A Comparative Study of an Aminopeptidase from Lactic Acid Bacteria

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Robyn Gillian Midwinter

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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.

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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.

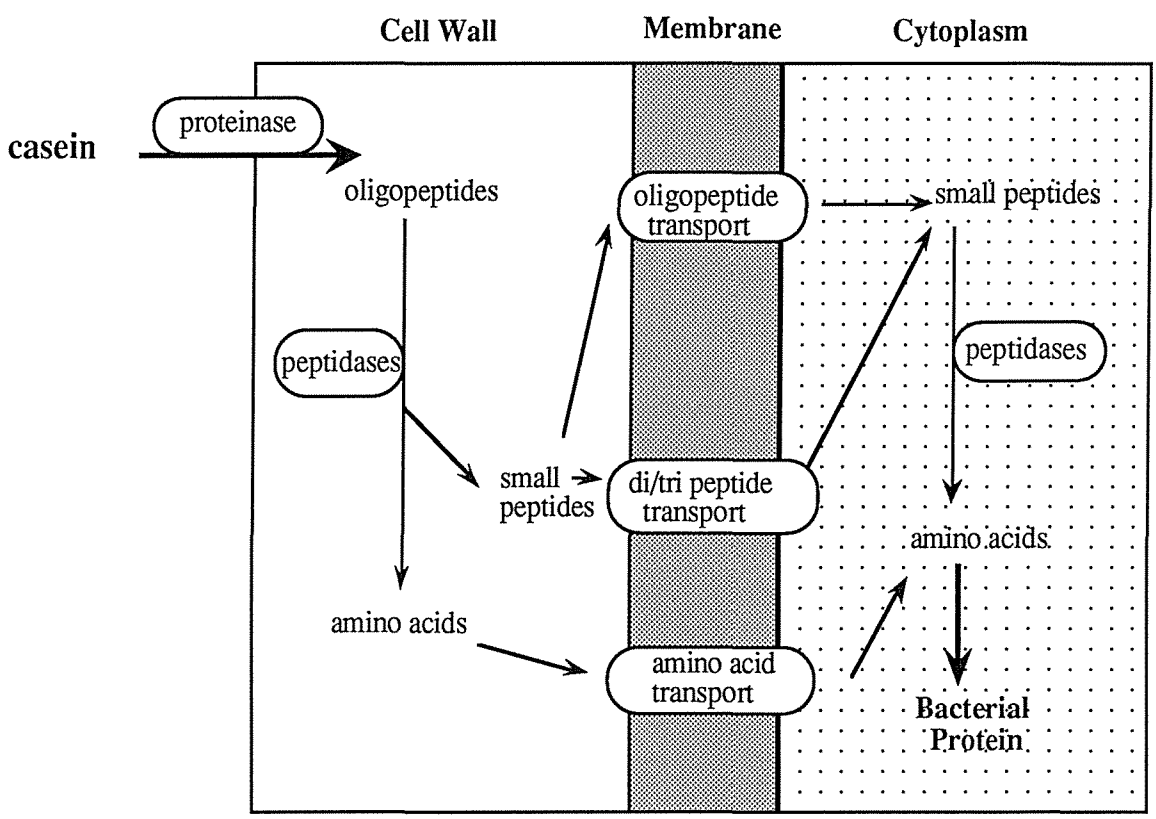


Figure 1.1: Proposed utilization of casein for growth of lactococci in milk.

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.

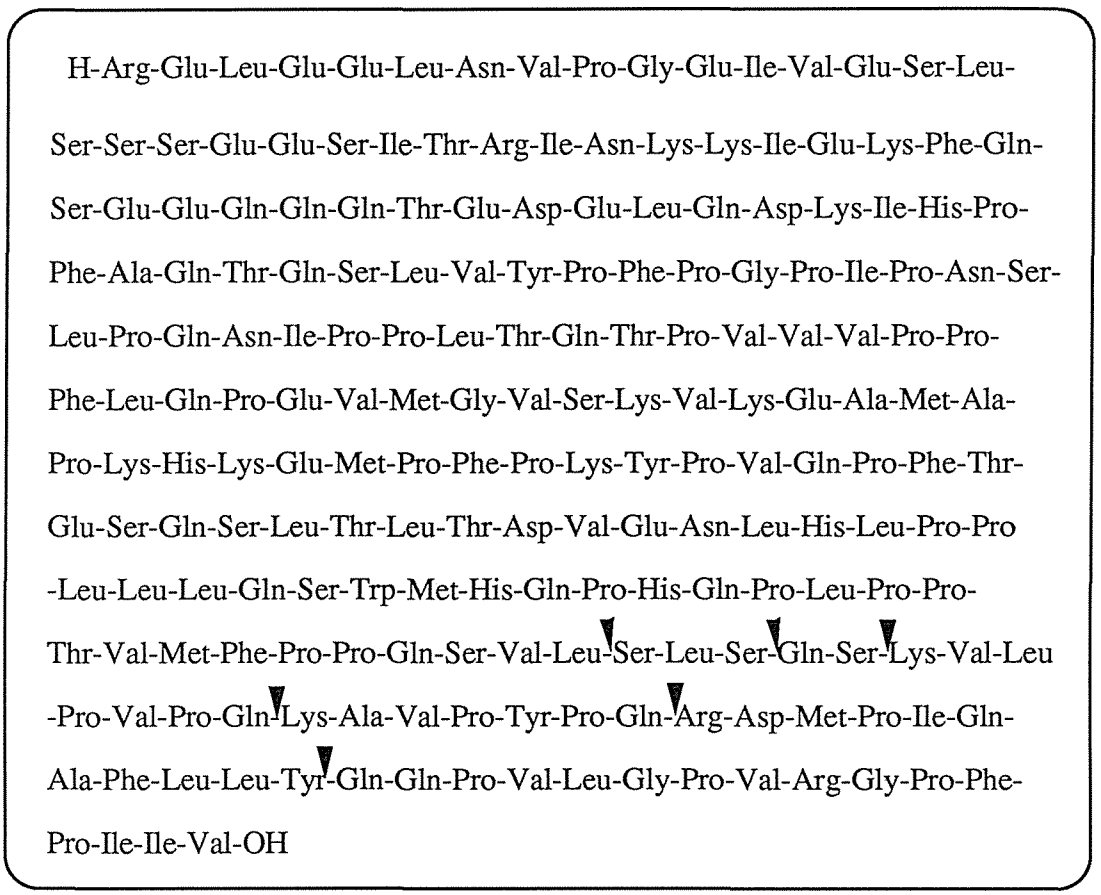


Figure 1.2: Sequence of β-casein showing sites at which β-casein is initially cleaved by the cell wall proteinase from several different strains of lactococci (see text).

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 55^0C 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*.

Chapter 2 Materials and Methods

2.1 Materials:

2.1.1 Bacterial Growth Media:

Yeast extract was obtained from Gibco laboratories; beef extract from BBL USA; Bio-trypticase from bioMérieux; D(+) lactose from Riedel-de-Haën. Low heat skim milk powder was provided by the New Zealand Dairy Research Institute and sodium β-glycerophosphate was from Sigma Chemical Co USA.

2.1.2 Substrates:

All peptides were supplied by Sigma as were all the p-nitroanilides except for L-phenylalanine pNA which was supplied by Bachem Inc. Amino-acyl AMC compounds were obtained from Sigma, or prepared from the BOC-amino acid derivatives obtained from Sigma (Lloyd, 1989). AMC was prepared by the Separation Science Unit, Massey University.

2.1.3 Chromatographic resins:

Diethylaminoethyl (DEAE) cellulose was supplied by Whatman Biochemicals,England. Sephacryl S-300, arginine-Sepharose 4B and Sephadex G-25 were obtained from Pharmacia.

2.1.4 Miscellaneous Materials:

NADH, SDS and ammonium persulphate were supplied by BDH Biochemicals, FBP, mutanolysin, lysozyme, Bis-Tris-Propane, and 2[N-Morpholino] ethanesulphonic acid (MES) were obtained from Sigma. Low molecular weight markers were from Bio-Rad.Acrylamide was supplied by Serva.
All other reagents were, wherever possible, of analytical grade.

2.2 Methods

2.2.1 Bacterial Strains:

The following strains of lactococci were obtained from the New Zealand Dairy Research Institute Palmerston North.

Lactococcus lactis subsp.*lactis* 4125 (a plasmid free strain of *L.lactis* spp *lactis* H1)

Lactococcus lactis subsp.*lactis* 4760 (A transconjugate strain derived from *L.lactis* 4125 into which, the lactose/proteinase plasmid from *L.lactis* H2 has been transferred by conjugation).

Lactococcus lactis subsp.*lactis* ML3

Lactococcus lactis subsp.*cremoris* H2 4409

Lactococcus lactis subsp.*cremoris* 4590

Lactococcus lactis subsp.*cremoris* SK11

Lactococcus lactis subsp.*cremoris* AM2

Streptococcus salivarius subsp.*thermophilus* 5109

2.2.2. Media

a) Lactose broth medium.

Lactose 10g	Magnesium sulphate 0.1g.
Peptone 5g	Manganese chloride 0.025g
Yeast extract 5g	
Beef extract 1g	
Potassium dihydrogen phosphate 2.5g	

This medium was made up to 500ml in deionised (Milli Q) water, autoclaved at 15psi for 15 minutes and stored at room temperature.

b) Reconstituted skim milk powder (RSM).

Low heat skim milk powder 30g was thoroughly mixed to a paste. After dilution to a suitable viscosity it was thoroughly mixed by blending for 10 seconds at high speed in a homogeniser. It was then made up to a final volume of 300ml with deionised (MilliQ) water and autoclaved at 10psi for 15 minutes.

To avoid coagulation of the medium by the lactic acid produced during culture growth, the RSM medium was buffered by adding 3ml of sterilised 2.5M sodium β-glycerophosphate per 100ml of media.

2.2.3. Growth of Bacteria:

Bacterial strains were maintained as milk cultures grown on RSM to the point of coagulation and stored at 4°C (short-term) or at -70°C (long-term). Short-term cultures were subcultured at monthly intervals.
In the case of *L. lactis* 4125, which lacks the lactose/proteinase plasmid, milk cultures were supplemented with 0.5% glucose and 0.2% trypticase.

2.2.4.Cultures grown on milk medium:

For preliminary studies cells were grown in 100ml of RSM media. The medium was buffered with Na-β–glycerophosphate and inoculated with 2ml of milk culture for every 100ml of medium. The culture was then incubated at 22°C overnight. The cells were harvested when the pH of the medium fell below 5.5 (approximately 14-16 hours). To harvest the bacteria the pH of the medium was brought to 7.0 by titrating with 1M NaOH. 6ml of 25% Na citrate per 100 ml were added and the culture was left to stand for 15 minutes in ice. This procedure complexed the Ca^{2+} and dispersed the protein micelles present in the milk.The culture was then centrifuged at 10,000g for 10 minutes at 4°C. The supernatant was discarded and the cells resuspended in 20ml of 0.05M phosphate buffer (pH 7.0) before recentrifugation. This wash was repeated and the pellet stored at -20°C until needed.

2.2.5.Cultures grown on lactose medium.

Broth cultures were grown in a 3 litre fermentor (LH Engineering Co.) at 30°C (37°C for the *S.thermophilus*) gassed with 5% CO_2 in nitrogen and maintained at pH6.0 by automatic addition of 2.5 M NaOH. The culture was stirred at 150 rpm.
The medium was inoculated with 40 ml of a 6 hour broth-grown culture. The cells were harvested when the culture had just reached the stationary phase, usually after 6 hours of growth.
The cells were harvested by centrifugation at 10,000g for 10 minutes. The supernatant was discarded, the pellet washed in 20 ml of 0.05M Na_2HPO_4/KH_2PO_4, pH 7.0 (subsequently referred to throughout this thesis as phosphate buffer) and recentrifuged.
For large scale purifications the cells were grown in 35-40 litre batches in a Fermacell Fermentor (New Brunswick Scientific Co U.S.A.) at the Biochemical Processing Centre DSIR, using the same conditions as for the smaller cultures. These cells were harvested in a continuous flow centrifuge and washed with cold phosphate buffer. The yield from both methods was approximately 10g of wet packed cells per litre of medium.
The cell pellet was stored at -20°C until needed.

2.2.6. Preparation of a cell free extract:

The frozen cell pellet was slowly thawed and resuspended in 40ml of cold 20mM phosphate buffer per 30g wet packed weight of cells. The cells were then disrupted by a single pass through a cooled French Press at a pressure of 38MPa. The homogenate were centrifuged at 23,500g for 15 minutes, and the supernatant decanted. The pellet was resuspended in a further 10 ml of phosphate buffer, and the supernatant pooled with that obtained previously. The combined supernatant was called the cell-free extract.

2.2.7. Enzyme assays:

i) Aminopeptidase assays.

Aminopeptidase activity was assayed routinely using amino acyl p-nitroanilides. The p-nitroaniline released by enzymatic hydrolysis produces a yellow colour that can be followed spectrophotometrically by an increase in absorbance at 405nm.

Peptidase activity towards p-nitroanilide (pNA) substrates was quantitatively measured using the following assay mixture. Measurements were carried out at room temperature in a 1ml cuvette with a pathlength of 1cm.

100mM MES buffer pH 6.8	0.85ml
Substrate 5mM	100µl
Sample	50µl

1 unit of the enzyme activity is defined as the amount of enzyme producing 1µmole of pNA per minute in this system.

Routine assays were carried out at room temperature using a Cecil CE292 spectrophotometer at a wavelength of 405nm. Kinetic parameters were determined at 30°C using an Aminco DW2a double beam spectrophotometer. An ε value [molar absorptivity] for the nitroaniline at this wavelength was 9900 (Machuga & Ives 1984).

To establish that the assay procedure described above gave a linear dependence of enzyme activity on enzyme concentration, activity was measured at several different concentrations of the purified aminopeptidase. Figure 2.1 shows that the activity is linearly related to the amount of enzyme present in the assay mixture

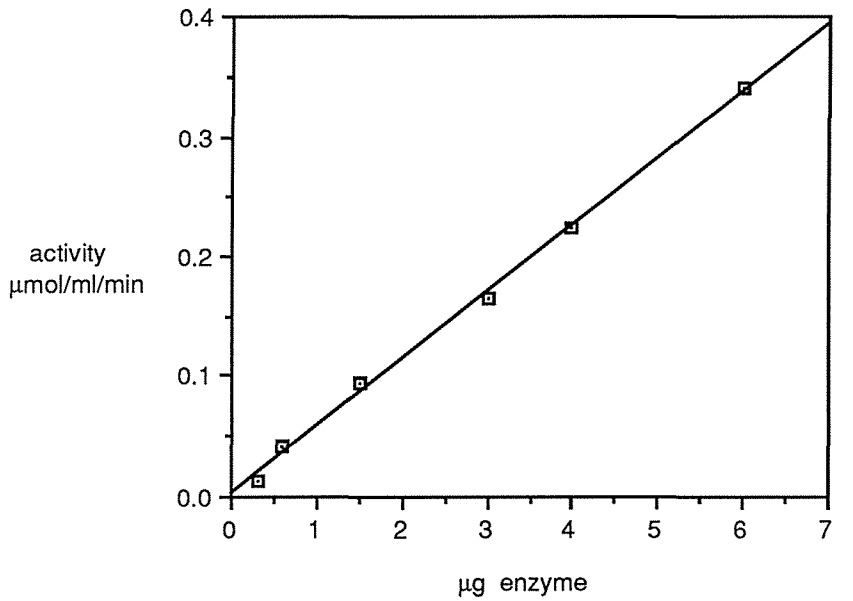


Figure 2.1. Linear dependenance of enzyme activity on enzyme concentration. Assay conditions are described in Section 2.2.7.

For rapid location of active fractions following column chromatography aliquots (100µl) of the column fraction and 50µl of 1mM lys pNA were mixed together in a micro titre plate.Where a yellow colour appeared a quantitative assay was used to define the amount of enzyme present in that fraction.

Amino-acyl derivatives yielding fluorescent products can also be used for assaying aminopeptidase activity. As fluorimetry is considerably more sensitive than spectrophotometry this method can be used to advantage when there are only small amounts of the enzyme available. The 7-amido-4-methylcoumarin (AMC) derivatives of amino acids have been widely used for peptidase assay (Kato et al.,1978).

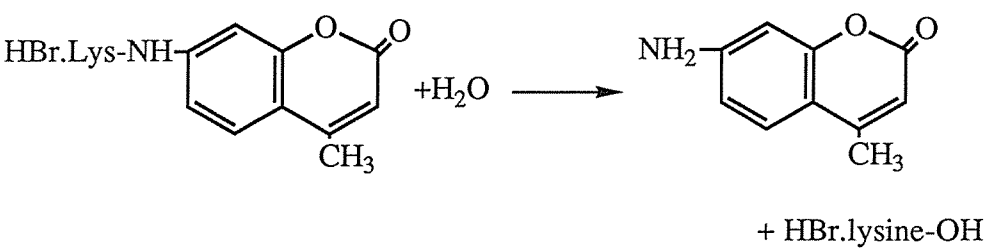


Figure 2.2 Hydrolyses of the Lys AMC substrate will produce 7-amino-4-methyl coumarin.

