A Comparative Study of an Aminopeptidase from Lactic Acid Bacteria

A thesis presented in partial fulfilment of the requirements for the degree of
Master of Science in Biochemistry at Massey University

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1992

Abstract:

Aminopeptidase enzymes from the proteolytic systems of *S.salivarius* subsp.*thermophilus* *Lactococcus lactis* subsp.*cremoris* and *Lactococcus lactis* subsp.*lactis* have been investigated.

An aminopeptidase was purified to near homogeneity from a crude cell free extract of *S.thermophilus* 5109. The enzyme had a native molecular weight of approximately 96kDa determined by gel-filtration, and a subunit molecular weight of 98kDa, determined by denaturing polyacrylamide gel electrophoresis, showing the native enzyme to be a monomer.

The aminopeptidase activity was optimal at pH 7.0 and 35°C. The enzyme was inactivated by p-chloromercuribenzoic acid, iodoacetic acid,the chelating agents EDTA and 1,10-phenanthroline and the divalent cations Cu^{2+}, Zn^{2+} and Co^{2+}. The aminopeptidase was not inhibited by the serine protease inhibitor PMSF and only minor inhibition occured with the inhibitor Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK). The aminopeptidase was capable of hydrolysing several amino-acyl amido methyl coumarin (AMC) and p-nitroanilide (pNA) derivatives, particularly those of lysine, arginine and leucine. The enzyme showed greatest activity with lysyl derivatives (and is therefore referred to in this thesis as a lys-aminopeptidase). The enzyme was able to degrade several oligopeptides by progressive cleavage of the peptide bond but did not hydrolyse peptides containing a proline or aspartic acid residue in the second position. The aminopeptidase activity was dependent on the size of the peptide in that generally only peptides with more than three amino acids were degraded. The aminopeptidase had no endopeptidase or dipeptidase activity.

Five different amino-acyl p-nitroanilides derivatives and two amido methyl coumarin derivatives were used to determine the kinetic parameters of the aminopeptidase. The Km values obtained for all the substrates tested were similar, with the exception of ala-pNA, for which the Km value was significantly higher.

On the basis of the distribution of activity between different cell-fractions the lys-aminopeptidase appears to be localised intracellularly.

An aminopeptidase was also partially purified from cell-free extracts from *Lactococcus lactis* subsp.*cremoris* AM2 and *Lactococcus lactis* subsp.*lactis* ML3.

The aminopeptidase from *L.cremoris* AM2 was shown to have a molecular weight of 106kDa and was a monomer. It showed optimal activity at a pH of 7.0 and 45°C. The aminopeptidase activity was inhibited by metal-chelators, SH group inhibitors and TLCK.

The aminopeptidase hydrolysed lysyl-, arginyl- and leucyl-p-nitroanilide derivatives, but had little or no activity with other pNA substrates.

The aminopeptidase from *L.lactis* ML3 had a molecular weight of 100-105kDa and was monomeric. The optimal activity for the aminopeptidase was at pH of 7.0 and 40°C. The enzyme was inactivated by metal-chelators, sulphydryl inhibitors and by TLCK. Like the aminopeptidases from the other two strains the ML3 aminopeptidase was very specific hydrolysing lysyl-, leucyl- and arginyl-pNA but with very little or no activity with other amino-acyl derivatives.

Acknowledgements:

This investigation would not have been possible without the help of many people within the Department and the Research Institutes.

I would like to thank the New Zealand Dairy Research Institute for providing the bacterial strains, cell lysates and milk powder used in this study. I am also indebted to the staff of the Biochemical Processing Centre, DSIR for carrying out large scale bacterial cultures.

I am most grateful to Dr N.Haggerty for providing the IMAC column; Mrs C. Flyger for carrying out the amino acid analyses and peptide sequences and Mr D.Poll who rode herd over my early efforts at FPLC and HPLC analysis.

I would like to express my thanks to the members of the laboratory particularly Julian Reid and Andrea Williams for their help during the last two years.

I am also greatly indepted to the inventor of the Apple Macintosh Dictionary.

Finally I would like to thank my supervisor, Dr. G.G. Pritchard for his unfailing patience, helpful advice and encouragement throughout this investigation.

List of Abbreviations:

AMC	7-amino-4-methyl coumarin
DEAE	diethylaminoethyl.
EDTA	ethylenediaminetetracetic acid
FPLC	fast performance liquid chromatography
HPLC	high performance liquid chromatography
IMAC	immobilized metal affinity chromatography
MES	2-(N-morpholino-) ethane sulphonic acid
PAGE	polyacrylamide gel electrophoresis
pCMB	p-chloromercuric benzoic acid
PMSF	phenyl methyl sulphonyl fluoride
pNA	p-nitroanilide
RSM	reconstituted skim milk medium
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N N N'N'-tetramethyl ethylene-diamine
TFA	trifluroacetic acid
TLCK	N-α-tosyl-L-Lysine chloro methyl ketone
TRIS	tris-(hydroxymethyl)-aminomethane

One Letter Code for Amino acids

A	Alanine	M	Methionine
C	Cysteine	N	Asparagine
D	Aspartic acid	P	Proline
E	Glutamic acid	Q	Glutamine
F	Phenylalanine	R	Arginine
G	Glycine	S	Serine
H	Histidine	T	Threonine
I	Isoleucine	V	Valine
K	Lysine	W	Tryptophan
L	Leucine	Y	Tyrosine

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Chapter 1: Introduction

1.1. Importance of Proteolytic Activity of Starter Bacteria:

The production of fermented milk products, such as cheese and yoghurt, is initiated by the addition of carefully selected individual starter strains, or a combination of different strains, of lactic acid bacteria. These strains belong to the species *Lactococcus lactis* subsp.*lactis* and *cremoris* and the related thermophilic bacteria *Streptococcus salivarius* subsp.*thermophilus, Lactobacillus bulgaricus* and *Lb.helveticus* . (For convenience the two lactococcal subspecies will subsequently be abbreviated to *L.lactis* and *L.cremoris* and *Streptococcus salivarius*subsp.*thermophilus* to its former name *S.thermophilus*).

