The Ester Hydrolytic and Synthetic Activities of X-Prolyl Dipeptidyl Peptidase from *Streptococcus thermophilus*

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Abstract

X-prolyl dipeptidyl peptidase (EC 3.4.14.11), or PepX, is a dipeptidase found in most dairy lactic acid bacteria that hydrolyses N-terminal dipeptides from larger peptides where proline is the residue penultimate to the scissile bond. It has recently been found that PepX will also catalyse the hydrolysis of some chromogenic esters and synthesise esters via an acyltransferase mechanism that uses ethanol as the acceptor molecule and tributyrin as the donor molecule.

In this study, the *pepX* gene from *Streptococcus thermophilus* strain B2513 was cloned and sequenced. This sequence was found to differ in several positions from the recently published *pepX* sequence of *S. thermophilus* strain ACA-DC4. None of the observed substitutions occurred in the catalytic domain of the enzyme, all being localised to the C-terminal ß-sheet domain.

An activity assay using a chromogenic peptide substrate with tributyrin as an was used to prove that PepX binds peptide substrates and acylglycerides at the same binding site, implying that the same catalytic machinery carries out both peptide hydrolysis and activities involving acylglycerides.

PepX was found to form esters only from the acylglyceride tributyrin, and was not active on any of the larger triglycerides tested. The chemical mechanism for this ester formation is proposed to involve the direct transfer of an acyl group from the donor to an acceptor, rather than acyl hydrolysis followed by the separate transfer of a carboxylic acid product onto an acceptor, as the enzyme does not form esters when provided with butyric acid and ethanol.

PepX was found to be incapable of hydrolysing milkfat and tributyrin in aqueous solution. This contrasts with the ability of PepX to hydrolyse the synthetic ester *p*-nitrophenyl butyrate, which probably is a reflection of the lability of the ester bond in this substrate.

The results of this study show that PepX is a peptidase that has a secondary acyltransferase activity, with no hydrolase activity on natural acylglyceride substrates.

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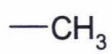
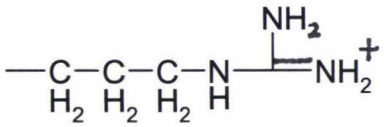
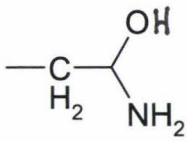
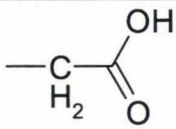
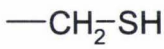
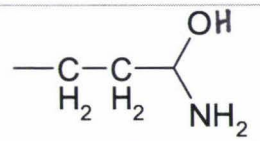
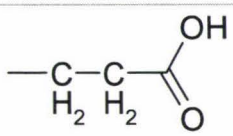
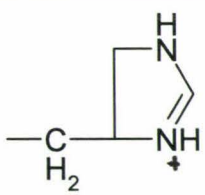
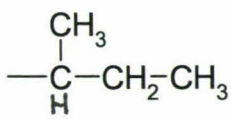
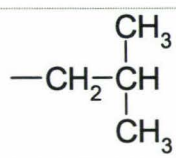
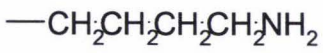
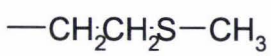
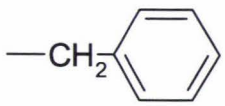
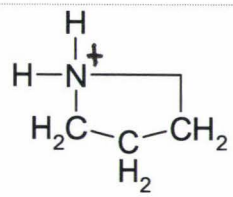
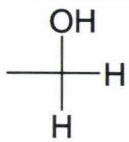
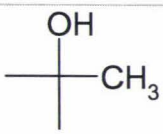
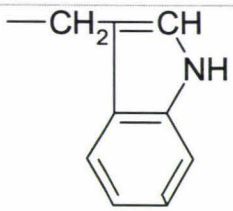
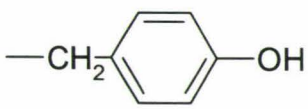
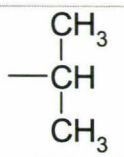
Abbreviations

Amp	Ampicillin
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
BTP	1, 3-Bis[Tris (hydroxymethyl) methylamino] propane
°C	Degrees Centigrade
CAPS	(3–[cyclohexylamino]–1–propanesulfonic acid)
CMC	Critical micelle concentration
ddH_20	Double distilled water
DEE	Diethyl ether
DIG	Digoxigenin
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNTP	Deoxyribose nucleotide triphosphate
ECL	Enhanced Chemiluminescence System (Amersham)
EDTA	Ethylene diamine tetraacetic acid
EtBr	Ethidium bromide
EtOH	Ethanol
FAA	Free amino acids
FFA	Free Fatty Acids
FID	Flame Ionisation Detector
FPLC	Fast Protein Liquid Chromatography
GLC	Gas Liquid Chromatography, Gas Liquid Chromatograph
HEPES	n–(2–hydroxyethyl)piperzine–n–(2–ethanesulfonic acid)
IEX	Ion Exchange Chromatography
IMAC	Immobilised Metal Affinity Chromatography
kDa	kilo Daltons
LB	Luria Broth
MCS	Multiple cloning site / polylinker (vector)
MWt	Molecular mass
NaCl	Sodium chloride

NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
NH_2	Amine
$(NH_4)_2SO_4$	Ammonium sulfate
OD	Optical Density
PCR	Polymerase Chain Reaction
pepX	X-prolyl dipeptidase gene
PepX	X prolyl dipeptidyl peptidase, X-prolyl dipeptidase protein
ppm	Parts per million
PVDF	Polyvinylidene difluorine
SDS-PAGE	Sodium Dodecyl Sulphate Polyacylamide Gel Electrophoresis
Sp.	Species
SPE	Solid Phase Extraction
SSC	Saline sodium citrate buffer
Subsp.	Subspecies
Tet	Tetracycline
TRIS	Tris (hydroxymethyl) Aminomethane
V_{max}	Maximum reaction velocity
X-Gal	5–bromo–4-chloro-3-indolyl-ß-galactoside

Amino Acid Abbreviations

Amino Acid	Sidechain	One letter code	Three letter code
Alanine		A	Ala
Arginine		R	Arg
Asparagine		N	Asn
Aspartate		D	Asp
Cysteine		C	Cys
Glutamine		Q	Gln
Glutamate		E	Glu
Glycine		G	Gly
Histidine		H	His
Isoleucine		I	Ile
Leucine		L	Leu

Lysine		K	Lys
Methionine		M	Met
Phenylalanine		F	Phe
Proline		P	Pro
Serine		S	Ser
Threonine		T	Thr
Tryptophan		W	Trp
Tyrosine		Y	Tyr
Valine		V	Val

Nucleic Acid Abbreviations

One Letter Code	Base(s) Represented
A	Adenosine
C	Cytosine
G	Guanine
T	Thymidine
U	Uridine
R	G or A
Y	T or C
K	G or T
M	A or C
S	G or C
W	A or T
B	G or T or C
D	G or A or T
H	A or C or T
V	G or C or A
N	Any

CHAPTER 1

Introduction and Literature Review

1.1 Cheese Manufacture

1.1.1 The Origins of Dairy Science

Cheese making is an art that has been practiced for centuries. It is believed that the process of fermenting milk into cheese was discovered accidentally, when milk was kept in bags made from animal stomachs. These early accidental cheese makers observed that the milk became a sludge, but was preserved from decay by a mysterious process that we now know as fermentation.

Since this time, the process of cheese production has changed from being a little understood phenomenon to a highly controlled industrial process. The factors that affect cheese production have been studied closely, and today a reasonable understanding of the processes that occur when milk becomes cheese has evolved. However, cheese manufacture is a complex process, and it is only relatively recently that the large array of complex factors that influence the subtle flavours of the final cheese product are becoming understood.

1.1.2 Cheese Manufacture

Cheese manufacture begins when so-called "starter" bacteria are added to pasteurised milk, along with rennet, which contains a protease that begins milk protein digestion. Sometimes lesser amounts of "adjunct" bacteria are also added, to produce altered flavours in the product. Both starter and adjunct species are collectively referred to as "dairy lactic acid bacteria" for their role in producing dairy products. The bacteria produce lactic acid as a by-product of the glycolytic cycle, as well as metabolising other milk compounds into different forms. Additionally most dairy products contain other species of lactic acid bacteria that are not starters or adjuncts, but inadvertently end up in the fermentation, and are referred to as Non Starter Lactic Acid Bacteria (NSLAB). The identity of all the species of the NSLAB is often unknown in any one

fermentation and NSLAB can either contribute to the product in desirable ways, or can cause flavour defects in the product that render it unfit for sale.

The lactic acid produced by these cultures causes the pH of the milk to decrease, leading to precipitation of milk proteins and expulsion of water from the solid curd. Ultimately the bacterial starter cells lyse in the cheese environment (low pH, low water activity and added salt), releasing bacterial intracellular enzymes directly into the immature curds. This results in an increase in certain enzymatic activities in the product as cheese maturation progresses, including lipolysis (Collins, 2003), and peptidolysis (Law *et al.*, 1974).

After the initial fermentation stage, cheese is stored for a period of time to mature. During this period, the enzymes released from the lysed starter bacteria slowly process milk compounds into the compounds that are responsible for the final flavour in the product. Whilst there are few intact starter bacteria remaining in a fully matured cheese product, the contribution made by released enzymes to the final product is very important.

1.1.3 Dairy Lactic Acid Bacteria

Dairy lactic acid bacteria belong to a group of species that cover a wide range of genera including *Lactococcus*, *Streptococcus* and *Lactobacillus*. They are all similar in that they produce lactic acid as a byproduct of their metabolism, and are generally harmless to humans. However, species differ in morphology and, more importantly, in their exact metabolism. They contain metabolic enzymes with a range of specificities that differs from species to species. The mix of enzymes plays an important role in producing subtle flavour components of dairy products. This means that the bacterial species added during cheese making can be varied to produce different cheese varieties.

In the current study, an enzyme from *Streptococcus thermophilus* was investigated. *S. thermophilus* is best known as a starter bacterium used in the manufacture of yoghurt, and is also used in the manufacture of European soft cheeses.

1.2 Cheese Flavour Components

1.2.1 Lactic Acid Bacteria Catabolism and Flavour

Many cheese flavour compounds are produced by the activity of enzymes from lactic acid bacteria on milk compound substrates. In particular, three major catabolic processes are implicated in cheese flavour. These are glycolysis, lipolysis and proteolysis.

The glycolytic process is an integral part of lactic acid bacteria metabolism, and results in the formation of lactic acid. Lactic acid is responsible for lowering the pH of the cheese, which prevents further bacterial growth and coagulates milk proteins to produce a solid product (curds). Glycolysis only occurs in live cells, where the cellular membrane is intact, so mainly occurs in the initial fermentation stage of cheese manufacture.

Lipolysis is the degradation of milk glycerides into fatty acid compounds, many of which have a strong flavour. Proteolysis is the breakdown of milk proteins into peptides and amino acids, both of which can also be flavour components. Lipolysis and proteolysis both occur in intact cells, but more importantly, the enzymes that perform these activities are released from the cell during lysis and continue to function, carrying out the flavour development processes that occur during cheese maturation. It has been noted that even enzymes that are weakly active in producing flavour compounds might have a significant impact on flavours over long maturation periods (Reiter *et al.*, 1966).

1.2.2 Control of Cheese Flavour

These flavour development processes have been well investigated, as it has been recognised that the study of the enzymes that are responsible for these flavour compounds leads to a better understanding and control of dairy product manufacture. For example, if it is understood how lipolysis and proteolysis work to produce flavours, it should be possible to control cheese flavour, and therefore develop new varieties of cheese.

Another potential advantage is the acceleration of the cheese ripening process, which in hard cheeses such as Cheddar, can take over nine months. Some investigations into

different ways of accelerating the cheese ripening process have already been undertaken, including the addition of exogenous enzymes to alter peptide and amino acid levels (Law and Wigmore, 1983). Cheese maturation is, however, a complex process to control, bearing in mind that proper cheese maturation is a process involving the interactions of hundreds of milk and microbially derived compounds, and tens, if not hundreds of enzymes. To date no attempt to produce a fast-matured cheese has been entirely successful in producing a product indistinguishable from a traditionally matured one.

1.3 Esterases as Dairy Flavour Factors

1.3.1 Ester Compounds

One group of cheese flavour compounds studied in recent times is esters. This is essentially a branch of the study of lipolysis, as lipids contain an ester bond. The compounds described as esters however, are generally esters of short chain acids and alcohols and are more water-soluble than lipids. Esterases, named for their ability to hydrolyse ester bonds, are enzymes found in a wide range of organisms.

In dairy lactic acid bacteria, ester bond hydrolase activities seem to be generally limited to the degradation of dairy-derived triacylglycerides containing short chain fatty acids to produce free fatty acids and glycerol, as these bacterial species apparently lack lipase enzymes. The esterases from lactic acid bacteria involved in cheese production are of particular interest to the dairy industry, as they control the quantity of ester based compounds present in the final product. Esters typically have a potent fruity flavour and aroma, which can be either desirable or undesirable depending on the product in question. It is therefore obvious that the type and relative amount of different lactic acid bacterial species that are present in the product during manufacture can influence the balance of these flavours. In addition, the cleavage of ester bonds can liberate free fatty acids, which can also have potent flavour and aroma. The contribution of milk lipoprotein lipase, which also releases fatty acids from acylglycerides, is regarded as important in raw milk cheeses, but not as critical in pasteurised milk cheeses where the enzyme is destroyed by heat treatment (Fox and Wallace, 1997).

1.3.2 Esterases and Lipases

In chemical terms lipases catalyse exactly the same type of chemical reaction as esterases, the hydrolysis of an ester bond (Figure 1.1) although they act on different substrates. Lipases are defined as acting on lipids that are water insoluble, whilst esterases act on generally shorter lipid substrates that are water soluble to a limited extent (Tsujita *et al.*, 1990). Most lipases and esterases can also be distinguished on the basis of whether they exhibit interfacial activation, a phenomenon observed mainly in lipases. Interfacial activation allows the lipase to act upon a dispersed insoluble substrate. The enzyme diffuses through solution until a lipid micelle is encountered. A hydrophobic loop on the surface of the enzyme associates and effectively dissolves into the lipid, opening the substrate binding site of the enzyme and allowing substrate molecules to enter. This means lipase activity actually increases when the critical micelle concentration (CMC) of a substrate is exceeded, unlike esterases that reach a maximum in activity either before or at solubility maxima. These differences in activity are easily observed by kinetic studies, since esterases exhibit the Michaelis-Menten kinetics of a fully soluble substrate, whilst lipases have a different concentration-dependant activity curve. Hypothetical plots of these different activities are shown in Figure 7.3.

Although interfacial activation can normally be used to distinguish esterases from lipases, Tsujita, *et al.* (1990) note that there are exceptions to these generalisations, such as rat hepatic triglyceride lipase that does not show any increase in activity when provided with a methyl butyrate substrate at a concentration exceeding solubility. These authors therefore define lipases as enzymes that act on long acyl group, water-insoluble ester substrates, and esterases as enzymes that act on short acyl, water-soluble ester substrates.

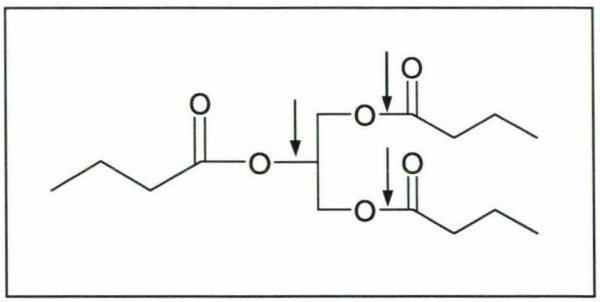


Figure 1.1 *Tributyrin, a triacylglyceride made up of three butyl groups ester bonded to a glycerol backbone. The three ester bonds of tributyrin are indicated by the arrows.*

1.3.3 Tributyrin Esterases

Tributyrin esterases are the most intensively studied esterases in the dairy industry as they are implicated in developing cheese flavours through the hydrolysis of butyl esters into potent butyl-based flavour compounds, such as butyric acid. This 4-carbon carboxylic acid has a characteristic odour described variably as cheese-like or rancid, depending on concentration. This changeability of the perception of dairy product odour and flavour according to concentration means it is of great importance to understand and control how esterase enzymes regulate the levels of fatty acid compounds in dairy products. Whilst tributyrin is not found in milk, 3.9% of the acyl groups found in milkfat (by weight) are butyl groups (MacGibbon, 1989) and these are likely to be a substrate for these enzymes.

In Cheddar cheese, butyric acid can represent 27% of the free fatty acids present after maturation, indicating the importance of this class of esterase (Forss and Patton, 1966). Butyric acid is also detectable at very low levels, and the threshold of detection has been found to alter depending in the pH of the product. At a pH value of 4.5, as found in most cheese products, butyric acid can be detected by even untrained sensory judges at just 1.9 parts per million (Baldwin and Cloninger, 1973). It is important that not too much butyric acid is present in a cheese product, as this will lead to a rancid type odour and flavour, similar to that observed in aged butter.

1.3.4 Esterases as Synthetic Enzymes

Besides having a role in the release of fatty acids from acylglycerides, the tributyrin esterases of *Lactococcus* species have the capacity to synthesise esters from an alcohol acceptor, and an acylglyceride. Alcohol esters generally have an odour described as "fruity", and ethyl butanoate, made from ethanol and tributyrin, has a characteristic pineapple-like odour. Ethyl butanoate is an extremely potent odour compound, and can be detected by untrained human subjects at a concentration of only 0.015 ppm in aqueous solution (Siek *et al.*, 1969). In some types of cheese, for example Cheddar, the presence of an ethyl butanoate derived "fruity" flavour is regarded as undesirable (Bills *et al.*, 1965). Different lactic acid bacteria starter species can lead to different levels of this type of flavour, for example *Lactococcus lactis* subsp. *lactis* is known to produce higher amounts of ethyl butanoate in cheese, compared to *Lactococcus lactis* subsp. *cremoris* (Perry, 1961), although both

Lactococcus lactis subspecies carry highly similar genes for tributyrin esterase (Fernandez *et al.*, 2000).

The phenomenon of ester synthesis by esterase enzymes has been investigated both *in vitro* (Shaw, 1999), and *in situ* in a simulated cheese mix (Holland *et al.*, 2002). In both cases it was shown that EstA, or tributyrin esterase, from *Lactococcus*, was able to produce alkyl ester compounds from alcohol and acylglycerides that were either provided *in vitro*, or were present in the cheese fermentation.

It has been reported more recently that EstA is responsible for the bulk of the ester synthetic activity in whole cell lysates of *Lactococcus lactis* (Nardi *et al.*, 2002), suggesting that this may be an important process to the cell in metabolic terms.

The synthetic activity of tributyrin esterase enzymes could potentially be utilised to control the level of certain esters in dairy products. Although, as mentioned above, whilst an intense "fruity" flavour is regarded as a defect in some types of cheese, limited ester formation by esterase enzymes may lead to the development of new cheese varieties, with desirable flavours, essential in the competitive markets of today.

1.3.5 Esterases in Different Species

Tributyrin esterases have been identified in the *Lactococcus lactis* cheese starter species (Holland and Coolbear, 1996), as well as in the cheese flavour associated adjunct species *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus casei* and *Streptococcus macedonii* (Gobbetti *et al.*, 1997a; Gobbetti *et al.*, 1997b; Castillo *et al.*, 1999; Georgalaki *et al.*, 2000). Additionally tributyrin esterase enzymes have been isolated from the cheese surface fungi *Debaryomyces hansenii* (Besancon *et al.*, 1995).

Esterases similar to *Lactococcus* tributyrin esterase have also been identified in other bacterial species, both biochemically, and by database searches on the basis of sequence homology with known tributyrin esterases. The monomeric molecular weight of these enzymes is variable: in *Lactococcus lactis* subspecies tributyrin esterase has a molecular mass of about 30kDa, whilst in *Lactobacillus* species the esterases identified appear to be larger, ranging from 67 to 85kDa.

The apparent difference in mass of known tributyrin esterase enzymes is large, and it is possible that the observed esterase activities in fact represent secondary activities of other enzymes. For example, an enzyme isolated from *S. thermophilus* which hydrolyses tributyrin was identified as glyceraldehyde-3-phosphate dehydrogenase (Liu *et al.*, 2001). This suggests that some esterases from lactic acid bacteria may, in time, be identified as enzymes that have other already characterized functions in the cell. Some of the tributyrin esterases that have been identified experimentally are summarised in Table 1.1.

1.3.6 Microesterases

It is interesting to note that two extracellular tributyrin esterase enzymes have been isolated from the fungal species *Emericella nidulans* and *Talaromyces emersonii* that have very low molecular mass, of 4.1kDa and 1.6kDa (Fan and Mattey, 1999).
Also a 5kDa esterase of 56 residues, that hydrolyses acylglycerides has also been isolated from *Candida lipolytica* (Mattey *et al.*, 1998). The *Candida* microesterase has a preference for butyl glycerides, and is thought to have a metal ion dependant acid catalysis mechanism, which is very different from the mechanism proposed for larger tributyrin esterases. This enzyme was found to be made up of 23% proline, which is far in excess of the average protein proline composition of 4.6% (Copeland, 1996).
Even more extreme in terms of size is the esterase isolated by Mattey, *et al.* (1998) from *Bacillus stearothermophilus* that is just 15 residues long. No sequence or structural information is available for these micro-enzymes to date, and it would certainly be interesting to elucidate how these tiny peptides can catalyse the hydrolysis of an ester substrate.
Whilst these enzymes are unlikely to resemble lactic acid bacterial tributyrin esterases in structure or mechanism, it is nevertheless interesting to know that evolution has developed different ways of carrying out the same chemical activity.

Species	Mass of enzyme monomer (kDa)	Reference
Lactococcus lactis subsp *lactis*	29	(Fernandez *et al.*, 2000)
Lactococcus lactis subsp. *cremoris*	29	(Holland and Coolbear, 1996)
Lactobacillus plantarum	85	(Gobbetti *et al.*, 1997a)
Lactobacillus fermentum	67	(Gobbetti *et al.*, 1997b)
Lactobacillus casei	28.9	(Fenster *et al.*, 2003)
	38	(Castillo *et al.*, 1999)
Debaryomyces hansenii	80	(Besancon *et al.*, 1995)
Streptococcus thermophilus	34 60	(Liu *et al.*, 2001)
Emericella nidulans *Talaromyces emersonii*	4.1 1.6	(Fan and Mattey, 1999)
Candida lipolytica *Bacillus stearothermophilus*	5 1.57	(Mattey *et al.*, 1998)

Table 1.1 *Enzymes that are known to hydrolyse either chromogenic butyl ester or tributyrin substrates.*

1.3.7 The Physiological Role of Esterase Enzymes

The actual role of esterases in bacteria has not yet been identified. They are not believed to play a major role in catabolism, as the enzyme activity is not high compared with lipases that have a metabolic role. Lactic acid bacteria in general have quite a low lipolytic activity (Meyers *et al.*, 1996), and therefore get most of their energy from catabolism of other compounds. In addition, it is not known if esterase-produced alcohol-based esters have any role in the cells of lactic acid bacteria, so the function of esterase catalysed ester formation in the cell, if it happens at all, is also unknown.

There is a possibility that some esterase enzymes may play a role in structural maintenance of the cell. This hypothesis is based on two facts. Firstly some *Lactococcus lactis* tributyrin esterase activity is found to be cell wall associated (Holland and Coolbear, 1996), although in light of the experimental work described in this thesis, it is possible that this is due to the peptidase enzyme PepX. Secondly Antigen 85C, a transferase enzyme from *Mycobacterium tuberculosis* with a 30% sequence similarity to *Lactococcus* tributyrin esterase, has a well-documented role in cell wall synthesis (Jackson *et al.*, 1999). It has also been proposed that lactic acid bacterial esterases may have a role in hydrolysing toxic metabolites as they have some sequence similarity to S-formylglutathione hydrolase, which is found in the formaldehyde detoxification pathway (Fernandez *et al.*, 2000).

1.4 Peptidases as Dairy Flavour Factors

1.4.1 Peptidases

Another class of enzymes that appear to have a significant impact on the final flavours present in dairy products are the peptidases. Unlike esterases, these enzymes play a significant role in the metabolism of lactic acid bacteria, because lactic acid bacteria obtain the nutrients necessary for cell growth from protein digestion. The uptake of peptides is therefore essential for the proliferation of these species, and so dairy lactic acid species have a diverse complement of peptidases.

These enzymes are implicated in flavour development in dairy products as they produce amino acids that either have distinct intrinsic flavour properties or that are used as precursors in the production of other flavour compounds.

Some peptides that contain high amounts of hydrophobic and acidic residues have been identified as producing flavours, mainly bitter, in cheese (Edwards and Kosikowski, 1983). Bitter peptides are in fact not the result of anabolic processes by the bacteria, but rather are produced by the degradation of proteins to bitter flavoured peptides. This has been demonstrated in work that found *Lactococcus lactis* subsp. *lactis* did not produce bitter flavours when grown in defined media with essential amino acids for growth added, but did produce these bitter components when grown in a casein based media (Harwalkar and Seitz, 1971). It is therefore most likely that it is the catabolic action of starter bacteria on milk proteins that is responsible for peptide derived bitter flavours.

Amino acids do not necessarily have a bitter flavour. For example, Swiss cheeses are characterised by a sweet flavour and have a relatively high concentration of free proline. It has been found that the addition of certain free amino acids to cheese can produce a product that ripens faster than normally, but has desirable flavour and texture properties (Wallace and Fox, 1997).

Some flavour compounds are also believed to be produced from anabolic processes that use free amino acids. The formation of flavourants and odourants from amino acids and carbonyl compounds has been demonstrated *in vitro* (Griffith and Hammond, 1989), with flavours similar to those found in Swiss-type cheeses produced. This suggests that amino acid based anabolism may also have a role in flavour production during maturation, at least in some varieties of cheese.

1.4.2 The Proteolytic System of Lactic Acid Bacteria

Lactic acid bacteria have an extensive proteolytic system, consisting of low specificity proteases that produce peptides for digestion by more specific peptidases. This is summarised in Figure 1.2.

Protein uptake in lactic acid bacteria begins with the secretion of an extracellular protease named lactocepin (in *Lactococcus*) or PrtS (in *Streptococcus*), which begins the digestion of proteinaceous material (Reid and Coolbear, 1998). This enzyme is generally non-specific in the sites it targets for cleavage, and degrades proteins into shorter peptides, up to 18 amino acid residues long (Detmers *et al.*, 1998).

After cleavage, peptides are transported into the cell using the oligopeptide transport system Opp. The Opp system consists of five proteins, OppB and OppC, which are membrane integral proteins, OppD and OppF, which are ATP binding proteins, and OppA, which binds to peptide substrates prior to uptake (Tynkkynen *et al.*, 1993). The Opp genes are arranged in an operon system that also includes PepO, an intracellular endopeptidase.

The peptide degradation and uptake system of *Lactococcus lactis* has been fairly well characterized (Tynkkynen *et al.*, 1993; Detmers *et al.*, 1998), and the more recently the extracellular protease of *Streptococcus thermophilus* has also been studied to a lesser extent (Fernandez-Espla *et al.*, 2000). Removal of the peptide uptake system by mutagenesis prevents lactic acid bacterial growth in milk, although the bacteria can still grow in media if it is supplemented with appropriate peptides shorter than 3 residues, as these are able to enter the cell via other means.

1.4.3 Intracellular Peptidases

Complementing the action of the extracellular protease, and completing peptide digestion is a set of intracellular peptidase enzymes, with substrate specificities that range from being quite general to highly specific. Some of these peptidases are endopeptidases, cleaving peptides at internal positions and some are exopeptidases that remove a single residue from the terminus of a peptide. In terms of cellular metabolism, these enzymes are thought to stay localised within the cytoplasm. They have however, an important role in dairy product production. As the lactic acid by-product of fermentation accumulates in the product the pH decreases and the bacterial cells lyse, releasing their intracellular peptidases into the product, where there is a rich

abundance of peptide substrates. As discussed above, proteolysis can have an impact on the flavours of some cheese varieties, and this is accelerated by the release of these enzymes during cellular lysis (Crow *et al.*, 1995).

1.4.4 PepO Aminopeptidase

The PepO endopeptidase is an example of an intracellular peptidase with general specificity. This 70kDa enzyme can hydrolyse peptides larger than 5 residues, and has a poorly defined substrate preference. As mentioned above, the gene that codes for PepO is located in the Opp peptide uptake operon, so it is likely that PepO provides a final general peptidolysis step to produce peptides that can then be more specifically digested by the other peptidases in the cell. Interestingly, cells that have been genetically manipulated to disrupt the PepO gene show a decrease in growth rate of less than 10% when compared with wild-type cells (Mierau *et al.*, 1996). This implies that there are other enzymes that can partially fulfil the role of PepO in the bacterial cell. Nevertheless, PepO is thought to have an important role in initial partial hydrolysis of peptides. This is especially important as many of the more specific peptidases in lactic acid bacteria act on terminal residues only. When PepO cleaves a peptide substrate in internal positions, it produces a number of new peptides which then act as substrates for exopeptidases.

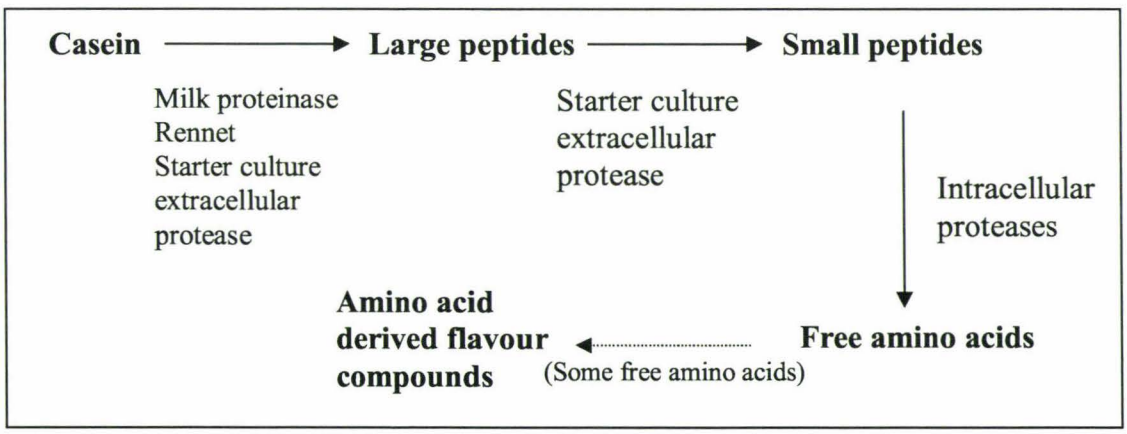


Figure 1.2 *Protein uptake and processing by dairy lactic acid bacteria. Figure based on Figure 2 (Sousa et al., 2001)*

1.4.5 General Aminopeptidases

Also included in the more general peptidases is a group of enzymes called aminopeptidases, which remove single residues from the N-terminal of peptides. These include PepN and PepC, which remove single residues with very little specificity for the type of residue involved. These peptidases are implicated in flavour development in dairy fermentations, as they release single amino acids, which, as mentioned above, are then available for the synthesis of amino acid based flavour molecules. The aminopeptidases have overlapping, but not identical, substrate specificities meaning that for effective peptide hydrolysis a full complement of peptidases is required. It should be noted, however, that cells are still capable of growth when several aminopeptidases are genetically deleted, so individually they cannot be considered essential, unlike some members of the PrtP and Opp peptide uptake system. Collectively however, deletion of up to five aminopeptidases (PepX, PepT, PepO, PepC and PepN) from *Lactococcus lactis* impaired cell growth in milk to 10% of normal rates (Mierau *et al.*, 1996). This demonstrates the importance of a reasonable complement of these enzymes for cell viability in dairy lactic acid bacteria.

1.5 X-prolyl Dipeptidyl Aminopeptidase

1.5.1 X-prolyl Dipeptidyl Peptidase Specificity

X-prolyl dipeptidyl peptidase, referred to as PepX, exhibits one of the most specific activities of all the lactic acid bacteria peptidases known. The enzyme is highly specific in its activity, as it hydrolyses dipeptides from the N-terminus of polypeptides, with the requirement that the residue on the N-terminal side of the scissile bond must preferentially be proline. PepX therefore cleaves in the pattern H_2N-X-Pro$\downarrow(X)_n$, where the arrow indicates the cleavage site, and X is any residue, except proline.

In experiments carried out to determine the specificity of *Lactococcus lactis* PepX activity, the presence of an N-terminal leucine residue caused a decrease in activity, yet activity was increased when a phenylalanine was present at the same position (Lloyd and Pritchard, 1991). Additionally PepX will remove dipeptides from the N-

terminus of proteins when both residues are proline, which is a highly unusual activity given the rarity of consecutive N-terminal proline residues in nature (Xin *et al.*, 2002). Because there are few peptide hydrolysing enzymes that will hydrolyse a bond where the imino acid proline is present, this enzyme has been extensively biochemically characterised.

Another aminopeptidase, PepP, has a complementary substrate specificity to PepX, cleaving X↓Pro-$(X)_n$, and releasing a single residue from the N-terminus of a peptide. PepP will also cleave X↓Pro-Pro-$(X)_n$, which again is unique amongst proline specific peptidases (Matos *et al.*, 1998). However Matos, *et al.* (1998) noted that genetic deletion of PepP does not seem to affect the growth of *Lactococcus* in milk media, an additional feature that differentiates this aminopeptidase from PepX.

1.5.2 Peptide Synthesis by PepX

PepX is also able to catalyse the formation of peptide bonds between amino acids under certain conditions. This phenomenon relies on the ability of most enzymes to carry out their reverse reaction, under suitable reaction. In these experiments amino acids were provided as *p*-nitroanilide derivatives, with the dipeptide acceptor at high concentrations. Also noted in these experiments was the ability of PepX to cleave amino acid esters (Yoshpe-Besancon *et al.*, 1994). PepX has also been used to synthesise enterostatin-amide (APGPR-NH_2), which has a proline at the second and fourth positions, also using a blocked *p*-nitroanilide donor (Houbart *et al.*, 1995). It is unlikely that peptide synthesis represents a physiological activity of PepX, as the synthetic ability has only been observed under non-physiological conditions with non-physiological substrates. Nevertheless, this activity of the enzyme is potentially a useful method for synthesising peptides where proline is present at alternate positions, especially as other peptidases have difficulty operating when proline is involved in the bond to be hydrolysed or synthesised.

1.5.3 Role of PepX in Lactic Acid Bacteria

The presence of X-prolyl dipeptidase activity was noted in lactic acid bacteria in the mid-1980's (Casey and Meyer, 1985), when it was realised that certain *Streptococcus* and related *Lactococcus* species need to have a peptidase capable of hydrolysing peptide bonds in short peptides containing proline. Milk casein contains up to 30% proline, so for the organism to survive on milk proteins it has to be capable of digesting proline rich peptides.

PepX has been isolated from a number of dairy related lactic acid bacteria, indicating that it has a ubiquitous role in the metabolism of this genera (Meyer and Jordi, 1987; Kiefer-Partsch *et al.*, 1989; Lloyd and Pritchard, 1991).

Since many proteases and peptidases have difficulties hydrolysing bonds where proline is involved, it is probable that PepX assists the digestion of proline rich substrates by removing "proline blockages" from the ends of peptides to allow further peptidolysis to continue. The essential role PepX plays in the cell has been demonstrated by studies where it has been mutagenised and deactivated. These studies show that *pepX* deletion mutants, whilst still able to grow in milk media, are less viable and their growth rates are reduced by about 10% (Mierau *et al.*, 1996; Guinec *et al.*, 2000). Also specific peptides have been shown to accumulate in cells where PepX has been deleted, showing that there is no alternative aminopeptidase that can perform the precise role of PepX (Kunji *et al.*, 1996). The presence of two proline-specific exopeptidases (PepX and PepP) in lactic acid bacteria illustrates the importance of the capacity to cleave proline peptide bonds to the survival of the organism in dairy fermentations.

1.5.4 Cellular Distribution of PepX

In dairy lactic acid bacteria, the cellular location of PepX is unclear. In *Lactococcus lactis* subsp. *cremoris* some authors report that due to the lack of an export signalling sequence, as well as antibody based localisation studies, PepX is likely to be associated with the inside of the cell membrane (Tan *et al.*, 1992). However these authors also note that the observed results do not exclude the possibility that PepX may hydrolyse peptides at the extracellular face of the membrane, although they concentrated the used growth media and used this to perform a western blot to show the absence secreted PepX enzyme. It is possible that, due to a low amount of PepX

expression that there was not enough PepX to be detected, even using western blotting.

Other authors have purified a PepX enzyme activity from the cell wall of *Lactococcus lactis* subsp. *cremoris*, without carrying out cellular lysis, implying that PepX is extracellular in dairy lactic acid species (Kiefer-Partsch *et al.*, 1989). It is possible however, that some cellular lysis may have occurred releasing enzymes into the media. These two studies were also performed on different *Lactococcus lactis* subspecies, so it may be that PepX is located in different cellular compartments in these different organisms. In turn this may explain some of the differences in cheese flavour characteristics noted for these two subspecies (Bills *et al.*, 1965).

It is also possible that these two studies have independently isolated different enzymes that have the same catalytic specificity. However both enzymes were approximately the same size (~90kDa monomer) as well as being found to exist as homodimers. As there have been no other aminopeptidases identified to date with the same catalytic specificity and physical characteristics, it seems more likely that they are in fact the same enzyme.

Adding a further dimension to the mystery of exactly where PepX is located in the cell system is the finding that PepX from the pathogen *Streptococcus gordonii* is extracellularly secreted to assist digestion of host tissue proteins (Goldstein *et al.*, 2001). It is possible that PepX plays different roles in dairy lactic acid species compared with its role in pathogenic *Streptococcus* species, and the distribution of the enzyme in the cell may reflect this. Thus PepX may be either cytoplasmic or secreted in dairy lactic acid bacteria dependant on species, or possibly certain environmental conditions trigger the excretion of PepX from the cytoplasm. It should be noted that sequence analysis of the *Lactococcus lactis* genome shows that the bacteria has only one gene coding for PepX. Therefore, if there are secreted and non-secreted PepX species, a single gene would be responsible for both forms. Such control could be mediated by an internal signalling sequence, or by some sort of activation by proteolytic cleavage, although no evidence exists for either of these scenarios.

1.5.5 PepX as a Dairy Flavour Factor

Free proline itself has been found to have a sweet flavour, and in some cheese varieties, such as Swiss, it is an important component of the overall cheese flavour (Mitchell, 1981). PepX itself does not release free proline, but it does release proline containing peptides that are then available as substrates for other aminopeptidases. PepX is therefore more likely to be important as an indirect flavour factor, producing peptides that are substrates for other aminopeptidases such as the proline-specific PepP.

1.6 Structural Features of Lipases, Esterases and Proteases

1.6.1 αß Hydrolase Enzymes

Although they catalyse the hydrolysis of different types of covalent bonds, esterase and peptidases use a similar catalytic mechanism, and their catalytic domains also have a similar tertiary arrangement, known as the αß hydrolase fold. This fold is conserved across a vast range of enzymes with large differences in primary sequence, and vastly different substrate specificities (Ollis *et al.*, 1992). Included in this tertiary family is acetylcholinesterase as well as various lipases, esterases and peptidases. Many enzyme structures have been solved for this large and growing tertiary structural family (Nardini and Dijkstra, 1999), allowing similarities in catalysis to be identified. Enzymes in this family are not necessarily hydrolases, for example *Mycobacterium tuberculosis* Antigens 85B and 85C are cell wall synthesising transferase enzymes, but still possess the characteristic αß fold (Ronning *et al.*, 2000; Anderson *et al.*, 2001). These transferase enzymes function *via* a similar mechanism to αß hydrolysis, but an alternative molecule to water is utilised as an acceptor. More recently PepX from *Lactococcus lactis* has been shown to have a catalytic αß hydrolase domain (Rigolet *et al.*, 2002). Sequence comparisons of *Lactococcus lactis* tributyrin esterase to *Mycobacterium tuberculosis* Antigen 85C and *Clostridium* feruloyl transferase also suggest that this enzyme is a member of the αß hydrolase family (Ronning *et al.*, 2000; Schubot *et al.*, 2001). Additionally cocaine esterase from *Rhodococcus* has a similar domain arrangement to PepX, and although it lacks a so-called N-terminal α-helical domain, the catalytic domain still has an αß hydrolase fold (Larsen *et al.*, 2002).

1.6.2 The αß Hydrolase Fold

The αß hydrolase fold was originally characterised as an 8-strand ß-sheet core, surrounded by α helices (Ollis *et al.*, 1992). The presence of a core ß sheet is highly conserved, whilst the arrangement and length of the helices tends to be more variable. αß hydrolase enzymes can have a single domain, such as *Mycobacterium* Antigen 85C, or they can have multiple domains of which one is an αß hydrolase catalytic domain. Examples of multidomain αß hydrolase enzymes include *Lactococcus* PepX and *Rhodococcus* cocaine esterase. In these multidomain enzymes, the ß-sheet of the αß hydrolase domain can consist of more than 8 strands (Larsen *et al.*, 2002; Rigolet *et al.*, 2002). Some proteins also have fewer than 8 ß strands in the central sheet, for example a lipase enzyme from *Psuedomonas aeruginosa* has only 5 ß strands in the central sheet (Nardini *et al.*, 2000). Figure 1.3 demonstrates the overall tertiary fold of *Mycobacterium* antigen 85C, a typical αß fold enzyme.

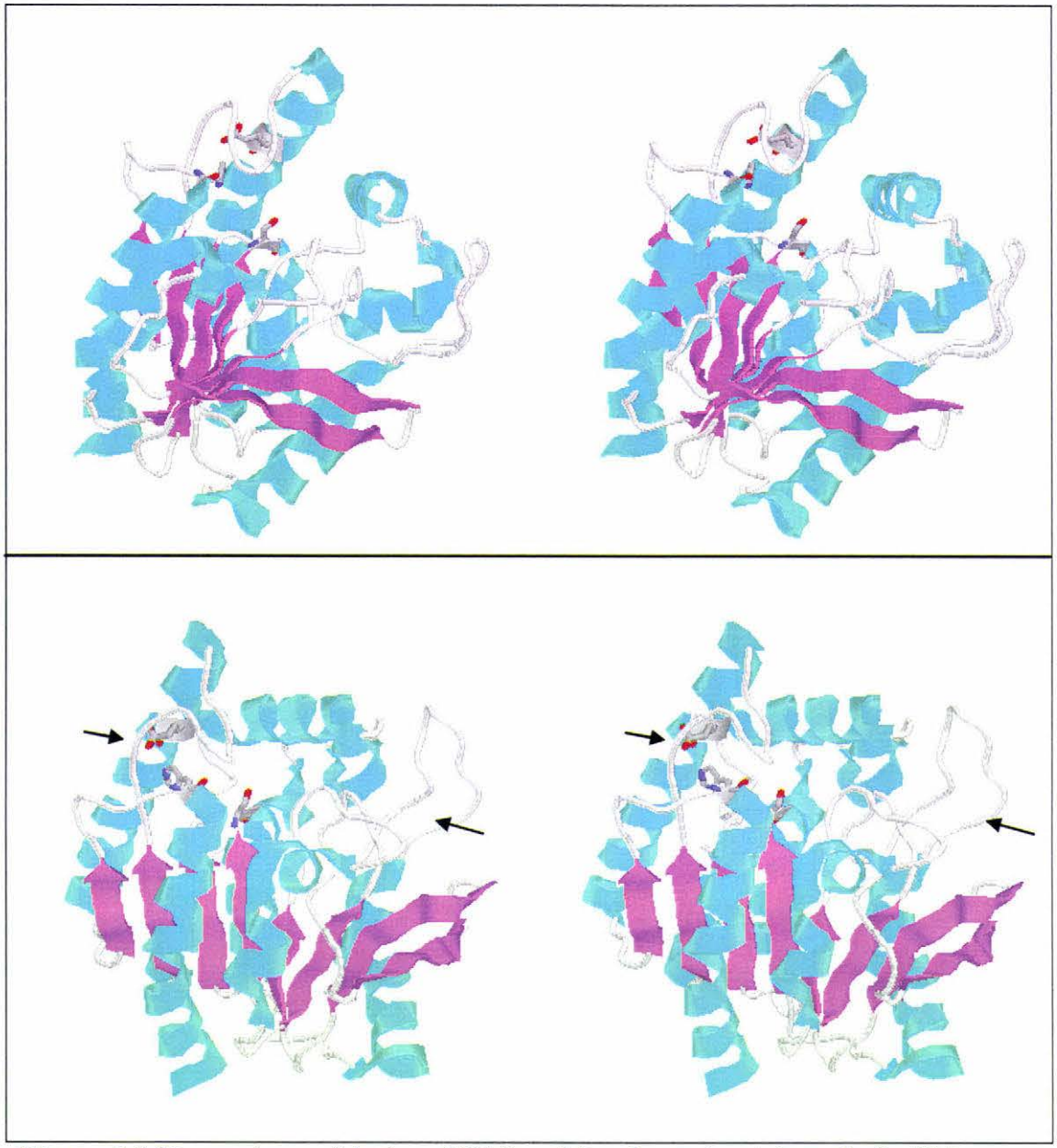


Figure 1.3 *Stereo views of Antigen 85C from* Mycobacterium tuberculosis. *This transferase enzyme exhibits a typical αß hydrolase fold. The catalytic triad residues are shown as sticks.*

Upper panel: Looking along the substrate binding cleft.

Lower panel: Molecule rotated to show the characteristic 8-strand twisted ß sheet. The substrate cleft runs from left to right across the top of the molecule as indicated by the arrows. Structural coordinates from PDB entry 1DQY (Ronning et al., 2000)

1.7 Serine Hydrolase Catalysis

1.7.1 The Esterase/Peptidase Catalytic Triad and Serine Motif

Most hydrolase enzymes, including esterases and peptidases, have a so-called "catalytic triad" of residues consisting of serine, a base (usually histidine), and an acid, usually either glutamic acid or aspartic acid (Cygler *et al.*, 1993).

Chemical modification and directed mutagenesis have confirmed the essential role played by the sidechains of all three amino acids in the active site of most lipases (DiPersio *et al.*, 1990; DiPersio *et al.*, 1991; DiPersio and Hui, 1993). The active site of esterases is thought to resemble that of lipases as they have common catalytic activities, and any differences in substrate preference are conferred by the substrate binding sites and not the catalytic mechanism. Enzymes that hydrolyse peptide bonds also use the same catalytic triad as esterases, and indeed most of the knowledge about catalytic triad catalysis has been gained from in-depth investigations of the mechanisms of proteases such as chymotrypsin (Stryer, 1995).

The residues of the catalytic triad are usually distant in primary sequence, but are located in close proximity to each other near the substrate-binding site of the enzyme in the folded protein. Sequence alignment of different lipases and esterases shows that whilst the sequences can be highly divergent over certain regions of the protein, the sequence around the catalytic residues, especially serine, tends to be more closely conserved (Cygler *et al.*, 1993). Figures 1.4 and 1.5 demonstrate the positioning of the catalytic triad residues in Antigen 85C, an ester transferase enzyme with 25% sequence similarity to *Lactococcus* tributyrin esterase (Anderson *et al.*, 2001).

Many of the bacterial alkyl esterases cloned and sequenced to date in fact have a characteristic consensus sequence that contains the catalytically active serine (Derewenda and Derewenda, 1991). This sequence consists of the pentapeptide (G-X-**S**-X-G), which in enzymes that have been structurally characterised has been shown to form a hairpin turn in the protein, with the active serine facing outward, and projecting towards the scissile bond of the substrate. This catalytic motif is preserved across a wide range of enzymes from various organisms, as can be shown from a similarity survey of the current NCBI non-redundant sequence database (Altschul *et al.*, 1997) using a bacterial esterase as a query sequence.

The functioning of these three residues is believed to facilitate a nucleophilic attack by the serine on the substrate, with an acyl-enzyme intermediate formed, similar to other serine hydrolase enzymes. This mechanism will be discussed in the following section.

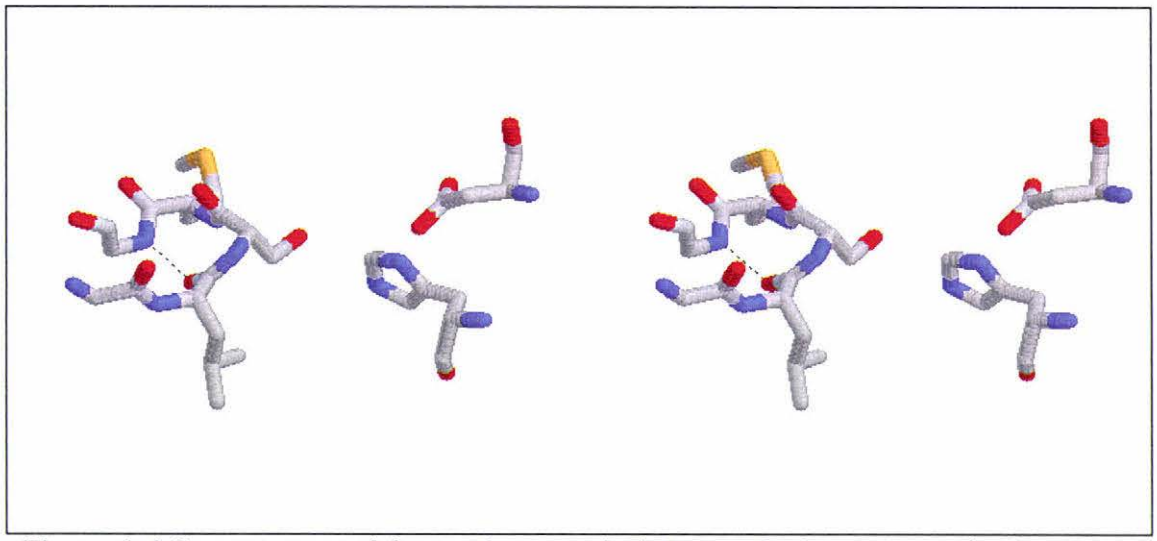


Figure 1.4 *Stereo image of the catalytic triad of Antigen 85C, a structural relative of tributyrin esterase, viewed horizontally along the substrate binding cleft.* *The serine hydrolase GXSXG active serine loop can be seen on the left, with a hydrogen bond (dotted) between the top and bottom of this loop. The active serine can be seen projecting into the centre of the picture. The catalytic histidine is at lower right, and the catalytic glutamic acid is at upper right. Other residues have been omitted for clarity. Structural coordinates from PBD entry 1DQY (Ronning et al., 2000).*

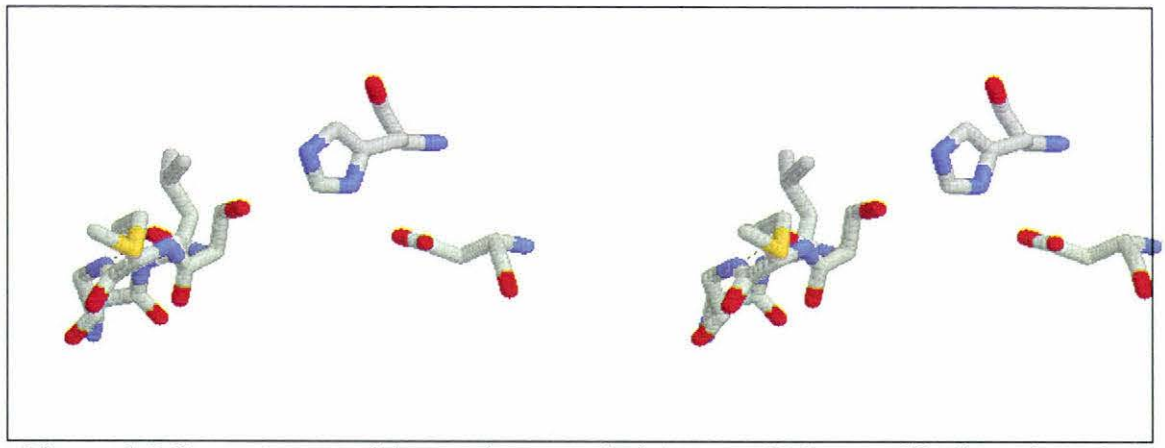


Figure 1.5 *Stereo image of the catalytic triad of Antigen 85C viewed looking directly into the substrate binding cleft.* *The catalytic serine loop is at left, with the loop oriented horizontally to the view. The catalytic histidine is top right and the glutamic acid is lower right. Other residues have been omitted for clarity. Structural coordinates from PDB entry 1DQY (Ronning et al., 2000).*

1.7.2 Proposed Mechanism of Tributyrin Esterase Hydrolytic Catalysis

Figure 1.6 depicts the proposed mechanism of catalysis for tributyrin esterase, based on the mechanism inferred from the structural analysis of acetylcholinesterase (Silverman, 2000), and chymotrypsin (Voet and Voet, 1995). It should be noted that this mechanism has no experimental basis, as no three-dimensional structure is available for this enzyme, but has been deduced from a comparison with other proposed esterase mechanisms, and from the results of kinetic studies (Shaw, 1999; Holland *et al.*, 2002).