Aminopeptidase assays using amino acyl AMC derivatives were carried out using an Aminco SP500 Ratio Spectofluorimeter. The reaction mixture was the same as that specified above for p-nitroanilides. The relative fluorescence ratio in the reaction mixture was measured at an excitation wavelength of 385 nm and an emission wavelength of 456nm. To quantitate the liberation of AMC in the reaction mixture the fluorescence was standardised using a 100µM solution prepared from recrystallised AMC.

ii) Other enzyme assays:

ii a) Dipeptidase Assay:

The assay for dipeptidase activity was based on the fluorometric determination of amino acids using an o-phthaldialdehyde reagent containing mercaptoethanol as a reducing agent (Roth,1971). The fluorescence yield of most dipeptides with this reagent is considerably lower than that of the free amino acids (Taylor & Tappel,1973) enabling this method to be used to follow the rate of dipeptide hydrolysis.

In the present study dipeptidase activity was determined by measuring the rate of hydrolysis of various dipeptides using the following reaction mixture.

0.95 ml of 20mM phosphate buffer (pH 6.8)
500µl dipeptide substrate (5mM)
50µl of enzyme

A 200µl sample was removed from this mixture at timed intervals, added rapidly to 100µl of 0.1M acetic acid and 2.7ml of the o-phthaldialdehyde reagent was added. The o-phthaldialdehyde reagent was prepared as follows.

90ml borate buffer 0.05M pH 9.5
1.5 ml mercaptoethanol (5µl ml^{-1} in ethanol)
1.5 ml o-phthaldialdehyde (10mgml^{-1} in ethanol)

This was read immediately on the fluorimeter at emission 455nm using an excitation wavelength of 340nm. Activity was quantitated by means of a standard curve prepared from mixtures containing different ratios of dipeptide and amino acid.

ii b) Tripeptidase assays: Tripeptidase activity was measured in the same way as dipeptidase activity except that the substrate used was 1mM leu-gly-gly or 1mM ala-ala-ala.

ii c) Lactate Dehydrogenase assay:

The FBP dependendent lactate dehydrogenase (used as a cytoplasmic marker enzyme) was assayed by the method of Crow & Pritchard (1977) replacing triethanolamine buffer with MES. The following reaction mixture was used in a total of 3ml.

Buffer 0.05 MES pH 6.9	2.6ml
NADH 5mM	0.1ml
Na pyruvate 150mM	0.1ml
FBP 3mM	0.1ml
Sample	0.1ml

The reaction was followed by the decrease in adsorbance at 340 nm. One unit of activity was definied as the amount of enzyme that oxidised 1μmol of NADH per min.

ii d) Assays for enzymes and protein used as molecular weight standards to calibrate the Sephacryl 300 gel permeation column:

β–amylase: Mr 200,000

100μl of the sample to be assayed was incubated with 0.5ml of 0.1% starch for 15 minutes. 100μl of saturated I_2 solution in 5% KI was added and the samples diluted to 2 ml. The extent of starch hydrolysis was determined from the decrease in absorbance at 600nm.

Alcohol Dehydrogenase: Mr 150,000

10μl of the sample was added to a mixture of 100μl of 0.1% (v/v) acetaldehyde in 100mM Tris/HCl (pH 7.0) 100μl of NADH 1mg/ml and 2ml of Tris/HCl (pH 7.0). Activity was followed by the decrease in absorbance at 340nm.

β-galactosidase: Mr 116,000

10μl of the fraction was added to 1ml of o-nitrophenyl β-D-galacto-pyranoside (3mg/ml) and 2ml of 100mM Tris pH 8.0. The activity was followed by measuring the increase in absorbance at 400nm.

Carbonic Anhydrase: Mr 29,000

100μl of the sample was added to a reaction mixture containing 0.5ml of p-nitrophenyl acetate (1mM) and 2ml 100mM tris buffer pH 8.0. The activity was followed by measuring the increase in absorbance at 400nm.

2.2.8 Protein Determination.

Protein was measured using the Coomassie Blue Dye-Binding method (Bradford 1976). 3ml of the reagent was added to 100µl of the sample and the absorbance read at 595 nm after 5 minutes (but before 1 hour). For a more sensitive assay 0.5 ml of the reagent was added to 100 µl of the sample. A standard curve was prepared using BSA.

2.2.9. Polyacrylamide Gel Electrophoresis:

The following stock reagents were prepared and stored at 4°C

30% Acrylamide:

Acrylamide	29.2g
Bis-acrylamide	0.8g
Milli Q water up to 100ml	

Running Gel Buffer (pH8.8)

1M HCL	40 ml
Tris	36.6g
Milli Q water up to 100ml	

Stacking Gel Buffer (pH6.8)

1M HCL	48 ml
Tris	5.98g
Milli Q water up to 100 ml	

Electrode Buffer (pH8.3)

Tris	12 g
Glycine	57.6 g
Milli Q water up to 4L	

Sample Buffer

10% Glycerol
0.0625 M Tris Buffer pH 6.7
4.2 ml water
5µl Bromophenol Blue

Mixtures used for preparation of 7.5% non-denaturing gels were as follows.

Running gel

30% acrylamide	12.7 ml
Running gel buffer	6.25ml
TEMED	50µl
Milli Q water up to	50ml
Ammonium persulphate	20mg

Stacking gel

30% acrylamide	1.7ml
Stacking gel buffer	1.25ml
TEMED	10µl
Milli Q water up to	10ml
Ammonium persulphate	5-10mg

Gels were prepared and run using a Pharmacia GE4 vertical slab gel apparatus and casting block.

Stain:

1% Coomassie Blue R-250 in water	12.5ml
Methanol	50ml
Acetic acid	10ml
Milli Q water up to	100ml

Destain 1

Methanol	500ml
Acetic acid	100ml
Milli Q water up to	1 litre

Destain 2

Methanol	140ml
Acetic acid	100ml
Milli Q water up to	2 litres

Samples for loading were mixed with an equal volumn of sample buffer.
Gels were run at a temperature of 15°C maintained by a water cooling system. The gels were run at 10mA until the sample had entered the stacking gel , when the current was increased to 20mA per gel until the dye band was 1cm from the bottom of the gel.

For SDS gels a final volume of 1% from a 10% stock solution of SDS was added to both the running and stacking gels and to the electrode buffer. To the sample buffer 3% SDS and 5% β-mercaptoethanol (final concentration) was added and the loading mixture was then boiled for 3 minutes.
The gels were stained for at least 2 hours in stain and then destained for 30 minutes in solution 1 followed by several washes with solution2.

2.2.9.High Performance Liquid Chromatography:

Separation of peptides and peptide digests was carried out on a Vydac 218 TP C18 reverse-phase column [10μ end capped silica 300 Å pore size with dimensions 250 x4.6mm] attached to a Philips PU4100 Liquid Chromatograph. Peptides were eluted with a linear gradient of water/acetonitrile in the presence of 0.1% trifluoroacetic acid. Details of the elution methods are given in the appropriate section of the results.
All solvents used were HPLC grade and filtered through a 0.45μm Millipore filter before use and degassed by sparging with helium.

2.2.10 Cell Fractionation.

The following procedure was used to study the cellular location of the aminopeptidase.
The cell pellet from a freshly harvested culture grown on RSM was resuspended in 30 ml of 50mM phosphate buffer pH6.4. This suspension was incubated at 30°C, in a water bath, for one hour to remove loosely associated cell wall proteins. The suspension was than centrifuged at 12,000g for 10 minutes using an SS34 rotor at room temperature. The supernatant was discarded.
The pellet was resuspended in 30 ml of 24% sucrose/10mM $MgCl_2$,then 36mg of lysozyme and 200μl (10,000 units per ml) mutanolysin was added. The mixture was incubated for 1 hour at 37°C then centrifuged at 20,000g for 10 minutes at 20°C.
The pellet was resuspended in 10 ml of cold 20mM Tris/HCL buffer (pH 7.0) and was incubated for 10 minutes at 37°C. 10ml of the Tris/HCL buffer containing 0.3mg of DNase (880 units mg) and 0.25mg of RNase (100 units mg) was added. The mixture was incubated for a further 30 minutes at 37°C. A further 10ml of the Tris buffer (pH7.0) was added. The mixture was incubated for another 10 minutes at 37°C and then centrifuged at 20,000g for 10 minutes at 4°C. The pellet was resuspended in 30ml of Tris buffer.
At each stage the supernatant was assayed for lys-aminopeptidase activity and for lactate dehydrogenase, which was used as an intracellular marker enzyme.

2.2.11.Amino Acid Composition and Sequence Analysis of Peptides.

Samples were dried down under vacuum. The dried peptides were than dissolved in 200µl of 6M glass distilled HCL containing 0.1% phenol and sealed in hydrolysis tubes under a vacuum of 0.1mm. Hydrolysis was carried out at 110 °C for 24 hours. The hydrolysis tubes were cracked and the contents were dried over P_2O_5 under vacuum. The hydrolysed peptide was dissolved in deionised water and loaded onto a Pharmacia LKB Alpha plus amino acid analyser. The analyser was calibrated with a Beckman calibration standard. Amino acid composition was calculated by peak-integration using a Spectra Physics 4290 integrator.

Amino acid sequences were obtained by the automated Edman method using an Applied Biosystems 470 A gas-phase protein sequencer. Any salt or phosphate buffer was removed from samples before application by repeated ultrafiltration of the sample in a Centricon 10 micro-concentrator. All sequencing programs included 3 cycles of prewash followed by the standard peptide sequencing program. PTH amino acid peaks were separated by direct HPLC and identified by retention time.

Chapter 3: Results.

3.1 Preliminary Studies:

Before investigating possible purification schemes for a "general" aminopeptidase some preliminary studies were carried out on various strains of the lactic acid bacteria, to compare the aminopeptidase activity found in the different starter strains and to determine the effect of different growth media on the level of aminopeptidase activity. It has been observed (Thomas &Mills,1981) that cells grown on complex media have much lower levels of proteinase activity than cells grown on milk. It was therefore decided to investigate the peptidase activity to see if it was similarly affected by the growth medium.

100ml batches of several strains of starter bacteria were grown in lactose broth and in RSM media. For the strain 4125 the RSM media was supplemented with 0.2% peptone and 0.5% glucose as strain 4125 lacks the lactose/proteinase plasmid.
Lysine p-nitroanilide (5mM) was used to assay for the "general" aminopeptidase activity in the cell-free extracts from all strains tested (prepared as described in section 2.2.6).

Organism		Reconstituted Skim milk	Lactose Broth
L.lactis	4760	0.04*	0.049
L.lactis	4125	0.05	0.02
L.cremoris	4409	0.18	0.13
L.cremoris	SK11	0.11	0.14
S.thermophilus	5109	0.038	0.029

Table 3.1 Specific activity of lys-aminopeptidase in cell-free extracts from several starter strains grown on different media.

* Activity is expressed as μmol of lys p-nitroanilide hydrolysed per minute per mg of protein.

A comparison of the specific activities in the cell free extracts (Table 3.1) suggests that the "general" aminopeptidase activity was similar irrespective of the growth medium used. The lactose broth media was therefore used in all subsequent experiments. An additional advantage of this medium is that large scale cultures could be grown more easily using the lactose medium. The data presented in Table 3.1 also shows that the specific activity of the

aminopeptidase was considerably higher in the two *cremoris* strains than in the *lactis* strains and in the*S.thermophilus*.

3.2 Purification of lys-aminopeptidase from *S.thermophilus* 5109:

The main aim of this project was to purify a "general" aminopeptidase (to be called lys-aminopeptidase throughout this thesis) and investigate its characteristics and possible role in the hydrolysis of the peptides produced by the proteinases.

Early investigations and more recent reports (Tan & Konings, 1989; Neviani et al.,1989) implied that lys-aminopeptidase was very unstable. In preliminary experiments attempts to decrease the loss of activity during the purification by the addition of 10-20% glycerol to the buffers to stabilize the protein were unsuccessful and produced a decrease in the flow rate of columns. Since many peptidases carry a metal ion at the active site it has been suggested that the loss of this essential factor during purification could be responsible for the rapid loss of enzymatic activity.
A series of metal ions (Co^{2+}, Zn^{2+}, Mg^{2+} and Ca^{2+}) over a range of concentrations (0.0125-2mM) were investigated in an attempt to improve the stability of the peptidase during purification. None of the metals showed any significant effect on stability; some (Co^{2+},Zn^{2+}) were inhibitory.

For large scale preparations,*S.thermophilus* was grown in a Fermacell fermentor in lactose broth.100 g of wet packed cells were unfrozen and mixed with (40ml) 20mM phosphate buffer (pH 7.0). Cells were then broken by a single passage through a cooled French press as described in section 2.2.6.

3.2.1 DEAE Cellulose Chromatography:

The cell free extract [20-40ml] was loaded directly onto a column of DEAE Cellulose (Whatman DE23), with dimensions 3x24 cm, which had been previously equilibrated with 20mM phosphate buffer pH7.0 until the pH and the conductivity of the effluent was the same as that of the running buffer. The column was washed with 100-200ml of the equilibration buffer to remove the unbound protein before the bound protein was eluted with a linear concentration gradient of 0-0.8M NaCl with a total elution volume of 400ml at a flow rate of 1ml min^{-1}. The elution profile is shown in Figure 3.1. Fractions of 5.4 ml were collected and tested for lys-aminopeptidase activity (section 2.2.7). The protein content of the fractions was followed by reading the absorbance at 280 nm, after dilution of the fraction. Fractions with high activity but low protein content were pooled for the next purification step. The aminopeptidase activity was eluted at ~ 12 mmho in a peak contaminated with dipeptidase activity (see Figure 3.1).

3.2.2 Sephacryl S-300 Gel Permeation Chromatography:

The fractions containing aminopeptidase activity were concentrated using an Aminco Diaflo concentrator with a PM30 membrane (cut off 30kDa), to a volume of 5-10ml. Assays of the filtrate showed that the peptidase was fully retained by this membrane.

The enzyme from the DEAE column purification step, in a volume of no more than 8ml, was then applied to a Sephacryl S-300 column (dimensions 2.6x90cm) which had been previously equilibrated with 20mM phosphate buffer (pH7). Elution was carried out with the same buffer at a flow rate of 0.3 ml min^{-1} and fractions of 4ml were collected. Fractions with the highest lys-aminopeptidase activity were combined. The Sephacryl step gave a 2 fold purification with up to 40% loss of the original activity (Table 3.2). Contrary to expectation, it was found that low molecular weight proteins were not adequately separated from the aminopeptidase fractions, possibly due to protein aggregates forming as a result of the high protein concentration of the applied sample in a relatively low ionic strength buffer (Figure 3.5).

3.2.3 Chromatography on arginine-Sepharose 4B:

A column of arginine-Sepharose 4B (Pharmacia) was used at this stage in the purification procedure in the hope that the lys-aminopeptidase, which had shown high activity towards lysine substrates, would bind specifically to the arginyl group. Arginine-Sepharose affinity chromatography had been successfully used to purify X-pro dipeptidyl peptidase from *Lactobacillus lactis* (Meyer & Jordi,1987) and from lactococci (Lloyd,1989).
The total pooled fractions eluted from the Sephacryl S-300 column were loaded on to an arginine-Sepharose 4B column (2cm x10cm) equilibrated with 20mM phosphate buffer pH7.0 containing 0.2M NaCl. The sample was brought up to the same molarity as the loading buffer before application. The column was washed with 60ml of loading buffer before the bound protein was eluted using a linear concentration gradient of 0.2-0.6M NaCl (in a total volume of 240ml). Fractions of 4ml were collected and the fractions with the highest lys-aminopeptidase activity were combined. The aminopeptidase peak activity was eluted at ~8.5 mmho (Figure 3.3).
The arginine-Sepharose was probably acting more as an ion exchange column retarding the elution of the aminopeptidase rather than specifically binding the enzyme by true affinity binding. Although spreading of the activity occurred, this method allowed the separation of lys-aminopeptidase from most other contaminating proteins and the large (30 fold) increase in purification warranted this step.
SDS-PAGE showed that the enzyme preparation after the arginine-Sepharose step still contained several minor contaminating bands (Figure 3.5). An FPLC step using ion exchange was used to remove these contaminating proteins.

3.2.4 FPLC on Mono Q:

The pooled fractions from the arginine-Sepharose column were desalted by applying to a 20ml column of G-25 Sephadex (2x6cm) equilibrated with 20mM Bis-Tris-Propane pH 7.0. The aminopeptidase was then concentrated to approximately 10ml using a Centricon 10 centrifugal concentrator (10kDa cut-off).

400µl aliquots of the enzyme were injected onto a high resolution Mono Q column HR 5/5 anionic exchanger [Pharmacia]. The aminopeptidase was eluted with a linear gradient from 20% to 100% B over 30 minutes at a flow rate of 0.5ml min^{-1}. Solvent A was 20mM Bis-Tris-Propane pH 7.0 and Solvent B was 20mM Bis-Tris-Propane pH 7.0 containing 1M NaCl. Peaks containing lys-aminopeptidase were collected and immediately cooled on ice (Figure 3.4).

This final FPLC step resulted in a preparation of lys-aminopeptidase which consisted of one major protein band on SDS-PAGE (Figure 3.5).

