatic attractions. Anion exchange resins have positively charged ligands, such as DEAE and QAE groups covalently linked to the matrix; while cation exchange resins have negatively charged ligands, such as carboxymethyl (CM) and sulphopropyl (SP) groups covalently link to the matrix. The charges on these columns are balanced by counterions such as Cl^- in the case of anion exchange, and Na^+ in the case of cation exchange. The net charge on the protein is the same as that of the counter ion, and protein displaces the counterions to bind to the ligand. This process can later be reversed by

eluting the protein with buffer containing either a higher concentration of the counter ion, or by changing the pH to neutralise the charge on the resin.

In the purification of the tributyrin esterase, IEX was used as the first chromatography step to reduce total amount of protein. The pellet from the 60% ammonium sulfate precipitation was redissolved in 45 mL of 20 mM BTP buffer pH7.0, and dialysed against several changes of 20mM BTP buffer, pH 7.0, over 4.5 h at 4°C. Particulate matter that remained undissolved was removed by centrifugation at 27000 g for 15 min and tested for activity. The supernatant was loaded on to a Q-Sepharose anion exchange column (20 cm x 10 mm, Pharmarcia LKB Biotechnology, Uppsala, Sweden) equilibrated in 20 mM BTP buffer, pH 7.0. The column was then washed with the loading buffer until all unbound material was eluted ($A_{280} < 0.03$). Bound protein was then eluted using a 2 L linear gradient of 0 - 1.0 M NaCl in 20 mM BTP buffer, pH 7.0, at a flow rate of 2 $mL \cdot min^{-1}$. Fractions (10 mL) were collected, and elution was monitored by measuring the absorbance of the eluant at 280 nm. Peak fractions were analysed for activity using microplate wells, and the active fractions analysed by SDS-PAGE. Those fractions that were active and showed least contamination with other proteins were pooled and concentrated by ultrafiltration using an Amicon 50 mL stirred cell, and a YM10 membrane (Amicon). In the first preparation, one anion exchange step was used, while in the second preparation, two anion exchange steps were necessary.

As a final polishing step, an ion exchanged chromatography using a Mono-Q column was tried. Mono Q is a strong ion exchange resin, and it was hoped that the smaller bead size and different characteristics of the column might remove the last contaminating protein.

2.4.4.2 Hydrophobic Interaction Chromatography (HIC)

HIC separates biomolecules on the basis of hydrophobicity due to the non-polar amino acids side chains at the surface of the protein. The hydrophobicity of the matrix results from either alkyl or aryl functional groups that are covalently linked to an inert matrix, such as sepharose or agarose. The adsorption of protein to these groups requires the presence of salt such as NaCl or $(NH_4)_2SO_4$. The salt ions decrease the availability of water molecules to the protein, removing ordered water from hydrophobic patches on the protein surface and enhancing hydrophobic interaction between the hydrophobic patches on the protein and the groups on the resin. Protein can be eluted by decreasing the salt concentration of the buffer, in a gradient or a stepwise manner. Lower pH values strengthen the interaction between the

protein and hydrophobic groups on the matrix, while lowering the temperature, increasing the pH, or including detergent, ethylene glycol or organic solvent in the elution buffer, weakens them (Scopes, 1994).

In the purification of tributyrin esterase, HIC was a very effective purification step that removed many contaminating proteins. The pooled, concentrated fractions from IEX chromatography were brought to 1.5 M with $(NH_4)_2SO_4$, and a maximum of 10 mg of protein solution was loaded on to an Alkyl Superose 10/10 column (Pharmarcia LKB Biotechonology, Uppsala, Sweden), which had been equilibrated in 20 mM BTP (pH 7.0) containing 1.5 M $(NH_4)_2SO_4$. The column was then washed with loading buffer until all unbound protein had been removed. Bound protein was eluted using a reverse gradient (80 mL) of 2.0 – 0.0 M $(NH_4)_2SO_4$ in 20 mM BTP (pH 7.0) at flow rate of 0.5 ml/min, and fractions (0.5 ml) were collected. Peak fractions were analyzed for activity and by SDS-PAGE. Fractions that showed esterase activity and had only one band on a SDS gel were pooled and concentrated by ultrafiltration as before. In both preparations, four columns were necessary to process all the material obtained from IEX chromatography.

In order to test whether alkyl sepharose gave the best seperation, Phenyl Sepharose, butyl sepharose 4 fast flow and Octyl Sepharose 4 fast flow were also tried, using a variety of conditions including those used for Alkyl Superose column. As mentioned above, the addition of various salts promotes ligand-protein interactions, but each type of salt differs in its ability to promote hydrophobic interactions. Various conditions including different pH, salt concentration and type of salt were tested for these columns. These conditions were:

(a) Phenyl-Sepharose HR 5/5 column at 4°C and pH 8.5 using 1.5 M $(NH_4)_2SO_4$;

(b) Octyl-Sepharose 4 fast flow at pH 8.0 (room temperature) using 1.5 M $(NH_4)_2SO_4$;

(c) Butyl-Sepharose 4 fast flow at pH 7.0 (room temperature) using 1.5 M $(NH_4)_2SO_4$;

(d) Butyl-Sepharose 4 fast flow at pH 6.0 (room temperature) using 1.5 M $(NH_4)_2SO_4$;

(e) Butyl-Sepharose 4 fast flow at pH 8.0 (room temperature) using 1.5 M $(NH_4)_2SO_4$;

(f) Alkyl Sepharose HR 5/5 column at pH 7.0 (room temperature) using 1.5 M $(NH_4)_2SO_4$;

(g) Alkyl Sepharose HR 5/5 column at pH 7.0 (room temperature) using 2.0 M or 3.0 M NaCl;

2.4.4.3 Gel Filtration Chromatography (SEC)

Gel filtration chromatography exploits the size and the shape of the protein. Proteins are separated on a matrix of evenly sized beads that contain a number of pores at limited size. The smaller molecules are able to penetrate the internal volume of pores and hence are the last to be eluted from the column. The large molecules are excluded from the pores and elute first from the column. Molecules of intermediate size will have access via diffusion to a portion of the pore depending on their size, and will move down the column at a rate dependent on this size.

In current studies, SEC was used as the final purification step. Gel filtratiion chromatography was carried out using Sephadex G-150 Superfine (90 cm x1.6 cm) or Superdex 75 (60 cm x 1.0 cm) (Pharmacia). The elution buffer was 50 mM BTP, pH 7.0, and a flow rate of 0.2 $mL \cdot min^{-1}$ was used to obtain optimal separation. Fractions of 1 mL were collected and analysed for activity and purity as before. Those containing esterase activity and showing only one band on SDS-PAGE were pooled and concentrated to 10 mg/mL for crystallization studies.

As superose 12 has a separation range from 100~300,000 Da, it was chosen to try to separate any multimers that may exist in solution (tetramers-hexamers). In SEC chromatography, interaction of packing materials with protein and protein-protein interactions, which include ion, hydrophobic and other non-specifical interaction will always affect the separation. To minimize these interactions, different ionic strength buffers (high and low salt concentration buffers) were tried.

2.4.4 Purification by Ultrafiltration

Membrane ultrafiltration is a pressure-modified, convective process that uses semipermeable membranes to separate species in aqueous solution on the base of size, and shape (Schratter, 1996). To try and separate any monomeric species from tetrameric or trimeric species, an Ultrafree-4 centrufugal filter device (Millipore, USA) with a 100 kDa moleular weight cut-off (NMWC) membrane, and a 2 mL Centricon centrufugal filter device (Centracon, USA) with a 50 kDa molecular weight cut-off (NMWC) membrane, were used.

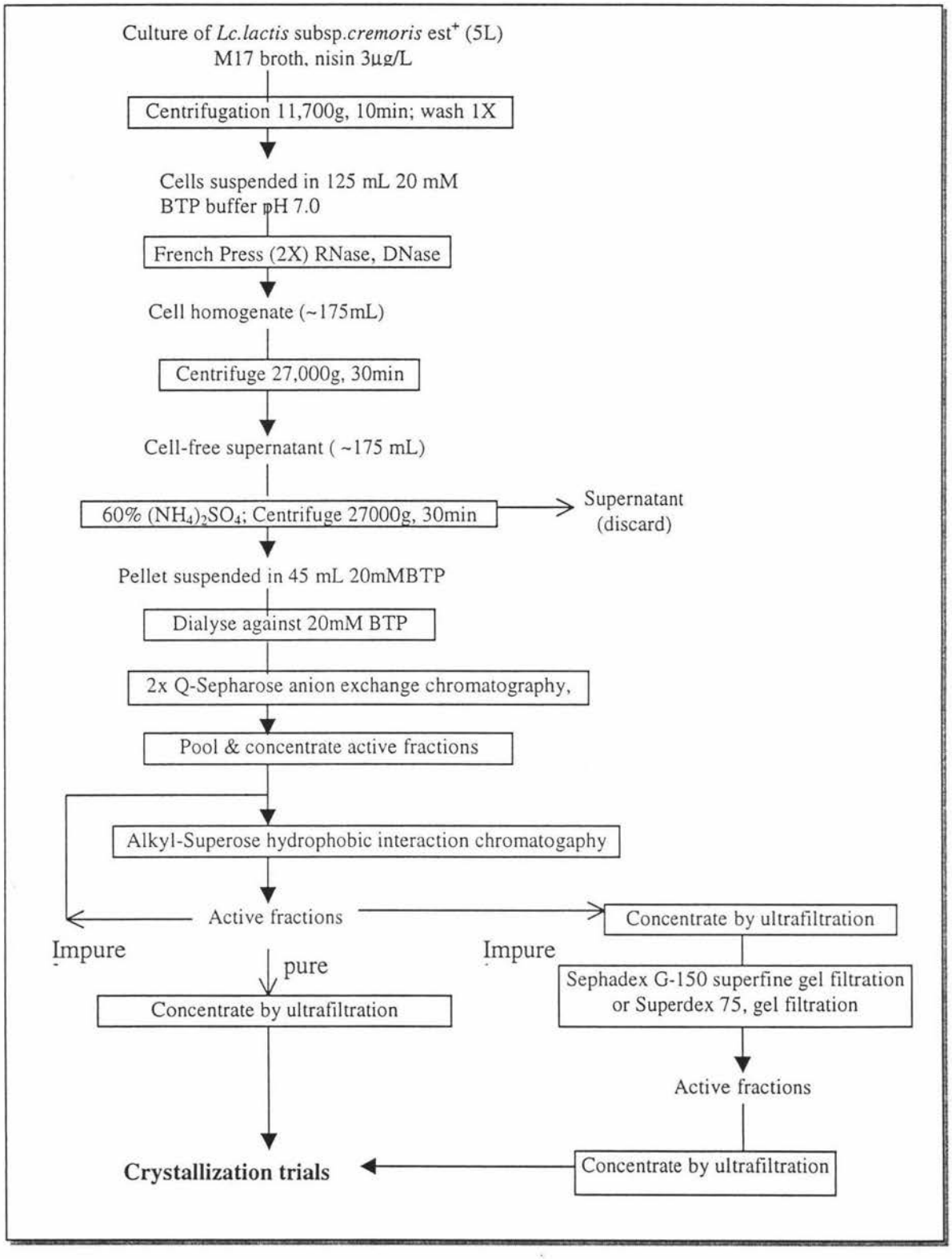


Figure 2.1 Summary of the scheme for purification of tributyrin esterase

2.5 Results and Discussion

2.5.1 Growth Curves

The growth of cultures of *Lc.lactis* subsp. *cremoris* strain est$^+$ is shown in Figure 2.2. The rate of growth of the culture slowed following the addition of nisin. About 0.042g/mL cells were harvested at the beginning of the stationary phase in each preparation.

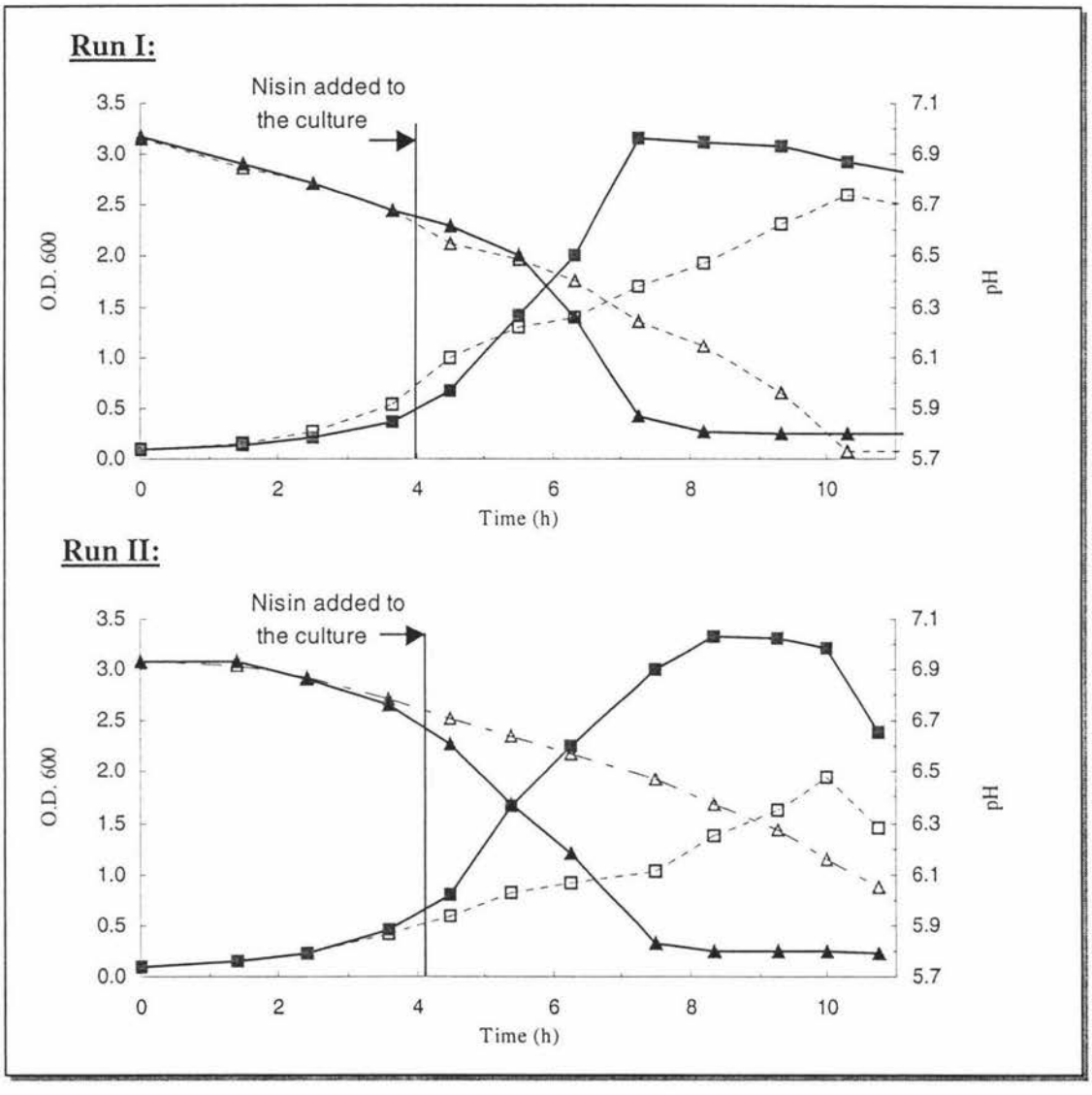


Figure 2.3: Growth Curve of *Lc. lactis* subsp. *cremoris* (RunI & Run II)
Growth cultures is in M17 broth. Cultures (500 ml) were incubated at 30°C.
Growth with addition of nisin (A_{600}: ---□--- and pH ---Δ---)
Growth without nisin (A_{600}: —■— and pH —▲—)

2.5.2 Determination of the Protein concentrations

Results obtained using both the Lowry and the BCA methods showed that they suffered significant interference from the buffer (Bis-Tris Propane). Therefore all measurements of protein concentration were carried out using the Bradford (Bio-Rad) method which

showed the least interference. However, the Bradford (Bio-Rad) method has been shown to give protein concentrations that are consistently lower than those obtained using other methods (Adney et al, 1995). As protein concentrations determined from measuring the absorbance at 280 nm (UV method) gave similar results to those obtained using the Bradford (Bio-Rad) method, and it was therefore used as the method to determine the protein concentration obtained from the final step of the purification.

2.5.3 Preliminary Study of Factors Affecting Enzyme Activity

(a) Protease inhibitor

"Complete" protease inhibitor (from Boehringer Mannhem) which contains serine, cysteine, metallo and aspartic protease inhibitors had an inhibitory effect on tributyrin esterase activity. When 1μL of 'Complete' protease inhibitor stock solution (one tablet dissolved in 2 mL of 100 mM phosphate buffer, pH7.0) was added to 50 μL of pure tributyrin esterase half of the activity was lost after one hour and total activity was lost after approximately 16 hours. These results suggest that tributyrin esterase may have a similar active site to one of these classes of proteases. Sequence analysis of tributyrin esterase showed that it has a GXSXG consensus sequence found in many hydrolases, including serine proteases. (Cygler, *et al.*1997).

(b) DTT

The esterase activity did not show any significant difference in the presence or absence of 10 mM DTT, indicating that free thiols are nonessential for activity.

(c) Buffer Type

Results in Figure 2.3 showed that buffer type had little effect on the esterase activity.

(d) Salt Concentration

The results in Figure 2.4 showed that NaCl concentration had some effect on the esterase activity over the range of 0~5 mM, as there was a significant drop in activity between 0 and 5 mm NaCl. This suggests that enzyme activity could be dependent on the association of monomer units, or on specific metal ions. In order to test these hypotheses, assays in the presence of 1 - 5 mM NaCl and different cations and anions need to be carried out. Results obtained from a preliminary study were affected by experimental error and further tests will need to be carried out to obtain reliable results. Nevertheless, it can be concluded that ion strength appears to have an effect on the activity of the enzyme, and that this effect needs further investigation.

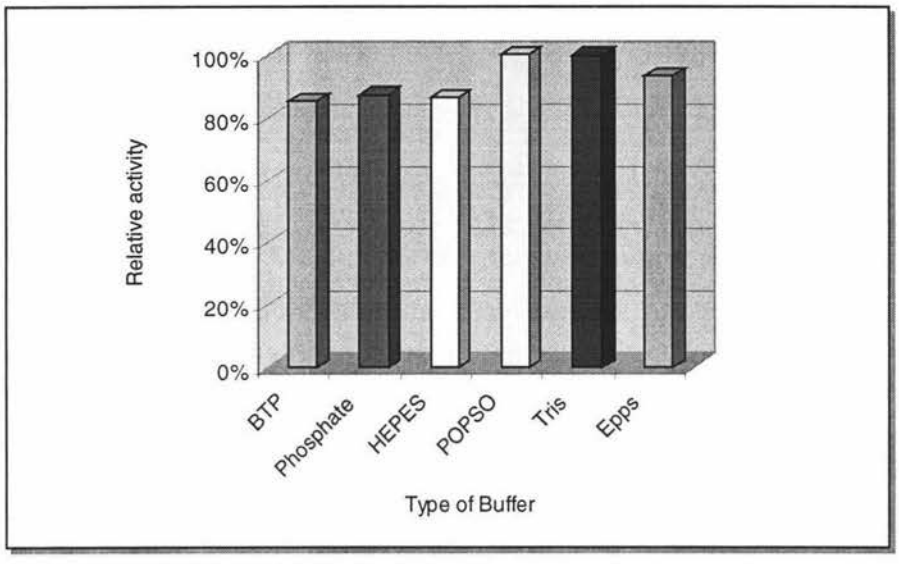


Figure 2.3: The effect of different buffer type on enzyme activity

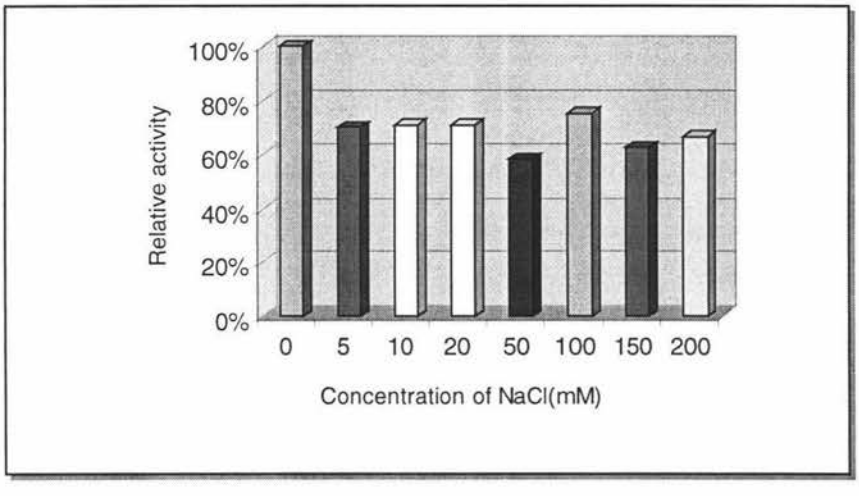


Figure 2.4: The effect of NaCl concentration on activity

2.5.4 Purification by Precipitation

2.5.4.1 Ammonium Sulfate Fractionation

Results from the ammonium sulfate fractionation are tabulated below (Table 2.1). The supernatant at 60% ammonium sulfate contained no esterase activity, which meant that most of the tributyrin esterase precipitated at this concentration along with many other contaminating proteins. The experiments carried out to investigate optimization of this step showed that the protein precipitated over a wide range of ammonium sulfate concentrations, so that purification could not be achieved through salt fractionation. This step was therefore used to concentrate the protein.

Table 2.1: Results of Ammonium Sulfate salt Fractionation

	Protein concentration (mg/ml)	Activity (unit/ml)	Specific Activity (unit/mg)	Total Protein (mg)	Total activity (unit)	Percentage
20% $(NH_4)_2SO_4$ Pellet	0.2	0	0	0.1	0	0
20% $(NH_4)_2SO_4$ Supernatant	2.87	4.6	1.6	2.3	36.8	100
40% $(NH_4)_2SO_4$ Pellet	1	1.15	1.15	0.5	5.8	16
40% $(NH_4)_2SO_4$ Supernatant	2.3	2.86	1.24	1.84	22.9	63
60% $(NH_4)_2SO_4$ Pellet	3.1	4.9	1.58	1.5	24.5	67
60% $(NH_4)_2SO_4$ Supernatant	0.465	0	0	0.37	0	0

2.5.4.2 Polyethylene glycol (PEG) Fractionation

The activity was distributed between the precipitate and the supernatant for both 3% and 4.7% PEG concentrations. There was therefore no advantage in using PEG fractionation. Also the pellet resulting from the PEG precipitation proved to be very hard to redissolve. This method of fractionation was therefore considered to be unsuitable for this enzyme.

Table 2.2: Results of Polyethylene glycol (PEG) Fractionation

	Protein concentration (mg/ml)	Activity (unit/ml)	Specific Activity (unit/mg)	Total Protein (mg)	Total activity (unit)	Percentage (%)
3% PEG Pellet	3.1	2.2	0.72	3.8	2.75	5.1
3% PEG Supernatant	2.25	4.5	2	20.25	40.5	75.7
4.7% PEG Pellet	2.98	1.9	0.64	3.43	2.2	5.4
4.7% PEG Supernatant	2.2	5.7	2.6	14.3	37.1	91

2.5.4.3 Precipitation by acidification

When a protein solution (3mg/mL) was slowly brought to pH 5.5, a precipitate was observed. Approximately 4% of the activity was recovered in the pellet, while 95% activity remained in the supernatant. This result suggested that the prediction of isoelectric point of 5.49 based on the amino acid sequence may not reflect that of the folded protein. Further investigation of the actual isoelectric point of tributyrin esterase is therefore necessary to determine whether this method could be used in the purification of tributyrin esterase.