As shown in panel 1 of Figure 1.6, the hydrolysis begins after the acylglyceride substrate binds to the enzyme. In some esterases the substrate is held in place through interactions with a charged region. An example of this type of substrate binding is seen in acetylcholinesterase, which has an anionic region near the active site that stabilises a quaternary ammonium ion of the substrate (Silverman, 2000), immobilising the substrate during hydrolysis. The substrates for acylglyceride esterases are hydrophobic, inferring that substrate binding in these enzymes may be mediated via a hydrophobic region in the substrate binding cleft.

The enzyme binds the substrate in a conformation that energetically and sterically favours a tetrahedral conformation about the scissile bond of the substrate. In the active site, the Nε of the histidine sidechain forms a hydrogen bond with the hydroxyl sidechain of the serine, polarising it so that it becomes a better nucleophile. The pK_a of the histidine side-chain is 6.0, so under physiological conditions at pH 7.0 this residue should be neutral, depending on its local environment. The histidine in turn forms a hydrogen bond with the third catalytic triad member, which is an acid, usually aspartic acid. The Asp is negatively charged at physiological pH, and has a polarising effect on the histidine that makes the transfer of a proton more energetically favourable. The OH of the serine sidechain carries out the nucleophilic attack on the scissile ester bond of the substrate and in the process loses its proton, which is accepted by the histidine residue, and a transient tetrahedral enzyme substrate complex is formed. This complex is highly unstable, but can exist in enzymes because the shape and architecture of the active site region exerts a stabilising force. This stability is partially mediated by the presence of an oxyanion hole, a region in the active site where polar protein mainchain groups are positioned to hydrogen bond with the oxyanion created in tetrahedral intermediate.

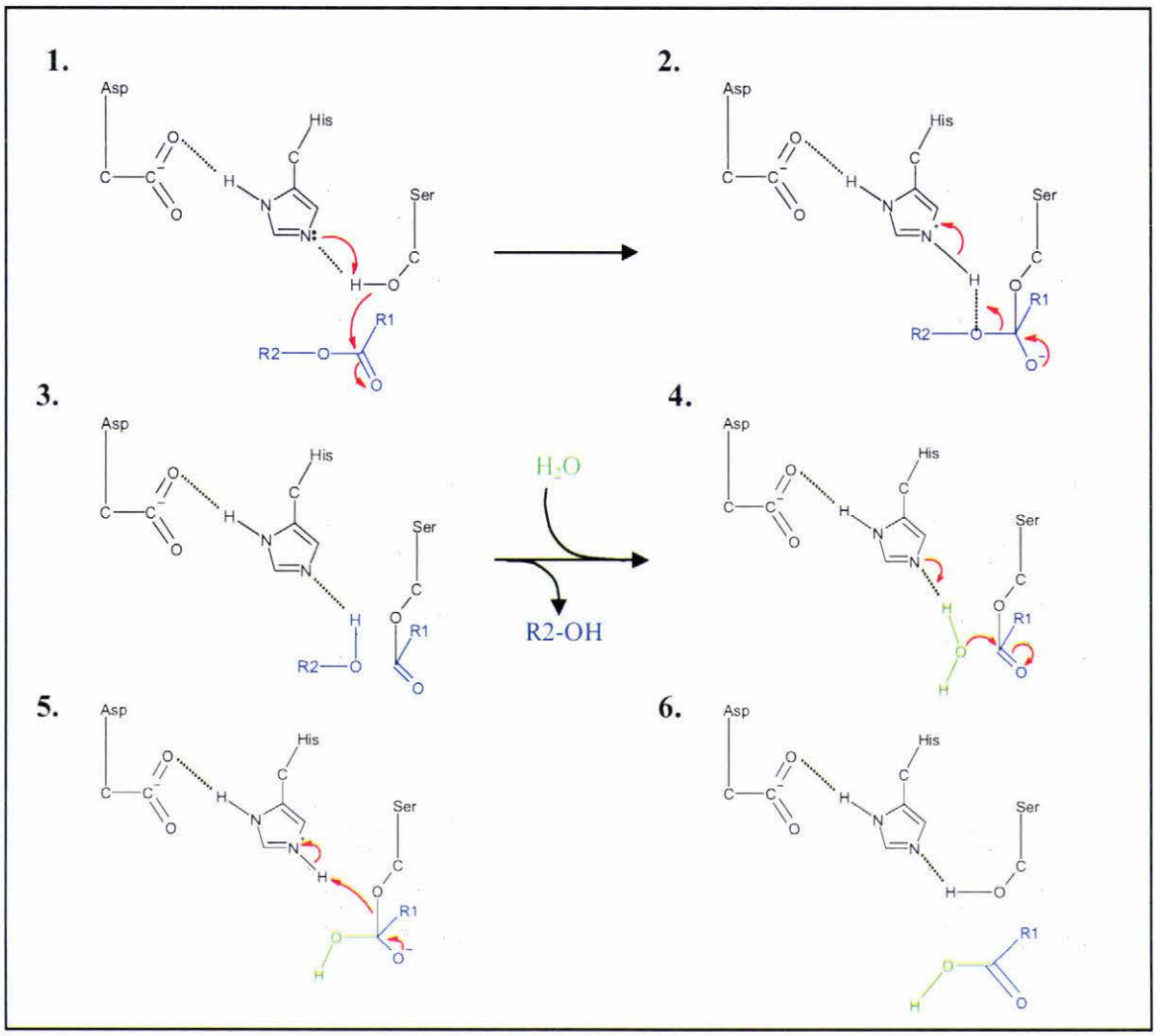


Figure 1.6 *Proposed catalytic mechanism of serine esterases. In the case of tributyrin esterase acting on tributyrin, R1 would be a propyl sidechain, and R2 would be the remainder of the tributyrin molecule.*

1) Acylation: The Michaelis complex, with substrate bound to the enzyme. The His donates electrons to the Ser and accepts the serine sidechain proton, as the Ser performs a nucleophilic attack on the carbonyl carbon.

2) The tetrahedral intermediate state, with the oxyanion transiently formed and stabilised by the oxyanion hole of the enzyme. Charge relay transfers electrons from the oxyanion back to the scissile bond thus breaking it.

3) The acyl-enzyme intermediate, with the carboxylic acid group covalently bound to the enzyme, and the alcohol ready for release.

4) Deacylation: Nucleophilic attack by a water molecule on the acyl enzyme forms a second tetrahedral transition state, with the oxyanion being again stabilised by the enzymes oxyanion hole. The His accepts a proton from the water molecule.

5) The second tetrahedral state. Charge relay to the His proton allows it to be transferred to the serine. This releases the acyl group from the enzyme, and returns the enzyme to its initial state of protonation.

6) The enzyme at rest state, with the product about to be released.

Diagrams based on Figure 14-23 (Voet and Voet, 1995)

The oxyanion is highly unstable, and quickly loses electrons by relaying them to the scissile bond. This results in the breakage of the scissile bond and the donation of a proton to the glyceride backbone, which is then released and diffuses away from the enzyme. The acyl group remains covalently bound to the enzyme's catalytic serine.
A water molecule then enters the active site of the enzyme, and is polarised by the formation of a hydrogen bond with the histidine. The water becomes a nucleophile and attacks the carbonyl carbon of the acyl group bound to the enzyme. The water loses a proton to the histidine sidechain and electrons are relayed from the carbonyl carbon to the carbonyl oxygen, forming an oxyanionic tetrahedral intermediate, which is again stabilised by the presence of the oxyanion hole in the enzyme.
In the deacylation reaction, the donation of a proton from the histidine to the serine oxygen results in the cleavage of the enzyme-substrate bond, and the carboxylic acid product is free to diffuse away from the enzyme.
Whilst no protein structure has been solved for any alkyl esterase from lactic acid bacteria, sequence analysis strongly suggests that the mechanism of these enzymes will be similar to the other esterase structures solved, and to other serine hydrolases, such as chymotrypsin.

1.7.3 Proposed Mechanism of Tributyrin Esterase Transferase Catalysis

As discussed in section 1.3.4, some tributyrin esterase enzymes have been found to be capable of synthesising esters when provided with an alcohol to act as an alternative acceptor to water. There are transferase enzymes that exhibit the same αß fold as tributyrin esterases, and these enzymes are, in all likelihood, evolutionarily derived from a similar ancestor. This close relationship between hydrolase and transferase enzymes is particularly evident from the high similarity in primary sequence (27%) of the *Mycobacterium* Antigen 85C cell wall transferase and *Lactococcus* tributyrin esterase. It is interesting to note that in the case of Antigen 85C, transferase activity begins with the substrates bound at two sites on the enzyme prior to the catalytic activity occurring (Anderson *et al.*, 2001).

Whilst the absence of structural information means no catalytic mechanism has been identified for tributyrin esterase, it is likely to proceed *via* a similar mechanism to that used by serine transferase enzymes, as summarised in Figure 1.7.

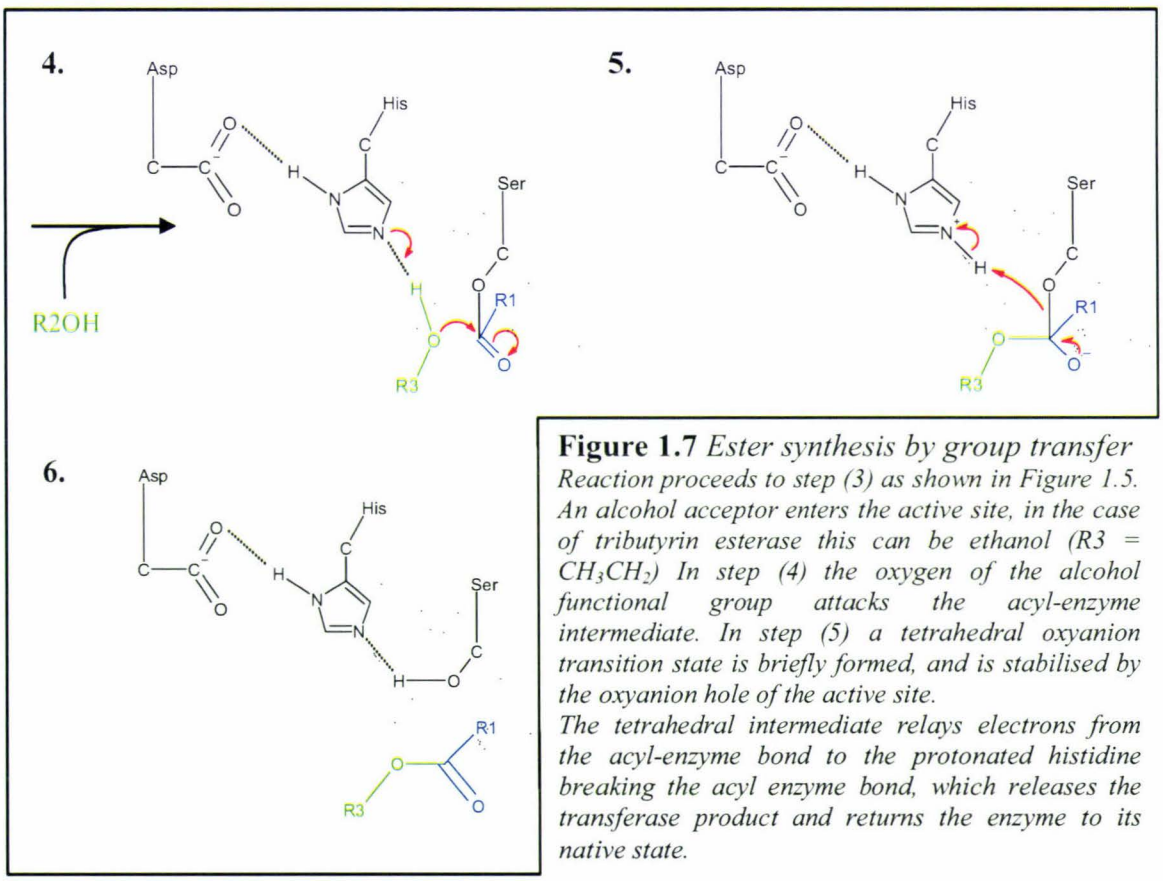


Figure 1.7 *Ester synthesis by group transfer*
Reaction proceeds to step (3) as shown in Figure 1.5. An alcohol acceptor enters the active site, in the case of tributyrin esterase this can be ethanol (R3 = CH_3CH_2) In step (4) the oxygen of the alcohol functional group attacks the acyl-enzyme intermediate. In step (5) a tetrahedral oxyanion transition state is briefly formed, and is stabilised by the oxyanion hole of the active site.
The tetrahedral intermediate relays electrons from the acyl-enzyme bond to the protonated histidine breaking the acyl enzyme bond, which releases the transferase product and returns the enzyme to its native state.

1.8 The Structure of PepX

1.8.1 General Overview

Recently the structure of PepX from *Lactococcus lactis* was solved (Rigolet *et al.*, 2002). This structure, to a resolution of 2.2Å, shows that PepX is a multidomain enzyme comprised of four distinct regions. The N-terminal domain is largely α helical, and is followed by the first half of the αß hydrolase catalytic domain. The third domain, a small linker, is mostly α helical in secondary structure, and joins the two halves of the catalytic domain. The C terminal domain of PepX is a ß sheet structure with the "jelly roll" topology. The linear arrangement of these domains is shown in Figure 1.8, and Figures 1.9 and 1.10 show a cartoon of the model derived from the crystal structure of the enzyme.

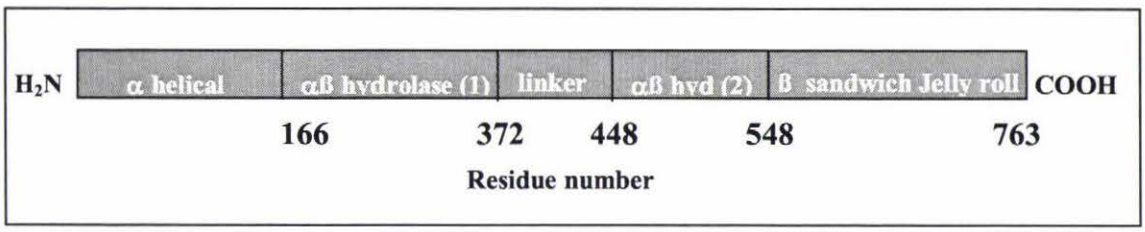


Figure 1.8 *The domain arrangement of Lactococcus lactis PepX. Domains are shown as defined by Rigolet, et al. (2002). Numbering is by amino acid residue.*

1.8.2 α Helical domain

A depression on the α helical domain participates in dimerisation of the enzyme, through interactions with a corresponding projection from the αß catalytic domain of the second enzyme molecule in the dimer. It is also postulated that the α helical domain has a role in substrate binding as dimerisation creates a cleft between the two monomers that is proposed to allow the substrate access to the active site of the enzyme. It is interesting to note that a protein with the α helical N-terminal domain genetically deleted is unable to dimerise, and will not hydrolyse a chromogenic peptide substrate that is normally a good substrate for the enzyme (M. Bennett, unpublished data). In addition, the inclusion of a GST purification tag to the N-terminus of a full-length version of PepX also results in an inactive enzyme (J. Ng, personal communication). These preliminary results support a role for the N-terminal

domain in enzyme activity, possibly by assisting in substrate binding, or indirectly by mediating dimerisation, which then allows substrate binding to other regions of the enzyme.

1.8.3 αß Hydrolase Catalytic Domain

The catalytic domain of PepX is arranged in an αß hydrolase fold, similar to many other peptidases, esterases and transferases. The ß sheet in PepX consists of 10 ß strands, rather than the 8 stands commonly seen in single domain αß hydrolases (Ollis *et al.*, 1992). The catalytic triad closely resembles the catalytic triad seen in other serine peptidases and esterases, although the sequence motif containing the active serine, GXSXXG is slightly different to the more common GXSXG. Interestingly, the αß hydrolase domain is not formed from a single continuous region of polypeptide; rather it is divided by a 76 residue linker domain insertion that forms an α helical structure. However the residues that form the catalytic triad are all found within the αß hydrolase domain.

1.8.4 αß Linker Domain

As discussed above, the αß hydrolase domain is formed from two sections of primary sequence, divided by a 72 residue linker sequence. A role for this linker in catalysis or dimerisation of PepX is not obvious, and at this stage it seems to be merely an extension of the αß hydrolase domain.

1.8.5 ß Sandwich Domain

The C terminal domain of PepX is made up of mainly ß strands that form a ß-sandwich structure in a "jelly-roll" arrangement (Rossman *et al.*, 1983). These classes of domain are often associated with a ligand binding function, and in the case of PepX it is interesting to note (as discussed above in section 1.5.4) that the enzyme is believed to be cell membrane associated (Tan *et al.*, 1992). It is possible that the association with the cell membrane is mediated through this domain. Jelly-roll domains are also known to bind sugar molecules in glycosyl asparaginase enzymes,

and although there is no evidence of a second catalytic site in this domain of PepX, it is possible that the enzyme could bind to sugars found in the cell wall. This could be relevant as some authors have reported PepX to be extracellular (Kiefer-Partsch *et al.*, 1989).

1.8.6 Other Features

One notable feature of PepX is a large loop that extends from the C-terminal domain across the dimerisation interface. It has been suggested that this loop might have a role in allowing substrate access to the active site. Another feature peculiar to PepX is a loop that extends right around the catalytic domain, going from the front to the rear of the molecule. The role of this loop is unknown, other than as a domain linker.

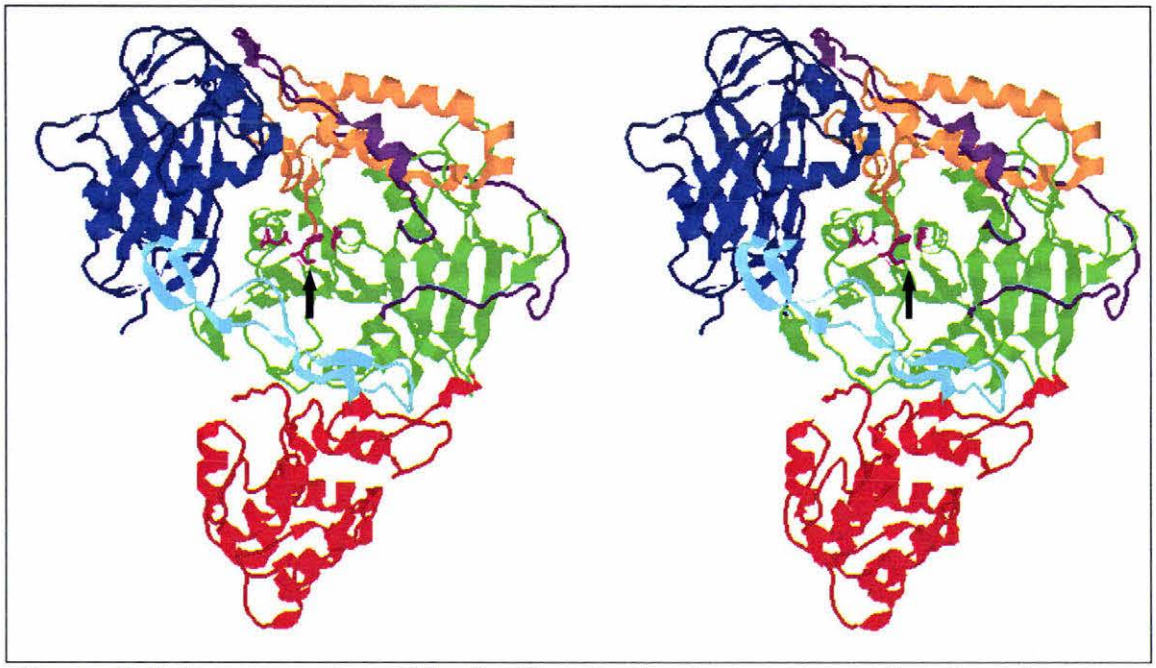


Figure 1.9 *Stereo overview of the PepX molecule. The domains are coloured as follows: α Helical N-terminus: red. Catalytic αß hydrolase: green. Hydrolase linker: orange. ß-sandwich C-terminal: blue. Also shown are three regions of interest: catalytic linker: purple. Substrate cleft cover: cyan. Putative catalytic residues are in magenta, indicated by the arrow. Diagram based on Rigolet, et al. (2002).*

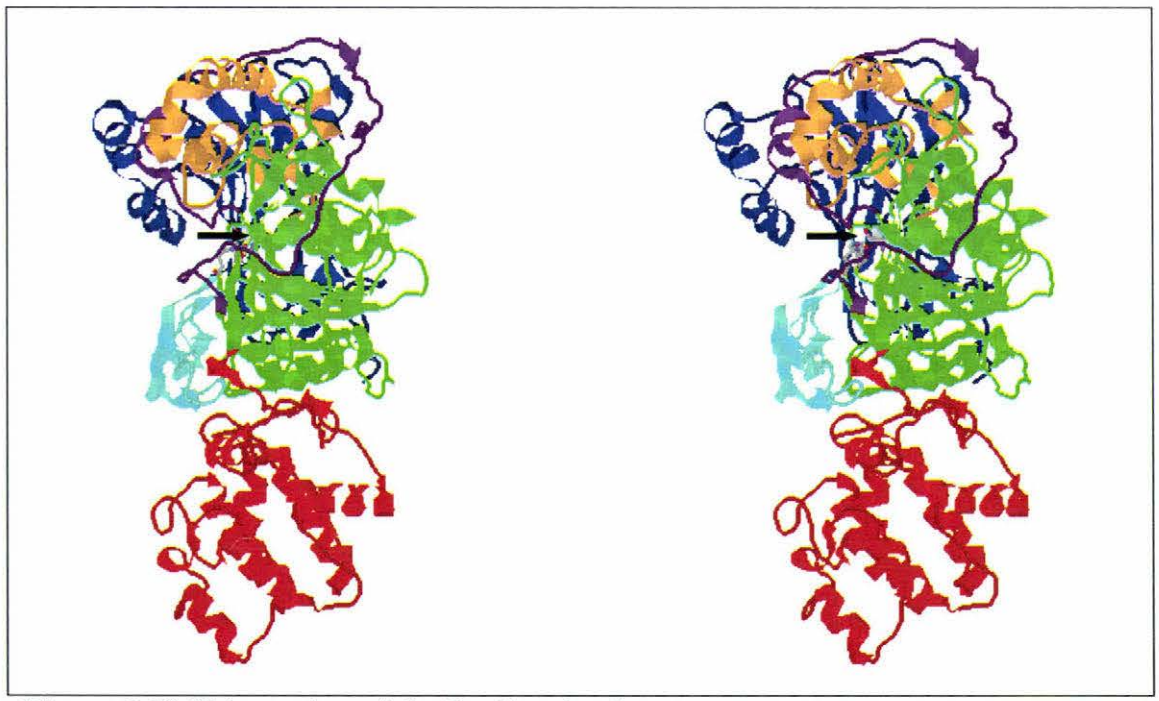


Figure 1.10 *Side-on view of the PepX molecule. The molecule is shown in an orientation rotated right 90° relative to that shown in Figure 1.9. Regions are coloured as described in Figure 1.9 above. The black arrow indicates the location of the putative catalytic triad.*

1.8.7 Comparisons with Structural Relatives

Of the protein structures solved to date, only one appears to have a high similarity in primary sequence to PepX. This is the enzyme cocaine esterase, from *Rhodococcus* solved by Larsen, *et al.* (2002), shown in Figure 1.11.

It is of interest to compare these two enzymes as this could lead to a better understanding of how protein structure affects function. Broadly speaking, cocaine esterase resembles PepX with the N-terminal domain removed. The αß hydrolase catalytic domain, the helical catalytic linker domain and the ß sandwich domain are all present.

As mentioned above, the N-terminal domain of PepX is involved in dimerisation, and the absence of this region is reflected in the quaternary structure of cocaine esterase, which is monomeric. Also, the loop that extends from the ß sandwich domain across the dimerisation interface is not present in cocaine esterase, nor is the unusual loop that extends around the catalytic domain of PepX.

The catalytic domain of cocaine esterase is broadly similar to that of PepX, with 10 ß strands in the central ß sheet, although there are some differences in the positioning of the α helices. Like PepX, the catalytic domain is made up of two halves separated in primary sequence by a linker domain reminiscent of the architecture of the catalytic domain of PepX. The catalytic triad is located in a very similar position to that of PepX, and has the same arrangement of active residues.

The linker domain of cocaine esterase, however, is larger than that found in PepX, comprising 95 residues, with more α helices. This linker is, in fact, the largest insertion into an αß hydrolase catalytic domain of any known protein structure (Larsen *et al.*, 2002).

The C-terminal domain of cocaine esterase is more barrel-like than that of PepX, which has a more flattened arrangement, although the ß sheets are still in a similar structural relationship to each other.

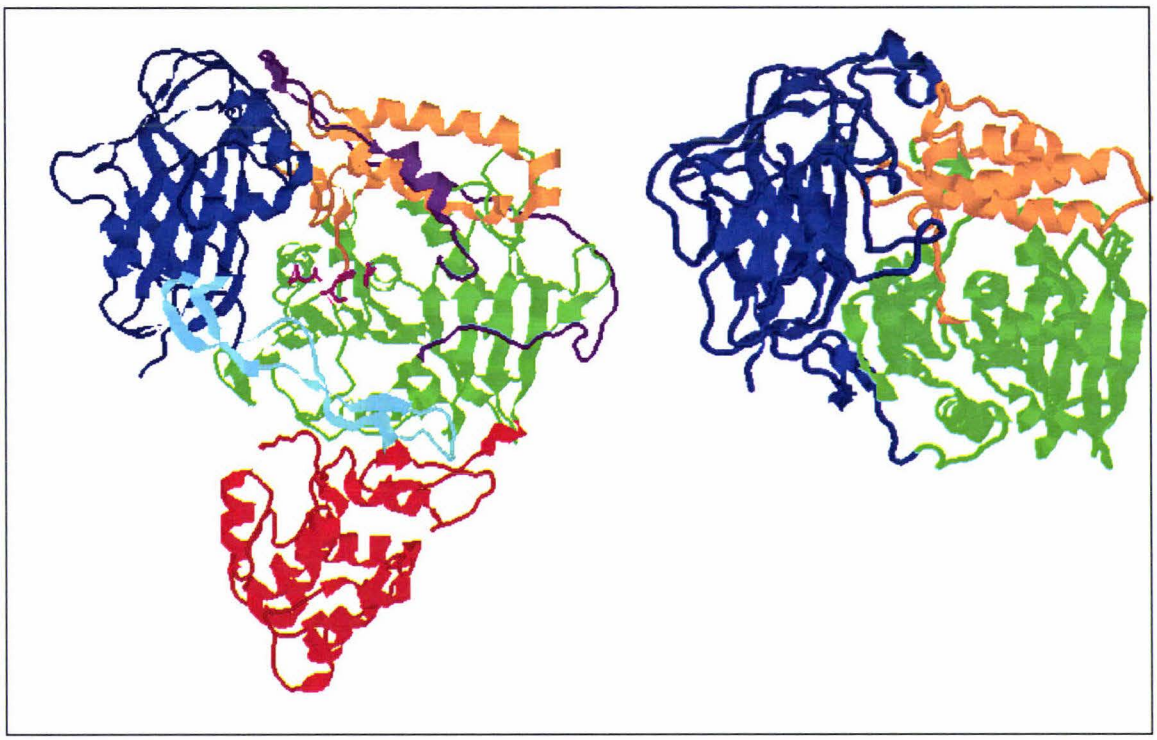


Figure 1.11 *Overall structure of PepX (left) and cocaine esterase (right). Domains are coloured as described in* ***Figure 1.9***

1.9 The Scope of This Project

The main aim of this project is to investigate Esterase II from *Streptococcus thermophilus*. This enzyme was one of three identified during a screen of this organism for enzymes capable of hydrolysing *p*-nitrophenyl butyrate, a chromogenic butyl ester substrate (Liu *et al.*, 2001). These authors did not identify a gene for this esterase, however, although an N-terminal sequence was obtained. Esterase II was also found to be capable of synthesising ethyl butanoate from tributyrin and ethanol under aqueous conditions. The hydrolytic activity of Esterase II was only examined using chromogenic substrates, and not acylglyceride substrates.

Early in this project, Esterase II was identified as X-prolyl dipeptidyl peptidase (PepX), for this reason a review of what is currently known about this enzyme has been given in the sections above. It is obvious that a specific dipeptide hydrolase activity and an ester hydrolase activity against chromogenic butyl ester substrates show an ability of the enzyme to recognise and hydrolyse very different substrates. The intention of this project was to characterise the esterase activity of PepX. Specifically the goals were to:

1) Use the recent release and annotation of several medically significant *Streptococci* genome sequences to attempt to identify the gene that codes for Esterase II (PepX), and to biochemically verify the presence of an ester hydrolytic activity.
2) Clone the Esterase II / PepX gene from *Streptococcus thermophilus* to allow the production of sufficient quantities of enzyme for kinetic studies to take place and to allow structural studies to be undertaken.
3) Investigate the hydrolytic activity of Esterase II / PepX on *p*-nitrophenol based chromogenic substrates, and use enzyme kinetics to investigate whether a single active site is performing both types of hydrolysis.
4) Characterise the ester synthetic transferase activity of Esterase II / PepX by determining the preferred substrate in terms of alkyl chain length, and number of acyl groups.

5) Characterise the ester hydrolytic activity of the enzyme on triacylglyceride substrates, and milkfat to investigate if the esterase activity of PepX has any relevance to a "natural" system.

This project reports the preliminary characterisation of PepX catalysed ester hydrolysis, and ester synthesis. The work undertaken was therefore essentially an investigation into the kinetics of these newly observed activities. The primary step required to produce enough enzyme for kinetic studies was to clone the gene for PepX from *Streptococcus thermophilus*, and develop an expression system to produce the large amounts of enzyme needed for this work.

This thesis consists of six experimental sections:

1) The identification of Esterase II by similarity and biochemical means.
2) The cloning of the *S. thermophilus pepX* gene.
3) The expression and purification of PepX protein from an *E. coli* expression system.
4) Kinetic studies of the hydrolytic activity of PepX of chromogenic ester substrates.
5) The investigation of the specificity of the ester synthetic activity of PepX using a series of different acylglycerides with an ethanol acceptor molecule.
6) The investigation of the hydrolytic activity of PepX on milkfat and tributyrin acylglyceride substrates.

CHAPTER 2

Materials and General Methods

2.1 Materials

The materials and chemicals used in this project were from obtained from the following companies:

Sigma-Aldrich Company, St Louis, USA :
Lysozyme (from hen egg white), Bis-Tris-Propane, CAPS, nickel chloride, dimethyl sulphoxide, ammonium persulfate, ampicillin, chloramphenicol, tetracycline, gum arabic, *p*-nitrophenyl butyrate, glycylprolyl *p*-nitroanilide, tributyrin,

United States Biochemical Company, Cleveland, USA :
Tris (enzyme and buffer grades), glycine, ethidium bromide, Coomassie Blue R-250, Coomassie Blue G-250

Asia Pacific Speciality Chemicals (Ajax), Seven Hills, Australia :
Ammonium sulfate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium chloride, potassium chloride, methanol (HPLC grade), orthophosphoric acid, ethanol (HPLC grade)

BDH Lab Supplies, Poole, England :
Lactose, glucose, imidazole, acetic acid, hydrochloric acid

Oxoid Ltd., Basingstoke, England :
Bacteriological agar

BioRad, California, USA :
Sodium dodecyl sulfate, acrylamide

Invitrogen, California, USA :

LB media base

Difco, Maryland, USA :

M17 media base

Scientific Supplies, Auckland, New Zealand :

Glycerol

2.2 Equipment

2.2.1 DNA Sequencing

DNA sequencing was carried out on either an ABI Prism 377 – 64 sequencer or an ABI Prism 3730 capillary sequencer, using BIGDYE dye labelled dideoxy chain termination chemistries (Applied Biosystems). DNA sequencing was provided by Massey University Allan Wilson Center for Molecular Evolution and Ecology Genome Service.

2.2.2 Protein Sequencing

Protein sequencing was carried out on an Applied Biosystems Model 476A Protein Sequencer. This machine uses automated Edman degradation chemistry to produce protein N-terminal sequence information.

2.2.3 Fast Protein Liquid Chromatography (FPLC)

FPLC was carried out using either a Waters 340 protein chromatography system, or a BioRad BioLogic protein chromatography system. Buffers and solvents for FPLC were prepared as described in the relevant sections and then filtered using 0.22 μM filter discs (Millipore Corporation, Bedford, USA).

2.2.4 Purified Water

Ultra pure water was obtained from a Sybron/Barnstead NANOpure II filtration system (Maryland, USA), containing two ion exchange and two organic filter cartridges. This filtered water is referred to as Milli-Q throughout this thesis. Double distilled water was obtained by filtering Milli-Q water through filter carbon and reflux distilling over powdered carbon. This water is referred to as double distilled water or ddH_2O in this thesis.

2.2.5 Filter Sterilisation Equipment

Sterile syringes were supplied by Terumo Corporation (Tokyo Japan) and sterile 0.22 μM syringe filters and filter membranes were obtained from Millipore.

2.3 General Methods

2.3.1 Storage of Cultures

Cultures of transformed *Esherichia coli* and *Streptococcus thermophilus* were stored as glycerol stocks at –70°C. To make these stocks, cells were streaked onto an agar plate of an appropriate media, containing antibiotic if necessary. Plates were grown overnight, and a single colony was picked with a sterile toothpick, and used to inoculate a liquid culture. This liquid culture was incubated at an appropriate temperature until the cells reached the mid-log phase of growth, as measured by optical density at 600nm. 0.8 mL of culture was then added to a sterile screwcap cryogenic storage tube containing 0.2 mL of sterile 80% (v/v) glycerol. The cryotube was then mixed by inversion, and snap-frozen in liquid nitrogen. The tubes were then directly transferred to storage at –70°C.

2.3.2 Media

S. thermophilus was grown in M17 medium (Difco), made according to the manufacturers instructions, but with 0.5% sterile glucose added instead of lactose. *E. coli* was grown in Luria Broth (LB), made from media base according to manufacturers directions (Invitrogen). Solid medium for agar plates was made by adding 1.5% agar to liquid media before sterilization. All media were sterilized by autoclaving at 121 °C and 15 psi for 20 minutes.

During transformation, *E. coli* cells were suspended in SOC broth medium, containing 20 $g.L^{-1}$ tryptone, 5 $g.L^{-1}$ yeast extract, 2 $mL.L^{-1}$ 5M NaCl, 2.5 $mL.L^{-1}$ 1M KCl and 10 mM glucose.

2.3.3 Antibiotic Stocks

Stock solutions of ampicillin and tetracycline were made in water or ethanol respectively, and filter sterilized prior to storage at –18°C. Ampicillin stock was 100mg/mL and tetracycline stock was 20mg/mL. Antibiotic stocks were stored at –18°C.

Ampicillin stock was added to media after autoclaving and cooling to a final concentration of 100 $\mu g.mL^{-1}$. Tetracycline stock was added to sterile cooled media to a final concentration of 12 $\mu g.mL^{-1}$.

2.3.4 Agarose Gel Electrophoresis

To separate DNA on the basis of fragment size, agarose gel electrophoresis was employed. All gels were run using 1X TAE buffer (40 mM Tris HCl, 20 mM acetic acid and 2 mM EDTA at pH 8.0). The agarose percentage of gels varied according to the purpose of the gel. For separation of PCR products, plasmid vectors and other small DNA species, gel concentration was typically 1%. To separate genomic material and digested genomic material an agarose concentration of 0.7% was used. To increase resolution, 0.5 $\mu g.mL^{-1}$ ethidium bromide was added to the gels, which were 20 cm long to ensure good separation of DNA fragments.

Gels for Southern blotting were run at a low voltage (typically 25 volts) for up to 15 hours to ensure an accurate mobility to fragment size ratio was obtained. During long runs, buffer depletion by electrolysis was avoided by recirculating tank buffer from the anode (+) to the cathode (-) reservoir, at a rate of 5 mL per minute.

DNA on the gels was visualized by staining the gel in a 0.5 $\mu g.mL^{-1}$ ethidium bromide bath for 20 minutes followed by destaining for 10 minutes in Milli-Q water. The DNA was illuminated by exposure to ultraviolet light at 302 nm. Gels were recorded from a video image using an Alpha Imager gel documentation system (Alpha Innotech, California USA)

2.3.5 Ethanol Precipitation of DNA

To allow the removal of salt, small oligonucleotide species and other contaminants from DNA solutions, ethanol precipitation was carried out. 0.2 volumes of 3 M sodium acetate–acetic acid, pH 5.0, was added to the DNA solution, followed by 2 volumes of ice-cold 100% ethanol. The solution was mixed by inversion several times, and the precipitated DNA was recovered by centrifugation at 9500 x g in a benchtop centrifuge at 4°C for 20 minutes. The supernatant was removed, and the pellet washed in 2 volumes of ice-cold 70% ethanol. The solution was centrifuged for

10 minutes at 9500g to pellet the precipitated DNA. The supernatant was removed, and the pellet allowed to air-dry at room temperature for approximately 30 minutes. The DNA was then re-suspended in either water or buffer, at a pH of 7.5 - 8.5, to ensure efficient resuspension.

2.3.6 Transformation of Competent *Esherichia coli* Cells

Plasmid vectors were introduced into *E. coli* cells using a heat shock protocol. A frozen stock of competent cells was thawed on ice. 50 µL of cells were transferred into an eppendorf tube, and an appropriate volume of plasmid solution was added. In the case of plasmid material that had been extracted from cells, 1 µL was added. In the case of ligations, the entire ligation mixture was added (up to 20 µL). The cells were incubated on ice for 20 minutes, before being heat shocked at 42 °C for 1 minute. After heat shock, the cells were immediately placed on ice for 20 minutes. 500 µL of prewarmed SOC media was then added, and the cells incubated at 37 °C for 45 minutes. 200 µL of transformed cells were then plated onto LB agar, with the appropriate antibiotic selection. Agar plates were incubated overnight, and a single transformant colony picked for further work, such as growth of a liquid culture for glycerol stocks, or for plasmid preparation.

2.3.7 Measurement of Optical Density of Cultures (OD)

A spectrophotometer (Cary 1, Varian) was set to 600nm and zero calibrated using a cuvette containing the appropriate sterile medium. The culture to be measured was sampled into a cuvette. The optical density of the sample was measured and recorded. If the OD of a sample is less than 0.3 or greater than 0.8, optical density readings are not accurate. Readings outside this range were avoided by diluting the culture as required with sterile medium. This was repeated as necessary until readings of the sample were within the 0.3 - 0.8 range.

2.3.8 Chromogenic Substrate for Butyl Esterase Activity Assays

Substrate for assessing butyl esterase activity was made by thawing an aliquot of *p*-nitrophenyl butyrate at room temperature until entirely liquid. 10 µL of *p*-nitrophenol butyrate was then dissolved in 1 mL of ice-cold HPLC grade methanol. The methanolic solution was then dissolved in 25 mL of 20 mM BTP at pH 7.0 made using purified water as described below in section 2.3.14, and kept on ice until required.

2.3.9 Chromogenic Substrate for X-prolyl Dipeptidase Activity Assays

A stock solution of 100mg/mL glycylprolyl *p*-nitroanilide was made in 20mM BTP pH 7.0 and stored at -18°C until required. A thawed aliquot of substrate stock was dissolved at 10µL.mL^{-1} in 20mM BTP at pH 7.0 made as described in section 2.3.14 below. Substrate was stored on ice until required.

2.3.10 Qualitative and Quantitative Analysis of Esterase and Peptidase Activity

For qualitative spot-testing of esterase and peptidase activity 200 µL of either *p*-nitrophenyl butyrate substrate or glycylprolyl *p*-nitroanilide substrate was pipetted into a microwell. 50 µL of protein sample was added and incubated at room temperature for up to 20 minutes, or until a colour change to yellow was observed. For quantitative testing of enzyme activity 990 µL of the appropriate substrate was pipetted into a cuvette, and the cuvette was placed in a temperature controlled Cary spectrophotometer (Varian). The cuvette was left to equilibrate to the correct temperature (37 °C) for at least 10 minutes. The assay was started by addition of 10 µL of appropriately diluted enzyme, and the initial rate of reaction was recorded. Reaction rates were converted into units of enzyme activity (µmoles of chromophore liberated per minute per mg of enzyme) using the extinction coefficients of 7626 L.mol^{-1}.cm^{-1} for *p*-nitrophenol at pH 7.0 and 8800 L.mol^{-1}.cm^{-1} for *p*-nitroaniline at pH 7.0, as described in Appendix 7.

2.3.11 Polyacrylamide Gel Electrophoresis

Proteins were separated on the basis of both mass and charge using polyacrylamide gel electrophoresis with and without sodium dodecyl sulfate, using the discontinuous buffer method described by Laemmli (1970). Gels were cast and run using a Mini-Protean II system (BioRad). Gels were typically run at 200 volts until the dye front reached the bottom of the gel. In some cases, such as during native gel electrophoresis, gels were run at a lower voltage on ice to provide a cold environment designed to minimize protein denaturation.

2.3.12 Coomassie Staining Polyacylamide Gels

Polyacrylamide gels were stained in a solution of 50% methanol (v/v) and 10% acetic acid (v/v) in water, with 1 $g.L^{-1}$ Coomassie Brilliant Blue R-250. Gels were stained with gentle agitation for 20 minutes. Excess stain was washed away with Milli-Q water, and the gels were then destained in the solution described above, with the Coomassie dye omitted. Gels were destained until the background staining had been removed, which varied from 30 minutes to several hours, depending on the level of background observed.

2.3.13 Activity Staining Polyacrylamide Gels for Peptidase and Esterase Activity

Non-denaturing and denaturing polyacrylamide gels were run as described above in section 2.3.11. They were then removed from the running apparatus, placed in a square petri-dish and rinsed briefly in Milli-Q water to remove excess buffer. 20mM BTP pH 7.0 was then added to the dish and the gels were shaken for 20 minutes to remove excess SDS (where applicable) and running buffer. This step was repeated to ensure full removal of high pH buffer that would otherwise cause the spontaneous hydrolysis of the *p*-nitrophenyl-based ester substrates.

The buffer was removed from the gel dish, and approximately 5mL of enzyme chromogenic substrate, made as described in sections 2.3.8 and 2.3.9, was added to the dish containing the gel. Colour was allowed to develop. Activity stained gels were placed between two plastic overhead transparency sheets and scanned using a computer scanner.

Gels were then Coomassie stained and compared with scanned activity stain images to allow mass estimation of protein bands that corresponded to enzyme activity.

2.3.14 Enzyme Assay Grade Buffer

Water for enzyme assays was produced by treating Milli-Q water with fine grade filter-carbon. After filtration to remove carbon particles, the water was distilled in a reflux distillation column. Water was then treated with Chelex mixed ion exchange resin (BioRad), and filtered using a 0.22 μM filter disc to remove chelex beads. The purified water was then used to make buffered solutions for enzyme assays as needed.

2.3.15 Colony PCR

Cell colonies were grown on an agar plate that contained an appropriate selection factor. Bacterial cells were removed from the plate by touching the surface of each colony with a sterile pipette tip. The pipette tip was then placed into a PCR tube containing prepared PCR mix (*Taq* DNA polymerase, buffer, dNTPs, and primers, made as recommended by the manufacturer), shaken several times to dislodge bacteria, and removed. PCR reactions were then performed as normal, with a 5 minute initial denaturation step to ensure lysis of cells, annealing at 55 °C for 1 minute, extension at 72 °C for 1.5 minutes, and a denaturation step at 95 °C for 1 minute. Products were visualized on a 1% TAE buffered agarose gel with ethidium bromide staining as described in section 2.3.4.

2.3.16 Densitometry of Gel Images

To determine the amount of protein present in a particular band, densitometry was performed on Coomassie stained polyacrylamide gels. Gels were destained as much as possible, without washing out minor bands, and photographed. The saturation tool provided with the Gel Documentation software (Alpha Innotech, California USA) was used to ensure the image was not saturated and exposure and aperture settings were adjusted if necessary. This step is important for accurate quantitation of proteins during densitometry analysis. If an image is oversaturated, large bands will appear to

have less density than is actually present. Gel images were then analysed using ImageJ software version 1.29 to compare the amount of material present in each band. By measuring the total protein present in a sample using the Bradford protein assay (Bradford, 1976), and measuring the percentage of total material present in a particular band, the amount of a particular protein in the mixture could be estimated.

2.3.17 Phosphate Buffer pH 7.5

Phosphate buffer was made by combining 4 parts of 0.1 M Na_2HPO_4 with 1 part of 0.1 M NaH_2PO_4. The pH of the buffer was adjusted as required with NaH_2PO_4. The buffer was filtered using a 0.2 μm filter membrane and stored at 4°C until required.

CHAPTER 3

Identification of Esterase II from *Streptococcus thermophilus* B2513

3.1 Introduction

During a screen for esterase activities in the dairy lactic acid bacterium *Streptococcus thermophilus*, three esterase activities were identified (Liu *et al.*, 2001). Two of the proteins responsible for these activities were subjected to Edman N-terminal sequencing. Subsequent database searches using bioinformatic methods identified one (Esterase I) to have an N-terminal sequence with high homology to a known enzyme. However the second esterase could not be identified and was named Esterase II by Liu, *et al.* (2001). Esterase II was known to have ester synthetic properties, but did not appear to belong to any of the other families of previously characterized esterases, based on both mass and N-terminal sequence. The identification of this new esterase was therefore of interest.

3.2 Objectives and Strategies

The primary goal of this study was to identify Esterase II by sequence similarity to other known proteins from bacterial species.

Before assuming a protein or enzyme is novel, a thorough search of the genetic sequence databases needs to be made to determine whether there is similarity to any other protein or protein family that has already been characterised. Database searching was carried out using computer sequence alignment algorithms to find protein sequences that had similar N-terminal sequences to the known N-terminal sequence of Esterase II. A match of the N-terminal sequence of Esterase II was made with the N-terminus of another protein would allow a tentative identification to be made.

Since the first isolation of Esterase II was carried out, a number of *Streptococcus* species have had their entire genomes sequenced. These species are generally investigated because they are human pathogens. However, they are still closely related

to non-pathogenic dairy lactic acid bacteria such as *Streptococcus thermophilus* and *Lactococcus* species. Thus the chances of identifying a candidate gene for Esterase II relied on there being some sequence similarity to a gene from the *Streptococcus* genera.

3.3 Methods

3.3.1 Software Used in Similarity Searches

Computer software used was from the National Center for Biotechnology Information (NCBI). Specifically, the Basic Local Alignment Search Tool (BLAST) algorithm was used from a website browser-based interface. The BLAST algorithm functions by taking a submitted query sequence, and searching a user selected database of genes to find similar sequences (Altschul *et al.*, 1997).

These sequences are then returned to the user as a summary that allows the user to establish the statistical significance of the results. The BLAST-P program, designed to search the Non-Redundant protein sequence database (nr) held by NCBI was used. Standard settings to identify "short nearly exact matches" were utilized. These settings were a word size of 2, and gap existence and extension penalties of 9 and 1 respectively. The small word size maximizes the chance of the algorithm finding less than exact matches to such a small query sequence.

The N-terminal sequence of "Esterase II" as identified by Liu, *et al.* (2001) was submitted as the database query sequence.

3.4 Results and Discussion

3.4.1 Database Search Results

The database search returned several potential matches to the query sequence. These are summarized in Table 3.1. From this information it could be seen that Esterase II had the greatest similarity to a predicted X-prolyl dipeptidyl aminopeptidase gene from *Streptococcus pyogenes*. This predicted gene was itself identified by its similarity to X-prolyl dipeptidyl peptidase from *Lactococcus lactis*, which has been identified by experimental methods (Mayo *et al.*, 1991; Nardi *et al.*, 1991). These similarities are summarised in Table 3.2. Based on these results, Esterase II was tentatively identified as X-prolyl dipeptidyl peptidase.

Species	**Similarity**
	** * ** # # **
Lactococcus Lactis	MRFNHFSIVD KNFDEQLAEL
Streptococcus pyogenes	MRYNQFSYIP TSLERAAEEL
Streptococcus thermophilus	MKFNQFSYIP VSPETAYQEL

Table 3.1 *N-terminal sequences of representative PepX proteins. * Denotes an exact match, # denotes similar residues.*

Species	**Identical residues**	**Similar residues**
Streptococcus pyogenes	65%	85%
Lactococcus lactis	35%	45%

Table 3.2 *Similarity of N-terminal sequences from cocci species to Esterase II.*

These results have interesting implications. X-prolyl dipeptidyl peptidase has already been extensively studied in the dairy industry because it is a proline specific peptidase that is important in generating dairy product flavours. The influence of PepX in isolation on flavour is difficult to gauge. The enzyme itself is not required for the survival of the organism as knockout mutants are viable (Mierau *et al.*, 1996; Guinec *et al.*, 2000), making it difficult to analyse of the precise role of PepX in the cell.

A minor esterase-like activity of PepX had been observed by other researchers, who had shown the enzyme could cleave ester bonded amino acids (Yoshpe-Besancon *et al.*, 1994), although no alkyl esterase activity had ever been attributed to this enzyme. Esterase II from *S. thermophilus* was ascribed a molecular mass of approximately 65 kDa from results obtained using SDS-PAGE, gel filtration, and mass spectrometry (Liu *et al.*, 2001). However the molecular masses of other PepX proteins, as well as the predicted masses from gene sequences, indicated that most of the known PepX enzymes would have molecular weights ranging from 80 kDa to 90 kDa (Meyer and Jordi, 1987; Nardi *et al.*, 1991; Meyer-Barton *et al.*, 1993). PepX had in fact been previously purified from *S. thermophilus* and found to have a monomeric weight of 80±5 kDa (Lloyd and Pritchard, 1991).

The reason for the disparity in PepX and Esterase II was unclear, but the N-terminal sequence similarity between Esterase II to *Streptococcus pyogenes* PepX was striking. A possible explanation for the smaller size of Esterase II is that the enzyme isolated by Liu, *et al.* (2001) may have been subjected to a specific partial proteolysis process at some point during experimental procedures, producing a stable truncated protein that still had esterase activity. This partial degradation most probably resulted from C-terminal cleavage, since the N- terminus produced a clear single signal during Edman sequencing, strongly indicative of a single N-terminal present (S. Liu, personal communication). Some authors have noted that PepX prepared from *Lactococcus lactis* does lose activity over time when stored in 20 mM Tris buffer with no salt added (Chich *et al.*, 1995).

A second possibility is that the strain of *Streptococcus thermophilus* used in prior studies had a point mutation resulting in a stop codon and therefore the expression of a truncated PepX protein. The strain of *S. thermophilus* used in previous studies was Fonterra strain B2513, isolated from a dairy source. If this had been the case, perhaps a truncated version of PepX would produce more desirable characteristics, and would therefore be selected during strain isolation. Further biochemical analysis was

required to support the computer prediction of Esterase II identity, and to investigate the apparent mass difference between Esterase II and other PepX enzymes.

3.5 Conclusions

The N-terminal similarity of Esterase II to several X-prolyl dipeptidyl peptidase enzymes suggested that this esterase enzyme was in fact the peptidase PepX. Further experiments therefore needed to be carried out to verify this prediction. A significant difference in mass between the Esterase II purified by previous researchers, and the PepX enzyme from *Streptococcus* and *Lactococcus* species was especially intriguing. This mass difference may have been due to a specific protein cleavage process that removed a C-terminal portion of the protein. Alternatively, a point mutation resulting in the insertion of a stop codon may have caused the expression of a truncated version of PepX in the strain of *S. thermophilus* studied by previous investigators. Further biochemical and genetic studies should provide an explanation for the size difference observed between Esterase II and all previously characterised PepX enzymes.

CHAPTER 4

Biochemical Identification of Esterase II from *Streptococcus thermophilus*

4.1 Introduction

In Chapter 3 the identification of a potential enzyme candidate for Esterase II was described. However this was a putative identification based on N-terminal sequence similarity. There was also some uncertainty about the molecular mass of Esterase II reported by Liu, *et al.* (2001). It was therefore necessary to biochemically characterise the enzyme from *Streptococcus thermophilus* to prove that the enzyme from this organism responsible for the esterase activity was in fact PepX. The work reported in this chapter is essentially a repeat of work carried out by the previous investigators (Liu *et al.*, 2001), although a different strain of *S. thermophilus* was used. The purification of Esterase II was undertaken to allow further biochemical characterisation of the enzyme, including investigations of alternative substrates for the enzyme.