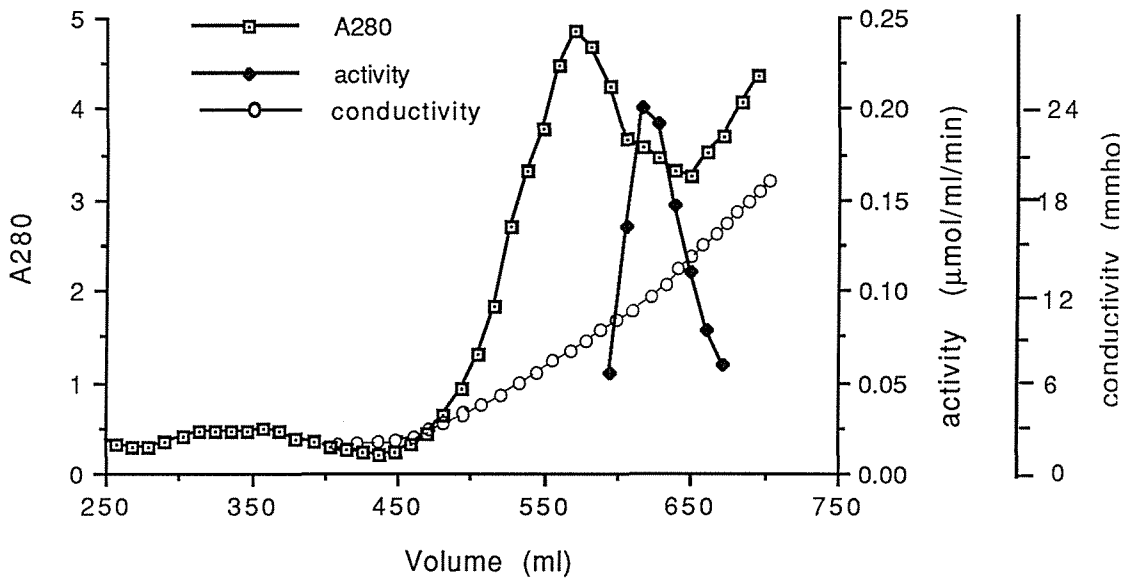


Figure 3.1. Elution profile of lys-aminopeptidase activity from *S.thermophilus* 5109 from a DEAE column.

The column was washed with 0.02M phosphate buffer pH 7.0 and eluted with a linear gradient of 0-0.8 M NaCl. Elution conditions as described in section 3.2.1.

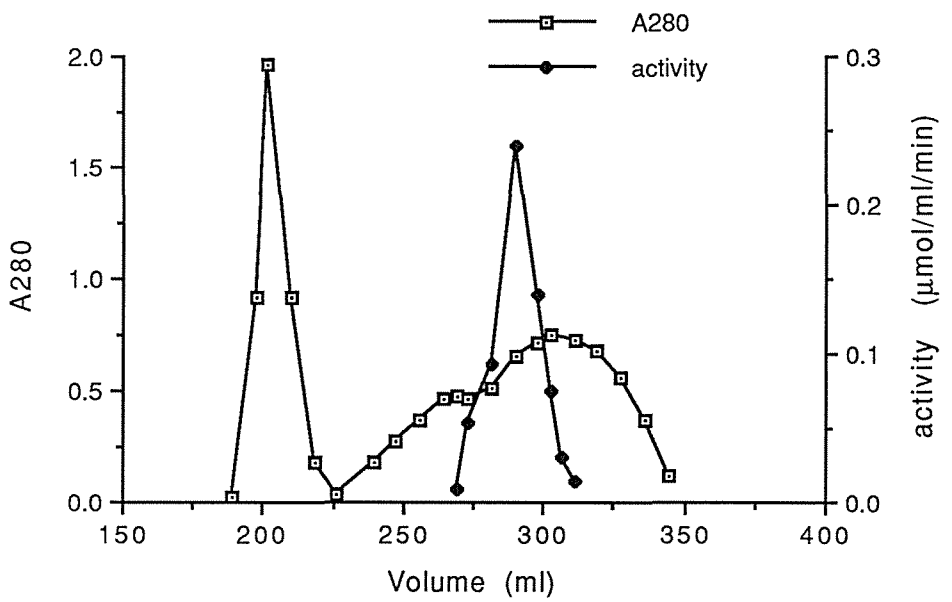


Figure 3.2. Elution profile of lys-aminopeptidase activity from a Sephacryl S-300 column.

The elution conditions are described in section 3.2.2

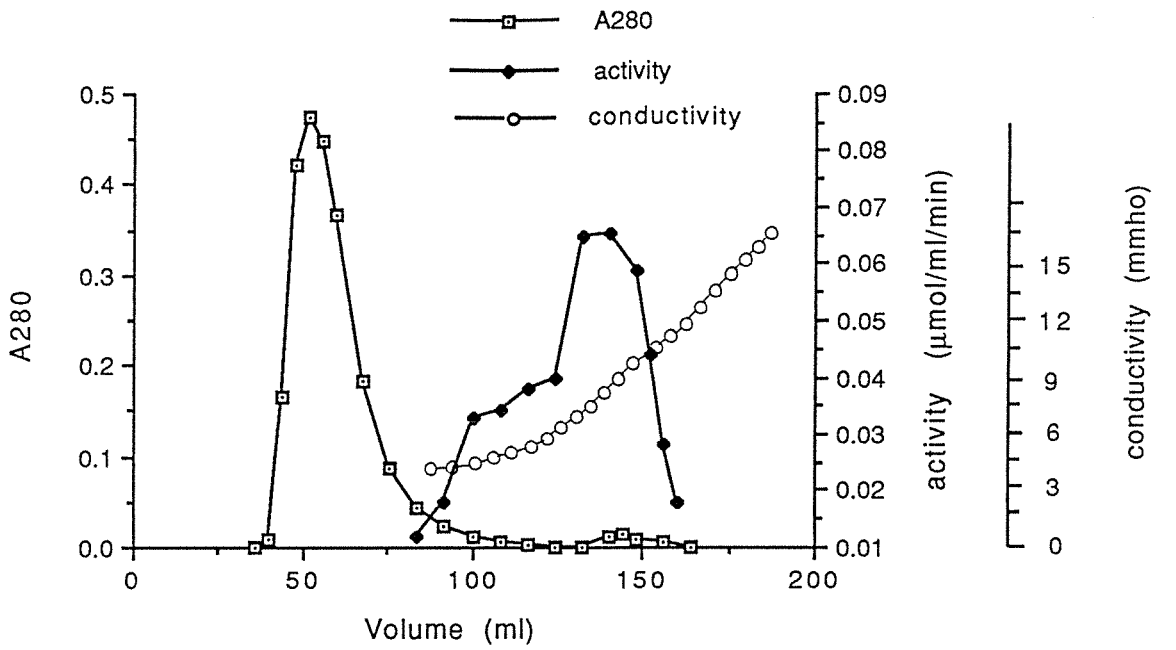


Figure 3.3. Elution profile of lys-aminopeptidase from an arginine-Sepharose 4B column. The column (dimensions 2x10cm) was washed with 20mM phosphate buffer pH 7.0 containing 0.2M NaCl. The aminopeptidase was eluted with a gradient of 0.2M-0.6M NaCl.

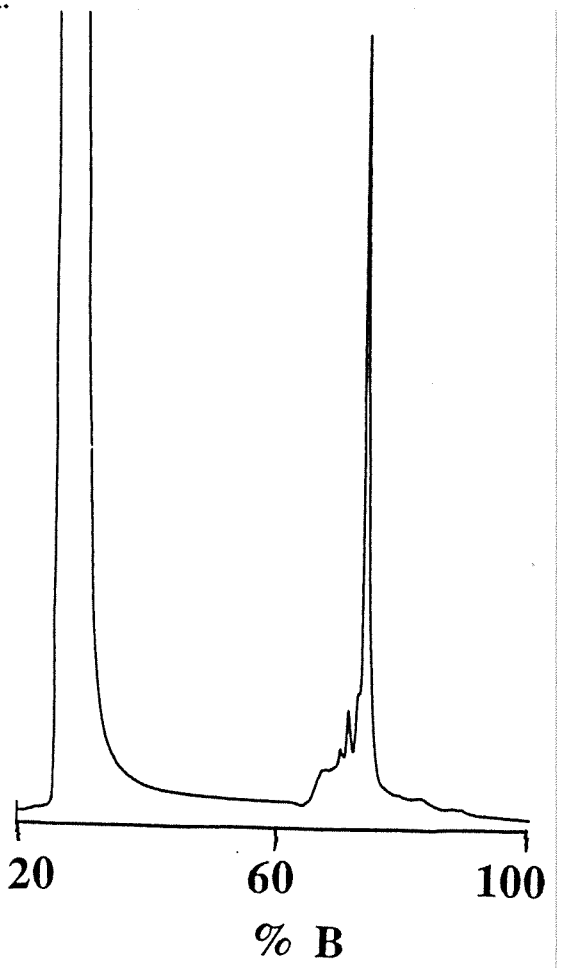
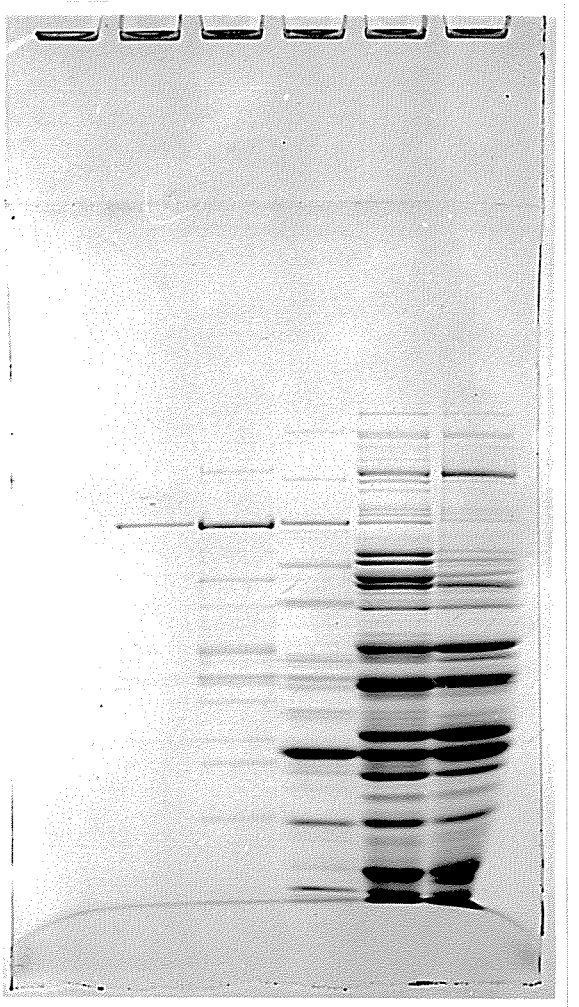


Figure 3.4 FPLC trace of the aminopeptidase run on a Mono Q anionic exchanager.



1 2 3 4 5

Figure 3.5. Photo of 7.5% SDS-PAGE showing the protein at each step from the purification of the lys-aminopeptidase for *S.thermophilus.*

Lane 1 FPLC sample 2 arginine-Sepharose sample 3 Sephacryl sample 4 DEAE 23 sample 5 cell-free extract of *S.thermophilus* 5109

3.2.5. Summary of the purification of lys-aminopeptidase from *S.thermophilus* 5109

The results of a typical purification protocol are shown in Table 3.2
A total of 0.2 mg of purified lys-aminopeptidase was obtained from 100g of*S.thermophilus* cells. An average final recovery of 7% of the original activity was achieved. The reliability of the protein determination at low protein concentrations was limited by the sensitivity of the Coomassie dye binding assay. Total purification values of greater than 300 fold were routinely accomplished in all large scale preparations.

An SDS polyacrylamide gel showing each stage of the purification is shown in Figure 3.5 Using this gel and a gel scanner attached to an integrator, the*S.thermophilus* lys-aminopeptidase was estimated to be better than 90% pure.

	Total Protein mg	Total Activity [a]	Specific Activity [b]	Recovery (%)	Purification Fold
CFE	1596	25.46	0.0159	100	1
DEAE	192.7	18.33	0.095	71	5.97
S-300	49.0	6.488	0.132	26	10
arg-Seph	1.26	6.0	4.77	24	300
FPLC	0.2	1.66	8.3	6.6	522

Table 3.2 Summary of the purification of lys-aminopeptidase from *S.thermophilus* 5109.
Quantities specified in this table are from 100g of wet packed weight of cells.
a)Total activity is expressed as μmoles of p-nitroaniline released per minute .
b) Specific activity is expressed as μmoles of p-nitroaniline released per minute per milligram of protein.

3.2.6 Investigation of Other Purification Methods.

Several attempts were made to find an alternative system for purification of the lys-aminopeptidase in the hope of cutting down purification time and enzyme activity loss.

Immobilised Metal Affinity Chromatography (IMAC):
IMAC was developed by Porath and co-workers in 1975 (Porath 1988). The method is not biospecific; proteins bind to an immobilized metal ion on a column matrix through easily accessible histidine, tryptophan and probably cysteine groups on the protein. Binding depends on the amino acid accessibility to the ions on the column. Selectivity can be accomplished by varying the type of metal ion on the column as well as the mode of elution. Zn^{2+} and Cu^{2+} are commonly used as the immobile ions. Elution is accomplished by changing the pH or ionic strength of the eluent or by using a stronger complexing agent such as glycine, or any combination of these. Elution can be made more specific by selecting a displacement compound similar to the structure of the metal binding site of the bound protein. Imidazole and histidine are such affinity displacers.

A small column containing 5ml of packed resin (Sepharose 6B with side arms of 0.5mmol imino diacetic acid per gram of dry weight of support) was supplied by Dr N.Haggerty of the Massey Separation Science Unit. The column was washed with 50mM EDTA to remove any ions, and then washed well with water. The column was washed with 0.4ml of 0.2M $CuSO_4$. This quantity was sufficient to load only the top part of the column allowing the unchelated resin in the lower half of the column free to rebind any Cu^{2+}that might be stripped off during elution.

The pooled fractions (20 ml, 0.18μmolml^{-1}min^{-1} in 20mM phosphate buffer pH 7.0) containing aminopeptidase activity from a DEAE column were applied to the Cu^{2+}column and eluted sequentially with the approximately 30 ml of each buffer;
(1) 20mM phosphate pH 7.0, (2) 25mM Na-acetate pH 6.5, (3) 50mM Tris/HCL pH 7.5, (4) 2mM imidazole pH 7.0 and finally 20mM imidazole. In the trials using this procedure, aminopeptidase activity was eluted in the Tris wash (Figure 3.6).

Initial studies appeared to be promising with a 12 fold purification using Cu as the immobilized ion. However the procedure was not reproducible in our hands. When the column was scaled up to 20ml the aminopeptidase did not bind and eluted in the first wash. A further problem was that leakage of Cu^{2+} from the column produced irreversible inactivation of the enzyme. Using Zn as the immobilized ion produced no purification of the enzyme and caused irreversible inactivation of the aminopeptidase.

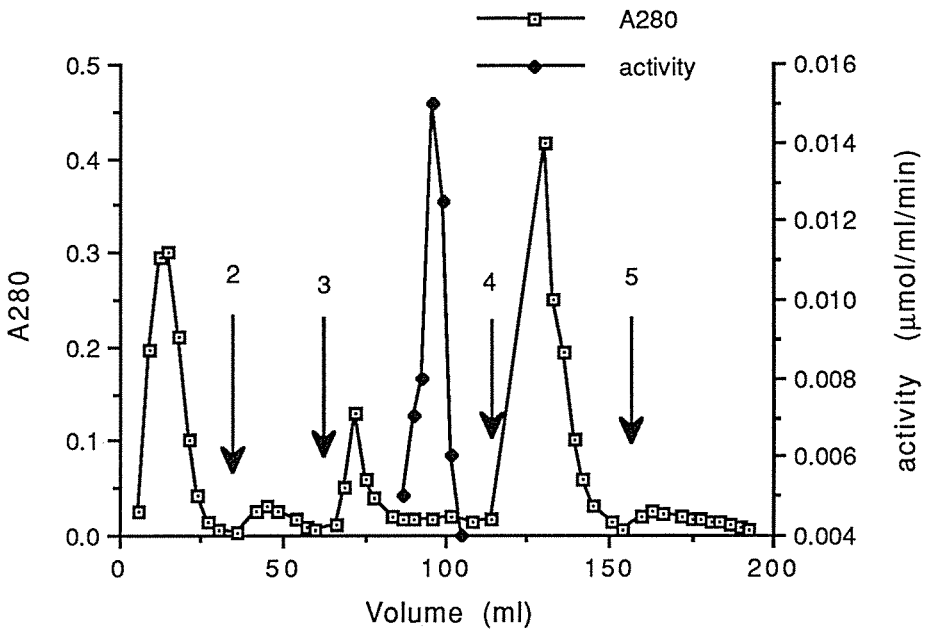


Figure 3.6 Aminopeptidase elution from a Copper IMAC column.

The eluting buffers used are numbered 1) 20mM phosphate pH 7;
2) 25mM Na-acetate pH6.5;
3) 50mMTris/HCL pH 7.5;
4) 2mM Imidazole pH 7.
5) 20mM Imidazole pH 7

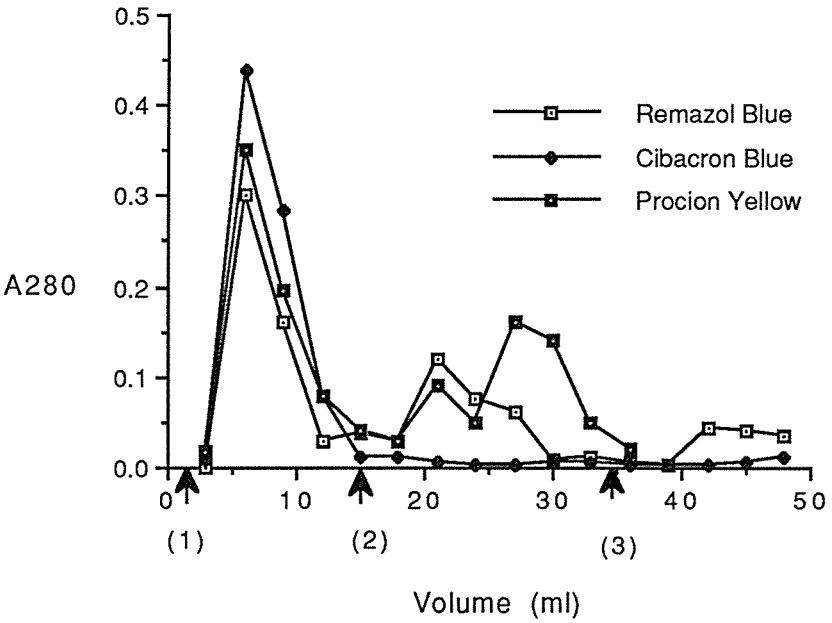


Figure 3.7 Lys-aminopeptidase elution from Dye-ligand Chromatography columns.