2.5.5 Chromatographic Purification

2.5.5.1 Ion Exchange Chromatography

(A) Q-Sepharose anion exchange chromatography

In both preparations, Q-Sepharose anion exchange chromatography was chosen as the initial step to reduce the total amount of protein. In preparation one, two peaks displaying esterase activity were obtained (Figure 2.5) although this result was not reproduced in the second preparation. Fractions 50 to 68 (containing the major peak of activity) were pooled for further purification (without SDS-PAGE gel verification).

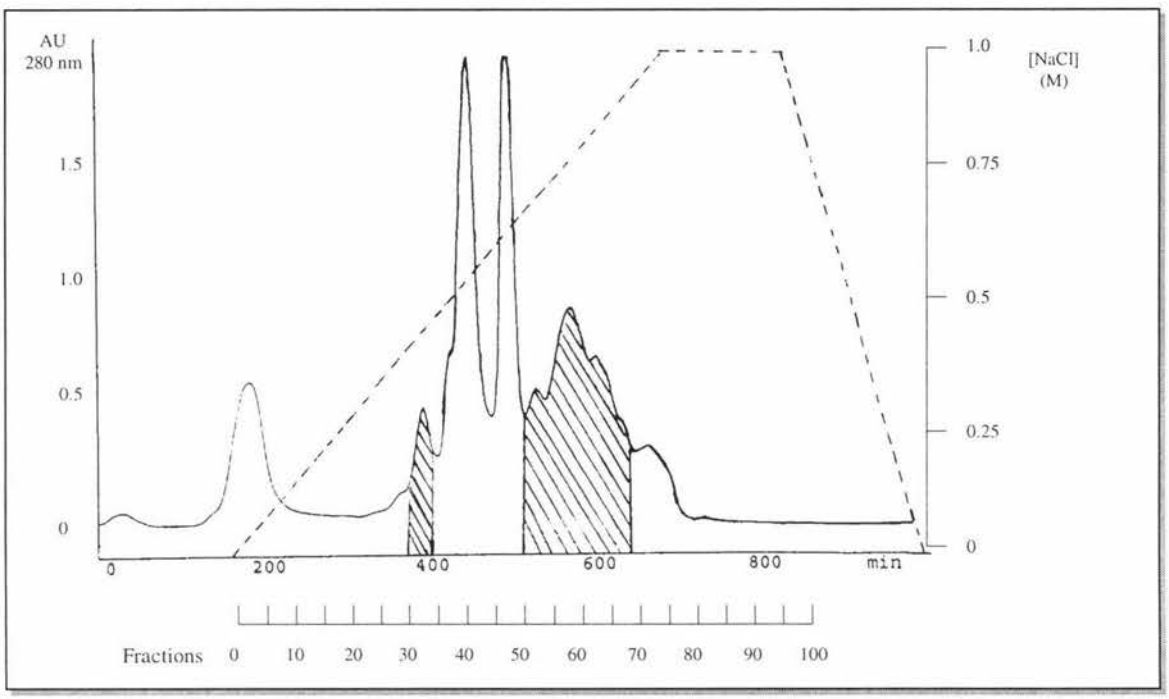


Figure 2.5: Chromatogram of elution from Q-Sepharose in Preparation-1

Buffer A: 20 mM BTP buffer, pH 7.0; Buffer B: 1.0M NaCl in 20 mM BTP buffer, pH 7.0; Flow rate: 2 $mLmin^{-1}$; Fraction size: 10 mL. Shade part indicate fractions with esterase activity; Key: -----: [NaCl]; —— : A_{280} ;

In preparation 2, the protein solutions were applied to the Q-Sepharose fast flow column in two batches. For batch-1, the salt concentration was increased in a stepwise manner as showing in figure 2.6-1. Fractions 29-42 displayed the esterase activity. For batch 2 (figure 2.7-1), a 1.2 L linear salt gradient was applied. Fractions 45-58 contained the esterase activity. Before pooling, the fractions within the active peaks were analysed by SDS-PAGE (Batch I: figures 2.6-2 and 2.6-3; Batch II: figures 2.7-2 and 2.7-3). While the differences in the gradient have some effect on the resulting elution pattern, the active fractions were all eluted at 0.6M~ 0.8M NaCl. After analysis of active fractions by SDS-PAGE, those with least contaminating proteins were pooled and kept separately from fractions with more contaminating proteins. Thus, in Batch I fractions 30-34 were combined as Pool 1 and

fractions 35-39 were combined as Pool 2. Similarly, in batch II, fractions 45-51 were combined as Pool 3 and fractions 52-58 were combined as Pool 4.

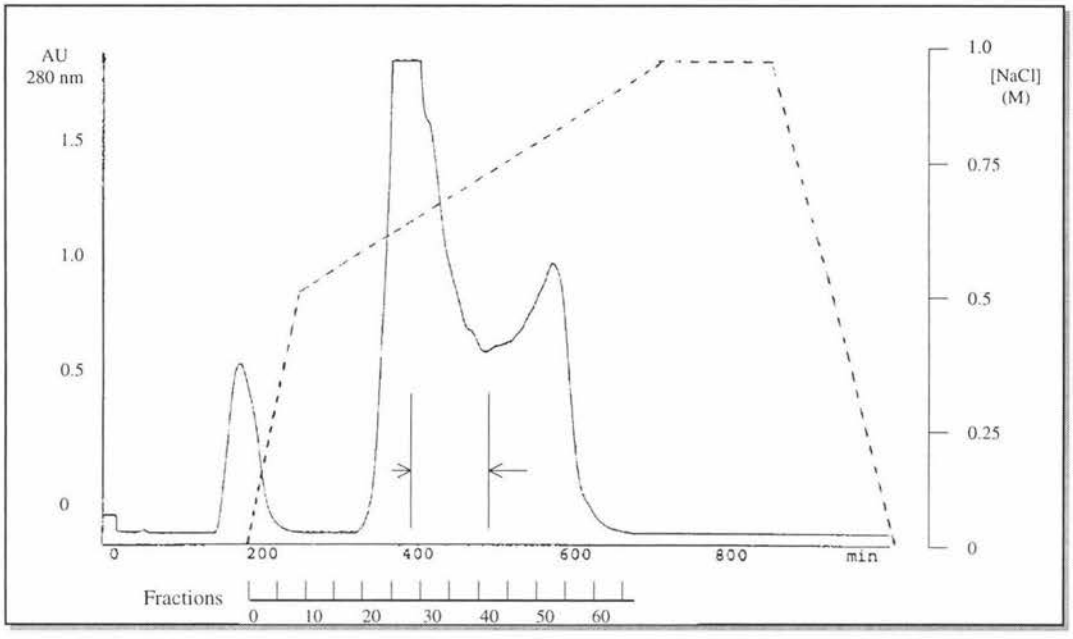


Figure 2.6-1: Chromatogram of elution from Q-Sepharose in Batch-1 in Preparation-2
BufferA: 20 mM BTP buffer, pH 7.0; Buffer B: 1.0M NaCl in 20 mM BTP buffer, pH 7.0; Flow rate: 2 mLmin^{-1}; Fraction size: 10 mL. Key: -----: [NaCl]; ——— : A_{280} ;

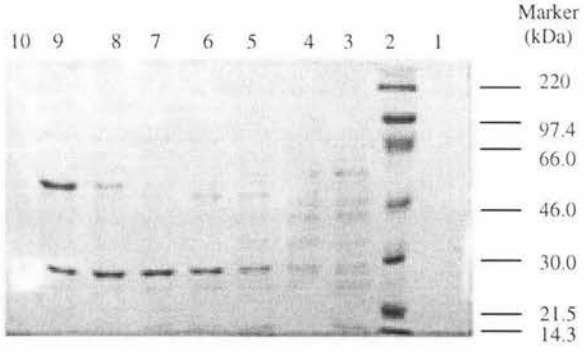


Figure 2.6-2: SDS gel of elution from Q-Sepharose in Batch-1 in Preparation-2
Lane1: Blank; Lane2: Marker; Lane3 to Lane 9: fractions 29 to 35;

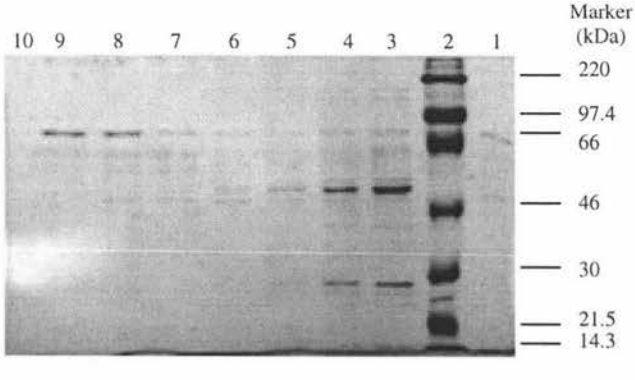


Figure 2.6-3: SDS gel of elution from Q-Sepharose in Batch-1 in Preparation-2
Lane1: blank; Lane2: Marker; Lane3 to Lane 9: fractions 36 to 42;

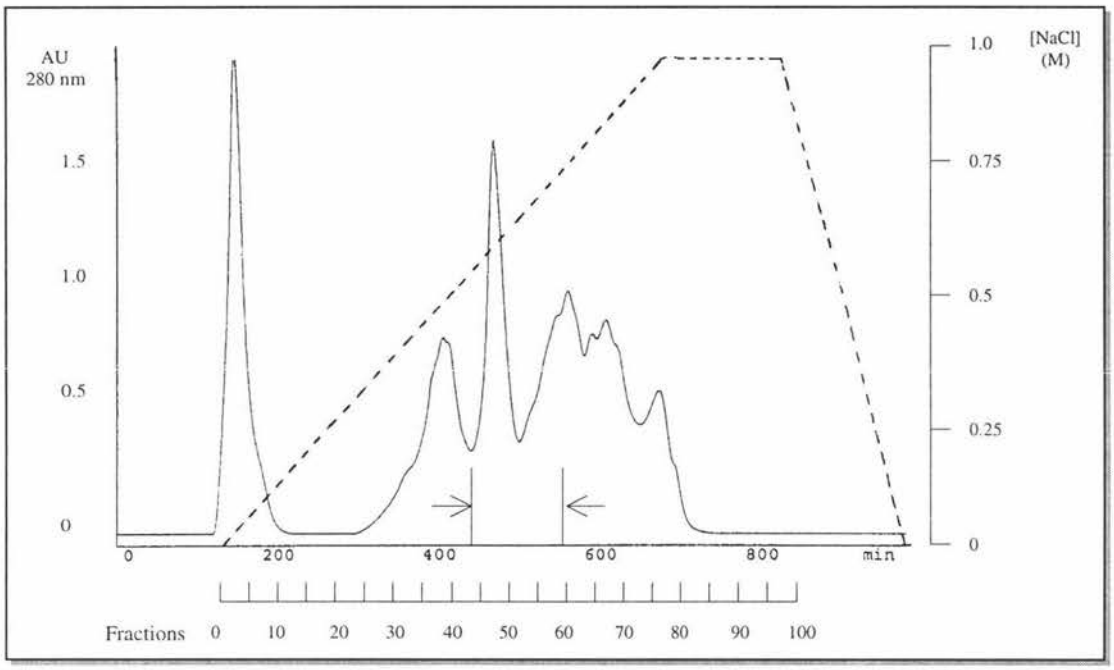


Figure 2.7-1: **Chromatogram of elution from Q-Sepharose in Batch-2 in Preparation-2**
BufferA: 20 mM BTP buffer, pH 7.0; Buffer B: 1.0M NaCl in 20 mM BTP buffer, pH 7.0; Flow rate: 2 $mLmin^{-1}$; Fraction size:10 mL. Key: -----: [NaCl]; —— : A_{280} ;

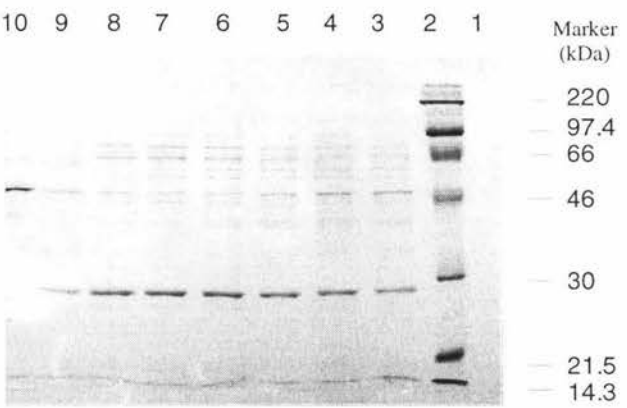


Figure 2.7-2: **SDS gel of elution from Q-Sepharose in Batch-2 in Preparation-2**
Lane1: Blank; Lane2: Marker; Lane3 to Lane 10: Fractions 44-51;

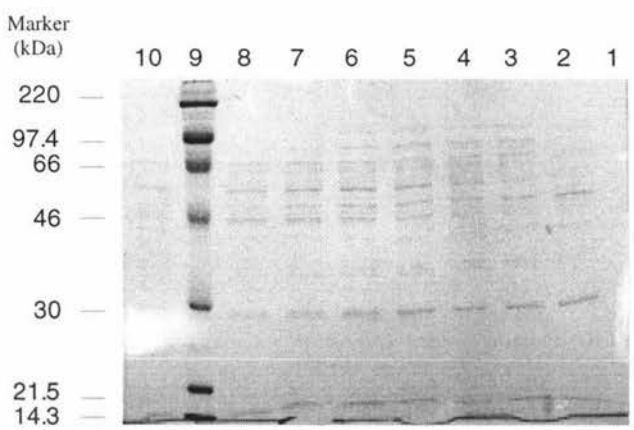


Figure 2.7-3: **SDS gel of elution from Q-Sepharose in Batch-2 in Preparation-2**
Lane 2 to Lane 8: Fractions 52 to 58; Lane9: Marker; Lane10 Fraction 59;

2.5.5.2 Hydrophobic Interaction Chromatography

(A) Alkyl-Superose column

Hydrophobic interaction chromatography was found to be the most efficient step in the purification of tributyrin esterase. Many contaminating proteins did not bind to the column under the conditions chosen, and the tributyrin esterase could be eluted as a reasonably sharp peak. In both preparations, the pooled active fractions from IEX chromatography were applied to Alkyl-superose column in four batches. A typical chromatogram is shown in figure 2.8-1. Activity was present in fractions 89-96, and after analysis of these fractions by SDS-PAGE, it could be seen that some active fractions were pure, while others contained a minor contaminating band at ~66KkDa (figure 2.8-2). Fractions 93-96 were pooled for use in crystallisation trials, while fractions 89-92 were pooled for further purification.

When the sample was eluted using a stepwise rather than a linear gradient, the separation was improved, as several shoulder peaks appeared (figure 2.9-1). However, analysis of active fractions by SDS-PAGE (figure 2.9-2) showed that the contaminating protein (~66kDa) was still present, although the relative concentration appeared to be low.

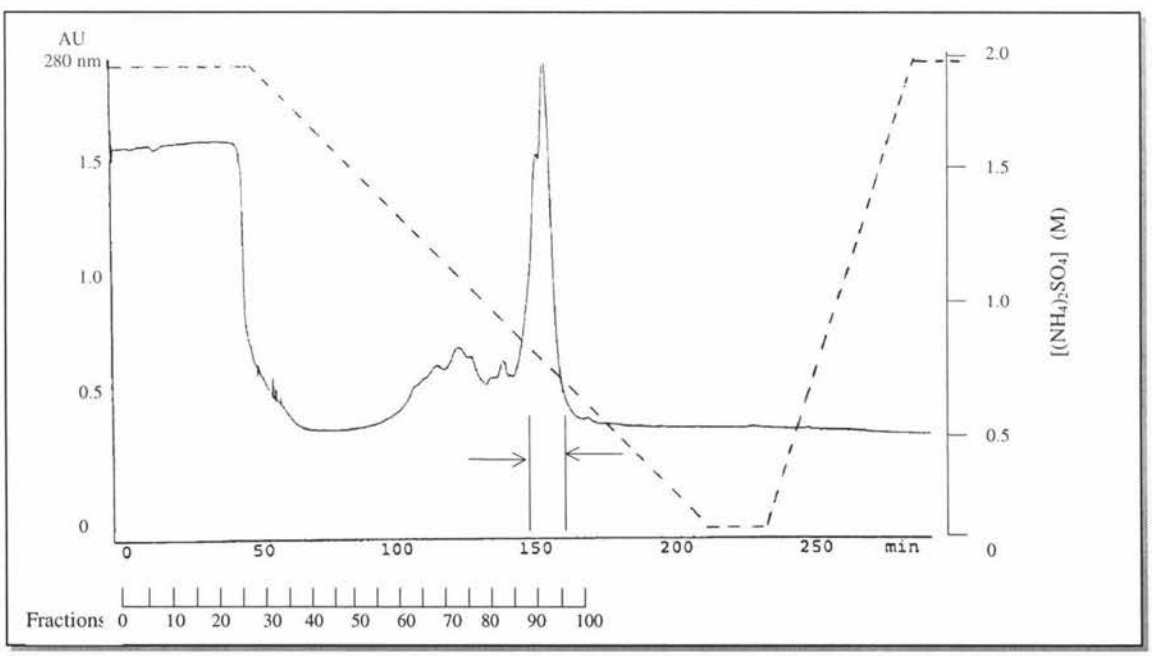


Figure 2.8-1: Chromatogram of elution from Alkyl-Superose in Batch-3 of Preparation-1
BufferA: 2.0M $(NH_4)_2SO_4$ in 20 mM BTP buffer, pH 7.0; Buffer B: 20 mM BTP buffer, pH 7.0; Flow rate: 0.5 mLmin^{-1}; Fraction size: 1 mL. Key: -----: $[(NH_4)_2SO_4]$ —— : A_{280} ;

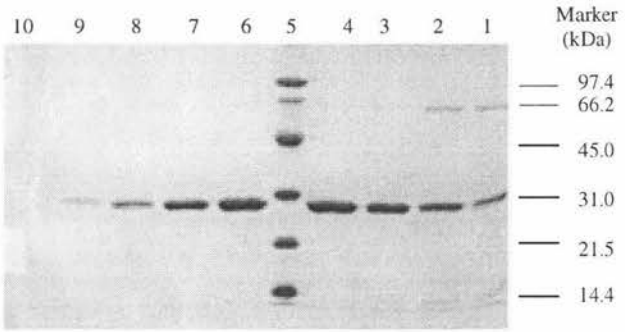


Figure 2.8-2: **SDS gel of elution from Alkyl-Superose in Batch-3 of Preparation-1**
Lane1 to Lane4: Fractions 89 to 92; Lane 5: Marker;
Lane6 to Lane 9: Fractions 93 to 96; Lane 10:Blank

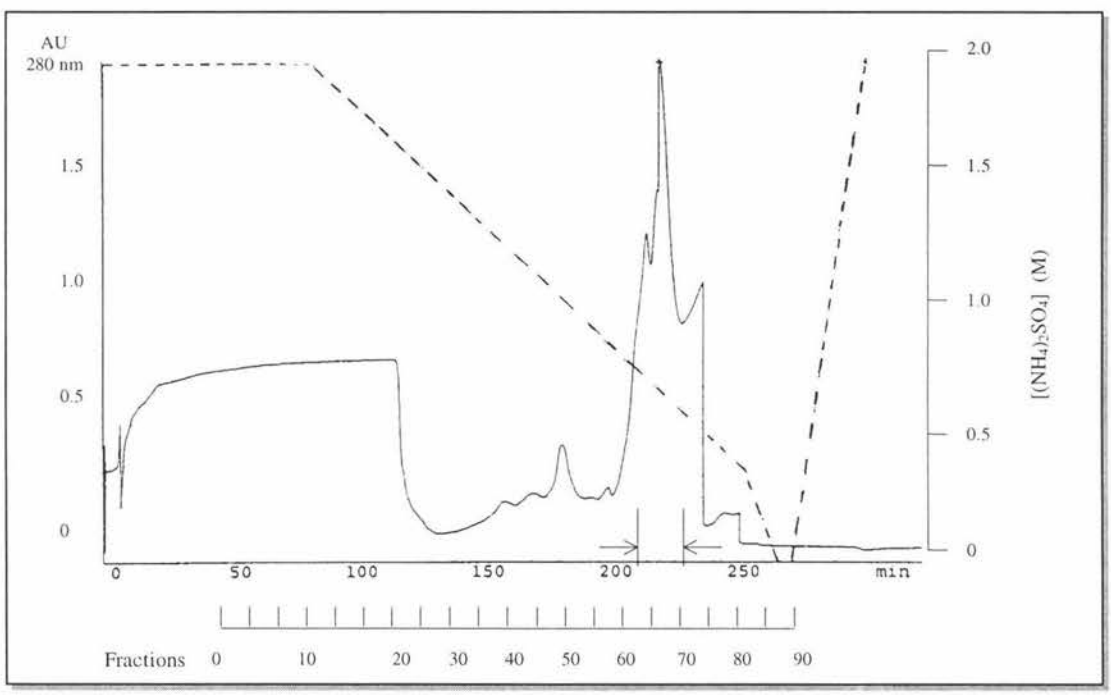


Figure 2.9-1: **Chromatogram of elution from Alkyl-Superose in Batch-1 of Preparation-2**
BufferA: 2.0M $(NH_4)_2SO_4$ in 20 mM BTP buffer, pH 7.0; Buffer B: 20 mM BTP buffer, pH 7.0; Flow rate: 0.5 $mLmin^{-1}$; Fraction size: 1 mL. Key: -----: $[(NH_4)_2SO_4]$ —— : A_{280} ;

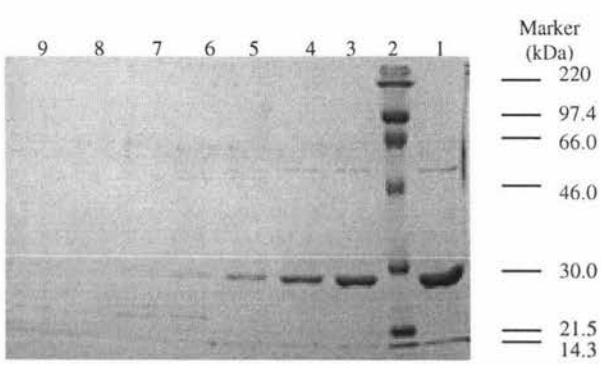


Figure 2.9-2: **SDS gel of elution from Alkyl-Superose in Batch-1 of Preparation-2**
Lane1: Fractions 68; Lane 2: Marker; Lane3 to Lane 9: Fractions 63 to 70;

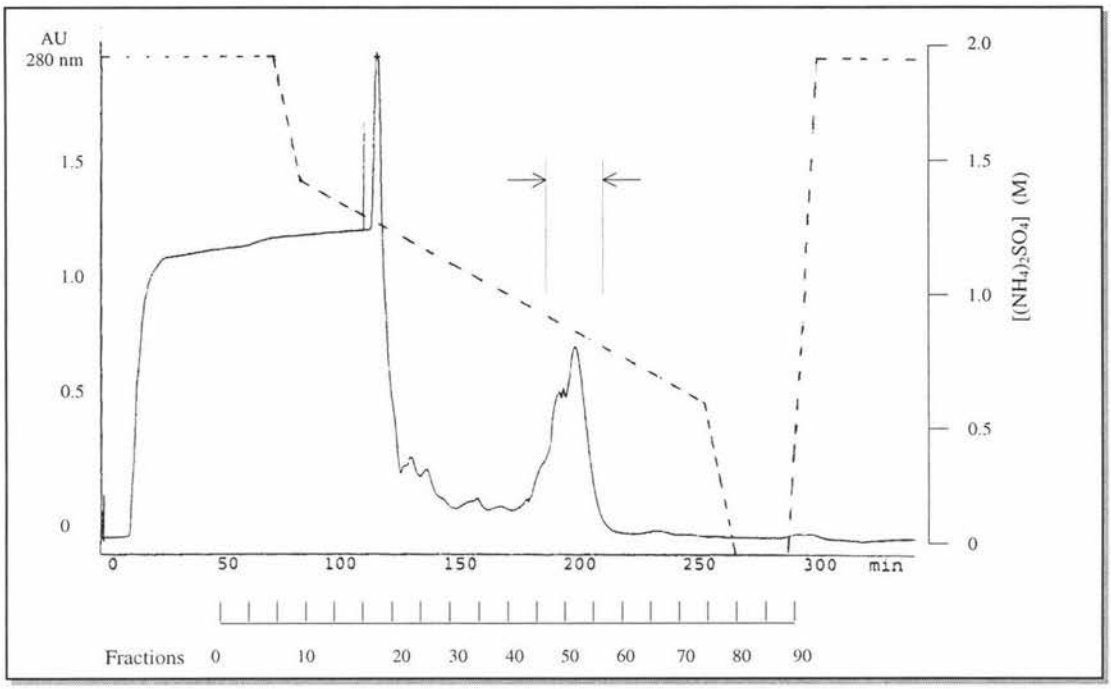


Figure 2.10-1: Chromatogram of elution from Alkyl-Superose in Batch-3 of Preparation-2
BufferA: 2.0M $(NH_4)_2SO_4$ in 20 mM BTP buffer, pH 7.0; Buffer B: 20 mM BTP buffer, pH 7.0; Flow rate: 0.5 $mLmin^{-1}$; Fraction size: 1 mL. Key: -----: $[(NH_4)_2SO_4]$ —— : A_{280} ;

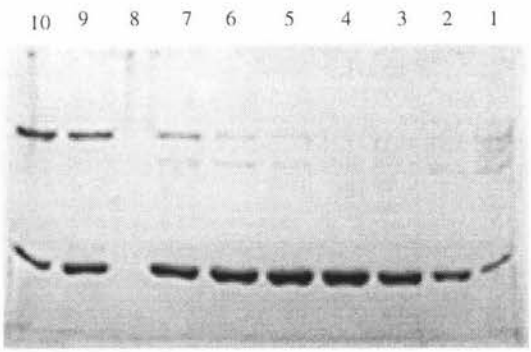


Figure 2.10-2 SDS gel of elution from Alkyl-Superose in Batch-3 of Preparation-2
Lane1 to Lane7: Fractions 47 to 53; Lane 8: Blank; Lane9-10: Fractions 54-55;

(B) Other Hydrophobic Ligands

The choice of hydrophobic ligand is of considerable importance in both selective binding of proteins to the resin and their subsequent elution. In general, HIC media fall into two groups, depending on their interactions with sample components. Straight alkyl chains (butyl, octyl) show a "pure" hydrophobic character, while aryl ligands (phenyl) show a mixed mode behaviour, where both aromatic and hydrophobic interactions play simultaneous roles. Phenyl Sepharose is generally less hydrophobic than octyl sepharose, where the ligands are straight alkyl chains, their length being proportional to the hydrophobicity. Generally, a C_8 resin will bind proteins more tightly than C_4 resins. However, binding is altered by pH, temperature and the presence of detergents, which

means optimisation of starting conditions and separations must be estimated by empirical methods.