4.2 Experimental Objectives

The objectives of this study were to investigate Esterase II from *Streptococcus thermophilus* to see if it had X-prolyl dipeptidase-like properties as well as butyl esterase properties. In detail these objectives were as follows:

1) Purify Esterase II from *Streptococcus thermophilus*
2) Characterize the esterase activity of Esterase II using artificial chromogenic substrates.
3) Confirm the dipeptidase activity of Esterase II using artificial chromogenic substrates.
4) Check the N terminal sequence of the purified enzyme to confirm its similarity to PepX and Esterase II.

4.3 Methods

4.3.1 Proteolysis Control

Ideally, protein preparations should be carried out under conditions that minimize the activity of proteases that would otherwise destroy the protein of interest.
Esterase enzymes are members of the serine hydrolase family, and the active site residues are thought to closely resemble serine proteases. Because a similar catalytic mechanism is used in both types of enzyme (Silverman, 2000), protease inhibitors have been found to interfere with esterase activity (Shaw, 1999). For this reason, no protease inhibitors were used at any stage of the preparation. To minimize potential proteolytic degradation, all solutions were kept refrigerated or on ice during use, and all chromatography steps were performed at 4 °C with the exception of hydrophobic interaction chromatography, which was carried out at room temperature.

4.3.2 Esterase II Purification

4.3.2.1 Growth of *Streptococcus thermophilus*

A glycerol stock of *Streptococcus thermophilus* was obtained from the Massey University culture collection (strain MU#17). A sterile nichrome loop was used to streak cells onto an M17 glucose agar plate, made as described in the general methods section. A single isolated colony was picked using a sterile toothpick, and used to inoculate a 10 mL tube of M17 glucose broth. This culture was incubated overnight at 37 °C under static conditions. 3 mL of this culture was then used to inoculate each of three main cultures of 3.3 L M17 glucose media. These main cultures were incubated at 37 °C for 48 hours under static growth conditions. A sample of each of the main cultures was taken, heat fixed to a glass slide and stained with crystal-violet, then observed by microscopy for cocci morphology to check the purity of the main cultures.

4.3.2.2 Harvesting and Lysis of Cells

The cells were harvested by centrifugation at 3800 x g for 25 minutes at 4 °C. Cells were washed once then resuspended in 20 mM Tris at pH 7.0. All centrifugation steps were carried out at 4 °C, and after the initial harvesting, all protein solutions were kept on ice.

Cells were lysed by two passes through a French press (Aminco) at 6 kPa. For Gram-positive species such as *Streptococcus*, sonication is not able to disrupt the thick cell wall. The French press disrupts cells by causing a sudden pressure drop as the cells are simultaneously forced through a small opening in a pressure chamber and is the best way to lyse the cells and keep the majority of cellular protein intact. The French press cylinder and plunger were cooled to 4 °C before use, and the cell suspension was kept on ice before and after lysing.

Cellular debris was removed by centrifugation at 7800 x g for 30 minutes at 4 °C. The cell free supernatant was retained and the remaining pellet discarded.

4.3.2.3 Ammonium Sulfate Precipitation of Proteins

Ammonium sulfate precipitation relies exploits the hydrophobic properties of proteins and results in the precipitation of proteins upon the addition of salt at higher than physiological concentrations. Different proteins will become insoluble at different salt concentrations, and this phenomenon can be exploited to either precipitate the protein of interest, or to precipitate contaminant proteins whilst leaving the protein of interest in solution. Both the type of salt added and the concentration used can be manipulated to achieve the best fractionation. Precipitated material can be removed by centrifugation, resuspended in buffer and resolubilised by dialysis or dilution.

Protein was precipitated from the cell free supernatant by the slow addition of finely ground solid ammonium sulfate to 40% saturation, with constant stirring. The salting-out was performed at 4 °C.

Precipitated material was removed by centrifugation at 17500 x g for 40 minutes at 4 °C. The supernatant was decanted from the pellet and both fractions were retained. The supernatant was brought to 80% saturation by the further addition of ammonium sulfate and the precipitated material again removed by centrifugation as before. The supernatant was decanted, and both supernatant and pellet were assayed for protein concentration and enzyme activity. Samples were assayed for esterase and X-prolyl

dipeptidase activity using the activity spot test described in Section 2.3.10, and protein concentrations were measured using the modified Bradford protein assay (Bradford, 1976). Pellets were resuspended in a minimum of 20 mM Tris-HCl at pH 7.0 and dialysed against several changes of 20 mM Tris-HCl pH 7.0 to reduce salt concentration prior to further steps.

4.3.2.4 Anion Exchange FPLC

Ion Exchange FPLC (IEX) separates proteins on the basis of differences in surface charge of the folded protein. In the case of anion exchange, negatively charged residues on the surface of the protein displace negative ions from positively charged groups attached to the column media, and bind to the media. By application of a gradient of increasing salt concentration, anions from the salt are exchanged with anionic protein residues, displacing the protein from the column. This allows protein species to be separated from one another.

A Q-Sepharose Hi Load column (Amersham Biosciences, New Jersey, USA) with a bed volume of 140 mL was used. The column was equilibrated in 20 mM Tris-HCl, pH 7.0, and loaded with the dialysed protein solution at a rate of 1 mL per minute. Unbound proteins were eluted by washing the column with 5 column volumes of loading buffer before bound protein was eluted with a 0 M to 1 M NaCl gradient, applied over 1000 minutes at a flow rate of 1 mL per minute. Eluate was collected in 8 mL fractions, the entire process being carried out at 4 °C.

The fractions were analysed for the presence of esterase and X-prolyl dipeptidase activities by spot testing. Active fractions were checked for purity using SDS-PAGE and those with the least impurities were pooled.

4.3.2.5 Hydrophobic Interaction Chromatography

Hydrophobic Interaction Chromatography (HIC) separates proteins on the basis of surface hydrophobicity. In aqueous solutions, hydrophobic groups tend to aggregate together, and HIC exploits this effect with hydrophobic groups on the column matrix binding the hydrophobic surface groups of protein. To increase hydrophobic interactions, HIC is carried out under high salt conditions. Hydrophobic interaction chromatographic separation is a temperature sensitive process, and affected by the type and concentration of the salt used. In previous esterase preparations, HIC at room

temperature had proven more effective at separating proteins from one another than HIC at 4 °C (Bennett, 2000). For this reason the alkyl superose chromatography was carried out at room temperature.

The pooled fractions from the IEX step were dialysed against several changes of 2 M $(NH_4)_2SO_4$, and loaded onto a pre-equilibrated alkyl superose 5/5 prepacked column (Amersham) in loading buffer (20 mM Tris-HCl with 2 M $(NH_4)_2SO_4$ at pH 7.0) at 0.4 mL per minute. Unbound proteins were eluted with 6 mL of loading buffer before a reverse salt gradient from 2 M $(NH_4)_2SO_4$ to 0 M $(NH_4)_2SO_4$ was applied at a flow rate of 0.5 $mL.min^{-1}$ over a period of 90 minutes. 0.5 mL fractions were collected during the run and spot tested for esterase and peptidase activities as before. Active fractions were analysed by SDS-PAGE to check for purity, pooled then dialysed against 20 mM Tris-HCl pH 7.0, to reduce the salt concentration.

4.3.3 Native Gel Electrophoresis and Activity Staining

The protein solution was concentrated using ultrafiltration (Centricon 10, Amicon). 20 µL samples were loaded onto several lanes of a native polyacrylamide gel, which was then run at 50 volts on ice until the dye front had reached the end of the gel. Each lane that contained protein was excised from the gel and washed in 20 mM Tris-HCl pH 7.0 before being stained with enzyme spot testing reagent. This washing step was repeated to ensure that the gel slice had a pH of 7.0, rather than 9.5 from the electrophoresis process. As spontaneous degradation of *p*-nitrophenyl butyrate had been observed at pH values above pH 7.5, it was important that these assays were run at or near a neutral pH to avoid spontaneous hydrolysis of the substrate upon contact with the gel slice.

The yellow colour, due to the release of the chromophore from the substrate, was allowed to develop before the gel slices were scanned using a colour scanner. After scanning, the gel slices were stained in Coomassie Blue R-250 PAGE stain, destained and photographed.

4.3.4 Blotting and Edman Sequencing

The protein solution was subjected to SDS-PAGE. Prior to electrophoresis the gel was stored at 4 °C overnight to allow complete polymerisation to occur. The gel was run using standard SDS-PAGE buffers, but with 2 mM sodium thioglycolate included in the cathode buffer to minimise the possibility of N-terminal blockage being caused by any remaining reactive oxygen species in the gel. After electrophoresis was completed, the gel was blotted onto a polyvinylidene difluoride (PVDF) membrane using a BioRad protein blotting cassette and a standard BioRad electrophoresis cell. The gel was sandwiched next to two pieces of PVDF membrane, followed by two pieces of 3MM filter paper (BioRad) on either side of the gel and membrane. The gel and membranes were assembled into the blotting apparatus according to manufacturers directions, with the gel nearest the cathode (negative).

The blotting was carried out in 10mM CAPS buffer with 5% methanol at pH 11 for 80 minutes at 60 volts. After blotting the membrane was lightly stained in Coomassie Blue R250 protein stain, destained and stored at –18 °C, prior to Edman protein sequencing.

Edman protein sequencing was carried out on the major band present on the blot using a model 476 protein sequencer (Applied Biosystems).

4.4 Results and Discussion

4.4.1 Purification of PepX

After the ammonium sulfate precipitation, significant butyl esterase activity was found in the 80% saturation pellet. No butyl esterase activity was found in any other fraction. This differed from the purification carried out by Liu, *et al* (2001), where most of the tributyrin esterase activity was observed to partition into the 80% $(NH_4)_2SO_4$ supernantant. There are several possible explanations for this. One is that strain differences between that used for this preparation and the B2513 strain used by Liu, *et al.* (2001) may lead to subtle changes in the protein amino acid composition and hence subsequent changes in salt precipitation properties. Another possibility is that a difference in protein concentration may have caused the proteins in each preparation to behave differently. Also the difference in size between Esterase II (Liu *et al.*, 2001) and other PepX proteins may cause the different precipitation properties observed.

After the anion exchange, esterase activity was found to elute from the column at an NaCl concentration of about 300 mM. These same fractions also had X-prolyl dipeptidase activity when assayed against glycylprolyl *p*-nitroanilide.

No precipitation was observed after dialysis against 2 M $(NH_4)_2SO_4$ to increase salt concentration for hydrophobic interaction chromatography.

After alkyl superose hydrophobic interaction chromatography, the butyl esterase activity still co-partitioned with X-prolyl dipeptidase activity, according to spot testing.

Spot testing was carried out across the active peak. The spot testing results were photographed and are shown in Figure 4.1.

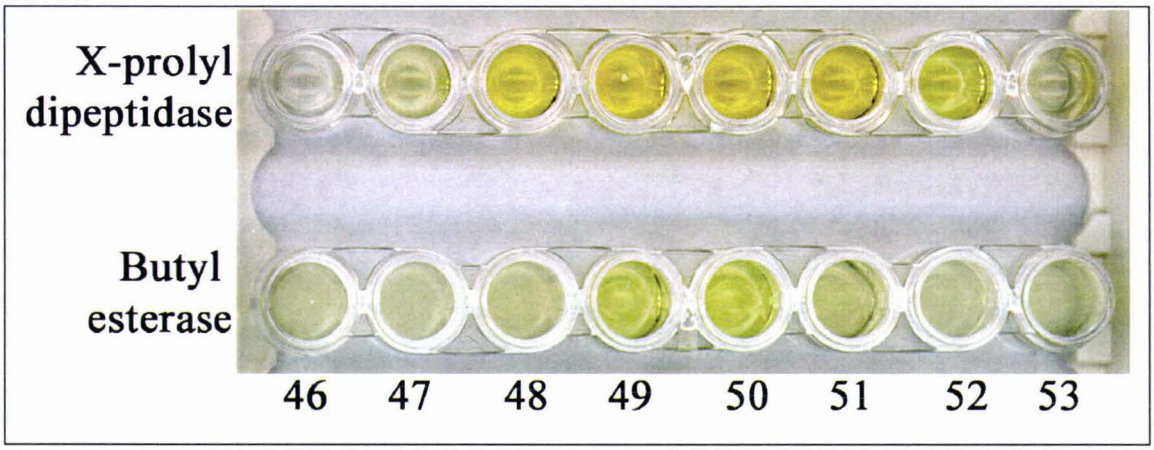


Figure 4.1 *Spot test across esterase active peak of the alkyl superose eluate fractions*

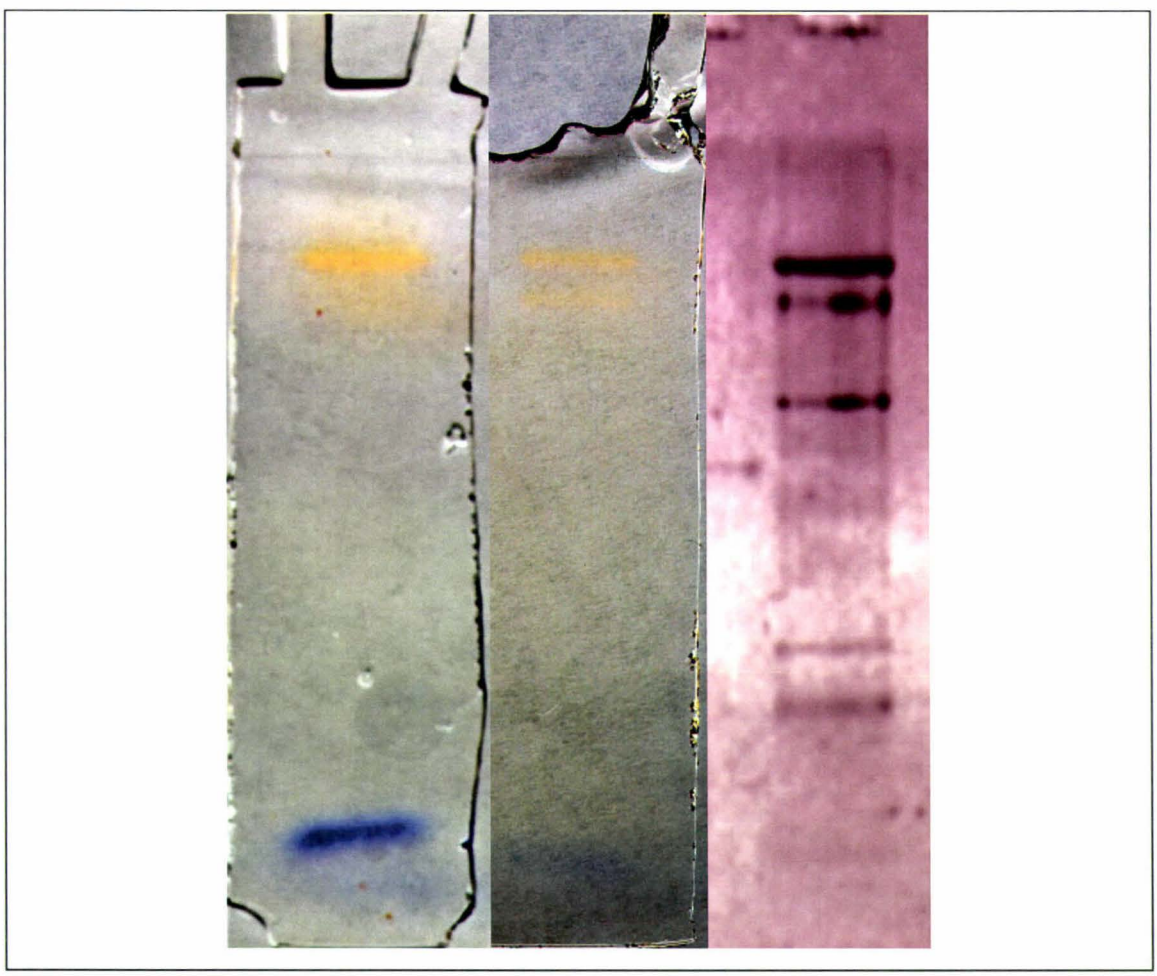


Figure 4.2 *Gel slices with activity and protein stains applied.*
Left: X-prolyl dipeptidase activity stain. Center: Butyl esterase activity stain. Right: Coomassie Brilliant Blue R-250 protein stain.

The observation of both butyl esterase and X-prolyl dipeptidase activities in fractions taken across a single peak from the column eluate suggests that one enzyme is

performing both catalytic activities. However, hydrophobic interaction chromatography occurs, by design, under conditions that promote hydrophobic interactions, and it is possible that this single peak consists of two separate enzymes hydrophobically associated with one another, rather than a single PepX enzyme capable of both peptidase and esterase activities. SDS-PAGE with Coomassie protein staining was unable to show if a single protein was present in these fractions, as the protein was too dilute to be stained by the Coomassie reagent.

4.4.2 Native Gel Electrophoresis

The activity-stained native gel produced from the concentrated pool of active fractions shown in Figure 4.2 shows one X-prolyl dipeptidase activity present. There are two esterase activities present, but the band of major esterase activity corresponds to the band of peptidase activity. The second esterase activity appears to be much less than the activity that corresponds to the peptidase activity band on the gel, as it took much longer to develop the yellow colour compared to the major esterase band. The identity of the second band is unknown; it may be another enzyme, or it is possible that some partial degradation of the enzyme occurred during the purification procedure, and this activity represents a partially degraded PepX. It is possible that this type of degradation may prevent the enzyme from carrying out peptide hydrolysis, whilst preserving some of ester hydrolase activity, albeit at a lower efficiency. The observation of this band may explain the apparent lower molecular weight of Esterase II observed by Liu, *et al* (2001), which was 65 kDa, compared to the 85 kDa average monomeric weight of other PepX enzymes. This may also explain the observation of a third esterase activity, "Esterase III" in *S. thermophilus*, by Liu, *et al.* (2001). It is suggested that Esterase III may represent an uncleaved form of PepX whilst Esterase II may be a cleaved version of PepX that has a C-terminal truncation due to proteolysis. This would also explain the observation that Esterase II was a monomer, when all known PepX enzymes form homodimers. It has been noted by other authors that *Lactococcus* PepX is unstable in aqueous buffers with low salt concentrations (Chich *et al.*, 1995). A solution to this problem may be to stabilise the protein, either by using a suitable solvent, or by addition of salt. It should also be noted that the additions of such substances are likely to interfere with certain purification

procedures, for example, salt will interfere with any process that relies on protein charge, such as ion exchange chromatography.

4.4.3 Coomassie Staining of Native Gel for Protein

Coomassie staining of the native gel shows that the peptidase/esterase active band is the major protein species of present, as it also shows the presence of some minor protein species. It should be noted that native gel electrophoresis does not occur under conditions that promote non-specific hydrophobic interactions, although proteins that associate specifically can be carried through the gel matrix as multimers. PepX is known to form homodimers, but in other purifications of this enzyme from *Streptococcus* species, no other protein species were observed that associate strongly with the PepX enzyme. This suggests that the PepX enzyme is responsible for both the peptidase and esterase activities observed on the native gel, and strongly reduces the possibility of weak, non-specific interaction with another protein responsible for the esterase activity.

An SDS-PAGE gel of the concentrated alkyl superose active pool showed that the major protein species present was approximately 85 kDa in mass. This is 20 kDa greater than the mass of Esterase II observed by previous investigators, but is within the size range of known PepX enzymes from *Streptococcus* species, including other strains of *S. thermophilus*. This discrepancy may be due to partial degradation occurring in earlier studies.

4.4.4 Edman Sequencing

The N-terminal sequencing of the major protein species present in the alkyl superose pool was found to be MRFNQ FRYNP. This differs from the N-terminal sequence of Esterase II at positions 2 and 9, and may be due to errors in the N-terminal sequencing, or to strain differences between the dairy derived *Streptococcus thermophilus* B2513 strain used by Liu, *et al.* (2001) and the Massey University culture collection strain used in this purification. Obtaining sequence beyond 10

residues proved impossible due to the small amount of protein present, and the large molecular mass of the protein.

There is a high similarity to the N-terminal of PepX enzymes from *Streptococci* species, as determined by BLAST similarity searches (Altschul *et al.*, 1997), and this N-terminal sequence. The possibility of an enzyme non-specifically associating with PepX and showing an esterase activity cannot be ruled out, since SDS-PAGE used to produce the blotted protein sample for sequencing would disrupt these sort of interactions and separate PepX from other proteins, unlike the native gel electrophoresis. However, when the N-terminal sequence of Esterase II was obtained in prior studies, no SDS-PAGE separation was used; instead a protein solution was used directly in the sequencing reactions (S. Liu, personal communication). If there had been other esterases associated with PepX during the Edman degradation process, a characteristic multiple N-terminal sequence would have been observed during Edman sequencing (T. Loo, personal communication). Only a single clean N-terminal was found during this work, which strongly suggests that the sample contained only a single protein species that was capable of both X-prolyl dipeptidase and butyl esterase activities.

The N-terminal sequence obtained from the protein band responsible for peptidase and esterase activities observed by activity staining is strong support for the hypothesis that Esterase II is X-prolyl dipeptidyl peptidase, and is a dual activity enzyme capable of butyl esterase activities, as well as the well documented dipeptidase activity.

4.5 Conclusions

The biochemical data gathered by partial purification of Esterase II from *Streptococcus thermophilus* strongly support the hypothesis that Esterase II is X-prolyl dipeptidyl peptidase. The X-prolyl dipeptidase activity co-purifies with a butyl esterase activity. The N-terminal sequence of the major protein product present in the partially purified protein is very similar to PepX from other *Streptococcus* species, and also to the Esterase II N-terminal sequence obtained by Liu, *et al* (2001). This work demonstrates that PepX from *Streptococcus thermophilus* exhibits both a well-characterised dipeptidase activity, as well as a previously unknown butyl esterase activity.

The observation of a second band that catalysed ester bond hydrolysis, but did not have an associated dipeptidase activity, may be due to the degradation product of PepX. This would fit with the smaller size of the Esterase II protein observed by the prior study of this enzyme undertaken by Liu, *et al.* (2001), and the known instability of the enzyme. This may explain why Esterase II was observed as a monomer by Liu, *et al.* (2001) when all known PepX enzymes are dimers, if a domain vital to dimerisation was lost due to degradation processes.

4.6 Further Work

The observation of a second band on the native gel (center panel, Figure 4.2) that hydrolysed the chromogenic butyl ester substrate raises the question of whether this band represents a second esterase in the preparation, or if it is a breakdown product of the PepX enzyme. It is interesting to note that when Esterase II was originally purified it was found to have a mass of 65 kDa, and to exist as a monomer. This could indicate that a site for precise protein cleavage exists in the protein and what Liu and co-workers in fact observed was the end result of complete cleavage at this position. Because of the N-terminal sequence information gained by Liu, *et al.* (2001), this cleavage must result in the loss of the C-terminus of the protein, since the N-terminal was found to be intact. It is possible therefore that the second esterase activity observed on the native gel is a caused by a partial breakdown product of the enzyme, and not a different esterase species contaminant. Liu, *et al.* also noted the presence of a third esterase in *S. thermophilus*, Esterase III. This protein was not investigated further but its presence was noted as it eluted at a slightly different salt concentration to Esterase II during hydrophobic interaction chromatography. It is possible that Esterase III represents the uncleaved form of PepX, but this possibility remains speculative in the absence of further information.

The identity of the minor esterase active band could be firmly established by N-terminal sequencing to see if it is PepX. Unfortunately at the conclusion of this study there was insufficient protein left to perform this analysis. Repurification could be undertaken under conditions that reduce proteolysis to examine if the minor esterase band is still present on a native gel. Additionally, certain organic solvents, including up to 90% glycerol, that are reported to stabilise the PepX enzyme from *Lactococcus* (Chich *et al.*, 1995), could be added to crude solutions during the purification process to reduce activity loss by degradation, although organic solvents may also make the material difficult to handle in certain situations.

CHAPTER 5

Cloning of PepX from *Streptococcus thermophilus*

5.1 Introduction

The enzyme X-prolyl dipeptidyl peptidase from *Streptococcus thermophilus* is of interest to the dairy industry because of its apparent ability to hydrolyse butyl esters. Although tributyrin is not found in natural dairy compounds, butyl-containing acylglycerides are present, and since tributyrin is relatively stable and readily available in purified form it is an ideal compound to use as a marker for this desirable activity. Butyl esters in milkfat are believed to be involved in flavour formation in dairy products, particularly cheese.

PepX is expressed at a relatively low level in *Streptococcus thermophilus*, and purification of this enzyme is a tedious task requiring large fermentations of bacteria and a minimum of four different separation procedures. As relatively large amounts of enzyme are required for complete biochemical characterisation, it was advantageous to clone the *pepX* gene to allow heterologous production of the protein in a host cell. An additional benefit in cloning the gene is the possibility of introducing purification tags on either the N or C terminus of the expressed protein, which can make the purification of the recombinant protein much easier.

5.2 Objectives and Strategies

5.2.1 Objectives

The objectives of this section of work were to:

1) Clone the *pepX* gene from *Streptococcus thermophilus* into a plasmid vector to allow DNA sequencing, using contemporary molecular biology techniques
2) Introduce the *pepX* gene into a plasmid vector suitable for expression of the enzyme, with a histidine tag system for ease of purification.

The strategies used to achieve these goals are described below.

5.2.2 *Streptococcus thermophilus* Genetics

The genome sequence for *S. thermophilus* has not yet been released into the public domain, and at the time of this study, the *S. thermophilus pepX* gene had not been sequenced. However, a public database of *S. thermophilus* translated open reading frames was available (Goffeau *et al.*, 2003), as well as the entire genome sequences of several pathogenic *Streptococcus* species.

5.2.3 Cloning Techniques Investigated

Two different approaches to cloning the *Streptococcus thermophilus* PepX gene were used. The first method attempted was based on that described in (Bewley *et al.*, 1996) and was essentially a restriction fragment library approach that used Southern blotting to identify the *pepX* gene. Genomic material was prepared from the target organism, and digested with two different restriction enzymes to generate fragments of genomic DNA. This process was repeated with combinations of five different enzymes. The digested genomic material was run on an agarose gel to separate fragments of different mass, and then the DNA fragments were transferred onto a nylon membrane by blotting. The membrane was probed using a piece of DNA complementary to a section of the *pepX* gene, which allowed the mass of the restriction fragment containing the gene to be estimated. Double digested genomic DNA was separated by agarose gel electrophoresis, and the DNA that matched the estimated mass of the restriction fragment was excised from the gel. The enzymes used to produce the restriction fragment were used to clone the excised DNA into a plasmid vector. A set of clones was produced containing genomic DNA from the organism in the appropriate mass range estimated from the Southern blot, and with the same restriction sites as the material used to produce the Southern blot.

In the average microbial genome, there will be between 10 and 30 fragments produced by two six base recognition site enzymes in the size range large enough to contain the gene (M. Patchett, personal communication). This means that PCR can be used to screen the cloned material for the gene of interest. Alternatively, *E. coli* colonies can be screened using the same DNA probe that was used to probe the Southern blot using a colony lift technique. A schematic representation of this cloning approach is shown in Figure 5.1.

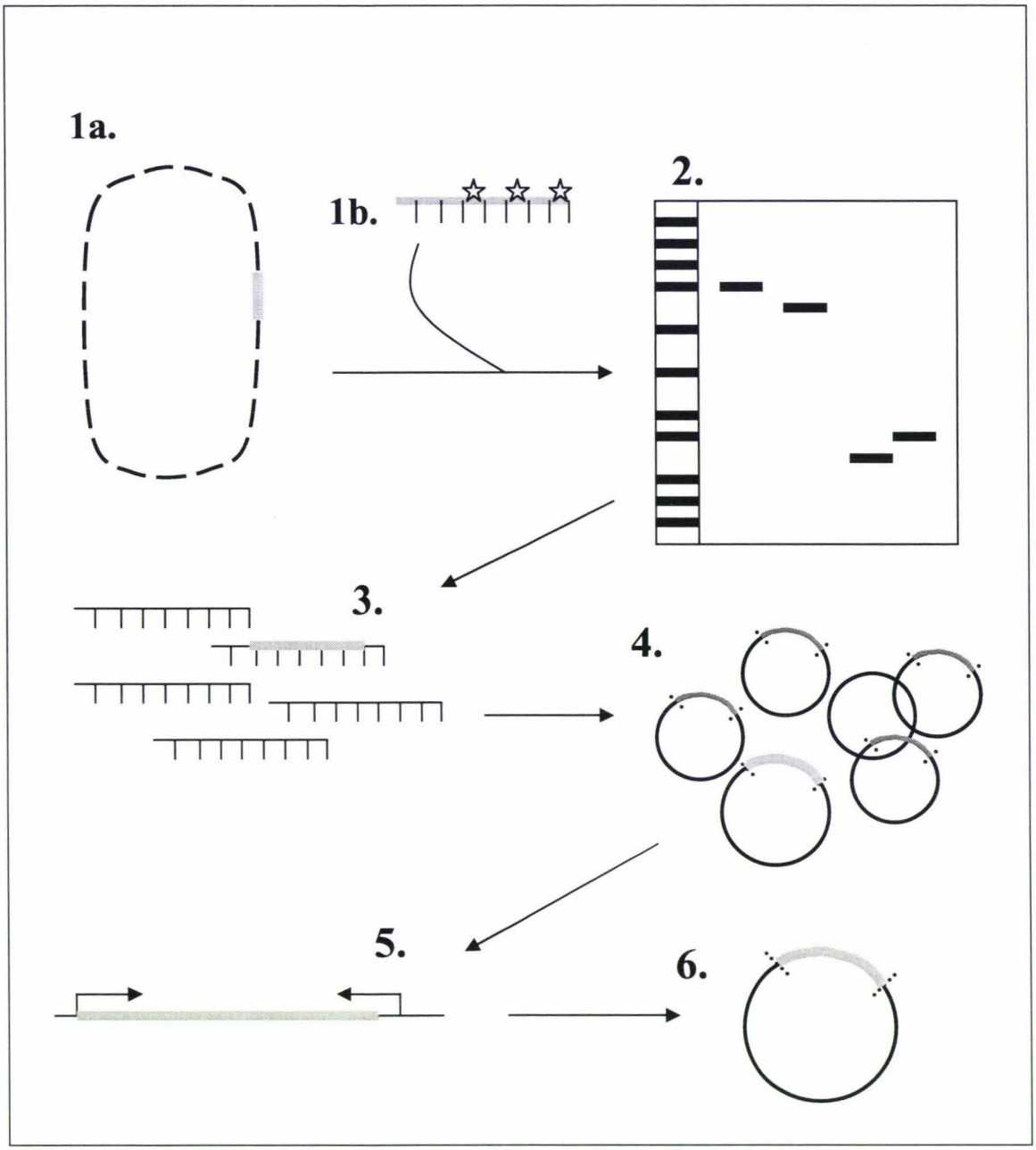


Figure 5.1 *Restriction fragment library cloning of specific genomic region.*

The gene of interest, in this case pepX, is shown in light grey. Representations are not to scale. ***1a:*** *Restriction endonuclease digestion of genomic material.* ***1b:*** *labelled gene-specific DNA probe.* ***2:*** *Southern blot of materials prepared in* ***1****.*
3: *Restriction fragments of genomic DNA, some containing gene of interest.* ***4:*** *Plasmid cloned genomic fragments* ***5:*** *PCR to identify genomic clone containing gene of interest.* ***6:*** *Plasmid containing gene of interest identified by PCR and DNA sequencing.*

The second method was based on the use of degenerate primer PCR, using publicly available protein sequence data to design DNA primers. Since protein sequences cannot be directly translated back into DNA sequence due to degeneracy in the

genetic code, primers were synthesized with up to four different bases in positions that vary in different codons coding for the same amino acid. This means essentially each primer synthesized is actually a series of primers that vary from one another at positions where there is degeneracy in the genetic code. The exact amount of degeneracy in a primer is described as the "degree of degeneracy". For example, a set of degenerate primers that differed from each other at one position only, where there are two possible bases at that position would have two degrees of degeneracy.

It is important that degenerate primers are not over-degenerate, as this may result in the primers annealing to other DNA sequences during PCR. Degeneracy can, however, be reduced by looking at the characteristics of coding DNA sequences, such as how often a codon for a particular amino acid is used. Where there is low codon usage (<~1%) for a particular amino acid the codon can usually be ignored, as the chances of the codon being present are negligible. Also, different codons are often used at different positions in a gene and such information about the way an organism uses different codons to code for the same amino acid allows the design of primers that will be most likely to anneal to the gene of interest during PCR whilst producing a minimum of other unwanted products. Once the gene has been amplified using PCR, it can be cloned into a vector for subsequent sequencing and protein expression.

5.3 Methods

5.3.1 Cloning of *PepX* by Restriction Fragment Screening

5.3.1.1 Genomic DNA preparation

A 500 mL overnight culture of *Streptococcus thermophilus* was grown in M17 medium at 37 °C. Genomic DNA was then extracted from the cells using a method adapted from a *Lactococcus* large plasmid extraction technique (Anderson and McKay, 1983), in combination with the DNA preparation reagent DNAzol™ (Life Technologies). The cells were pelleted by centrifugation at 5000 x g for 15 minutes. The cells were resuspended in 30 mL of 50 mM Tris, 1mM EDTA with 6.7% sucrose (w/v), pH 8.0. The solution was warmed to 37 °C and 7.5 mL of 10 $mg.mL^{-1}$ lysozyme in 25 mM Tris at pH 8.0 was added. The cell suspension was incubated for 5 minutes at 37 °C before 2.25 mL of 20% SDS and 20 mM EDTA in 50 mM Tris-HCl at pH 8.0 was added and mixed by gentle inversion. Cell lysis was completed after 5 minutes.

1 volume of DNAzol™ reagent was added to precipitate protein material from the lysed cells, and the precipitate was removed by centrifugation at 10000 x g for 30 minutes, at 4 °C. DNA was precipitated by the addition of 0.5 volumes of ice-cold ethanol to the supernatant. The precipitate was pelleted by centrifugation at 10000 x g, and then washed with 95% ethanol. The DNA was repelleted, washed again, and air-dried for 15 minutes. The DNA was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), and stored at 4 °C.

Techniques likely to damage large DNA molecules such as the use of vortex mixers and freezing of samples were avoided when handling the genomic material. Solutions containing whole genomic material were therefore stored at 4 °C in TE buffer to minimize the activity of nuclease enzymes on the DNA.

5.3.1.2 Restriction Digests

DNA was digested using restriction endonuclease enzymes according to the manufacturers directions. All digests were performed in volumes of 50 µL. Where large amounts of DNA were digested, a large volume of solution containing all

ingredients was made, and divided into 50 µL aliquots to maximize heat transfer into the mixture.

5.3.1.2.1 Digestion of Genomic DNA

Genomic DNA was obtained as described above. The DNA was then digested with two different restriction endonucleases using different combinations of several restriction endonuclease enzymes as shown in Table 5.1.

To check if digestion was complete, the cleaved DNA was analysed by gel electrophoresis on a long agarose gel, to maximize fragment size resolution. The DNA bands were visualized by UV illumination, after staining with ethidium bromide.

5.3.1.3 Generation of a DNA Probe for *pepX* Gene

A forward primer was designed based on the known N-terminal sequence of the *Streptococcus thermophilus* PepX protein. Using computer-based similarity searches of putative and biochemically confirmed *pepX* sequences in *Streptococcus* species, a reverse primer was designed based on a region of conserved gene sequence. This conserved region was located approximately 550 base pairs into the gene. These primers were named Peptidase_probe_FWD and Peptidase_probe_RVS respectively. The sequences of these primers can be found in Appendix 3.

Using the primers described above, a PCR reaction was performed to generate a 550 bp DNA product. This PCR reaction was carried out using a denaturation step at 95 °C for 1 minute, followed by annealing at 45 °C for 1 minute, then a polymerase extension step at 72 °C for 1.5 minutes. 50 cycles of PCR were carried out using *Taq* DNA polymerase (F-Hoffman, La Roche Ltd, Switzerland). PCR for such a large number of cycles using *Taq* polymerase should normally be avoided, because of the likely generation of PCR induced errors into the DNA product due to the fact that *Taq* polymerase has no proofreading 3′ exonuclease activity. In this case however, a true replication of the sequence was not important, as the DNA probe should be sufficiently similar to hybridise to the target DNA sequence during the blot probing process, even with several PCR induced sequence errors present.

In addition to using a large number of PCR cycles to generate more DNA product, up to 10 PCR reactions of 50 µL each were carried out in parallel to generate sufficient

DNA product for the planned experiments. The reaction product was then gel purified using a gel extraction kit (Qiagen Inc., Valencia, California, USA).

5.3.1.4 Cloning of PCR Product

Several approaches were used to try and clone the PCR product into a plasmid vector that could then be transformed into the appropriate cell line, in order to increase the yield of the DNA probe, since very little probe was produced in each PCR reaction. Plasmid material containing the DNA probe was isolated, purified and digested with the appropriate restriction endonuclease to release the probe from the plasmid. Finally the liberated DNA probe was gel-purified and used to probe the Southern blotted *S. thermophilus* genomic DNA.

5.3.1.5 Non-cohesive Ended Cloning

Blunt (non-cohesive) end cloning was attempted, to introduce the *pepX* probe DNA into a pGEM 3zf– plasmid vector (Promega, Madison, Wisconsin, USA). Although *Taq* polymerase is well known for generating 3′ poly-thymidine overhangs when it completes the replication of a strand of DNA, a significant percentage of these ends are blunt, and can therefore be cloned into a non-cohesive restriction site (M. Patchett, personal communication).

The vector was digested with *Sma* I (New England Biolabs Inc., Beverly, Maryland, USA), according to manufacturers directions to generate non-cohesive DNA ends in the multiple cloning site of the plasmid vector. The digested plasmid was precipitated with ethanol, and then resuspended in sterile ddH_2O. The plasmid DNA was then dephosphorylated using calf thymus alkaline phosphatase (Roche) according to manufacturers instructions. The dephosphorylated vector was purified using a PCR cleanup kit (Qiagen), to remove residual alkaline phosphatase enzyme, a step that is essential in cloning into a single restriction site as it prevents self-ligation of the digested vector ends.

A ligation mixture containing 1 µL of digested plasmid DNA, 8 µL of probe DNA, 2 µL of DNA ligase buffer, 1 µL of T4 DNA ligase (Roche) and 8 µL of H_2O was mixed by inversion, and incubated at 16 °C overnight.

The ligation mixture was used to transform a thawed frozen stock of heat shock competent XL-1 *E. coli* cells, using the heat shock transformation method described in general methods (Section 2.3.6)

Cells were plated onto LB agar containing ampicillin, IPTG and X-gal, incubated overnight then examined for colony growth. The pGEM 3zf- plasmid contains a multiple cloning site (MCS) inserted into the gene that processes X-gal. Thus colonies with no insert in the plasmid are able to process the X-gal synthetic substrate into a coloured product that makes them appear blue, whilst colonies with an insert in the MCS appear white. This makes it easy to select colonies harbouring plasmids with an insert in the MCS.

5.3.1.6 Restriction Mapping of DNA Probe

Test digests of the DNA probe were undertaken in an attempt to empirically locate restriction endonuclease sites that could be used for cohesive ended cloning of the probe, as this was judged to be more likely to succeed than non-cohesive cloning, due to the cohesive insert and plasmid ends having an affinity for each other. Restriction digests were carried out in a total volume of 20 µL, with 1 µL of endonuclease added. The restriction endonuclease enzymes *Eco* RI, *Bam* HI, *Hin*d III, *Kpn* I and *Sal* I (Roche) were incubated with the DNA probe using conditions recommended by the manufacturer and the DNA was analysed on a 1% TAE buffered agarose gel to see if digestion had occurred.

5.3.1.7 Southern Blotting of Genomic DNA

5.3.1.7.1 Overview of Southern Blotting Process

Southern blotting was carried out using standard protocols as described in (Sambrook *et al.*, 1989).

Double digested genomic DNA from *Streptococcus thermophilus* B2513 was separated using a 20 cm long 0.7% agarose TAE buffered gel. The gel was run overnight at 25 volts, with buffer recirculation to prevent ionic depletion, until the dye front had run ¾ of the length of the gel. The outer lane, loaded with 1 kb plus DNA size standards (Invitrogen), was removed from the gel with a clean scalpel blade, EtBr stained and photographed for future reference. The gel was soaked in two changes of denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 2 x 20 minutes, washed briefly in Milli-Q water, then soaked in 2 changes of neutralisation solution (1 M ammonium acetate, 20 mM NaOH) for 2 x 20 minutes. After denaturation and neutralization, the gel was soaked in 20X SSC buffer (3 M NaCl, 0.3 M Na citrate pH 7.0) for 20 minutes before blotting. A glass plate was laid across a buffer reservoir and two layers of thin filter paper were placed on top of it. Four pieces of parafilm were used to form a "window" on the filter paper through which buffer could be drawn. The gel was carefully placed on top of the parafilm layer, in contact with the filter paper, and flooded with excess 20X SSC buffer to remove any bubbles trapped between the gel and filter paper.

A nylon membrane (Hybond, Amersham), pre-wetted in 20X SSC, was placed on top of the gel, followed by two pieces of 3 mm filter paper. A stack of paper towels was placed on top of the stack and finally a 1L Schott bottle containing water to a final weight of 400 g was added to hold the stack together. The buffer reservoir was filled to ensure that a constant flow of buffer occurred during the blotting process. The Southern blot apparatus was left overnight to allow the DNA to be transferred from the gel to the membrane by capillary action. After DNA transfer was complete, the stack was disassembled and the gel was stained in EtBr, and photographed under UV illumination to check for transfer of DNA. The membrane was dried and wrapped in plastic film prior to probing and detection.

5.3.1.7.2 Probe Labelling

The DNA probe was labelled with horseradish peroxidase using the ECL system (Amersham). The probe was diluted to 10 ng.μL^{-1}, then 100 ng (10 µL) was boiled for 5 minutes, before being quickly cooled by plunging into ice. This boiling step separates the DNA duplex while the fast cooling prevents the helices reassociating. 10 µL of labelling solution was added to the DNA, mixed, and the solution spun briefly in a microcentrifuge to settle the tube contents. 10 µL of glutaraldehyde solution was added to the DNA/label mixture, which was then mixed and centrifuged as before. The solution was then incubated at 37 °C for 10 minutes. Incubation at this temperature causes glutaraldehyde-mediated crosslinks to form between the horseradish peroxidase enzyme and the DNA probe. After incubation, the probe was stored on ice prior to addition to the hybridisation mixture. The ECL system relies on the peroxidase enzyme being directly conjugated to the DNA probe, in contrast to other systems that use antibodies to tether the peroxidase to DIG (Digoxigenin) labelled DNA. For this reason care must be taken not to expose the labelled probe to conditions that may reduce enzyme activity, such as temperatures above 42 °C.

5.3.1.7.3 Hybridisation of Probe to Blot

The hybridisation buffer, as supplied with the ECL kit, was prewarmed to 42 °C in a waterbath. The genomic DNA blot was prewetted in 5X SSC buffer, rolled and placed into a hybridisation tube. The blot was unrolled until it lay flat against the sides of the tube. Bubbles were removed from between the blot and the wall of the tube, by flushing with 5X SSC buffer. The 5X SSC was removed by decanting, and 20 mL of prewarmed hybridisation buffer was added to the tube. The blot was then placed in a motorised hybridisation oven (Hybaid, Germany) at 42 °C and rotated at 30 rpm for 20 minutes. After prehybridisation the labelled probe was added to the buffer reservoir, and hybridisation was performed at 42 °C for 18 hours at 30 rpm.

The hybridisation buffer was discarded and 50 mL of 5X SSC added to the tube. The tube was rotated in the oven at 42 °C for 5 minutes to remove traces of hybridisation buffer.

The 5X SSC was removed from the tube, and the tube was filled 1/3 with prewarmed (42 °C) primary wash buffer (6 M Urea, 0.4% SDS and 0.5X SSC). After further

rotation in the oven for 20 minutes, a second primary wash step was carried out for a further 10 minutes with fresh primary wash buffer.
The blot was removed from the hybridisation tube and unrolled into a glass dish, then covered in secondary wash buffer (2X SSC) and agitated for 5 minutes. This step was repeated with fresh secondary wash buffer.

5.3.1.7.4 Detection of Probe Hybridisation

After the stringency washing steps had been completed, the membrane was placed in a clean glass dish, and premixed ECL detection reagent was added. The final reagent is a mixture of two proprietary solutions that produce Chemiluminescence as they are acted upon by the horseradish peroxidase conjugated to the DNA probe.
The blot was drip dried, wrapped in Clingfilm then placed in an autoradiography cassette with a sheet of X-ray film (Kodak). 3 separate exposures were made for 1 minute, 2 minutes and 50 minutes and the film developed in an automatic developer (Agfa). The autoradiograph was then compared with the 1kb ladder excised from the gel earlier, to allow the estimation of the molecular size of the bands that the probe had hybridised to.

5.3.1.8 Cloning of Genomic Restriction Fragments

The digested genomic DNA was separated by agarose gel electrophoresis. A 1 cm length of each lane, corresponding to the approximate size of the restriction fragments identified by Southern blotting, was excised with a scalpel. The DNA was extracted from the gel slice using a gel extraction kit (Qiagen), as described in general methods, then ligated into a suitably prepared pGEM 3fz- plasmid vector using standard molecular biology protocols (Sambrook *et al.*, 1989). Transformants were selected using ampicillin and X-gal, and the presence of a *pepX* gene insert confirmed by PCR using a vector sequencing primer, and one of the primers used to generate the DNA probe. Vectors that produced a PCR product were sequenced and the sequence examined for similarity to other known streptococcal *pepX* sequences.

5.3.2 Cloning of *pepX* Gene by Degenerate Primer PCR

5.3.2.1 Primer Design

The protein sequence for a putative *S. thermophilus pepX* gene was obtained by searching the publicly available *S. thermophilus* genome sequencing database (Goffeau *et al.*, 2003) for similar sequences. At the time this work was carried out, a protein sequence for PepX was the only sequence available. Other streptococcal *pepX* and putative *pepX* nucleotide sequences were obtained by similarity searches from the non-redundant (nr) section of the GenBank database at NCBI (Altschul *et al.*, 1997). Comparison of these nucleotide sequences, in conjunction with a codon usage table for *S. thermophilus* provided the basis for the design of a pair of degenerate primers complementary to both the N and C termini of the *S. thermophilus pepX* gene, called PepX_deg_Fwd and PepX_deg_RVS respectively.

The primers that were designed were longer than would normally be used for PCR at 23 nt long (PepX_deg_Fwd) and 24 nt long (PepX_deg_RVS). Because there is a likelihood of primer/template mismatches occurring during PCR, the annealing temperature of the primers will be effectively raised. A longer complementary region to the target sequence will help to overcome such potential mismatches. The sequences of these primers can be found in Appendix 3.

5.3.2.2 Degenerate Primer PCR

A series of PCR reactions were carried out using undigested genomic DNA as a template and *Taq* DNA polymerase (Roche). Decreasing annealing temperatures were applied until a temperature was found that gave a reasonable yield of the desired product with minimal background. A large scale PCR was performed using this optimised temperature regime in 10 x 50 µL volumes. The protocol included a denaturation step of 95 °C for 1 minute, an annealing step of 42 °C for 1 minute and extension at 72 °C for 1.5 minutes, for 40 cycles.

A PCR product the expected size of the *pepX* gene (based on comparison with other *pepX* gene sequences) was identified by agarose gel electrophoresis. This product was excised from the gel, purified using a gel slice purification kit (Qiagen), then subjected to digestion with a range of restriction endonuclease enzymes. Several enzymes that did not cut within the PCR product were identified and more primers

were designed to incorporate terminal recognition sites for these enzymes that would allow the cloning of the PCR product into a plasmid vector. The sites chosen were *Nco* I (forward primer) and *Sal* I (reverse primer), since these enzymes both cut within the plasmid vector multiple cloning site in an appropriate position to allow directional cloning of the *pepX* gene in the correct orientation. These primers were named PepXDegFWDNcoI and PepXDegRVSSalI, and these sequences can be found in Appendix 3.

PCR was then carried out using the primers containing restriction sites. After optimisation of the annealing temperature, the gene coding region was amplified from genomic material using five PCR cycles with *Taq* polymerase. After 5 cycles the reaction products were ethanol precipitated, and resuspended. This product was then used as a template for another PCR reaction of 30 cycles using *Pwo* DNA polymerase (Roche). This approach was necessary as a PCR reaction using a genomic DNA template, *Pwo* enzyme and degenerate primers gave no amplified product.

5.3.2.3 Digestion and Ligation of PCR Product in Plasmid Cloning Vector

The restriction sites introduced into the ends of the gene during PCR were digested using the appropriate restriction endonucleases to produce cohesive DNA ends. The digested insert was then ligated into pre-prepared double digested pProEx HtB vector (InfoTech) using T4 DNA ligase, at 16 °C overnight. *E. coli* XL-1 cells were transformed using heat shock, and any antibiotic resistant transformants containing a plasmid insert of the correct size were identified by PCR using a vector forward sequencing primer (M13 pUC Reverse) and a custom primer designed to anneal to the 3′ region of the plasmid MCS in a reverse orientation (HtB_Reverse). This was performed using colony PCR as described in Section 2.3.15. The sequence of these primers is recorded in Appendix 3.

5.3.2.4 Clone Selection and Sequencing

A clone containing an insert of the expected size of the *pepX* gene was selected, grown in liquid culture, and the plasmid material extracted. This material was submitted for sequencing. The sequencing reactions were primed using the plasmid vector's forward sequencing primer, and the HtB_Reverse primer based on the 3′ region of the plasmid MCS. Gaps in the middle of the gene beyond the sequence

obtained with the vector primers were sequenced using primers based on the sequence of the 5′ and 3′ ends of the initially obtained sequences, and were located in a region of unambiguous sequence about 400 bp from each end of the gene.
The complete sequence of the insert was submitted to the NCBI website for BLAST analysis to confirm the insert was the PepX gene.

Some of the remaining plasmid vector was used to transform competent *E. coli* BL-21 cells, using the heart shock protocol described in the general methods section. A single transformant colony was selected and grown in LB broth with ampicillin selection. The presence of the insert in the transformant cell line was verified using PCR. Glycerol stocks were made from the LB culture and these were stored at –80 °C until required in subsequent investigations.

5.4 Results and Discussion

5.4.1 Southern Blotting

Figure 5.2 shows the Southern blot produced by probing double restriction endonuclease digested genomic DNA from *S. thermophilus* with a probe complementary to the PepX gene. Table 5.1 shows the enzymes used to digest the genomic DNA analysed on the blot.

In most lanes, the probe has hybridised to a discrete band except for lane 8. In this case, the probe appears to have hybridised in a smear. This is most likely to be due to incomplete digestion of the genomic DNA in spite of the fact that genomic digests were allowed to run overnight to ensure complete digestion occurred. Some lanes have faint regions indicating other hybridisation may have occurred. These may be due to non-specific background interactions, shearing of genomic DNA during preparation, or non-specific nuclease digestion of DNA due to residual nuclease activity in the DNA preparation. Lanes 1, 2 and 3 of the blot appeared to have restriction fragments of the same size that hybridised to the probe. These lanes were also all cut with the *Eco* RI enzyme in combination with another endonuclease, as indicated in Table 5.1. Similarly in lanes 5 and 6, an identically sized region has hybridised to the probe. It is possible that these bands represent different restriction fragments that are the same size by coincidence. A more likely explanation however is that the identical size of these bands indicates that the DNA fragment containing the *pepX* probe sequence has no *Bam* HI or *Sal* I cutting site within it, and is instead bordered by two *Eco* RI sites. This could have been confirmed by probing a Southern blot of genomic DNA that had been digested with one restriction endonuclease only. Due to time and resource constraints, this was not done. Alternatively, the probe itself could be sequenced in order to identify any restriction sites. This was not attempted because cloning of the probe to enable sequencing to be done from a plasmid was unsuccessful, and dideoxy termination DNA sequencing is difficult to achieve when degenerate primers are used (C. Matthews, personal communication). The fragments identified by probing the Southern blot produced by *Eco* RI in lanes 1-3 and by *Kpn* I in lanes 5 and 6 were long enough, at approximately 4kb, to contain the entire *pep*X gene. The other fragments identified were too small to contain the entire *pep*X gene. The *Eco* RI and *Kpn* I fragments were therefore used for the subsequent cloning procedure.