These starter bacteria use the lactose present in milk to produce pyruvic acid, NADH, and ATP via the glycolytic pathway. The pyruvic acid is then reduced to lactic acid, which results in a reduction of the pH of the milk to 4.5-5.0. The low pH prevents the growth of undesirable microorganisms and contributes to casein precipitation which is the initial step in cheese production.

A common feature of lactic acid bacteria is their fastidious nutritional nature. They are unable to synthesize most of the amino acids essential for their continued growth. Milk has a low free amino acid level (Thomas & Mills,1981). Therefore, for the bacteria to grow to the high cell density essential for lactose fermentation, they need to hydrolyse the milk proteins, primarily casein, into small peptides and amino acids which they can then use for protein synthesis (Laan et al.,1989).

Proteolysis is important not only for starter growth but also in the subsequent ripening process. Cheese ripening involves texture changes, aroma and flavour development brought about, at least in part, by the enzymic hydrolysis of casein. It has been claimed that large peptides give brothy background flavours in Swiss cheese and that proline and small peptides, in combination with Ca^{2+} and Mg^{2+}, contribute towards the sweet flavours found in cheese (Biede & Hammond, 1979). As well as contributing to desirable flavours, products of proteolysis can cause off-flavours. In particular a common cheese defect causing bitterness in the cheese has been attributed to the presence of peptides containing a high proportion of hydrophobic residues (leu, phe, pro) produced by the action of proteolytic enzymes on casein (Visser et al.,1983).The bitter flavour defect has been attributed to peptides derived from both α-casein (Richardson & Creamer,1973) and from β-casein (Visser et al.,1983).

For these reasons the proteolytic enzymes produced by the lactic bacteria are of considerable interest to the dairy industry.

Hydrolysis of milk proteins during the manufacture of cheese is due to the action of two major groups of proteolytic agents.

a) Rennet, a proteolytic enzyme added to produce clotting.

and b) Proteolytic enzymes produced by the starter culture and subsequently by adventitious non-starter bacteria.

The degradation of casein into small peptides and free amino acids by starter bacteria involves the combined action of two types of enzymes (Thomas & Pritchard,1987).

Proteinases -hydrolyse casein into large oligopeptides. These enzymes are predominantly located near or on the cell surface of the starter bacteria (Hugenholtz et al.,1984).

Peptidases -degrade oligopeptides produced by the proteinases into small peptides and free amino acids. They may be located in the cell wall, attached to the cell membrane or within the cell.

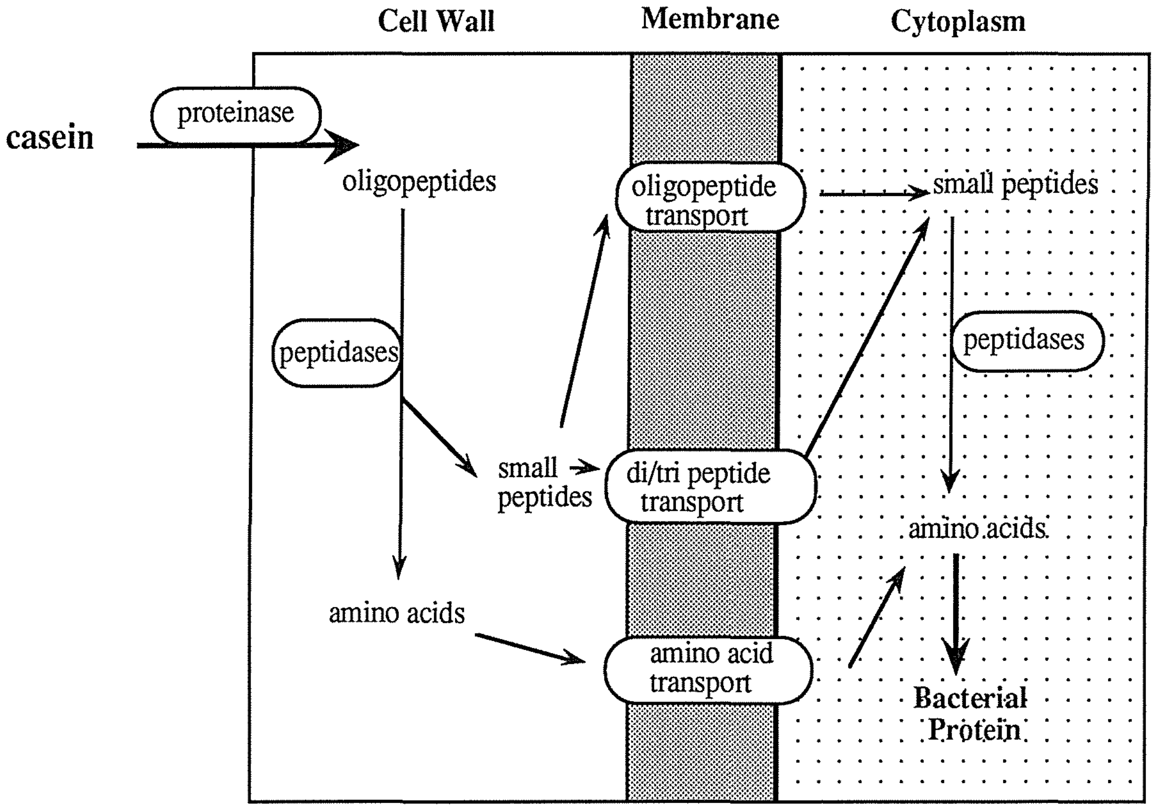

Figure 1.1: Proposed utilization of casein for growth of lactococci in milk.