A series of different hydrophobic interaction columns were therefore tested using different conditions. These include: phenyl sepharose, butyl sepharose 4 fast flow and octyl sepharose 4 fast flow. Holland (personal communication) found that tributyrin esterase could not be eluted from phenyl sepharose at room temperature and pH 6.5. As hydrophobic interaction are weakened at high pH, and because most proteins gain a net charge becoming more hydrophilic under mildly alkaline conditions, binding is expected to weaken at higher pH. Reducing the temperature also decreases binding. However, even at pH 8.5 and 4 °C, tributyrin esterase could not be eluted from phenyl sepharose in the current study. Whether the enzyme was still bound to the column or had been eluted in an inactivated state has not however been established.

The chromatogram obtained from butyl sepharose 4 fast flow is shown in figure 2.11-1. The fractions were analyzed by SDS-PAGE (figure 2.12). The separation was improved to some extent, compare to that achieved by alkyl sepharose in that the contaminating protein was mainly in the first fractions. It thus seems very promising, although further investigation is necessary to find the optimised conditions for the separation (type of salt, pH, gradient). In contrast, separation obtained using octyl-sepharose 4 fast flow (figure in 2.12-1 and 2.12-2) shows that the separation has not been improved.

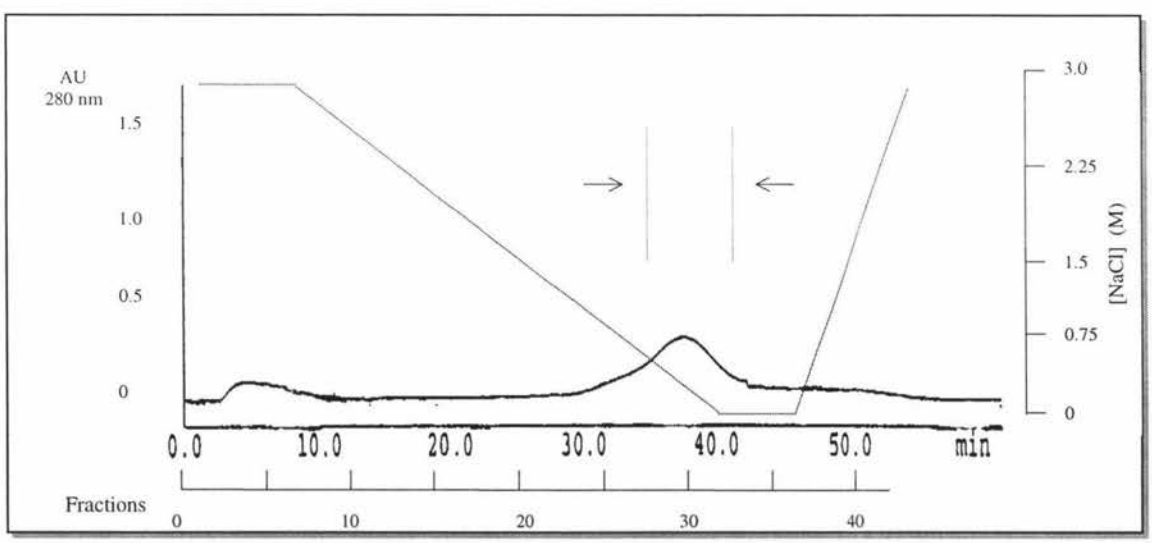


Figure 2.11-1: Chromatogram of elution from Butyl-Sepharose 4 fast flow at pH 8.0
Buffer A: 3.0M NaCl in 20 mM Tris-HCl buffer, pH 8.0;
Buffer B: 20 mM Tris-HCl buffer, pH 8.0; Flow rate: 0.5 $mLmin^{-1}$; Fraction size: 0.5 mL.
Key: -----: [NaCl] —— : A_{280} ;

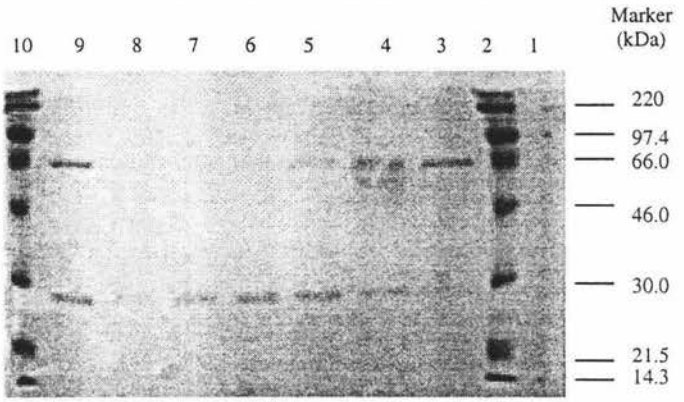


Figure 2.11-2: SDS gel of elution from Butyl-Sepharose 4 fast flow at pH 8.0
Lane 2 & 10: Marker ; Lane 3 to Lane8: fractions 27 to 32; Lane 9: Sample Load

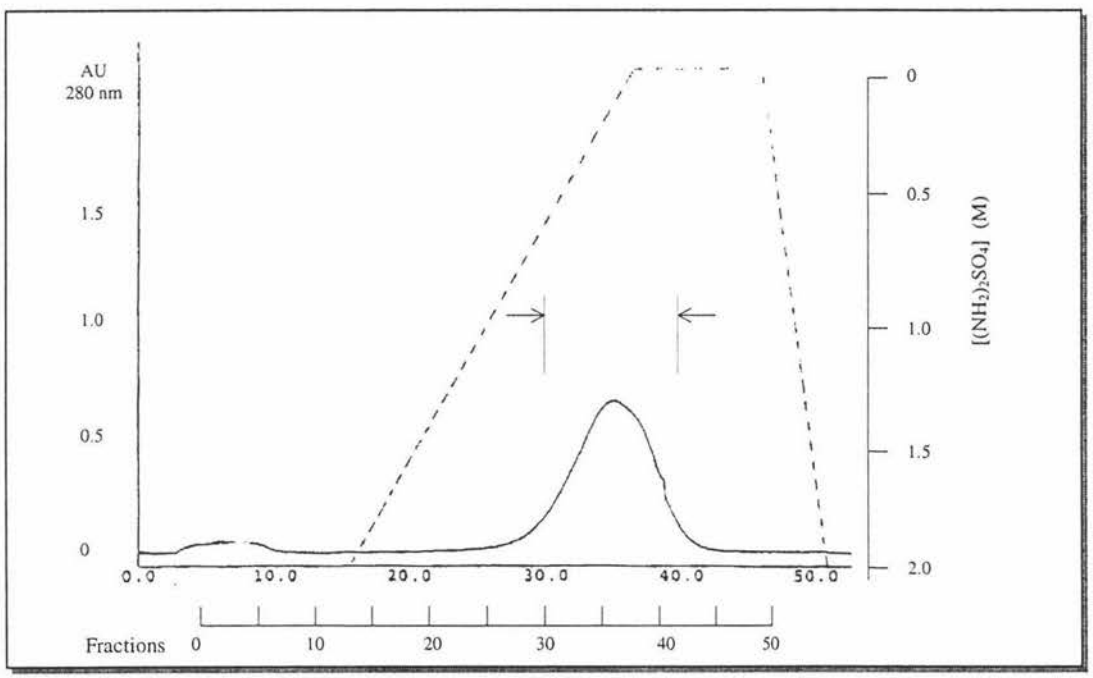


Figure 2.12-1: Chromatogram of elution from Octyl-Sepharose at pH 7.0
Buffer A: 2.0M $(NH_4)_2SO_4$ in 20 mM BTP buffer, pH 7.0; Buffer B: 20 mM BTP buffer, pH 7.0; Flow rate: 0.5 $mLmin^{-1}$; Fraction size: 1 mL. key: ----: $[(NH_2)_2SO_4]$; ——— : A_{280}

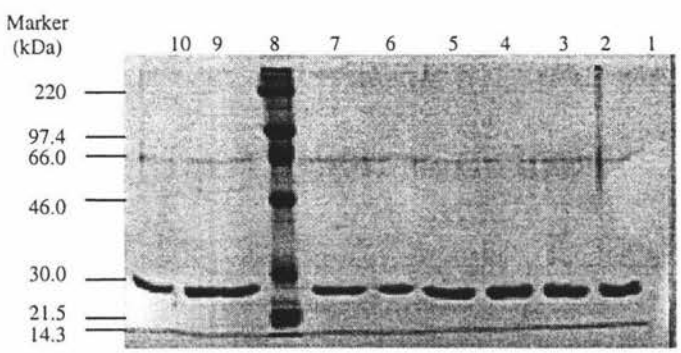


Figure 2.12-2: SDS gel of elution from Octyl-Sepharose at pH 7.0
Lane1: Blank; Lane2 to Lane7: Fractions 33 to 38: Lane 8: Marker;
Lane9: Sample loaded; Lane 10: Fraction 39;

2.5.5.3 Gel Filtration Chromatography

(A) Superdex 75 Gel Filtration Chromatography

Gel filration chromatography was used as the final purification step to polish the product. The pooled Alkyl Superose elution fractions with minimal contaminating protein were applied to a Superdex 75 column (10/16, Pharmacia) using a FPLC with 50 mM BTP (pH7.0) as elution buffer. The results of chromatography and the SDS-PAGE analysis of fractions are shown in figure 2.13-1 and figure 2.13-2.

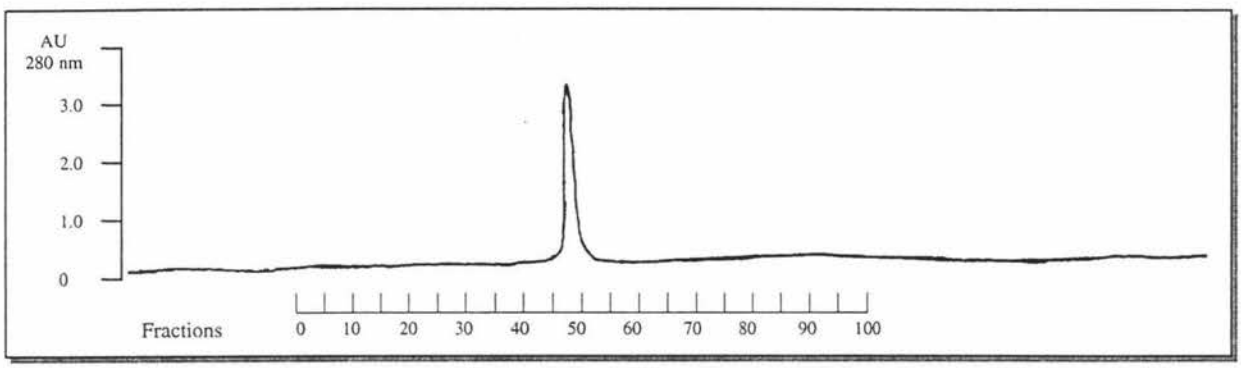


Figure 2.13-1:Chromatogram of elution from Superdex 75
Flow rate: 0.05mL min^{-1}; Fraction size: 0.5mL.Buffer: 50 mM BTP (Bis-tris propane) pH 7.0;

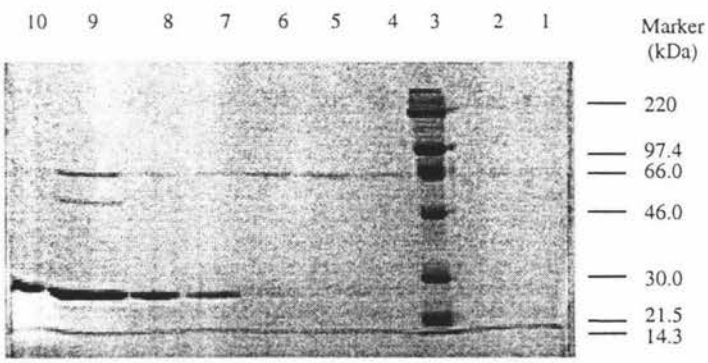


Figure 2.13-2 SDS-PAGE analysis of elution from Superdex 75
Lane 1 and 2: Fractions 45 and 46; Lane 3: Marker; Lane 4 to Lane 8: Fractions 47 to 51; Lane 9: Sample load; Lane 10: Fraction 52;

A single sharp peak was obtained that contained two bands, the tributyrin esterase at ~30 kDa, the impurity at ~66 kDa, although there was some separation as shown in Figure 2.13-2. However, the contaminating protein could not be completely removed. Some possible reasons for this are discussed below. In SEC chromatography, interaction of the packing materials with the protein and protein-protein interactions, through ionic, hydrophobic and other non-specific interactions will always affect the separation. For example, partial adsorption of the protein under specific buffer conditions results in the protein eluting from the column later than would be expected for its molecular size. Most interactions of an

ionic nature can be eliminated by increasing the ionic strength of the buffer. Typically a concentration of 0.15 M NaCl is recommended to avoid ionic interactions in the case of Sephadex gels. Therefore, the gel filtration chromatography on Superdex 75 using 150 mM NaCl in 50 mM BTP as elution buffer was tested. The result obtained was similar to that obtained under the low salt elution buffer conditions (data not shown), and suggests that the interaction between the contaminating protein and trybutyrin esterase is not of an ionic nature. As it was shown by the results obtained in previous sections, tributyrin esterase is quite hydrophobic. Acetonitrile was added to the buffer in an attempt to minimize any hydrophobic interactiions. The results are presented in the following section.

(B) Comparison of SEC with and without acetonitrile in the elution buffer

Trials were carried out using a Superose 12 column on the SMART system (Pharmacia), because of its ability to effect a better separation of a mixture of proteins due to the smaller, more even bead size and small sample load. When the buffer used to elute the protein contained no NaCl and no acetonitrile, the tributyrin esterase eluted near the void volume (Figure 2.14B). From alignment of the peaks with standard proteins running at the same condition (Figure2.14 A), the molecular weight of tributyrin esterase was estimated to be 135 kDa (Figure 2.15). As the protein appeared to have molecular weight of 29 kDa on SDS-PAGE (see section 2.5.6), this would suggest the protein exist as a tetramer or pentamer. However, when using a molecular weight of 25.8 kD as monomer, based on the sequence, the protein could be either a pentamer or hexamer. However, this estimation was based on only one experiment and the value from two protein standards, and further experiments will need to be done to obtain a more accurate result.

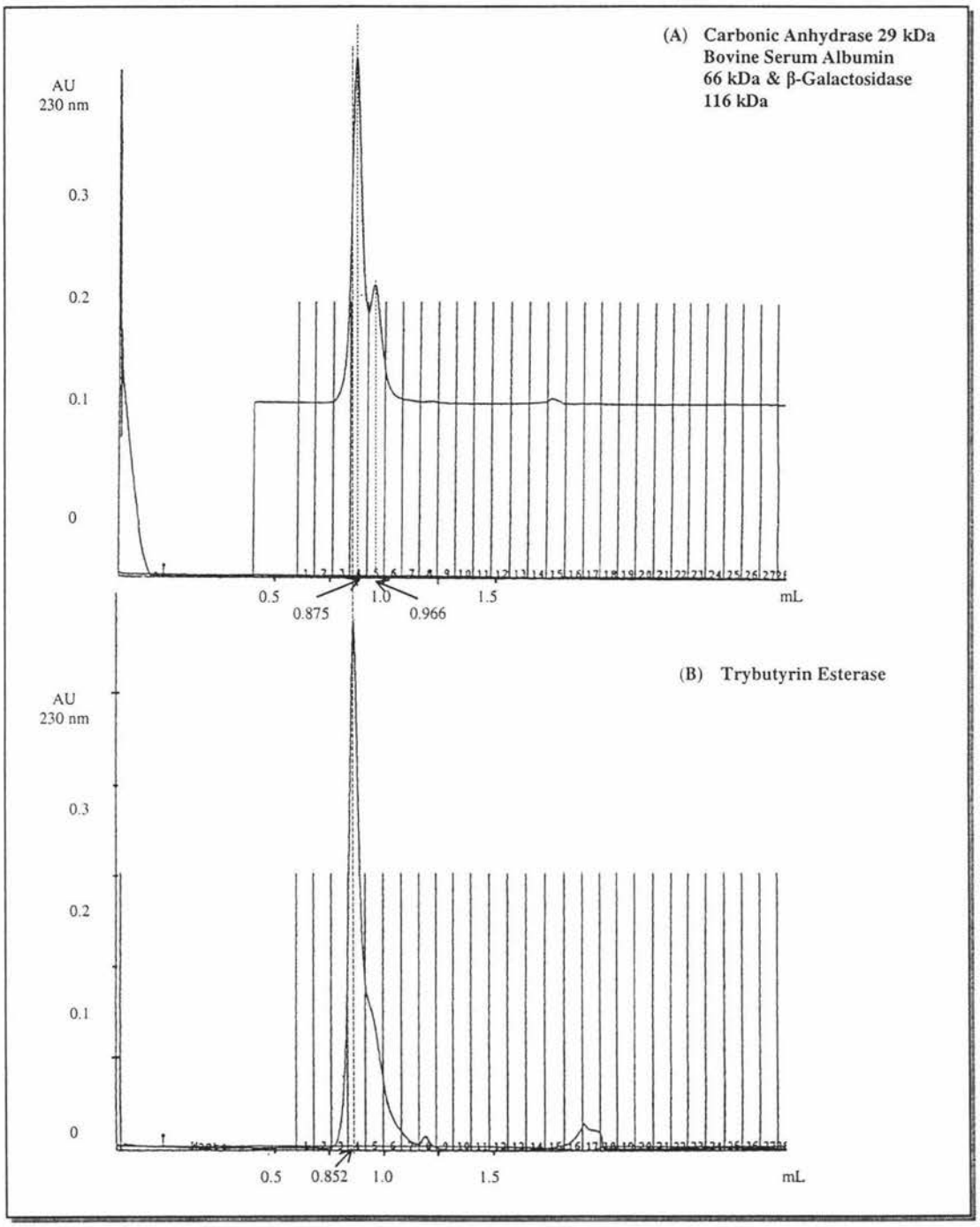


Figure 2.14 : Alignment of Chromatogram of elution of tributyrin esterase & standard protein eluted from Superose 12 on SMART System at low salt buffer conditions
Flow rate: 100µL min^{-1}; Fraction size: 100µL. Buffer: 50 mM BTP
(A) Carbonic Anhydrase (29 kDa), Bovine Serum Albumin (66 kDa) & β-Galactosidase (116 kDa); (B) Tributyrin Esterase

Table 2.3 Summary of Data of molecular weight and elution volume of protein marker

Molecular weight (Mr)	Log Mr	Elution volume (mL)
116,000	5.06	0.875
66,000	4.82	0.966

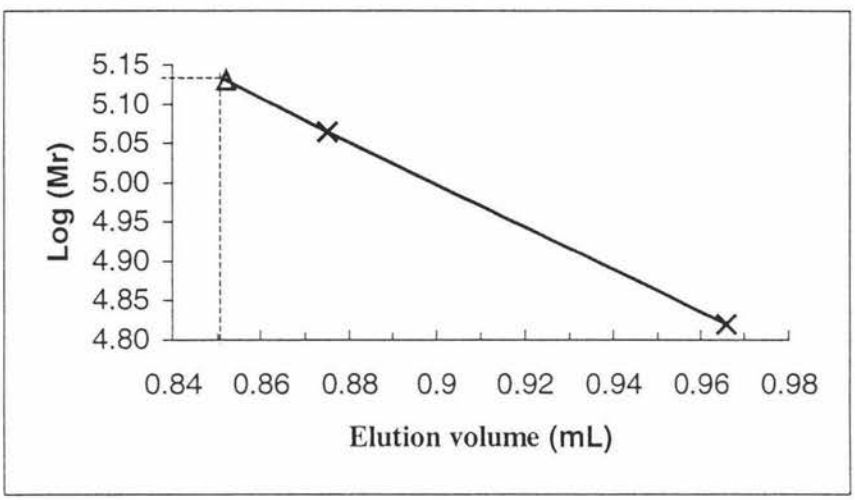


Figure 2.15 Plot of log relative molecular weight against elution volume

* Log (Mr) for tributyrin esterase is 0.857

When acetonitrile was added to the elution buffer to minimize possible hydrophobic interaction between proteins, two peaks were obtained (figure 2.16). The contaminating protein was very close to the first peak. The alignment with three standard proteins is shown in figure 2.17. One of the two peaks corresponded to the molecular weight at about 30 kDa, the other corresponded to a molecular weight ~60 kDa. They were thus estimated as monomer and dimer respectively. The results suggest that, once the hydrophobic interaction was minimized, the protein quaternary structure was changed.