Digest	Enzymes used
R1	*Eco* RI + *Kpn* I
R2	*Eco* RI + *Bam* HI
R3	*Eco* RI + *Sal* I
R4	*Eco* RI + *Hin*d III
R5	*Kpn* I + *Bam* HI
R6	*Kpn* I + *Sal* I
R7	*Kpn* I + *Hin*d III
R8	*Bam* HI + *Sal* I
R9	*Bam* HI + *Hin*d III
R10	*Sal I* + *Hin*d III

Table 5.1 *Enzymes used to digest genomic DNA for each lane of the Southern blot*

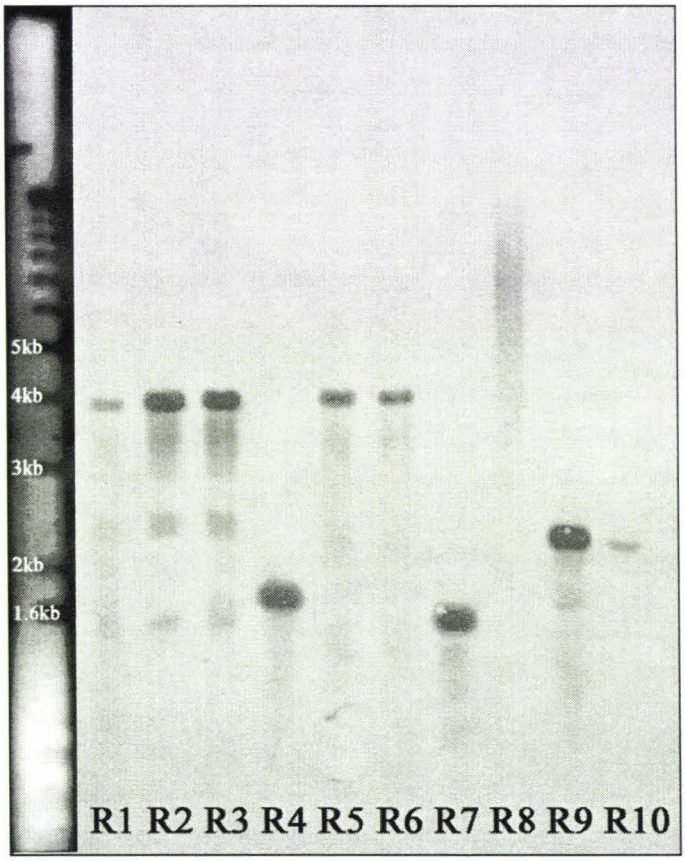


Figure 5.2 *Southern blot of digested genomic material probed with PepX gene. Table 5.1 lists the enzymes used to produce R1 – R10, the sample in each lane.*

Several attempts were made to clone appropriately sized restriction fragments from *S. thermophilus* genomic DNA. Approximately 30 clones containing inserts were identified by X-Gal α-complementation selection. One of these inserts produced PCR

products when used as a template in a PCR reaction with either one or both of the probe primers and the vector primers, suggesting that at least part of the *pepX* gene was present. Subsequent sequencing of this clone showed that whilst *S. thermophilus* genomic material had been cloned, the *pepX* gene was not present. Analysis of the clone sequence was carried out using CLUSTAL X sequence alignment software (Thompson *et al.*, 1997) to align the probe primer sequences to the DNA sequence. This showed that the probe primers had most likely annealed to other sequences located in the cloned *S. thermophilus* genomic material that had a high similarity to the primer sequence, but were not the *pepX* gene. If PCR had been carried out using the two probe primers, no product would have been produced, as the primer binding sites in the cloned DNA actually faced outwards from each other (Figure 5.3). A PCR reaction using both of the probe primers was not done as it was considered unlikely to work effectively given the low efficiency of the PCR reaction used to produce the DNA probe using these primers.

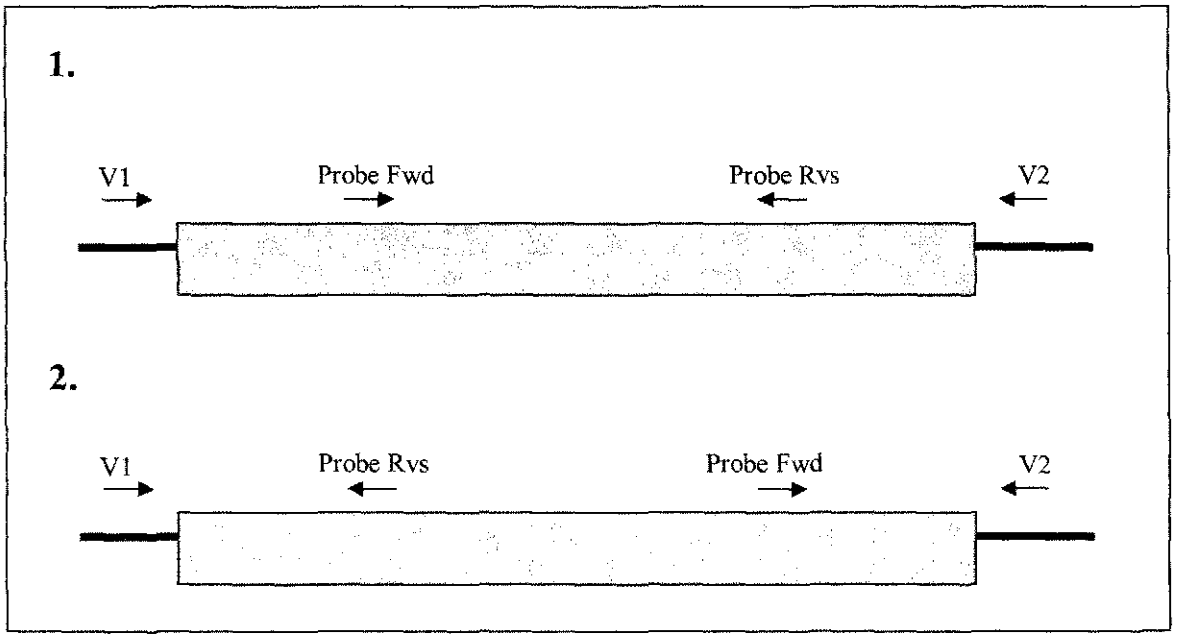


Figure 5.3 *PCR to identify fragment containing probe sequence from restriction fragment library.*

The grey box represents the cloned genomic DNA, and the black lines represent the MCS of the plasmid vector. V1 and V2 represent the vector based primers T7 Forward and SP6 Rvs. Probe Fwd and Probe Rvs represent the primer set used to generate the DNA probe used in Southern blotting and later used to attempt to identify the genomic fragment containing the pepX gene. Panel 1 illustrates the anticipated positions of primers if the pepX gene was present, and panel 2 demonstrates how the primers annealed to give a false positive identification of the pepX gene as binding occurred to a random genomic fragment. PCR reactions were performed using V1 and Probe_Rvs, and V2 and Probe_Fwd.

The reason for the failure to clone the *pepX* gene using this method may be explained using simple statistics. A restriction endonuclease with a 6 base pair recognition sequence cuts DNA on average every 4^6 bases (4096 bases). If two 6-base recognition sequence restriction endonucleases are used in tandem, the expected size of the fragments generated would be, on average, half that of the fragments produced by a single enzyme, approximately 2048bp. Assuming a normal distribution of fragment sizes about a mean of 2048bp, a 4kb DNA fragment that is a product of digestion by two different enzymes would be relatively rare, and the number of fragments of this size in the genome would be relatively low. This means only a few fragments would be selected during the cloning process, and it would be easy to screen for the fragment

of interest using techniques suited to analysing small numbers of clones, such as PCR, as opposed to techniques more suited to large-scale analyses, such as colony probing. Statistically it is very difficult to estimate the number of fragments expected from the restriction digestion of a particular genome, although theoretically, fragment size from a single digest should be normally distributed about a mean of 4096bp.

The difficulty in estimating the number of fragments produced from a genomic digestion occurs for a number of reasons. For example the GC content of the genome will affect how often a particular enzyme cuts the DNA, since the recognition sites for 6 base cutting enzymes are generally palindromic, and therefore must be at least 2/3 GC or 2/3 AT. In addition, factors that alter the randomness of DNA, such as coding sequences, will cause the distribution of the fragment size to deviate from the norm. Assuming a restriction endonuclease does cut, on average every 4096bp, if the 1.8Mb genome of *S. thermophilus* was digested with a single 6bp recognition-site restriction enzyme approximately 440 fragments of 4096bp would be the expected product. Selecting the appropriate gene fragment would therefore be a more difficult task as large numbers of clones would have to be screened to locate one that carries the desired gene.

Since it seemed likely that a pair of Eco RI sites flanked the pepX probe sequence, the number of clones to be screened would in theory, be in the hundreds. This may explain the failure of this approach to select a genomic clone containing the *pepX* gene. In the case of this study of *S. thermophilus*, an organism with a high genomic AT content, the use of restriction enzymes with a preference for AT rich recognition sites might have resulted in a more fragmented genome, and subsequent ease of selection of a clone containing the desired sequence.

5.4.2 Degenerate Primer PCR

After temperature optimisation of the PCR reaction, a large PCR was performed to prepare material for restriction mapping to empirically identify restriction sites contained within the *pepX* sequence. The DNA produced by this reaction was gel purified and digested using several restriction endonucleases. The product of the restriction digestion was run on a gel and examined for a reduction in the product size, or for the presence of 2 or more bands. Table 5.2 shows the results of these restriction digests.

Enzyme	Digestion
Sal I	No
Not I	No
Hind III	Yes
Nco I	No
Bam HI	No
Eco RI	Yes
Kpn I	Yes

Table 5.2 *Empirical identification of restriction endonuclease sites in PepX gene. Digestion of the PepX gene was judged by analysis of DNA using agarose gel electrophoresis*

As discussed in the methods section of this chapter, problems were encountered in carrying out the PCR reaction with restriction site incorporating primers and *Pwo* proofreading DNA polymerase. However this reaction could be made to work with *Taq* DNA polymerase. It is possible that the 3′ proofreading exonuclease activity of the *Pfx* enzyme used interfered with the first few cycles of the PCR reaction. While the primer sequence designed to complement the target sequence in the template anneals to the template, the restriction sites and terminal enzyme tethering bases do not. Such a large degree of mismatching may prevent the enzyme from either attaching to the DNA molecule or elongating the template. The PCR reaction to generate enough restriction site incorporating gene insert was eventually performed using a short PCR reaction of five cycles with *Taq* polymerase followed by a 30 cycle PCR reaction using *Pwo* DNA polymerase as outlined in the methods section of this

chapter. This reaction worked at a low efficiency, but enough template was generated for the cloning procedure. This use of two polymerases in sequence was designed to take advantage of the ability of *Taq* to replicate a template with mismatched primers to produce enough DNA molecules to act as a template for *Pwo*, which, because it has a 3′ proofreading activity, is less likely to introduce errors into the cloned sequence. It was reasoned that the products from a low cycle PCR would be a better template for the *Pwo* catalysed PCR reaction, as there would be no mismatches between the primer and the template. This approach proved to be successful.

Vector DNA was prepared for ligation by digestion with *Nco* I and *Sal* I restriction endonucleases as directed by the enzyme manufacturer, with ethanol precipitation being used between digestions to change the buffer present, as described in Methods section 2.3.5. Digestion was followed by agarose gel purification to remove any undigested plasmid.

After ligation of the amplified insert into the pProEX expression vector, colonies were selected and the presence of an insert was verified by PCR using the vector based primers M13/pUC Reverse and pProEX_Ht_Rvs, followed by agarose gel electrophoresis as shown in Figure 5.4. Nineteen colonies were screened and four were identified that contained an insert of the expected size. A further four colonies harboured plasmids with inserts of a smaller size than the *pepX* gene. This was possibly due to non-specific endonuclease activity producing a shortened PCR product that was subsequently ligated into the vector. The remaining 11 colonies contained no insert but did harbour plasmids as shown by the presence of low molecular weight PCR products that are likely to be the plasmid MCS which lies between the vector sequencing primers. This is an unusually high background, and may be due to incomplete digestion of the plasmid during one of the vector digestion steps. In hindsight, this could have been avoided by dephosphorylation of the vector with alkaline phosphatase. A dephosphorylation step was judged to be unnecessary, because this was a cohesive ended ligation, and the plasmid material had been gel purified to remove any entire plasmid and MCS linker material. It is still possible that some plasmid remained that was only cut with one enzyme. Such material cannot be removed using gel purification since it is very similar in size to double cut plasmid and cannot be resolved on a 1% agarose gel. In this case however, the PCR screen

was successful in identifying clones that contained a plasmid that had self-ligated with no insert.

The sequence returned from the pooled plasmid DNA of these colonies was an almost exact match to an open reading frame from the *S. thermophilus* partial genome sequence, and was very similarity to other *pepX* genes from *Streptococcus* species. A single clone was selected (colony 9), grown in liquid culture and the plasmid material extracted using a plasmid miniprep kit (BioRad). The gene insert in the plasmid was then sequenced entirely, using the vector primers M13 pUC Reverse and pProEx Ht Rvs, and primers internal to the *pepX* gene, PepX_int_Fwd and PepX_int_Rvs, that were designed from the sequence obtained with vector primers. The sequences of these primers can be found in Appendix 3. A schematic diagram of where the primers annealed relative to the *pepX* gene is shown in Appendix 2.

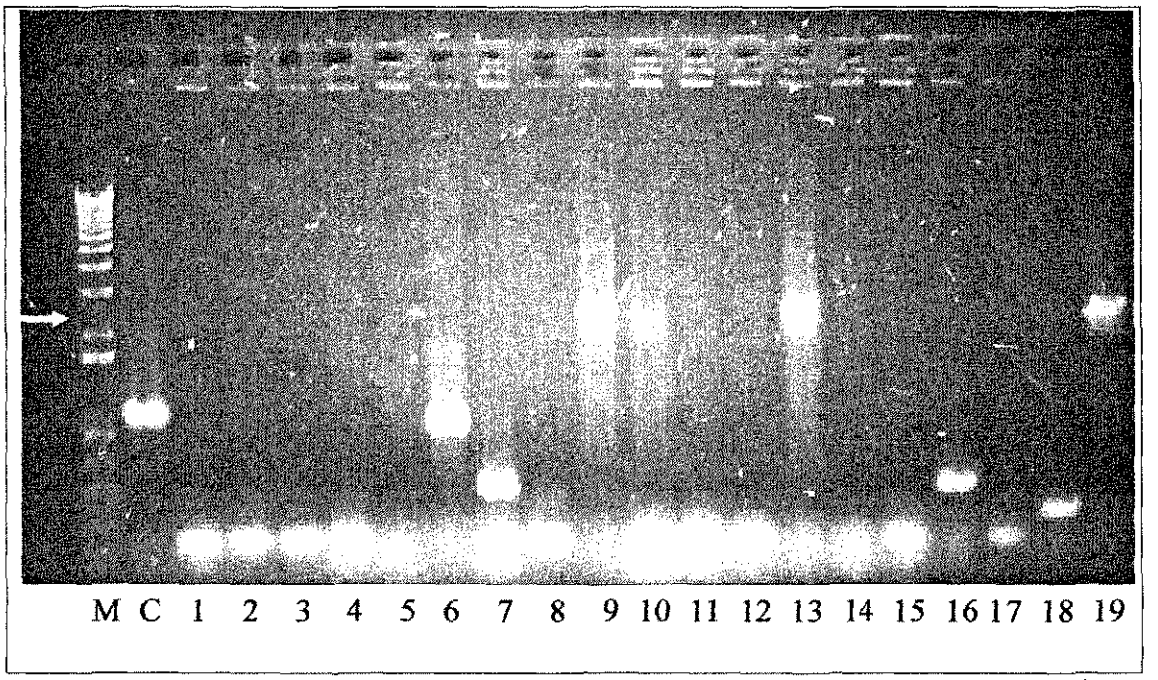


Figure 5.4 *An agarose gel showing the products of the colony PCR used to identify* E. coli *clones carrying a gene insert. Lane M contains DNA size marker (1kb plus ladder, as detailed in Appendix 11). Lane C is a control PCR reaction performed on cells hosting a plasmid with an insert of known size. The white arrow, at left, indicates the expected position of the pepX gene on this gel, at 2.25kb.*

5.4.3 Sequence of Cloned *pepX* Gene

The entire clone insert was sequenced (Appendix 4), as described in Section 2.2.1. The pProEX HtB plasmid vector containing the *pepX* coding sequence insert was named PepX HtB. The sequence was compared with the gene sequence of *pepX* from the ACA-DC4 strain of *S. thermophilus* that was published after this *pepX* gene cloning process was complete (Anastasiou *et al.*, 2002). A comparison of these sequences is shown in Appendix 5. The protein translation of the nucleotide sequence was also compared with the PepX protein sequence from the ACA-DC4 and LMG18311 strains of *S. thermophilus* (Anastasiou *et al.*, 2002; Goffeau *et al.*, 2003). This comparison is shown in Appendix 6.

5.4.3.1 Nucleotide Sequence

The nucleotide sequence of *S. thermophilus* B2513 *pepX*, as shown in Appendix 5, was found to differ from that of ACA-DC4 *pepX* in 15 positions that did not include the degenerate primer induced alterations to the sequence. In addition, PepX from ACA-DC4 appears to have an extra amino acid residue located at the C-terminus of the molecule.

It is difficult to tell whether these differences are due to PCR induced errors or strain differences. However, the distribution of the nucleotide differences does not appear to be random, as would be expected with PCR induced nucleotide substitutions, but appear to be clustered towards the 3′ half of the gene. This suggests that the changes are not PCR induced substitutions, and are more likely to reflect real differences in sequence in the different strains of *S. thermophilus*. For gene cloning from both strains, PCR was utilized in the gene isolation process, so it is possible that some of the differences are due to PCR errors in one or both sequences. It is impossible to tell if this is the case, however, without further information such as direct sequencing of the gene from genomic material, since the genomic sequence of *S. thermophilus* is not yet available from the group currently sequencing this genome. Genomic sequences could be obtained by using a library screening method to isolate the gene from genomic material. As described above, this type of approach was preferred and originally attempted in the cloning of the *pepX* gene from *S. thermophilus* B2513, but this attempt failed (Section 5.4.1). Alternatively, limited genomic sequence could be obtained directly from genomic material using capillary electrophoresis DNA sequencing. In the case of this study, time was a limiting factor for carrying out such sequencing.

The use of degenerate primers to amplify the gene has also resulted in some PCR induced nucleotide substitutions. However these primers were designed to preserve the protein sequence of the gene product and so these substitutions result in the same amino acid residue in the protein product. There is one exception in the forward primer. The second codon of this primer was deliberately altered to facilitate the introduction of a restriction site in the correct frame. This was intended to reduce the length of the primer used by minimizing the introduction of extra base pairs that would not actually anneal to the template during PCR. This change in the N-terminal region of the protein was judged to have minimal effect on the enzyme activity.

5.4.3.2 Protein Sequence

The translated protein sequence of the *S. thermophilus* strain B2513 *pepX* gene contains seven residue substitutions compared to the consensus PepX protein sequence from *S. thermophilus* strains ACA-DC4 and LMG18311, shown in Appendix 6. This number discounts the deliberate change made by the forward primer and discussed in the section above. Once again, the distribution of changes in the protein sequence is clearly not random, but strikingly clustered around the C terminus of the molecule. Also of note is the fact that the single nucleotide substitution in the 5' half of the gene does not lead to a change in the amino acid residue for which the triplet codes, so the N terminal 2/3 of PepX from the two strains compared is identical. There are also some changes between PepX from LMG18311 and ACA-DC4, so all three strains do have some protein sequence differences.

The PepX protein is known to consist of four domains, with the αß hydrolase catalytic domain being located in the N terminal half of the protein chain (Rigolet *et al.*, 2002). It is interesting that no sequence changes are present in the catalytic or the α helical N-terminal domains of the protein, but all seven of the residue changes are located in the C terminal part of the molecule, which has no documented catalytic role. The C terminal domain of the molecule consists of a ß sheet "jelly roll" domain of unknown function. These types of domains have been associated with binding proteins and virus capsid coats (Rossman *et al.*, 1983), but no such ligand has been identified as associating with X-prolyl dipeptidase. PepX is noted to associate with the membrane region in *Lactococcus*, so it is possible that this domain acts as a linker to tether the protein in this position (Tan *et al.*, 1992).

Whether the changes in the C-terminal of the molecule reflect changes in the functional specificity of this domain is difficult to predict without knowing the identity of the ligand for the domain, if any. It is also possible that this domain is no longer essential to the functioning of the enzyme, and the higher rate of nucleotide changes in this part of the protein may reflect the fact that there is no longer genetic pressure to conserve this region.

It should also be noted that the strains used for comparison in this study are all derived from different sources, and are selected for different properties. It is possible that the changes in protein sequence observed represent subtle changes in strain properties. X-prolyl dipeptidase does not have an essential role in the main

metabolism of the cell and deletion mutants of *pepX* remain highly viable as other peptidase enzyme are thought to have overlapping activity specificities. Nevertheless, PepX is thought to contribute to amino acid flavour components in dairy products, as it removes proline from peptides to facilitate further peptidolysis, so it is possible that selection for these properties has led to the differences in PepX sequence observed in these strains. With the sequencing of a variety of *Streptococcus* and *Lactococcus* species, it may be of interest to investigate if inter-specific changes are biased towards the C-terminal domains of the PepX protein, or whether this is a phenomenon observed only in *Streptococcus thermophilus*.

5.5 Conclusions

In this section the *pepX* gene, which produces the X-prolyl dipeptidase enzyme, was cloned from *Streptococcus thermophilus* strain B2513. Initial attempts to clone this gene using a restriction fragment screening approach were unsuccessful, but the degenerate primer PCR approach was successful.

It is obvious from the similarity of the sequence of the cloned gene to previously identified *pepX* sequences that the correct sequence has been cloned. There are a few differences in the nucleotide and translated protein sequence when compared with other *S. thermophilus* strains, although some nucleotide differences merely result in a different codon for the same amino acid, and hence no alteration to the protein product at that position. There are some nucleotide changes that lead to amino acid residue changes in the C-terminal half of the molecule. It is suggested that these changes may be due to strain variations, and not PCR errors, as these changes do not occur within the known catalytic domain of the molecule, and are distinctly non-random in distribution. Comparison of the protein sequence obtained by conceptual translation of the DNA sequence of the cloned product with two other PepX sequences from different strains of *S. thermophilus* shows that no amino acid differences between these PepX proteins occurs within the catalytic region. It is suggested that this may reflect genetic pressure on the organism to conserve the catalytic domain of the enzyme, whilst the C-terminal domains may not be under pressure to retain a constant sequence. Alternatively it is possible that the C-terminal domains may be under selective pressure during strain isolation, which may have led to these changes in amino acid sequence.

5.6 Further Work

It may be of value to investigate the sequences of the *pepX* from other strains of *S. thermophilus*. Analysis of different sequences would be of interest to see if inter-strain differences only occur in the non-catalytic regions of the protein, or if they are distributed throughout the sequence. With sequencing primers based on known highly conserved regions of the *pepX* gene, and the use of modern capillary electrophoresis DNA sequencing techniques, analysis of *pepX* genes could be performed directly from genomic material derived from different *S. thermophilus* strains.

CHAPTER 6

Expression and Purification of Recombinant PepX

6.1 Introduction

As discussed in previous chapters, PepX is expressed at low levels in *Streptococcus thermophilus*. To obtain useable quantities of protein, the gene was cloned and introduced into an *Esherichia coli* host cell to allow heterologous expression of the PepX protein.

Expression in heterologous hosts can be problematic, especially if the host cell varies greatly from the native organism. This can be due to several factors. One is different intracellular conditions affecting the folding, solubility and stability of the protein product. The codon usage and population of tRNAs in the host cell can also affect the translation of a protein from a different species, as a shortage of a particular tRNA can cause protein translation to stall, generating shortened, and often insoluble, protein products. Expression systems for *Lactococcus* species are available, although protein expression levels in tend to be low. Because of the availability of a number of well developed expression systems for *E. coli*, this was the system chosen for initial work. Although the metabolism of *E. coli* and lactic acid bacterial species is quite different, the expression of esterase enzymes from *Lactococcus* species in *E. coli* has been successful in prior studies (T. Loo, personal communication). In addition codon usage frequencies in *Streptococcus* and *E. coli* are similar, so translation of a heterologous gene in *E. coli* is unlikely to be problematic.

6.2 Objectives and Strategies

The objectives of this section of work were:

1) Express the recombinant PepX enzyme in *E. coli* BL-21 cells.
2) Purify the PepX protein from the cells exploiting the histidine tag introduced into the expressed product during cloning, and traditional chromatographic techniques.

6.3 Methods

6.3.1 Revival and Growth of Genetically Modified *Esherichia coli*

E. coli cells hosting the PepX HtB plasmid were obtained from either a frozen glycerol stock, or by transforming cells with the PepX HtB plasmid, and inoculating 10 mL of LB-Amp media with a single colony from a plate of transformants. 50 mL of LB-Amp medium were inoculated with 1 mL of either glycerol stock or transformed cells, and grown at 37 °C overnight on a shaker. All of the 50 mL starter culture was used to inoculate a preheated 2.5 L fermenter, which was incubated at 37 °C with stirring at 250 rpm and aerated at 2 L/min until the absorbance of the culture at 600 nm (A_{600}) was 0.5. This took a period of approximately 3 hours. All growth was carried out in LB medium with ampicillin selection added, made as described in Sections 2.3.2 and 2.3.3.

6.3.2 Induction of Protein Expression

The expression of genes located in the MCS of pProEX-HtB is under control of the *lac* promoter, and is therefore inducible by the addition of lactose or IPTG. IPTG is a gratuitous inducer of the promoter; it activates gene expression, but is not metabolised by the cell, as it is a non-physiological analogue of lactose. Whilst lactose will induce the *lac* promoter, it is metabolised by the cell, so consequently must be added in greater amounts to maintain similar expression levels to IPTG. Lactose has the

advantage of costing far less than IPTG, which is a consideration when large cultures are being induced for protein expression.

Once at an OD of 0.5, measured as described in section 2.3.7, the culture was induced by the addition of lactose to a final concentration of 20 mM. The lactose was dissolved in heated Milli-Q water before being filter sterilized immediately prior to use.

The culture was then incubated for three hours under the same conditions as before and the growth monitored by measuring the optical density (OD) of the culture at 600 nm, as described in Section 2.3.7, to ensure the recombinant protein was not toxic to the cells.

6.3.3 Harvesting and Lysis of Cells

The culture was transferred to GS-3 centrifuge bottles, and centrifuged at 4200 x g for 45 minutes in a GS-3 rotor (Sorvall) at 4 °C to harvest the cells.

The cells were resuspended in cold 20 mM Tris buffer at pH 7.0, and re-pelleted by centrifugation to wash away residual media. The cells were then resuspended in 50 mM Tris with 0.5 M NaCl, pH 7.0. The suspension was passed through a French press to lyse the cells (2 passes at 6 kPa). Cellular debris was removed by centrifugation at 17000 x g for 60 minutes in a SS-34 rotor at 4 °C. The remaining cell-free supernatant was retained and kept on ice until required for purification steps.

6.3.4 Immobilised Metal Affinity Chromatography

Immobilised Metal Affinity Chromatography (IMAC) is a purification process that uses a metal chelating chromatographic matrix to coordinate a bond between a metal ion, usually nickel or zinc, and histidine residues on a protein in a specific fashion. Using this process, proteins that have had a histidine tag added during expression can be preferentially retained, whilst other proteins are removed from the matrix by washing the column with an appropriate buffer. The washing step is usually performed with buffers containing an increasing concentration of imidazole or free histidine. Imidazole chemically resembles the sidechain of histidine, and displaces bound histidines from the matrix, as it competes with the histidine sidechain for

binding sites on the matrix. In this way, proteins with few surface histidines are eluted with a low concentration of imidazole, while proteins with a tag of several histidines are retained until high concentrations of imidazole are reached.

5 mL of Chelating Sepharose matrix (Amersham) was packed into a 1 cm x 10 cm low pressure Econo-Pac column (BioRad). The matrix was equilibrated in loading buffer (50 mM Tris, 0.5 M NaCl, pH 7.0) then charged with a 100 mM solution of nickel chloride made in the same buffer. Buffers were applied to the column using an Econo peristaltic pumping system (BioRad), running at 1 $mL.min^{-1}$. The column and buffers were kept on ice during the separation procedure. Excess NiCl was removed from the column by washing in loading buffer before the protein solution was then loaded onto the column at a flow rate of $0.75mL.min^{-1}$. Unbound protein was removed by washing the column with five column volumes of loading buffer at the same flow rate. Once all unbound protein had been removed (confirmed by measuring the absorbance of the eluate at 280 nm), the bound protein was eluted from the column by applying successively higher concentrations of imidazole in loading buffer using a step gradient. The imidazole concentrations used were 10 mM, 20 mM, 50 mM, 100 mM, 200 mM and 250 mM, and 8 column volumes (40 mL) of buffer was applied at each step.

After applying the highest concentration of imidazole, any remaining bound protein was eluted by stripping the bound metal ions from the chelating media using loading buffer containing 200 mM EDTA. Eluates from all the washing steps were collected in separate fractions and stored on ice.

Fractions were analysed by SDS-PAGE on a 12% gel, and by spot testing for esterase and X-prolyl dipeptidase activities, and the fractions showing activity and a band with the expected mass (~89kDa) on an SDS-PAGE gel were retained for further purification. Retained fractions were pooled and dialysed against several changes of 20 mM Tris buffer, pH 7.0, to remove salt and imidazole.

6.3.5 Q-Sepharose Anion Exchange Chromatography

The conditions used to carry out IMAC purification of histidine tagged proteins are designed to increase hydrophobic effects. This helps the histidine residues in the His-tag bind to the Chelating Sepharose matrix. However, an increase in hydrophobicity can also cause proteins with surface histidine residues to bind to the column medium. In addition, proteins with hydrophobic surface regions can bind to other proteins, and be "piggy backed" through the column. These events can lead to contaminants being co-eluted from the column with the protein of interest.

A second step was required in this preparation to remove these contaminants. The procedure chosen was anion exchange chromatography, which is typically carried out under conditions designed to minimize hydrophobic forces.

A Q-Sepharose Hi-Load 3 cm x 13 cm column (Amersham) was equilibrated in 20 mM Tris pH 7.0. Protein solution that had been equilibrated in the same buffer by dialysis was loaded onto the column at a flow rate of 1 $mL.min^{-1}$. Unbound protein was eluted with 5 column volumes of 20 mM Tris buffer, before bound protein was eluted from the column by applying a linear gradient of 20 mM Tris pH 7.0 containing from 0 M to 1 M NaCl, over a volume of 600 mL, at 4 $mL.min^{-1}$. 4 mL fractions were collected and all steps were carried out at 4 °C.

Fractions were analysed for peptidase and esterase activity by spot testing, and any active fractions were then subjected to SDS-PAGE (12% gel). Fractions that showed activity, and had the least impurities, were pooled and stored at 4 °C.

6.3.6 Concentration and Storage of Enzyme

Once a protein has been purified it is usually necessary to store it under conditions that minimize denaturation and degradation until it is required for use. In this case the method chosen was storage at −80 °C. In order to minimize any loss of activity on freezing, it is best to keep the protein concentration high, and to store the protein in a buffer that is amenable to temperature changes (Scopes, 1994). The buffer used for the purification process was Tris buffer. Tris is an ideal buffer for using in large amounts due to its low cost, but decreases in pH as temperature is reduced. Bis Tris propane (BTP) on the other hand is much more amenable to temperature changes, and is therefore better suited as a buffer in which to freeze proteins.

The pooled enzyme fractions were concentrated in a Centricon ultrafiltration device (Amicon), with a 10 kDa cutoff, operated as directed by the manufacturer. The pooled material was added sequentially to the Centricon and centrifuged to remove low molecular mass contaminants and water. The flowthrough was retained and tested for activity. Once the entire pool of material had been concentrated, the buffer was exchanged by adding high purity 20 mM BTP buffer pH 7.0 made as described in general methods section 2.3.14. The protein solution was concentrated as before, and the buffer addition process was repeated 6 times to ensure complete buffer exchange. The protein concentration was measured using the Bradford protein assay (Bradford, 1976), and once the protein had been concentrated to 2 $mg.mL^{-1}$ or higher, it was dispensed into 200 µL tubes, snap-frozen in liquid nitrogen, and stored at –80 °C until required for use.

6.3.7 Gel Filtration

The protein produced from the Q-sepharose ion exchange chromatography step was estimated to be 95% pure PepX. This is adequate for kinetic studies but not pure enough for crystallography. The protein was subjected to size exclusion chromatography using a Superdex 75 HR 10/10 column (Amersham) in order to further purify the protein.

This column is capable of resolving proteins from 3 kDa to 100 kDa. PepX is about 160 kDa in native form, so falls outside the resolving range of this column. However, as PepX was the largest protein in the preparation, as estimated by SDS-PAGE, it was hoped that this column would remove contaminants by retarding them as they passed through the column, whilst the larger PepX enzyme would elute in the void volume. The sample injection volume was 200 µL and the column was run at a flow rate of 0.25 $mL.min^{-1}$. Two buffers were trialled for this separation: 20 mM Tris at pH 7.0, and the same buffer with the addition of 0.02% Nonidet detergent, to attempt to reduce protein-protein interactions.

1mL fractions were collected, and the column was run for a period of 2 hours between injections to ensure all material had been eluted. Fractions that showed enzyme activity, as detected by spot testing, were analysed by SDS-PAGE.

6.3.8 Hydrophobic Interaction Chromatography

The protein solution was also subjected to hydrophobic interaction chromatography using a HR 10/10 alkyl superose column (Amersham), as this procedure had proved to be a highly effective purification step for other esterase purifications.

Hydrophobic interaction chromatography requires samples to be loaded onto the column at a high salt concentration to promote hydrophobic interactions between proteins and chromatographic media, but it is important that the protein of interest is not "salted out" by the high concentrations of salt used. Finely ground $(NH_4)_2SO_4$ was slowly added to the dialysed (no salt) IMAC pooled material, to bring the solution to a known concentration of salt. Any precipitated protein was removed by centrifugation at 26000 x g for 40 minutes.

Salt concentrations used ranged from 0.5 M to 2.5 M $(NH_4)_2SO_4$, in intervals of 0.5 M. However the recombinant protein precipitated between 1.5 M and 2.5 M $(NH_4)_2SO_4$, and did not bind to the column at salt concentrations of below 1.5 M $(NH_4)_2SO_4$. The hydrophobic interaction chromatography step was therefore abandoned.

6.3.9 Measurement of Enzyme Activities

Samples from throughout the purification were assayed for both butyl esterase and X-prolyl dipeptidase activity using the procedures described in section 2.3.10. All assays were carried out at 37°C, which is the optimal growth temperature of *Streptococcus thermophilus*. Control assays were also carried out using column buffers in the place of active enzyme solutions. This was particularly important when measuring the esterase activity in the IMAC column eluates, as imidazole, added to the buffers to displace histidine-tagged proteins from the column, was observed to cause the spontaneous hydrolysis of *p*-nitrophenyl butyrate to *p*-nitrophenol and butyric acid in the absence of any enzyme. In this case, the activity due to the buffer alone was subtracted from the activity measured in the eluate containing buffer and enzyme to ascertain the contribution of the enzyme to the total esterase activity.

6.4 Results and Discussion

6.4.1 Expression of Recombinant PepX Protein in *E. coli*

Expression of the PepX enzyme appeared to be successful. The lysed cells exhibited both butyl esterase and X-prolyl dipeptidase activity, as indicated by spot testing with the appropriate chromogenic substrates. Cell growth did not appear to be inhibited by the induction of PepX expression indicating that the recombinant protein was not toxic to the cells.

6.4.2 Immobilised Metal Affinity Chromatography

The immobilized metal affinity chromatography purification appeared to be highly successful. Only minor X-prolyl dipeptidase activity was detected in the IMAC column flowthrough, indicating that the recombinant PepX protein was being bound to the column with a high level of efficiency. Some butyl esterase activity was detected in the flowthrough, but as discussed in Section 6.4.5 below, purification data suggests that this is due to an endogenous *E. coli* butyl esterase activity. A Coomassie blue stained 12% SDS-PAGE gel of the IMAC column eluates is shown in Figure 6.1. Recombinant PepX eluted from the column in both the 200 mM imidazole and 250 mM imidazole fractions, although the majority of the activity was in the 200 mM fraction. The PepX enzyme was visible as a large band that corresponded to a molecular mass of 85 kDa, estimated by comparison with protein size standards run on the same gel. The molecular mass standards used are shown in Appendix 11.
The 200 mM imidazole eluate was estimated to be 69% pure PepX, using densitometry analysis of the Coomassie stained gel shown in Lane 3 of Figure 6.1. Some slight activity was eluted from the column during stripping of the nickel ions from the chelating sepharose medium, and could be seen as a faint band on a Coomassie blue stained SDS-PAGE gel. The 200 mM and 250 mM imidazole fractions contained most of the enzyme activity and were pooled for subsequent purification steps.
The purity of the histidine-tagged PepX protein from the immobilized metal affinity chromatography was not as high as expected. Generally there are few proteins in nature that have enough histidine residues to bind specifically to Chelating Sepharose

media. It is possible that this can occur, however, if a protein has a reasonable number of histidine residues in close proximity. Another explanation is that contaminant proteins are binding to PepX itself and being carried through the column. PepX is known to have a ß-sheet ligand binding domain with an unknown function, so it is quite possible that *E. coli* proteins are binding non-specifically to this or other parts of the protein.

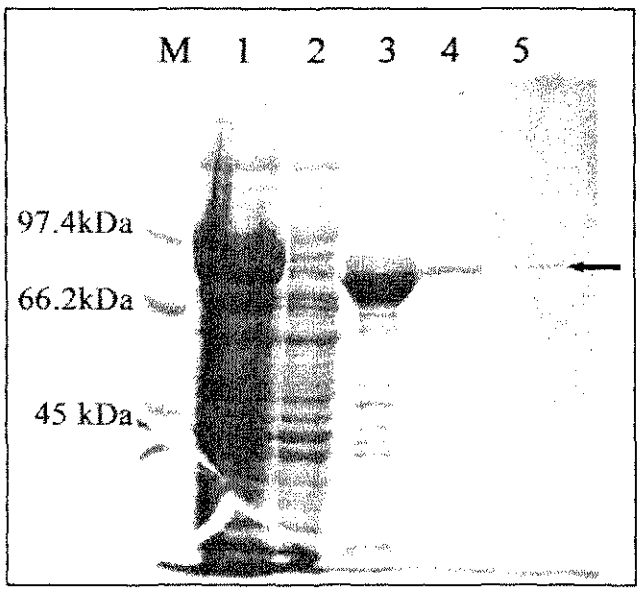


Figure 6.1 *12% SDS-PAGE of IMAC column eluates. Lane M shows protein molecular mass standards, with sizes indicated on the left Lane 1, cell-free supernantant. Lane 2, column loading flowthrough. Lane 3, 200mM imidazole eluate. Lane 4, 250mM imidazole eluate. Lane 5, EDTA stripping buffer eluate. The position of the recombinant PepX protein is indicated by the arrow at right*

6.4.3 Anion Exchange Chromatography

To remove contaminating proteins, anion exchange chromatography was used. No protein was detected in the column flowthrough during the loading process, indicating that all protein applied to the column was bound.

Several peaks were observed to elute from the column during the salt gradient application by UV absorbance at 280 nm. Figure 6.2 shows a plot of the 280 nm and 214 nm absorbance levels during the column run. There was one major peak observed and X-prolyl dipeptidase and butyl esterase activities were found to be located in this peak. A minor shoulder peak eluted shortly after the major peak, which contained a protein of less than 30 kDa as deduced by SDS-PAGE.

Analysis of active fractions from the Q-sepharose separation by SDS-PAGE showed that fewer contaminating proteins were present after this purification step as shown in Figure 6.3.

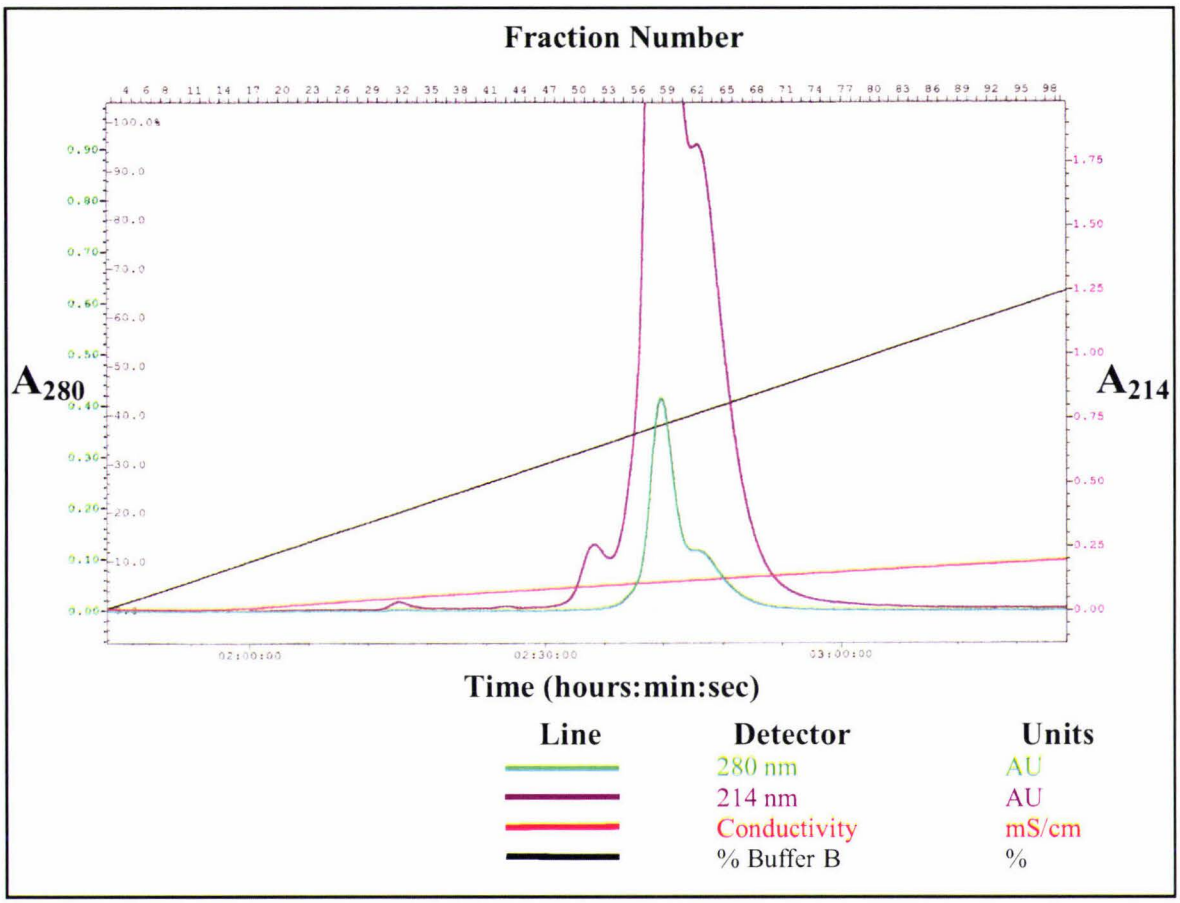


Figure 6.2 *UV, conductivity and salt gradient monitor chart of a typical anion exchange chromatography run, using a Q-sepharose HiLoad column (3 x 13 cm) (Amersham). A_{280} scale is from 0-1 absorbance units and the A_{214} scale is from 0-2 units. Esterase and peptidase activity was identified in fractions 55-65 and activity levels were approximately proportional to the amount of protein present as indicated by the A_{280} monitor.*

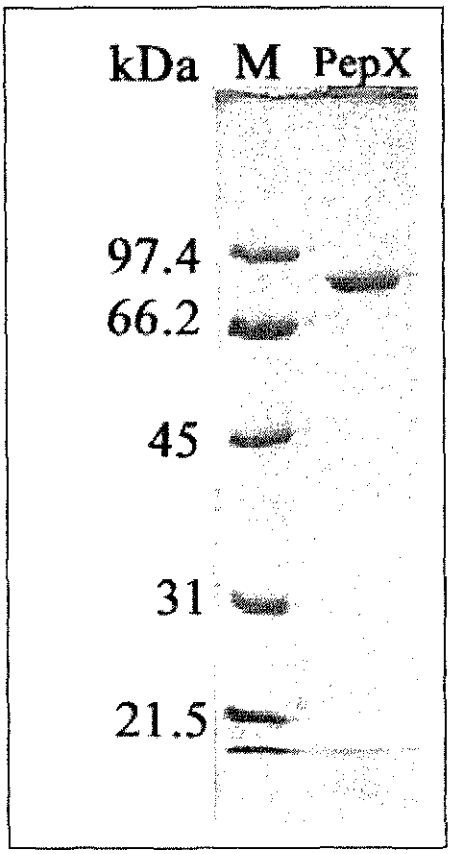


Figure 6.3 *SDS-PAGE analysis of Q-sepharose pool, with Coomassie blue stain applied. A prominent band can be seen in the second lane, representing the recombinant PepX enzyme.*

The pooled fractions from anion exchange were estimated to be 85% pure as judged by densitometry analysis. Whilst this was not pure enough to use in crystallization trials, it was judged to be pure enough for use in kinetic work given the lack of native X-prolyl dipeptidase activity in *E. coli* and the apparent removal of native butyl esterase activity during the initial IMAC purification. This was verified in later studies by kinetic studies of the recombinant enzyme.

It is interesting that whilst only one protein peak that contained activity was eluting from the column, this peak contained the PepX protein as well as other minor contaminant proteins, as judged by SDS-PAGE analysis. This suggests that the *S. thermophilus* PepX protein is non-specifically binding these *E. coli* proteins. An additional shoulder eluting just after this main peak showed that the column was separating some contaminants from the recombinant PepX. This conclusion is also supported by the results summarised in the purification table (Table 6.1), which shows that enzyme specific activity increased during this purification step.

PepX was found to elute from the column at a salt concentration of about 0.3M NaCl, which should be sufficient to disrupt any nonspecific ionic interactions, suggesting that the binding of non-specific proteins to PepX is likely to be due to hydrophobic interactions.

Since these proteins are from the *E. coli* host cell, it is unlikely that they represent specific heterodimeric partner proteins for PepX. It is noteworthy however that PepX is reported to possess a C-terminally located putative ligand-binding domain with a currently unknown function (Rigolet *et al.*, 2002). This domain is not located near the catalytic region of the enzyme, so if this domain is responsible for the non-covalent attachment of contaminants, they will be unlikely to interfere with the activity of the enzyme. Another possibility is that the protein contaminants are bound to the peptide-binding site of the enzyme. This does not seem likely, however, as the enzyme is highly specific in the peptides it targets and therefore is unlikely to bind non-target proteins in such a tight-binding fashion.

The protein was concentrated to at least 3.4 $mg.mL^{-1}$ using ultrafiltration and frozen in liquid nitrogen for storage at –80 °C until required for further use.

6.4.4 Gel Filtration

Size exclusion chromatography was carried out as a final purification step. This was done with the rationale that the contaminant proteins were generally smaller than PepX and should be able to be separated based on size.

Initially this separation was done in 20 mM Tris buffer at pH 7.0, resulting in the separation of several minor peaks from the major peak containing the PepX enzyme activities. However SDS-PAGE analysis of the major peak showed that the PepX was still associating with the same contaminant proteins as in the previous anion exchange step. A second gel filtration purification was attempted using 20 mM Tris buffer containing 0.02% Nonidet detergent to disrupt any hydrophobic interactions. The gel of the active fractions from this purification was similar to the run with no detergent. The contaminant proteins observed were smaller than PepX and should have been retarded in the gel filtration column because of their smaller size. This suggests that the contaminants travelled through the column bound to the recombinant PepX protein, or that they are multimers with a large number of subunits and consequently a molecular mass similar to PepX. Given that some of the contaminants observed were

in the size region of 10 kDa monomers, aggregation would be necessary to produce a native molecule of such a high molecular mass.

6.4.5 Purification Table

A purification table for a typical enzyme preparation is shown in Table 6.1. Enzyme activity is expressed in units where one unit is the amount of enzyme required to hydrolyse 1μmole of substrate per minute.

There are some discrepancies in this table in terms of comparing the yield of enzyme activities between purification steps. For example the yield of esterase activity after the immobilised metal affinity chromatography step is 32%, yet the yield of X-prolyl dipeptidase activity is 67%. Some esterase activity was observed in the IMAC column loading flowthrough in some preparations (data not shown), which did not have a corresponding X-prolyl dipeptidase activity. Although *E. coli* does not have an X-prolyl dipeptidase activity (Law and Haandrikman, 1997), it is possible that some host ester hydrolytic activity is present that may account for this activity. This is supported by the fact that the enzyme exhibiting esterase activity does not bind to the IMAC media, and is therefore unlikely to be a histidine-tagged recombinant enzyme.

The purification that occurs during the Q-sepharose anion step is similar for both the esterase and peptidase activities at 2.20 and 2.28 fold, suggesting that no *E. coli* native butyl esterase activity is present after the IMAC step.

In samples where the protein level was high (cell lysate, cell free supernatant and IMAC loading flowthrough), dilutions of up to 100 fold were made in water before Bradford protein concentration assays were carried out. This has the disadvantage of potentially introducing experimental errors into measurements. All measurements made, however, seemed to be in reasonable agreement with other samples. In particular, although there appears to be 175 mg of protein unaccounted for in the IMAC column eluates, this is most likely to have been lost during the imidazole buffer washing steps to elute non-specifically bound proteins. These low stringency washes were not retained since during early IMAC studies they had been found to contain no detectable recombinant enzyme activity.

Purification Step	Total Protein (mg)	Total butyl Esterase activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Cell-free Supernatant	1075	65.5	0.061	100	1
IMAC column flowthrough	900	-	-	-	-
IMAC eluate pool	41	21.22	0.517	32	8.4
Q-sepharose eluate	14	15.57	1.13	23	18.5
Purification Step	**Total Protein (mg)**	**Total peptidase activity (units)**	**Specific activity (units/mg)**	**Yield (%)**	**Purification (fold)**
Cell-free Supernatant	1075	3854	3.585	100	1
IMAC column flowthrough	900	762	-	-	-
IMAC eluate pool	41	2581	62.95	67	17.5
Q-sepharose eluate	14	2009	146	52	40

Table 6.1 *Summary of the purification of recombinant* S. thermophilus *PepX enzyme expressed in* E. coli. *Activities were measured with chromogenic esterase and peptidase substrates.*

6.4.6 Densitometry Analysis

Densitometry analyses were performed on the IMAC column pool and the anion exchange pool, as described in Section 2.3.16. The proportion of PepX protein present at these stages was estimated and the amount of PepX present was calculated. These results are recorded in Table 6.2.

Purification Step	Total protein (mg)	Estimated percentage PepX	Estimated PepX (mg)
Immobilised metal affinity	41	69%	28.29
Anion exchange	14	85%	11.9

Table 6.2 *Densitometry analysis of purification steps from SDS-PAGE.*

6.4.7 Summary

Immobilized metal affinity chromatography that exploited the six-histidine purification tag introduced onto the N-terminus of the recombinant PepX protein was used as a first purification step. This step was highly effective at removing *E. coli* proteins from the cell free supernatant. In addition, the X-prolyl dipeptidase activity detectable in the cell free supernantant prior to this step was depleted in the eluate of this column, indicating efficient binding of the His-tagged recombinant protein to the column medium.

Protein eluted from the IMAC column was found to be approximately 69% pure as estimated from an SDS-PAGE analysis.

Anion exchange chromatography removed some additional proteins, but contaminants still remained.

Gel filtration steps, in the absence and presence of detergent were unable to further remove contaminant proteins, suggesting they were strongly hydrophobically associated with the recombinant PepX protein.

Protein was frozen and stored for kinetic studies to verify the recombinant enzyme had the same kinetic parameters as the native enzyme, and for further kinetic characterization.

6.5 Conclusions

A procedure was developed to purify recombinant *Streptococcus thermophilus* PepX protein from an *Escherichia coli* hosted expression system. This procedure was successful in its primary goal of producing enzyme of a sufficient purity for kinetic investigations.

Despite attempts to produce protein of a higher purity through the application of hydrophobic interaction chromatography and size exclusion chromatography, contaminant proteins could not be removed from the preparation. This meant protein of a sufficient purity for crystallization trials was not made.

The reason for the difficulty in removing the contaminant proteins appears to be that the proteins are binding in a non-specific hydrophobic manner to the recombinant enzyme. It is suggested that these proteins may be binding to the C-terminal ß-sheet domain of PepX, which would position them distal to the substrate binding and catalytic regions of the protein, and thus make this binding, although a problem for purifying crystal grade protein, unlikely to interfere with enzymatic activity.

6.6 Further Work

The difficulty observed in separating recombinant PepX protein from other *E. coli* proteins suggests further work is needed to develop an expression and purification system to produce cleaner enzyme suitable for crystallography experiments.