The properties of these enzymes have been extensively investigated in the mesophilic lactococcal strains used in cheese manufacture. Much less is known about the enzymes in non-starter lactic acid bacteria and the thermophilic bacteria. It is known that lactobacilli tend to be more active in proteolysis than *Streptococcus thermophilus* although the activity of both groups is rather weak (Tourneur,1972).

1.2. Proteinases:

These enzymes have the ability to hydrolyse the native caseins producing large peptides.Whey proteins are thought not to act as substrates for starter proteinases (Thomas & Pritchard,1987) although detailed studies on whey protein degradation by starter bacteria have not been carried out.

The wide variety of species and strains studied and the variety of methods used for extraction and purification, makes comparison between the proteinases of different strains difficult. However the lactococcal proteinases do show many similarities: an apparent molecular weight of 140-160kDa, an isoelectric point of 4.5, an optimum pH for activity of 6.0 (Laan et al.,1989), activation or stabilization by Ca^{++} and sensitivity to serine protease inhibitors (Kok,1990).

Unlike most other proteolytic bacteria the lactococci do not seem to secrete high levels of proteinase into the medium. The proteinases responsible for degradation of extracellular protein usually remain largely bound to the cell wall (Thomas & Mills,1981; Hugenholtz et al.,1984) although considerable release into the medium has been found with lactococci grown in media other than milk (Nissen-Meyer & Sletten,1991).
The existence of intracellular proteinase activity has been reported although there is uncertainty about its role.Two distinct intracellular proteinases have been found in lactococci (Akuzawa et al.,1983,1985) one with an optimal temperature of 30-40^{o}C the other of 5-10^{o}C. The release of such enzymes from lysed bacteria may contribute to the ripening and flavour development of the cheese products.

One as yet unresolved question concerns the number of distinct cell wall proteinases produced by different strains of lactococci. The proteinases from different strains of lactococci have been classified according to several criteria; pH and temperature optima, (Exterkate,1976) immunological cross reactivity (Hugenholtz et al.,1984) and specificity towards the casein components of bovine milk (Visser et al.,1986). On the basis of specificity of action in hydrolysing casein, two distinct patterns of action are currently recognised, designated P_I-type and P_{III}-type proteinases (Exterkate & de Veer,1989). The P_I-type (or HP type) proteinase preferentially degrades β–casein. The particular peptide bonds cleaved in the β-casein by P_I-type proteinases have been determined for the enzymes from *L.lactis* NCDO763 (Monnet et al.,1986,1989) and several strains of *L.cremoris* (Visser et al.,1988; Ng,1988; Reid,1991). While there are some differences between the proteinases from these strains with respect to the bonds cleaved in β-casein during long-term digestion, a consistent pattern has been found for the initial sites of cleavage. These sites are mainly located in the C-terminal 53-residue region of the β-casein molecule.

H-Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Ser-Leu-
Ser-Ser-Ser-Glu-Glu-Ser-Ile-Thr-Arg-Ile-Asn-Lys-Lys-Ile-Glu-Lys-Phe-Gln-
Ser-Glu-Glu-Gln-Gln-Gln-Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys-Ile-His-Pro-
Phe-Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-
Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln-Thr-Pro-Val-Val-Val-Pro-Pro-
Phe-Leu-Gln-Pro-Glu-Val-Met-Gly-Val-Ser-Lys-Val-Lys-Glu-Ala-Met-Ala-
Pro-Lys-His-Lys-Glu-Met-Pro-Phe-Pro-Lys-Tyr-Pro-Val-Gln-Pro-Phe-Thr-
Glu-Ser-Gln-Ser-Leu-Thr-Leu-Thr-Asp-Val-Glu-Asn-Leu-His-Leu-Pro-Pro
-Leu-Leu-Leu-Gln-Ser-Trp-Met-His-Gln-Pro-His-Gln-Pro-Leu-Pro-Pro-
Thr-Val-Met-Phe-Pro-Pro-Gln-Ser-Val-Leu▼Ser-Leu-Ser▼Gln-Ser▼Lys-Val-Leu
-Pro-Val-Pro-Gln▼Lys-Ala-Val-Pro-Tyr-Pro-Gln▼Arg-Asp-Met-Pro-Ile-Gln-
Ala-Phe-Leu-Leu-Tyr▼Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-
Pro-Ile-Ile-Val-OH

Figure 1.2: Sequence of β-casein showing sites at which β-casein is initially cleaved by the cell wall proteinase from several different strains of lactococci (see text).

The P_{III}-type (or AM1 type) of proteinase degrades β-casein at different sites from those of the P_I-type and also significantly digests α_s-casein and κ-casein (Visser et al.,1986). The peptide bonds in both α- and β-casein cleaved by the proteinase from *L.cremoris* SK11 (a P_{III}-type proteinase) have recently been determined in this laboratory (Reid et al, 1991a, 1991b).