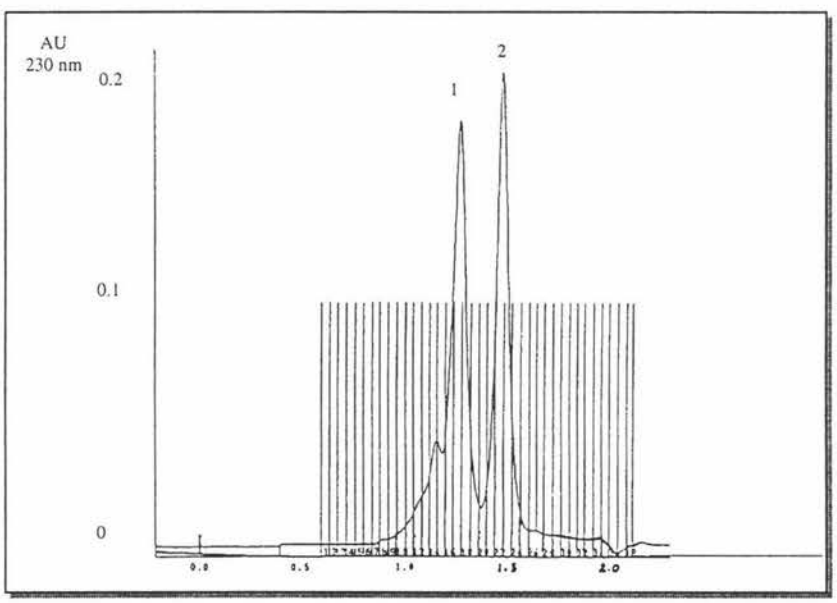


Figure 2.16 **Chromatogram of elution from superose 12 on SMART System at 20% CH_3CN buffer conditions**
Flow rate: 100µL min^{-1}; Fraction size: 100µL.
Buffer: 20% CH_3CN 50 mM BTP ; Sample: Tributyrin Esterase

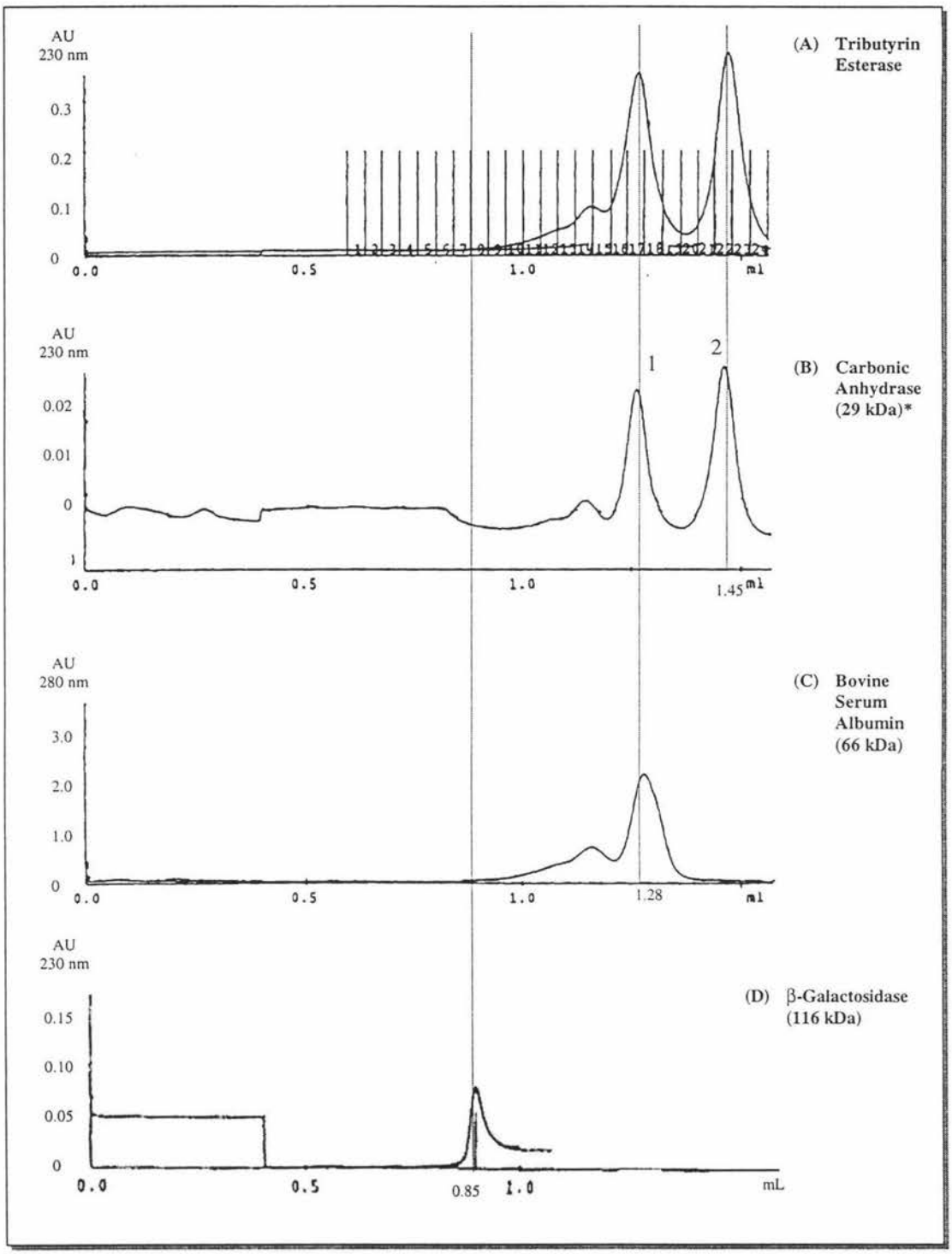


Figure 2.17 Alignment of Chromatogram of tributyrin esterase & standard protein eluted from Superose 12 on SMART System using buffer containing 20% CH_3CN

Flow rate: 100μL min^{-1}; Fraction size: 100μL

Buffer: Buffer: 20% CH_3CN in 50 mM BTP (Bis-Tris propane)

(A) Tributyrin Esterase

(B) Carbonic Anhydrase (29 kDa)

* The first peak might be the dimeric form of Carbonic Anhydrase

(C) Bovine Serum Albumin (66 kDa)

(D) β-Galactosidase (116 kDa)

(C) Superose-12 Gel Filtration Chromatography on FPLC

Having shown that the tributyrin esterase may exist as multimers (tetramers-hexamers) in solution, as Superose 12 was chosen for size exclusion chromatography (separation range from 100~300,000 Da). It was hoped that this technique would separate the multimers from contaminating proteins. The results of the gel filtration using Superose-12 are shown in figure 2.18-1. Although one main peak and two minor peaks were eluted, analysis of the peak fractions by SDS-PAGE (figure 2.18-2) showed that the contaminating protein, molecular weight is 66kDa, was still not separated from the tributyrin esterase.

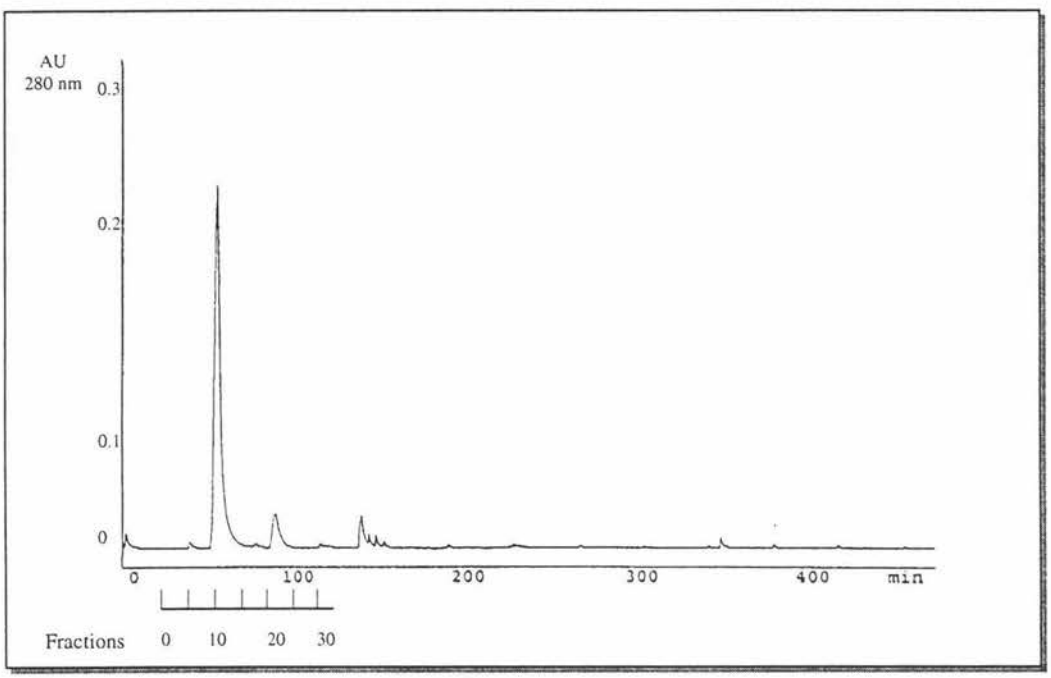


Figure 2.18-1: Chromatogram of elution from Superose 12
Flow rate: 0.05mL min^{-1}; Fraction size: 0.5mL.Buffer: 50 mM BTP (Bis-tris propane) pH 7.0;

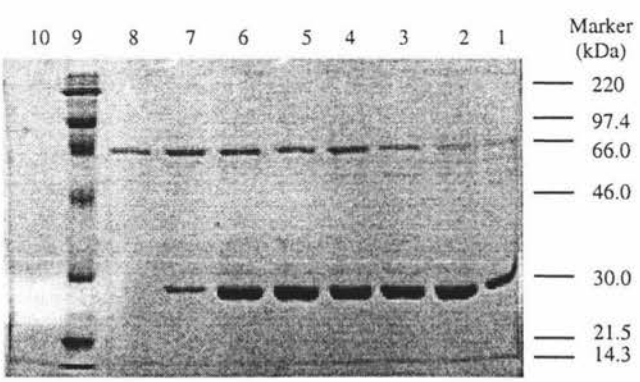


Figure 2.18-2: SDS-PAGE analysis of elution from Superose 12
Lane 1 to Lane 8: Fraction 13 to 20; Lane 9: Marker; Lane 10: Blank;

(D) Sephadex G-150 Gel Filtration Chromatography

Size exclusion chromatography on Sephadex G-150 superfine gave the results shown below (figure 2.19-1). SDS-PAGE analysis of the peak (Figures 2.19-2&3) showed that there was one major and one very minor protein band in some of fractions. Most of fractions were free of any contaminating proteins. These pure fractions (fraction 35-43) were pooled for crystallization studies. The contaminating protein was shown to be larger than 66 kDa, which was earlier determined as the molecular weight of contaminating protein (see previous section). This can not be explained so far.

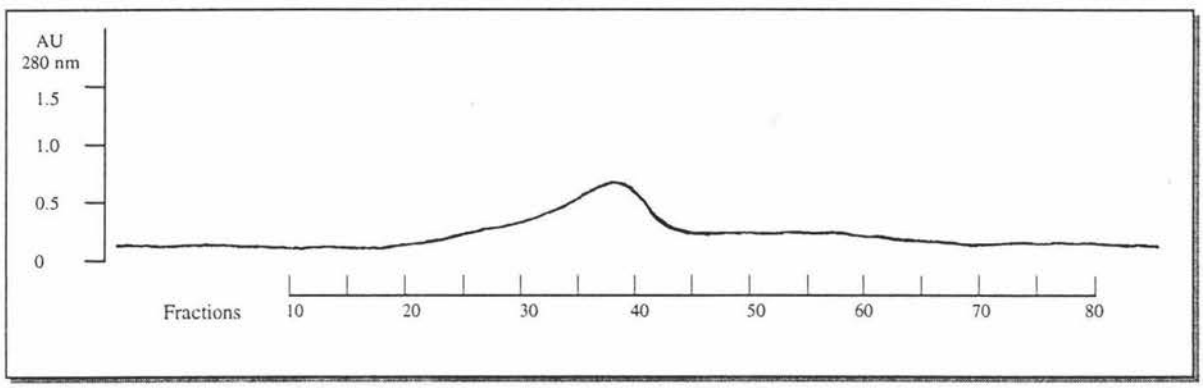


Figure 2.19-1 Chromatogram of elution from Sephadex G-150 superfine
Flow rate: 0.2mL min^{-1}; Fraction size: 0.5mL.Buffer: 50 mM BTP, pH 7.0;

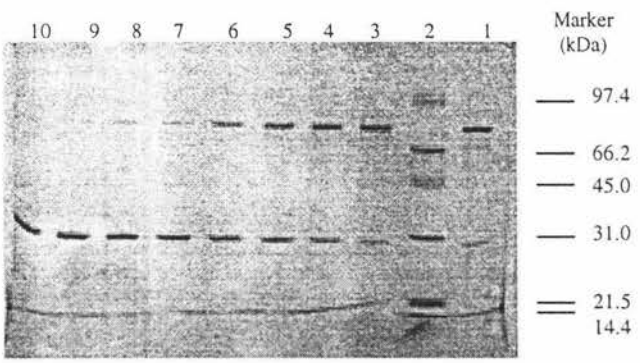


Figure 2.19-2 SDS-PAGE (Gel-1) of elution from Sephadex G-150 superfine
Lane 1: Fraction 25; Lane2: Marker; Lane3 to Lane10: Fraction 27 to 34;

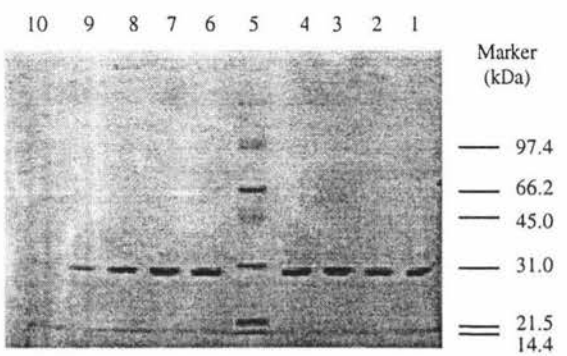


Figure 2.19-3 SDS-PAGE (Gel-2) of elution from Sephadex G-150 superfine
Lane 1 to Lane 4: Fraction 35 to 38; Lane 5: Marker; Lane 6 toLane10: Fraction 39 to 43;

To summarize the results obtained from SEC chromatography, it has been shown that size exclusion chromatography using Sephadex G-150 superfine (90 × 1.6 cm), and a low flow rate of 0.2 ml/min is the most efficient way to separate tributyrin esterase from the contaminating protein.

2.5.6 Molecular Weight Estimation

In a typical SDS-PAGE gel, a lane containing molecular weight standards (Bio-Rad), was loaded alongside the protein samples. A standard curve was drawn in figure 2.20 by measuring the distance of migration (R_f) and using known molecular weights marker. The data is summarized in table 2.4. The log (Mr/1000) value of tributyrin esterase was determined to be 1.47 from the curve. Therefore, the molecular weight of tributyrin esterase was estimated to be ~ 29 kDa.

Table 2.4 Summary of data of molecular weight and mobility of protein markers

Molecular weight (Mr)	Mr/1000	Log Mr/1000	Distance of migration (cm)
97,400	97.4	1.9886	0.80
66000	66	1.8195	1.25
45000	45	1.6532	2.00
31000	31	1.4914	3.00
21500	21.5	1.3324	3.85
14500	14.5	1.1614	4.80

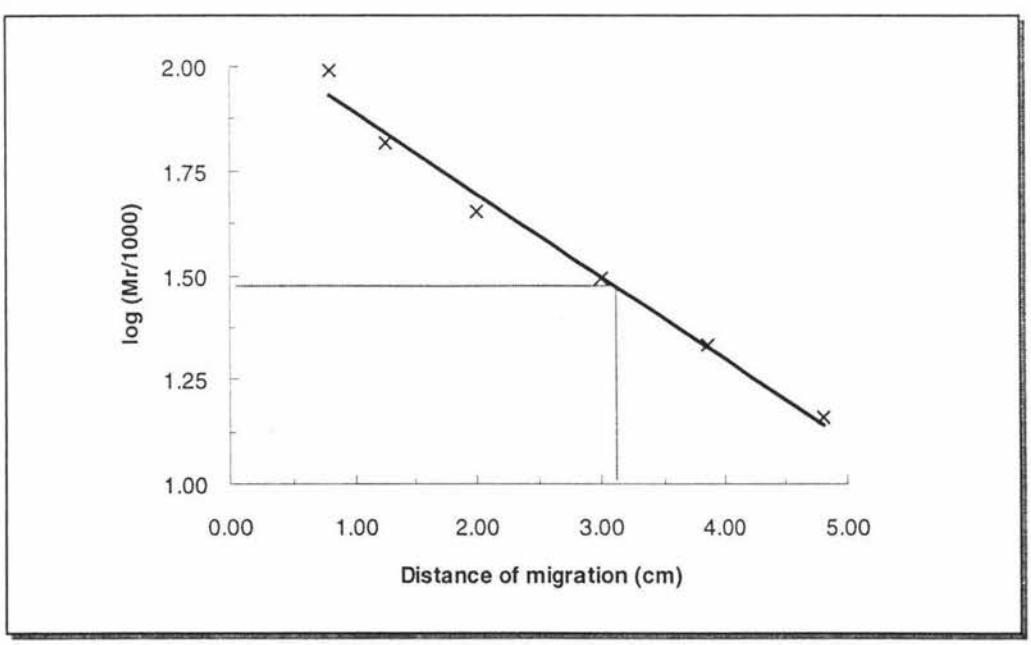


Figure 2.20 Plot of log relative molecular weight against distance of migration
Log (Mr/1000) for tributyrin esterase is 1.46

2.5.7 Polishing the final product

(A) Purification by ultrafiltration

Although the tributyrin esterase was estimated to be 29 kDa on SDS-PAGE, it seems to be able to exist as monomer, dimer, tetramer or hexamer under different conditions. The contaminating protein is 66kDa. If the protein is in its monomer form, it can be separated from the contaminating protein by ultrafiltration using a 50 kDa molecular weight cut-off (NMWC) membrane. The results of the experiment using such a membrane are shown in figure 2.21-B. Analysis of both the filtrate and the concentrate by SDS-PAGE showed that both the tributyrin esterase and the contaminating protein were retained in the concentrate. Interestingly, when the protein solution was subjected to ultrafiltration in a Centricon using a membrane with 100-kDa molecular weight cut-off (NMWC), analysis of the filtrate and the concentrate showed that again, both the contaminating protein and the tributyrin esterase were present in the concentrate (lane 2 in figure 2.21A). This is unexpected, as the 66kDa protein should pass through a 100kDa cut-off membrane if it is a monomer as indicated by native electrophoresis (figure 2.22-A). There are several reasons may explain why it did not. Firstly, the shape of the protein will affect whether it can pass through the pores; secondly, the pore size is not ideally uniform and will show a normal distribution around the mean pore size. Thirdly, the protein aggregation under the conditions used for the filtration, i.e. these proteins may be non-covalently linked in some way. However, in the filtrate, only the tributyrin esterase is present, suggesting that some of the protein exists in the monomeric form and could be separated from the contaminating protein under current conditions.

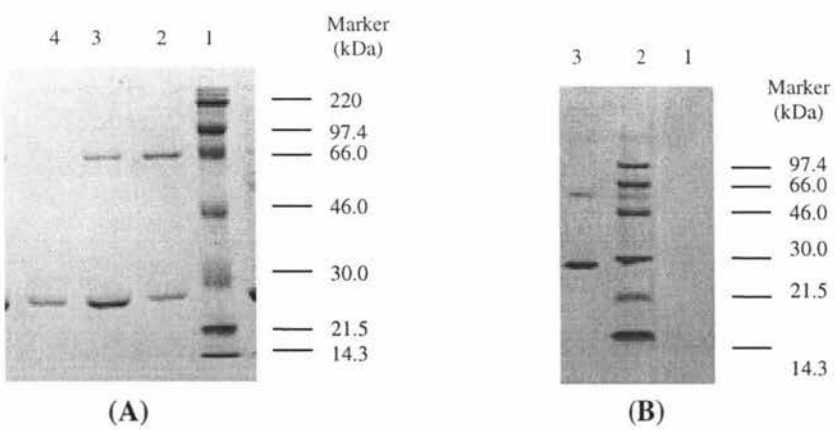


Figure 2.21 SDS gel of elution from Purification by ultrafiltration
(A) Results of ultrafiltration with a 100kD NMWC membrane
Lane1: Marker; Lane2: Sample tested; Lane3: concentrate; Lane4: filtrate;
(B) Results of ultrafiltration with a 50kD NMWC membrane
Lane1: filtrate; Lane2: Marker; Lane3: concentrate;

(B) Mono Q anion exchange

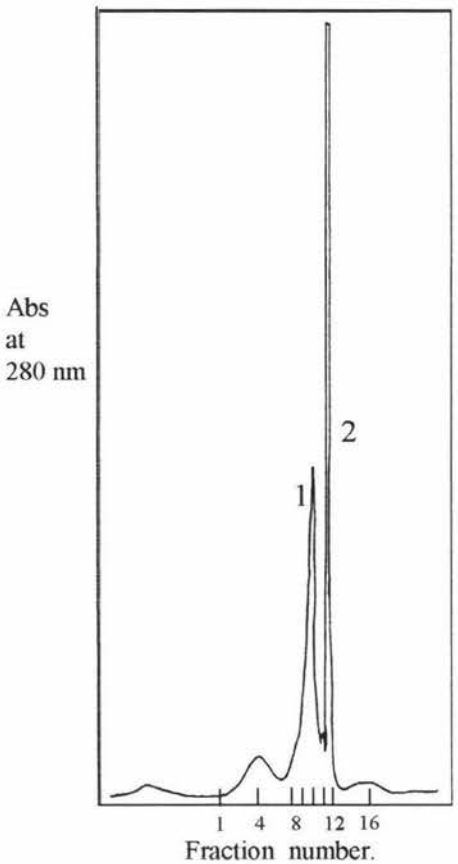
A Mono-Q strong anion exchange column was investigated as a measure of removing the troublesome containminant at ~66kDa that had proved to be very difficult to separate by the other methods described in previous section. An impure sample from the hydrophobic interaction chromatography step was used, which contained the two bands as shown by SDS-PAGE, to be a major band (the esterase) at ~30kDa, and a minor band at ~66 kDa (Lane1 in figure 2.22B). Two peaks were eluted from the column (figure 2.22A) and were analysed by SDS-PAGE (figure 2.22B). Peak 1 fractions (Lane2 & 3 in figure 2.22B) did not stain with Comassie Blue, even when concentrated, indicating it probably did not contain protein. Peak 2 shows one major band at 29 kDa, and one very minor band at 66 kDa. These fractions were further analysed by native-PAGE (figure 2.22c). Two bands were also found on this gel, one of which ran at a position representing a high molecular weight and showed esterase activity when stained with *p*-nitrophenyl butyrate (lane 4 in figure2.22D), and one at lower molecular weight that showed no activity.

2.5.8 Overall Purification of Tributyrin Esterase

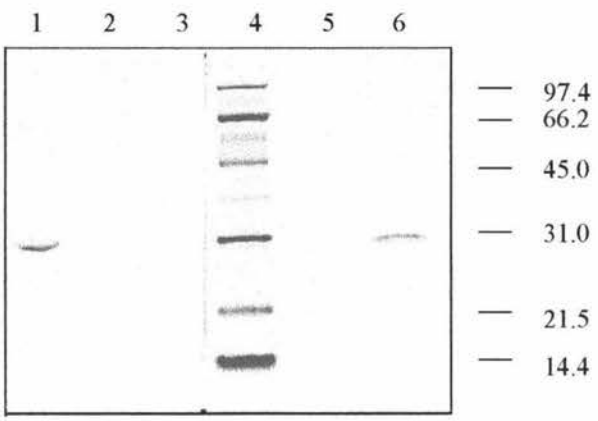
The overall results are shown in Table 2.5. Samples were taken at various stages of the purification to determine both the protein concentration and esterase activities. A summary of the data collected is given in Table 2.5. After the final gel filtration steps (Superdex 75 and Sephadex G-150 superfine), approximately 6 mg of purified tributyrin esterase was recovered with an overall ~35-fold purification which yielded a 14% recovery of tributyrin esterase activity. The purified tributyrin esterase had a specific activity of ~140 U/mg. The largest losses of enzyme occurred during the initial separation of the enzyme from the cell debris, as a result of ammonium sulfate precipitation and during dialysis prior to Q-Seharose loading. In each case, the losses were approximately 30% percent of activity from the previous step. These results are similar to those reported by Holland (1997), except that the final specific activity in the current study is significantly higher. Further investigations of substrate specificity and the factors affecting activity assay may reveal the reasons for the difference.