Eluting buffers used are numbered 1)30mM phosphate pH 6.0, 30mM NaCl, 2mM $MgCl_2$
2)30mM phosphate pH 6.5, 30mM NaCl,
3)30mM phosphate ,100mM NaCl.

Dye Ligand Chromatography:

Dyes often have an affinity for proteins, offering a variety of groups which can bind to proteins through H-bonding, hydrophobic or electrostatic interactions (Scopes 1986).
The large number of dyes presently in use has meant that dye adsorbents are now classified into five groups according to their ability to bind proteins. Group 1 dyes have the weakest affinity for protein while Group 5 dyes exhibit the greatest affinity (Scopes 1986).
The ability of the following dyes, (immobilised on a column) to bind lys-aminopeptidase was investigated:

Remazol Brilliant Blue R a group 3 dye,
Procion Yellow H-E4R a group 5 dye,
Cibacron Blue a group 5 dye.

A 5ml column of each gel was equilibrated with 30mM phosphate pH6.0 containing 2mM $MgCl_2$ and 30mM NaCl . Protein containing aminopeptidase activity was loaded onto the column (15mg), then eluted sequentially with the following buffers:

30mM phosphate pH6.0, with 30mM NaCl, 2mM $MgCl_2$
30mM phosphate pH6.5, with 30mM NaCl
30mM phosphate pH6.5, with 100mM NaCl.

The lys-aminopeptidase did not bind to any of the columns (Figure 3.7) even when the pH of the buffers was lowered to 6.0 to maximise binding and 2mM $MgCl_2$ (to increase protein binding to the dye) was added.The Procion Yellow H-E4R did bind other proteins, so some purification of the aminopeptidase did occur. However the purification achieved was not sufficient to warrant further investigation at this stage.

Phenyl-Sepharose:

Separation of proteins on Phenyl Sepharose is based on the differing strengths of hydrophobic interaction of proteins with the phenyl groups of the matrix without any interfering ionic effects. Elution of the bound protein is accomplished by selective desorption by varying the strength of the hydrophobic interactions between the proteins and the matrix. This may be achieved by lowering the ionic strength, raising the pH or including a detergent in the eluting buffer.
Pooled fractions containing aminopeptidase activity from a DEAE column (10ml containing $0.07\mu molml^{-1}min^{-1}$)were applied to a small column of phenyl Sepharose with dimensions 1.5x 17 cm, and eluted with a gradient from 4.0-0.0M NaCl buffered with 20mM phosphate pH 7.0.The high salt concentration caused irreversible inhibition of the enzyme and no purification was accomplished.

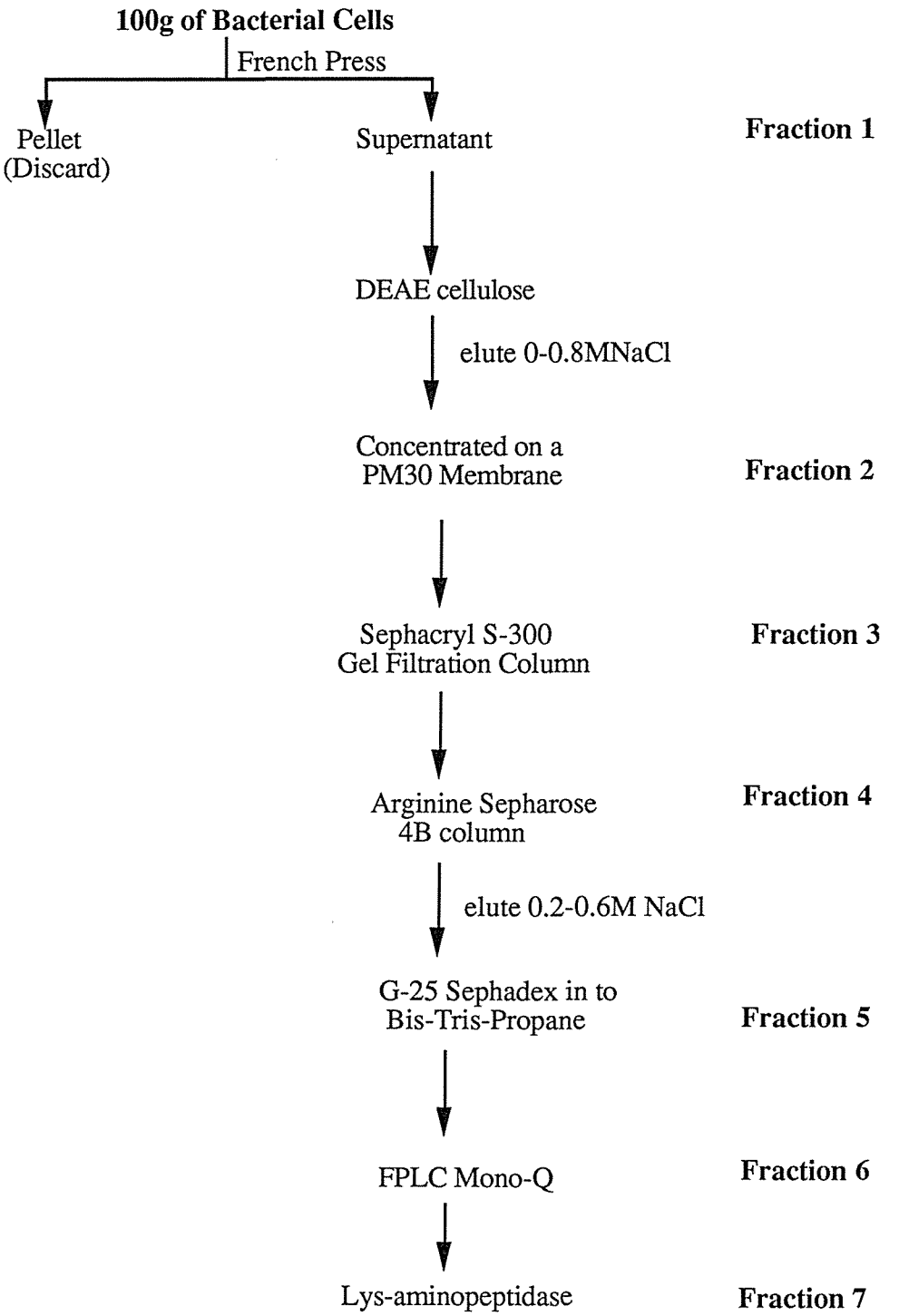


Figure 3.8: Protocol for the purification of lys-aminopeptidase from lactic bacteria.

3.3 Characterization of the lys-aminopeptidase:

3.3.1 Molecular Weight Determination by SDS-PAGE:

The subunit molecular weight of the purified aminopeptidase was determined by SDS polyacrylamide gel electrophoresis, as described in section 2.2.9. The purified enzyme gave only one major band on SDS-PAGE. The molecular weight of the lys-aminopeptidase was estimated to be approximately 98,200Da ±900 by averaging the results obtained from four separate gels using protein from three different purifications (Figure 3.9, Figure 3.11).

3.3.2 Molecular weight determination by gel permeation on a Sephacryl S-300 column:

Molecular weight determination of the native lys-aminopeptidase was carried out by gel filtration on a Sephacryl S-300 column (2.6x90cm) equilibrated in 0.1M phosphate buffer pH 7.0 with 0.1M KCl present to prevent aggregation of the molecular weight standards and run at a flow rate of 0.5ml per minute. The molecular weight of the aminopeptidase was determined by its elution volume from the calibrated Sephacryl S-300 column (Figure 3.10). The molecular weight [determined using enzyme from the Sephacryl S-300 step (step 3)] yielded a value of 96,000 Da based on an average from three runs [92kDa-98kDa].

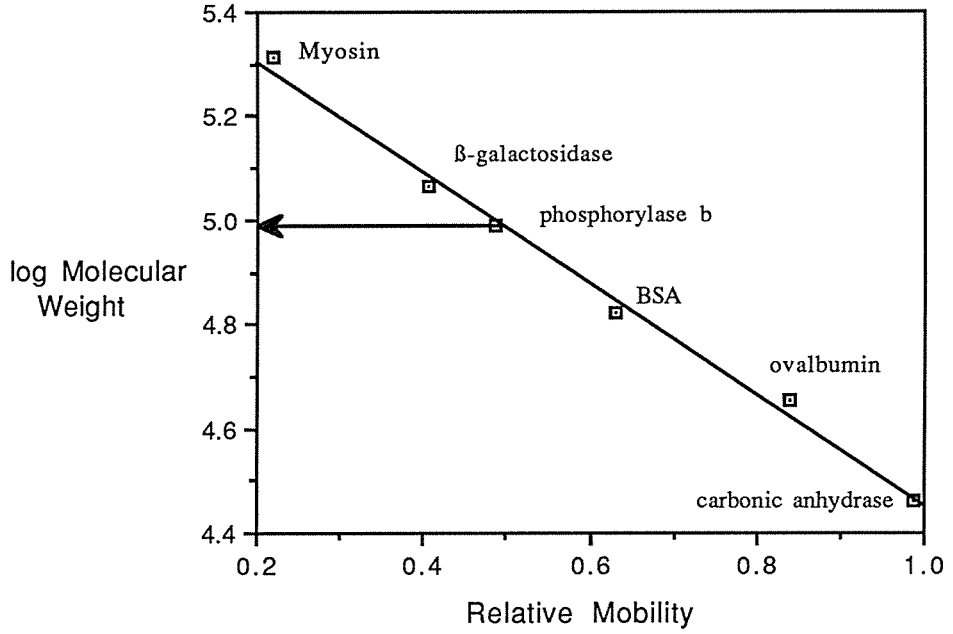


Figure 3.9 Graph showing the Molecular weight determination by SDS PAGE.

Log subunit molecular weight of lys-aminopeptidase is shown by the arrow. The following markers were used: Myosin 205kDa, β-Galactosidase 116kDa, Phosphorylase b 97.4kDa, Bovine serum albumin 66kDa, Ovalbumin 45kDa and Carbonic anhydrase 29kDa.

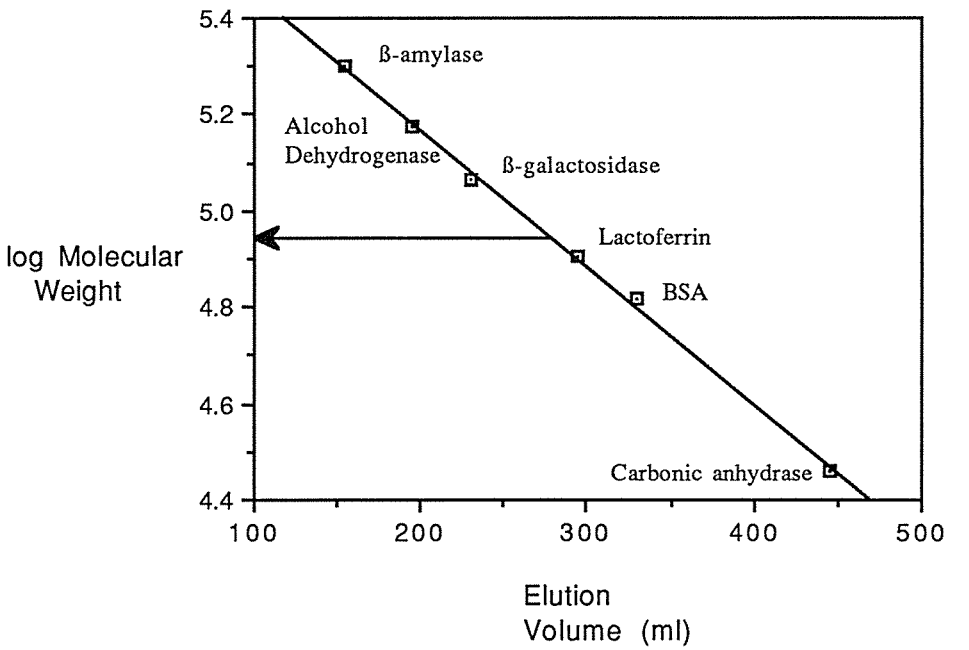


Figure 3.10 Calibration graph from S-300 gel filtration column.

Marker proteins used in the Molecular weight determination were:β-amylase 200kDa, Alcohol Dehydrogenase 150kDa, β-galactosidase 116kDa, Lactoferrin 80kDa, Bovine serum albumin 66kDa, Carbonic Anhydrase 29kDa. The position of the aminopeptidase is shown by the arrow.

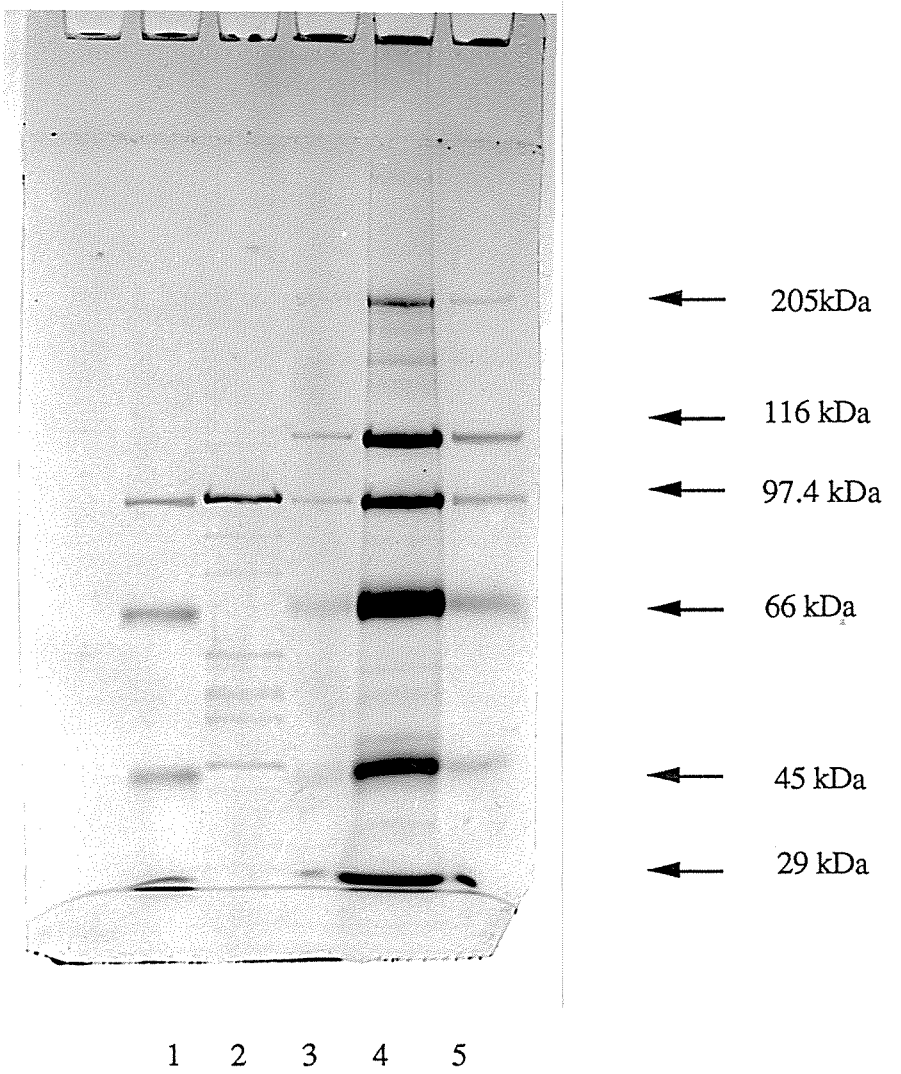


Figure 3.11. SDS gel of the molecular weight of the lys-aminopeptidase from *S.thermophilus* 5109

Lane 1,3,4,5 molecular weight markers lane 2 FPLC sample.

3.3.3 Effect of Temperature and pH on the Activity of the Enzyme:

The optimal pH range for the aminopeptidase was determined by using enzyme from the arginine-Sepharose purification step (step4), over a pH range of 4.0 to 9.5.The buffers used were 100mM MES,with a pH range of 4.5-7.5 and 100mM Bis-Tris-Propane pH range 4.9-9.5. All buffers were checked by a pH meter that had been calibrated at two pH's with freshly prepared standards. The reaction mixture was checked before and after the addition of the enzyme and no pH change was detected. The assay was carried out as described in section 2.2.7.
The optimal pH for the aminopeptidase activity (Figure 3.12) was, pH 6.8-6.9 using MES buffer and pH7.0 using Bis-Tris-Propane. Below a pH of 4.5 and above that of 8.5 the hydrolysing activity was lost. There was a rapid fall off in enzyme activity on either side of the optimal pH. The pH range where there was greater than 50% of the peak activity was quite narrow covering the pH range 5.8-7.8.

The optimal temperature range for the lys-aminopeptidase activity was investigated using a Gilford 260 Spectrophotometer with a Haake circulating water bath D1-L. The enzyme was equilibrated with the buffer at a set temperature for ten minutes, after which the activity was determined by the addition of lysine-p-nitroanilide.
The optimal temperature for hydrolytic activity of the lys-aminopeptidase (Figure 3.13) was found to be 35^{o}C. The activity had a broad temperature range 25-50^{o}C with no activity above 65^{o}C and very low activity below 10^{o}C.