Strains exhibiting only P_{III} activity do not produce the bitter "off" flavours that are encountered when P_I proteinases are used (Exterkate &deVeer,1989).

Genetic studies of the proteinase has led to considerable clarification of previous information on the complexity of proteinase types.

The proteinase gene is located on a plasmid. Mapping of the proteinase gene led to the discovery that many, if not all lactococci carry essentially the same gene for the cell-wall associated proteinase with only minor sequence differences.The major difference is a duplication of 60 amino acids near the C-terminal end of the P_{III}-type which is missing from the P_I-type, and also certain substitutions of residues close to the active site.These small differences in primary structure are responsible for the different specificity of the proteinase (Kok 1990).

The apparent multiplicity of proteinases from a single strain (Hugenholtz et al.,1984; Cliff &Law,1985) can probably be explained by proteinase autolysis. The proteinase size predicted from the nucleotide sequence was estimated to be 200kDa (Kok,1990). Autoproteolysis was suggested to account for the difference between this predicted molar mass and that of the isolated enzyme (140-160 kDa).

Proteolytically active degradation products of 60-80kDa have been isolated.These could be products of proteinase self-digestion (Laan & Konings,1991) or parts of a heterodimer proteinase comprised of 60kDa and 80kDa subunits (Exterkate & de Veer,1989). Relatively little work has been carried out on the proteolytic enzymes of the thermophilic starter *S.thermophilus*. This species has proteinase activity associated with the cell wall, but its proteinase levels are low in comparison to those of the lactococci (Shankar & Davies, 1977).When grown in milk *S.thermophilus* behaves like a Prt^- (proteinase negative) variant of *L.cremoris* (Thomas &Mills,1981).

1.3. Peptidases:

Degradation products of casein resulting from the proteinase activity are further degraded by peptidases to small peptides and amino acids (Figure 1.1). The full peptidase complement of any single lactococcal strain has not been studied, hence the nature of the synergistic action between the different peptidases leading to hydrolysis of oligopeptides has not yet been defined.

A wide variety of peptidases have been reported from different strains of lactic acid bacteria. These can be classified as endo- or exo- peptidases.

1.3.1.Endopeptidases-:

These are peptidases capable of hydrolysing specific peptide bonds some distance from the N- or C-terminal of the protein or oligopeptide.
The endopeptidase action reported in *L.cremoris* HP (Exterkate,1975) was attributed to two endopeptidases P_{37} and P_{50} which had different pH and temperature optima.These were detected using the chromophoric substrate N-glutaryl phenylalanine p-nitroanilide in which the glutaryl moiety was thought to block exopeptidase activity. It was later shown (Exterkate & de Veer,1987b) that a membrane-bound aminopeptidase A was responsible for the cleavage of the N-terminal glutaryl residue while one or more phenylalanine aminopeptidases released the p-nitroanilide.This highlights the care that must be taken in interpretation of results based on the use of synthetic substrates.

Two different endopeptidases which may contribute to the hydrolysis of casein oligopeptides have been found in the cell free extract of *L.cremoris* H61 (Yan et al.,1987 a,b). One of the endopeptidases, LEP-II, is a metallo-endopeptidase dimer with a molecular weight of 80kDa. This enzyme shows a specificity for peptide bonds involving hydrophobic residues and only hydrolyses peptides of less than 3.5kDa molecular weight. It has been shown to cleave the N-terminal 23 residue oligopeptide derived from α_s-casein. This endopeptidase could be involved in the early stages of casein oligopeptide breakdown by providing small peptides for further degradation by exopeptidases (Figure 1.1). However it is probably located in the cytoplasm,and Yan et al.(1987a) suggest that its function may be to degrade signal peptides which are hydrophobic and of a suitable size to be a substrate for the enzyme.

Another endopeptidase designated LEP-I was found in the same strain. This was also a metallo-enzyme with a molecular weight of 98kDa. Like LEP-II it had no detectable hydrolytic activity with intact milk caseins. However it did show activity towards α-casein derived peptides. The maximum substrate size LEP-I could hydrolyse was even smaller than the largest peptide hydrolysed by LEP-II. It is possible that LEP-I may act in conjunction with the extracellular proteinase to hydrolyse oligopeptides into amino acids and peptides small enough to enter the cell. Further work to establish the cellular location of these two enzymes is important to understand their possible role in casein oligopeptide breakdown.

An endopeptidase from *Streptococcus thermophilus* was reported (Desmazeaud,1974) with a molecular weight of 39kDa which specifically cleaved bonds involving the α-amino group of hydrophobic residues in X-leu or X-phe bonds. The enzyme was claimed to be located intracellularly

1.3.2 Exopeptidases:

These enzymes catalyse the cleavage of one or two amino acids from the N or C-terminal of the peptide chain. Exopeptidases can be further classified into:

-aminopeptidases, which hydrolyse amino acids from the N-terminal end of a peptide and
-carboxypeptidases, which hydrolyse amino acids from the C-terminal .

There have been no reports to date of carboxypeptidase activity in starter lactococci even though many strains have been tested (Kaminogawa et al.,1984). However the non-starter lactic acid bacteria *Lactobacillus.casei* and *Lb.plantarum* do contain a carboxypeptidase (Kok,1990).

The following main types of aminopeptidase have been purified and characterised from lactic acid bacteria.

1.3.3. "General" Aminopeptidases:

There have been numerous reports of an aminopeptidase in lactic acid bacteria which is active in the cleavage of a broad spectrum of amino-acyl derivatives. It is frequently referred to as a "general" aminopeptidase. However systematic and quantitative studies of its substrate specificity have not been carried out nor has its activity towards oligopeptides been investigated.