Addition of detergent to separation procedures might be able to disrupt the hydrophobic binding of proteins to the PepX enzyme. There is a risk of causing denaturation by addition of detergents. However PepX is known to be resistant to sodium dodecyl sulfate, and does in fact become active again after being run on an SDS-PAGE gel and upon removal of SDS by dilution, provided boiling of samples is avoided.

Alternatively the addition of a small semi-soluble hydrophobic molecule, such as tricaproin that will displace hydrophobically bound protein from the enzyme, and can then be removed by dialysis, may also be successful in improving purification.

It is apparent that during purification from native sources, PepX does not strongly adhere other proteins. This is evident from the fact that *Lactococcus lactis* PepX can be purified enough from a native source to produce crystals for crystallography (Rigolet *et al.*, 2002). It is therefore suggested that purification of PepX with the intention of crystallography be performed from a native source, rather than heterologous expression in *E. coli*. This was avoided in this study, as discussed in Section 6.1 above, due to the low yields produced in the native cell, and to the lack of a requirement for extremely high purity enzyme.

Alternatively, the *pepX* gene could be expressed using a *Lactococcus lactis* inducible expression system. These systems have been used to express esterase enzymes in *Lactococcus* (Fernandez *et al.*, 2000), and could potentially be improved for large-scale preparations by development of a histidine-tagged expression vector to aid purification.

CHAPTER 7

Kinetic Characterisation of PepX Using Synthetic Substrates

7.1 Introduction

The study of the kinetic parameters of an enzyme can lead to a better understanding of how the enzyme functions. In the previous chapters, the cloning and production of purified X-prolyl dipeptidase enzyme from a recombinant source were described.
In the case of PepX, it was of interest to investigate the previously unknown esterase activity of the enzyme, as well as to verify the kinetic properties of the peptidase activity of the recombinant protein.
Kinetics can be used to examine whether or not the enzyme is a true esterase, displaying Michaelis-Menten kinetics with a soluble substrate, or whether it exhibits the characteristic kinetics that result from interfacial activation as seen with lipases. Kinetic studies can also be used to compare both peptidase and esterase activities, and determine whether these two hydrolysis reactions are both carried out at the same active site, or at separate active sites found in different domains of the protein. In the case of the recombinant PepX protein, kinetics can also be used to ensure that the heterologous enzyme has the same kinetic parameters as the wild-type enzyme.

7.2 Objectives and Strategies

The objectives of this section of work were:

1) To examine the ester hydrolytic activity of X-prolyl dipeptidyl peptidase on a chromogenic ester substrate to determine whether the enzyme is a true esterase or exhibits interfacial activation characteristic of a lipase
2) To confirm the kinetic parameters of the recombinant enzyme from an *E. coli* expression system to verify the enzyme is functionally identical to that purified from the native *Streptococcus thermophilus*.

3) To use kinetics to investigate if the peptidase activity of the enzyme can be competitively inhibited with an ester substrate, thus inferring a single catalytic site is responsible for both activities.

7.3 Kinetic Analysis

7.3.1 Use of Chromogenic Substrates

Analysis of peptidase and esterase enzymes on natural substrates, such as peptides and acylglycerides, is a time consuming process and hydrolysis of these substrates cannot be measured directly with a spectrophotometer. As a result, discontinuous assays have to be performed, which can introduce an extra element of error into measurements. In addition, assays for peptides and glycerides tend to require complicated and time-consuming extraction steps to prepare reaction components for analysis, and these steps are a further opportunity for experimental errors to arise.

Fortunately, artificial chromogenic substrates can be used to assay both X-prolyl dipeptidase and butyl esterase activities. These substrates release the chromophores *para*-nitroaniline or *para*-nitrophenol respectively during enzymatic hydrolysis of a scissile bond. The liberation of these chromophores by the enzyme can be measured by visible light absorption at 410nm using a spectrophotometer, making the measurement of the rate of enzyme activities quick and easy. However, the use of artificial substrates to investigate hydrolytic enzymes has some disadvantages. The chromogenic substrate does not necessarily behave in exactly the same way as a natural substrate, and for this reason he kinetic parameters for the hydrolysis of these substrates may not reflect what happens with the natural substrate. An example of these differences can be seen by comparing the chromogenic substrate for X-prolyl dipeptidase activity, glycylprolyl *p*-nitroanilide, with the natural substrate peptides with proline adjacent to the scissile amide bond. Whilst the glycylprolyl part of the molecule is identical to the natural substrate, the *p*-nitroaniline group occupies an area in the enzyme substrate-binding site that would normally be occupied by the rest of the peptide substrate. Similarly *p*-nitrophenyl butyrate, a butyl glyceride analogue, has a *p*-nitrophenol group positioned where the glycerol backbone would lie in a glycyl substrate such as tributyrin.

The *p*-nitrophenol group obviously has different properties from those of the natural glyceryl substrates of these reactions. For this reason the rates of reaction observed using chromogenic substrates are often different from those seen when non-chromogenic substrates are used. However artificial chromogenic substrates remain an ideal way to test enzyme activity in the presence of inhibitors, as any decrease in activity with these substrates usually reflects a similar decrease in activity with physiological substrates.

Characterisation of the activities of the recombinantly expressed enzyme using *p*-nitrophenyl butyrate as a substrate was necessary to confirm that the recombinant enzyme was identical to the native enzyme previously studied by earlier investigators. A single deliberate amino acid change at position 2 of the protein was made to this enzyme during cloning by the mutation of a lysine to an aspartate. This change was made to facilitate easier cloning and, based on modelling and examination of the structure of PepX from *Streptococcus thermophilus* (Rigolet *et al.*, 2002), was expected to have minimal effect on the protein product.

7.3.2 Characterisation of PepX Active Site

The observation that PepX is capable of carrying out hydrolysis on two very different substrates (in terms of both the steric shape of the substrate and the type of bonds involved) raises the question as to whether one active site catalyses both reactions. The PepX protein is large by bacterial enzyme standards, and has four recognized domains (Rigolet *et al.*, 2002), so it is perhaps possible that a second active site exists that is responsible for the hydrolysis of ester substrates. In addition to these hydrolyses, PepX can catalyse the transfer of butyl groups from tributyrin onto an ethanol acceptor molecule. For such a transfer to occur, the existence of a second binding site for the acceptor alcohol during acyl group transfer is implied.

To address the question of whether one active site performs both hydrolytic reactions, an assay system was devised that involved adding an authentic substrate to a chromogenic substrate assay. If one active site was performing both hydrolytic activities, a decrease in the rate of chromophore release that could be overcome by a high enough concentration of substrate was expected. This is a characteristic of competitive inhibition. In this assay system, the authentic substrate would be competing with the artificial substrate.

In addition, some trials were undertaken that involved adding ethanol and/or methanol to both esterase and peptidase chromogenic assays. This was intended to show whether the presence of alcohol resulted in a reduction or loss of activity. Any loss of activity would imply that a specific binding site existed for the acceptor substrate, rather than a mechanism involving random diffusion into the active site. Verification that the *p*-nitrophenol butyrate was being hydrolysed, as opposed to the butyl group being transferred to an alcohol acceptor was important as methanol is present at low concentrations in the ester chromogenic substrate solution, and ester synthesis was not considered in these reactions. This experiment was important in light of the finding that PepX will not hydrolyse tributyrin to butyric acid (Chapter 9).

There are a few technical considerations in this competitive assay system. One is that the authentic substrate must not absorb light at similar wavelengths to the artificial substrate. In the case of these assays, the authentic substrates used were tributyrin (a colourless oily liquid), ß-casomorphin (a short soluble peptide), and ethanol. None of these substances absorb light at 410nm. However both the *p*-nitrophenol and the *p*-nitroaniline groups, released by cleavage of the scissile bond by the enzyme, do absorb at this wavelength, allowing easy detection of the hydrolysis products of the enzyme, with minimal or no interference from the competing substrate.

7.3.3 Kinetic Analysis Methods

One of the key features of competitive inhibition is that the K_m of the enzyme is changed in the presence of inhibitor, but the V_{max} of the enzyme remains the same. The rationale behind this is that in the presence of enough substrate the inhibitor becomes so diluted that its effect on enzyme activity is negligible. This can be observed using a simple plot of substrate concentration against reaction velocity. At a high enough substrate concentration the inhibited enzyme will reach V_{max}. However, it is not always practical to use such a high concentration of substrate. Also, substrate inhibition and substrate insolubility can mean that the enzyme kinetic parameters deviate from classic Michaelis-Menten kinetics when high concentrations of substrate are used.

Data transformations have been devised, however, that allow V_{max} and K_m to be determined from activity measurements without the need to carry out assays at V_{max}.

7.3.3.1 The Lineweaver-Burke Transformation

The most commonly used transformation for kinetic data is the double reciprocal, or Lineweaver-Burke transformation, where 1/S is plotted against 1/V. The y intercept of this plot represents $1/V_{max}$, and the x intercept represents $1/K_m$. These kinetic parameters can be directly determined by examining the plot. In the case of competitive inhibition, where V_{max} is constant, the best-fit lines of different inhibitor concentration should all intercept at the same point on the y-axis.

However, the reciprocal nature of the Lineweaver-Burke transformation means that the smallest data values receive the highest weighting during the transformation. These represent the lowest substrate concentrations, and due to the inherent difficulty of both accurate and precise measurement of small quantities, these points have the highest experimental error. This can skew best-fit lines, making Lineweaver-Burke based results inconclusive.

7.3.3.2 The Hanes-Woolf Transformation

A transformation that circumvents the data weighting problems associated with Lineweaver-Burke is the Hanes-Woolf transformation of S against S/V. This does not place high weightings on lower substrate values, and produces a linear plot with a reduced spread of data. From a Hanes-Woolf plot, the slope of the line of best-fit is $1/V_{max}$, and the y intercept is $-K_m$. This means that in competitive inhibition, the best-

fit lines for different inhibitor concentrations should be parallel, and intersect the y axis at different points, since ideally V_{max} will be identical but K_m will be different for each concentration of inhibitor assayed.

7.3.3.3 The Eadie-Hofstee Transformation

Another transformation that linearises the Michaelis-Menten equation is the Eadie-Hofstee plot of V against V/S. This also produces a straight line where the y intercept is equal to V_{max} and the slope of the line is equal to $-K_m$. There are some problems associated with the use of this transformation to present experimental data. The main problem is that the dependent variable, in this case the reaction velocity (V), is used to transform both axes, so that any experimental uncertainties are introduced onto both axes of the plot, magnifying experimental error. Any deviations from Michaelis-Menten behaviour are obvious, so this transformation is ideal for highlighting non-Michaelis-Menten behaviour.

7.4 Methods

7.4.1 Measurement of Butyl Esterase Activity

Measurement of the ester hydrolytic activity of PepX was carried out by measuring the rate of hydrolysis of *p*-nitrophenyl butyrate in the presence of purified enzyme. Although, as mentioned previously, the enzyme kinetics with this substrate do not necessarily reflect the kinetics on a glyceride substrate such as that found in a natural system, these experiments were carried out to gain a baseline for further experiments involving inhibitors and effectors of enzyme activity.

p-Nitrophenyl butyrate (Sigma) was pipetted into a 1.5 mL eppendorf tube, and weighed. The amount used was dependant on the final concentration of substrate required for the experiment. 1 mL of ice-cold HPLC grade methanol was added, and the substrate was dissolved by inverting the tube several times. To obtain lower substrate concentrations than could be accurately measured with an autopipettor (typically below 1 µL), substrate was weighed out and dissolved in methanol as before then serial dilutions were made as required in methanol, to a final volume of 1 mL. The final concentrations of *p*-nitrophenol butyrate used ranged from 0.056 mM to 5 mM.

The substrate methanol solution was pipetted into a 25 mL standard flask. Any remaining substrate mixture was washed from the eppendorf tube into the standard flask with cold enzyme grade BTP buffer (20 mM BTP, pH 7.0, in Chelex treated ddH_2O), made as described in the methods section. The standard flask was filled with 20 mM BTP until the bottom of the meniscus was level with the volume indicator. The contents of the flask were mixed, by inverting the flask carefully, and the substrate was stored on ice until required.

990µL of substrate solution was pipetted into a new 2 mL disposable cuvette. The cuvette was then placed into a preheated spectrophotometer (Varian Cary model 1) at 37 °C for 5 minutes to warm the substrate to the assay temperature. After heating to 37 °C, 10 µL of enzyme was added and the cuvette contents mixed by vigorous pipetting. The spectrophotometer lid was closed, and the rate of *p*-nitrophenol release was measured at 410 nm. The slope of the initial reaction rate was measured and recorded. After the reaction, the entire cuvette was discarded to prevent *p*-nitrophenol damage to the cuvette interfering with subsequent measurements. The hydrolytic

activity was measured over a range of substrate concentrations, and each measurement was repeated at least 5 times to ensure reproducibility. From these measurements, the kinetic parameters of the hydrolysis of the artificial substrate could be calculated to generate a baseline for further studies.

Reaction rates were converted into μmoles/min per mg of enzyme using Beere's law. The pK_a of the chromophore released by enzymatic hydrolysis, *p*-nitrophenol, has a pK_a of 7.15 and only the anionic form of *p*-nitrophenol absorbs light at 410 nm. This means the exact extinction coefficient must be calculated for use of the substrate at pH 7.0. This was carried out based on the Henderson-Hasselbach equation and the extinction coefficient of *p*-nitrophenol at pH > 8 which is 18400 $L.Mol^{-1}.cm^{-1}$. The coefficient calculated was 7626 $L.Mol^{-1}.cm^{-1}$. This was derived as follows:

$pH = pKa - \log[HA/A^-]$

$\log[HA/A^-] = pKa - pH$

$\log[HA/A^-] = 7.15 - 7.0$

$= \text{inverse log } (0.15)$

$= 1.41 : 1\ [HA/A^-]$

so if 18400 $L.Mol^{-1}.cm^{-1}$ represents the extinction coefficient of completely ionised *p*-nitrophenol, then:

18400 / (1.41+1) x 1 equals the coefficient due to the remaining A^- ionic form.

$= 7634\ L.Mol^{-1}.cm^{-1}$

The pH of the assays was carefully controlled by buffering at exactly pH 7.0 and in addition the enzyme solution added to the assays was stored in the same buffer as used for the assays.

7.4.2 Measurement of X-prolyl Dipeptidase Activity

Measurement of the X-prolyl dipeptidase activity of PepX was carried out on glycylprolyl *p*-nitroanilide, an artificial dipeptide substrate. Hydrolysis of this substrate can be measured using a spectrophotometer to monitor *p*-nitroaniline release at 410nm, as the enzyme hydrolyses the scissile bond between the glycylprolyl part of the molecule, and the *p*-nitroanilide chromophore.

A stock of glycylprolyl *para*-nitroanilide was made by weighing solid substrate into an eppendorf tube and adding 20 mM BTP, pH 7.0, (made in ddH_2O) to a final concentration of 100 $mg.mL^{-1}$. This stock was stored at –18 °C until required. Substrate stock was thawed on ice. An appropriate volume of substrate was added to a new 2 mL plastic cuvette containing 20 mM BTP pH 7.0, to a final volume of 990 µL. The substrate was equilibrated to assay temperature by incubation in a pre-warmed spectrophotometer at 37 °C for 5 minutes.

10 µL of appropriately diluted enzyme solution was added to the cuvette, and quickly mixed with a clean disposable pipette. The spectrophotometer was closed, and the release of *p*-nitroaniline was monitored at 410 nm. The initial rate of reaction was measured and recorded. These measurements of rate of activity were carried out across a range of substrate concentrations to generate a Michaelis-Menten plot of enzyme activity rates.

Unlike the *p*-nitrophenol chromophore, *p*-nitroaniline has a stable extinction coefficient between pH 2 and 10. This means calculating a pH specific extinction coefficient is unnecessary at the pH used in this assay system.

7.4.3 Measurement of Dipeptidase Activity in the Presence of Tributyrin

To investigate if the addition of glyceryl ester substrates for the enzyme inhibited peptidase-type hydrolysis, the chromogenic assay was modified. Fixed concentration solutions of tributyrin of 0.125 µL per mL, and 0.05 µL per mL, made in 20 mM BTP, pH 7.0, were used for assays containing glycylprolyl *para*nitroanilide as the substrate. Assays were carried out over the same range of substrate concentrations as the uninhibited reaction to allow a comparison to be made, and were repeated at least 4 times to ensure reproducibility.

7.4.4 Measurement of Esterase Activity in the Presence of Peptide Substrate

Trials were performed using the short peptide ß-casomorphin, a bovine casein derivative, and a known X-prolyl dipeptidyl peptidase substrate, to inhibit the hydrolysis of *p*-nitrophenyl butyrate. This seven-residue peptide contains a proline at every second position from the N-terminus, as shown in Figure 7.1. Some of the peptide cleavage products therefore become substrates for the PepX enzyme during PepX catalysed hydrolysis. There is one exception: the C-terminal bond may not be efficiently cleaved because of its proximity to the C-terminus, and the presence of isoleucine next to the scissile bond. It has been previously observed that mammalian dipeptidase IV, which has a substrate specificity similar to that of PepX, is inhibited by the presence of isoleucine adjacent to the scissile bond (Callebaut *et al.*, 1993). It was nevertheless hoped that this substrate would compete with the chromogenic esterase substrate and inhibit chromophore release, as it is known to be at least partially cleaved by PepX.

In practise, however, no significant decrease in esterase activity was measured in the presence of ß-casomorphin, even at concentrations up to 0.1 $mg.mL^{-1}$. The explanation for this is not clear. It is possible that the concentration used was not high enough to inhibit the enzyme effectively. Another explanation is that the enzyme is so much more efficient at peptide hydrolysis than ester hydrolysis that the peptide is hydrolysed so quickly that binding and hydrolysis of the chromogenic substrate is not affected. A sufficiently high concentration of peptide may overcome this problem, however ß-casomorphin is expensive, and available in small amounts only.

Another peptide was also tested for inhibitive properties in the same way as ß-casomorphin. This was the short peptide GlyProGlyGly. This is a known substrate of human dipeptidase IV. No inhibition of esterase activity was observed in the presence of this peptide either. An attempt to see if PepX actually hydrolysed GlyProGlyGly was made by digesting the peptide with PepX and analysing using HPLC, but the instrument was unable to separate any hydrolytic products from unhydrolysed substrate, suggesting that this peptide is not a substrate for PepX.

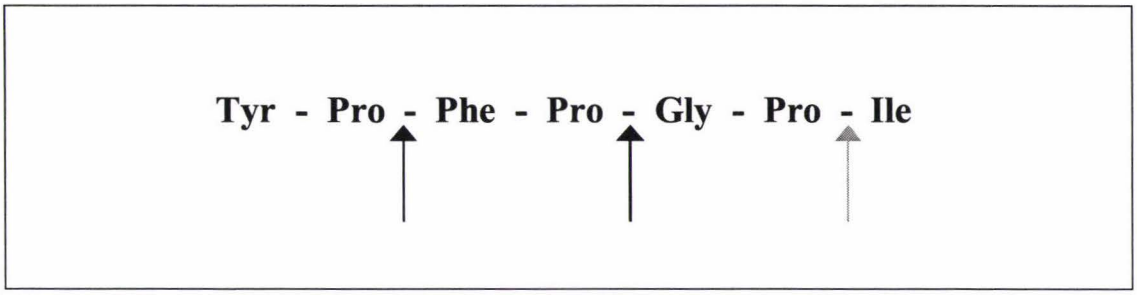


Figure 7.1 *ß-casomorphin sequence and PepX cleavage sites. The C-terminal cleavage site is shown in grey, as it may not be cleaved due to the proximity of the peptide end, and the presence of isoleucine.*

7.4.5 Measurement of Enzyme Activities in the Presence of Alcohol

To investigate if ethanol affected the rate of reaction the esterase and peptidase activity of PepX was assayed in the presence of alcohol.

This was to examine the possibility that rather than hydrolysing the *p*-nitrophenyl butyrate substrate to *p*-nitrophenol and butyric acid, the enzyme was transferring the butyl group to the methanol that was present in the assay substrate at a concentration of 1 M.

This situation would be difficult to detect kinetically unless the concentration of acceptor (methanol) was altered or replaced with a known acceptor, such as ethanol, since these assays measure the release of *p*-nitrophenol, which would still be released during a group transfer reaction.

Chromogenic substrate solutions for esterase and peptidase activities were made as described in sections 7.4.1 and 7.4.2, respectively, at concentrations of 2 mM (esterase) and 5 mM (peptidase). These concentrations were in excess of those required to reach V_{max} to ensure that any decrease in activity was due to inhibition and not due to a decrease in substrate concentration from dilution upon addition of the alcohol.

10 µL of enzyme solution was added to each assay. For esterase assays, undiluted PepX at 3.4 $mg.mL^{-1}$ was used. For peptidase assays, the enzyme was diluted 50 fold in 20 mM BTP, pH 7.

Assays were started by the addition of enzyme solution, and the initial slope was measured. 40 µL of ethanol was then added, and the solution was mixed by aspiration as before. The slope was measured again. The addition of ethanol was repeated several times to observe activity changes at different ethanol concentrations.

In order to verify that methanol was not affecting the hydrolytic activity of the enzyme, assays were performed as described above, but with the addition of 40 μL of methanol. This control was important as the esterase substrate reagent contains 40 μL of methanol per mL, which, because of its similarities to ethanol may be able to act as an acceptor for hydrolysis products rather than water. As a further control, esterase chromogenic reagent was made as described in section 7.4.1, but using 1 mL of DMSO instead of methanol to ensure no potential alcohol acceptor molecules were present.

Attempts to make chromogenic esterase reagent with ethanol were unsuccessful, as it appeared that the *p*-nitrophenyl butyrate was not soluble in ethanol and underwent some spontaneous degradation.

7.4.6 Recording of Results and Processing of Data

The spectrophotometer (Varian Cary 1) was used to record the rate of chromophore release from the synthetic substrates at 410 nm. This chromophore release was also visible as a yellow colour produced in the cuvette. The "Ruler – least squares" tool in the spectrophotometer operating software (WinUV, Varian) was used to measure the slope of the initial part of the reaction. This was generally measured after the initial burst but during the first 20 seconds of the reaction. Measurements were taken only where there was a linear relationship between absorbance and time. If the signal was unsteady, usually due to air bubbles inside the cuvette, the cuvette was quickly tapped and a reading taken at a slightly later time, but still in the linear part of the reaction. Any reaction that had excessively noisy signal was discarded and repeated.

Data was recorded in the form of a spreadsheet (Excel 2000, Microsoft 1999). The spreadsheet software was used to transform data as necessary and to generate plots of enzyme activity.

7.4.7 Definition of Enzyme Activity Unit

One unit of enzyme activity was defined as the amount of enzyme in milligrams required to hydrolytically liberate either 1 μmole of *p*-nitrophenol per minute or 1 μmole of *p*-nitroaniline per minute, respectively, for esterase and peptidase activities.

7.5 Results and Discussion

7.5.1 *p*-Nitrophenyl Butyrate Assays for Esterase Activity

Measurements were made for substrate concentrations ranging between 0.05 mM and 4.7 mM, almost a hundred-fold range of substrate concentrations. At the highest substrate concentration measured (4.7 mM) some substrate insolubility was observed. However once heated to 37 °C (the reaction temperature) it appeared to be fully soluble, as the solution in the cuvette became colourless and transparent. The data points measured at 4.7 mM were used to confirm that the maximum reaction velocity (V_{max}) had been reached and were not used in subsequent calculations as they were outside the range of useful data for determination of other kinetic parameters, such as K_m. The data gathered were used to produce a Michaelis-Menten plot of enzyme activity versus substrate concentration, shown in Figure 7.2.

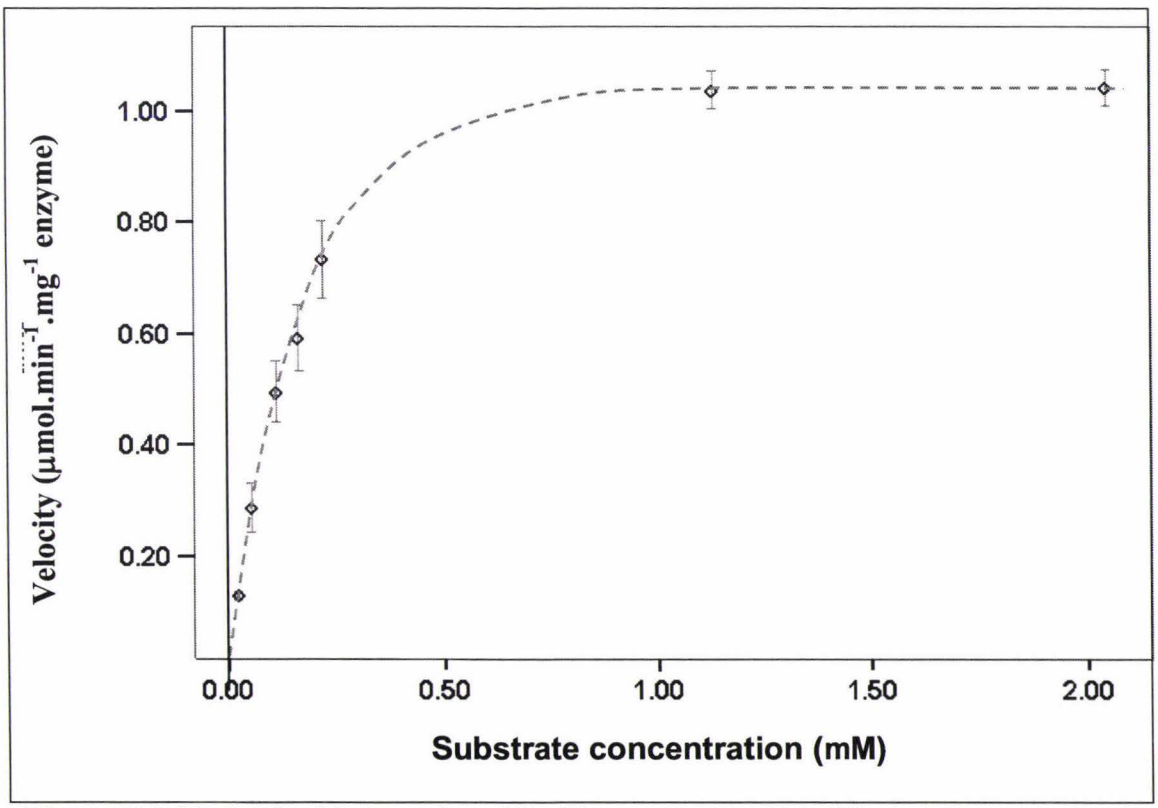


Figure 7.2 *Michaelis-Menten plot of PepX using* p-*nitrophenyl butyrate as a substrate. Error bars represent 1 standard deviation from the mean of each substrate concentration.*

The plot shows an exponential curve typical of an enzyme acting on a fully soluble substrate, with no indication of a sigmoidal curve, characteristic of interfacial

activation. Lipases are characterised by a low activity at low substrate concentrations where lipids are water soluble, which increases as substrate concentration becomes high enough for the substrate to form micelles and induce interfacial activation, before levelling off at V_{max} as the enzyme becomes saturated with substrate. Figure 7.3 contrasts the expected Michaelis-Menten plots of enzymes with and without interfacial activation. For this work, the assays were carried out largely at concentrations where the substrate used was completely soluble. Activity at these concentrations steadily increased and peaked at a V_{max} slightly below the limit of solubility for the substrate at the temperature of the assay. Insolubility was determined by cloudiness observed above this concentration. In order to be sure V_{max} had been reached several assays were carried out at much higher substrate concentrations where the substrate was no longer soluble. The enzyme activity in these assays was the same as the lower substrate concentration assays where an apparent V_{max} had been reached. If the enzyme had been a lipase, higher activity would have been expected at the non-soluble, micelle forming substrate concentrations, than at concentrations where the substrate is fully soluble.

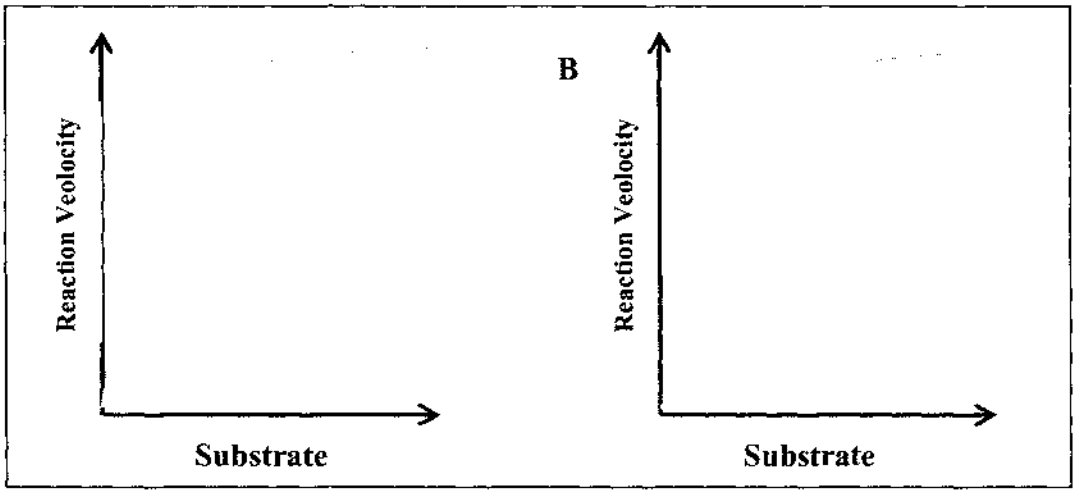


Figure 7.3 *Kinetics of normal and interfacially activated enzymes. Panel A shows the typical Michaelis-Menten kinetics of an enzyme acting on a fully soluble substrate, whilst panel B shows the characteristic concentration vs velocity plot of an interfacially activated enzyme.*

This indicates that the butyl ester hydrolase activity of PepX is a true esterase activity, and is in line with the observation by Liu, *et al.* (2001) that Esterase II prefers *p*-nitrophenyl butyrate as a substrate to longer chain esters. Short chain esters are more soluble in aqueous solution than long chain esters, and therefore form micelles much less readily.

7.5.1.1 Lineweaver-Burke Transformation

A Lineweaver-Burke (Reciprocal) plot was produced from the data and is shown in Figure 7.4.

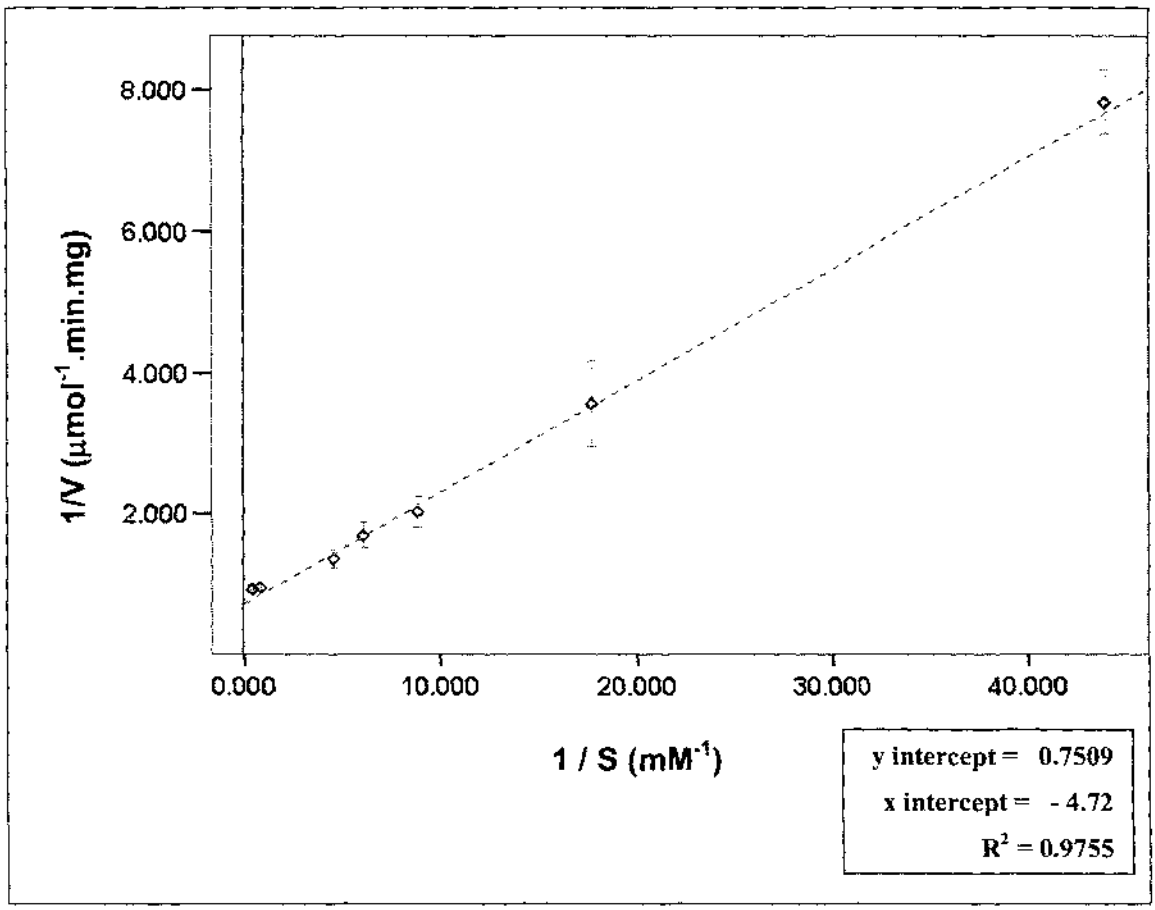


Figure 7.4 *Lineweaver-Burke plot of* p-*nitrophenyl butyrate hydrolysis by PepX. Error bars represent 1 standard deviation from the mean of each substrate concentration.*

The kinetic parameters K_m and V_{max} were calculated from the intercepts of the slope of the double reciprocal plot.

Using the Lineweaver-Burke plot, V_{max} and K_m can be calculated.

$1/V_{max}$ = y intercept

so $1/V_{max} = 0.7509$

$\mathbf{V_{max} = 1.33\ \mu mol.min^{-1}mg^{-1}\ enzyme}$

$-1/K_m$ = x intercept

so $1/K_m = 4.72$

$\mathbf{K_m = 0.21\ mM}$

As previously discussed (section 7.3.3.1), the Lineweaver-Burke plot is prone to being skewed by errors in measurements, particularly at the higher weighted low substrate concentrations. This can be seen on the plot shown above, where the data towards the top end of the line-of-best-fit has the largest spread amongst replicates. These data represent the measurements made at lower substrate concentrations, which are the most sensitive to experimental errors. The points at the top of the line are difficult to make conclusions about as this substrate concentration was only assayed twice, before it was realized that difficulties in measuring such a low amount of substrate would cause unacceptably large experimental errors. The line plotted on the graph fits the data acceptably, however, with an R^2 value of 0.9755. The R^2 value can be lowered on Lineweaver-Burke plots by excluding the data at the lowest, most error prone substrate concentrations. This is commonly done to produce more accurate measurements of K_m and V_{max}. However, in carrying out a selective removal of data points there is a risk of missing unusual kinetic behaviour at lower substrate concentrations. Other data transformations can be applied that produce more accurate kinetic measurements, and are designed to avoid placing high weightings on the more error prone measurements (Cornish-Bowden, 1995).

7.5.1.2 Hanes-Woolf Transformation

A plot that is recognized to produce a better measurement of K_m and V_{max} than Lineweaver-Burke is that of S against S/V, the Hanes-Woolf plot. This type of plot is less prone to deviations in the line-of-best-fit caused by experimental errors and should give a more accurate value of K_m and V_{max}. The Hanes-Woolf plot for the data is shown in Figure 7.5. This data points on this plot should lie on a straight line, and

this is clearly the case for the kinetic measurements of PepX ester hydrolysis. The errors of these data points are obviously less than those on the Lineweaver-Burke plot, as shown by the error bars on this plot.

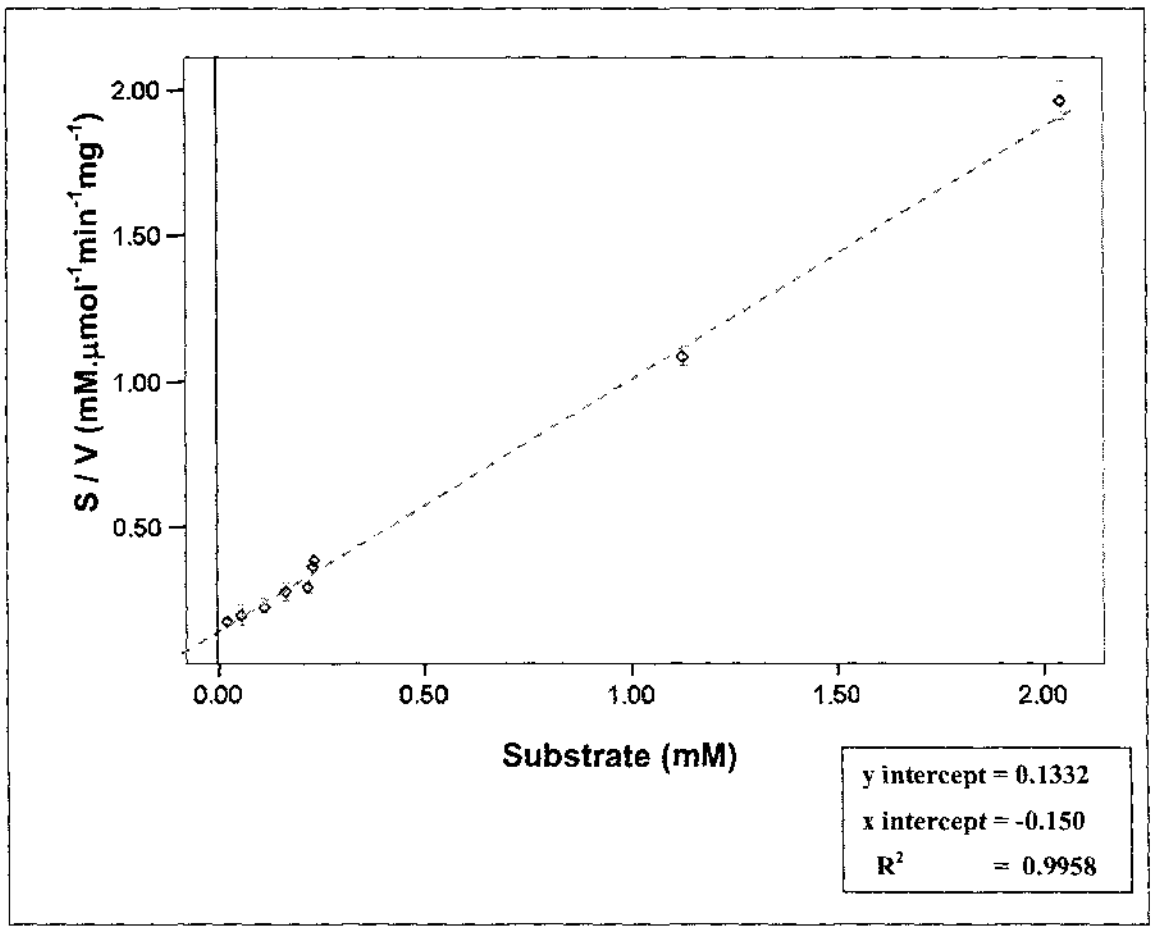


Figure 7.5 *Hanes-Woolf plot of p-nitrophenyl butyrate hydrolysis by PepX. Error bars represent 1 standard deviation from the mean of each substrate concentration.*

Using the Hanes-Woolf data transformation, V_{max} and K_m can be calculated.

$-K_m$ = x intercept

so **K_m = 0.150 mM**

K_m/V_{max} = y intercept

so K_m / y intercept = V_{max}

0.150 / 0.1332 = V_{max}

V_{max} = 1.13 μmol.min^{-1}mg^{-1}

The value of K_m measured from this plot agrees more closely with that observed by Liu et. al. (2001), which was 0.11 mM.

7.5.1.3 Eadie-Hofstee Transformation

A plot of V versus V/S, also known as the Eadie-Hofstee plot, was produced from the dataset and is shown in Figure 7.6. The nature of the transformation applied to the data, where the dependant variable V is represented on both axes, causes the unusual spread of data points in a diagonal from the mean of each point. The graph shown in Figure 7.6 therefore does, in fact, represent a typical Eadie-Hofstee plot in terms of spread of the data points due to experimental error (Cornish-Bowden, 1995).

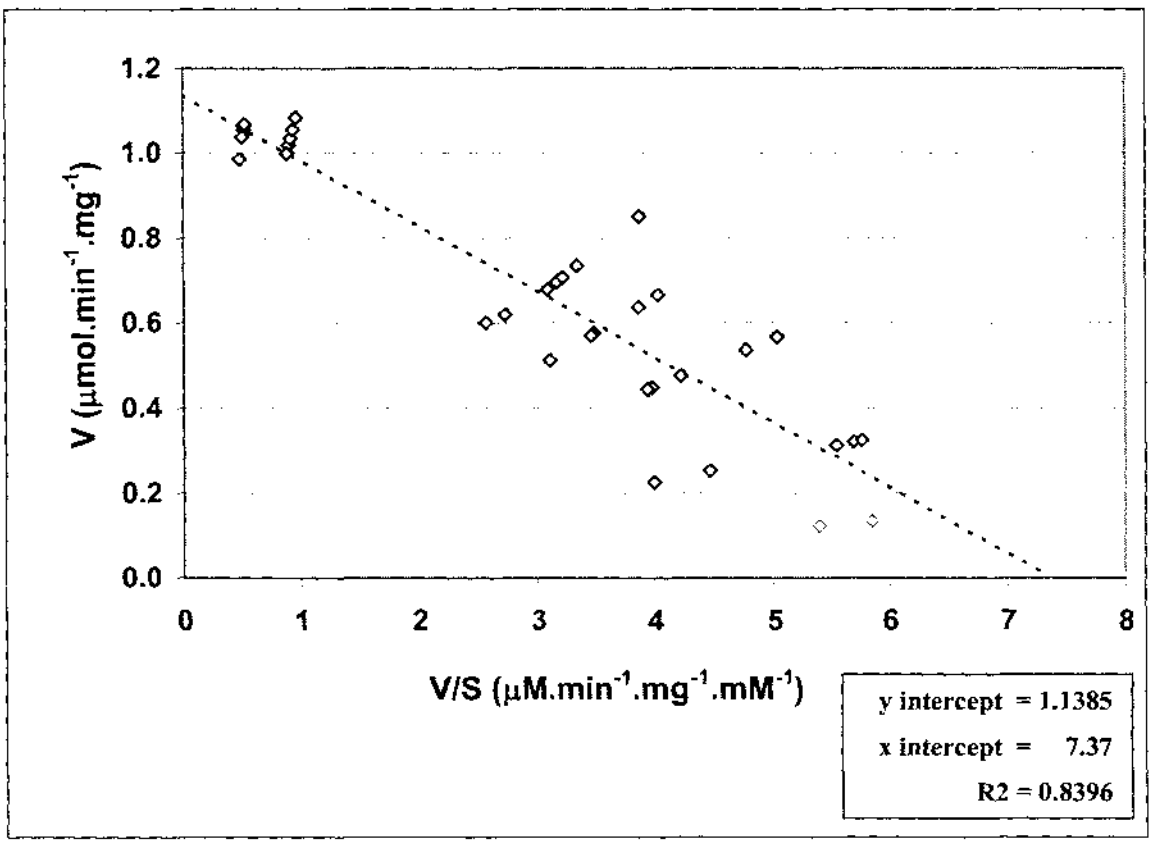


Figure 7.6 *Eadie-Hofstee plot of* p-*nitrophenyl butyrate hydrolysis by PepX*

Using the Eadie-Hoffstee transformation, K_m and V_{max} can be calculated.

V_{max}/K_m = x intercept

$-K_m$ = slope

V_{max} = y intercept

so **K_m = 0.154 µmol**

so V_{max}/K_m = 7.37

$V_{max}/0.154$ = 7.37

V_{max} = 1.13 µmol.min^{-1}mg^{-1}

Or from the y intercept:

V_{max} = 1.14 µmol.min^{-1}mg^{-1}

From the plot it can be seen that the data do not quite lie in a straight line. However the two main outlying data points, shown in grey at lower right, represent

measurements made of enzyme activity on the lowest substrate concentration assayed. There were only two measurements made at this concentration due to problems with reproducibility at low substrate concentrations, as discussed above. It is therefore difficult to gauge where the true line of best fit lies in the absence of further data points. Additionally the R^2 value of how well this line fits the observed data is low, at 0.86, and ideally needs to be higher to be certain that the data can be fitted to a straight-line relationship. Further experiments may rectify this problem, but might be futile due to problems in accurately measuring substrate at such a low concentration.

7.5.1.4 Summary of Data Transformations (Esterase Activity)

In the sections above, the data obtained during the experimental hydrolysis of *p*-nitrophenyl butyrate were transformed in several ways to examine the validity of the measurements made. Table 7.1 gives a summary of the measurements made by different methods.

Transformation	**K_m (μmol)**	**V_{max} (μmol.min^{-1}mg^{-1})**
Lineweaver-Burke	0.21	1.33
Hanes-Woolf	0.150	1.13
Eadie-Hofstee	0.154	1.13*
Native PepX (Liu, et al. 2001)	0.11	2.9

Table 7.1 *Summary of kinetic parameters calculated using various transformations*

**average of both methods used*

The measurements made with the Hanes-Woolf and Eadie-Hofstee transformations agree closely, whilst those made using the Lineweaver-Burke method are somewhat higher. It should be noted however that, as discussed, the Lineweaver-Burke plot is prone to being skewed by the higher weightings placed on the more error-prone low substrate concentration measurements.

The Hanes-Woolf and Eadie-Hofstee values agree reasonably with the values for K_m and V_{max} experimentally measured for Esterase II (Liu *et al.*, 2001). In addition it should be noted that the Eadie-Hofstee plot tends to increase errors in the data. The fact that the values measured from this plot are similar to those measured by the Hanes-Woolf plot as well as the K_m value obtained from Esterase II (Liu *et al.*, 2001), indicates the data set obtained is an accurate reflection of the kinetic parameters of the enzyme. It should be noted that the assays performed by Liu, *et al.* (2001) took place under different reaction conditions to those used in this study. Whilst this study was carried out at in 20 mM BTP buffer at pH 7.0, Liu, *et al.* (2001) used 100 mM HEPES buffer at pH 7.5. Some experimental evidence was obtained during this study that suggested the optimal pH for the esterase activity of PepX was around pH 7.5, but this

pH was not used when it became apparent that *p*-nitrophenyl butyrate spontaneously hydrolysed above pH 7.5 (data not shown). The apparent two-fold difference in V_{max} between this work and that of Liu, *et al.* (2001) may be due the enzyme being more active as an esterase at higher pH values than those used in this study. Further assays to determine V_{max} at pH 7.5 could establish whether this is in fact the case.

The K_m value found in the current study suggests that it is likely the recombinant *E. coli* expressed enzyme is correctly folded, since it has kinetic properties that are nearly identical to those of the native enzyme.

As mentioned in the introduction, the kinetics of the PepX enzyme on artificial substrates does not necessarily reflect the activity on peptide or glyceride substrates. However the characterization of the enzyme activity on these substrates allows further studies to be carried out, such as investigations of enzyme inhibitors, with these values of V_{max} and K_m used as a baseline.

7.5.2 Glycylprolyl *p*-nitroanilide Assays for Dipeptidase Activity

Assays were carried out over a substrate range from 0.07 mM to 6 mM. The data points measured for a substrate concentration of 6 mM were used to confirm that V_{max} had been reached, and were discarded in subsequent analyses.

A Michaelis-Menten plot of the data (Figure 7.7) shows that the hydrolysis of peptide bonds takes place at a much greater rate compared with that of the butyl esterase substrate. In other words, PepX is more efficient as a peptidase than as an esterase. The raw data has been plotted on this graph to demonstrate the spread of measurements made during these assays.

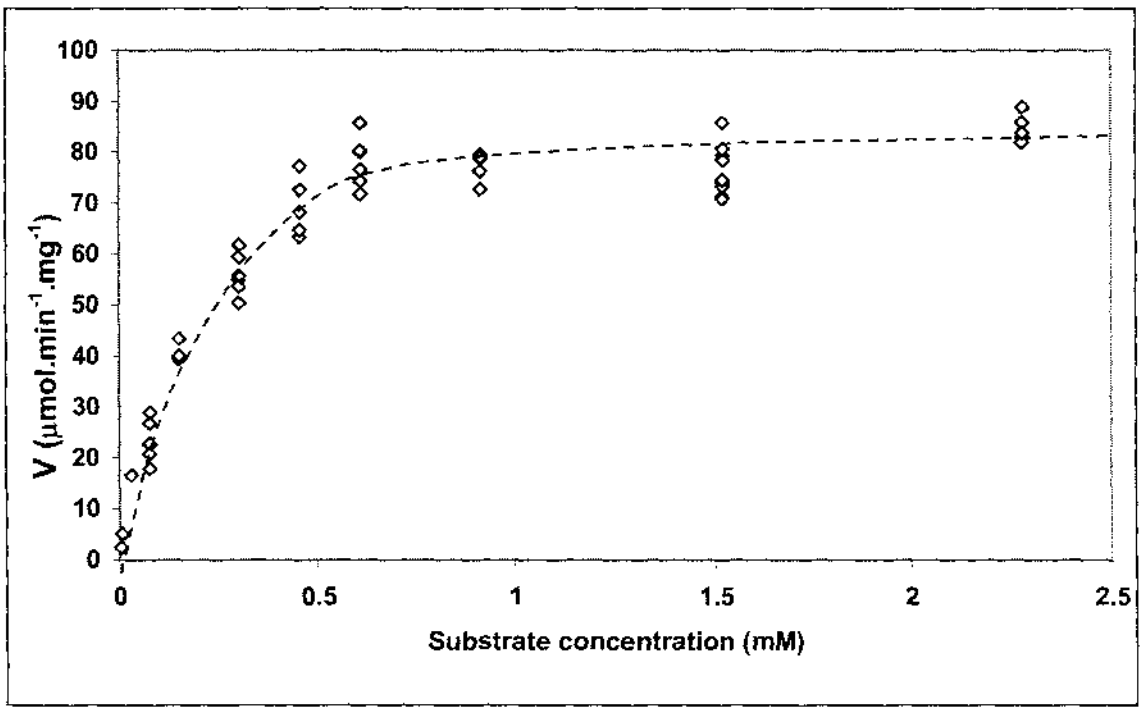


Figure 7.7 *Michaelis-Menten plot of glycylprolyl* p-*nitrophenol anilide hydrolysis by PepX.*

Initial trials performed by addition of tributyrin to the peptidase chromogenic assay indicated that the tributyrin caused a decrease in peptidase activity. Further assays for a range of glycylprolyl *p*-nitroanilide concentrations and two fixed concentrations of tributyrin were carried out. The data gathered from these assays are plotted in Figure 7.8. From this graph it is evident that the addition of tributyrin causes a decrease in enzymatic hydrolysis of the chromogenic substrate. A greater amount of tributyrin added causes a greater decrease in activity. In addition, for the case where tributyrin concentration was 0.05 µL per mL of buffer, the loss in activity could be overcome by the addition of a higher concentration of glycylprolyl *p*-nitroanilide. This was evident

at a concentration of 6 mM substrate, where the data points for the inhibited assay, and the uninhibited assays have similar values (data not shown). This suggests that the type of inhibition observed is competitive inhibition between the two enzyme substrates.