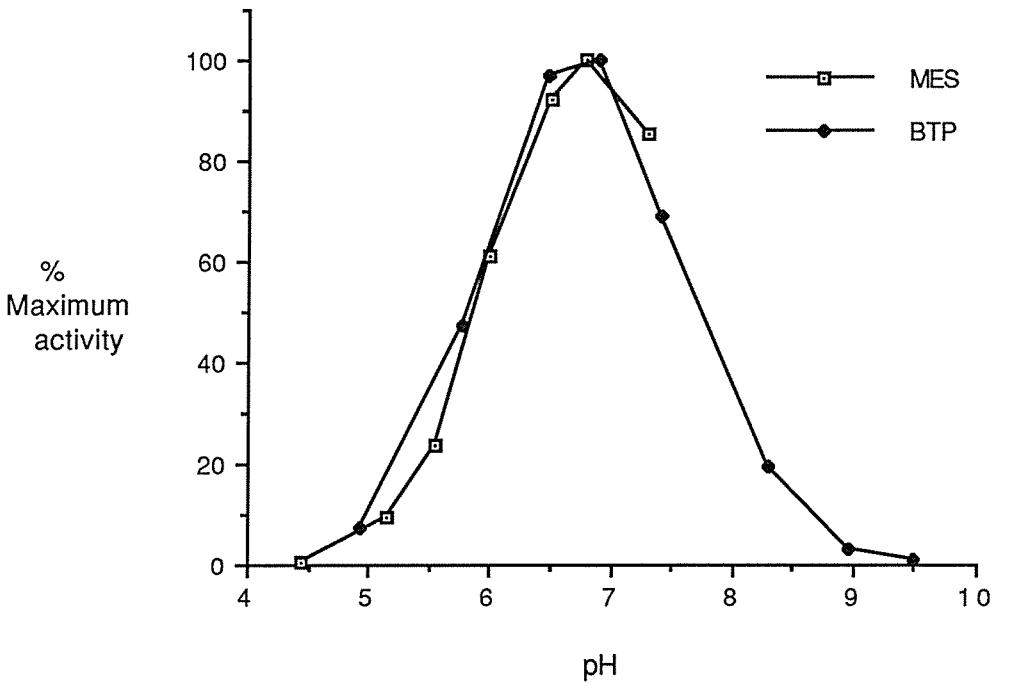
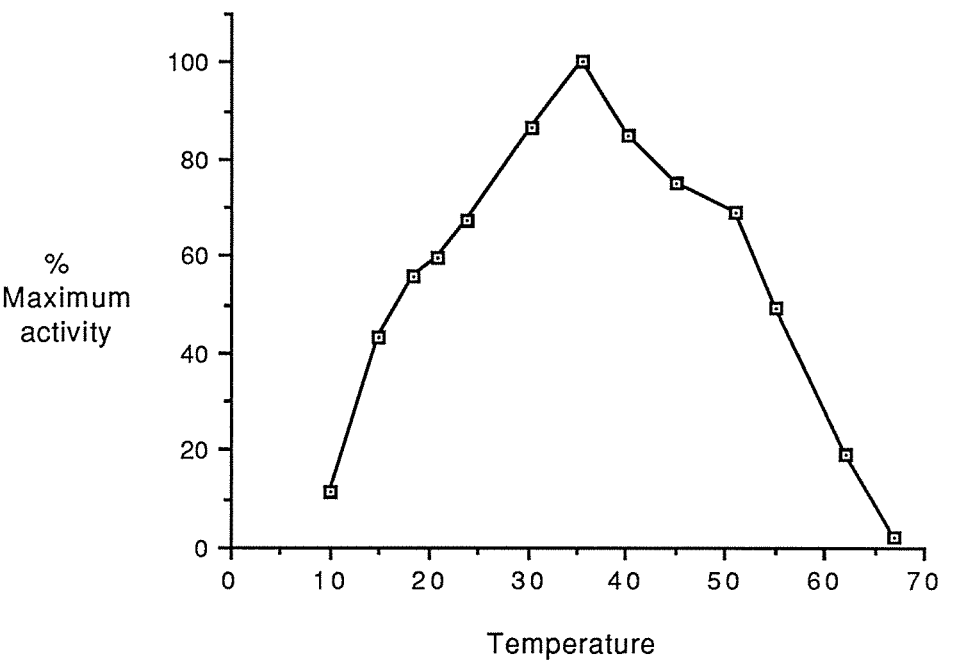


Figure 3.12. pH activity curve of the lys-aminopeptidase from *S.thermophilus* 5109 over a pH range of 4.5-9.5 using 100mM MES and Bis-Tris-Propane.
Conditions are as described in section 3.3.3



.Figure 3.13: Temperature curve of the lys-aminopeptidase over a temperature range of 10-68 ºC.

The enzyme was incubated for 10 minutes in a water bath at the set temperature before the reaction was transferred to a thermostated spectrophotometer and the reaction was initiated by the addition of 0.5mM (final concentration) of lys-pNA.

3.3.4 Inhibition Studies:

Many of the peptidases purified from the lactic acid bacteria have been found to be metallo-enzymes. Investigations were carried out on the*S.thermophilus* 5109 lys-aminopeptidase to determine its activity when in the presence of various cations and inhibitors of metallo-enzymes.
The metal chelators EDTA and 1,10-phenanthroline,which chelate a range of divalent cations, were incubated at concentrations ranging from 0-2mM with the purified enzyme for 10 minutes at room temperature. Enzyme activity was than determined using the standard assay. Both metal chelators inhibited lys-aminopeptidase activity (Table 3.3 and Figure 3.14).

The effects of the sulphydryl enzyme inhibitors iodoacetic acid and pCMB were investigated by incubating the enzyme with the inhibitor for 10 minutes at room temperature before assaying the activity of the enzyme. The inhibitor pCMB totally inhibited the enzyme at low concentrations (0.8mM) while iodoacetic acid also strongly inhibited the activity (figure 3.14).This suggested that the enzyme has a cysteine residue either at the active site or in a position of possible structural importance to the enzyme.

Inhibition of the lys-aminopeptidase activity by serine protease inhibitors was also investigated. Incubation of the enzyme with 1mM PMSF (phenyl methyl sulphonyl fluoride) for two hours produced no inhibition.and actually activated the enzyme by up to 20% compared to a non PMSF containing control. Incubation with N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK) [an inhibitor of the serine protease trypsin and the cysteine protease papain,by the specific binding to a histidine group] for two hours gave only limited inhibition. This suggests that the enzyme does not belong to the serine protease family.

The effect of several divalent cations on the enzyme activity was also studied.
All metals ions were added as chlorides to prevent any influences of the anion on the reaction. Zn^{2+}, Co^{2+}, and Cu^{2+} were all inhibitory at low concentrations, while the ions Mn^{2+},Mg^{2+} and Ca^{2+} showed little or no significant inhibitory or stimulatory effects on the lys-aminopeptidase activity over the range of concentrations used (Figure 3.15 and Table 3.3).

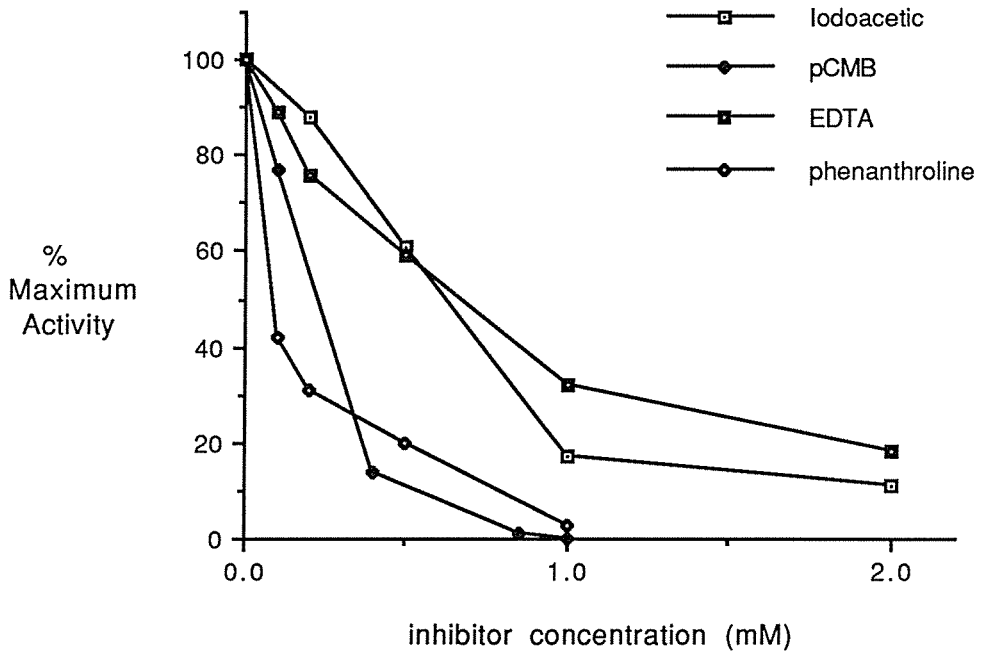


Figure 3.14: Inhibition of the lys-aminopeptidase activity by various inhibitors.
The aminopeptidase was incubated for ten minutes at room temperature in the presence of the inhibitor before the reaction was initiated by the addition of (0.5mM) lys-pNA. Activity is expressed as a percentage of the activity of a lys-aminopeptidase control without any inhibitor present.

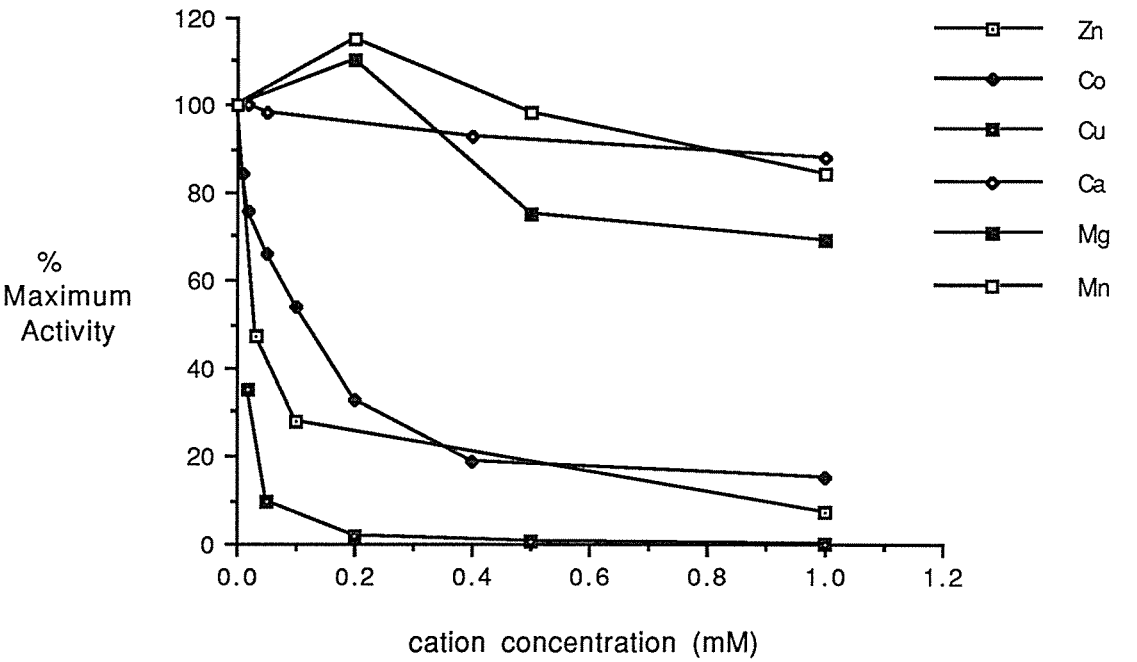


Figure 3.15: Effect of divalent cations over a concentration range of 0-1mM on the activity of lys-aminopeptidase.
Conditions as described in section 2.2.6. Activity is expressed as a percentage of the activity of the peptidase without any metal.

Reagent	activity with 1mM of reagent % activity
no addition	100
1,10-phenanthroline	3
EDTA	32
Iodoacetic acid	17
pCMB	0
N-TLCK (10min)	67
(30min)	61
(60min)	76
(120min)	77
PMSF	119
Mn^{2+}	84
Ca^{2+}	88
Co^{2+}	15
Zn^{2+}	7
Cu^{2+}	0
Mg^{2+}	69

Table 3.3 Effect of cations and inhibitors on lys-aminopeptidase activity

Activity is expressed as a percentage of the activity in a control with no inhibitor. All assays were carried out at 20°C and pH 6.8.With a final concentration of 1mM reagent and 0.5mM of lys-pNA.

3.3.5 Specificity:

The primary objective of the characterization of the lys-aminopeptidase was to determine its specificity towards various substrates and to investigate its possible role in the degradation of the oligopeptides produced from casein by starter cell proteinase during the maturation of dairy products, since this is particularly relevant to its potential role in the degradation of milk proteins.

3.3.5.1 Relative activity with different aminoacyl derivatives:

The specificity of the aminopeptidase was investigated using various amino-acyl AMC and pNA substrates. Activity towards a range of dipeptides, a tripeptide and X-prolyl dipeptidyl AMC was also investigated. The highest purity enzyme was used for these studies (Table 3.4).

Substrate	Relative activity(%)	Substrate	Relative activity(%)
Lys pNA	100	Lys AMC	100
Arg pNA	39	Leu AMC	38
Leu pNA	38	Ala AMC	36
Ala pNA	6.3	Phe AMC	10
Phe pNA	5.1	Tyr AMC	7
Val pNA	1.9	Ser AMC	4
Gly pNA	<1	Glu AMC	<1
Pro pNA	0	Pro AMC	0
Leu-Gly	0	Leu-Pro AMC	0
Leu-Ala	0	Lys-Pro AMC	0
Lys-Ala	0	Gly-Pro AMC	0
Ala-Ala	0		
Leu-Gly-Gly	low		
Ala-Ala-Ala	low		

Table 3.4 Relative activity of lys-aminopeptidase with various amino-acyl substrates. All substrates were at a final concentration of 0.5mM. Activities are shown as a percentage of the activity with lys pNA or lys AMC ($(ala)_3$ and leu-gly-gly could not be given as a percentage of lys pNA or AMC due to the differing substrates) .Assays were carried out at 20°C and a pH of 6.8 as described in sections 2.2.7 i and ii

Among the pNA and AMC derivatives tested activity was highest with the lysyl derivatives but relatively high activity was also obtained with leu-pNA and AMC, with arg-pNA and with ala AMC (at the standard 0.5mM substrate concentration used). Low levels of activity were obtained with phe,val,ser and tyr derivatives, very little activity with gly-pNA and glu AMC and no activity with pro-pNA or AMC. Thus the enzyme, while catalysing the hydrolysis of a range of amino-acyl derivatives, has a quantitatively fairly restricted specificity.

Dipeptides and X-pro-AMC substrates were not hydrolysed indicating that the preparation was not contaminated by X-pro dipeptidyl peptidase, and that the aminopeptidase had no dipeptidase activity. The slow hydrolysis of the tripeptide leu-gly-gly may indicate tripeptidase contamination or could be due to low aminopeptidase activity with tripeptide substrates (see below).

3.3.5.2 Kinetic parameters of lys-aminopeptidase with amino-acyl derivatives:

From the results of the preliminary survey of relative activity, a more detailed investigation of the specificity of the aminopeptidase towards selected acyl-pNA and AMC substrates was carried out by determining the Km and Vmax values with the various substrates. Km and Vmax values were determined using Lineweaver-Burke plots (Table 3.5). Non-linear regression analysis of the experimental data was carried out using the programme Enzfitter version 1.05 (Leatherbarrow, 1987). Substrate concentrations of 0.005-2mM were used to determine the rates of the reaction. Consistent Km and Vmax values were obtained for each substrate using several different enzyme preparations. Rate determinations were all carried out at least in triplicate for each substrate. Examples of the rate versus substrate concentration and Lineweaver Plots are shown in Figure 3.16 et seq.

Substrates	Km (mM)	Vmax (μmol min^{-1} mg^{-1})
lys pNA	0.20±0.01	15±0.03
leu pNA	0.17±0.02	9.7±0.6
arg pNA	0.094±0.003	4.8±0.06
phe pNA	0.11±0.02	1.6±0.1
ala pNA	1.4±0.2	8.4±0.9
lys AMC	0.060±0.005	5.7±0.2
leu AMC	0.025±0.003	4.0±0.2

Table 3.5 Kinetic parameters for purified lys-aminopeptidase from *S.thermophilus* for several amino-acyl pNA and AMC substrates.