One of the first reports of a broad specificity aminopeptidase was by Mou et al. (1975) who found, during fractionation of cell free extracts from lactococci by gel filtration, a single peak active towards arginyl, glutamyl, alanyl and histidinyl-β-naphthylamides.They concluded that this activity was that of a "general" aminopeptidase of wide specificity and distinct from the activity responsible for prolyl-β-naphthylamide hydrolysis.

Kaminogawa et al.(1984) carried out a survey of the aminopeptidase activities from the cell-free extracts of eleven strains of *L.lactis* and *L.cremoris* .They classed the strains into three groups based on their aminopeptidase activities. After partial purification by ion exchange chromatography they tentatively divided the fractions showing aminopeptidase activity into four types based on their specificity profiles and their pH optima. One type was found to be active against a wide variety of amino-acyl p-nitroanilides. The activity profiles of this fraction in the presence of various metal ions and inhibitors and the pH optimum were the same with different substrates.They concluded that the activity was due to a "general" aminopeptidase with a wide specificity, rather than a mixture of several enzymes with different specificities.

Subsequent to these early studies several aminopeptidases have been purified from dairy starter bacteria. The properties of these purified enzymes showed considerable differences between strains. An aminopeptidase from *L.cremoris* AC1 (Geis et al.,1985) had a molecular weight of 36kDa, it rapidly hydrolysed lysyl p-nitroanilide (lys-pNA) and to a lesser extent leu-pNA, ala-pNA and ala-ala-pNA. The enzyme was active over a pH range of 5.5-8.0 with an optimum at pH 7.0 and was strongly inhibited by 1mM EDTA. A partially purified lys-aminopeptidase from *L.lactis* 4760 (Lloyd,1989) showed the enzyme to have a molecular weight of 78kDa and an optimum pH of 6.5. It was inhibited by 1,10-phenanthroline and pCMB. Its specificity was limited, being active only against lys-,arg- and leu-, but not gly-,pro-,ala-,phe-,tyr- or ser- amidomethylcoumarin (AMC) substrates.

The most recent reports of the properties of purified "general" aminopeptidases are from *L.cremoris* strains AM2 (Neviani et al.,1989) and Wg2 (Tan & Konings,1990). The aminopeptidases from these two strains had the same optimum pH and temperature, 7.0 and 40°C respectively and both had broad specificity, degrading dipeptides as well as amino-acyl chromophoric substrates. The aminopeptidase from the strain Wg2 did not hydrolyse dipeptides containing N-terminal alanine, phenylalanine or proline. However oligopeptides containing 3-6 alanyl residues were substrates suggesting a preference for the hydrolysis of peptides with more than two amino acids. The enzyme from the Wg2 strain was a monomer with a molecular weight of 95kDa while that from the AM2 strain had a molecular weight of 300 kDa and was reported to be a hexamer. The Wg2 aminopeptidase was inhibited by metal chelating agents such as EDTA and 1,10-phenanthroline while the AM2 enzyme was not. Both enzymes were inhibited by thiol inhibitors. The AM2 enzyme also was inhibited by Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) an inhibitor of certain serine proteases.

The properties of the aminopeptidases from these lactococcal strains and from other lactic acid bacteria are summarized in Table 1.1. It is evident that aminopeptidases with broad specificities are common in lactic acid bacteria.

1.3.4. Aminopeptidase A

The "general" aminopeptidases so far purified have had very low or no hydrolytic activity towards substrates with N-terminal acidic amino acids, i.e. aspartyl and glutamyl residues. An exception to this seems to be the *L.lactis* AM2 aminopeptidase (Neviani et al.,1989) which had a hydrolysing activity for glu-β-naphthylamide (β-NA) of 80% relative to that obtained for lys-β-NA.

An aminopeptidase A was purified from *L.cremoris* (Exterkate & de Veer,1987) it hydrolysed N-terminal glutamic and aspartic residues. It was thought to be a trimer with a relative molecular weight of 130,kDa. The enzyme was completely inhibited by chelating agents, Cu and dithiothreitol. Another aminopeptidase A has recently been characterised from *L.lactis* NCDO 712 (Niven,1991). It was specific against N-terminal aspartyl and glutamyl residues and was also active against acidic tripeptides. It had a reported molecular weight of 245kDa and was a hexamer and a metalloenzyme.

The Aminopeptidase A may be significant in taste development of cheeses as glutamate salts are thought to have flavour-enhancing properties (Kirmura et al.,1969). In view of the high content of aspartyl and glutamyl residues in β-casein (10% of the total residues) and the low activity of the general aminopeptidase towards peptides with N-terminal acidic residues, the aminopeptidase A would be needed to ensure the degradation of casein into amino acids for continued cell growth.

1.3.5. Dipeptidases:

One of the earliest reports of the purification and characterization of a dipeptidase was that of a dipeptidase from *Streptococcus thermophilus* 160 (Rabier and Desmazeaud,1973). The dipeptidase preferentially hydrolysed dipeptides that had an N-terminal hydrophobic amino acid; it would not hydrolyse dipeptides if a proline was in the first or second position.