Figure 2.22 Results of Mono-Q anion exchange chromatography

(a) Chromatogram of the elution from Mono-Q

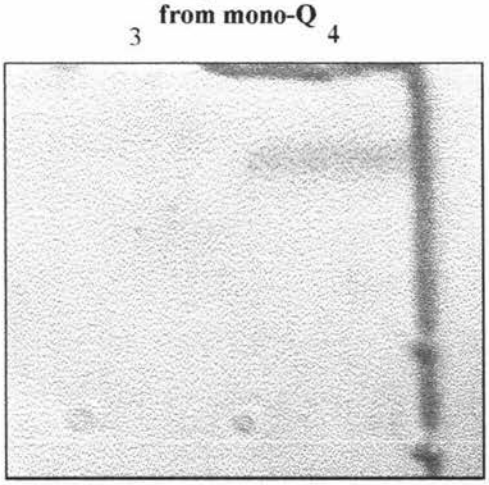


(b) SDS PAGE of Fractions from Mono-Q Column



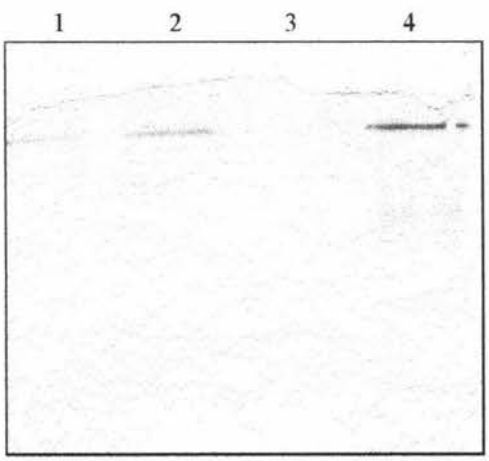
Lane 1, load; Lane 4, marker;
Lanes 2, 3, fractions 8 and 9 ;
Lanes 5 , 6, fractions 11 and 12

(d) Portion of the activity stained native gel of fractions from mono-Q



Lane 3, blank; Lane 4, Fraction 12.

(c) Native PAGE of fractions from Mono-Q



Lane 1, fraction 10 ; Lane 2 fraction 11;
Lane 3, blank; Lane 4, Fraction 12.

Table 2.5 Overall purification of tributyrin esterase in preparation 2

Purification step	Total activity (units)	Specific activity (unit mg^{-1})	Recovery (%)	Purification (-fold)	Actual Volume (mL)	Protein Content (mg/mL)	Total Protein (mg)
1a Culture Supernatant	-	-	-	-	10,000	-	-
1 Whole cells	5252	6.8	-	-	165	4.7	776
2 Homogenate	5896	4.1	100	1.0	160	8.9	1426
3 Cell pellet	2020	2.0	34	0.5	345	3.0	1035
4 Cell free supernatant	3863	2.8	66	0.7	345	4.0	1380
5 $(NH_4)_2SO_4$ pellet	2741	3.8	47	0.9	90	8.0	720
5a $(NH_4)_2SO_4$ supernatant	425	0.5	8	0.1	340	2.5	850
6 Dialysis	1862	4.0	32	1.0	90	5.2	466
7 Q-Sepharose	1650	23	27	5.8	180	0.4	72
8 Alkyl Superose	1260	115	21	28	53	0.2	11
9 Sephadex G150 superfine	840	140	14	34	12	0.5	6

2.6 Conclusion and Discussion

The aim of this section of work was to prepare enough tributyrin esterase for crystallisation studies. Despite efforts to optimise the purification procedure by using different techniques or modifying established ones, tributyrin esterase was difficult to purify to homogeneity by all the methods tested, resulting in only a 14% recovery of pure enzyme. Recombinant DNA technology is therefore the best method likely to facilitate protein purification, as it is possible to add tags for affinity purification that are cleavable after purification has taken place. From the amino acid sequence (Holland, personal communication), the isoelectric point of tributyrin esterase is calculated to be 5.49 and molecular mass 25.8kDa. However, the apparent monomeric molecular weight indicated from SDS-PAGE is 29 kDa. Thus the protein may behave anomalously because of its amino acid sequence, its shape or there may even be some post-translation modification, although this would be unlikely.

In the purification procedure, no protease inhibitor was added in the early stages of the purification, because some protease inhibitors were shown to have an inhibitory effect on tributyrin esterase activity (see 2.5.3 A). Although, no obvious protein degradation has been observed by SDS-PAGE, trybutyrin activity was lost at the early stage of purification in one preparation (data not shown). This may have been due to protease although again, it seems unlikely.

In the future, modification of the purification procedure may involve missing out the ammonium sulfate precipitation step, where large losses occurred, and using ion exchange chromatography at lower pH. Such a protocol was used by Chich *et al.*, (1997) to purify esterase from *lactococcus latics* subsp. *lactis* NCDO 763, an esterase similar to that used in this study.

Chapter 3 CRYSTALLIZATION OF TRIBUTYRIN ESTERASE

As mentioned in the introduction (Chapter1), the three-dimensional structure determination of tributyrin esterase using X-ray crystallographic techniques is the long-term goal of this project. This requires the production of large single crystals. Establishing the correct conditions for the crystallisation is an empirical process that typically uses techniques and reagents that have proven successful in other cases. For these reasons, the aim of this section of work was to find the optimum conditions for growing diffraction quality crystals of tributyrin esterase.

3.1 Materials and Methods

All the polyethleneglycol was specially purified by Hampton research; all other chemicals used were analytical grade, from BDH, Ajax chemicals, United States Biochemical Corporation and Sigma. All precipitant and protein solutions were filtered using Millipore 0.22 μm filters and all pipette tips, containers, crystallization trays were dusted with a compressed clean gas, "blow-hard" (Hampton research), before use to remove possible nuclection sites. The hanging-drop technique for crystallization which depends on the slow equilibration of the protein solution with the precipitant solution through the vapour phase (Wlodawer and Hodgson, 1975) was used. 24 well tissue culture trays (Linbro) were used to set-up the crystallization trials as follows: A 0.6ml reservoir solution of buffered precipitant solution was transferred into each well. 1μl of 12 mg/ml purified tributyrin esterase in 20 mM BTP and 1 μl of reservoir buffer solution were combined on each siliconized cover-slip and gently mixed by pipetting up and down to constitute the hanging drop. The cover slips were placed drop-side down over each well and sealed with Petroleum jelly. The trays were stored at 4° undistriburbed for 1 week. After 1 week, weekly monitoring of crystal growth was carried out by examination of the drops under a light microscope.

All cover slips used were siliconized using the following protocol:

- Wash the cover slips by soaking in a solution of hot detergent for 20 min;
- Rinse well with double distilled water;
- Soak in 50% HNO_3 for 20 min;
- Rinse well with several changes of double distilled water and dry at room temperature;

- Soak in 5 % dichlor-dimethy-silan in CH_2Cl_2 for 30 min;
- Rinse well in water, blow off water and dry at 100°C for 2 hours;

3.2 Crystallization trials using reduced matrix methods

The first crystal screen used the sparse matrix screening method for protein crystallization developed by Jancarik & Kim (1991). The second crystal screen was that developed by Cudney, (1994). Compositions of solutions used are shown in Appendix-1 and Appendix-2. The protein concentration used in all screen was 12 mg/ml. The third crystallization screen used was part of an incomplete factorial PEG Screen developed by Kingston, et al., (1996). The conditions used were based around the isoelectric point of tributyrin esterase, and solutions used are also listed in Appendix-3. All crystal trials were carried out at 4°C.

The fourth, fifth and sixth crystallization trials were carried out to optimize the crystallisation conditions that were identified from the first experiment. These were conditions 21 & 23 from crystal screen-I (Appendix-1) and condition 14 from crystal screen-II (Appendix-2).

Small crystals were obtained from 30 % PEG 400, 0.1M Na Heppes, pH 7.5 and 0.2 M $MgCl_2$ as precipitant, (run 21 in crystal screen I) within one week. They slowly grew a little larger over the next week, but did not increase further in size. Conditions were slightly varied as shown in table 3.1-3.3 in the hope to increase their size. These conditions are listed in Appendices-4, 5, and 6. These experiments were repeated at room temperature.

Table 3.1 Scheme for Screen 4:

Concentration of Precipitant (PEG 400) ↕	pH →					
Run No.	7.0	7.2	7.4	7.6	7.8	8.0
20%	1	2	3	4	5	6
25%	7	8	9	10	11	12
30%	13	14	15	16	17	18
35%	19	20	21	22	23	24

Table 3.2 Scheme for Screen 5:

Additives (0.2M) →

Concentration of Precipitant (PEG 400) ↕

Run No.	$MgCl_2$	$CaCl_2$	NaCl	$(NH_4)_2SO_4$	Na Acetate	Na citrate
20%	1	2	3	4	5	6
25%	7	8	9	10	11	12
30%	13	14	15	16	17	18
35%	19	20	21	22	23	24

Table 3.3 Scheme for Screen 6:

$[MgCl_2]$ →

Concentration of Precipitant (PEG 400) ↕

Run No.	0.2	0.18	0.16	0.14	0.12	0.1
20%	1	2	3	4	5	6
25%	7	8	9	10	11	12
30%	13	14	15	16	17	18
35%	19	20	21	22	23	24

The seventh crystallisation screen was carried out at 4°C using MPD as precipitant, and the conditions that produced crystals at a similar shape to those produced using PEG as precipitant, but smaller in size. The percentage of MPD, precipitant, pH and salt concentration were varied, as shown in Table 3.4. The solutions are listed in Appendix -7.

Table 3.4 Scheme for Screen 7:

salt concentration → pH →

Concentration of Precipitant (MPD) ↕

Run No.	0.21	0.25	0.3	6.0	6.7	7.0
20%	1	2	3	4	5	6
25%	7	8	9	10	11	12
30%	13	14	15	16	17	18
35%	19	20	21	22	23	24

Crystals obtained at 2.0M ammonium sulfate, 0.1M Sodium Citrate pH5.6, 0.2M K/Na Tartrate (conditions 14 in crystal screen II) gave the best diffraction. Therefore, in trials eight and nine, these conditions were varied to optimize crystal growth and quality, and the drop size was increased in order to produce more and bigger crystals. The ammonium sulfate concentration, pH, K/Na tartrate concentration and additive type were varied in the eighth and ninth screens. The schemes are listed in Tables 3.5 and 3.6. Experiment 8

was carried out at 4°C using the solutions listed in appendix-8, and experiment 9 (solutions in Appendix 9) was carried out in duplicate at room temperature by using different sources of protein. In plate-1 of screen 9, the proteins obtained from preparation 1 (12.5mg/mL) were used; in plate-2 of screen 9, the proteins obtained from preparation 2 (10mg/mL) were used.

Any crystals that grew to suitable size were mounted in a glass capillary in order to test whether they diffracted, and preliminary X-ray data was collected for those that did.

Table 3.5 Scheme for Screen 8:

Concentration of Precipitant $(NH_4)_2SO_4$ ▲	pH ◄►			[K/Na Tartrate] ◄►		
Run No.	5.6	6.5	7.0	0.15	0.18	0.25
1.2M	1	2	3	4	5	6
1.6M	7	8	9	10	11	12
2.0M	13	14	15	16	17	18
2.4M	19	20	21	22	23	24

Table 3.6 Scheme for Screen 9:

Concentration of Precipitant $(NH_4)_2SO_4$ ▲	Additive ◄►					
Run No.	K/Na Tartate	Na/K Phasphate	$MgCl_2$	NaCl	$CaCl_2$	Size
1.6 M	1	2	3	4	5	6 (1/1)
1.8 M	7	8	9	10	11	12 (1/1)
2.0 M	13	14	15	16	17	18 (2/2)
2.2 M	19	20	21	22	23	24 (5/5)

3.3 Results and Discussion

Of the 150 crystallization conditions tested in first three screen searches, a solution of 0.2M Mg Chloride, 0.1M Sodium Hepes (pH 7.5) containing 30% PEG 400 as a precipitant (condition in run 23 in Appendix-1) produced several rod like crystals (type I) with hexagonal cross section (Figure 4.1) after one week. The size of the crystals was ($0.02 \times 0.02 \times 0.07$ mm). After two months, a solution of 0.2M Mg acetate, 0.1M sodium Cacodylate (pH 6.5) containing 30% MPD as a precipitant (condition in run 21 in Table 3.1) also produced several similarly shaped crystals, but of smaller size. Those crystals obtained from PEG 400 were found to diffract, but only to very low resolution (8Å). Freezing the crystals did not improve the diffraction. Crystallisation trials in screen 4 for

the type I crystals resulted in the production of bigger, more regular shaped crystals which have not, as yet, been tested for their ability to diffract X-rays.

Four crystals (type-II) were grown using 0.2MK/Na Tartrate, 0.1M Sodium Citrate, (pH 5.6) containing 2.0 M Ammonium Sulfate (run14 in Table 3.2) at 4 °C. These crystals differ greatly in appearance and shape from the crystals grown under the other conditions (figure 4.2). They have been found to diffract to 3.0 Å and belong to the space group C222, with cell dimensions a= 76.4 Å, b=179.9Å, c= 176Å; $\alpha = \beta = \delta = 90°$. Calculation of V_m based on the molecular weight of tributyrin esterase gave a value of V_m=2.0 with 6 molecular in the asymmetric unit. This would suggest a solvent content that is low for a protein crystal. If the asymmetric unit contains 4 molecules, then V_m=3.0, which is quite high for a protein crystal (Mathews, 1968).

So far, experiment-9 (plate-1) has only yielded one crystal (type-III). The conditions from which the crystal grew are 2.0M ammonium sulfate, 0.1 M Na Citrate pH 5.6, 0.2 M $NaPO_4$. Although only a single crystal grew, it had a different morphology to type I and type-II crystals, and was shown to belong to the space group I222 with cell dimensions a= 69.3 Å, b=93.5 Å, c= 218.2Å; $\alpha = \beta = \delta = 90°C$. Calculation of V_m gave a value of 2.4 with 3 molecules in the asymmetric unit.

Overall, the preliminary attempts to crystallize the tributyrin esterase have been successful, suggesting the protein is readily crystallised. The crystals grew over a pH range from 6~8, and buffer type appears not to be a critical determinant in the growth of these crystals. At the time of writing this thesis, X-ray diffraction studies have been begun by Dr Gill Norris (May, 1998), and the preliminary results show that tributyrin esterase is most likely to exist as a trimer or hexamer. However, the results obtained from SEC, SDS-PAGE and native PAGE would indicate the protein exists as a dimer or tetramer. Interestingly, an intracellular esterase from *L. lactis* subsp. *lactis* NCDO that has strikingly similar N-terminal sequence to tributyrin esterase has a molecular mass of 91kDa and apparent molecular mass of 29 kDa, suggesting that it exists as a trimer (see introduction). This esterase also appears to be able to exist in a dimeric form with molecular mass of 59 kDa (Chich et al., 1997). This suggests that both esterases can have different aggregation states under different conditions. The exact correlation between the form of aggregation and the conditions needs to be further investigated. Gary and Jane

(1996) suggest that salt concentration is a major factor, as the salt concentration increases the interactions between protein molecules. They also suggest that salt ions bind to the surface of the protein and changes the dielectric constant of the medium, inducing dissociation. It remains to be seen what the "active" conformation of tributyrin esterase is, and whether it is indeed a dimer, trimer, or multimer of these two forms in its native active conformation.

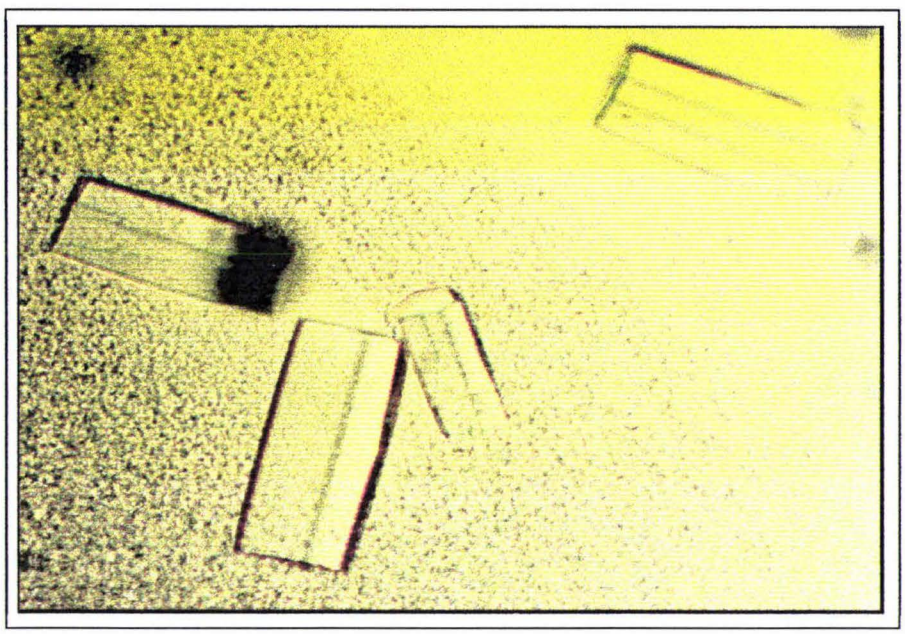


Figure 3.1 Crystals grown in PEG 400 at 4 ° C. (Type I)

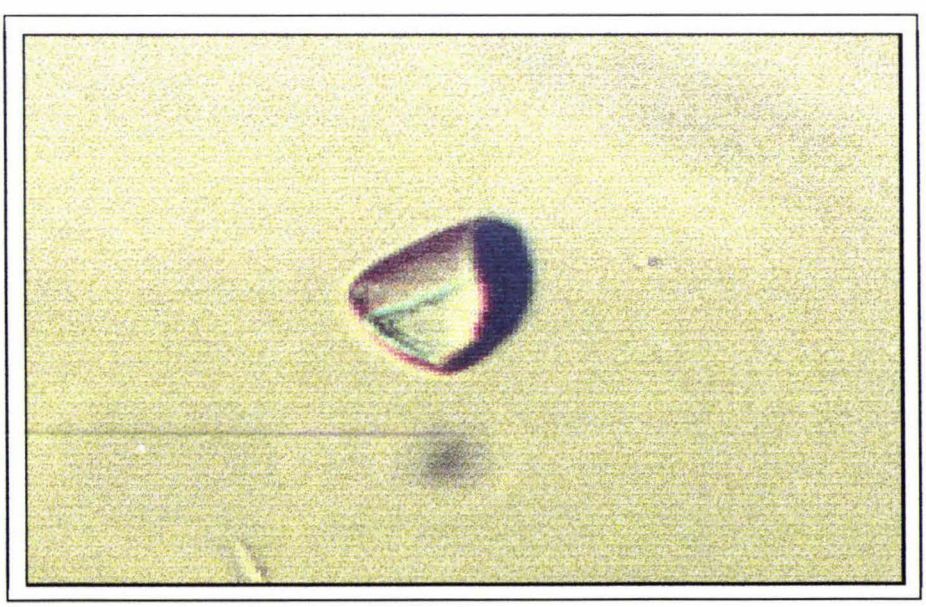


Figure 3.2 Crystals grown in 1.6 M Ammonium sulfate at 4 ° C. (Type II)

Chapter 4 Expression of Lactococcal Tributyrin Esterase in *E. coli*: Transfer of tributyrin esterase coding sequences to an *E. coli* Expression vector

4.1 Experimental Objectives and Strategies

The aim of the work described in this section was to remove the coding sequences for the tributyrin esterase from the lactococcal vector and transfer the gene to a suitable vector system for expression in *E. coli*. The immediate goal was to develop an expression system suitable for over-expressing and purifying tributyrin esterase in *E. coli*, and thus provide a convenient and abundant source of pure enzyme, free of any contaminating protein, as required by crystallization studies. Previous work (Chapter 2) had shown it was very difficult to completely get rid of some contaminating proteins in the purification of tributyrin esterase from the lactococcal overexpression strain. Furthermore, a longer-term goal of this work is to probe structure-function relationships of tributyrin esterase using site-directed mutagenesis. The overexpression of tributyrin esterase in *E. coli* would provide a foundation for mutagenesis of the enzyme, which is relatively harder to achieve in the original lactococcal overexpression system, due to the difficulty in manipulating the vector construct in a lactococcal host.

The overall strategy of cloning is summarized in figure 4.3. The gene encoding the tributyrin esterase---called "lipase gene" (refer to Appendix-10) was obtained from the pNZ9306 plasmid from *Lactococcus lactis* subsp. *cremoris* B1079 (est^+). The expression vector used was the proEX HTa prokaryotic expression system, which contained six histidine codons upstream of the multiple cloning site (MCS) (see map in Figure 4.1). This allows the expression of the protein as a fusion with a his-tag, which greatly facilitates purification. The vector also encodes the sequence for a 7-amino acid spacer arm and the Tobacco Etch Virus (TEV) protease cleavage site (4-8), which can be recognize by rTEV protease to remove the histidine tag from the fusion protein.

Two primers were designed to create suitable restriction sites to clone the lipase gene into the proEX HTa vector (see figure 4.3). The entire lipase gene was amplified by PCR using these primers and pNZ9306 as template. This will yield a product with the entire coding sequence with an in-frame *Bam HI* site at the 5' (N-terminal) end and a *Sal I* site

at the 3' (C-terminal). Both vector and PCR product were double digested with *Bam HI* and *Sal I* restriction endonuclease to create cohesive ends. The digested PCR product (insert) was then ligated into proEX HTa. The ligated products were used to transform a suitable *E. coli* host cell (DH 5α). Finally, the transformant carrying the desired plasmid construct --called proEX-lip (containing the lipase gene) was identified by digestion of the plasmid DNA with diagnostic restriction endonucleases. During the course of the experiment the initial strategy was slightly changed. The 3' (C-terminal) end of the insert could not be completely digested with *Sal I*. To eliminate this problem, the 3' end of the PCR product was end-filled by Klenow DNA polymerase plus dNTP's to create a blunt end. A blunt end was also created in the proEX HTa vector through digestion with *Stu I*. The PCR product was then ligated into proEX HTa following the described procedure. The insert with *Bam HI* and blunt end was also cloned into the pGEM-3Z vector, which contained the *Sma I* (blunt) and *Bam HI* sites (see map in Figure 4.2).