Each assay contained 0.05μg of purified enzyme in a total volume of 2ml. Assays were carried out at 30^0C at a pH of 6.8 (see section 2.2.7)

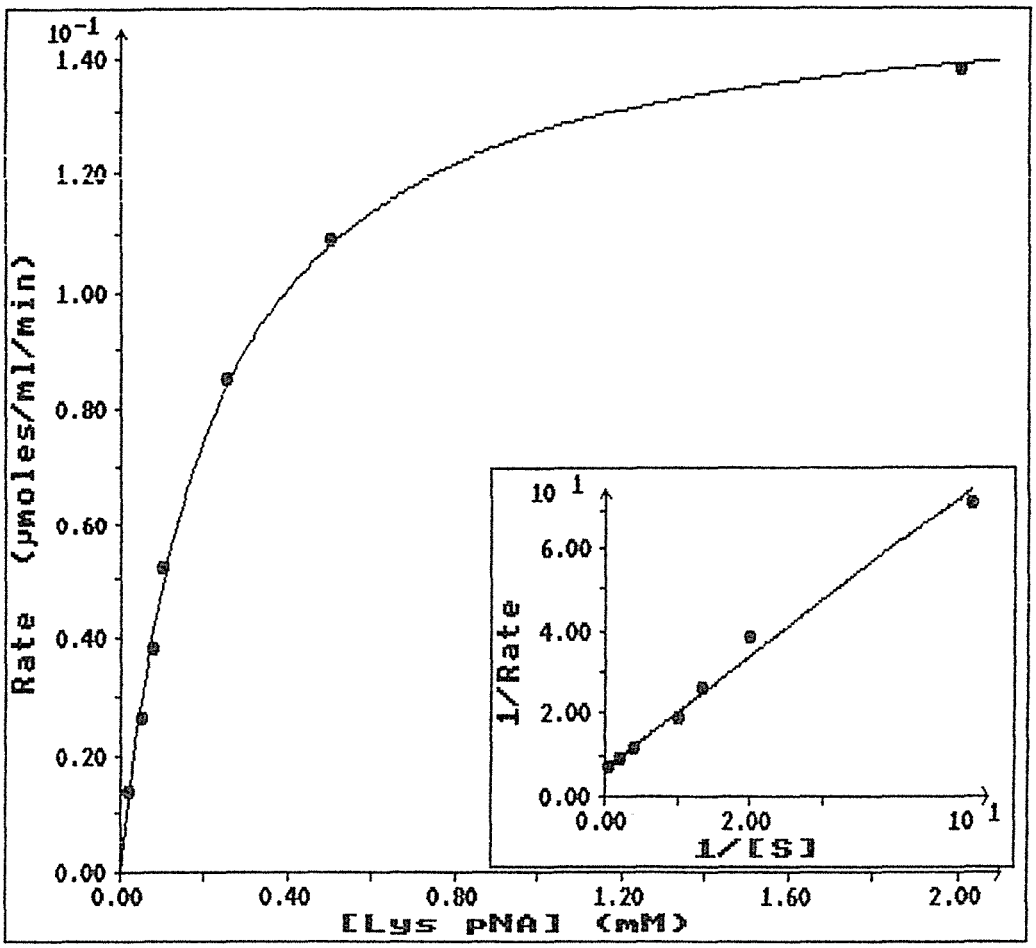
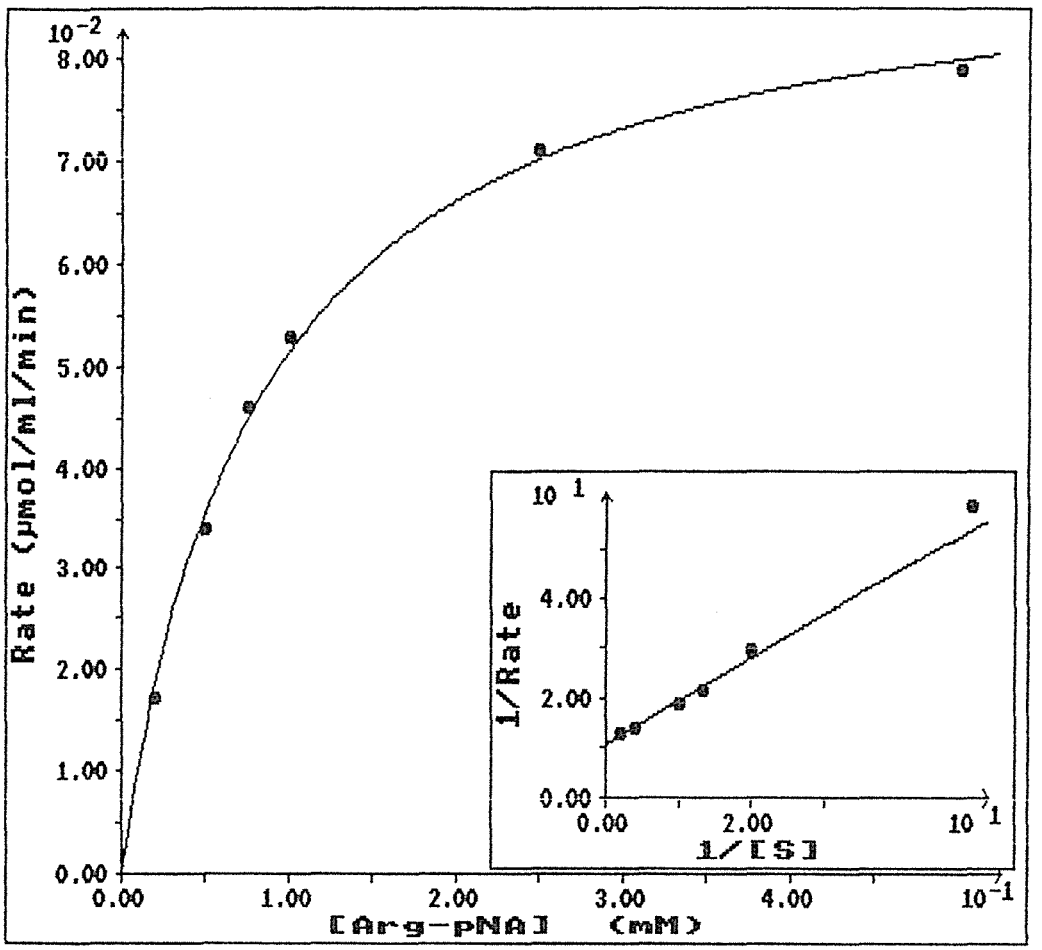
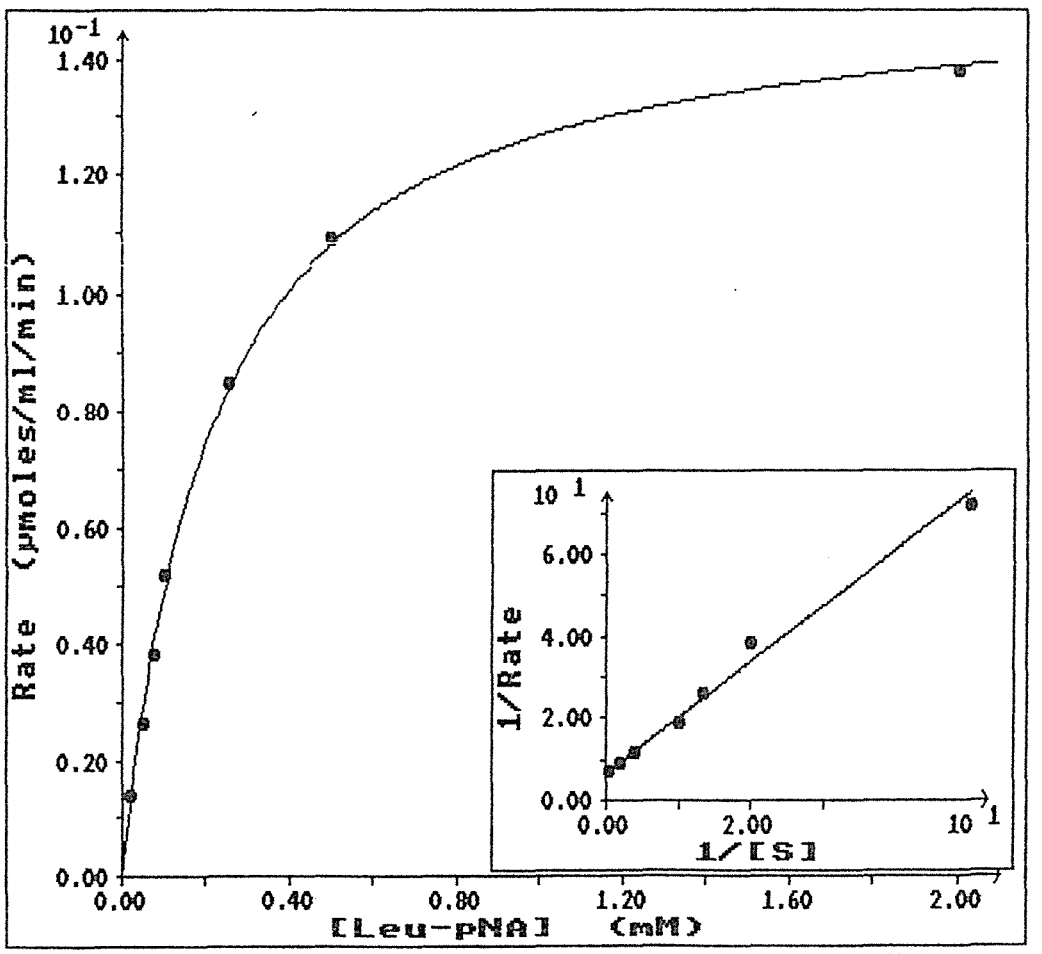


Figure 3.16 Rate versus concentration plots and Lineweaver-Burke plots of the 1) lys-pNA,2) arg-pNA, 3) leu-pNA, 4) phe-pNA, 5) ala-pNA, 6) lys AMC and 7) leu AMC substrates for lys-aminopeptidase from *S.thermophilus*.. Each assay contained 0.05μg of purified lys-aminopeptidase in a total volume of 2ml. Assays were carried out in 100mM MES buffer pH 6.8 at 30°C using substrate concentrations over the range of 0.005-2mM.

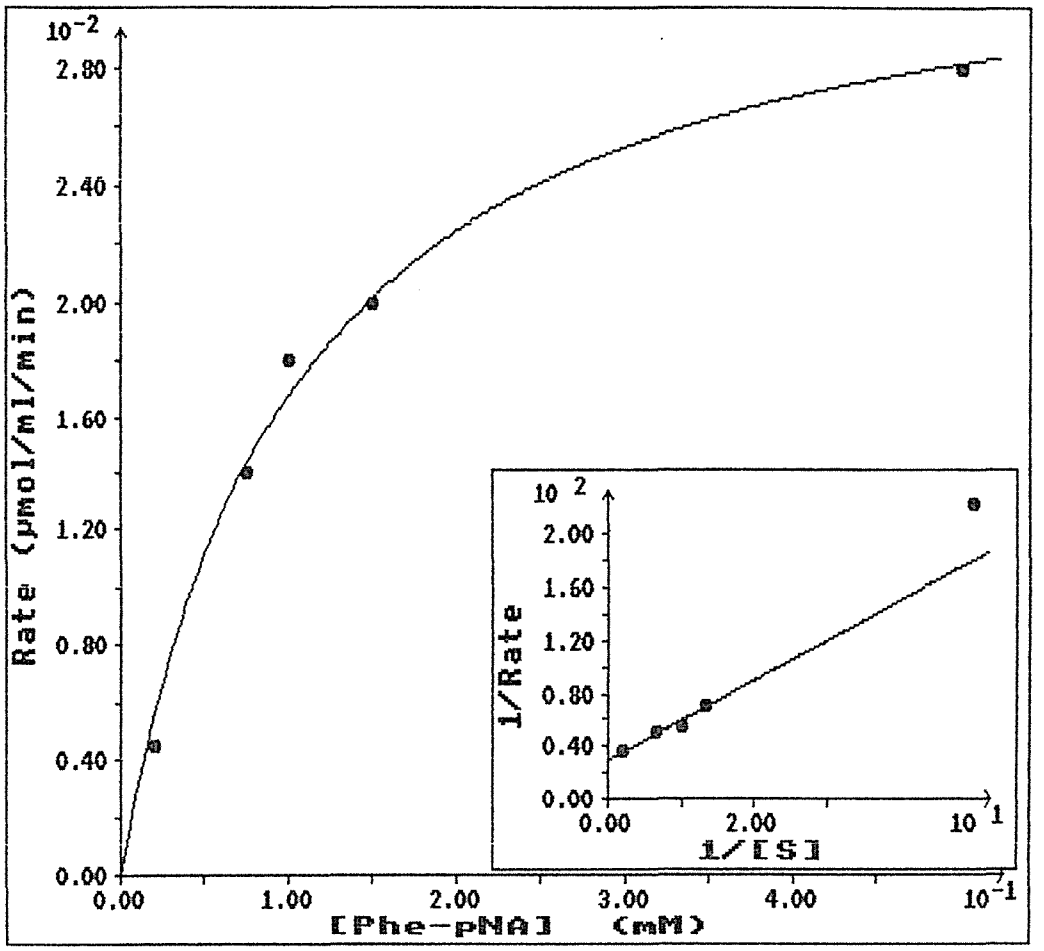
2


10^{-2}
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Rate (µmol/ml/min)
0.00
1.00
2.00
3.00
4.00
10^{-1}
[Arg-pNA] (mM)
10 1
4.00
2.00
0.00
1/Rate
0.00
2.00
10 1
1/[S]

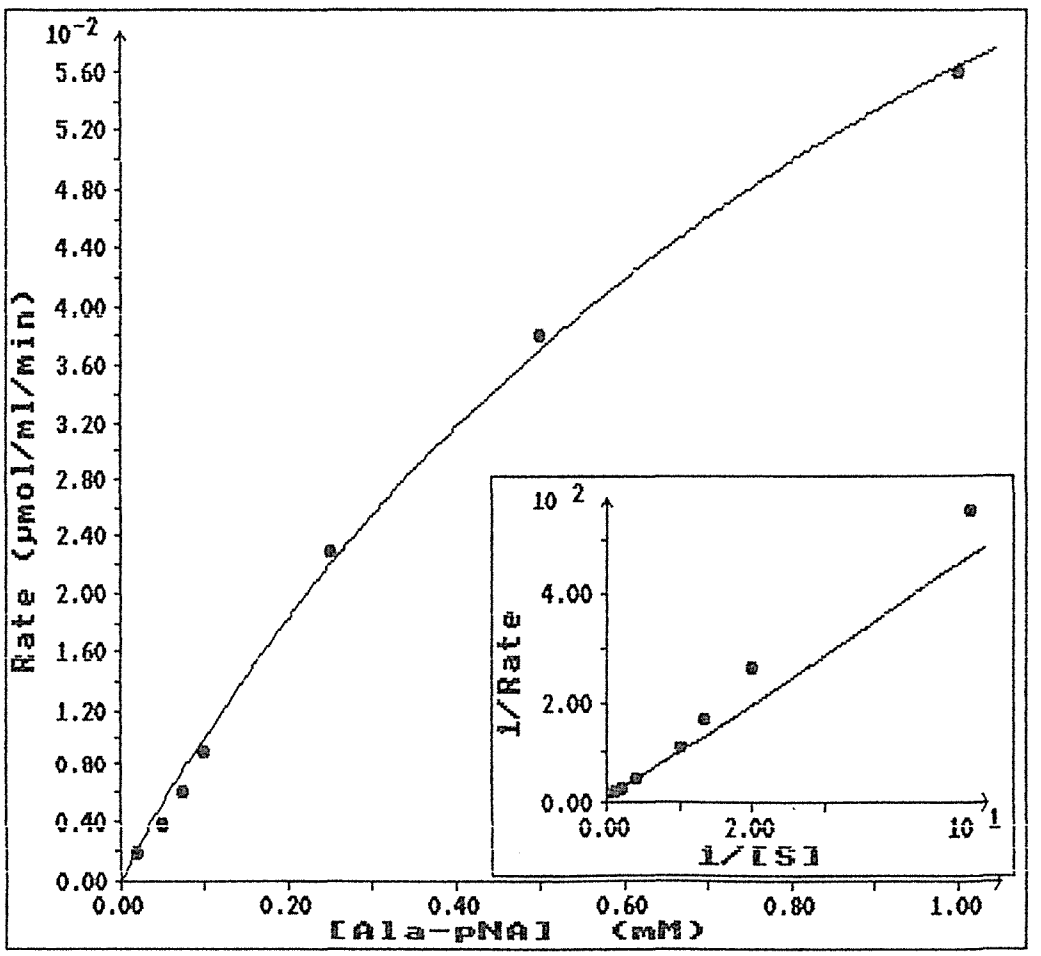
3


10^{-1}
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Rate (µmoles/ml/min)
0.00
0.40
0.80
1.20
1.60
2.00
[Leu-pNA] (mM)
10 1
6.00
4.00
2.00
0.00
1/Rate
0.00
2.00
10 1
1/[S]