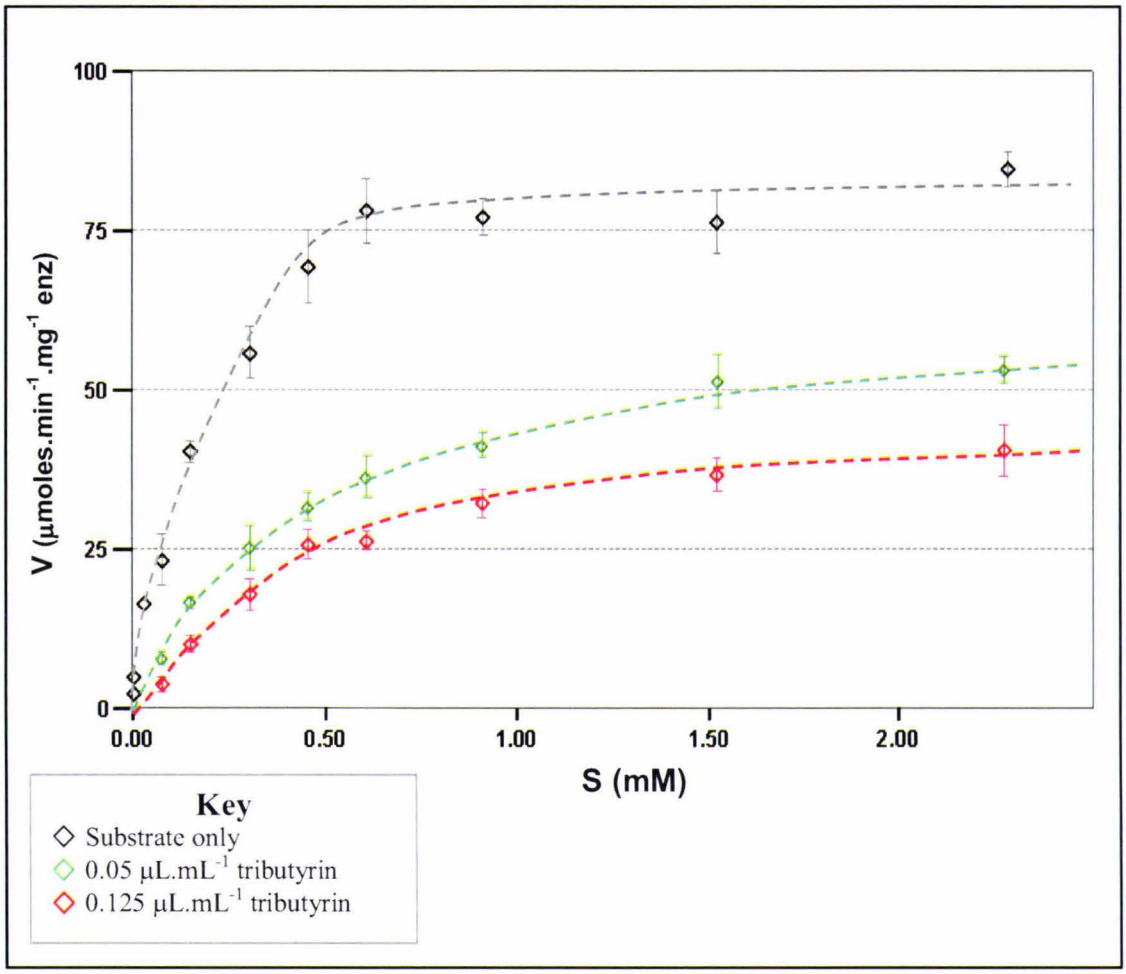


Figure 7.8 *Michaelis-Menten plot for glycylprolyl* p-*nitroanilide hydrolysis by PepX in the presence of tributyrin.*

7.5.2.1 Lineweaver-Burke Transformation

A Lineweaver-Burke reciprocal function was performed on the data and is shown in Figure 7.9. Lineweaver-Burke plots are an ideal way to identify competitive inhibition. On a Lineweaver-Burke plot, $1/V_{max}$ can be obtained directly from the y intercept of the plotted data, therefore a series of assays carried out in the presence of different concentrations of a competitive inhibitor should all intercept the y axis at the same point.

The overall Lineweaver-Burke plot of the data, shown in the top panel of Figure 7.9, is satisfactory. The data points lie in a straight line, as expected, and the R-squared

values for the "substrate only" and "0.05 $\mu L.mL^{-1}$ tributyrin" series show acceptable goodness of fit at 0.9894 and 0.9635 respectively. For the "0.125$\mu L.mL^{-1}$ tributyrin" series the R^2 value is less acceptable at 0.8474. The lines of best fit for each series do not intercept precisely on the y axis, possible due to this slight variation from an ideal straight fit. However, the lines cross very close to the axis in terms of experimental error. The main disadvantages of the Lineweaver-Burke plot have been discussed previously. Because the lowest substrate concentrations and are the most prone to experimental errors, it is common practice to omit lower substrate concentrations when plotting Lineweaver-Burke plots (Cornish-Bowden, 1995). When the lowest concentration was omitted in this study, the best-fit lines all crossed just inside the +x, +y quadrant of the plot, but no lines intersected directly on the y-axis (plot not shown).

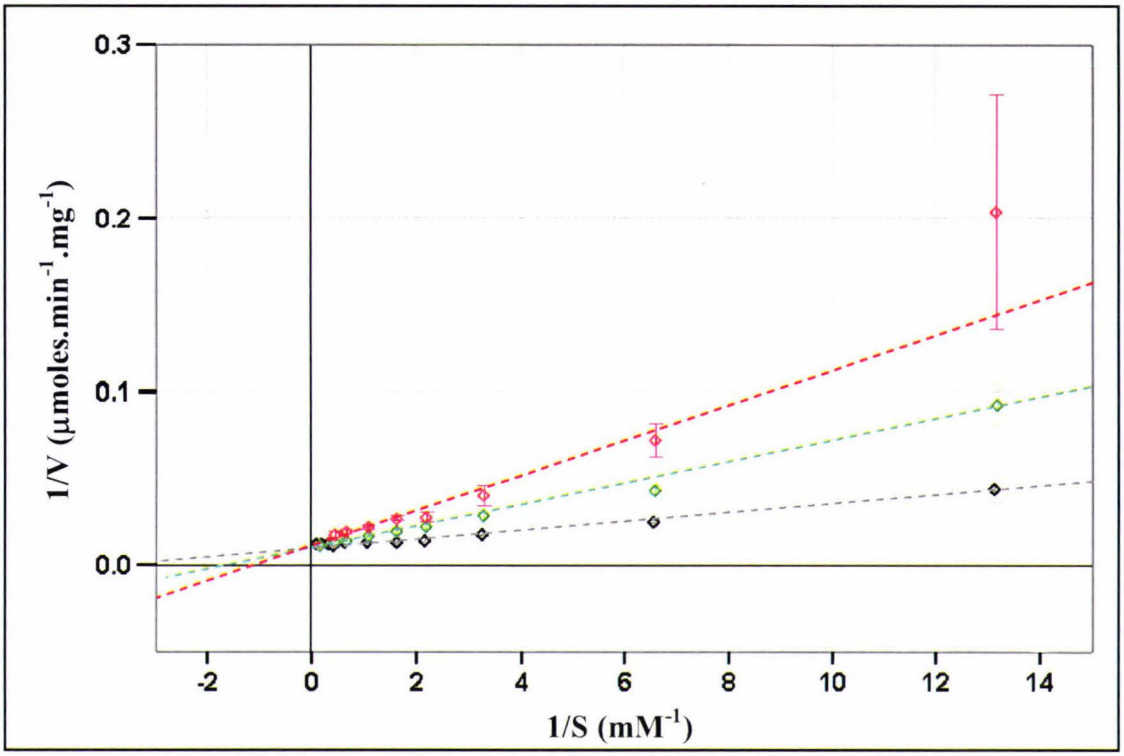


Figure 7.9 *Lineweaver-Burke reciprocal plot of glycylprolyl* p-*nitroanilide hydrolysis by PepX in the presence of tributyrin. Series colours are the same as that used in Figure 7.8. Error bars represent 1 standard deviation from the mean.*

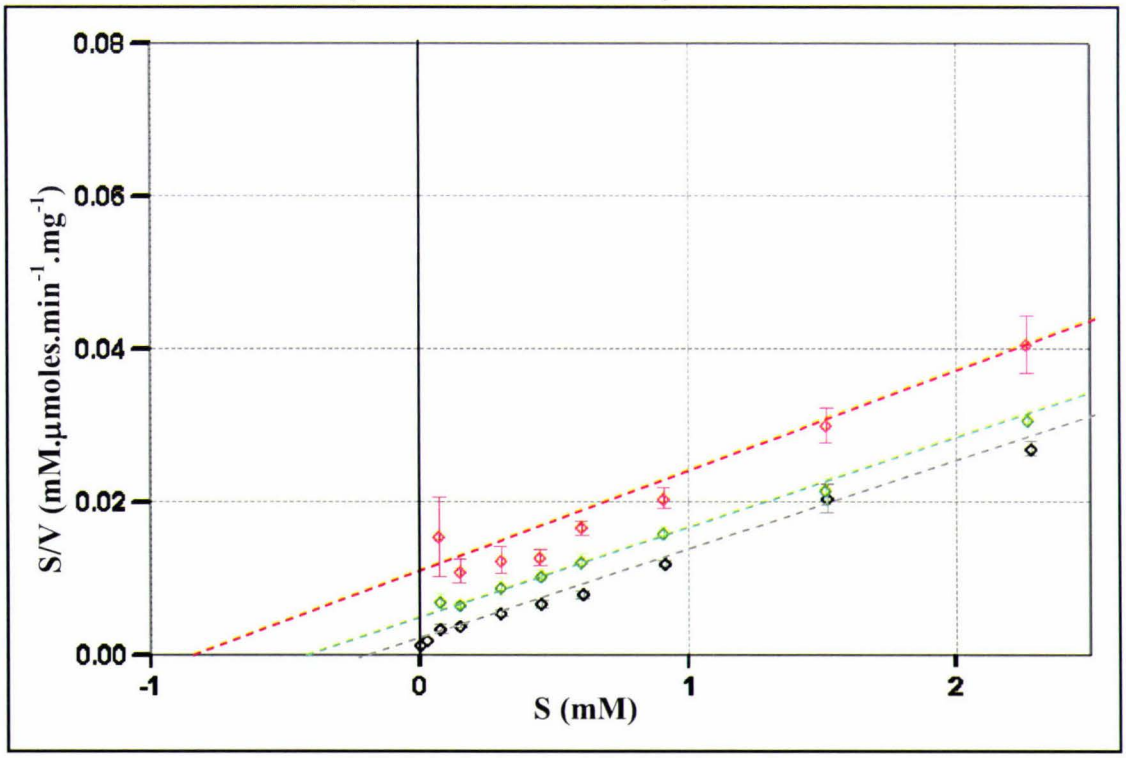


Figure 7.10 *Hanes-Woolf plot of glycylprolyl* p-*nitroanilide hydrolysis by PepX in the presence of tributyrin. Series colours are the same as that used in Figure 7.8. Error bars represent 1 standard deviation from the mean*

7.5.2.2 Hanes-Woolf Transformation

As discussed previously the plot of S/V versus S, the Hanes-Woolf transformation, is a better way to obtain kinetic data, as it does not apply disproportionately high weighting to any measured values, unlike the Lineweaver-Burke plot. The Hanes-Woolf plot for the data is shown in Figure 7.10

From the equations of the best-fit lines (not shown on plot for clarity) the x and y intercepts were calculated. As mentioned previously, in the case of the Hanes-Woolf plot, the slope of the best-fit line is $1/V_{max}$, and the x intercept is $-K_m$

Fit Line	Slope	y intercept	x intercept	K_m	V_{max}
Substrate only	0.0113	0.0028	-0.179	0.179	88.4 $\mu M.mg^{-1}$
Substrate + 0.125 µL/mL tributyrin	0.0133	0.0131	-0.708	0.708	75.1 $\mu M.mg^{-1}$
Substrate + 0.05 µL/mL tributyrin	0.0110	0.0076	-0.500	0.500	90.9 $\mu M.mg^{-1}$

Table 7.2 *Kinetic parameters for glycylprolyl* p-*nitroanilide hydrolysis by PepX derived from the Hanes-Woolf transformation*

The kinetic parameters calculated from this plot show that the presence of tributyrin appears to alter the K_m of the enzyme, but does not alter the V_{max} significantly. In the case of the 0.125 µL/mL tributyrin series, V_{max} appears to be somewhat less than that calculated for the other two series. Re-examination of Figure 7.10 shows that some of the data points for the lowest substrate concentration are higher up the S/V axis (y axis) than would be ideal for the straight line fit (0.125 $\mu L.ml^{-1}$, closest to the y axis). The spread of these points due to experimental error between replicates is greater than for any other set of replicates on the plot, suggesting a relatively high amount of experimental error across these measurements. This is not surprising as they represent the lowest substrate concentrations of the series, perfectly illustrating the difficulties experienced in kinetic analysis. If these data values were more closely spaced, the slope of the best-fit line would be decreased, and become similar to the other best-fit lines, bringing the calculated value of V_{max} in line with the other values obtained.

7.5.2.3 Eadie-Hofstee Transformation

As a further check of the measurements made using the method above, an Eadie-Hofstee transformation was applied to the data. The plot of the Eadie-Hofstee transformation, V versus V/S is shown in Figure 7.11.

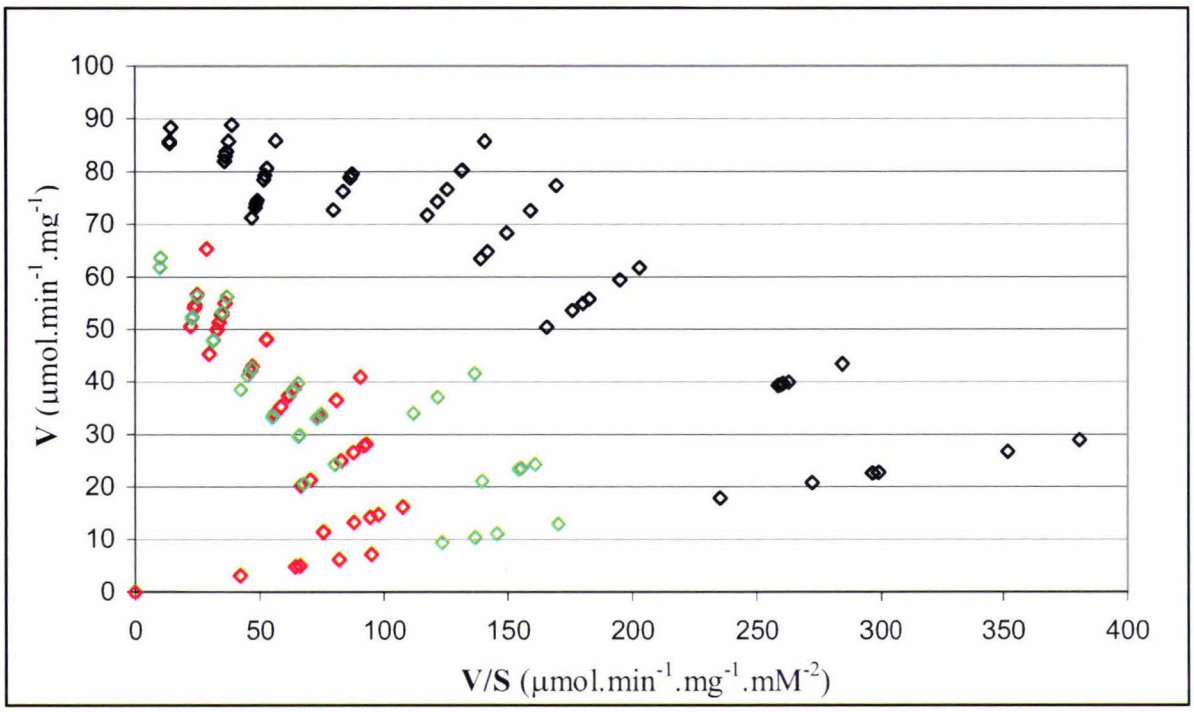


Figure 7.11 *Eadie-Hofstee plot of Glycylprolyl p-nitrophenol anilide hydrolysis by PepX in the presence of tributyrin. Series colours are the same as those used in Figure 7.8*

As mentioned in section 7.5.1.3, the Eadie-Hofstee transformation tends to amplify any errors that cause experimental values to deviate from the Michaelis-Menten ideal. This is evident from the plot of these data. The data points for each series should fall in a straight line; however on this plot this is not the case. For this reason, no best-fit lines have been plotted, as they would be of little value in obtaining kinetic data. The deviation of the data points from a straight line on the Eadie-Hofstee plot does not necessarily mean that non-Michaelis-Menten kinetics are being observed. Given the previously well-characterized action of X-prolyl dipeptidase on its authentic peptide substrate (Meyer and Jordi, 1987), it is possible that this deviation from a straight line is caused by an artefact of enzyme activity on the chromogenic substrate. With the Eadie-Hofstee plot the measurements made of enzyme velocity affect both axes of the plot, rather than just one axis. This means any experimental errors cause deviations on both axes, so it is also possible that this non-linearity is caused by experimental errors.

7.5.2.4 Summary of Data Transformations (Peptidase Activity)

Kinetic parameters for the hydrolysis of glycylprolyl *p*-nitroanilide were obtained for assay conditions of 37 °C at pH 7.0, conditions that have not been examined before with this particular substrate. The value of V_{max} observed in this study was lower than that found by other authors using different substrates(Meyer and Jordi, 1987) but this is not unexpected as V_{max} is dependent on a range of conditions, including pH, and also varies with different substrates.

The data measured for the hydrolysis of the synthetic dipeptidase substrate in the presence of tributyrin was transformed in several ways to examine the validity of the data and to determine if tributyrin inhibits the PepX enzyme in a competitive fashion. Although deriving kinetic parameters from the Eadie-Hofstee transformation was impossible due to experimental errors, the kinetic parameters obtained using the Hanes-Woolf transformation suggest strongly that tributyrin competitively inhibits the dipeptidase activity of PepX.

7.5.3 Esterase and Peptidase Activity in the Presence of Ethanol

To test if ethanol, a substrate for the ester synthetic transferase activity of PepX, inhibits the hydrolytic activities of PepX, chromogenic substrate assays were carried out using both ester and peptide substrates. The rationale behind these assays was to investigate the possibility that, rather than hydrolysing *p*-nitrophenyl butyrate to *p*-nitrophenol and butyric acid, the enzyme was transferring the butyl group to methanol (which is present in the standard assay system for esterase activity) to produce the ester methyl butanoate. For all assays the concentration of ethanol used was not enough to denature the enzyme. This is proven by the results in Chapter 8 of this thesis that demonstrate the enzyme is most active at an ethanol concentration of 2.8 M.

7.5.3.1 Ester Hydrolysis in the Absence of Alcohol

The assays carried out using DMSO to dissolve the substrate, rather than the usual methanol, indicate that the substrate is being hydrolysed not transferred. This is because *p*-nitrophenol was released in the absence of any alcohol acceptor. If an acyl group transfer was occurring, either no chromophore release would occur, as the substrate would not be acted upon in the absence of an acceptor, or limited chromophore release would be observed until the all the enzyme molecules in the solution had formed acyl intermediates in the absence of an alcohol acceptor. The formation of a stable acyl intermediate is highly unlikely, as these complexes are, due to the nature of enzyme catalysis, unstable. In cases where a stable intermediate forms, it is usually an irreversible process, resulting in permanent inactivation of the enzyme.

7.5.3.2 Inhibition of Esterase Activity by Ethanol

As shown in Figure 7.12, inhibition of esterase activity was observed in the presence of ethanol only when DMSO was used to dissolve the chromogenic substrate during substrate preparation. In the assay carried out using methanol to dissolve the substrate, limited inhibition was observed initially, but did not increase when ethanol was subsequently added to the reaction mix to a final concentration of 1.8 M. The reason for the lack of inhibition by ethanol in the presence of methanol is unclear, especially as in methanol-based assays there is a lower concentration of methanol (1 M) than ethanol present, and some inhibition by high concentrations of ethanol would be

expected. Methanol does not appear to inhibit the esterase activity of PepX on the chromogenic substrate, even when the concentration of methanol is increased to 1.8 M.

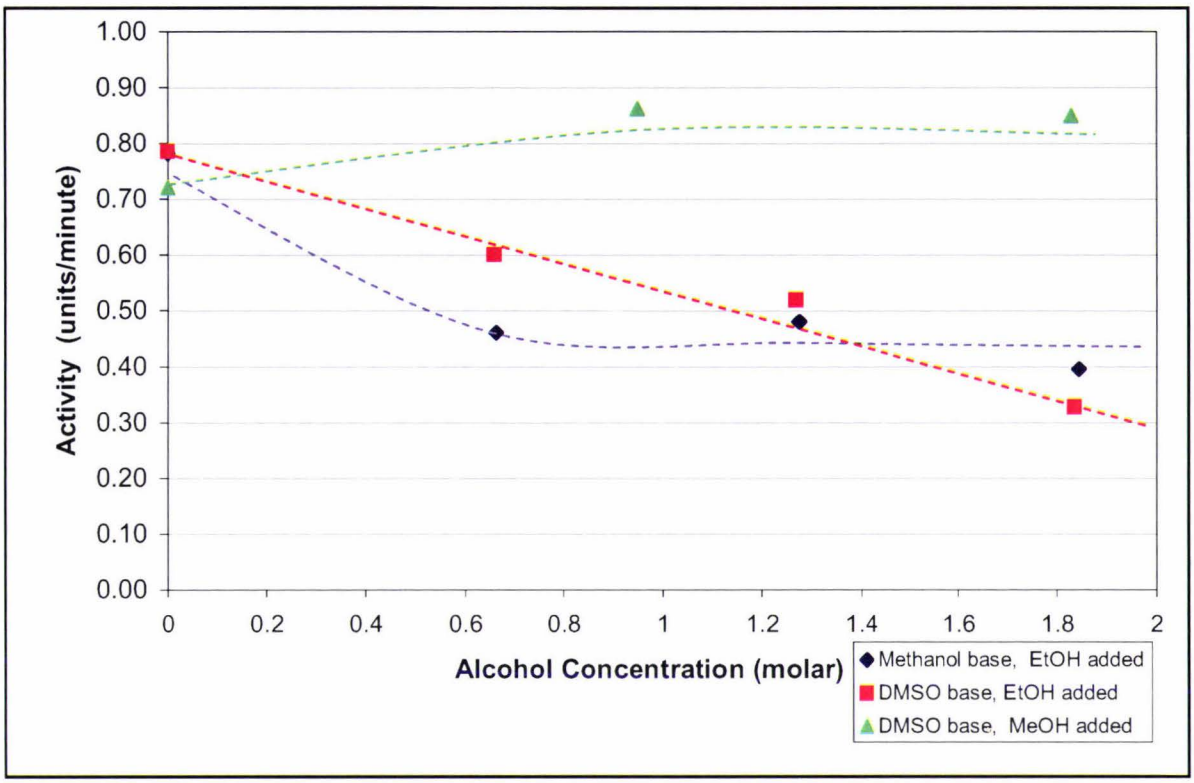


Figure 7.12 *Ester hydrolase activity of PepX in the presence of alcohols. The solvent used in substrate preparation is indicated in the legend.*

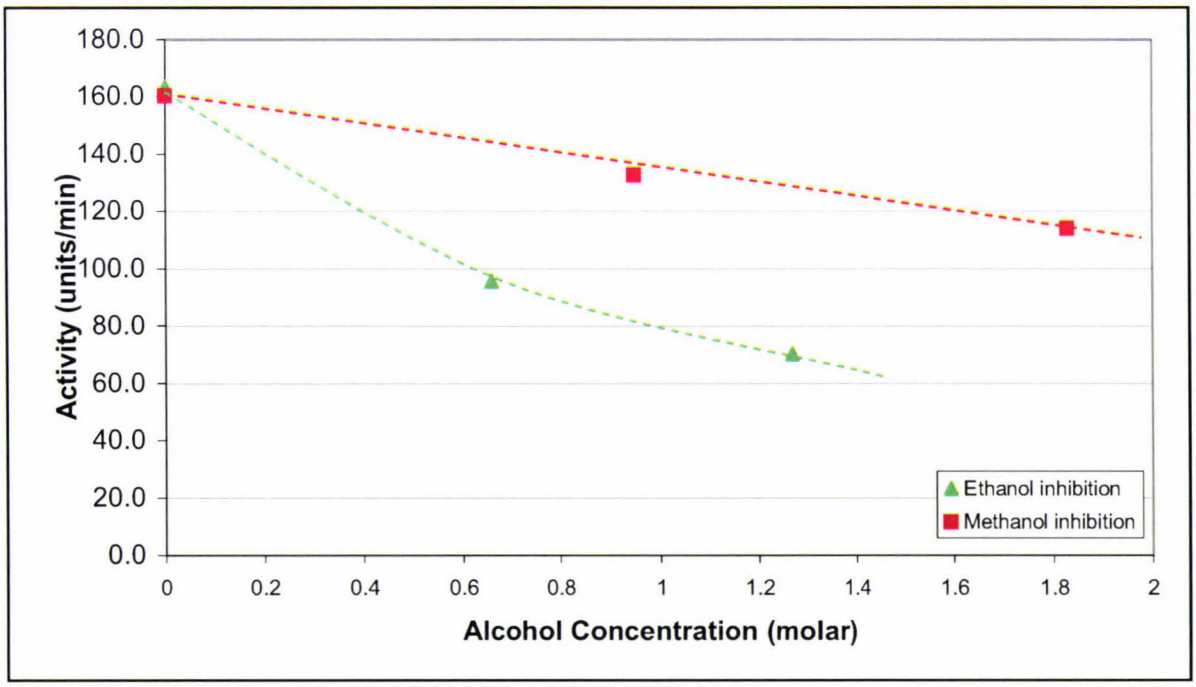


Figure 7.13 *Peptide hydrolase activity of PepX in the presence of alcohols.*

7.5.3.3 Inhibition of Peptidase Activity by Alcohol

The effect of adding different concentrations of ethanol and methanol to the chromogenic assay for dipeptidase activity was examined, and is shown in Figure 7.13. From this plot of activity versus alcohol concentration, it can be seen that the dipeptidase activity of PepX is inhibited by the addition of both ethanol and methanol, although less inhibition is observed with methanol.

7.5.3.4 Interpretation of Inhibition Results

The results of the alcohol addition experiments appear to disprove the hypothesis that *p*-nitrophenyl butyrate is participating in a transferase reaction, rather than a hydrolytic reaction, since the addition of ethanol, a known acceptor for the transferase reaction of PepX, does not increase the release of the chromophore group. In fact it has the opposite effect inhibiting the hydrolytic reaction. This was totally unexpected and a model, shown in Figure 7.14, is proposed to explain these results.

In the model, during esterase activity, methanol will bind to PepX preferentially over ethanol, and is not able to be displaced by ethanol. Methanol is small enough to allow water to access the active site and complete the hydrolysis of the ester substrate. As the sole alcohol present, ethanol will bind to the active site, and since it is larger than methanol, prevents the substrate from binding.

During peptidase activity, both alcohols bind to the enzyme in such a way as to prevent the substrate from binding and being hydrolysed, possibly by occupying a binding site. Methanol is smaller than ethanol, and is consequently less effective at blocking the peptide substrate from the active site.

These results support the hypothesis that one active site performs esterase and peptidase activities, as well as group transfer reactions, since peptide hydrolysis is blocked by a known acyltransferase acceptor molecule.

It should be noted this model is only putative, and requires the structural information of how the enzyme binds its substrates to be verified. The layout of the substrate binding sites shown in this model is not intended to necessarily be representative of the layout in PepX.

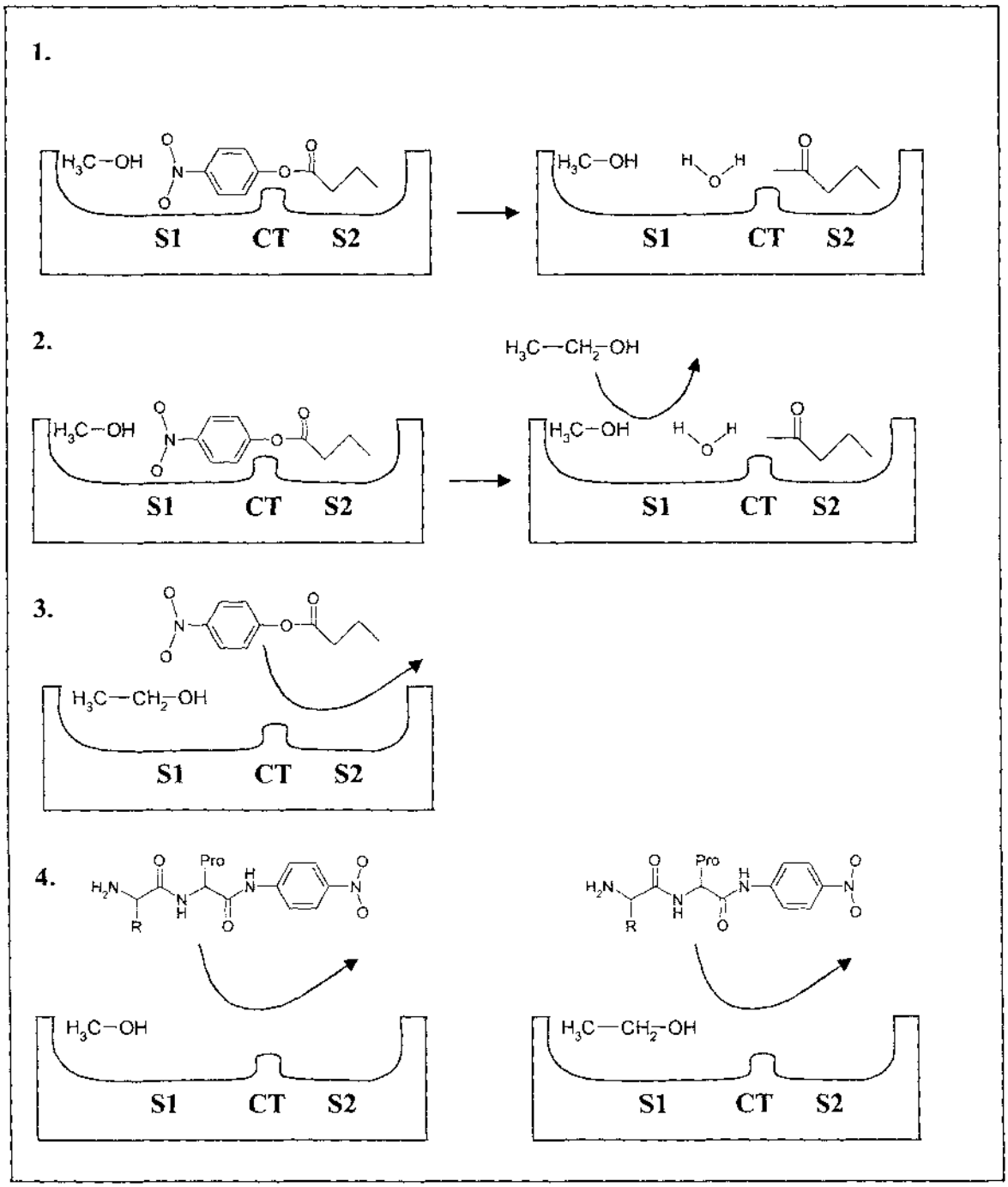


Figure 7.14 *Proposed model of PepX alcohol inhibition. Shown is PepX with two substrate binding sites (S1 & S2), and the catalytic triad (CT).*
In (1), methanol binds at site 1, but is small enough that the chromogenic ester substrate p-*nitrophenyl butyrate is still able to bind, and be hydrolysed.*
In (2), methanol is present and binds at site 1. This prevents ethanol from binding to this site, so the substrate can still be hydrolysed.
In (3) ethanol is present only, and binds to site 1. This prevents the binding of p-*nitrophenyl butyrate to the enzyme.*
In (4) the presence of ethanol and methanol in site 1 prevents the binding of glycylprolyl p-*nitroanilide to the enzyme.*

7.6 Conclusions

The purpose of the study detailed in this chapter was to determine if the recombinant version of PepX exhibited a true esterase activity with identical kinetic parameters to those of the native enzyme, and to investigate whether both of the observed hydrolase activities (peptidase and esterase) were carried out at a single active site.

Analysis of kinetic assays over a large range of concentrations of synthetic esterase substrate showed that the esterase activity of the enzyme is indeed a pure esterase activity, with no indication of the interfacial activation characteristic of lipases. This is supported by both the fit of the enzyme activity curve to the Michaelis-Menten equation for fully soluble substrates, and the observation that maximum reaction velocity is reached whilst the substrate is fully soluble, and is not increased at concentrations where substrate micelles are observed visually as clouding in the solution.

The study confirmed that the K_m for PepX in esterase mode, as measured by hydrolysis of *p*-nitrophenyl butyrate, was similar to the K_m measured from the native enzyme (Liu *et al.*, 2001). However, the V_{max} for this enzyme differed from that found by these authors, being less by a factor of two. It should be noted that different reaction conditions were used in these two studies, and this may explain the apparent difference in kinetic parameters observed. The earlier study by Liu, *et al.* (2001) was carried out at pH 7.5, and there was some evidence gathered during this project that the enzyme was most active against the synthetic ester substrate between pH 7.5 and 8.0. The PepX enzyme was found to be most active on synthetic dipeptide substrates around pH 8.0 by the authors of prior work (Meyer and Jordi, 1987).

It should be noted that *p*-nitrophenyl butyrate is unstable at pH values over 7.5, and for this reason full examination of the pH maxima of esterase activity is difficult, especially when coupled with the fact that the liberated chromophore group, with a pKa of 7.15, is only detectable at 410nm in anionic form. In addition, PepX from *S. thermophilus* does not have significant activity on longer chain ester substrates (Liu *et al.*, 2001).

The examination of the kinetic parameters of the PepX enzyme hydrolysis of glycylprolyl *p*-nitroanilide produced a baseline for further studies of PepX peptide hydrolysis. This particular artificial substrate has not been used to assay for this enzyme at this pH and temperature before, and this work therefore produces a baseline for further studies of this enzyme under these conditions.

The work carried out on the synthetic dipeptide substrate with and without the addition of an ester substrate showed that it is likely that one active site performs both types of hydrolytic activity, as the dipeptide hydrolysis was inhibited by the presence of the ester substrate in a competitive fashion.

The study of esterase and peptidase activities in the absence of methanol proved that the activities seen on these substrates are purely hydrolase activities with water being used as an electron acceptor. It was important to verify this because the activity seen with these chromogenic substrates does not necessarily represent the physiological activity of the enzyme, and because PepX has been shown to use alcohols as acceptors in ester synthesis.

This study also showed that ethanol inhibits the esterase activity of the enzyme, possibly by blocking substrate binding. This inhibition does not occur if methanol is added first and may be due to methanol occupying a binding site in the enzyme, that does not block substrate binding.

Ethanol and methanol both inhibit the dipeptidase activity of PepX. While there is currently no structural information available about substrate binding in this enzyme it is hypothesised that this may be due to these alcohols occupying a binding site that is required for peptide recognition and/or binding.

7.7 Further Work

The observed kinetic data fit with the crystallographically determined structure of PepX from *Lactococcus lactis*, which shows the enzyme has a single recognizable catalytic site, located in the αß hydrolase domain. The results of the kinetic studies could be further supported by mutagenesis studies to alter the active site residues, to examine if this halts both types of hydrolysis. Using an ester chromogenic substrate, such as *p*-nitrophenyl butyrate, and inhibiting the enzyme using a peptide substrate would also be of interest and support the conclusions made in this study, although as discussed in Section 7.4.4, obtaining a suitable peptide substrate may be difficult. Exactly how the enzyme performs hydrolysis on what are, from a steric point of view, two very different types of substrate is not known. If suitable non-reversible inhibitors could be identified and obtained, the mechanism by which the enzyme binds an ester and peptidase substrate could be identified structurally, through solving the crystal structure of the enzyme complexed with the inhibitor. This would offer final evidence that a single active site catalyses both types of hydrolysis, assuming the results of this chapter can be extended to acylglyceride substrates.

Further investigation could be undertaken to understand the inhibition of hydrolase activity by methanol and ethanol. This could possibly be done using enzyme kinetics, to investigate the blocking effect methanol appears to have on ethanol inhibition. Structural studies will be necessary to completely unravel this enigma however, once suitable inhibitors have been identified.

CHAPTER 8

Specificity of PepX Transferase Activity

8.1 Introduction

8.1.1 Ester Synthesis

An interesting property of some alkyl esterases is their ability to synthesize ester compounds when provided with the appropriate substrates, and under the appropriate conditions. Like most of the alkyl esterases previously identified in lactic acid bacteria, PepX appears to be able to carry out ester synthesis as well as ester hydrolysis (Holland *et al.*, 2002). Unlike previously identified esterases however, PepX appears to have a higher ester synthetic activity than ester hydrolysis activity, when comparing a chromogenic ester substrate and an ethanol plus tributyrin synthetic reaction. (Liu *et al.*, 2001; Holland *et al.*, 2002).

The makeup of the esters produced by an enzyme is dictated by both the acylglycerides that are available to the enzyme, and the preference the enzyme has for the size of the acyl group of the donor glyceride.

It is of interest to investigate the specificity of this synthetic capability as this has the potential to lead to a better understanding of how ester flavours develop in dairy products, and how the ester components of dairy products could be controlled.

8.1.2 Transferases, Synthases and Reverse Hydrolysis

In chemical terms the reaction catalysed by a transferase enzyme is similar to that performed when a hydrolytic enzyme is driven in the reverse direction by the presence of a high concentration of substrates and a low concentration of water. In reverse hydrolysis, lack of water causes the enzyme to substitute hydrolytic reaction products as acceptors thus catalysing the union of two products, rather than the hydrolysis of one substrate into two products.

A transferase reaction, whilst chemically similar to the reversal of hydrolysis, has the important distinction of occurring when the concentration of water present is relatively high. Therefore a transferase type enzyme will preferentially use an

acceptor molecule other than water. Glyceride transferases operate by transferring a fatty acid group from a glyceride directly onto an acceptor molecule, rather than transferring a free fatty acid onto an acceptor.

In practice, a transferase will carry out transfer reactions on relatively low concentrations of substrate, whereas reverse hydrolysis will only occur at high substrate concentrations, where water is excluded from the reaction. Water exclusion can also be achieved by the inclusion of non-aqueous non-reactive organic solvents that will lower the concentration of available water. In some cases these solvents also change the hydrophobic properties of substrate binding groups, which can alter the specificity of the enzyme.

Dairy lactic acid bacteria-derived esterase enzymes with transferase activities all appear to be capable of performing ester synthesis at low substrate concentration and in the presence of water, indicating that the synthesis is a transferase reaction and is not observed because of reverse hydrolysis.

Synthase reactions are similar to transferase reactions in that they occur when two substrates are joined into one product. However in a synthase reaction the donor molecule is joined in its entirety to the acceptor molecule. In a transferase reaction, the donor group is removed from a larger molecule and then transferred directly onto an acceptor molecule.

All currently known dairy lactic acid bacterial alkyl esterase catalysed transferase activity only occurs when substrates are provided as esters such as acylglycerides, and not as carboxylic acids, which are the product of ester hydrolysis (R. Holland, personal communication). This defines the reaction catalysed by esterases as a transferase reaction rather than a synthase reaction.

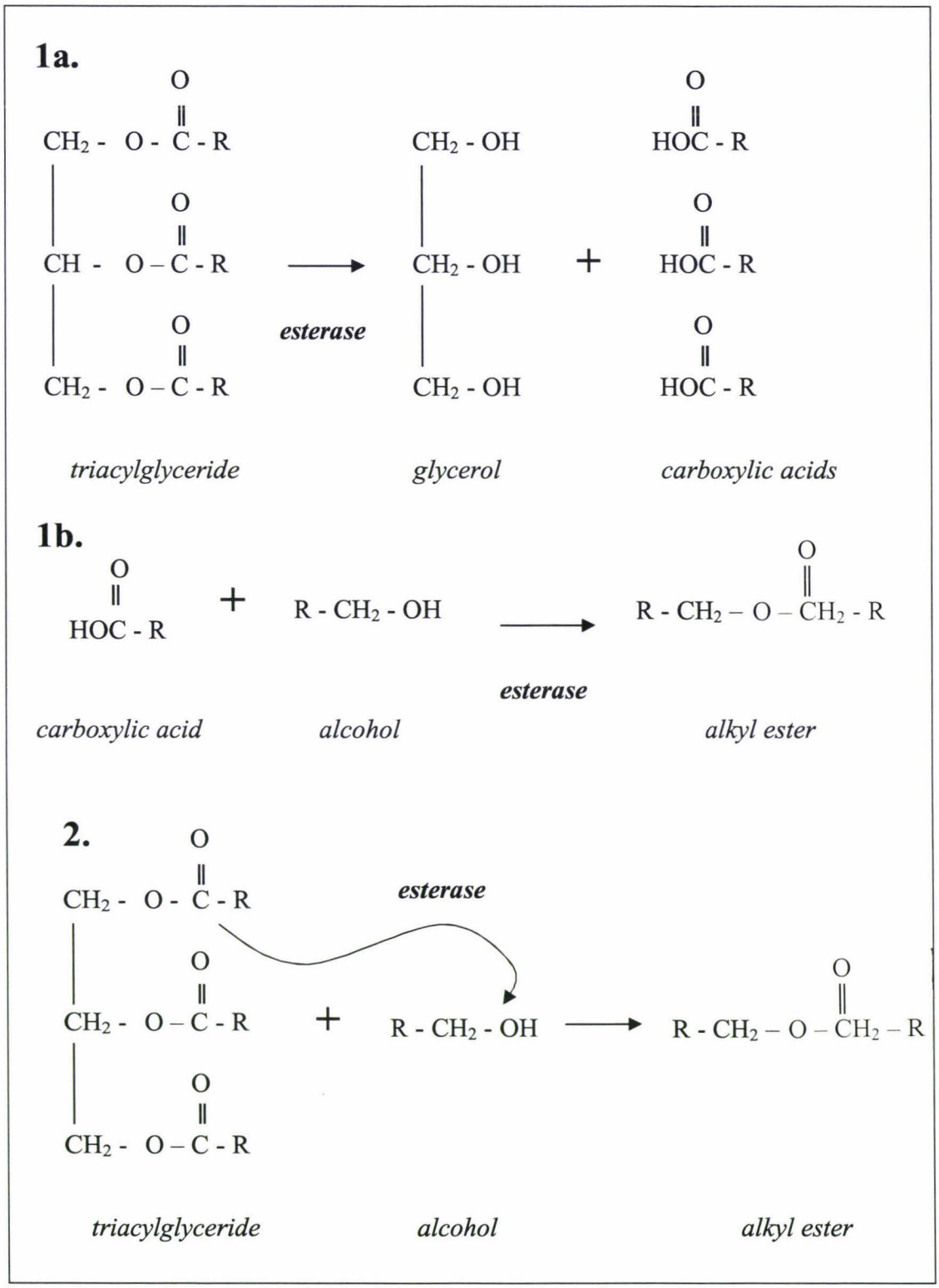


Figure 8.1 *Mechanisms of ester synthesis.*
1a, enzymatic hydrolysis of acylglyceride to glycerol and carboxylic acids and dissociation of products from enzyme.1b, Binding and enzymatic transfer of carboxylic acid to alcohol.
2. Direct enzymatic transfer of acyl group from acylglyceride to alcohol.

8.2 Objectives and Strategies

The experimental objectives of this section were to investigate the ester synthetic ability of PepX from *Streptococcus thermophilus*, using a discontinuous assay that involved extraction and analysis of reaction products after a set reaction time. Specifically these objectives were to:

1) Determine the optimal substrate concentrations for ester synthesis, in particular the ethanol concentration, which would allow assay results to be easily compared.
2) Determine an appropriate incubation time for the assays. This time needs to be long enough to allow a sufficient amount of ester product to be produced, but not so long that substrate becomes depleted by the enzyme activity, resulting in an artificially decreased activity rate that will affect the accuracy of measurements.
3) Investigate the acylglyceride substrate preference of PepX to identify the preferred number of carbon molecules in the acyl group, and to determine how the number of acyl groups affects this substrate preference.
4) Study the mode of ester synthesis by PepX, specifically to determine if this occurs over two reaction steps *via* a carboxylic acid intermediate product, or if esters are produced by direct transfer of an acyl group from a donor acylglyceride to an alcohol acceptor.

The strategy to achieve these objectives consisted of performing enzyme assays on a variety of acylglycerides and ethanol, in the presence of the PepX enzyme, with separation and quantitation of reaction products using gas chromatography.

8.3 Methods

8.3.1 Ester Synthesis Assay

To identify the preferred length of carbon chain for glyceride donors, assays to measure the rate of PepX catalysed transferase activity were carried out using a variety of acylglycerides as donor substrates.

A stock solution of substrate was made by weighing out the appropriate amount of substrate into a universal bottle. In the cases of substrates that were partially solid at room temperature, the substrate was fully melted by brief incubation in a 37 °C waterbath to produce a liquid compound that was easier to manipulate.

100 µL DMSO was added per mL of stock and the solution swirled to fully dissolve the substrate. Control assays were carried out without DMSO and it was found that the presence of this solvent had no measurable effect on the reaction rate. Gum arabic was added at 100 mg per mL of stock to stabilize the acylglyceride/buffer emulsion produced.

900 µL of 100 mM PO_4 buffer, pH 7.0 (made as detailed in Section 2.3.17) was added per mL of stock to bring the stock to the correct volume. The substrate stock was sonicated (level 4, 50% duty cycle) to fully solubilise the substrate. Care was taken during sonication to ensure the substrate was fully dispersed. In some cases this involved adjusting the position of the sonication probe in the stock to dislodge material from the bottom of the vessel. This was particularly important with longer chain glycerides that were greasy and in some cases difficult to fully disperse.

Ethanol was diluted in sterile Milli-Q water as required to reach the appropriate final concentration in the assay.

Reaction assays were then set up in either a maxi-assay or a mini-assay format. Table 8.1 summarises the components of each assay type.

Assay volume	Glyceride stock	Ethanol volume	Buffer	Total
Maxi assay	830μL	830μL	3.34mL	5mL
Mini assay	250μL	250μL	1mL	1.5mL

Table 8.1 *Reaction formats and compositions*

Maxi assays were used to determine appropriate incubation times as the larger volume allows multiple samples to be taken. Mini assays were used where replicate assays were required to ensure reproducibility.

Several maxi assays were performed to determine the appropriate amount of enzyme to use and the appropriate incubation. An incubation time of 2 hours was used for the substrate specificity mini assays, with 4 μL of PepX enzyme at 3.4mg.mL^{-1} added. After determining the length of incubation needed to gain sufficient ester production, while still remaining within the linear range of the reaction (using maxi assays), mini assays were carried out in triplicate for 2 hours. In addition, two control reactions were carried out; one contained no enzyme, and the other contained the same substrate and enzyme components as the normal reactions, but was sampled after 1 hour. The latter control reaction was intended to ensure the 2 hour samples were still in the linear range of the reaction.

After incubation at 37 °C a 1 mL sample was removed from each assay and added to a screw capped test tube containing 100 μL of 2.5 M HCl to denature the enzyme and halt ester synthesis. The sample and stop solution were mixed by shaking, and the tubes were capped to prevent ester evaporation prior to extraction.

8.3.2 Ester Extraction

To separate the esters present in the sample from the aqueous solvent of the reaction mix, 2mL of diethyl ether (DEE) was added to each stopped reaction. All further liquid transfers were carried out using glass graduated pipettes, which are not dissolved by DEE.

The tubes were re-capped and the reaction products were extracted into the organic solvent by shaking for three minutes. The tubes were then centrifuged at 3000 x g for eight minutes to separate the aqueous and organic phases. 1 mL of organic phase was removed from each tube and pipetted into a fresh screw-cap test tube containing 1mL of internal standard. This internal standard was 220 ppm propyl butyrate in DEE. The sample and internal standard were mixed by gentle agitation for a few seconds.

A vacuum extraction tank (Alltech) was loaded with test tubes to collect flowthrough from the manifold. The apparatus was then assembled by positioning the manifold on top of the tank, and loading the manifold with 500 mg silica-based NH_2 substituted solid phase extraction (SPE) cartridges (Bond Elut, Varian). The cartridges were conditioned by loading with 2 mL of DEE and allowing the solvent to flow through the cartridge bed and into the collection tubes. The assay product/internal standard mixtures were loaded into the SPE cartridges, and 500 µL was allowed to flow through into the collection tubes to complete column conditioning and to ensure any remaining conditioning DEE was displaced into the waste tubes. The collection tubes in the extraction tank were replaced with 1mL GC vials, and the remaining ester samples were passed through the SPE column and collected in the GC vials. The vials were capped with Teflon vial seals, and the samples were either analysed immediately, or stored at –18 °C wrapped in parafilm until analyses were performed within 24 h.

8.3.3 Gas Chromatographic Analysis

Gas liquid chromatography (GLC) analysis separates compounds based on their differential partitioning into and out of a mobile gas phase into a stationary polymer phase in a long thin column.

Samples are only mobile whilst in the gas phase, so samples that partition easily into the solid phase are retained in the column longer than those that spend more time in the gas phase. The column is usually heated to help compound partitioning, and a heating gradient is usually applied to optimise the separation of compounds. In this way compounds with a similar volatility at one temperature can be separated during the GC process by heating to a temperature where separation is maximized.

The gas phase is an inert gas such as helium or nitrogen, as oxygen or air will cause oxidative damage to the column at the temperatures used for analysis. Samples are injected into the gas phase at the column inlet and partition in and out of the stationary liquid phase on the column surface before they appear at the column to the outlet. Samples in this experiment were detected at the column outlet using a flame ionisation detector (FID), which heats compounds to the point of ionisation in an ion free hydrogen/oxygen flame. Usually the volume of carrier gas used in the column is too low for a flame ionisation detector to function accurately, so a make-up gas is added just before the FID to increase gas volume through the detector.

The conductivity across the detector is increased in the presence of ions, and this is recorded by the instrument as a plot of voltage against retention time. Compound peaks are usually identified by their retention time against the retention times of a set of known standard compounds that are used to calibrate the column prior to analysis. Sample compounds are quantitated by comparing the peak area of a particular compound with the peak area of a known quantity of internal standard added to the sample before analysis.

8.3.3.1 Sample Analysis

Extracted ester samples were analysed using a Shimadzu GC-15A gas chromatograph with a fused silica capillary column (solid phase DB-1, J&W Scientific, USA) that had a length of 30 m with an internal diameter of 0.25 mm and a solid phase film thickness of 1.0 μm. The solid phase, DB-1, is composed of dimethyl polysiloxane.

The oven temperature for each run was programmed at 45 °C for 5 minutes, followed by an increase to 50 °C over 1 minute, with a further increase to 270 °C over 11 minutes. The temperature was then held at 270 °C for 8 minutes. The injector temperature was held at 250 °C and the FID at 275 °C during the entire run. The carrier gas used was helium (0.9 $mL.min^{-1}$) and the make-up gas pressure at the column exit was gaseous nitrogen at 2 kg per cm^2.

The sample injection volume was 3 µL for each run, and every 5 runs the column was cleaned with two blank runs of 3 µL DEE. Prior to each set of assays to be analysed, 3 µL of external standard was run on the instrument. The peaks produced by GC analysis of each sample were identified by comparing the peak retention times with the peak retention times of the components of the external standard. The components of the external standard are documented in Table 8.2. The compound identifications made by the GC analysis software were verified by comparing ester identifications with the expected ester product of the reaction, deduced from the substrate used. Once the identity of the peaks corresponding to the ester compounds of interest were verified, the amount of ester reaction product present was quantitated using the Class VP analysis software (Shimadzu). The software compares the area of the peak of interest with the area of a peak of a known amount propyl butyrate internal standard and uses this ratio to accurately calculate how much of the ester product is present in the sample.