Another dipeptidase was purified from *L.cremoris* H61 (Hwang et al.,1981). The specificity was broad but the enzyme was not active against dipeptides with N-terminal proline or glycine or with tripeptides or CBZ-peptides (carbobenzoxyl-peptide).The specificity of this dipeptidase was further characterized by kinetic studies (Hwang et al.,1982).The substrates hydrolysed could be divided into three groups. Neutral dipeptides such as leu-gly, leu-ala had Km values of 4-6.6mM. Group two dipeptides which had aromatic amino acids at either the N- or C- position such as leu-phe,phe-ala, leu-tyr had lower Km values of 1-2.4 mM. Group three dipeptides with acidic or basic amino acids at the N-terminal such as his-ala, glu-val had higher Km values of 10-20mM

van Boven et al. (1988) purified a dipeptidase from *L.cremoris* Wg2 which was strongly inhibited by thiol reducing reagents such as DTT and mercaptoethanol, but the enzyme was not inhibited by sulphydryl reagents. It was active towards dipeptides with hydrophobic and neutral N-terminal amino acids but inactive against dipeptides containing pro,his,glu or gly. Tri- or larger peptides were not hydrolysed.

Current experimental data suggests that dipeptidases are produced both extracellularly and intracellularly (Law, 1979). The studies on purified dipeptidases suggest that a single enzyme with a broad specificity is responsible for hydrolysis of most dipeptides. However, there are specific enzymes for the hydrolysis of proline-containing dipeptides (Section 1.3.7.).

1.3.6. Tripeptidase:

Tripeptidase activity was established by Kaminogawa et al. (1984) to be distinct from either di- or amino-peptidase activity. Using 11 strains of *L.lactis* the cell-free extracts were partially purified on DEAE cellulose. A tripeptidase with an apparently broad specificity, and a neutral pH optimum was found in all strains. Tripeptidase activity was completely inhibited by metallo-agents such as EDTA and 1,10-phenanthroline.

As in the case with dipeptidase there is some evidence that the intracellular tripeptidase exists as a distinct type differing from the extracellular tripeptidase.Using polyacrylamide gels Kolstad and Law, (1985) found a cell wall tripeptidase fraction which had a similar specificity to the intracellular tripeptidase but ran at a lower mobility on the gels.

Recently a tripeptidase was purified from *L.cremoris* Wg2 (Bosman et al.,1990). It was a dimer with two identical subunits of molecular weight 52kDa. It had an optimum temperature of 55^{0}C and an optimum pH of 7.5. The specificity was broad in that it could hydrolyse all tripeptides except those with proline as the second amino acid. Dipeptides,tetrapeptides or oligopeptides were not hyrolysed. Only the N-terminal amino acid of the tripeptide was cleaved so for complete hydrolysis of tripeptides the combined action of a tripeptidase and a dipeptidase would be required. EDTA inhibited the enzyme while sulphydryl group reagents such as pCMB, mersalyl, pCMBS (p-chloromercuribenzene sulphonate) stimulated the activity two-fold. Reducing agents such as DTT and mercaptoethanol strongly inhibited enzyme activity. The tripeptidase was therefore a metalloenzyme that was only active in the oxidised state. As yet there is no data available on tripeptidases in other species of lactic acid bacteria.

1.3.7. Proline-specific Peptidases:

β-casein has an unusually high proline content ;18% in contrast with 5-6% in most globular proteins. The peptidases described above are not able to hydrolyse bonds involving proline residues. This has lead to a search for starter peptidases that act specifically on proline-containing peptides which would be important in the degradation of casein. The degradation of proline-containing peptides is also relevant to the bitter defect often found in cheeses. This defect has been attributed to peptides derived from casein containing a high proportion of hydrophobic groups, in particular, proline residues (Sullivan & Jago,1972).

At least five types of peptidases showing specificity for bonds containing proline have been reported from lactobacilli and lactococci.

aminopeptidase P	X↓Pro-Y....
proline iminopeptidase	Pro↓X....
iminodipeptidase (prolinase)	Pro↓X
imidopeptidase (prolidase)	X↓Pro
dipeptidyl-peptidase	X-Pro↓Y....

Mou et al.(1975) fractionated extracts from lactococci on a Sephadex G-200 column and found a proline iminopeptidase, a proline iminodipeptidase and an aminopeptidase P in *L.cremoris* SK11, *L.lactis* C2 and *L.diacetylactis* DRC1. The presence of proline iminopeptidase in conjunction with the aminopeptidase P could lead to the complete hydrolysis of any proline-containing peptides. However there has been relatively little further work on either of these peptidases in lactococci. The purification of a proline iminopeptidase was reported in a recent conference proceedings (Baankreis & Exterkate,1990) but details have not yet been published.

The most thoroughly studied of the proline-specific peptidases is the X-prolyl dipeptidyl aminopeptidase, catalysing cleavage of N-terminal X-prolyl residues.This enzyme was first reported in a survey of 21 strains of lactic acid bacteria tested, using gel electrophoresis. It was distinguished as a different enzyme from proline iminopeptidase (Casey &Meyer,1985). X-prolyl dipeptidyl peptidase was subsequently purified and characterised in *Lactobacillus lactis* and *Streptococcus thermophilus* (Meyer & Jordi,1987). The enzymes from both organisms were dimers with the same molecular weight (165kDa) and were serine proteases. The *Lb.lactis* enzyme was also sensitive to SH-blocking agents although the *S.thermophilus* enzyme was not. Both enzymes were insensitive to metal-chelating agents. Both enzymes were specific for peptides containing proline at the second position. X-prolyl dipeptidyl peptidases have now been thoroughly characterised from a range of lactococcal strains (Meyer,1987; Kiefer-Partsch et al.,1989; Zevaco et.al.,1990 ;Lloyd & Pritchard,1991).