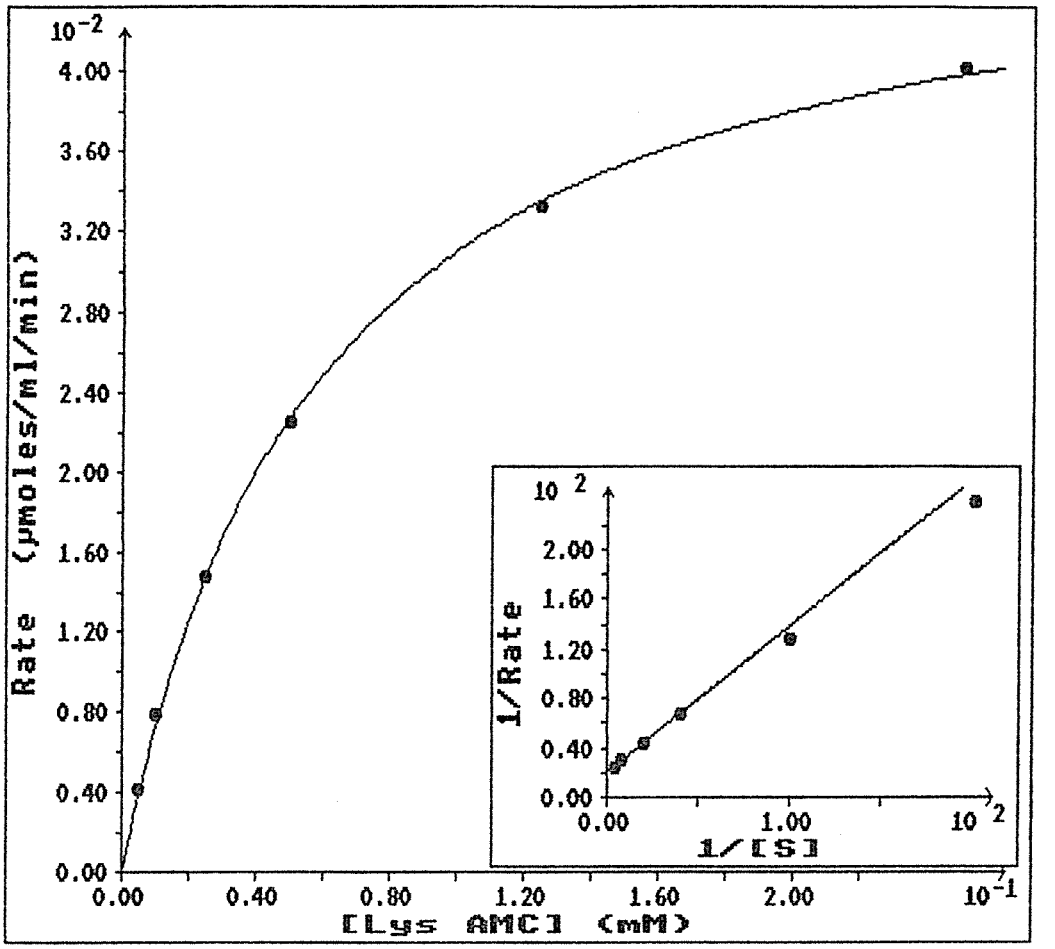
4


10^{-2}
2.80
2.40
2.00
1.60
1.20
0.80
0.40
0.00
Rate (µmol/ml/min)
0.00
1.00
2.00
3.00
4.00
10^{-1}
[Phe-pNA] (mM)
10^{2}
2.00
1.60
1.20
0.80
0.40
0.00
1/Rate
0.00
2.00
10^{1}
1/[S]

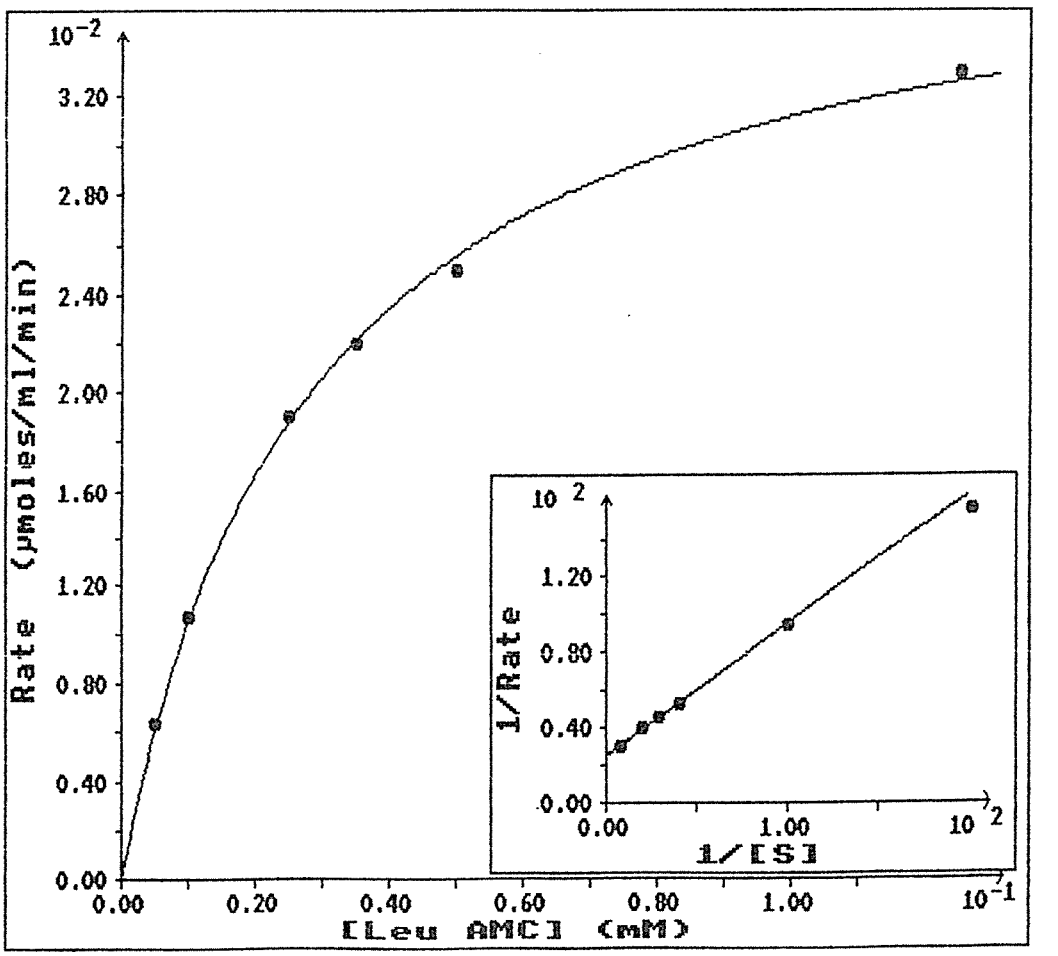
5


10^{-2}
5.60
5.20
4.80
4.40
4.00
3.60
3.20
2.80
2.40
2.00
1.60
1.20
0.80
0.40
0.00
Rate (µmol/ml/min)
0.00
0.20
0.40
0.60
0.80
1.00
[Ala-pNA] (mM)
10^{2}
4.00
2.00
0.00
1/Rate
0.00
2.00
10^{1}
1/[S]

6


10^{-2}
4.00
3.60
3.20
2.80
2.40
2.00
1.60
1.20
0.80
0.40
0.00
Rate (µmoles/ml/min)
0.00
0.40
0.80
1.20
1.60
2.00
10^{-1}
[Lys AMC] (mM)
10^{2}
2.00
1.60
1.20
0.80
0.40
0.00
1/Rate
0.00
1.00
10^{2}
1/[S]

7


10^{-2}
3.20
2.80
2.40
2.00
1.60
1.20
0.80
0.40
0.00
Rate (µmoles/ml/min)
0.00
0.20
0.40
0.60
0.80
1.00
10^{-1}
[Leu AMC] (mM)
10^{2}
1.20
0.80
0.40
0.00
1/Rate
0.00
1.00
10^{2}
1/[S]

The Km values for the p-nitroanilides were similar except for that of ala-pNA, which was 7 times higher than that for lys-pNA. Of the p-nitroanilide substrates activity was highest with lys-pNA. The Vmax with ala-pNA was comparable to that of the leu-pNA. The much lower relative rate for ala-pNA shown in table 3.5 is a consequence of the higher Km for this substrate which is considerably greater than the 0.5mM substrate concentration used for the initial comparison. The Vmax values for the AMC derivatives were somewhat lower than those for the corresponding pNA derivatives while the Km value for the AMC substrates were lower than those of the corresponding pNA substrates.

3.3.5.3. Activity of the lys-aminopeptidase towards peptides:

In the published studies of the properties of purified "general" aminopeptidase from lactococci little quantitative information is available on the activity towards peptides (as opposed to amino-acyl chromophoric or fluorogenic substrates).

The aminopeptidases from lactococcal strains have been found to have dipeptidase activity (Tan & Konings, 1990; Neviani et.al., 1989; Rabier & Desmazeaud, 1973). This is not the case with the aminopeptidase from *S.thermophilus* 5109 which showed no activity towards any of the dipeptides tested (Table 3.4). However the aminopeptidase did have low tripeptidase activity with leu-gly-gly. This suggested that the aminopeptidase might require a minimum chain length to be fully active against peptides. To investigate this possibility enzyme activity was studied using a series of poly-alanyl peptides.

10µl of purified enzyme was added to 240µl of the substrate [2mM $(ala)_x$]in 20mM phosphate pH 7.0. The system was incubated at 25^0C and 50µl samples were removed at intervals and stopped by adding to 25µl of 3% TFA. Each sample was analysed by HPLC.and the areas under each peak determined with a Spectra Physics SP4290 integrator. Peaks were identified by their retention time which was obtained from the appropriate zero-time analysis. It was found that there was no activity towards the dipeptide ala-ala and very low activity towards the tripeptide $(ala)_3$ (Figure 3.17), confirming earlier observations with the fluorometric assay. However with $(ala)_4$ there was considerable activity. After one hour almost all of $(ala)_4$ had been degraded with a proportionate accumulation of $(ala)_3$ (Figure 3.18). With $(ala)_5$ hydrolysis of the N-terminal residue was even more rapid (Figure 3.19). A transient accumulation of $(ala)_4$ was detected but, as expected this was further degraded to $(ala)_3$ which accumulated. The accumulation of $(ala)_3$ was consistent with the very low tripeptidase activity of the aminopeptidase. The hydrolysis of $(ala)_6$ was much slower than the rate with $(ala)_5$. After 3 hours 20% of the $(ala)_6$ was still undegraded (Figure 3.20).

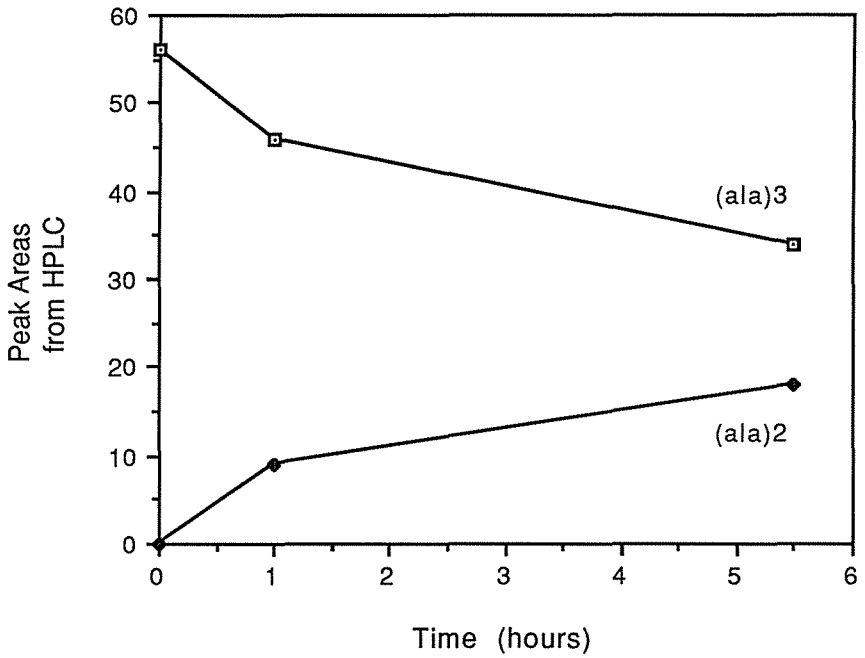
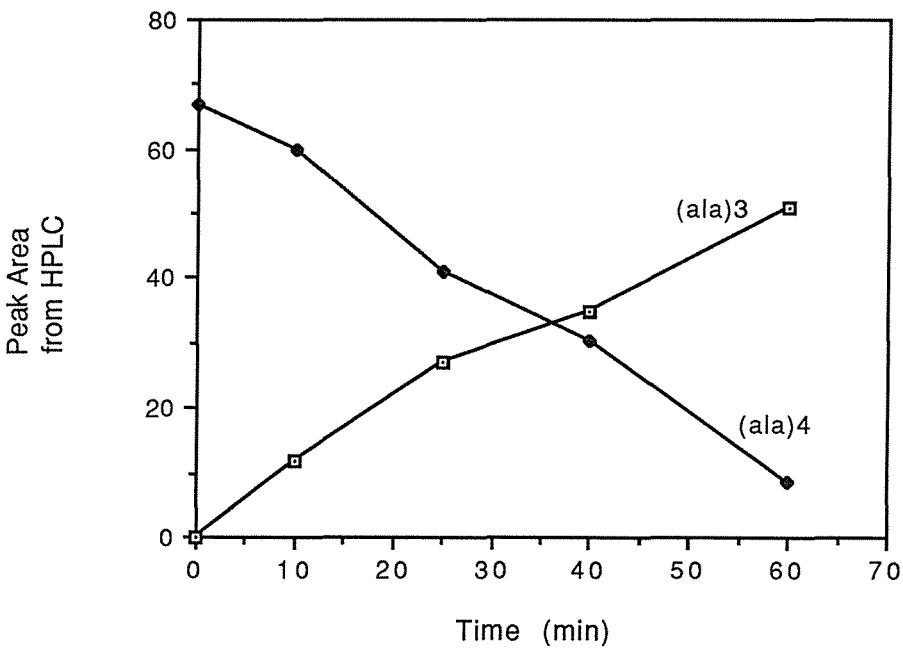


Figure 3.17 Hydrolysis of $(ala)_3$ by lys-aminopeptidase.

Separation of peptides was performed by HPLC on a Vydac 218 TP C18 column. Solvent system: solvent A = 0.1% TFA in water;solvent B = 0.08% TFA in acetonitrile.

Separation was obtained using a linear gradient of 0% B to 10% B over 15 minutes at a flow rate of 1ml min^{-1}. Peptides were detected by measuring their absorbance at 220nm



.Figure 3.18 The hydrolysis of $(ala)_4$ by lys-aminopeptidase.

HPLC conditions as in Figure 3.17.

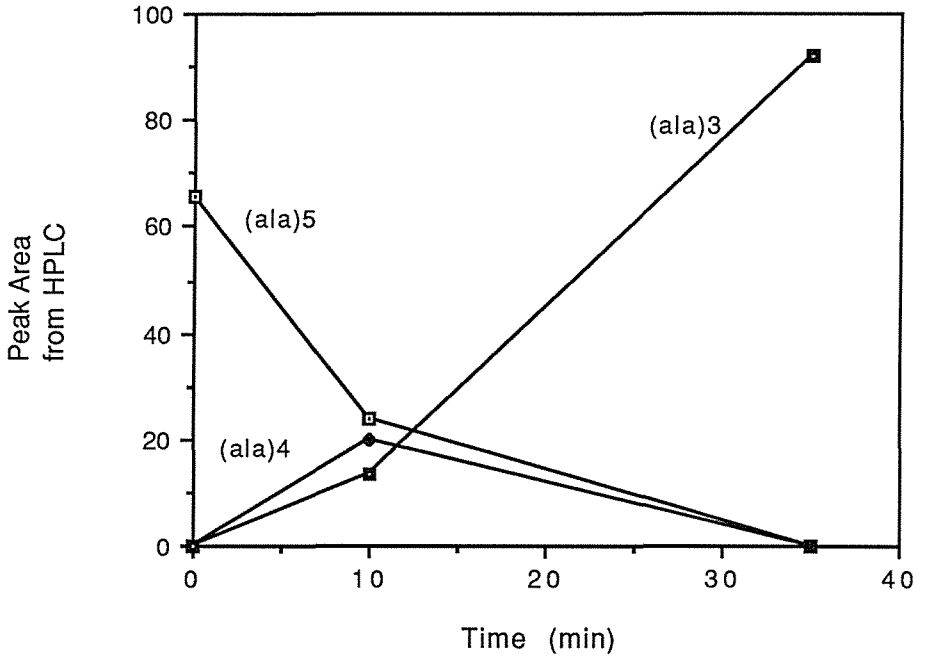


Figure 3.19 The hydrolysis of $(ala)_5$ by lys-aminopeptidase.
HPLC conditions as in Figure 3.17

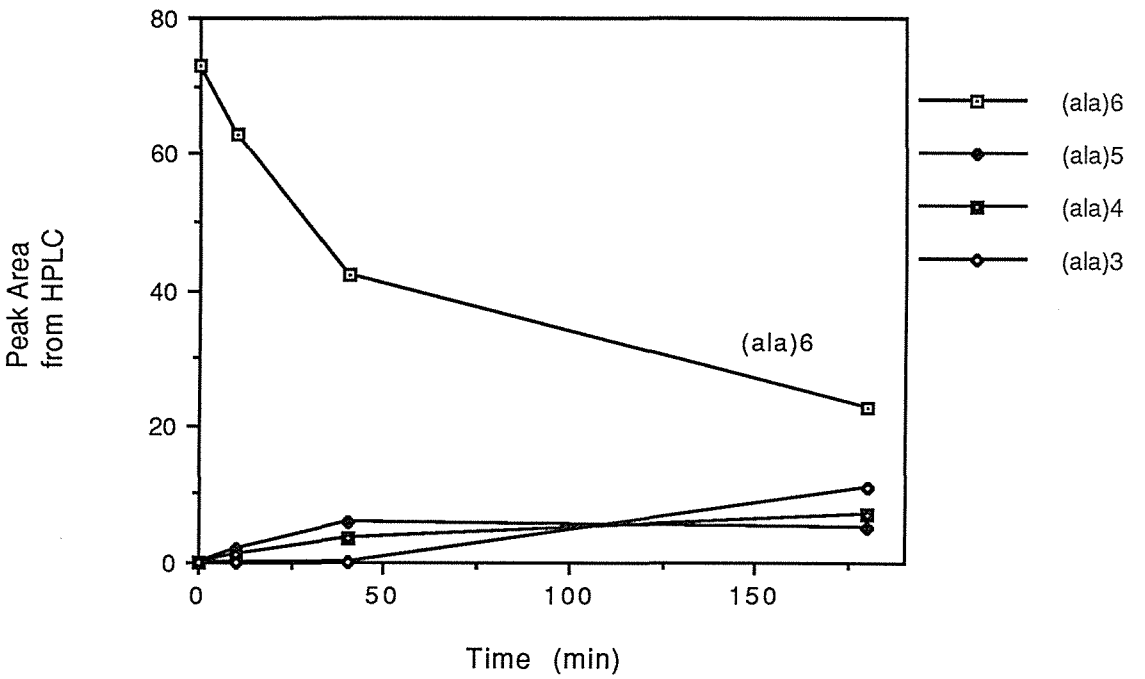


Figure 3.20: The hydrolysis of $(ala)_6$ by lys-aminopeptidase.
HPLC conditions as in Figure 3.17