Ester Standard	Concentration (ppm)
Ethyl acetate	225
Ethyl butyrate	110
Propyl butyrate	**220**
Ethyl hexanoate	109
Ethyl octanoate	110
Ethyl decanoate	107.5
Ethyl laurate	108.4
Ethyl myristate	107.5
Ethyl palmitate	105
Ethyl stearate	105
Ethyl oleate	107.5

Table 8.2 *External Standard compounds used to calibrate the GC column. Propyl butyrate (in bold type) is the quantitative standard for the other external standard compounds, so all amounts are normalised to this standard.*

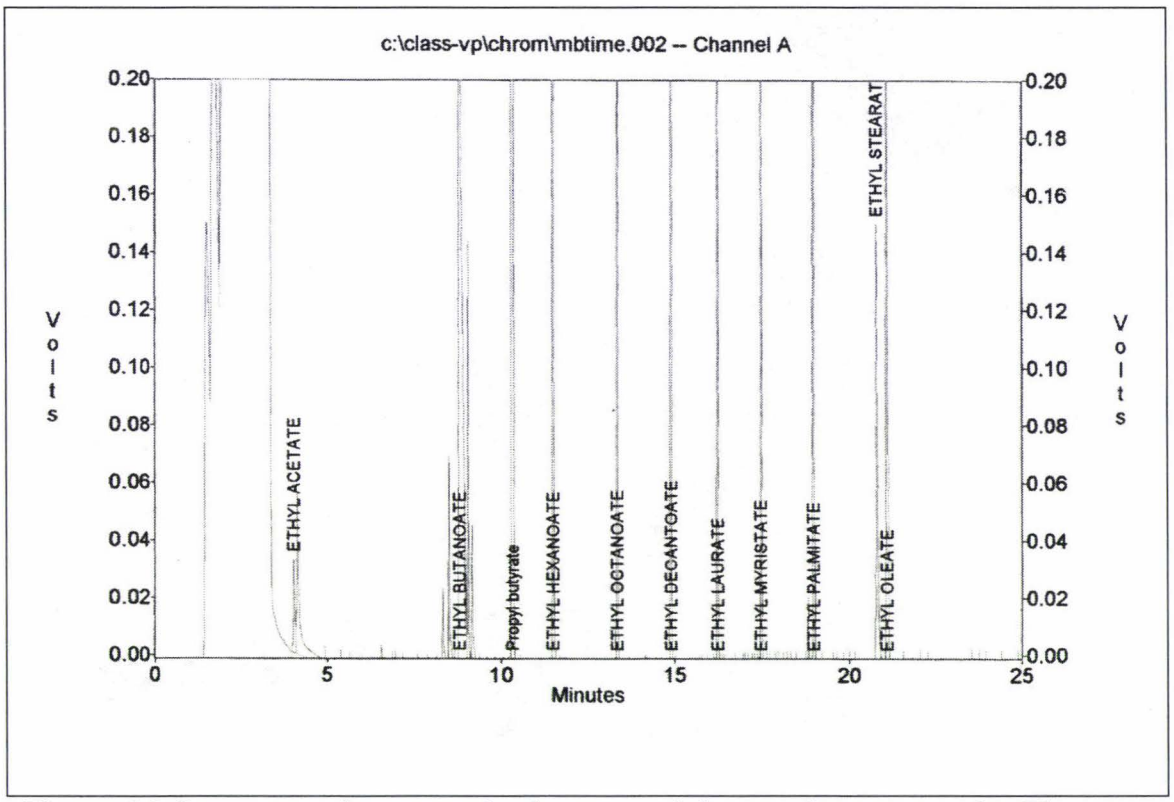


Figure 8.2 *Separation of ester standard compounds by gas chromatography. The x axis represents retention time of the compound and the y axis is the voltage across the FID detector of the instrument. Each peak is labelled with the identity of the compound as determined by the instrument software based on retention time.*

8.3.4 Optimisation of the Assay for Ester Synthesis

To analyse the substrate preference of the PepX enzyme in transferase mode, it was first necessary to find out the optimal concentration of reactants to use. By identifying the maximum velocity of the reaction, and performing all reactions at this V_{max}, the rate of synthesis using different substrates can be accurately compared. If reactions are performed at substrate concentrations that give a lesser reaction rate than V_{max}, large changes in activity could occur with small differences in substrate concentration, leading to anomalous measurements of activity. This is demonstrated in Figure 8.3. By ensuring all assays were carried out at saturating concentrations, this situation was avoided.

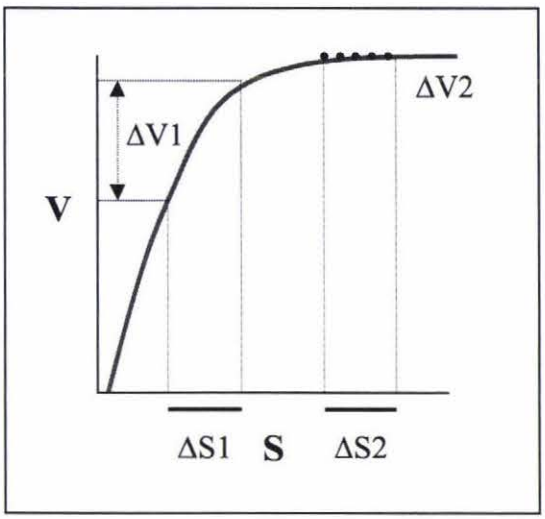


Figure 8.3 *Rationale for choosing a high substrate concentration. At low substrate concentration values, the change in V (ΔV1) can be quite high for low differences in S (ΔS1). At substrate concentrations at or above V_{max} depicted by ΔS2, even large differences in substrate concentrations result in small changes to V(ΔV2) , if any. This feature of Michaelis-Menton kinetics was used to reduce potential errors by ensuring all assays were run at the V_{max} of the enzyme.*

8.3.4.1 Optimisation of Glyceride Concentration

Glyceride substrates were used in the assay at a concentration exceeding solubility. In the case of tributyrin (the shortest glyceride surveyed, and therefore the most water soluble), a concentration of 60 mM was used to ensure saturation was maintained. Larger and therefore less soluble glycerides were utilized at 30 mM. Since PepX has been shown to be an esterase, not an interfacially activated lipase (Section 7.5.1), it is incapable of binding and using insoluble substrates, so the amount of substrate in an insoluble form is irrelevant for the purposes of the assay. Because the solubilised substrate is in equilibrium with the non-soluble substrate (in the form of a micelle) as the concentration of substrate decreases due to enzymatic activity, more substrate will be removed from the micelles into solution. Thus the glyceride concentration in maintained at a point where the assay solution is saturated. The continued presence of insoluble glyceride substrate can be verified by the presence of cloudy reagent at the end of the assay. In addition, internal checks of the reaction linearity were carried out to ensure substrate had not been depleted enough to cause a drop in activity during the course of the assay.

8.3.4.2 Optimisation of Alcohol Concentration

The optimal concentration of alcohol used was determined by performing trial reactions at different alcohol concentrations. Initially ethanol was added from a stock of 30% (w/v), but trials showed that this was far lower than maximal velocity, so in subsequent trials ethanol was added from a stock of 100% (w/v).

Ethanol concentrations up to 3.4 M were tested to identify a maximum rate of enzyme transferase activity.

8.3.5 Glyceride Substrate Specificity

To establish the donor substrate preference of PepX in terms of carbon chain length and number, mono- di- and triglyceride substrates of a range of carbon chain lengths from C4 to C10 were assayed using ethanol as an acceptor molecule. Table 8.3 details the substrates trialled.

Substrate	Carbon chain length	Number of acyl carbon chains
Tributyrin	C4	3
Tricaproin	C6	3
Monocaprylin	C8	1
Dicaprylin	C8	2
Tricaprylin	C8	3
Monocaprin	C10	1

Table 8.3 *Acylglyceride substrates trialled.*

Dicaproin was not trialled as it was not available at the time the assays were done. Some acylglyceride compounds spontaneously form esters by a non-enzyme catalysed pathway when combined with alcohol, in particular those larger than butyl glycerides. Ester formation that was not due to enzyme activity was measured in control reactions where all the components apart from enzyme were present. The rates of spontaneous synthesis measured by this control were subtracted from the final rates of synthesis in the enzyme-containing reactions to gain a measurement of the rate of ester formation due to enzyme activity.

8.3.6 Verification of Transferase Activity

Previously characterised dairy esterases have been shown to be capable of butyl ester hydrolysis as well as ester synthesis when provided with tributyrin and an ethanol acceptor, so there are two possible mechanisms of activity available to these enzymes to synthesise esters (Shaw, 1999; Holland *et al.*, 2002).

In the first mechanism, tributyrin would first be hydrolysed to produce glycerol and butyric acid, with both products being released from the enzyme. Butyric acid would then be rebound to the enzyme with an alcohol acceptor and transferred to this acceptor to form an ester.

The second mechanism is a pure transferase mode, where the acyl group is directly transferred from tributyrin to the alcohol acceptor in one step. All known esterase enzymes that are capable of ester synthesis are thought to operate by this direct transfer mode.

The mode of ester synthesis utilized by PepX was investigated by monitoring the production of esters from tributyrin and ethanol, and butyric acid and ethanol. If the enzyme was in fact hydrolysing tributyrin prior to transferring the liberated butyric acid groups onto ethanol, then ester synthesis would be seen in the assay with butyric acid and ethanol. These assays were made up as detailed in Table 8.4

Ethanol	2.85M	Tributyrin	60mM
Ethanol	2.85M	Butyric Acid	50mM

Table 8.4 *Components of assays to investigate the mechanism of ester synthesis by PepX.*

The pH of assays containing butyric acid was checked to verify that the buffering capacity of the phosphate buffer had not been exceeded by addition of butyric acid. The substrate concentrations for the butyric acid assay were chosen to be at a high enough concentration to produce detectable product in a reasonable incubation period and to avoid reducing the pH of the buffer used. In theory, 60 mM of tributyrin could, when fully hydrolysed, yield 180 mM of butyric acid, but this was judged to be unlikely as previous assays using tributyrin had shown no sign of tributyrin depletion.

It was also expected that if the enzyme was in fact hydrolysing tributyrin to butyric acid, any liberated butyric acid would be used by the enzyme reasonably quickly in order to produce esters at the rates previously measured.

Control assays were also carried out in the absence of enzyme to measure any background ester formation. All enzyme assays were initiated with 4 μL of PepX at 3.4 $mg.mL^{-1}$. Assays were incubated for 60 minutes at 37 °C and were stopped and ester products were extracted as described in Section 8.3.3 above.

8.4 Results and Discussion

8.4.1 Optimisation of Ethanol Concentration

The effect of ethanol concentration on the enzyme was investigated. The enzyme showed a remarkable tolerance of relatively high concentrations of ethanol. A definite dependant relationship between activity and ethanol concentration was observed. The ester synthetic rate for different concentrations of ethanol is presented in Figure 8.4. The calculations used to convert the GC results (ppm) into $\mu mol.min^{-1}.mg^{-1}$ are presented in Appendix 10.

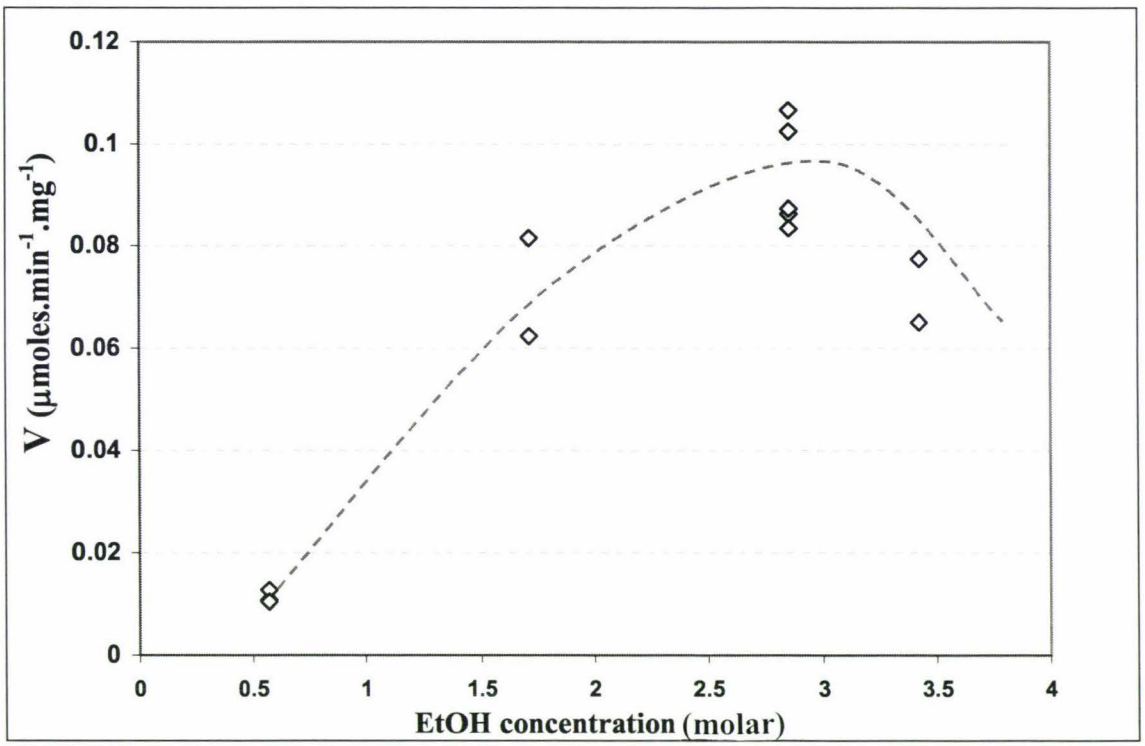


Figure 8.4 *Optimisation of ethanol concentration for formation of ethyl butanoate by PepX. Five replicates are shown for a concentration of 2.85M as further experiments were undertaken once this concentration had been established as* V_{max}.

From the plot it can be seen that the optimal ethanol concentration was found to be about 2.85M. Above this concentration a drop in ester synthesis was seen. This is likely to reflect ethanol-induced denaturation of the protein leading to activity loss. Although this is a high concentration of ethanol, at 16.7% (w/v) or 2.85 M, the

concentration of water present is still 46 M. This is a water to ethanol ratio of 16:1, so a high concentration of water is still available to the enzyme. This implies a transferase reaction is occurring, not a concentration driven reversal of hydrolysis.

8.4.2 Verification of Reaction Linearity

To verify the linearity of the reaction over the period of incubation used, a maxi assay was carried out and sampled after 15, 30 and 120 minutes. The amounts of product in each sample are compared in Figure 8.5. From this graph it can be seen that the reaction was still in a linear phase at an incubation time of 2 hours. This validates the length of time chosen to run further assays, which was 2 hours also, shows that the measurements made are for the enzyme with no activity reduction due to substrate depletion.

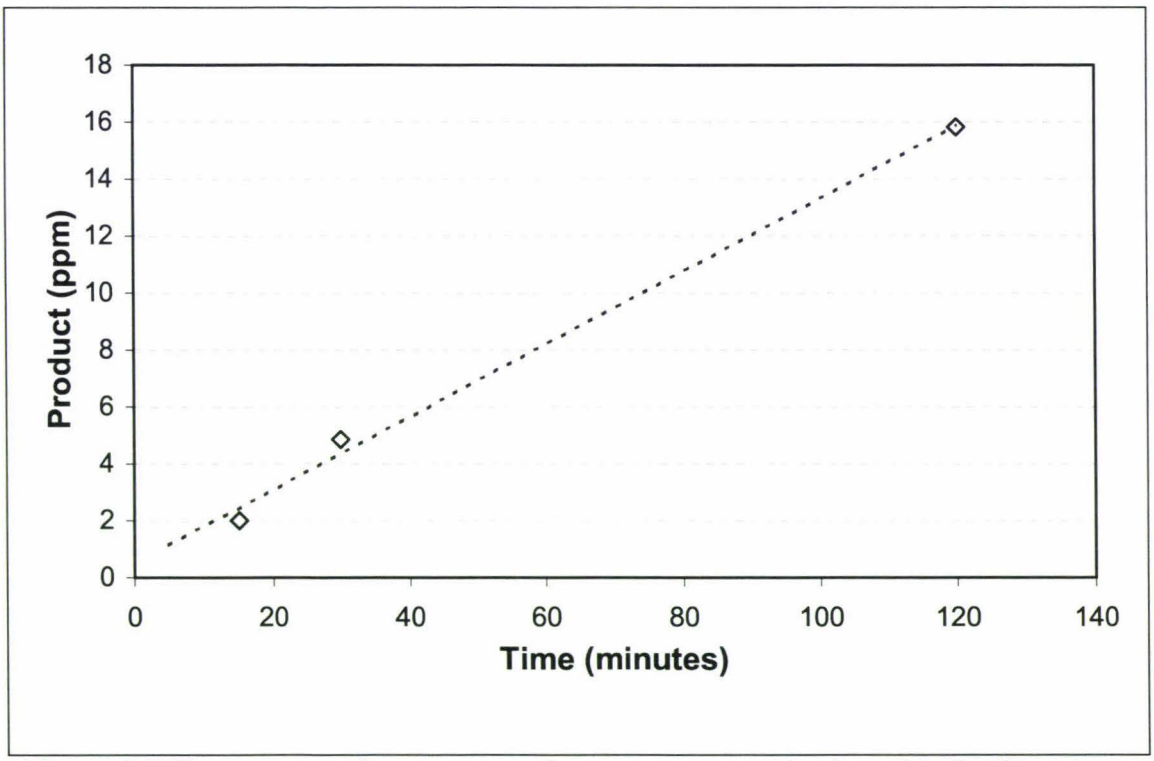


Figure 8.5 *Time course of enzyme transferase reaction, with ethanol and tributyrin substrates producing ethyl butanoate.*

8.4.3 Glyceride Substrate Specificity

The glyceride substrate preference of PepX ethyl ester production was investigated by incubating ethanol with various glycerides in the presence of PepX. The amount of ester product produced was quantitated by gas chromatography as described above. Spontaneous ester formation was observed in some of the control reactions when no enzyme was present. This was quantitated and subtracted from the amount of ester product produced in the enzyme assays.

The main observation made during these experiments was that the PepX enzyme appeared to only catalyse a transfer reaction when tributyrin was provided as a donor molecule. For the other glyceride donors, no significant ester formation was observed, other than that which occurred spontaneously. The data obtained for ester formation observed with different acylglycerides are shown in Table 8.5 and summarized in Figure 8.6

Acyl donor	Carbon chain length	Amount of ester (ppm)
Tributyrin	4	10.8
Tricaproin	6	-0.0029*
Monocaprylin	8	0.21
Dicaprylin	8	-0.019*
Tricaprylin	8	-0.42*
Monocaprin	10	-0.47*

Table 8.5 *Ester products produced in assays using ethanol and a range of acyl donors. The amounts shown are the mean of three triplicates less any spontaneous ester formation as measured by enzyme-free assays. Negative values for ester content indicated by * are due to the averaging process and experimental errors in GC measurements. Assays were incubated for a time period of 2 hours*

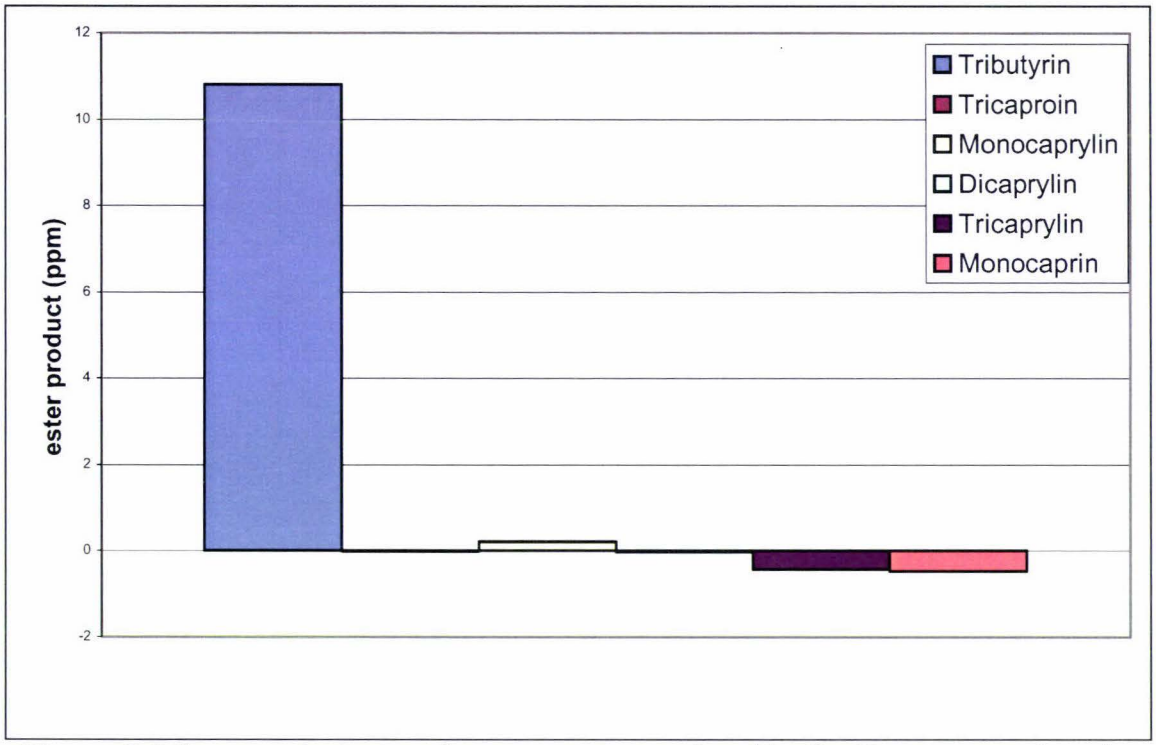


Figure 8.6 *Ester synthetic transferase reaction catalysed by PepX acting on ethanol and glycerides*

Intriguingly the results show that not only is PepX acting as a transferase, but also it is very selective for acyl substrates, as it will only transfer a butyl fatty acid, and not larger fatty acids, from donor acylglyceride molecules. There is some slight apparent activity with monocaprylin, but this is within experimental error of zero. The negative results calculated are also indicative of the experimental error inherent in the system. Apparent negative ester synthesis measurements occur when the amount of ester production measured in the enzyme free control reaction is slightly more than the amount measured from the triplicate reactions with enzyme present. When the control reaction is subtracted from the mean of the triplicates, a negative value is produced. The high specificity for tributyrin as an acyl donor is unusual. Other esterases examined in previous studies had some capacity to use glycerides with larger acyl chains than tributyrin as acyl donors in ester synthetic reactions (Shaw, 1999). However this preference for butyl compounds is reflected in the hydrolytic activity of PepX on *p*-nitrophenol based alkyl substrates with different sized alkyl groups (Liu *et al.*, 2001). In these experiments PepX was found to have only 6% activity with caproate (C6) based chromogenic substrates, where 100% activity is defined as the

activity with *p*-nitrophenyl butyrate. In this previous study, PepX showed no detectable hydrolytic activity on artificial substrates larger than C6 (Liu *et al.*, 2001). The high specificity contrasts with tributyrin esterase from *Lactococcus lactis* subsp. *cremoris*, which has a substrate acyl length preference that does not appear to be as specific. Tributyrin esterase will use acyl groups up to 18 carbons long in hydrolysis reactions, and prefers monoacylglycerides as donors over triacylglycerides (Holland *et al.*, 2002). Unfortunately the smallest monoacylglyceride and diacylglyceride commercially available is C8. The inherent instabilility of shorter glyceride compounds makes testing PepX for a mono or di butyl preference impossible.
The transferase activity of PepX is compared to *Lactococcus lactis* subsp. *lactis* tributyrin esterase (EstA) in Table 8.6. It should be noted that the synthetic activity of PepX and EstA with tributyrin and ethanol is different. The activity of PepX with ethanol and tributyrin, at 0.6 M and 60 mM, respectively, is 0.0054 μmol product.min^{-1}.mg^{-1} enzyme, while for EstA at an ethanol concentration of 0.6 M the activity on tributyrin (30 mM) was 0.038 μmol product.min^{-1}.mg^{-1} of enzyme (Shaw, 1999). Although the synthesis rate of PepX is about one seventh of that of EstA per milligram of enzyme, the rate of ester synthesis per μmole of enzyme of PepX is half that of EstA. These comparisons are summarized in Table 8.7. This table does not take into account any quarternary structure of these enzymes as the quarternary state of EstA is unknown.

It is difficult to identify a reason for PepX having such a high specificity for tributyrin from a physiological point of view. Tributyrin is an artificial compound, which does not exist in natural systems. Acylglyceride compounds that are found in dairy products do contain approximately 3.9% butyl groups, but these are mixed with other larger sized acyl groups (MacGibbon, 1989).
It is likely that butyl groups on mixed acylglycerides would be accessible to the enzyme, although it is possible that large acyl groups on the end positions of the glycerol backbone may sterically block the internal acyl group. The effect of the position of a monoacyl group on the glycerol backbone (whether in the inner position or on the two outer positions) on the activity of esterase enzymes during transferase activity has not been investigated.

Acyl donor	Carbon chain length	Transferase Activity (%)	
		PepX	*EstA*[†]
Tributyrin	4	100	100
Tricaproin	6	<0.02*	0
Monocaprylin	8	1.9	0
Dicaprylin	8	<0.02	4700
Tricaprylin	8	-3.8*	0
Monocaprin	10	-4.35*	11000

Table 8.6 *Comparison of the transferase activities of PepX and* Lactococcus *EstA using ethanol and acylglycerides. 100% is defined as the activity on tributyrin and ethanol. Values denoted with * are negative due to experimental errors, and are most likely to demonstrate the enzyme has no activity on these substrates as the relative activities are less than the generally accepted error of 5% in GC analysis systems.*

[†]*Values for EstA are derived from Shaw, (1999).*

Esterase	Mass of enzyme kDa[†]	Ester synthesis rate $\mu M.min^{-1}.mg^{-1}$ enzyme	Ester synthesis rate $\mu M.min^{-1}.\mu mol^{-1}$ enzyme
PepX	88.1	0.0054	0.47
EstA	29.4	0.038	1.12

Table 8.7 *Comparison of ester synthesis rates of PepX and EstA using ethanol and tributyrin.* [†]*Molecular masses are predicted based on protein sequence information*

8.4.4 Mode of Ester Synthesis

As discussed in section 8.3.6, all previously characterized lactic acid bacterial alkyl esterases that synthesise esters appear to achieve this by a transferase mechanism (Shaw, 1999; Holland *et al.*, 2002).

In the presence of tributyrin and ethanol, ethyl butanoate was produced at the rate discussed in section 8.4.3 above. When the PepX enzyme was incubated with butyric acid and ethanol, under the same conditions as the normal ester synthesis assays, no ethyl butanoate was detectable in any of the assays.

This is strong evidence that the PepX enzyme, like other ester synthetic enzymes from lactic acid bacteria, synthesize esters by direct transfer of an acyl group from an acylglyceride onto an alcohol acceptor, rather than by hydrolysing an acylglyceride into carboxylic acids and glycerol, followed by rebinding of the a carboxylic acid and transfer to an alcohol.

This makes the mechanism of ester synthesis exhibited by PepX the same as in previously investigated ester synthesizing esterase enzymes.

8.4.5 A Role For PepX as a Transferase

The ability to make alcohol butyl esters may have been physiologically relevant for this organism prior to domestication, although it may no longer be physiologically relevant in a dairy system.

Ethanol is not known to be a byproduct of *Streptococcus thermophilus* metabolism, and BLAST comparison with other microbial alcohol dehydrogenases shows that no alcohol dehydrogenase gene is present in the partially completed *S. thermophilus* genome (Goffeau *et al.*, 2003). The human pathogen *Streptococcus mutans* can produce ethanol under oxygenated conditions using pyruvate-formate lyase (Takahashi *et al.*, 1991), so it is possible that an alternative ethanol production mechanism also exists in *S. thermophilus*, although, like alcohol dehydrogenase, there is no identifiable homologue of this enzyme in the partial *S. thermophilus* genome.

It is possible that this enzyme activity exists as a means of de-toxification of ethanol produced by other species in mixed species environments. Ethanol can be present in mixed species fermentations such as in a cheese system and there is some evidence that it is produced by certain strains of *Lactococcus lactis* subsp. *lactis* (Bills *et al.*, 1965). Additionally yeasts are often found in unpasteurised milk that may provide

another source of ethanol. There is little information available as to the toxicity of ethyl butanoate, or whether lactic acid bacteria have a means of metabolising this product.

Alternatively, it is possible that PepX, like other αß domain-containing transferase enzymes (such as *Mycobacterium* Antigen 85C), may have a role in cell-wall maintenance (Jackson *et al.*, 1999). This could be relevant as bacterial cell walls contain a high proportion of cross-linked amino acids, although PepX is possibly too specific in terms of amino acid preference to fulfil this role effectively.

8.5 Conclusions

From the studies undertaken to investigate the ability of PepX to synthesise esters new insights into the enzyme have been gained.

PepX was found to have a high specificity for tributyrin as an acyl donor during ester synthesis. No ester synthetic activity was observed when tributyrin was substituted for other glycerides with longer acyl chains. It was noted that the specificity of the enzyme for butyl glycerides was reflected by the preference for butyl based artificial ester substrates during hydrolysis experiments carried out on Esterase II from *S. thermophilus* (Liu *et al.*, 2001). The specificity of PepX for butyl based acyl donor compounds is far greater than the specificity of tributyrin esterase from *Lactococcus lactis*, which is active over a range of acyl chain lengths on chromogenic substrates (Fernandez *et al.*, 2000).

The rate at which ester synthesis occurs was found to be comparable with tributyrin esterase from *Lactococcus lactis* as calculated by other authors (Shaw, 1999), although reaction conditions were not identical to this study.

The mode of ester synthesis was investigated to determine whether esters were produced by direct transfer of an acyl group from a glyceride donor to an alcohol, or by hydrolysis of glycerides into carboxylic acids and glycerol, followed by rebinding of the carboxylic acids and transfer to an alcohol acceptor. The ester synthetic process was found to occur by the former mechanism, direct transfer, as the enzyme was incapable of using butyric acid and ethanol as substrates to produce an ester product. All esterase-catalysed synthetic reactions by lactic acid bacterial enzymes that have been investigated to date have proceeded by this mechanism (R. Holland, personal communication). The role of the transferase activity of the PepX enzyme in the cell remains unclear, but it is possible that it may play a role in detoxification of ethanol produced by neighbouring species in mixed species microbial communities, by turning the ethanol into ethyl butanoate. Alternatively, PepX may, like other αß transferase enzymes, have a role in cell-wall maintenance.

8.6 Further Work

This investigation has provided an indication of the specificity of the PepX enzyme but has raised many more questions. For example, the specificity of PepX for acyl length is far more specific than any other esterase enzyme investigated to date. This also raises the question as to what role the esterase/ester synthetic activity of PepX plays in the cell. Some of these questions could be answered through the use of GC enzyme activity assays using different compounds as trial substrates. This could potentially involve an extensive screening process, since the physiological substrates for PepX ester synthesis are unknown. It may, however, be possible to look at secondary metabolite esters found in dairy products, and use this knowledge to predict potential alcohol acceptors for PepX before trials are undertaken. The preference of PepX for different alcohols could be investigated. This rationale could identify a physiological acceptor molecule for the enzyme, assuming that this is not ethanol.

CHAPTER 9

Hydrolytic Activity of PepX on Acylglyceride Substrates

9.1 Introduction

With the transferase activity of the PepX enzyme on acylglyceride substrates partially characterized in the previous chapter, the next investigation undertaken was into the ability of PepX to hydrolyse tributyrin and milkfat substrates. Although the hydrolytic activities of this enzyme with substrates such as chromogenic esters (Chapter 7 and Liu, *et al.* 2001), peptides, and chromogenic peptides are documented (Kiefer-Partsch *et al.*, 1989; Lloyd RJ and Pritchard G, 1991), the activity of the enzyme on acylglyceride substrates that better represent natural substrates for the esterase activity of the enzyme had not been reported.

It was established in this study that PepX has a high specificity for butylglycerides acting as the donor molecule for ester synthesis via a transferase mechanism. As noted by other authors, the enzyme has a high specificity for butyl-based artificial *p*-nitrophenyl ester substrates, compared with ester substrates with longer acyl groups when acting as an ester hydrolase (Liu *et al.*, 2001). It was therefore of interest to see if this specificity extended to the hydrolytic activity of this enzyme with acylglyceride substrates.

The specificity of the enzyme for different sized acylglycerides can be examined by investigating the ability of PepX to release different sized fatty acids from a milkfat-based substrate. Such a substrate has the advantage of being made of acyl groups with a range of different sizes, allowing a concommitant investigation of substrate preference. This approach also allows the investigation of the enzyme in a system that more closely resembles what would occur in a cheese making system, where the enzyme would be used in a mixture of acylglyceride species.

Additionally, it is of interest to examine if the enzyme will hydrolyse tributyrin in the presence of a water acceptor molecule (hydrolysis) and ethanol (acyl transfer), to determine if a preference for a particular acceptor is exhibited.

Examination of the activity of PepX with acylglycerides, which more closely resemble a dairy substrate for the enzyme than the *p*-nitrophenol based ester substrates used in earlier investigations should give an indication of the *in situ* function of the enzyme in cheese, that is, whether it is likely to act as an esterase or a transferase.

9.2 Objectives and Strategies

The objectives of this section of work were to:

1) Determine the ability of PepX to liberate free fatty acids from milkfat substrate preparations.
2) Determine the ability of PepX to liberate butyric acid from tributyrin, both in the presence and absence of ethanol as an alternative acceptor group to water.

The strategy for carrying out this work consisted of performing assays on a variety of substrates with PepX using the assay system described in Chapter 8.

9.3 Methods

9.3.1 Milkfat Hydrolysis Assays

Milkfat substrate was made up by first weighing out 1.217 g of milkfat. The milkfat was melted in a 37 °C waterbath until liquid, and 0.8 g of gum arabic was added, with 20 mL of pre-warmed 100 mM phosphate buffer (made as described in section 2.3.17). The substrate was sonicated for 2 minutes (level 4, 50% duty cycle) to emulsify the lipids. 5 mL of this emulsion was then transferred into a universal bottle and warmed in a waterbath to ensure the substrate was at 37 °C, and then 10 μL of thawed PepX enzyme at

3.8 $mg.mL^{-1}$ was added to each assay to initiate the enzymatic reaction.

The substrate was prepared with both non-lipolysed and partially lipolysed milkfat. The lipolysed milkfat contained a higher proportion of mono- and diglycerides than natural milkfat, but had the majority of the free fatty acids liberated during lipolysis removed. In the case of the lipolysed milkfat preparation used for these experiments, 54.5% of the fats present were triacylglycerides, 0.84% of the substrate was free glycerol, and the remainder of the milkfat was made up of mono- and diacylglycerides.

Control assays were also performed, where 10 μL of buffer was added to the mixture in the place of the enzyme.

The assays were incubated in a 37 °C waterbath with gentle shaking, and 0.5 mL samples were taken at various timepoints, and pipetted into a 15 mL screwcap test-tube containing 100 μL of 5 M H_2SO_4 plus 1.0 mL of absolute ethanol. Samples were stored sealed with parafilm at –18 °C until fatty acid extraction.

9.3.2 Tributyrin Hydrolysis Assays

To investigate the ability of PepX to hydrolyse the artificial butylglyceride tributyrin, assays were performed with a tributyrin substrate in the presence and absence of ethanol.

A stock solution of 180 mM tributyrin with 0.1 $mg.mL^{-1}$ gum arabic was made in 100 mM phosphate buffer. The tributyrin was dissolved and emulsified by sonication for 2 minutes (level 4, 50% duty cycle).

1 mL of tributyrin stock was measured into a universal bottle, with either 1 mL of absolute ethanol or 1 mL of buffer. The assays were made up to a final volume of 6 mL with 100mM $NaPO_4$ buffer, pH 7.0, then preheated to 37 °C in a waterbath before the addition of 10 µL of PepX enzyme at 3.8mg.mL^{-1}. A control assay where enzyme was replaced with buffer was carried out at the same time. The assays were incubated at 37 °C and 0.5 mL samples for free fatty acid analysis were taken at 1 h, 2 h, 4 h and 24 h, and stopped by the addition of 100 µL 5 M H_2SO_4, and 1 mL of ethanol. Samples were stored at
–18 °C until processing for analysis. 1 mL samples for ester analysis were pipetted into 100 µL 2.5 M H_2SO_4 and were immediately extracted for analysis.

9.3.3 Free Fatty Acid Extraction

Frozen samples were thawed, and 3 g of sodium sulfate was added to each tube. 10 mL of heptane/diethyl ether 1:1 was then added to each tube, followed by 100 µL of a C13 internal standard at 2 mg.mL^{-1} to each tube.
Products were extracted from aqueous solution by vortexing and manual shaking for 2 minutes. This step also agitates the sodium sulfate to ensure efficient water absorption.
Samples were centrifuged at 3000 x g for 5 minutes to pellet the sodium sulfate and separate the aqueous and organic phases. The organic top phase was removed and transferred to a fresh 16 mL screwcap tube containing 1 g sodium sulfate. The tubes were shaken and recentrifuged to pellet the sodium sulfate. The solvent phase was then transferred into a new tube. All manipulations were performed using glass pipettes and measuring equipment, which are not affected by organic solvents.
Automated fatty acid extraction was carried out using an ASPEC sample preparation robot (Gilson). During this procedure, extracted samples were transferred into an NH_2-propyl solid phase extraction (SPE) cartridge. Fatty acids will bind to the column media, whilst esters and acylglycerides pass through the column and are removed. Remaining neutral lipids and other contaminants were washed form the column with a solvent composed of 2:1 chloroform:IPA (iso-propyl alcohol).
The bound material was then eluted from the column using 6% formic acid in 2:1 heptane:DEE and collected into a GC vial for analysis.

9.3.4 Ester Extraction

Extraction of ester compounds from assay samples was carried out using the method described in Section 8.3.2. Briefly the ester reaction product was extracted into diethyl ether and centrifuged to separate aqueous and organic phases. Esters were then separated from any free fatty acids using an NH_2 SPE column, before being analysed by GC as described in Section 8.3.3.

9.3.5 Gas Chromatographic Analysis of Free Fatty Acids

Gas chromatography using flame ionisation detection (Shimadzu GC-15A) was used to identify compounds by column retention time against an external standard of different sized free fatty acids. The column used was an EC-1000 (J&W Scientific, USA), with dimensions 30 m long and an internal diameter of 0.53 mm, with a film of polyethylene glycol and diepoxide esterified with terephthalic acid of 1.2 μm thickness.

1 μL of sample was injected onto the column (injector temperature 220 °C), and the separation of compounds was achieved using the following temperature regime: 100 °C held for 1.5 min, then 10 °C/min to 245 °C and held at 245 °C for 24 min. The detector temperature was set at 245 °C.

The column was washed with an injection of heptane:diethyl ether 1:1 with 6% formic acid every 8 runs to ensure that good resolution and a low background were maintained.

9.4 Results and Discussion

9.4.1 Release of Free Fatty Acids from Milkfat

The release of fatty acids from a milkfat preparation in the presence of PepX was analysed by gas chromatography. The results of this experiment are shown in Figure 9.1.

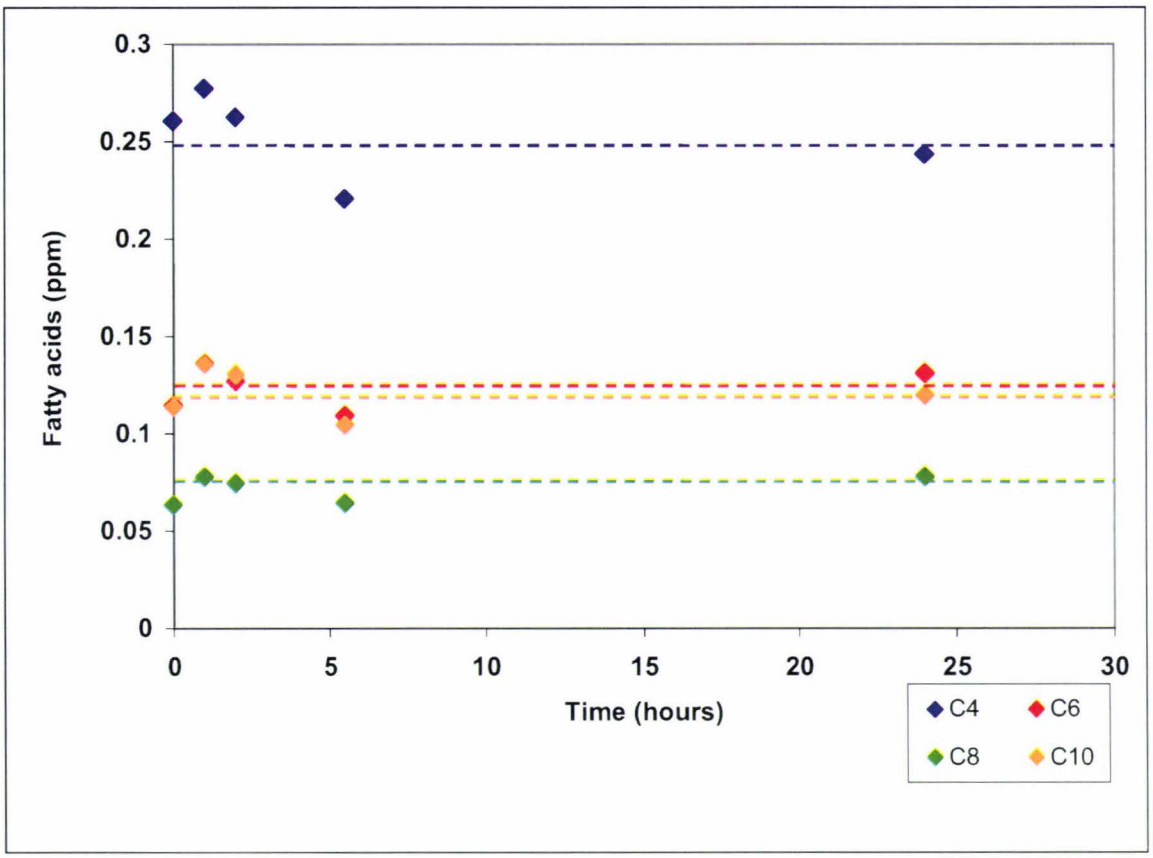


Figure 9.1 *Fatty acid release from milkfat in the presence of PepX. Values shown are from duplicate 2 of lipolysed milkfat plus PepX*

From the graph it can be seen that there is no significant change in the fatty acid content of the assay, even after 24 hours. This was surprising as it was expected that PepX would have been capable of hydrolysing butyl groups from partially lipolysed glycerides, even if some glycerides remained inaccessible to the enzyme due to the presence of larger acyl groups causing steric problems. Figure 9.2 summarises the butyric acid levels in all assays carried out. This figure shows that in non-lipolysed milkfat free butyric acid was undetectable, whilst in lipolysed milkfat, butyric acid levels showed no significant change up to an assay period of 24 hours, regardless of whether PepX was present or not. Apparent slight deviations from a straight line are

seen in the 1 and 2 hour samples in Figure 9.1, but these are most likely to be due to experimental error, and are not significant. The preparation of samples for free fatty acid analysis by GC requires several manipulations, and these are the most likely source of experimental error.

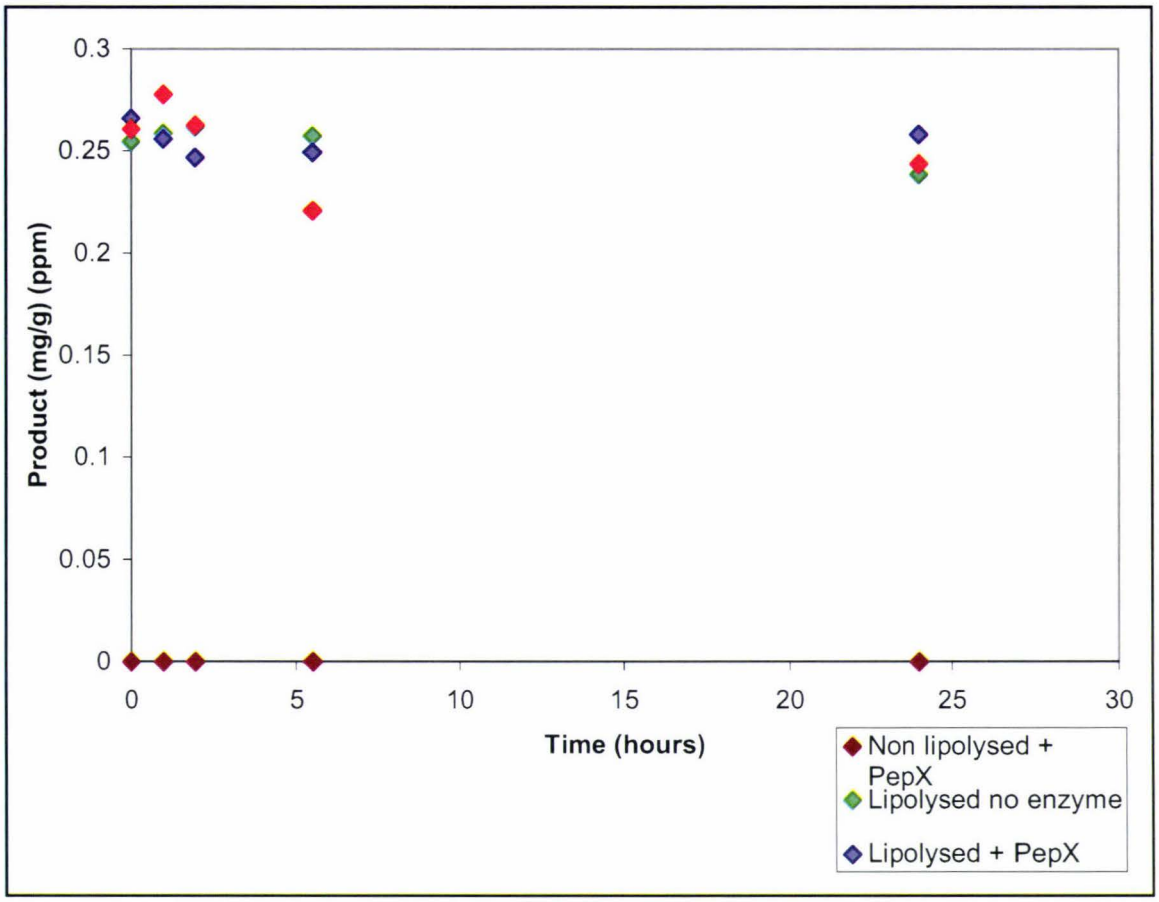


Figure 9.2 *Butyric acid release from milkfat in the presence of PepX.*

The results of this experiment provide evidence that, under the conditions used, PepX did not hydrolyse milkfat acylglycerides into fatty acids.

9.4.2 Release of Butyric Acid from Tributyrin

The release of butyric acid from a tributyrin substrate in the presence of PepX was investigated using gas chromatography analysis of butyric acid. Figure 9.3 summarises the findings of this study.

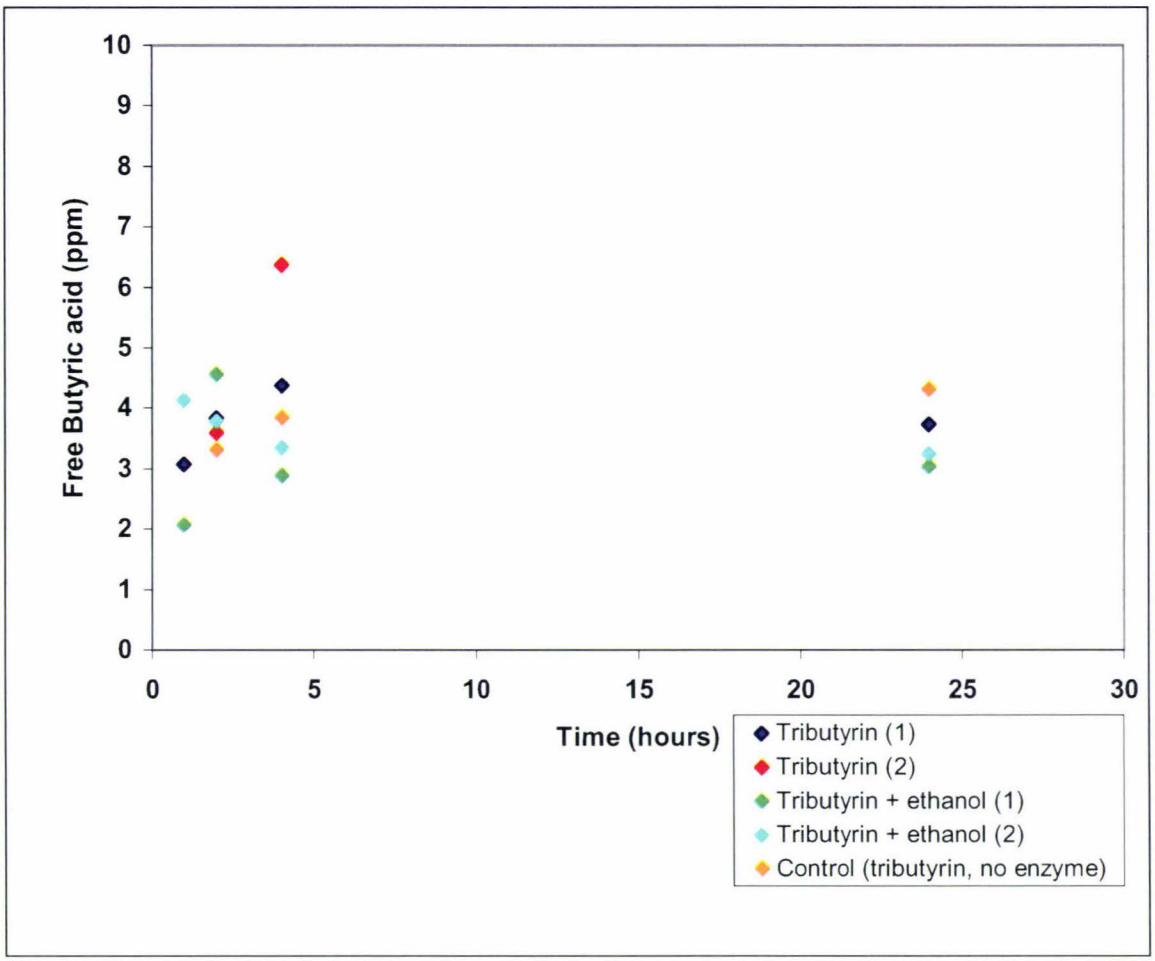


Figure 9.3 *Butyric acid release from tributyrin in the presence of PepX.*

The experimental findings show no significant change in butyric acid levels in the tributyrin-containing assays in the presence of the PepX enzyme. Although the second replicate containing tributyrin only seems to show an increasing amount of butyric acid from 2 to 4 hours, it is likely that this is simply due to experimental errors due to sampling or processing samples prior to GC analysis. A further sample would have been useful to confirm or refute this trend. This sample was lost during processing for analysis.

There are several potential reasons for the observation that PepX did not release butyric acid from tributyrin in this experiment. One is that an error in sample processing, for example, incorrect extraction methods, may have lost free fatty acids from the samples. However this seems unlikely, and it was noted at the time of assaying the enzymes that no characteristic butyric acid smell was observed. This fits with the limit of detection of butyric acid by the human olfactory senses, which is about 6ppm (R. Holland, personal communication). Had the PepX enzyme been able to hydrolyse tributyrin, it was anticipated that after 24 hours the concentration of butyric acid liberated from the substrate would be in the range of tens to hundreds of parts per million. All samples were treated identically during processing prior to analysis, so even if a large proportion of the free fatty acid content had been lost a relative increase would still be expected between samples containing PepX and enzyme free controls, especially after a 24 hour incubation period. This evidence all suggests that the GC analysis of the assay products is accurate, and that PepX is not capable of liberating free fatty acids from tributyrin under the conditions used.

Given the data reported in Chapter 7, that demonstrated PepX was capable of hydrolysing a butyl ester chromogenic substrate, these results were unexpected. However it has been noted over the course of these studies that the *p*-nitrophenyl butyrate chromogenic substrate is chemically unstable, and can spontaneously hydrolyse to butyric acid and *p*-nitrophenol, especially at pH values above 7.0 or at temperatures above 4 °C. This suggests that the ester bond found in *p*-nitrophenyl butyrate is more labile than the ester bonds found in tributyrin, which is more pH and temperature resistant.

The presumed chemical mechanisms of serine hydrolase enzymes involve acid and/or base catalysis, along with the substrate being bound in a favourable conformation for a chemical intermediate to be formed (Silverman, 2000). It is likely that upon binding to the active site of the PepX enzyme, the chromogenic substrate is forced to adopt a trigonal conformation that spontaneously disrupts the labile ester bond to form an acyl intermediate with the enzyme, albeit at a low efficiency. Like most acyl-enzyme intermediates this is highly unstable, and is quickly transferred non-specifically onto any available acceptor molecule, which in aqueous solution is water.

It is likely therefore that the enzyme does not hydrolyse the more chemically stable tributyrin, even though it is capable of binding to the enzymes active site, as demonstrated by the competitive kinetics reported in Chapter 7 and the ester synthetic reactions reported in Chapter 8 of this work. As demonstrated in Chapter 8, tributyrin is, however, transferred to ethanol to produce an ester. This implies the enzyme has a mechanism will only activate catalysis once a suitable acceptor is present in the substrate binding site. This activation mechanism is usually a conformational change, and is common in transferase enzymes, and essential to ensure that the donor group is transferred to an acceptor other than water (Cornish-Bowden, 1995).

This model fits the approximately 50 fold decrease in chromogenic ester substrate hydrolysis by the enzyme, compared with chromogenic peptide substrate hydrolysis and is evidence that the hydrolysis of *p*-nitrophenyl butyrate is fortuitous, rather than representing a genuine secondary esterase activity. This shows that caution must be applied when using chromogenic substrates to assign activities to enzymes, and care must be taken to verify the presence of activities using authentic substrates where possible.

9.4.3 Ester Formation From Tributyrin and Ethanol

As a confirmation of enzyme transferase activity, samples were taken from the tributyrin plus ethanol reactions and analysed for ethyl butanoate content. The results for these experiments are presented in Figure 9.4.

From this graph it is obvious that the synthesis of ethyl butanoate is being carried out in these reactions, whilst the data shown in Figure 9.3 demonstrate that no butyric acid is being released during this process. Although this is effectively a repeat of experiments carried out in Chapter 8, the reaction was repeated and included for completeness.

This implies that PepX is not actually an esterase, but is a transferase. This experiment also shows that ethyl butanoate is not a substrate for PepX, or else the release of butyric acid from this enzyme product would have been observed in these experiments.