All X-prolyl dipeptidyl aminopeptidases so far characterised have been dimers with identical subunits and a molecular weight of 160-180kDa. They are serine protease types and are not affected by metallo-enzyme inhibitors. All specifically degrade X-pro-Y·· peptides although the enzymes also show low activity with X-ala-Y··· peptides.

Recently, with the cloning and DNA sequence analyses of the X-prolyl dipeptidyl aminopeptidase from *L.lactis* NCDO 763 (Nardi et al.,1991) and *L.cremoris* P8-247 (Mayo et al.,1991), more information about the enzyme has been obtained. It was found that amino acid sequences of both enzymes showed a high degree of homology. Both enzymes had 763 residues of which only 7 residues were different in the two strains, indicating that the enzyme was highly conserved in *Lactococcus* strains. Because of inhibition of the enzymes by serine protease inhibitors it was expected that it would have some degree of homology with the serine protease family. However there was no sequence homology with any of the known serine proteolytic enzymes even in the region of the active site.This result suggested an early evolutionary divergence for the X-prolyl dipeptidyl aminopeptidase from the serine protease family.

The only other proline-specific peptidase characterised from lactococcal strains is a prolidase catalysing hydrolysis of dipeptides with a proline at the C-terminal position. Prolidases have been purified from *L.cremoris* H61 by Kaminogawa et al.(1984) and from *L.lactis* AM2 by Booth et al. (1990).In both cases the enzyme was shown to have a molecular weight of 42-45 kDa, to be sensitive to metal-chelating inhibitors and to be active against a wide range of X-Pro dipeptides.

Strain	Reference	Optimum temperature °C	Optimum pH	Type of Enzyme[a]	Mw(10^3) mono/native	Reported Location	Specificity[b]
L.cremoris Wg2	Tan&Konings 1990	40	7.0	metallo sulphydryl	95	Not determined	Broad,not dipeptides with pro ala phe
L cremoris AM2	Neviani at al. 1989	40	7.0	sulphydryl TLCK	50/300	Intracellular	Broad except pro
L.cremoris AC1	Geis et al. 1985	40	7.0	metallo	36	Cell wall	lys,leu ala ala-ala
S thermophilus 160	Rabier & Desmazeaud 1973	35	6.4	metallo	62		All pNA except phe or dipeptidespro,phe,gly,his
L.diacetylactis 267	Desmazeaud & Zevaco 1979	35	6.5	metallo sulphydryl	85		Broad
Lb.casei NCDO151	El Soda et al. 1978	45	6.5	metallo	---	Intracellular	Broad
Lb.lactis 1183	Eggimann & Bachmann 1980	47.5	6.2-7.2	metallo	78-81	Surface bound	Broad prefer N-terminal aromatic or basic
Lb.helveticus CNRZ 32	Khalid&Marth 1990	45	6.5	metallo sulphydryl	97	Intracellular	Broad not pro

Table 1.1: General aminopeptidases found in lactic acid bacteria.

[a] Metallo enzymes are inhibited by agents such as EDTA or 1,10-phenanthroline Sulphydryl inhibition is produced by sulphydryl blocking agents p-chloro-mercuribenzoate, iodoacetate or iodoacetamide, TLCK, is a serine and thiol protease inhibitor.

[b] Broad specificity = the general aminopeptidase acts on amino acyl-derivatives, certain di-and tri- peptides. It does not show carboxypeptidase or endopeptidase activity.

1.4. Location of Peptidases:

Establishing the cellular location of the various peptidases is essential for understanding their role during starter growth in milk and also for their possible contribution to the ripening and maturation of cheese. Despite many attempts, few of the above mentioned peptidases have been unequivocally assigned a cellular location.

Using conventional cell fractionation methods it is often difficult to establish that the extracellular activity is not due to the release of intracellular peptidases through lysis of the cell. In many cases where a cellular location is claimed, adequate evidence using appropriate marker enzymes or other cellular components is not presented.

It is important that the techniques used in establishing the cellular location of the peptidases satisfy rigorous criteria since the enzyme can appear in different cellular fractions depending on the fractionation method used. Difficulties are often encountered in preparing clean cell fractions of lactic acid bacteria. Even in the cases where no intracellular marker enzymes were released it is possible that either solubilisation of the cell wall was limited or that lysis did occur and the marker used was unstable under the experimental conditions. To overcome these problems, release of marker enzymes such as aldolase and lactate dehydrogenase should be followed in every experiment. Other techniques such as microscopy can also be used to detect if lysis has occurred.

Since many of the oligopeptides produced by the cell wall proteinase are too large to enter the cell (Smid et al.,1991; Rice et al.,1978) it seems logical that the cell has extracellular peptidase(s) to break down the large peptides into smaller fragments which can then be transported across the cell membrane.

Some studies of the general aminopeptidase have suggested an extracellular location (Geis et al.,1985; Tan & Konings,1990). An aminopeptidase from *L.cremoris* AC1 (Geis et al.,1985) was suggested as being located at the outer boundary of the bacterial cell wall on the basis of the observation that the enzyme was extracted by gentle washing of the cells, under conditions where no lysis of the cell could be seen by microscopy and the level of the cytoplasmic marker phospho-β-galactosidase in the extract was low. However, no measurement of total aminopeptidase activity was reported so it is not known whether the released enzyme was only a small proportion of the total activity present. Similar evidence was cited for the aminopeptidase from *L.cremoris* Wg2 (Tan & Konings, 1990) but again no quantitative data was given. The aminopeptidase from *L. cremoris* AM2 (Neviani et al.,1989), from the purification method used, appeared to be intracellular.