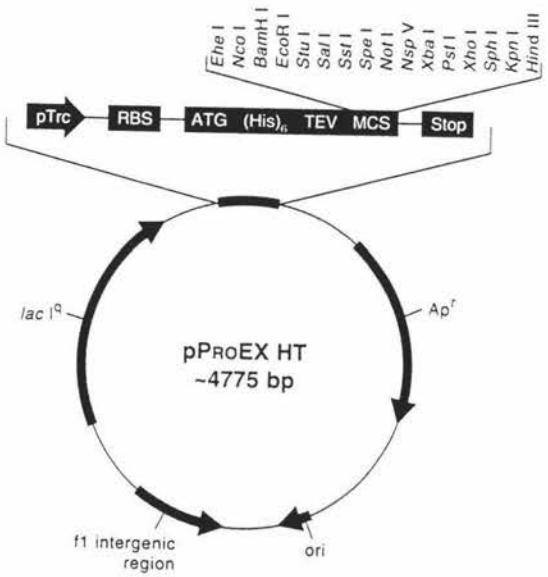


Figure 4.1 Map of proEX HTa Vector

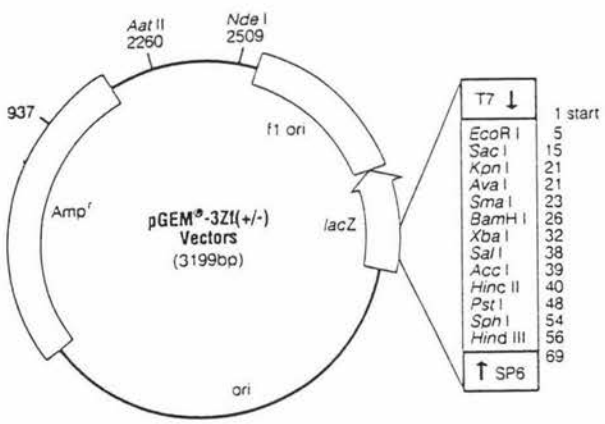
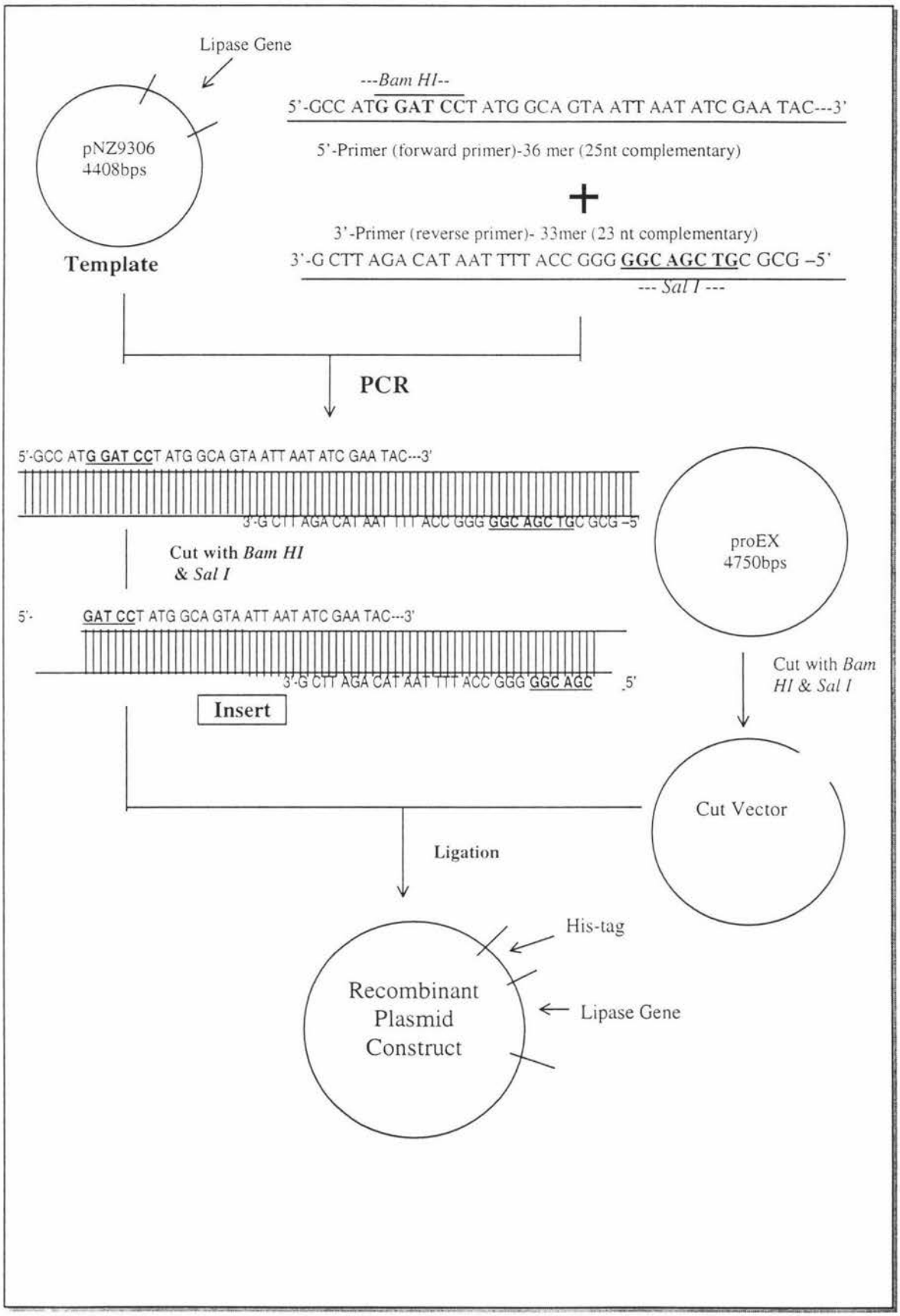


Figure 4.2 Map of pGEM-3z Vector

Figure 4.3 Overall Strategy of cloning



4.2 Materials

4.2.1 Bacterial Growth

The LB media and bacteriological agar used for the growth of *E. coli* were supplied by Difco Laboratories. Ampicillin (Sigma) was prepared as 100 mg/mL stock solution in Milli-Q water and filter-sterilized using Millex-GS 0.22 μm filters (Millipore).

4.2.2 DNA Preparation and Analysis

The plasmid in *Lactococcus lactis* subs. *cremoris* B1079 (est')--- pNZ 9306 was obtained from NZDRI. The stock culture containing proEX HTa vector was kindly provided by Prasad (personal communication). The pGEM-3Z vector was kindly provided by Catherine Day (personal communication). The QIAGEN Plasmid DNA preparation Kit was from Qiagen Pty Ltd., (Victoria, Australia). The Prep-A-Gene DNA purification kit and mini-plasmid prep kit were from Bio-Rad Laboratory (CA, USA). Agarose (Sigma) was used for horizontal agarose gel electrophoresis. All the restriction endonucleases and buffers were from New England Biolabs Inc. (MA, USA). T4 DNA ligase, ligase buffer, Klenow DNA polymerase, thermosensitive alkaline phosphatase (TsAP) accompanying reaction buffers and the 1 kb DNA ladder were from Gibco BRL (USA). The PCR primers (Lip-F and Lip-R) and M13/pUC reverse and forward 23-base sequencing primer were synthesized by Gibco BRL. Lysozyme and RNase were from Sigma (USA). *Taq* DNA polymerase and the accompanying reaction buffers and $MgCl_2$ solutions were from Promega (USA). Standard reagents used in the manipulation of DNA, such as neutral phenol, were prepared as described by Sambrook et al. (1989).

4.3 Methods

4.3.1 Agarose gel electrophoresis.

Agarose gel electrophoresis was performed in a 1x TAE buffer containing 40 mM Tris, 20 mM acetic acid, and 2 mM Na_2EDTA (pH8.0), as described by Sambrook et al., (1989). A 1% agarose gel was prepared by making up 40 mL of 1% agarose mix (0.4 g agarose in 40 mL 1x TAE buffer). The agarose slurry was melted in a microwave, cooled to 45°C, and 1.5 μL of ethidium bromide (10 mg/mL) stock solution was added immediately before pouring the gel. After agarose gels had set, they were transferred to a Bio-Rad "sub-mini" electrophoresis unit tank submerged in 1x TAE buffer. 5~10 μL

sample was loaded with 5× loading buffer. The electrophoresis was performed at 100 V (3,6V/cm) for 1 h. Gels were photographed under UV light on Gel Doc (model TM 20, UVP).

4.3.2 Isolation of Lactococcus Plasmid (pNZ9306) Containing Lipase Gene

The protocol for purifying plasmid DNA from the lactococcal strain followed the method described by Anderson (1983) with some modification in the latter stage. All the steps were listed in table 4.1. The preparation was performed in a 250-ml centrifuge bottle.

Table 4.1 Lysis Protocol for *Lactic Streptococci*

1) Grow *Lactococcus Lactis* subsp. *cremoris* B1079 (600 ml culture) under the conditions described in chapter 2; Harvest cells by centrifugation.
2) Resuspend pelleted cells with 30 mL 6.7% sucrose-50 mM Tris-1 mM EDTA, pH8.0, and warm to 37°C;
3) Add 7.5 mL Lysozyme (10 mg/ml) in 25 mM Tris, pH8.0), and incubate for 5 min at 37 °C;
4) Add 3.75 ml 0.25 M EDTA-50mM Tris, pH8.0
5) Add 2.25 ml sodium dodecyl sulfate (20% [wt/vol] in 50 mM Tris-20 mMEDTA, pH 8.0)
6) Mix immediately by swirling
7) Incubate for 5 to 10 min at 37°C to complete lysis
8) The chromosomal DNA was sheared by dispensing 13- to 15-ml portions of the lysate into screwcap test tubes (25 mm by 150 mm). Each tube was vortexed at full speed for 30 S, and the sheared lysates were pooled before denaturation.
9) Add 2.4 mL fresh 3.0N NaOH;
10) Mix gently by intermittent inversion for 5 min;
11) Add 3.9 mL 2.0 M Tris-hydrochloride, pH 7.0 and continue gentle mixing for 3 min;
12) Add 5.7 mL 5.0 M NaCl;
13) Add 55.8 mL phenol saturated with 3% NaCl; Mix thoroughly and centrifuge at 5,000 rpm for 10 min;
14) Remove upper phase and extract with chloroform-isoamyl alcohol (24:1)
15) Remove upper phase, add 1 vol of isopropanol; incubate at 0° for at least 1 hour to precipitate the plasmid DNA.
16) Centrifuge at 8,000 rpm for 20 min;
17) Remove excess isopropanl and resuspended the plasmid DNA in 1mL 10 mM Tris-1 mM EDTA, pH 7.5.

The plasmid DNA obtained from the above preparations could not be digested with restriction enzymes. Therefore, a washing step was carried out by using the following procedure:

- Take 100 µL of plasmid solution;
- Add 50 µL phenol and 50 µL chloroform;
- Centrifuge 5 min to separate the solvent and aqueous phases. Re-extract the aqueous (upper) phase with chloroform-isoamyl alcohol (24:1).
- Remove lower aqueous phase and add 0.1 volume of 3 M ammonium acetate.
- Add 2.5 volume of 95% ethanol and incubate at -20°C for 1-2 hours.
- Centrifuge 5 min to recover precipitated DNA, Wash pellet once with 80% ethanol, incubate at –20 °C for 20 min.
- Centrifuge for 4 min and re-dissolve pellet in 25 µL 10 mM Tris-1 mM EDTA, pH 7.5 (TE buffer).

The plasmid DNA was capble of restriction endonuclease digestion after this treatment.

4.3.3 The Amplification of Lipase gene by PCR

The oligonucleotide PCR primers LipF [$^{5'}$GCC ATG GAT CCT ATG GCA GTA ATT AAT ATC GAA TAC $^{3'}$] and LipR [$^{3'}$G CTT AGA CAT AAT TTT ACC GGG GGC AGC TGC GCG $^{5'}$] were designed from the known sequence of the lipase gene (see appendix-10). The optimum set of conditions for the amplification of the gene coding for the lipase was obtained by setting up trial PCR experiments in which the concentration of Mg^{2+}, and annealing temperature were varied. A typical PCR, with a total reaction volume of 50 µL in a 0.5mL Eppendorf tube, was set up by adding 5 µL of $MgCl_2$ (25 mM) to give a final concentration of 2.5 mM, 5 µL 10 x *Taq* DNA polymerase buffer (Mg^{2+} free), 5 µL of 3 mM dNTP's (15 nmoles), 5 µL of each primers (50 ng/ µL), 1.5 µL of 10-fold diluted lipase plasmid template, 0.5 µL of *Taq* DNA polymerase (5U/ µL) and made up to 50 µL total volume with sterile Milli-Q water. The thermo-cycling program that gave the best results was 5 minutes at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C.

4.3.4 Large Scale Isolation of proEX HTa Plasmid DNA

A glycerol stock of *E. coli* in -70 °C containing proEX HTa vector was plated on LB/Ap plates (100 μg/mL). A single colony was inoculated into a 5 ml LB Broth containing ampicillin and grown overnight at 37°C. All of this culture was used to inoculate a flask containing 500 ml of LB Broth with ampicillin, which was then incubated overnight at 37°C. The QIAGEN Plasmid kit was used to produce large scale plasmid DNA as described in the QIAGEN plasmid handbook. This method is based on alkaline lysis of bacteria, followed by neutralization to precipitate chromosomal DNA and protein. Plasmid DNA was purified by anion exchange chromatography using a modified DEAE-silica gel (QIAGEN instruction manual).

4.3.5 Preparation of Insert and Vector for Ligation

Four ligation experiments were carried out. The treatment of insert and vector was varied in each experiment to determine the most critical factor for the ligation and transformation.

(A) Experiment I

Both insert and vector used in the first ligation reaction were prepared by the following steps: The 700 bp PCR product and 4.75 kb vector were first digested with restriction endonuclease *Bam HI* at 37°C for 2 hours. The buffer was changed by ethanol precipitation following the method used in section 4.3.2. Both insert & vector were then digested with restriction endonulease *Sal I* at 37°C for 2 hours. Before the ligation, both the insert and vector were gel purified following electrophoresis using the Prep-A-Gene DNA purification kit (Bio-Rad) according to manufacturers instructions.

(B) Experiment II

The insert and vector used in experiment II were initially digested with the restriction endonuclease *Sal I* at 37°C for 2 hours, after changing to *Bam HI* buffer using the same method in experiment I. The PCR and vector were then digested with the restriction endonuclease *Bam HI* at 37°C for 2 hours. Furthermore, the vector was dephosphorylated by adding 0.2 μL of thermosensitive alkaline phosphatase (TsAP) and 5 mM $MgCl_2$. The reaction was undertaken at 65°C for 15 minutes and then stopped by adding 4μL of stop

buffer (200 mM EDTA, pH 8.0) and incubated at 65°C for 15 minutes. Gel purification was also carried out for both vector and insert before ligation.

(C) Experiment III

The PCR product used in experiment III was first treated with the Klenow DNA polymerase to ensure that the blunt end (at the 3' end of the lipase gene) required for the ligation was present. The reaction mixture was set up by adding 33 μM each dNTP's, 1unit Klenow per μg DNA and was incubated for 15 minutes at 25°C; the reaction was stopped by adding EDTA to 10 mM final concentration. After changing buffer, the PCR product was digested with the restriction endonuclease *Bam HI* to create a cohesive end at the 5' end of the lipase gene. Two vectors (proEX and pGEM) were used in experiment III. The proEX was digested with restriction endonulease *Stu I* and *Bam HI* at 37°C for 2 hours. The pGEM was digested with restriction endonulease *Sma I* and *Bam HI* at 37°C for 2 hours, to create a blunt end and a *Bam HI* cohesive end that corresponded the sticky end of the PCR product. The vectors were then dephosphorylated as before. All the inserts and vectors were gel purified using the same method as above.

(D) Experiment IV

In experiment IV of ligation, the insert was prepared only through digestion with *Bam HI* without the treatment with Klenow. The proEX vector was digested with Stu I and *Bam HI*. A phenol/chloroform precipitation step rather than gel purification was carried out for both vector and insert before ligation to remove the small DNA fragment obtained from restriction digestion. The dephosphorylated vector and phosphorylated vector were compared. The ligation was verified by PCR using the ligation mixture as the template and M13/pUC reverse 23-base sequencing primer as the forward primer and Lip R as the reverse primer.

4.3.6 Ligation of Vector and Insert

Three different ratios of insert and vector were tried in ligation reactions (i.e vector : insert was 1:1; 1:3 and 3:1) . The ligation reaction was set up as follows: For vector: insert ratio at 1:3, 15 ng of insert (treated PCR product) and 100 ng of treated proEX vector were taken, and 2 μl T4 ligase buffer (5x), 1 μL T4 ligase and 7 μl H_2O were

added to 10 µL of total volume. The reaction mixture was incubated at room temperature for 3 hours to allow ligation to occur.

4.3.7 Transformation

4.3.7.1 Preparation of Competent Cell

Ultra-competent DH5α cells were produced by the Institute of Molecular BioScience using the following method. DH5α cells from frozen stock (in LB/7% DMSO) were streaked onto LB plates and incubated overnight. 10- 12 large colonies were inoculated to 250 ml of SOB medium (20 g/l tryptone, 5 g/l yeast extract, 0.5 g/l N) in a 2-liter flask, and grown to an A_{600} of 0.6 at room temperature, with vigorous shaking. The cells were then placed on ice for 10 minutes and spun down at 2500 g for 10 min at 4°C. The pellet was resuspended in 80 ml of ice-cold TB (10 mM PIPES, 15 mM $CaCl_2$, 250 mM KCl pH 6.7, 55 mM $MnCl_2$ H_2O), incubated in an ice bath for 10 min, and spun down as above. The cell pellet was gently resuspended in 20 ml of TB, and DMSO was added with gentle swirling to a final concentration of 7%. After incubating in an ice bath for 10 min, the cell suspension was dispensed in 1-2 ml aliquots into tissue-culture cell-freezing tubes and immediately chilled by immersion in liquid nitrogen.

4.3.7.2 Transformation

Transformation of ligated plasmid into DH5α cells was carried out according to Hiroaki et al., (1990) as follows: 200 µL of competent cells were thawed at room temperature, 5 µL of the ligation reaction mixture were added, and the cells were incubated in an ice bath for 30 min. They were then heat-pulsed without agitation at 42°C for 30 seconds and transferred to an ice bath. 0.8 mL LB (without ampicillin) was added, and the tubes were incubated at 37 °C for one hour. The cells were then spun down and resuspended in 100 µL LB and poured onto LB plates containing 100 µg Ap/ml. Colonies were counted after overnight incubation at 37 °C.

4.3.8 Preparation and Analysis of Plasmid DNA from Transformants

'Mini-Prep' isolation of plasmid DNA was carried out by using the alkaline lysis method (Holmes and Quigley, 1981). Single colonies of ampicillin-resistant bacteria were grown overnight in 5 mL LB Broth. 1.5 ml of the culture was pelleted for 5 minutes at 13,000 g. The pellet was resuspended in 100 µL of lysis solution I (Glucose 50 mM, Tris/HCl pH

8.0, 25 mM EDTA 10 mM and Lysozyme 0.1 g/25 ml). After mixing by vortex at room temperature for 5 minutes, 200 µL of lysis solution II (0.2 M NaOH, 1% SDS) were added, the solutions were mixed by inversion and placed on ice for 5 minutes. Finally, 150 µL of ice cold lysis solution III was added (Glacial acetic acid 28.5 mL, 60 mL potassium acetate and 11.5 mL H_2O). The resulting mixture was vortexed gently and placed on ice for 5 minutes. The gelatinous pellet was removed by centrifugation at 13,000 g for 5 minutes. The supernatant was washed according to the method described in section 4.3.2. The lyophilised DNA pellet was dissolved in 20 µL of TE buffer and stored at -20°C.

The 'mini-prep' plasmid DNA samples were analysed for the presence of a PCR insert in proEX by electrophoresing undigested plasmid DNA samples alongside the 1 kb DNA ladder on a 1% agarose gel. The larger plasmids containing inserts were digested with diagnostic restriction endonucleases, e.g. *Hind III* and *Bam HI* (see map in figure 4.1). The linearized plasmids were further analyzed by electrophoresis on a 1% agarose gel.

In experiment IV of ligation, the transformant carrying the desired plasmid construct (proEX-lip) was first identified by sub-plating a single colony and using the bacterial transformant to provide the DNA template directly in a PCR using the LipF and LipR primers. The 'Mini-Prep' isolation of plasmid DNA (only for transformant contain proEX-lip) was carried out by using the mini-plasmid prep kit from Bio-Rad following the protocol described in the handbook. The analysis of plasmid DNA followed the same procedure as above.

4.4 Results and Discussion

4.4.1 Isolation of Lactoccocus Plasmid (pNZ9306) Containing Lipase Gene

After preparation, the plasmid DNA could not be digested with restriction enzymes. Following the further washing with phenol/chloroform, the plasmid could be digested with restriction endonuclease *EcoR I*. The original plasmid (lane 2 & 3) and digested plasmid (lane 4) were analysed on a 1% agarose gel (Figure 4.3). The linearized plasmid runs in between the 4 kb and 5 kb molecular size marker in the 1 kb plus ladder (Gibco BRL) run in lane 1. The observed plasmid size is consistent with the size of lactoccocus plasmid (pNZ9306) 4.4 kb (refer to Appendix-11).

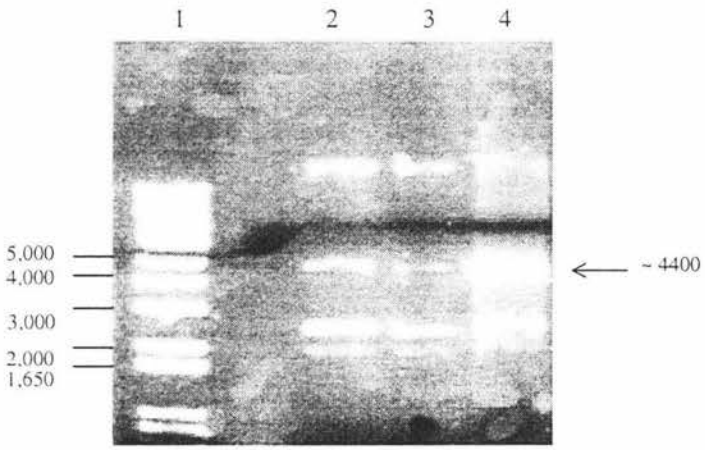


Figure 4.4: Agarose gel of isolated lactoccocus plasmid (pNZ9306)
Lane 1: 1 kb plus ladder molecular size markers;
Lane 2 & 3: original plasmid; Lane 4: digested plasmid

4.4.2 Amplification of the Lipase Gene by PCR

The optimum set of conditions for the amplification of the gene coding for the lipase was obtained by setting up trial PCR experiments in which the concentration of Mg^{2+}, and annealing temperature were varied. Figure 4.5 shows the PCR product that was obtained in the first experiments. Lane 2 shows the PCR product running as a smear, suggesting that there were many non-specific PCR products due to the lower annealing temperature. After increasing the annealing temperature from 55°C to 60°C, only one PCR product of about 700 bp was obtained (data not shown). The effect of changing the concentration of Mg^{2+} was tested. Figure 4.5 shows the PCR products obtained from 1 mM (lane2), 2 mM (lane 3), 2.5 mM (lane 4), 3mM (lane 5), 3.5 mM (lane 6) Mg^{2+}. It is clearly shown that 2.5 mM is the optimal concentration of Mg^{2+}. Lane 7 shows that the PCR product

digested with diagnostic restriction endonuclease *EcoR I* gives two fragments of ~600 bp and ~150 bp as predicted. This verified that the PCR product obtained was probably correct, although DNA sequencing will be required to eliminated the possibility that errors were introduced during the PCR process (refer to the sequence of lipase gene in appendix).