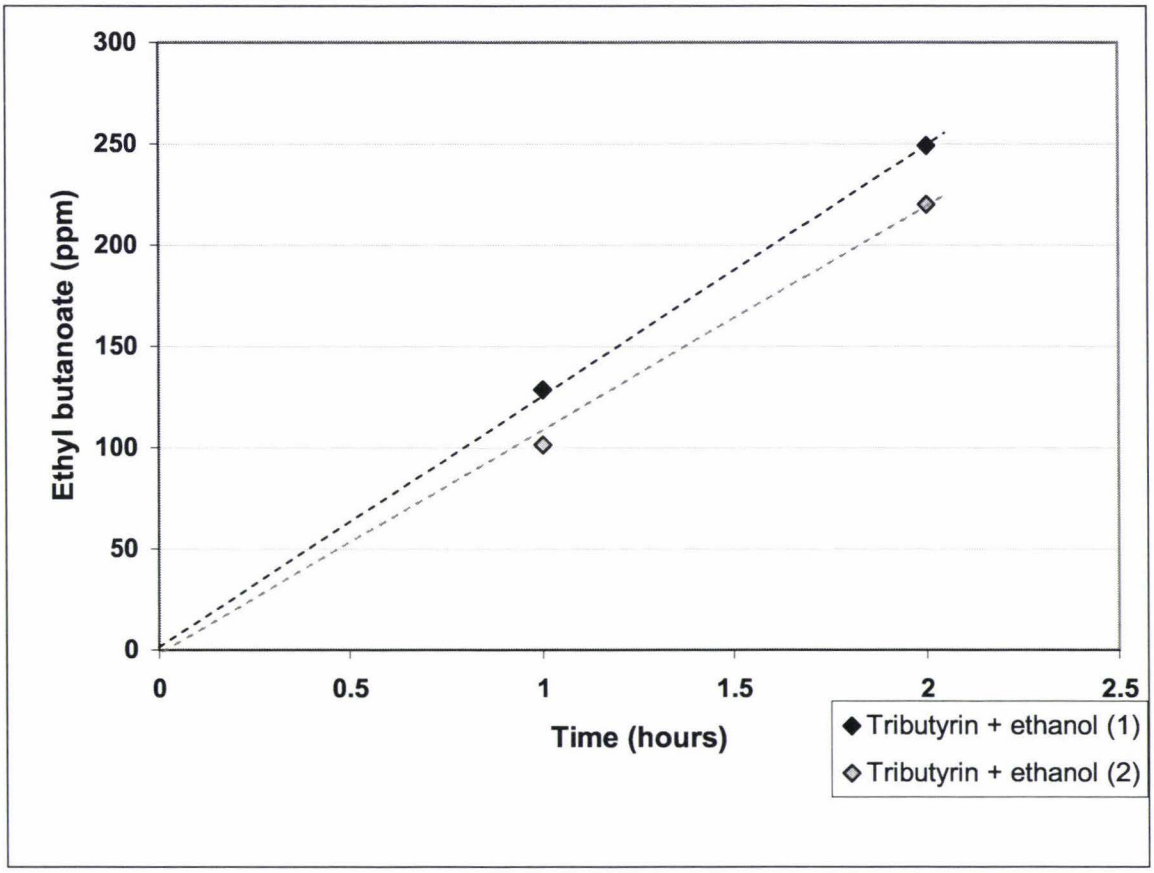


Figure 9.4 *Ethyl butanoate formation from tributyrin and ethanol in the presence of PepX.*

The physiological significance of this is not immediately clear and may be simply serendipitous. PepX is known to have a significant role in peptide hydrolysis during cellular metabolism. It is possible that the ester synthetic capacity of this enzyme represents a secondary function in the cell, although it seems unlikely that ethanol would be a readily available acceptor molecule, as it is not produced by *Streptococcus thermophilus*. It is difficult to imagine what physiological role ethyl butanoate might have in a cell. The identification of a physiological acceptor molecule would be of interest as it may reveal the role of the enzyme *in vivo*, if it is indeed of metabolic significance. The Kyoto Encyclopaedia and Genes and Genomes (KEGG) database has no listing for ethyl butanoate as a substrate of any known enzymatic reaction of physiological significance.

Alternatively, as discussed in Chapter 8, it is possible that PepX may have played a role, prior to domestication of *S. thermophilus,* by detoxifying ethanol produced by other species in mixed microbial communities, with ethyl butanoate then being removed from the cell by an as yet unknown metabolic mechanism.

9.5 Conclusions

The findings of the investigations into tributyrin hydrolysis by PepX, coupled with data gathered for milkfat hydrolysis by the same enzyme, are strong evidence that PepX is a peptidase enzyme with a dual role as an alkyl transferase.

The enzyme has been shown to bind tributyrin, as demonstrated by competition studies in Chapter 7, and additionally by the ability to produce ethyl butanoate when provided with ethanol and tributyrin. This shows the enzyme must be able to bind ethanol as a prerequisite to forming butyl esters. The enzyme does not appear to hydrolyse tributyrin is the absence of an acceptor however.

Additionally, the enzyme is capable of hydrolysing peptides, so it must use water as an acceptor molecule in these reactions. Intriguingly PepX has also been shown to be capable of synthesizing peptide bonds under certain conditions (Yoshpe-Besancon *et al.*, 1994).

It is potentially of interest to investigate the molecular switch that selects between these multiple activities, to learn how the enzyme can use water as an acceptor in peptide hydrolysis, but will not use water as an acceptor during ester hydrolysis.

9.6 Further Work

The finding that PepX is incapable of hydrolysing tributyrin was unexpected, and has raised more questions about this enzyme than it has answered.

It would be of interest to study how the enzyme selects between water and ethanol as an acceptor molecule during the different activities of peptide hydrolysis, and ester synthesis. This could potentially be demonstrated using substrate studies, specifically to see if ethanol can be used as an acceptor molecule during peptide hydrolysis. It would be interesting to find out whether water or ethanol was selectively used during peptide hydrolysis, especially in light of the discovery that only ethanol is used as an acceptor for butyrate transfer, and not water as in other lactic acid bacterial esterases (Shaw, 1999).

Structural studies of how the enzyme binds different substrates, already proposed in Section 7.7 could be undertaken to examine for a selective mechanism that allows the enzyme to use water or ethanol for different catalytic tasks. The discovery that the enzyme binds tributyrin, but does not hydrolyse it in the absence of ethanol, means tributyrin is a potential candidate molecule to be used as an enzyme inhibitor, provided suitable crystallization conditions can be identified, that is conditions that do not require the presence of ethanol, or other acceptors.

CHAPTER 10

Conclusions

10.1 Introduction

The work described in this thesis has involved an investigation of the esterase and ester synthetic activities of X-prolyl dipeptidyl peptidase from *Streptococcus thermophilus*. This study has made several interesting discoveries about these newly identified properties of an already well characterised peptidase.

10.2 PepX Gene Sequence

The cloning of the PepX gene, as described in Chapter 5, has allowed comparisons to be made between PepX genes from three strains of *Streptococcus thermophilus*. The main finding from this analysis is that the PepX sequence is not absolutely conserved between the three sequences available. Nucleotide and protein sequence differences were identified between all three sequences. However these changes in sequence were found to be largely limited to the C-terminal half of the protein, and the single nucleotide change observed in the 5′ part of the gene was degenerate, and codes for the same amino acid in the expressed protein. The nucleotide substitutions in the 3′ half of the gene do lead to some changes in the sequence of the expressed protein, but these changes are not contained within the catalytic domain, and seem localised in the C-terminal ß sheet "jelly roll" domain. In the absence of a structure for PepX from *S. thermophilus*, certain inferences can be made based on the structure of PepX from *Lactococcus lactis*, but it is difficult to map the residue changes observed to a precise position in the protein. However sequence comparison of these two proteins suggests that the domain architectures are likely to be similar.

This pattern of substitution and conservation suggests that the N-terminal half of the protein is more essential for the viability of the organism than the C-terminal region. This is supported by the structural work of Rigolet, Metchin, *et al.* (2002), that showed that the N-terminal and catalytic domains of PepX (both located in the N-terminal region of the molecule) are important for dimerisation of PepX, and that this dimerisation is important for substrate binding.

10.3 Active Site Characterisation

The results reported in Chapter 7 of this thesis support the hypothesis that the peptidase and esterase activities of PepX on chromogenic substrates are carried out at one active site. This is demonstrated by the finding that adding an ester substrate to a chromogenic peptidase assay causes the inhibition of the peptidase substrate hydrolysis in a competitive fashion. Competitive inhibition is only observed in enzymes when an inhibitor (in this case tributyrin), binds to the enzyme in such a way as to prevent either substrate binding, or to prevent substrate access to the catalytic site.

Ethanol was found to inhibit the hydrolysis of *p*-nitrophenyl butyrate, but interestingly methanol blocked this inhibition, although it seemed to have no measurable effect on hydrolytic activity in isolation. The fact that the esterase activity was not diminished in the presence of methanol shows that the enzyme is hydrolysing *p*-nitrophenyl butyrate, rather than carrying out a transferase reaction and using methanol as the acceptor. Furthermore, the fact that this esterase activity still occurs in the complete absence of an alcohol acceptor suggests that true hydrolysis is being observed.

Other results presented in Chapter 7 show that both ethanol and methanol inhibit the hydrolysis of a peptide substrate.

A model has been proposed in this chapter (Figure 7.14) to explain these results. This model suggests that methanol and ethanol both bind to the enzyme in such a way as to prevent the binding of the chromogenic peptide substrate to the enzyme.

10.4 Transferase Activity

The transferase activity of PepX using various acylglycerides and an ethanol acceptor is described in Chapter 8. This study found PepX was highly specific for the acyl group on the donor molecule, the specificity being that the donor acyl group must be strictly a butyl in size. This reflects the ester hydrolytic activity of this enzyme observed by Liu, *et al.* (2001) on chromogenic ester substrates, which suggested PepX had a high specificity for butyl esters, and almost no activity against larger molecules, even six-carbon esters. Although these experiments were carried out using synthetic acylglycerides, which are not found in milk fermentations, milk does contain butylglycerides that might be substrates for the enzyme.

PepX was found to only form ethyl esters when provided with a glyceride substrate. The enzyme was unable to form esters from butyric acid and ethanol, showing a transferase mechanism was operating, rather than a concentration driven reverse hydrolysis.

It is not known whether ethanol represents a true physiological acceptor for the transferase activity of PepX, as ethanol is not produced by *Streptococcus thermophilus*, although might be present in mixed species fermentations, as found in wild microbial communities, likely to have been inhabited by *S. thermophilus* prior to this species use as a dairy starter. It is proposed that the enzyme activity of transferring a butyl group onto ethanol may constitute a detoxification mechanism. The toxicity of the reaction product, ethyl butanoate, compared with the reaction substrate, ethanol, is not known. However it is possible that the cell has a mechanism to remove ethyl butanoate, although no such enzyme has ever been characterised.

10.5 Mechanism of Ester Synthesis by PepX

The evidence collected during this study suggests PepX uses a two-stage substrate binding process during the synthesis of esters from tributyrin and ethanol. This model is summarised in Figure 10.1. In this model, the binding of the donor molecule (tributyrin) causes a conformational change that allows an acceptor other than water to bind at a second substrate binding site. The model is supported by the experimental evidence that tributyrin will bind to PepX in the absence of ethanol (Chapter 7), and that PepX will catalyse the formation of esters from tributyrin and ethanol. In chemical terms ethanol must bind to the enzyme prior to catalysis occurring to ensure the acyl group is transferred to the acceptor. Once the enzyme has reached the highly unstable acyl-enzyme intermediate transfer to an acceptor occurs rapidly, and if the desired acceptor is not bound in a suitable position for this to occur, the acyl group is instead transferred to a water molecule. This was shown in the fortuitous esterase activity of PepX on *p*-nitrophenyl butyrate, where once an intermediate has formed due to the instability of this substrate, hydrolysis to water proceeds in the absence of an alcohol acceptor. The binding of a donor substrate then an acceptor substrate is a common mechanism for transferase enzymes (Cornish-Bowden, 1995), and is necessary to avoid hydrolysis of donor substrates using water as an acceptor. As no detectable acylglyceride hydrolysis was catalysed by PepX, the alcohol acceptor must be in a highly favourable position for acyl transfer to occur, rather than a transfer to water.

The conformational change mechanism has been characterised in αß hydrolases other than PepX by structural studies, such as in *Mycobacterium* Antigen 85B mycolyl transferase, which employs a conformational change to ensure cell wall components are joined rather than hydrolysed (Anderson *et al.*, 2001). It seems likely that a similar mechanism exists in PepX, to ensure catalysis does not occur until a suitable acceptor is in the appropriate position.

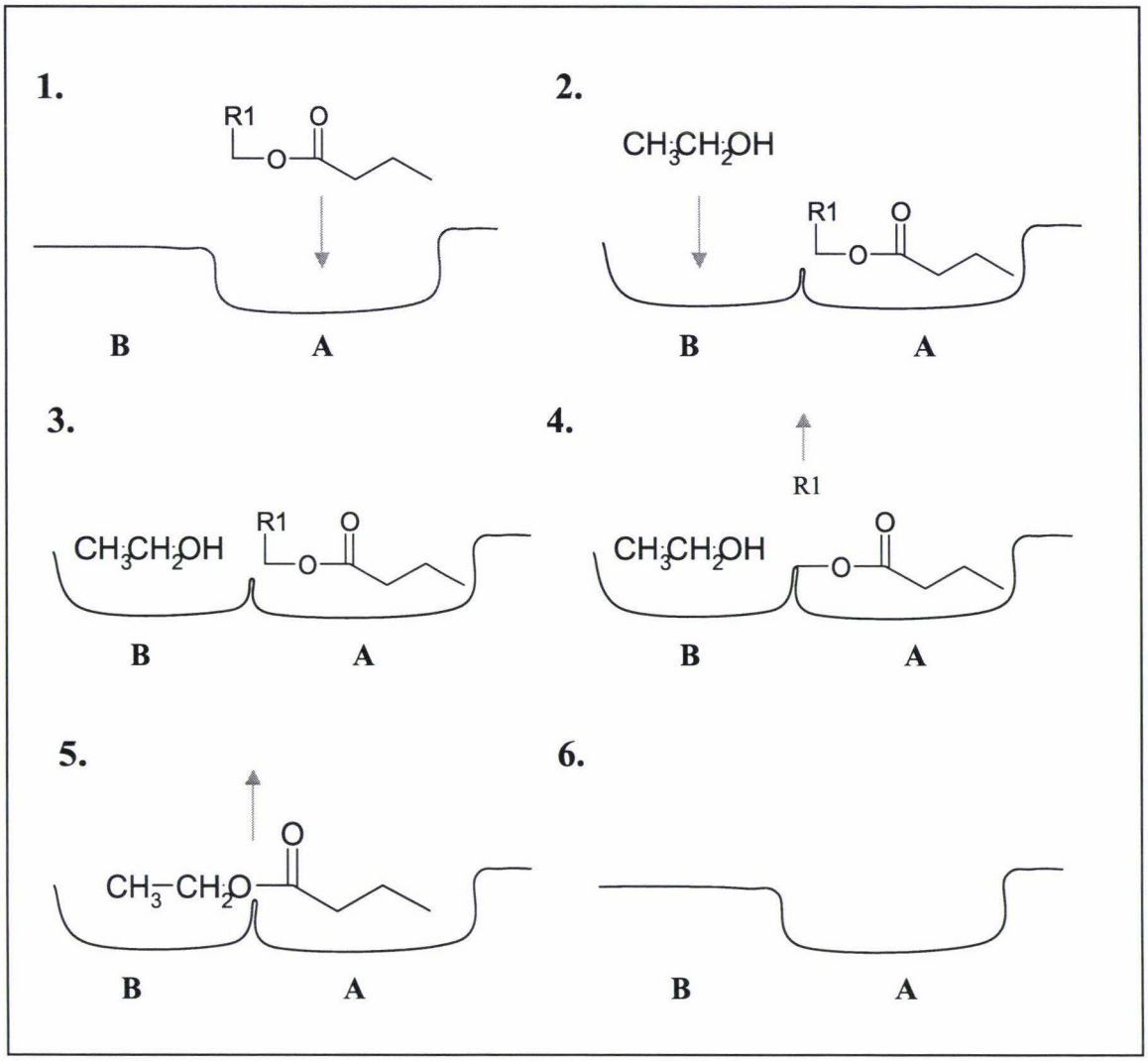


Figure 10.1 *Proposed mechanism of PepX ester synthesis.*

(1) Tributyrin binds to enzyme binding site A. One ester group is shown, and the rest of the tributyrin molecule is represented by R1 (2) Binding in site A causes a conformational change that opens site B. (3) An acceptor molecule, in this case ethanol, binds in site B. (4) A nucleophilic attack occurs, resulting in an acyl-enzyme intermediate. The remainder of the acyl donor diffuses away from the enzyme. (5) The acyl group is transferred from the enzyme to the acceptor, and the product diffuses out of the active site. (6) The enzyme adopts the original conformation, ready to bind another donor molecule. Diagram adapted from Figure 6.2 (Cornish-Bowden, 1995).

10.6 Hydrolase Activity on Acylglycerides

The hydrolase activity of PepX on tributyrin and milkfat acylglycerides was investigated in Chapter 9. This work showed that, whilst able to catalyse the hydrolysis of a chromogenic butyl ester substrate (Chapter 7), surprisingly PepX does not catalyse the hydrolysis of acylglycerides into glycerol and carboxylic acids. This result was observed with both tributyrin and milkfat (with both intact and partial glycerides) as substrates. This evidence suggests that the hydrolysis of the chromogenic ester substrate is a fortuitous activity due to the nature of the ester bond of *p*-nitrophenyl butyrate, rather than a true acylester hydrolytic activity, and supports the conclusion that PepX, as well as a dipeptidase, is an acyltransferase.

10.7 Further Work

The findings of this thesis have begun to answer some of the questions associated with the newly identified acyltransferase activity of X-prolyl dipeptidyl peptidase. However many aspects of this activity remain to be investigated, especially as to how an enzyme that hydrolyses peptides also can specifically transfer an acyl group to an alcohol acceptor without any hydrolysis occurring. Perhaps one of the most conclusive ways to investigate these dual activities would be through structural studies. This work is currently underway, and several conditions that promote the crystallisation of PepX have been identified. These crystals have been found to diffract to better than 2Å resolution and are a promising start to further studies of the structural features of PepX that allow its unique activity. The goal of future structural work is to ultimately solve several structures of PepX with substrate analogues bound, to examine how the enzyme conformation changes during substrate binding, and also to identify how the enzyme is able to bind acylglycerides with a high specificity, whilst binding peptides with a lesser preference. Other authors have identified tributyrin analogues that irreversibly form an acyl intermediate with tributyrin binding enzymes (Longhi *et al.*, 1997), and the use of these compounds is planned, in order to understand how substrates bind to PepX. As well as leading to an understanding of the catalytic mechanism of PepX, it may lead to an insight into how other dairy related esterases might be modified to produce more desirable industrial characteristics.

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APPENDIX 1

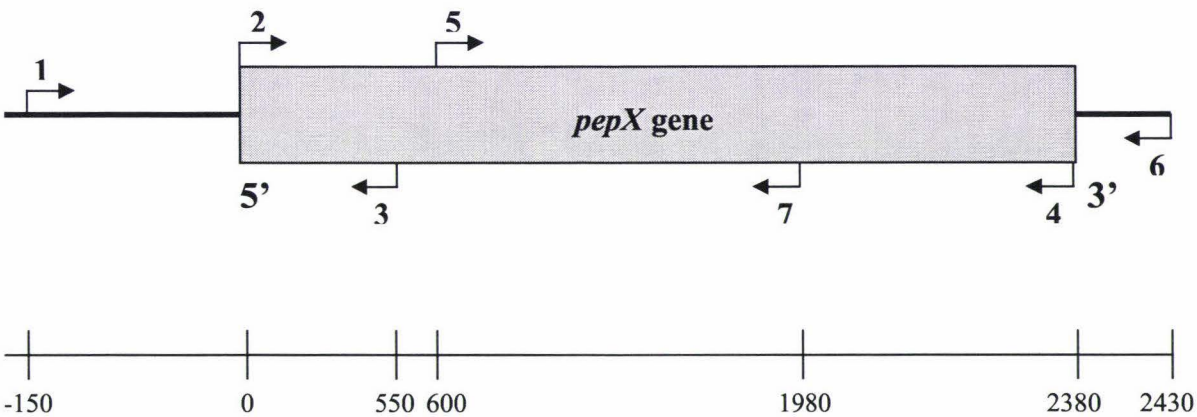
The Genetic Code

The following translation table was used to generate predicted protein translations from DNA sequence data.

1st Position 5'	2nd Position U	2nd Position C	2nd Position A	2nd Position G	3rd Position 3'
U	UUU ***Phe***	UCU ***Ser***	UAU ***Tyr***	UGU ***Cys***	U
	UUC ***Phe***	UCC ***Ser***	UAC ***Tyr***	UGC ***Cys***	C
	UUA ***Leu***	UCA ***Ser***	UAA **Stop**	UGA **Stop**	A
	UUG ***Leu***	UCG ***Ser***	UAG **Stop**	UGG ***Trp***	G
C	CCU ***Leu***	CCU ***Pro***	CAU ***His***	CGU ***Arg***	U
	CUC ***Leu***	CCC ***Pro***	CAC ***His***	CGC ***Arg***	C
	CUA ***Leu***	CCA ***Pro***	CAA ***Gln***	CGA ***Arg***	A
	CUG ***Leu***	CCG ***Pro***	CAG ***Gln***	CGG ***Arg***	G
A	AUU ***Ile***	ACU ***Thr***	AAU ***Asn***	AGU ***Ser***	U
	AUC ***Ile***	ACC ***Thr***	AAC ***Asn***	AGC ***Ser***	C
	AUA ***Ile***	ACA ***Thr***	AAA ***Lys***	AGA ***Arg***	A
	AUG ***Met***	ACG ***Thr***	AAG ***Lys***	AGG ***Arg***	G
G	GUU ***Val***	GCU ***Ala***	GAU ***Asp***	GGU ***Gly***	U
	GUC ***Val***	GCC ***Ala***	GAC ***Asp***	GGC ***Gly***	C
	GUA ***Val***	GCA ***Ala***	GAA ***Glu***	GGA ***Gly***	A
	GUG ***Val***	GCG ***Ala***	GAG ***Glu***	GGG ***Gly***	G

APPENDIX 2

PepX Expression Construct and Primer Annealing Positions



This construct was hosted in the pProEX HtB plasmid expression vector.

Key to Primer Numbers:

1) ***M13 pUC Reverse*** **(vector sequencing primer)**

2) ***Peptidase_Probe_FWD***
PepX_deg_Fwd
PepXDegFWDNcoI

3) ***Peptidase_Probe_RVS***

4) ***PepX_deg_RVS***
PepXDegRVSSalI

5) ***PepX_int_Fwd***

7) ***PepX_int_Rvs***

8) ***pProEX Ex HtB_Rvs*** **(custom designed vector primer)**

APPENDIX 3

Primer Sequences

For primers used in sequencing and PCR

Primer Name	Length (nt)	Tm (°C)	Sequence (5′ - 3′)
M13/pUC Reverse	23	65.8	AGC GGA TAA CAA TTT CAC ACA GG
Peptidase_Probe_FWD	33	63.5	ATG AAA TTT AAT CAA TTT TCT TAT ATT CCA GTT
PepX_deg_Fwd	23	53.2	ATG GAR TTY AAY CAR TTY TCN TAY AT
PepXDegFWDNcoI	35	72.8	TTT GGC GCC ATG GAR TTY AAY CAR TTY TCN TAY AT
Peptidase_Probe_RVS	27	58	ATC AAG TTG GCC ATC TTG ATC GGT ATC
PepX_deg_RVS	24	64.2	TTA TGG CAA NGT CAT YTT WGA YTG
PepXDegRVSSalI	33	73.9	TTT GTC GAC TTA TGG CAA NGT CAT YTT WGA YTG
PepX_int_Fwd	16	52.6	AGC TGA CAC ATG ATG G
PepX_int_Rvs	18	52.9	CTC ACT TGC TGA GAA CTC
pPRO Ex Ht_Rvs	22	69.4	TTC TCT CAT CCG CCA AAA CAG C

APPENDIX 4

Gene Sequence of *pepX* and Conceptual Translation

From *Streptococcus thermophilus* strain B2513 (ST-1)

```
tatcaccatcaccatcacgattacgatatccaacgaccgaaaacctgtatttcagggc
 Y  H  H  H  H  H  D  Y  D  I  P  T  T  E  N  L  Y  F  Q  G
gccatggagttcaaccaatttcgtacatcctgttagtcctgaaacggcttatcaggaa
 A  M  E  F  N  Q  F  S  Y  I  P  V  S  P  E  T  A  Y  Q  E
ctccgttcttttgggctttgaagtctctctagatgctagtgccaaagccaatttgaatcc
 L  R  S  L  G  F  E  V  S  L  D  A  S  A  K  A  N  F  E  S
tttgttcgcaagtacttccttttctttgaagatactgatttggcttgaaaaattggatt
 F  V  R  K  Y  F  L  F  F  E  D  T  D  L  A  L  K  N  W  I
gccgatccagagacagacctcctgtccttttttccaatcagaccgtccttgactgctgaa
 A  D  P  E  T  D  L  L  S  F  F  Q  S  D  R  P  L  T  A  E
gtctttggcttagtagcccttcaactgctaggatttgtccctaatgtggactttaccgac
 V  F  G  L  V  A  L  Q  L  L  G  F  V  P  N  V  D  F  T  D
agtgttgcttttccttgagaaaaatggccttccccaattgctttgatggtagccttaataac
 S  V  A  F  L  E  K  M  A  F  P  I  A  F  D  G  S  L  N  N
cttcaccagttgttagcgacacgcacacaatctggcaatacctaattgatcagttagta
 L  H  Q  L  L  A  T  R  T  Q  S  G  N  T  L  I  D  Q  L  V
gcccaagatttaatcccaataagtaatgactactgtcttctttaatggtaagagcttggca
 A  Q  D  L  I  P  I  S  N  D  Y  V  F  F  N  G  K  S  L  A
acatttgacactaatcagcttcaccgtgaagtggtttatgtcgaaacacctgtagatact
 T  F  D  T  N  Q  L  H  R  E  V  V  Y  V  E  T  P  V  D  T
gacaaagatgggctgttagacctagtcaaggtgactatcttacgtcctaatgttgacttc
 D  K  D  G  L  L  D  L  V  K  V  T  I  L  R  P  N  V  D  F
ccagttccagccatgatgaccgcagcccttatcaacaagggactaatgaacctcctct
 P  V  P  A  M  M  T  A  S  P  Y  Q  Q  G  T  N  E  P  S  S
gataaactgacccacaagatggaggagacttgctcgtcaaaccagctggtaagatttct
 D  K  L  T  H  K  M  E  G  D  L  L  V  K  P  A  G  K  I  S
cttagccgtccagaaattaaagcaccagaggcagacttacgcctatcaaccctgtcaca
 L  S  R  P  E  I  K  A  P  E  A  D  L  T  P  I  N  P  V  T
aaggctgaggagcgtttgctcacactgatacctatacacttaacgattacatgttggct
 K  A  E  E  R  F  A  H  T  D  T  Y  T  L  N  D  Y  M  L  A
cgtggtgtggcttctatctatgtatctggtgtcggtacttcaactcggaaggttcatg
 R  G  V  A  S  I  Y  V  S  G  V  G  T  F  N  S  E  G  F  M
acctctggagactatcaacaagtttttggcctataaagctgtcattgactggctcaatggt
 T  S  G  D  Y  Q  Q  V  L  A  Y  K  A  V  I  D  W  L  N  G
cgtgcacgcgcctttactagtcgtagcagacagcatacaatcactgctgattgggcttct
 R  A  R  A  F  T  S  R  S  R  Q  H  T  I  T  A  D  W  A  S
ggtaaggtgactactactggacttttcatattttgggtaccatgtccaacgcccttgctaca
 G  K  V  T  T  T  G  L  S  Y  L  G  T  M  S  N  A  L  A  T
actggtgttgacggttttggagatggttattgctgaagcgggtatttcttcttggtacgac
 T  G  V  D  G  L  E  M  V  I  A  E  A  G  I  S  S  W  Y  D
tattatcgtgaaaatggactcctctcgttagtcctggagggtacctggtgagatctcgat
 Y  Y  R  E  N  G  L  L  V  S  P  G  G  Y  P  G  E  D  L  D
accttgactgaattcacctattctcgtgccctattagctggagaatacctacgccaccaa
 T  L  T  E  F  T  Y  S  R  A  L  L  A  G  E  Y  L  R  H  Q
aaagactacgaggcctacctcaatgagctaagcacagctattgatcgtaagcatggggat
 K  D  Y  E  A  Y  L  N  E  L  S  T  A  I  D  R  K  H  G  D

tataaccagttctggcatgaccgcaactatgtgcaattcgcagaccgtgtcaaagctact
 Y  N  Q  F  W  H  D  R  N  Y  V  Q  F  A  D  R  V  K  A  T
gtagtctttacacacggtagccaggattggaatgtcaaaccaattaatgtctatcaaatg
 V  V  F  T  H  G  S  Q  D  W  N  V  K  P  I  N  V  Y  Q  M
```

ttcagagcccttcctaaatctctcgaaaaacacctcttcttccataatggtgcccacgtt
F R A L P K S L E K H L F F H N G A H V
tacatgaatgcctggcagtctattgacttccgagaaagcatgaacgccttgatttgtcag
Y M N A W Q S I D F R E S M N A L I C Q
aaactccttggttagatgatggtacacactgcctacagtcatctggcaaaacaaccag
K L L G L D N G Y T L P T V I W Q N N Q
tctgaacagacctggaagttctcgataactttggtcatgataatggtaaacacattcag
S E Q T W E V L D N F G H D N G K H I Q
cttggtaaggcagaagctagtatcgctaaccattacgaagaggaaatctttgctaagtac
L G K A E A S I A N H Y E E E I F A K Y
agtaagacttatcaaagctttaaggatgacctctttatggataaagccaatgccatcact
S K T Y Q S F K D D L F M D K A N A I T
ctagactttgaactggaccaagacatccaaatcaatggtcgtgtccacctagagctcagg
L D F E L D Q D I Q I N G R V H L E L R
gtcaagtctagcacaaaccgtggtctcatttcgcccaagttctggaaatgggagataaa
V K S S T N R G L I S A Q V L E M G D K
aaatacctagctccaatacctgaattaaaacgcatgaatgtagacaacggccgtctcttc
K Y L A P I P E L K R M N V D N G R L F
aaggaggaagccttacgtgaattgccattcaagcaggcaaaatactcgtgtcattaccaaa
K E E A L R E L P F K Q A K Y R V I T K
ggacacctcaacctgcaaaatcgcaaagatctccttagcattgaggaatgtcactccaaat
G H L N L Q N R K D L L S I E N V T P N
gaatggatgactatcggttggacttacaaccaaccatctacaaactcaacaaggggcgac
E W M T I G L D L Q P T I Y K L N K G D
aaactccgactcgttctctataccacagactttgatcacactattcgtgacaatagtgac
K L R L V L Y T T D F D H T I R D N S D
tatgaagtgactgtggacctaagccagtctaaaatgacattgccataagtcgacgagctc
Y E V T V D L S Q S K M T L P - V D E L
actagtcgcggccgctttcgaatctagagcctgcagtctc
T S R G R F R I - S L Q S

APPENDIX 5

PepX Nucleotide Sequence Comparisons

```
                 905        915        925        935        945        955        965        975        985        995
PepX_ST1         tctggagact atcaacaagt tttggcctat aaagctgtca ttgactggct caatggtcgt gcacgcgcct ttactagtcg tagcagacag catacaatca
PepX_ACA_DC4     tctggagact atcaacaagt tttggcctat aaagctgtca ttgactggct caatggtcgt gcacgcgcct ttactagtcg tagcagacag catacaatca

                1005       1015       1025       1035       1045       1055       1065       1075       1085       1095
PepX_ST1         ctgctgattg ggcttctggt aaggtgacta ctactggact ttcatatttg ggtaccatgt ccaacgccct tgctacaact ggtgttgacg gtttggagat
PepX_ACA_DC4     ctgctgattg ggcttctggt aaggtgacta ctactggact ttcatatttg ggtaccatgt ccaacgccct tgctacaact ggtgttgacg gtttggagat

                1105       1115       1125       1135       1145       1155       1165       1175       1185       1195
PepX_ST1         ggttattgct gaagcgggta tttcttcttg gtacgactat tatcgtgaaa atggactcct cgttagtcct ggagggtacc ctggtgagga tctcgatacc
PepX_ACA_DC4     ggttattgct gaagcgggta tttcttcttg gtacgactat tatcgtgaaa atggactcct cgttagtcct ggagggtacc ctggtgagga tctcgatacc

                1205       1215       1225       1235       1245       1255       1265       1275       1285       1295
PepX_ST1         ttgactgaat tcacctattc tcgtgcccta ttagctggag aatacctacg ccaccaaaaa gactacgagg cctacctcaa tgagctaagc acagctattg
PepX_ACA_DC4     ttgactgaat tcacctattc tcgtgcccta ttagctggag aatacctacg ccaccaaaaa gactacgagg cctacctcaa tgagctaagc acagctattg

                1305       1315       1325       1335       1345       1355       1365       1375       1385       1395
PepX_ST1         atcgtaagca tggggattat aaccagttct ggcatgaccg caactatgtg caattcgcag accgtgtcaa agctactgta gtctttacac acggtagcca
PepX_ACA_DC4     atcgtaagca tggggattat aaccagttct ggcatgaccg caactatgtg caattcgcag accgtgtcaa agctactgta gtctttacac acggtagcca

                1405       1415       1425       1435       1445       1455       1465       1475       1485       1495
PepX_ST1         ggattggaat gtcaaaccaa ttaatgtcta tcaaatgttc agagcccttc ctaaatctct cgaaaaacac ctcttcttcc ataatggtgc ccacgtttac
PepX_ACA_DC4     ggattggaat gtcaaaccaa tgaatgtcta tcaaatgttc agagcccttc ctaaatctct cgaaaaacac ctcttcttcc ataatggtgc ccacgtttac

                1505       1515       1525       1535       1545       1555       1565       1575       1585       1595
PepX_ST1         atgaatgcct ggcagtctat tgacttccga gaaagcatga acgccttgat ttgtcagaaa ctccttggct tagataatgg ttacacactg cctacagtca
PepX_ACA_DC4     atgaatgcct ggcagtctat tgacttccga gaaagcatga acgccttgat ttgtcagaaa ctccttggat tagataatgg ttacacactg cctacagtca

                1605       1615       1625       1635       1645       1655       1665       1675       1685       1695
PepX_ST1         tctggcaaaa caaccagtct gaacagacct gggaagttct cgataacttt ggtcatgata atggtaaaca cattcagctt ggtaagtcag aagctagtat
PepX_ACA_DC4     tctggcaaaa caaccagtct gaacagacct gggaagttct cgataacttt ggtcatgata atggtaaaca cattcagctt ggtaagtcag aagctagtat

                1705       1715       1725       1735       1745       1755       1765       1775       1785       1795
PepX_ST1         cgctaaccat tacgaagagg aaatctttgc taagtacagt aagcttatc aaagctttaa ggatgacctc tttatggata aagccaatgc catcactcta
PepX_ACA_DC4     cgctaaccat tacgaagagg aaatctttgc taagtacggt aagcttatc aaagctttaa ggatgacctc tttatggata aagccaatgc catcactcta
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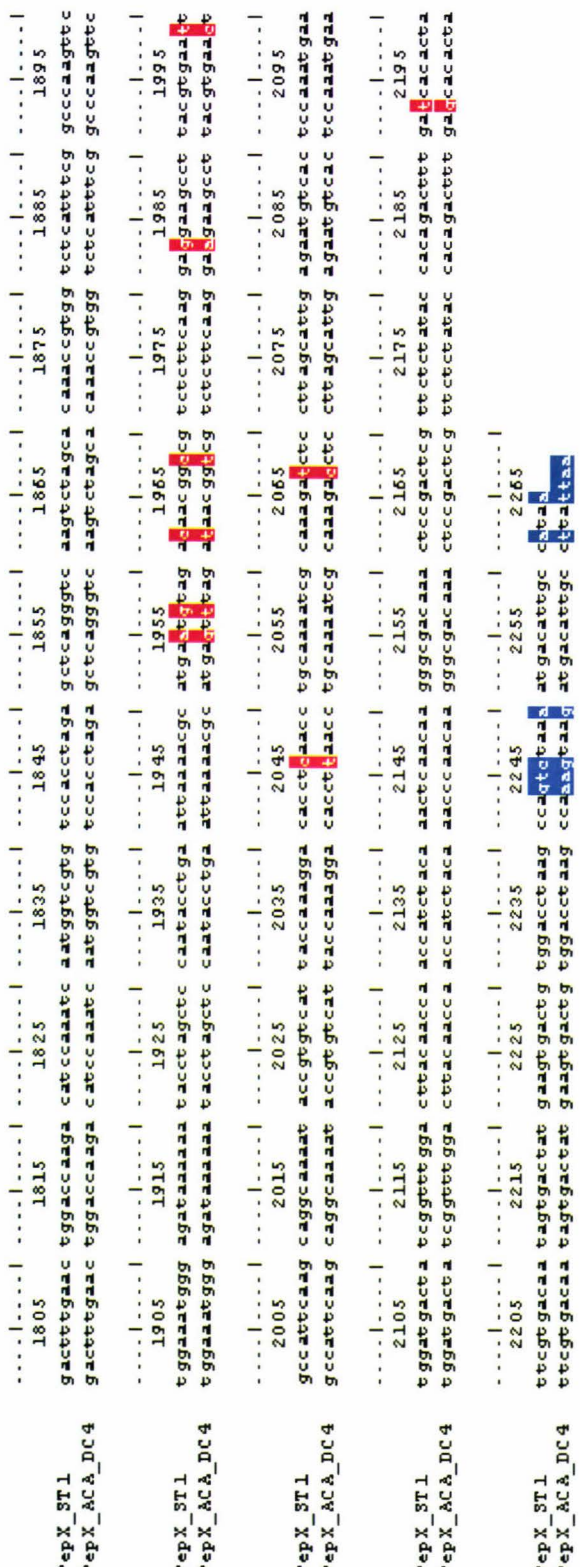


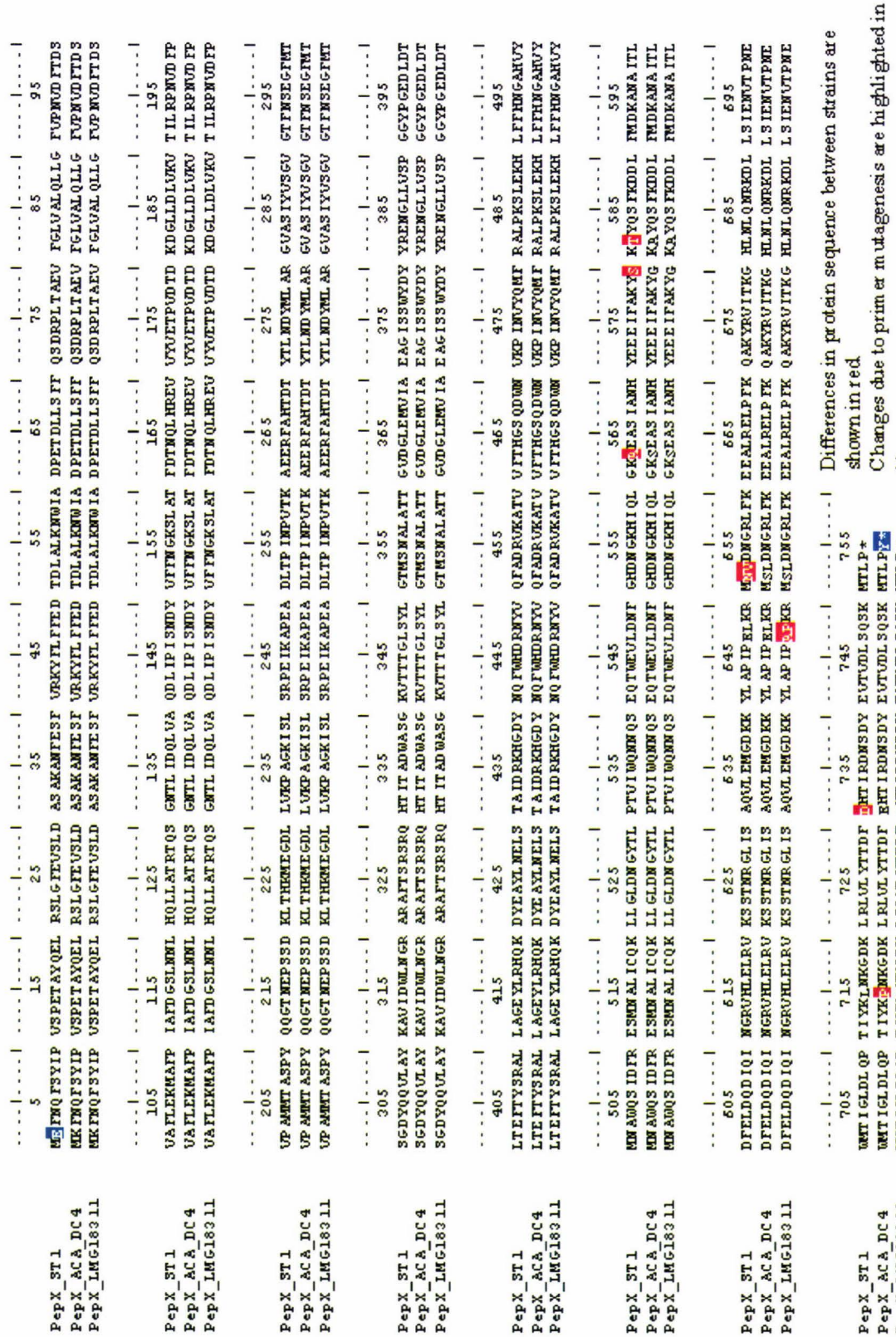
Notes:

ST-1 is the strain used in the current study. **ACA DC4** is the strain used by (Anastasiou *et al.*, 2002) to clone the PepX gene.

Changes in sequence between strains are highlighted in **red**.
Changes due to primer induced mutagenesis during PCR cloning are highlighted in **blue**.

APPENDIX 6

PepX Protein Sequence Comparisons



APPENDIX 7

Kinetics Data

Ester hydrolysis using *p*-nitrophenyl butyrate:

Substrate concentration (mM)	Reaction rate (μmoles.min^{-1}.mg^{-1} enzyme)
2.046	1.054
2.046	1.066
2.046	1.068
2.046	1.039
2.046	0.986
0.165	0.574
0.165	0.636
0.165	0.570
0.165	0.512
0.165	0.664
0.220	0.695
0.220	0.735
0.220	0.707
0.220	0.678
0.220	0.851
0.113	0.476
0.113	0.567
0.113	0.537
0.113	0.449
0.113	0.445
0.056	0.225
0.056	0.312
0.056	0.320
0.056	0.324
0.056	0.252
1.128	1.084
1.128	1.018
1.128	0.999
1.128	1.034
1.128	1.055

Peptidase hydrolysis using glycylprolyl *p*-nitroanilide and tributyrin inhibitor:

No inhibitor		0.125μL/mL tributyrin		0.05μL/mL tributyrin	
Substrate concentration (mM)	Reaction rate ($\mu moles.min^{-1}.mg^{-1}$)	Substrate concentration (mM)	Reaction rate ($\mu moles.min^{-1}.mg^{-1}$)	Substrate concentration (mM)	Reaction rate ($\mu moles.min^{-1}.mg^{-1}$)
0.30	50.30	0.30	21.39	0.15	24.35
0.30	61.66	0.30	20.22	0.15	23.51
0.30	54.81	0.30	26.57	0.15	21.16
0.30	55.65	0.30	27.90	0.15	23.34
0.30	59.32	0.30	25.07	0.08	12.90
0.30	53.48	0.30	28.24	0.08	9.38
0.15	39.46	0.15	14.78	0.08	11.06
0.15	39.63	0.15	16.29	0.08	10.39
0.15	43.30	0.15	14.28	0.30	41.61
0.15	39.29	0.15	13.27	0.30	37.10
0.15	39.96	0.15	11.42	0.30	34.10
0.08	22.57	0.08	7.20	0.30	24.35
0.08	26.74	0.08	6.20	0.30	20.61
0.08	17.89	0.08	5.03	0.45	33.92
0.08	28.92	0.08	3.18	0.45	33.07
0.08	22.73	0.08	4.86	0.45	29.92
0.08	20.73	0.61	37.60	0.45	29.68
0.61	85.73	0.61	37.43	0.61	39.78
0.61	76.54	0.61	38.77	0.61	38.33
0.61	80.05	0.61	34.09	0.61	33.63
0.61	71.69	0.61	35.43	0.61	33.39
0.61	74.20	0.45	33.76	0.91	42.67
0.61	80.21	0.45	33.76	0.91	42.67
0.91	79.55	0.45	36.61	0.91	38.57
0.91	72.69	0.45	40.98	0.91	41.22
0.91	78.71	0.45	33.25	1.52	56.17
0.91	78.88	0.91	42.11	1.52	47.97
0.91	76.20	0.91	42.95	1.52	53.28
0.46	72.56	0.91	47.96	1.52	47.73
0.46	63.37	0.91	42.28	2.27	56.33
0.46	77.24	0.91	48.13	2.27	52.33
0.46	64.70	1.52	54.98	2.27	51.97
0.46	68.22	1.52	51.30	2.27	52.21
2.28	85.77	1.52	45.29	6.08	61.84
2.28	88.78	1.52	52.81	6.08	63.65
2.28	82.93	1.52	49.97		
2.28	81.93	2.27	65.33		
2.28	83.77	2.27	54.59		
1.52	73.86	2.27	54.07		
1.52	71.19	2.27	50.55		
1.52	73.20	2.27	56.60		
1.52	78.37				
1.52	85.83				
1.52	74.50				
6.08	85.73				
6.08	88.23				
6.08	85.39				

Calculation of Reaction Rate from Absorbance of Chromophore at 410nm

Chromophores used:
p-nitrophenyl butyrate releases *p*-nitrophenol upon hydrolysis
Glycylprolyl *p*-nitroanilide releases p-nitroaniline upon hydrolysis

Convert reaction rate absorbance to μmole units:

$$\frac{\Delta Abs.min^{-1}}{(\varepsilon / 1000)} \times \frac{Volume\ of\ enzyme\ (\mu L)}{1000} \times Total\ assay\ volume(mL) \times Dilution\ factor$$

Where ε_{410nm} **(p-nitrophenol) = 7626**
or ε_{410nm} **(p-nitroaniline) =8800**

= *Reaction rate* ***(μmoles.min^{-1}.mL^{-1})***

Convert from mL of enzyme to mg:

$$\frac{Reaction\ rate\ (\mu moles.min^{-1}.mL^{-1})}{Protein\ concentration\ (mg.mL^{-1})}$$

= *Reaction rate* ***(μmoles.min^{-1}.mg^{-1})***

APPENDIX 8
Gas Chromatograph Data from Ester Synthesis Assays

Substrate	Sample incubation time (min) / control	Product (μg/g)
Tributyrin (C4)	120	10.85
	120	11.02
	120	10.53
	120 / no enzyme	0
Tricaproin (C6)	120	-
	120	1.08
	120	1.00
	120 / no enzyme	1.05
Monocaprylin (C8)	120	22.37*
	120	14.03
	120	16.21
	120 / no enzyme	14.90
Dicaprylin (C8)	120	5.36
	120	5.00
	120	6.13
	120 / no enzyme	5.84
Monocaprin (C10)	120	25.48
	120	26.27
	120	23.88
	120 / no enzyme	25.68

- = no analysis (sample lost)

** = discarded as an outlier*

For $\mu g.g^{-1}$ to $\mu moles.mg^{-1}$ enzyme conversion see Appendix 10

APPENDIX 9
Gas Chromatograph Data from Ester Hydrolysis Assays

Data for tributyrin hydrolysis:

Substrate	Butyric acid product (ppm)			
	1 h	2 h	4 h	24 h
Tributyrin (1)	3.08	3.80	4.38	3.74
Tributyrin (2)	4.14	3.60	6.37	-
Tributyrin + EtOH (1)	2.08	4.57	2.89	3.03
Tributyrin + EtOH (2)	4.14	3.79	3.36	3.26
Tributyrin (no enzyme)	-	3.32	3.84	4.31

- = no analysis (sample lost)

Data for milkfat hydrolysis:

Acid Acyl size	Sample	Product (ppm)				
		0 h	1 h	2 h	5.5 h	24 h
C4	Non lipolysed, no enz	0	0	0	0	0
	Non lipolysed + enz	0	0	0	0	0
	Lipolysed, no enz	0.2545	0.2585	0.2617	0.2572	0.2386
	Lipolysed + enz	0.2661	0.2561	0.2469	0.2495	0.2582
	Lipolysed + enz	0.2607	0.2777	0.2627	0.221	0.2438
C6	Non lipolysed, no enz	0	0	0	0	0
	Non lipolysed + enz	0	0	0	0	0
	Lipolysed, no enz	0.1212	0.1247	0.1185	0.1228	0.1321
	Lipolysed + enz	0.1297	0.1259	0.1213	0.1279	0.1386
	Lipolysed + enz	0.1147	0.1361	0.1272	0.1097	0.1311
C8	Non lipolysed, no enz	0	0	0	0	0
	Non lipolysed + enz	0	0	0	0	0
	Lipolysed, no enz	0.0696	0.0733	0.0666	0.0721	0.0809
	Lipolysed + enz	0.0761	0.0737	0.0716	0.0777	0.0831
	Lipolysed + enz	0.0635	0.0778	0.0746	0.0644	0.0779
C10	Non lipolysed, no enz	0	0	0	0	0
	Non lipolysed + enz	0	0	0	0	0
	Lipolysed, no enz	0.1283	0.1287	0.1133	0.1215	0.1293
	Lipolysed + enz	0.137	0.1295	0.0716	0.1315	0.1359
	Lipolysed + enz	0.1137	0.1357	0.1306	0.1046	0.1197

APPENDIX 10

Part per million to μmol Conversions

From GC raw data (ppm or $\mu g.g^{-1}$)

Assuming 1 mL of sample weighs 1g, then

$\mu g.g^{-1}$ = μg per mL of sample.

Using 10.8 $\mu g.g^{-1}$ where product was ethyl butanote (MwT = 116.18)

Convert μg to g

$= 10.8 \times 10^{-6}$

Convert g to moles using molecular mass of product

$= (10.8 \times 10^{-6}) / 116.18$

$= 9.295 \times 10^{-8}$ moles.mL^{-1} sample

convert to moles.mg protein

In total assay (1.5 mL) there was 4 μL of enzyme, only sampled 1 mL so

(1/1.5) x 4 μL = 2.6 μL per 1 mL sample

Protein concentration was 3.4 $mg.mL^{-1}$

so there was (2.6 / 1000) x 3.4

$= 9.06 \times 10^{-3}$ mg per 1mL sample

converting to moles product/mg protein

$= 9.295 \times 10^{-8} / 9.06 \times 10^{-3}$

$= 1.026 \times 10^{-5}$ moles.mg protein

converting to μmoles

$= 1.026 \times 10^{-5}$ moles.mg $\times 10^{6}$

= 10.26 μmoles.mg over 2 h assay period

converting to $\mu moles.min^{-1}.mg^{-1}$

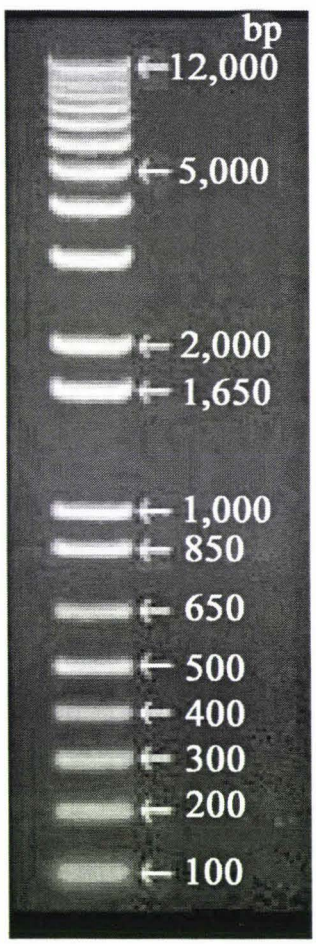
= 10.26 / 120

= 0.085 $\mu moles.min^{-1}.mg^{-1}$ enzyme.

APPENDIX 11

DNA and Protein Markers

1kb plus DNA ladder (Invitrogen) 1% agarose gel



Protein low-range molecular mass markers (BioRad) on 12% polyacylamide gel