Current data suggest that dipeptidases are produced both extracellularly and intracellularly (Law, 1979).The dipeptidase activity released from the cell wall in lysozyme-treated cells of *L.lactis* and *L.cremoris* differed from the dipeptidase found intracellularly. All dipeptidases were inhibited by EDTA but reversal of this inhibition was accomplished by different metal ions depending on the location of the enzyme. Intracellular dipeptidases were reactivated by Co^{2+}, Mn^{2+} and to a lesser extent by Zn^{2+} while dipeptidases released from whole cells by lysozyme were reactivated only by Mg^{2+}. Mercaptoethanol completely inhibited the internal dipeptidases from *L.cremoris* but did not affect the extracellular dipeptidase (Law, 1979).

Most reports on the X-prolyl dipeptidyl aminopeptidase agree on an intracellular location, although an extracellular location had been postulated (Kiefer-Partsch et al.,1989). With the cloning and subsequent sequencing of the X-prolyl dipeptidyl aminopeptidase the location was tentatively placed as intracellular. The N-terminal sequence of the purified enzyme was found to be identical to the sequence predicted by DNA sequence analysis, indicating that the enzyme had not undergone any post-translational modification at the N-terminus and it was not exported through the processing of a signal peptide (Nardi et al.,1991).

More work in this area is needed before an unequivocal assignment of cellular location of peptidases in starter bacteria can be made.

1.5. Transport:

Transport of amino acids and peptides in lactic acid bacteria is usually an active process that requires a source of energy; electrochemical, ATP or a pH gradient. However some amino acids can enter the cell by passive diffusion (Marshall and Law, 1984). Active transport of free amino acids and external peptides ensures that a high level of essential amino acids is maintained within the cell. Lactococci possess separate transport systems for amino acids, di /tripeptides and for oligopeptides (Smid et al.,1989).

Four types of amino acid transport system have been identified in lactococci: (Konings et al.,1989)

1) Uptake of amino acids coupled to a proton motive force. The amino acids met, leu,ile,val, ser,thr,ala,gly and lys are transported in this way.
2) Transport coupled to phosphate bond energy where ATP or a derived metabolite drives the transport. Glu,gln,asp and possibly asn are transported in this manner.
3) Exchange transport e.g. where arginine uptake is driven by ornithine excretion.
4) Free proline is very slowly taken up by passive diffusion (Smid & Konings,1990).

Peptide transport was at one stage thought to be dependent on peptide hydrolysis. However Smid et al.(1989a) using membrane vesicles of *L.lactis* with an intact pmf-generating system, showed that dipeptide transport occurred in the absence of peptidase activity. There was, however, slow dipeptide transport in de-energised cells. This was thought to occur via a secondary transport system driven by a chemical gradient, generated as a result of the degradation of the peptides inside the cell.

Dipeptide transport is an essential component of casein utilization by the bacteria during growth (Smid et al., 1989b). *L.lactis* ML3 mutants with a deficiency in di- and tripeptide transport were unable to grow on a chemically defined medium with casein as the sole nitrogen source, whereas growth could be restored by the addition of free amino acids. *L. lactis* could not accumulate free proline, therefore this amino acid must enter the cell as a peptide and be hydrolysed within the cell to give free proline.

L.lactis ML3 possesses two different peptide transport systems:

1) A proton motive force-dependent peptide carrier which has an affinity for di- and tripeptides but not for peptides containing more than three amino acids (Smid et al., 1989a).
2) A metabolic energy-dependent oligopeptide transport system with an affinity for peptides with 2-6 amino acids.

This oligopeptide transport system has a much lower activity than the di- tripeptide transport system. Oligopeptides containing proline or glutamic residues are not utilised by this system indicating that the oligopeptide transport system has a narrow substrate specificity (Smid 1991).The oligopeptide transport system is not able to transport peptides containing more than six amino acids confirming an earlier finding by Rice et al. (1978). This upper size limit implies that extracellular peptidase activity must be present since the oligopeptide products of the cell wall proteinase are considerably larger than hexapeptides (see Figure 1.2)

1.6. Objectives of the Present Study:

The aim of the present investigation was to make a comparative study of the "general" aminopeptidase from different starter bacteria with particular reference to its specificity.

As discussed earlier the published studies of this enzyme in starter bacteria revealed some differences between the aminopeptidases from different strains with respect to such properties as molecular weight, subunit composition, specificity and sensitivity to inhibitors. An aminopeptidase has previously been partially purified from *L.lactis* H1 in this laboratory (Lloyd, 1989) but the preparation was contaminated by endopeptidase activity and traces of other peptidases precluding definitive studies of its properties. It proved to be an unstable enzyme resulting in low yields following purification.

The immediate aims of this project were to purify and characterise a "general" aminopeptidase from *Streptococcus thermophilus* and to compare and contrast the purified aminopeptidase with those purified from strains of *Lactococcus lactis* subsp.*cremoris* and *Lactococcus lactis* subsp.*lactis* .

The aminopeptidase from *S.thermophilus* was chosen for initial study since preliminary work showed it to be more stable then those from the lactococci. *S.thermophilus* is used in the manufacture of yoghurt and hard cheeses; despite this very little investigation has been carried out on its proteolytic system.

The specific aims of this project were:

a) To develop a suitable purification protocol..

b) To characterise the general properties of the aminopeptidase.

c) To determine the specificity of the enzyme towards different synthetic substrates and peptides including oligopeptides from casein produced by the activity of the proteinase

and d) To compare the general aminopeptidase from *S.thermophilus* with that found in *L.cremoris* and *L.lactis*.