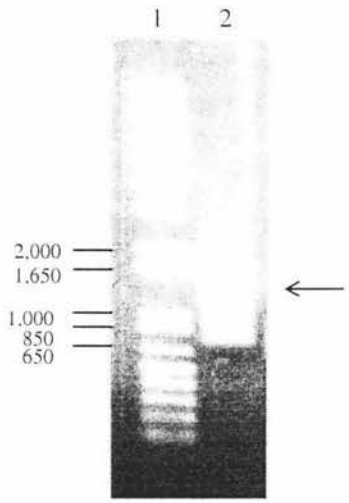


Figure 4.5: Agarose gel of PCR product obtained at annealing temperature 55°C
Lane 1: 1 kb plus ladder molecular size markers; Lane 2: PCR product

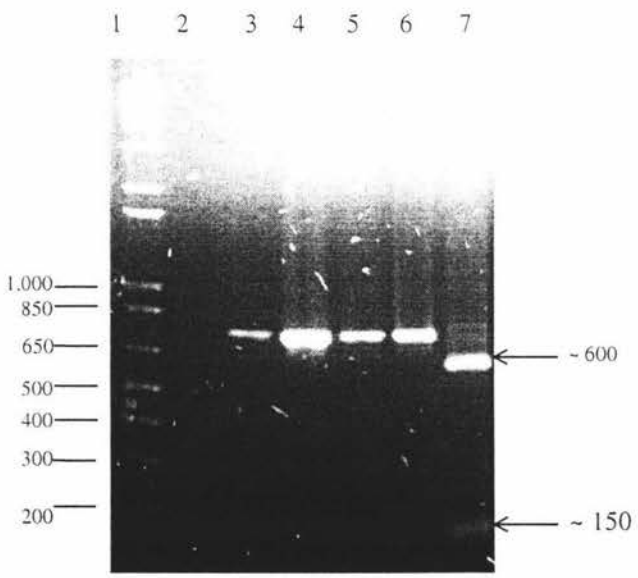


Figure 4.6: Agarose gel of PCR product obtained at different Mg^{2+} concentration

Lane 1: 1 kb plus ladder molecular size markers; Lane 2: 1 mM Mg^{2+}; Lane 3: 2 mM Mg^{2+}; Lane 4: 2.5 mM Mg^{2+}; Lane5: 3 mM Mg^{2+}; Lane 6: 3.5 mM Mg^{2+}; Lane 7: EcoR I digested PCR product

4.4.2 Preparation of Insert from PCR Product

The digestion of the PCR product to create cohesive ends can not be verified by gel electrophoresis, because the difference between digested and undigested PCR products was only a few base pairs. After two failed attempts to ligate the insert into proEX vector, the digestion of PCR product was verified by ligating the digested PCR product. Since the end of the primers does not contain phosphate, only successfully digested PCR product can be ligated. Lane 2 in Figure 4.7a shows, that *Bam HI* digested PCR product can be ligated to form a dimer, suggesting the digestion was successful. However, the *Sal I* digested PCR product did not form any dimer after ligation (Lane2 Figure 4.7b). Furthermore, the method itself was proved to be a good tool to verify the efficiency of digestion and production of cohesive ends.

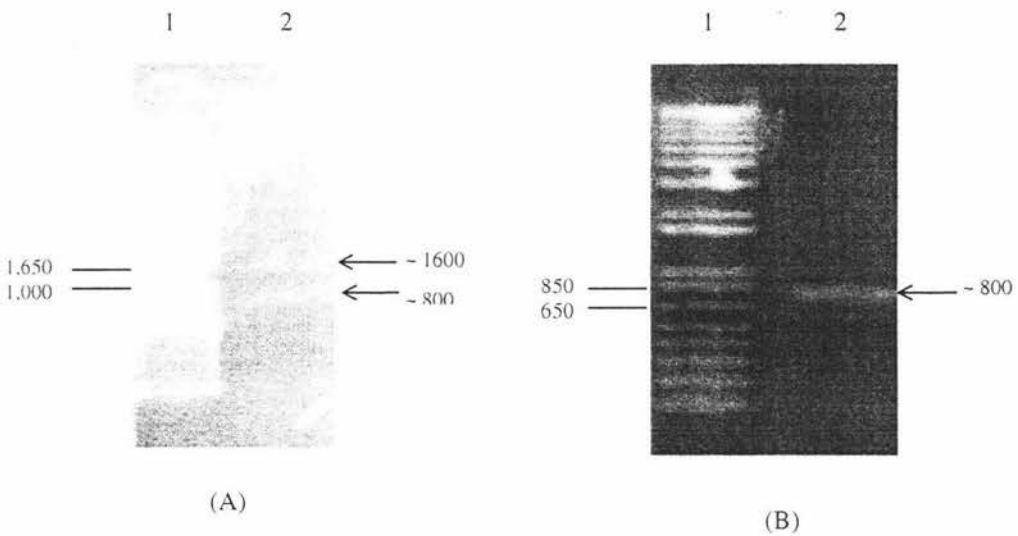


Figure 4.7: Agarose Gel of self ligated BamHI digested PCR product and Sal I digested PCR product

Lane 1 in (A) and (B) : 1 kb plus ladder molecular size markers; (A) Lane 2: self-ligated *Bam HI* digested PCR productt; (B) Lane 2: self-ligated the Sal I digested PCR product;

4.4.3 Ligating the PCR product into proEX Vector

Before transformation experiment IV, ligation of the PCR product into the proEX vector was verified by PCR using the ligation mixture as the template and M13/pUC reverse 23-base sequencing primer [AGC GGA TAA CAA TTT CAC ACA CAGG] as the forward primer and Lip R as the reverse primer. Because M13/pUC reverse 23-base sequencing primer is complementary to part of vector sequence, and Lip R is complementary to part of insert sequence, only the successfully ligated plasmid can produce PCR product of the size of the insert. As the result in figure 4.7 shows, in all ligation mixtures, a ~800 bp

PCR product was obtained, suggesting the ligation had been successful. The other PCR product shows ~100 bp size, probably was due to the low specificity of the annealing step in PCR reaction. However, this result only show that the insert and vector were ligated at one end, it does not shows whether they were also ligated at the other end. When PCR was carried out using both M13/pUC reverse and forward primers, only product that was observed was ~100bp which was much smaller than expected if the insert had been cloned (data not shown). From this result, it was presumed that only a small portion of vector was ligated with the insert at both ends and this method was not suitable to verify the ligation.

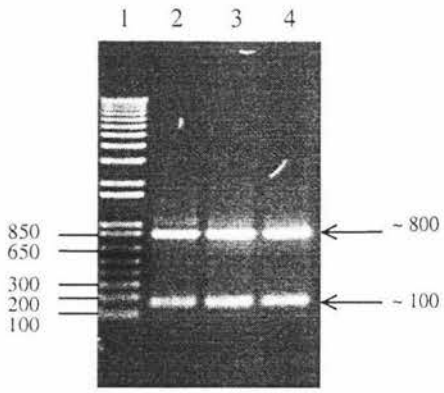


Figure 4.8: Agerose Gel of verification of ligation by PCR

Lane 1: 1 kb plus ladder molecular size markers. Lane 2: 1:1-ratio ligation mixture as template; Lane 3: 1:3-ratio ligation; Lane 2: 3:1-ratio ligation;

4.4.4 Transformation and Analysis of Plasmid DNA from Transformants

In transformation experiment I, both vector & insert was digested with *Bam HI* first and then Sal I, and finally purified from the gel. As the result, many colonies were obtained on all ampicillin resistant plates (both transformants with and without insert), which indicated that many of the transformed cells did not contain a vector with insert. It was almost impossible to identify the transformant carrying the desired plasmid construct (proEX-lip) with such a high background. It can be concluded from these resulsts that some self ligation may have occurred in the vector only control reaction mixture. This result could also be due to the Sal I digestion of vector being incomplete. The digestion of Sal I sites close to the end of DNA molecule has been shown to be difficult (Promega catalog, 1995).

In transformation experiment II, the insert was treated the same as before, the vector was digested with Sal I first and then digested *Bam HI* after ethanol precipitation. In addition, the vector was dephosphorylated. The results obtained are shown in Table 4.3. The positive and negative control plates showed that the transformation system was working well, although the number of colonies in the no insert transformants plate was low. Too few colonies were obtained from the vector with insert transformants plate. After all single colonies were inoculated to 5 mL overnight culture, only four of them grew. This may have been due to improper technique when transfering cells from the plates to liquid culture. After preparation and digestion of plasmid DNA, only one mini-prep showed the plasmid band after agarose gel electrophoresis, no insert was found in the digestion of this plasmid (data not shown).

Table 4.3 Transformation results in experiment II

Cut proEX vector : insert 1:3	7 *
Cut proEX vector : insert 1:1	3
Cut proEX vector : insert 3:1	3
No ligase cut proEX vector + insert	0
No insert cut proEX vector only	8
Original proEX vector (uncut)	Too many to count
Cells+water	0

In transformation experiment III, the pGEM vector was digested with SmaI and BamHI endorestriction nuclease and dephosphorylated. The proEX vector was digested with *Stu I* and *Bam HI* restriction endonucleases and dephosphorylated. The insert was treated with Klenow to fill the end and was then digested with *Bam HI.* Both the vector and insert were gel purified. The results obtained are shown in Table 4.4. Few colonies were obtained in the vector plus insert transformation plate. This suggested that the treatment of DNA was causing a very low efficiency of transformation. After all single colonies had been inoculated to 5 mL overnight culture, only four of them grew. After preparation and digestion of plasmid DNA from liquid cultures, one mini-prep show a plasmid band larger than the others (Lane 3 in figure 4.9). However no insert was found after the digestion of plasmid (Lane 11).

Table 4.4 Transformation results in experiment III

pGEM3Z vector: insert 1:1	5 **
proEX vector : insert 1:1	4 *
proEX vector : insert 1:3	1 *
proEX vector : insert 3:1	0
No ligase cut pGEM vector	1
No ligase cut proEX vector	2
No insert cut pGEM vector	0
No insert cut proEX vector	0
Original vector	Too many
Cells+water	0

* Plasmid obtained

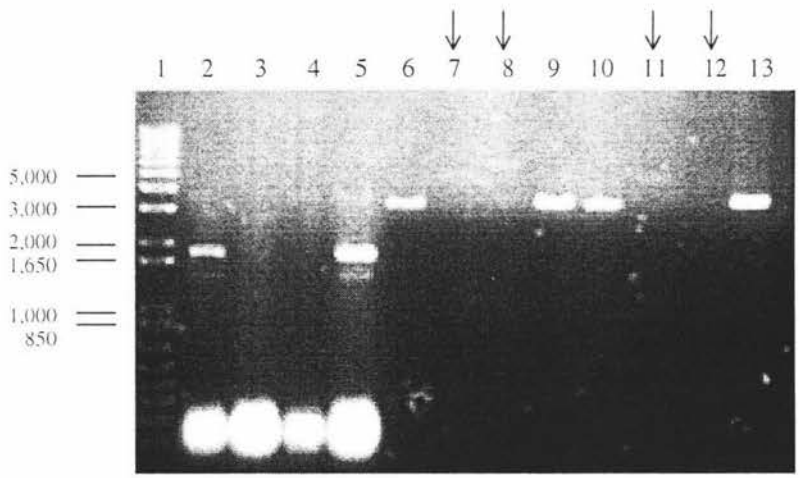


Figure 4.9: Agarose gel of sreen of plasmi 'mini-prep' in experiment III

Lane 1: 1 kb plus ladder molecular size markers;

Lane 2 to Lane 5: original plasmid;

Lane 6 to Lane 9: single digested plasmid (with EcoR I);

Lane 10 to 13: plasmid double digested with (Bam HI & Sal I)

In trasformation experiment IV, the proEX vector was digested with Stu I and *BamH I* and then treated with phenol/Chloroform to remove the small DNA fragment (without gel purification). The insert was digested with *BamH I* without the treatment with Klenow or gel purification. The results from previous section had confirmed that the ligation was successful. The results of this transformation are shown in Table 4.5.

Table 4.5 Transformation results in experiment IV

Cells+water	0
Original vector (uncut)	>300
phosphorylated vector : Insert 1:1	80~90
dephosphorylated vector : Insert 1:1	3
phosphorylated vector : Insert 1:3	>90
No insert phosphorylated vector	40~50

The number of colonies obtained on the ligation of phosphorylated vector and insert plate was much higher than in the ligation of dephosphorylated vector and insert. This result suggested that the dephosphorylation of the vector may have damaged the ends of the vector, causing the failure of the ligation.

ProEX-lip transformants were screened by PCR. Twenty colonies were transferred by a tooth-pick to another plate and grown overnight to prevent the contamination of the unligated insert. The transformant plasmid was then amplified by PCR using the LipF and LipR as primers and the transformant bacterial cell as the template. The PCR product was identified by agarose gel electrophoresis. As the result in figure 4.10 shows, two out of the twenty colonies contained a PCR product ~800 bp in size, suggesting that these transformants probably contain the desired plasmid construct (proEX-lip) (transformant 9 & 13).

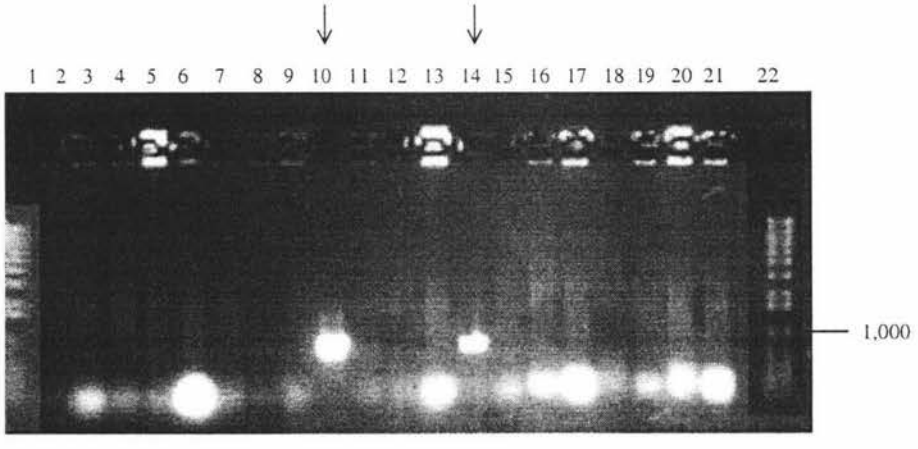


Figure 4.10: Agarose gel of PCR transformnts

Lane 1& Lane 22: 1 kb plus ladder molecular size markers; Lane 2 to Lane 21: some transformants from phosphorylated vector plus insert transformation plate

A 'Mini-Prep' isolation of plasmid DNA was carried out on transformants containing proEX-lip. The 'mini-prep' plasmid samples were analysed for the presence of a PCR insert in proEX by comparing undigested and digested plasmid DNA samples by gel electrophoresis. The results showed that the undigested plasmid DNA of transformant 9 (Lane3) was slightly larger than the original proEX plasmid DNA (in Lane2). Lane 4 shows the plasmid DNA of this transformant digested with *Bam HI* & *Hind* restriction endonucleases. The presence of a ~800 bp insert fragment and a ~5 kb proEX vector fragment confirmed that this transformant contained the correct plasmid construct. The double digested and undigested plasmid DNA of transformant 13 in lane 5 and 6 showed the same size, suggesting it is not right plasmid construct.

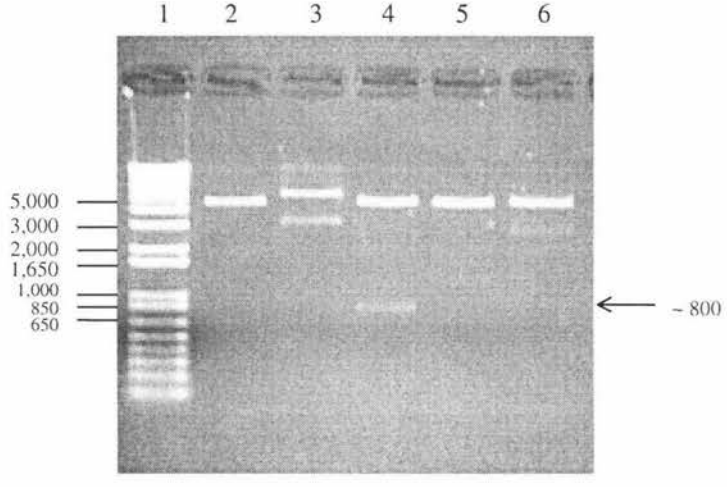


Figure 4.11: Agarose Gel of 'mini-prep' plasmid samples in experiment IV

Lane 1: 1 kb plus ladder molecular size markers;
Lane 2: original proEX vector;
Lane 3: undigested plasmid of transformant 9;
Lane 4: *Bam HI* & *Hind III* digested plasmid of transformant 9;
Lane 5: undigested plasmid of transformant 13;
Lane 6: *Bam HI* & *Hind III* digested plasmid of transformant 13;

4.4.5 Conclusion and Discussion

After several attempts the ligation and transformation finally worked, albeit at low efficiency. One out of the twenty transformants contained desired plasmid construct (proEX-lip). The treatment of insert and vector was found to be the most critical factor to determine the efficiency of ligation and transformation.

For future work, because the lipase gene inserted into the proEX expression vector has been produced by PCR, it will be necessary to check the sequence of the lipase gene in

proEX-lip to guard against the possibility of errors having been introduced by infidelity of *Taq* DNA polymerase in the PCR amplification process. It will also be critical to characterize the recombinant lipase, because the addition of a his-tag to the protein may influence the enzyme activity and the tertiary protein structure (see review in section 1.6).

CHAPTER 5 Summary and Final Discussion

In summary, having obtained some crystallographic-grade pure tributyrin esterases, the preliminary attempts to crystallize the tributyrin esterase have been successful and native data sets have been collected on two crystal forms of tributyrin esterase. While these crystals diffract, they do so only to moderate resolution. More protein will be required to improve their quality and size and to prepare heavy-atom derivatives that will be necessary to solve the structure because of the lack of any suitable models for molecular replacement studies.

However, the way is now clear to provide pure recombinant protein in sufficient quantities for crystallization trials. Expressing and characterizing the recombinant protein from initially developed *E.coli* expression system are next steps to achieve this aim.

It is reasonable to hope that an X-ray crystal structure might be available for this tributyrin esterase in the next two years. The exact correlation between the form of aggregation and the conditions and the "active" conformation of tributyrin esterase would be important to reveal the catalytic mechanism and the substrate specificity. The successful structure solution of this protein will hopefully answer many questions:

- Is tributyrin esterase an esterase, a lipase or a hydrolase belonging to some other family of enzyme?
- What is its nature active conformation?
- What is its substrate *in vivo*?
- What is the basis of its interaction with other proteins?

A high-resolution crystal structure and well developed expression system in *E.coli* of this enzyme will also open the way for rational site directed mutagenesis and protein engineering to produce an enzyme specifically tailored to the dairy industry and cheese manufacture.

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Appendix-1: Conditions of Crystal General Screen I

Run	Precipitant	Buffer	(pH)	Salt
1	30% MPD	0.1 M Sodium Acetate	4.6	0.02Mcalcium chloride
2	0.4 M K,Na Tartrate	none		none
3	0.4 M Ammonium Phosphate	none		none
4	2.0 M Ammonium Sulphate	0.1M Tris-HCl	8.5	none
5	30% MPD	0.1M Sodium Hepes	7.5	0.2Msodium Citrate
6	30% PEG 4000	0.1 M Tris-HCl	8.5	0.2M Mg Chloride
7	1.4M Sodium Acetate	0.1M Sodium Cacodylate	6.5	none
8	30% 2-Propanol	0.1M Sodium Cacodylate	6.5	0.2M Na Citrate
9	30% PEG 4000	0.1M Sodium Citrate	5.6	0.2M Ammonium acetate
10	30% PEG 4000	0.1 M Sodium Acetate	4.6	0.2M Ammonium acetate
11	1.0M Ammonium Phosphate	0.1M Sodium Citrate	5.6	none
12	30% 2-Propanol	0.1M Sodium Hepes	7.5	0.2M Mg Chloride
13	30% PEG 400	0.1M Tris HCl	8.5	0.2M Sodium Citrate
14	28% PEG 400	0.1M Sodium Hepes	7.5	0.2M Calcium Chloride
15	30% PEG 8000	0.1M Sodium Cacodylate	6.5	0.2M Ammonium sulfate
16	1.5 M Li Sulfate	0.1M Sodium Hepes	7.5	none
17	30% PEG 4000	0.1M Tris-HCl	8.5	0.2 M Lithium Sulfate
18	20% PEG 8000	0.1M Sodium Cacodylate	6.5	0.2M Mg Acetate
19	30% 2-Propanol	0.1M Tris-HCl	8.5	0.2M Ammonium acetate
20	25% PEG 4000	0.1 M Sodium Acetate	4.6	0.2M Ammonium sulfate
21	30% MPD	0.1M Sodium Cacodylate	6.5	0.2M Mg Acetate
22	30% PEG 4000	0.1M Tris HCl	8.5	0.2M Sodium Acetate
23	30% PEG 400	0.1M Sodium Hepes	7.5	0.2M Mg Chloride
24	20% 2-Propanol	0.1 M Sodium Acetate	4.6	0.2M Calcium Chloride
25	1.0 M Sodium Acetate	0.1M Imidazole	6.5	none
26	30% MPD	0.1M Sodium Citrate	5.6	0.2M Ammonium acetate
27	20% 2-Propanol	0.1M Sodium Hepes	7.5	0.2M Sodium Citrate
28	30% PEG 8000	0.1M Sodium Cacodylate	6.5	0.2M Sodium Acetate
29	0.8 M K, Na Tartrate	0.1M Sodium Hepes	7.5	none
30	30% PEG 8000	0.2M Ammonium Sulfate		none
31	30% PEG 4000	0.2M Ammonium Sulfate		none
32	2.0Ammonium Sulfate	none		none
33	4.0 Sodium Formate	none		none
34	2.0M Sodium Formate	0.1 M Sodium Acetate	4.6	none
35	1.6M Na, K Phosphate	0.1M Sodium Hepes	7.5	none
36	8% PEG 8000	0.1M Tris HCl	8.5	none
37	8% PEG 4000	0.1 M Sodium Acetate	4.6	none
38	1.4 M Sodium Citrate	0.1M Sodium Hepes	7.5	none
39	2% PEG 400 + 2.0M Ammonium	0.1M Sodium Hepes	7.5	none
40	20% PEG 4000 + 20% 2-Propanol	0.1M Sodium Citrate	5.6	none
41	20% PEG 4000 +10% 2-Propanol	0.1M Sodium Hepes	7.5	none
42	20% PEG 8000	0.05M Potassium Phosphate		none
43	30% PEG 1500	none		none
44	0.2 M Magnesium Formate	none		none
45	18% PEG 8000	0.1M Sodium Cacodylate	6.5	0.2 M Zinc Acetate
46	18% PEG 8000	0.1M Sodium Cacodylate	6.5	0.2M Calcium Acetate
47	2.0 M Ammonium Sulpate	0.1 M Sodium Acetate	4.6	none
48	2.0 M Ammonium Phosphate	0.1M Tris HCl	8.5	none
49	2% PEG 8000	1.0 M Lithium Sulfate		none
50	15% PEG 8000	0.5 M Lithium Sulfate		none

Appendix-2: Conditions of Crystal General Screen II

Run	Precipitant	Buffer	(pH)	Salt
1	10% PEG 6000	none		0.2M Sodium Chloride
2	0.2M Sodium Chloride, 0.01M Magnesium chloride	none		0.01 M Cetyl trimethyl-ammonium bromide
3	25% Ethylene glycol	none		None
4	25% Dioxan	none		None
5	5% Isopropanol	none		2.0M Ammonium sulfate
6	1.0M Imidazole pH7.0	none		None
7	10% PEG 1000, 10% PEG 8000	none		None
8	10% Ethanol	none		1.5M Sodium Chloride
9	2.0M Sodium Chloride	0.1 M Sodium Acetate	4.6	None
10	30% MPD	0.1 M Sodium Acetate	4.6	0.2M Sodium Chloride
11	1.0M 1,6 Hexanediol	0.1 M Sodium Acetate	4.6	0.01M Cobalt Chloride
12	30% PEG 400	0.1 M Sodium Acetate	4.6	0.1M Cadmdium Chloride
13	30% PEG-mme 2000	0.1 M Sodium Acetate	4.6	0.2M Ammonium Sulfate
14	2.0M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2MK/Na Tartrate
15	1.0M Lithium Sulfate	0.1M Sodium Citrate	5.6	0.5M Ammonium Sulfate
16	4% Polyethyleneimine	0.1M Sodium Citrate	5.6	0.5M Sodium Chloride
17	35% tert-butanol	0.1M Sodium Citrate	5.6	None
18	10% Jeffamine M-600	0.1M Sodium Citrate	5.6	0.01M Ferric Chloride
19	2.5M 1,6 Hexanediol	0.1M Sodium Citrate	5.6	None
20	1.6M Magnesium sulfate	0.1 M MES	6.5	None
21	2.0M Sodium Chloride	0.1 M MES	6.5	0.2M Na/K Phosphate
22	12% PEG 20000	0.1 M MES	6.5	None
23	10% Dioxane	0.1 M MES	6.5	1.6M Ammonium Sulfate
24	30% Jeffamine M-600	0.1 M MES	6.5	0.05M CesiumChloride
25	1.8M Ammonium Sulfate	0.1 M MES	6.5	0.01 M Cobalt Chloride
26	30% PEG-mme 5000	0.1 M MES	6.5	0.2M Ammonium Sulfate
27	25% PEG-mme 550	0.1 M MES	6.5	0.01M Zinc Sulfate
28	1.6M Sodium Citrate pH6.5	none		None
29	30% MPD	0.1M Sodium Hepes	7.5	0.5M Ammonium Sulfate
30	10% PEG 6000, 5% MPD	0.1M Sodium Hepes	7.5	None
31	20% Jeffamine M-600	0.1M Sodium Hepes	7.5	None
32	1.6M Ammonium Sulfate	0.1M Sodium Hepes	7.5	0.1m Sodium Chloride
33	2.0M Ammonium Formate	0.1M Sodium Hepes	7.5	None
34	1.0M Sodium acetate	0.1M Sodium Hepes	7.5	0.05M Cadmium Sulfate
35	70% MPD	0.1M Sodium Hepes	7.5	None
36	4.3M Sodium Chloride	0.1M Sodium Hepes	7.5	None
37	10% PEG 8000, 8% Ethylene glycol	0.1M Sodium Hepes	7.5	None
38	20% PEG 10000	0.1M Sodium Hepes	7.5	None
39	3.4M 1,6 Hexanediol	0.1M Tris HCl	8.5	0.2M Magnesium Chloride
40	25% tert-butanol	0.1M Tris HCl	8.5	0.1M Calcium Chloride
41	1.0M Lithium Sulfate	0.1M Tris HCl	8.5	0.01M Nickle Chloride
42	12% Glycerol	0.1M Tris HCl	8.5	1.5M Ammonium Sulfate
43	50% MPD	0.1M Tris HCl	8.5	0.2M Ammonium Phosphate
44	20% Ethanol	0.1M Tris HCl	8.5	None
45	20% PEG-mme 2000	0.1M Tris HCl	8.5	0.01M Nickle Chloride
46	30% PEG-mme 550	0.1M Bicine	9.0	0.1M Sodium Chloride
47	2.0 M Magnesium Chloride	0.1M Bicine	9.0	None
48	10% PEG 20000	0.1M Bicine	9.0	2% Dioxane

Appendix-3: Conditions of PEG Screen

Run	Precipitant	Buffer	pH
1	7% PEG 6000	0.2 M Citric acid/KOH	4.9
2	14% PEG 6000	0.2 M Acetic acid/KOH	4.9
3	21% PEG 6000	0.2 M Citric acid/KOH	4.9
4	28% PEG 6000	0.2 M Acetic acid/KOH	4.9
5	7% PEG-mme 5000	0.2 M Citric acid/KOH	4.9
6	14% PEG-mme 5000	0.2 M Acetic acid/KOH	4.9
7	21% PEG-mme 5000	0.2 M Citric acid/KOH	4.9
8	28% PEG-mme 5000	0.2 M Acetic acid/KOH	4.9
9	7% PEG 6000	0.2 M Succinic/KOH	5.5
10	14% PEG 6000	0.2 M Malic acid/KOH	5.5
11	21% PEG 6000	0.2 M Succinic/KOH	5.5
12	28% PEG 6000	0.2 M Malic acid/KOH	5.5
13	7% PEG-mme 5000	0.2 M Succinic/KOH	5.5
14	14% PEG-mme 5000	0.2 M Malic acid/KOH	5.5
15	21% PEG-mme 5000	0.2 M Succinic/KOH	5.5
16	28% PEG-mme 5000	0.2 M Malic acid/KOH	5.5
17	7% PEG 6000	0.2 M Cacodylic acid/KOH	6.1
18	14% PEG 6000	0.2 M MES/KOH	6.1
19	21% PEG 6000	0.2 M Cacodylic acid/KOH	6.1
20	28% PEG 6000	0.2 M MES/KOH	6.1
21	7% PEG-mme 5000	0.2 M MES/KOH	6.1
22	14% PEG-mme 5000	0.2 M Cacodylic acid/KOH	6.1
23	21% PEG-mme 5000	0.2 M MES/KOH	6.1
24	28% PEG-mme 5000	0.2 M Cacodylic acid/KOH	6.1
25	7% PEG 6000	0.2 M PIPES/KOH	6.7
26	14% PEG 6000	0.2 M Bis-Tris Propane HCl	6.7
27	21% PEG 6000	0.2 M PIPES/KOH	6.7
28	28% PEG 6000	0.2 M Bis-Tris Propane HCl	6.7
29	7% PEG-mme 5000	0.2 M Bis-Tris Propane HCl	6.7
30	14% PEG-mme 5000	0.2 M PIPES/KOH	6.7
31	21% PEG-mme 5000	0.2 M Bis-Tris Propane HCl	6.7
32	28% PEG-mme 5000	0.2 M PIPES/KOH	6.7
33	7% PEG 6000	0.2M MOPS/KOH	7.3
34	14% PEG 6000	0.2M HEPES/KOH	7.3
35	21% PEG 6000	0.2M MOPS/KOH	7.3
36	28% PEG 6000	0.2M HEPES/KOH	7.3
37	7% PEG-mme 5000	0.2M HEPES/KOH	7.3
38	14% PEG-mme 5000	0.2M MOPS/KOH	7.3
39	21% PEG-mme 5000	0.2M HEPES/KOH	7.3
40	28% PEG-mme 5000	0.2M MOPS/KOH	7.3
41	7% PEG 6000	0.2M EPPS/KOH	7.9
42	14% PEG 6000	0.1M Tris HCl	7.9
43	21% PEG 6000	0.2M EPPS/KOH	7.9
44	28% PEG 6000	0.1M Tris HCl	7.9
45	7% PEG-mme 5000	0.1M Tris HCl	7.9
46	14% PEG-mme 5000	0.2M EPPS/KOH	7.9
47	21% PEG-mme 5000	0.1M Tris HCl	7.9
48	28% PEG-mme 5000	0.2M EPPS/KOH	7.9

Appendix-4: Conditions of Screen 4 (PEG 400 screen I)

Run	Precipitant	Buffer	(pH)	Salt
1	20% PEG 400	0.2 M Bis-Tris Propane HCl	7.0	0.2M Mg Chloride
2	20% PEG 400	0.2M MOPS/KOH	7.2	0.2M Mg Chloride
3	20% PEG 400	0.2M HEPES/KOH	7.4	0.2M Mg Chloride
4	20% PEG 400	0.2M HEPES/KOH	7.6	0.2M Mg Chloride
5	20% PEG 400	0.2M EPPS/KOH	7.8	0.2M Mg Chloride
6	20% PEG 400	0.1M Tris HCl	8.0	0.2M Mg Chloride
7	25% PEG 400	0.2 M Bis-Tris Propane HCl	7.0	0.2M Mg Chloride
8	25% PEG 400	0.2M MOPS/KOH	7.2	0.2M Mg Chloride
9	25% PEG 400	0.2M HEPES/KOH	7.4	0.2M Mg Chloride
10	25% PEG 400	0.2M HEPES/KOH	7.6	0.2M Mg Chloride
11	25% PEG 400	0.2M EPPS/KOH	7.8	0.2M Mg Chloride
12	25% PEG 400	0.1M Tris HCl	8.0	0.2M Mg Chloride
13	30% PEG 400	0.2 M Bis-Tris Propane HCl	7.0	0.2M Mg Chloride
14	30% PEG 400	0.2M MOPS/KOH	7.2	0.2M Mg Chloride
15	30% PEG 400	0.2M HEPES/KOH	7.4	0.2M Mg Chloride
16	30% PEG 400	0.2M HEPES/KOH	7.6	0.2M Mg Chloride
17	30% PEG 400	0.2M EPPS/KOH	7.8	0.2M Mg Chloride
18	30% PEG 400	0.1M Tris HCl	8.0	0.2M Mg Chloride
19	35% PEG 400	0.2 M Bis-Tris Propane HCl	7.0	0.2M Mg Chloride
20	35% PEG 400	0.2M MOPS/KOH	7.2	0.2M Mg Chloride
21	35% PEG 400	0.2M HEPES/KOH	7.4	0.2M Mg Chloride
22	35% PEG 400	0.2M HEPES/KOH	7.6	0.2M Mg Chloride
23	35% PEG 400	0.2M EPPS/KOH	7.8	0.2M Mg Chloride
24	35% PEG 400	0.1M Tris HCl	8.0	0.2M Mg Chloride

Appendix-5: Conditions of Screen 5 (PEG 400 screen II)

Run	Precipitant	Buffer	(pH)	Salt
1	20% PEG 400	0.1M Sodium Hepes	7.5	0.2M Mg Chloride
2	20% PEG 400	0.1M Sodium Hepes	7.5	0.2M Calcium Chloride
3	20% PEG 400	0.1M Sodium Hepes	7.5	0.2M Sodium Chloride
4	20% PEG 400	0.1M Sodium Hepes	7.5	0.2M Ammonium sulfate
5	20% PEG 400	0.1M Sodium Hepes	7.5	0.2M Sodium Acetate
6	20% PEG 400	0.1M Sodium Hepes	7.5	0.2M Sodium Citrate
7	25% PEG 400	0.1M Sodium Hepes	7.5	0.2M Mg Chloride
8	25% PEG 400	0.1M Sodium Hepes	7.5	0.2M Calcium Chloride
9	25% PEG 400	0.1M Sodium Hepes	7.5	0.2M Sodium Chloride
10	25% PEG 400	0.1M Sodium Hepes	7.5	0.2M Ammonium sulfate
11	25% PEG 400	0.1M Sodium Hepes	7.5	0.2M Sodium Acetate
12	25% PEG 400	0.1M Sodium Hepes	7.5	0.2M Sodium Citrate
13	30% PEG 400	0.1M Sodium Hepes	7.5	0.2M Mg Chloride
14	30% PEG 400	0.1M Sodium Hepes	7.5	0.2M Calcium Chloride
15	30% PEG 400	0.1M Sodium Hepes	7.5	0.2M Sodium Chloride
16	30% PEG 400	0.1M Sodium Hepes	7.5	0.2M Ammonium sulfate
17	30% PEG 400	0.1M Sodium Hepes	7.5	0.2M Sodium Acetate
18	30% PEG 400	0.1M Sodium Hepes	7.5	0.2M Sodium Citrate
19	35% PEG 400	0.1M Sodium Hepes	7.5	0.2M Mg Chloride
20	35% PEG 400	0.1M Sodium Hepes	7.5	0.2M Calcium Chloride
21	35% PEG 400	0.1M Sodium Hepes	7.5	0.2M Sodium Chloride
22	35% PEG 400	0.1M Sodium Hepes	7.5	0.2M Ammonium sulfate
23	35% PEG 400	0.1M Sodium Hepes	7.5	0.2M Sodium Acetate
24	35% PEG 400	0.1M Sodium Hepes	7.5	0.2M Sodium Citrate

Appendix-6: Conditions of Screen 6 (PEG 400 screen III)

Run	Precipitant	Buffer	(pH)	Salt
1	20% PEG 400	0.1M Sodium Hepes	7.5	0.2 M Mg Chloride
2	20% PEG 400	0.1M Sodium Hepes	7.5	0.18 M Mg Chloride
3	20% PEG 400	0.1M Sodium Hepes	7.5	0.16 M Mg Chloride
4	20% PEG 400	0.1M Sodium Hepes	7.5	0.14 M Mg Chloride
5	20% PEG 400	0.1M Sodium Hepes	7.5	0.12 M Mg Chloride
6	20% PEG 400	0.1M Sodium Hepes	7.5	0.10 M Mg Chloride
7	25% PEG 400	0.1M Sodium Hepes	7.5	0.2 M Mg Chloride
8	25% PEG 400	0.1M Sodium Hepes	7.5	0.18 M Mg Chloride
9	25% PEG 400	0.1M Sodium Hepes	7.5	0.16 M Mg Chloride
10	25% PEG 400	0.1M Sodium Hepes	7.5	0.14 M Mg Chloride
11	25% PEG 400	0.1M Sodium Hepes	7.5	0.12 M Mg Chloride
12	25% PEG 400	0.1M Sodium Hepes	7.5	0.10 M Mg Chloride
13	30% PEG 400	0.1M Sodium Hepes	7.5	0.2 M Mg Chloride
14	30% PEG 400	0.1M Sodium Hepes	7.5	0.18 M Mg Chloride
15	30% PEG 400	0.1M Sodium Hepes	7.5	0.16 M Mg Chloride
16	30% PEG 400	0.1M Sodium Hepes	7.5	0.14 M Mg Chloride
17	30% PEG 400	0.1M Sodium Hepes	7.5	0.12 M Mg Chloride
18	30% PEG 400	0.1M Sodium Hepes	7.5	0.10 M Mg Chloride
19	35% PEG 400	0.1M Sodium Hepes	7.5	0.2 M Mg Chloride
20	35% PEG 400	0.1M Sodium Hepes	7.5	0.18 M Mg Chloride
21	35% PEG 400	0.1M Sodium Hepes	7.5	0.16 M Mg Chloride
22	35% PEG 400	0.1M Sodium Hepes	7.5	0.14 M Mg Chloride
23	35% PEG 400	0.1M Sodium Hepes	7.5	0.12 M Mg Chloride
24	35% PEG 400	0.1M Sodium Hepes	7.5	0.10 M Mg Chloride

Appendix-7: Conditions of Screen 7 (MPD screen)

Run	Precipitant	Buffer	(pH)	Salt
1	30% MPD	0.1M Sodium Cacodylate	6.5	0.21 M Mg Acetate
2	30% MPD	0.1M Sodium Cacodylate	6.5	0.25 M Mg Acetate
3	30% MPD	0.1M Sodium Cacodylate	6.5	0.3 M Mg Acetate
4	30% MPD	0.2 M MES/KOH	6.0	0.2M Mg Acetate
5	30% MPD	0.2 M Bis-Tris Propane HCl	6.7	0.2M Mg Acetate
6	30% MPD	0.2M HEPES/KOH	7.0	0.2M Mg Acetate
7	33% MPD	0.1M Sodium Cacodylate	6.5	0.21 M Mg Acetate
8	33% MPD	0.1M Sodium Cacodylate	6.5	0.25 M Mg Acetate
9	33% MPD	0.1M Sodium Cacodylate	6.5	0.3 M Mg Acetate
10	33% MPD	0.2 M MES/KOH	6.0	0.2M Mg Acetate
11	33% MPD	0.2 M Bis-Tris Propane HCl	6.7	0.2M Mg Acetate
12	33% MPD	0.2M HEPES/KOH	7.0	0.2M Mg Acetate
13	36% MPD	0.1M Sodium Cacodylate	6.5	0.21 M Mg Acetate
14	36% MPD	0.1M Sodium Cacodylate	6.5	0.25 M Mg Acetate
15	36% MPD	0.1M Sodium Cacodylate	6.5	0.3 M Mg Acetate
16	36% MPD	0.2 M MES/KOH	6.0	0.2M Mg Acetate
17	36% MPD	0.2 M Bis-Tris Propane HCl	6.7	0.2M Mg Acetate
18	36% MPD	0.2M HEPES/KOH	7.0	0.2M Mg Acetate
19	40% MPD	0.1M Sodium Cacodylate	6.5	0.21 M Mg Acetate
20	40% MPD	0.1M Sodium Cacodylate	6.5	0.25 M Mg Acetate
21	40% MPD	0.1M Sodium Cacodylate	6.5	0.3 M Mg Acetate
22	40% MPD	0.2 M MES/KOH	6.0	0.2M Mg Acetate
23	40% MPD	0.2 M Bis-Tris Propane HCl	6.7	0.2M Mg Acetate
24	40% MPD	0.2M HEPES/KOH	7.0	0.2M Mg Acetate

Appendix-8: Conditions of Screen 8 (Ammonium sulfate screen I)

Run	Precipitant	Buffer	(pH)	Salt
1	1.2M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2MK/Na Tartrate
2	1.2M Ammonium Sulfate	0.2 M MES/KOH	6.5	0.2MK/Na Tartrate
3	1.2M Ammonium Sulfate	0.2 M Bis-Tris Propane HCl	7.0	0.2MK/Na Tartrate
4	1.2M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.15MK/Na Tartrate
5	1.2M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.18MK/Na Tartrate
6	1.2M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.25MK/Na Tartrate
7	1.6M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2MK/Na Tartrate
8	1.6M Ammonium Sulfate	0.2 M MES/KOH	6.5	0.2MK/Na Tartrate
9	1.6M Ammonium Sulfate	0.2 M Bis-Tris Propane HCl	7.0	0.2MK/Na Tartrate
10	1.6M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.15MK/Na Tartrate
11	1.6M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.18MK/Na Tartrate
12	1.6M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.25MK/Na Tartrate
13	1.8M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2MK/Na Tartrate
14	1.8M Ammonium Sulfate	0.2 M MES/KOH	6.5	0.2MK/Na Tartrate
15	1.8M Ammonium Sulfate	0.2 M Bis-Tris Propane HCl	7.0	0.2MK/Na Tartrate
16	1.8M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.15MK/Na Tartrate
17	1.8M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.18MK/Na Tartrate
18	1.8M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.25MK/Na Tartrate
19	2.0M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2MK/Na Tartrate
20	2.0M Ammonium Sulfate	0.2 M MES/KOH	6.5	0.2MK/Na Tartrate
21	2.0M Ammonium Sulfate	0.2 M Bis-Tris Propane HCl	7.0	0.2MK/Na Tartrate
22	2.0M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.15MK/Na Tartrate
23	2.0M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.18MK/Na Tartrate
24	2.0M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.25MK/Na Tartrate

Appendix-9: Conditions of Screen 9 (Ammonium sulfate screen II)

Run	Precipitant	Buffer	(pH)	Salt	Size
1	1.6M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M K/Na Tartrate	1µ L+1µL
2	1.6M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M K/Na phosphate	1µ L+1µL
3	1.6M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M $MgCl_2$	1µ L+1µL
4	1.6M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M NaCl	1µ L+1µL
5	1.6M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M $CaCl_2$	1µ L+1µL
6	1.6M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M K/Na Tartrate	1µ L+1µL
7	1.8M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M K/Na Tartrate	1µ L+1µL
8	1.8M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M K/Na phosphate	1µ L+1µL
9	1.8M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M $MgCl_2$	1µ L+1µL
10	1.8M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M NaCl	1µ L+1µL
11	1.8M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M $CaCl_2$	1µ L+1µL
12	1.8M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M K/Na Tartrate	1µ L+1µL
13	2.0M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M K/Na Tartrate	1µ L+1µL
14	2.0M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M K/Na phosphate	1µ L+1µL
15	2.0M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M $MgCl_2$	1µ L+1µL
16	2.0M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M NaCl	1µ L+1µL
17	2.0M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M $CaCl_2$	1µ L+1µL
18	2.0M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M K/Na Tartrate	2µ L+2µL
19	2.2M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M K/Na Tartrate	1µ L+1µL
20	2.2M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M K/Na phosphate	1µ L+1µL
21	2.2M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M $MgCl_2$	1µ L+1µL
22	2.2M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M NaCl	1µ L+1µL
23	2.2M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M $CaCl_2$	1µ L+1µL
24	2.2M Ammonium Sulfate	0.1M Sodium Citrate	5.6	0.2M K/Na Tartrate	5µ L+5µL

APPENDIX 10: TRIBUTYRIN ESTERASE GENE SEQUENCE

(Sequence for *Lactococcus lactis* tributyrin esterase read in from disk from Ross Holland)

```
AAGTTAGATAAATGAAGGAAAAAGTGAGCAAAAGACATCTATAAAATGTTACAATAAAGA
---------+---------+---------+---------+---------+---------+
1                                                          60

AGACGTTGAAGGCCTGTAGAAACGTAAAAATACTACCATTATTTGATTTCTAATGTACCC
---------+---------+---------+---------+---------+---------+
61                                                         120

CAAAAATAAAATGGAGTAATGACTTCACAGCGCACACTTTGAAAAAAAGTGAAAGAAGGT
---------+---------+---------+---------+---------+---------+
121                                                        180

CAGAATAAAAGAGAAAGGAAATTTCAAAATTAAGGGATTGAAGAAAATCCACTTTTAATA
---------+---------+---------+---------+---------+---------+
181                                                        240

GGTAGAGAGTTGTCAACAAATGATAACAACATAAATTTAATTAAGGAGATATTTTATGGC
---------+---------+---------+---------+---------+---------+
241                                                        300

AGTAATTAATATCGAATACTATTCAGAAGTTCTTGGGATGAATCGAAAAGTTAATGTTAT
---------+---------+---------+---------+---------+---------+
301                                                        360

TTATCCTGAATCAAGTAAAGTGGAAGATTTTACCCAAACAGATATTCCGGTGCTTTATCT
---------+---------+---------+---------+---------+---------+
361                                                        420

TTTGCATGGAATGAGCGGAAACGAGAATTCTTGGATAATTCGTTCAGGAATTGAACGATT
---------+---------+---------+---------+---------+---------+
421                                                        480

GATTCGGCATACTAATTTAGCAATTGTCATGCCCTCAACGGATCTTGGTTTTTATGTGAA
---------+---------+---------+---------+---------+---------+
481                                                        540

CACCACATATGGTATGAATTATTTTGATGCGATTGCTCATGAACTTCCCAAAGTAATTAA
---------+---------+---------+---------+---------+---------+
541                                                        600

CAATTTTTTCCCAAATCTATCCACAAAAAGAGAAAAAAACTTTATCGCTGGTTTATCAAT
---------+---------+---------+---------+---------+---------+
601                                                        660

GGGAGGATATGGAGCTTATCGTCTCGCATTAGGAACTGACTATTTTAGTTATGCGGCTAG
---------+---------+---------+---------+---------+---------+
661                                                        720
```

```
TTTGTCTGGCGTTTTGACTTTTGATGGAATGGAAGAAAATTTCAAAGAAAATCCAGCTTA
---------+---------+---------+---------+---------+---------+
721                                                       780

CTGGGGAGGAATTTTTGGAAATTGGGAAACTTTTAAAGGGTCAGATAATGAAATTTTATC
---------+---------+---------+---------+---------+---------+
781                                                       840

TTTGGCAGACAGAAAACAAGAAAACAAACCCAAACTTTATGCTTGGTGTGGGAAACAAGA
---------+---------+---------+---------+---------+---------+
841                                                       900

TTTTCTTTTTCCAGGAAATGAATACGCGACAGCCGAGTTGAAAAAATTAGGTTTCGCATA
---------+---------+---------+---------+---------+---------+
901                                                       960

TTACTTATGAAAGCTCAGACGGCGTTCATGAATGGTATTATTGGACCCAAAAAATCGAAT
---------+---------+---------+---------+---------+---------+
961                                                      1020

CTGTATTAAAATGGCCCCCAATAAATTATAAACAAGAAGAACGCTGAGCTAATCTAAAAA
---------+---------+---------+---------+---------+---------+
1021                                                     1080

AATTCTCTGTTTATTCACTACAAAATTAAAAATTTGTCTATCAAACGCTTACAAAGACAA
---------+---------+---------+---------+---------+---------+
1081                                                     1140
```