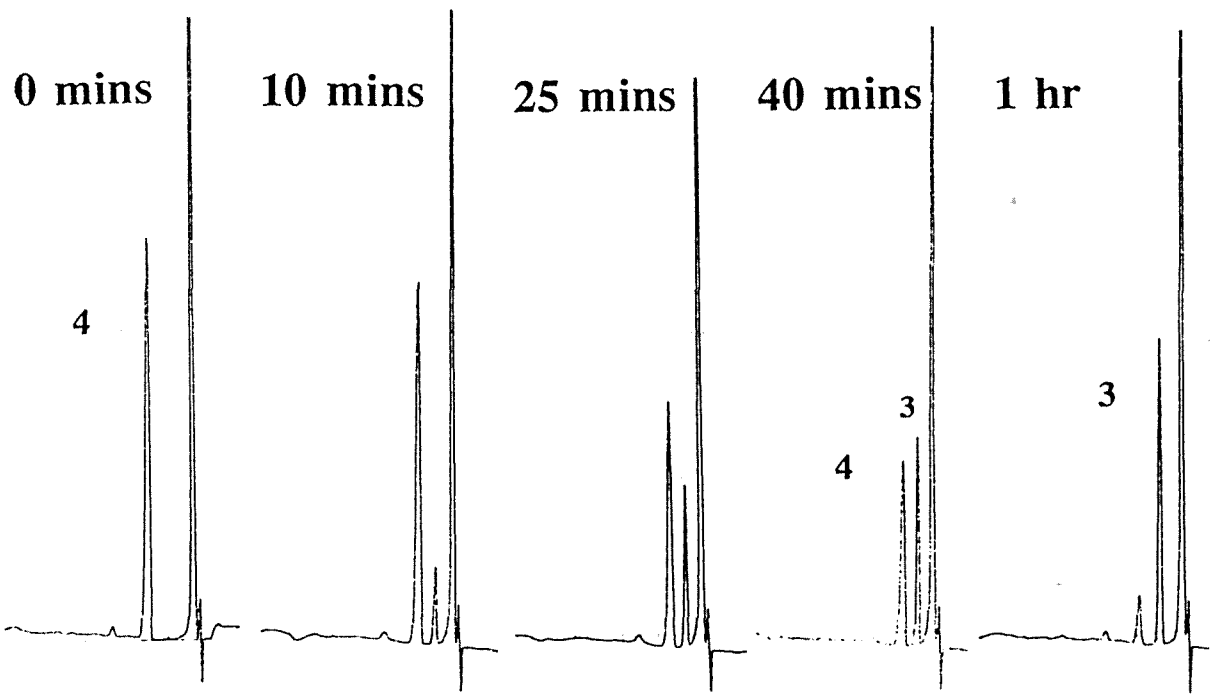


Figure 3.21 The breakdown of $(ala)_4$ by the aminopeptidase from *S.thermophilus*.
HPLC conditions; 0-5% B over 15 minutes, 10µl of the purified enzyme was added to 240µl of 2mM $(ala)_4$. 50µl of the reaction mixture were added to 25µl of 3% TFA to stop the reaction at timed intervals.
Peak 4 is $(ala)_4$ Peak 3 is $(ala)_3$
See section 3.3.5.3 for results.

Several small peptides with an N-terminal lysine or arginine were used to investigate the action of the lys-aminopeptidase. Earlier studies described above (Table 3.4) had demonstrated high activity with lysyl and arginyl derivatives. The effect of adjacent residues could not be determined from these studies. It is possible that the nature of subsequent residues could influence the aminopeptidases activity.
Degradation of each peptides was followed during incubation using HPLC. The peaks corresponding to degradation products were collected and identified by sequencing or amino acid analysis as specified in section 2.2.11.

Bradykinin: arg-pro-pro-gly-phe-ser-pro-phe-arg:
230µl of 3.3 mg ml^{-1} bradykinin were incubated with 1.4µg of purified aminopeptidase in 20mM phosphate buffer pH 7.0 for 24 hours at 25°C. 50µl samples were removed at 0,1,6 and 24 hours and the digestion was stopped by mixing each aliquot with 25µl of 3% TFA. 25µl of each sample was analysed by HPLC using a Vydac 218 TP column and a gradient running from 100% A (water with 0.1% TFA) to 70% A (solvent B acetonitrile containing 0.08% TFA) over 30 minutes.
After 24 hours of digestion the N-terminal arginine was still intact. It appears that the presence of the second proline applies sufficient steric restraint to prevent the hydrolyes of the arg-pro- peptide bond.

Thymopoeitin fragment: (32-36) arg-lys-asp-val-tyr:
200µl of 3mgml^{-1} thymopoeitin was incubated with 20µl (1.4µg) of the purified aminopeptidase in 20mM phosphate buffer pH 7.0. 50µl samples were removed at 0, 1, 2 and 5 hours. The samples were analysed by HPLC with a gradient 0-30% B over 30 minutes.
This peptide was degraded after one hour. The only detected product in the HPLC elution profile was a peptide with the sequence asp-val-tyr, the arg and the lys having been rapidly removed leaving a tripeptide that was not degraded, even after five hours (Figure 3.22).

β-lipotropin fragment: (88-91) lys-lys-gly-glu:
3.3µg of β-lipotropin in 200µl of phosphate buffer pH 7.0 was incubated with 20µl (1.4µg) of aminopeptidase for 5 hours at 30°C. Samples were removed after 0, 1, 2 and 5 hours and analysed by HPLC with a gradient from 0-10% B over 15 minutes. The single peak detected after one hour incubation was collected and analysed by amino acid analysis. It was found to be a tripeptide of composition glu 1.0 gly 1.0 and lys 1.0 corresponding to the C-terminal tripeptide lys-gly-glu. No further digestion could be detected after 5 hours digestion.
The degradation pattern of these three peptides confirms the previous observation that lys-aminopeptidase is relatively inactive against tripeptides and is unable to hydrolyse X-pro-peptide bonds.

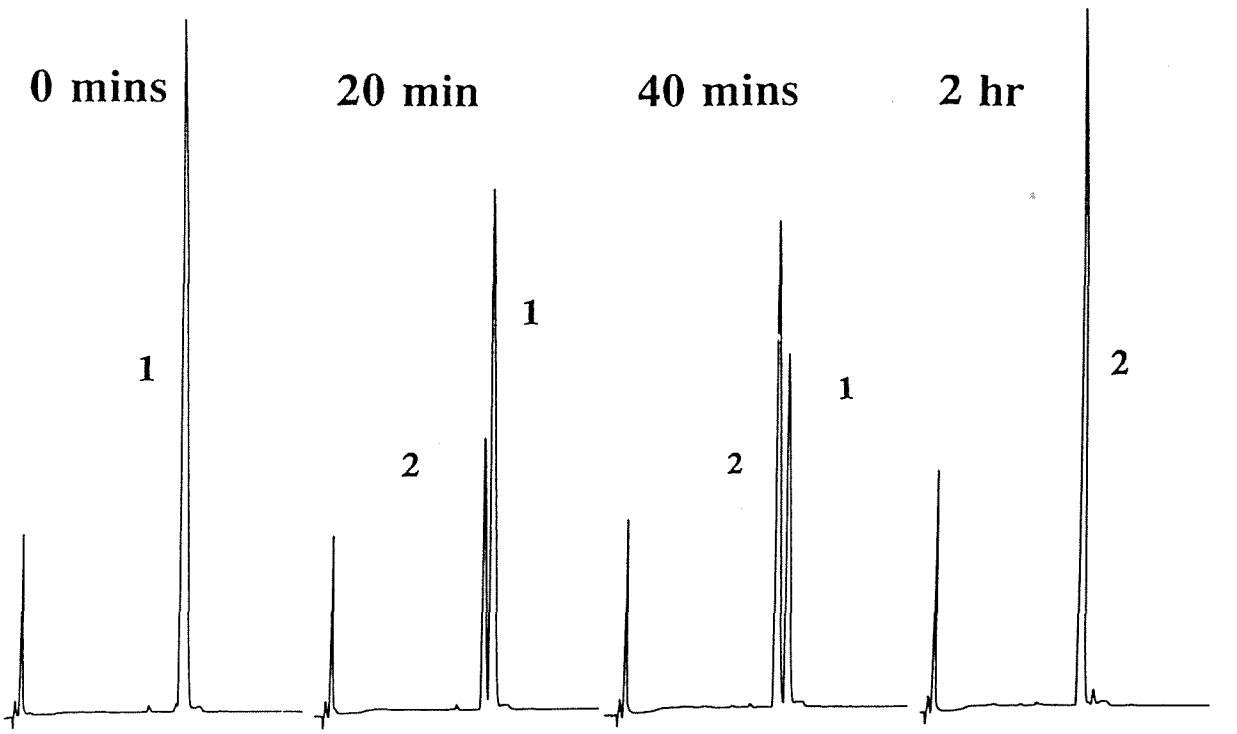


Figure 3.22 Degradation of Thymopoeitin fragment (32-36) by the aminopeptidase from *S.thermophilus*

Thymopoeitin was eluted with a gradient of 0-30% B over 30 minutes.

Peak 1 arg-lys-asp-val-tyr Peak 2 lys-asp-val-tyr

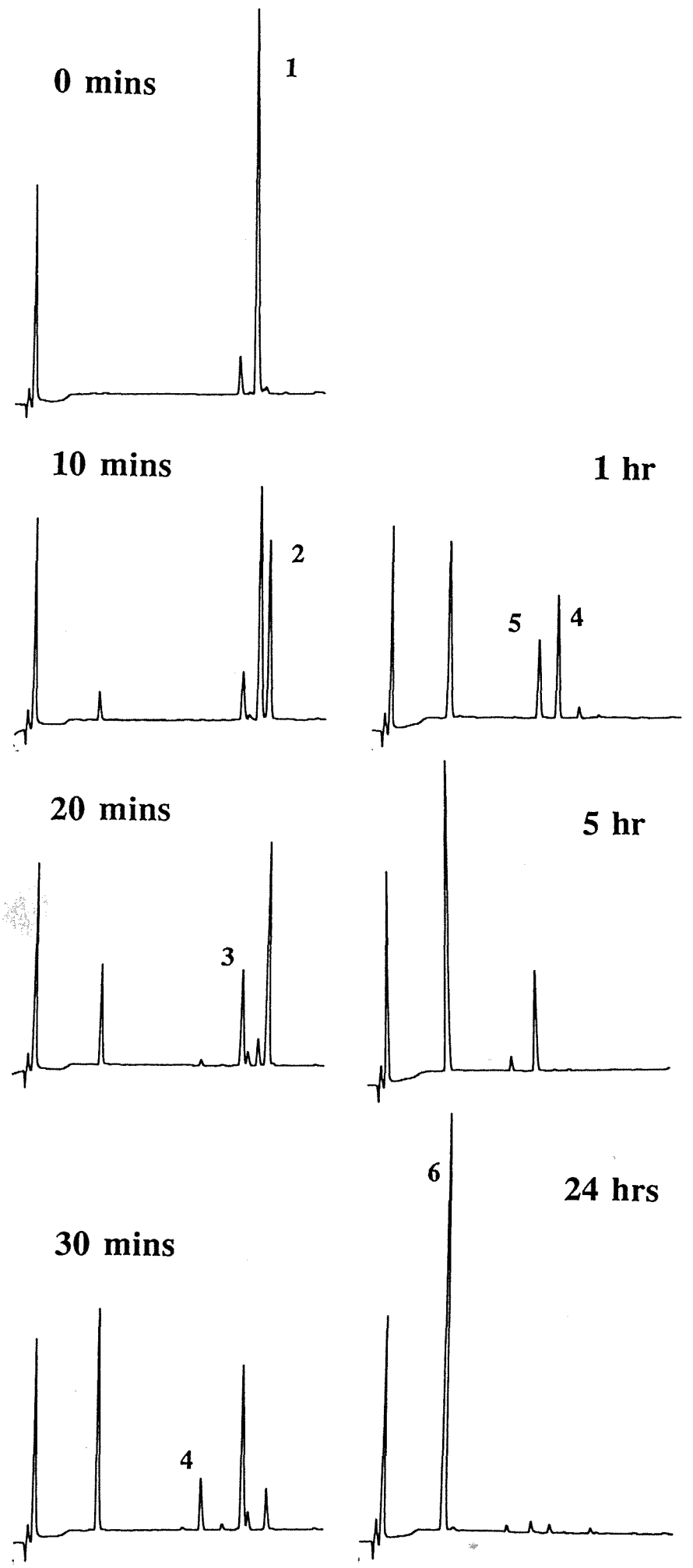
α–casein : fragment (90-95) arg-tyr-leu-gly-tyr-leu:

220μg of α-casein fragment in 250μl of 20mM phosphate buffer pH 7.0 were incubated with 1.4μg (20μl) of purified lys-aminopeptidase at 25°C, 25μl samples were removed from the digest at 0, 10, 20, 30 and 60 minutes and 5 and 24 hours and stopped by adding to 25μl of 3% TFA. 40μl from each sample was analysed by HPLC using a linear gradient from 0-50% B (Solvent A: water-0.1% TFA and B: Acetonitrile-0.08% TFA) over 30 minutes at a flow of 1 ml min^{-1}. Each peak was collected and identified by sequence.
The degradation of the peptide was more extensive than expected(Figure 3.23) in that after an initial rapid removal of the first three residues over sixty minutes (Figure 3.24) there was a further slow hydrolysis of the remaining tripeptide into its constituent amino acids (Figure 3.25). This result was unexpected as the previous experiments had indicated that the enzyme had limited or no capacity to degrade tripeptides or dipeptides.
Tyrosine was followed as the end product of hydrolysis rather than leucine as free leucine gave no signal at 220nm during HPLC analysis where as the tyrosine produced a strong signal.

Figure 3.23 Degradation of α–casein fragment (90-95) by the aminopeptidase from *S.thermophilus*

Peak 1 arg-tyr-gly-tyr-leu
Peak 2 tyr-leu-gly-tyr-leu
Peak 3 leu-gly-tyr-leu
Peak 4 gly-tyr-leu
Peak 5 tyr-leu

Samples were run on a gradient of 0-50% B over 30 minutes, at a flow rate of 1ml min^{-1} and at an absorbance of 220nm.
HPLC separation was carried out on a Vydac TP 18 column.
Solvent A Water-0.1% TFA
Solvent B Acetonitrile-0.08% TFA


0 mins
1
10 mins
2
1 hr
5
4
20 mins
3
5 hr
30 mins
4
6
24 hrs

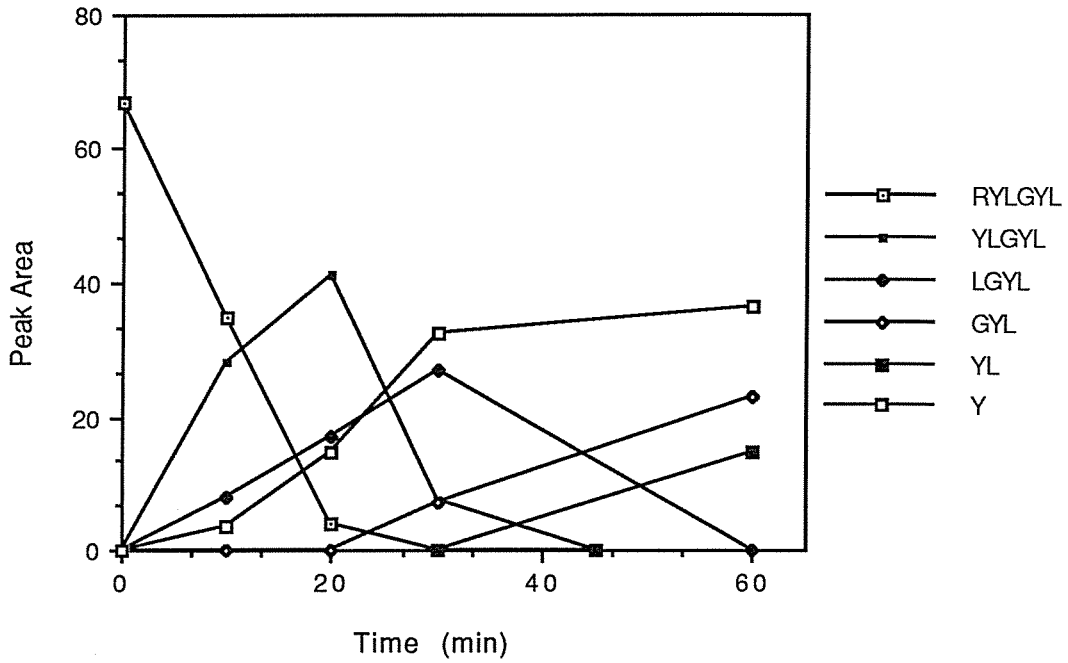


Figure 3.24 α-casein fragment (90-95) degradation by lys-aminopeptidase.

Separation of peptides by HPLC as in Figure 3.17 except that a linear gradient of 0-50% B was used over 30 minutes

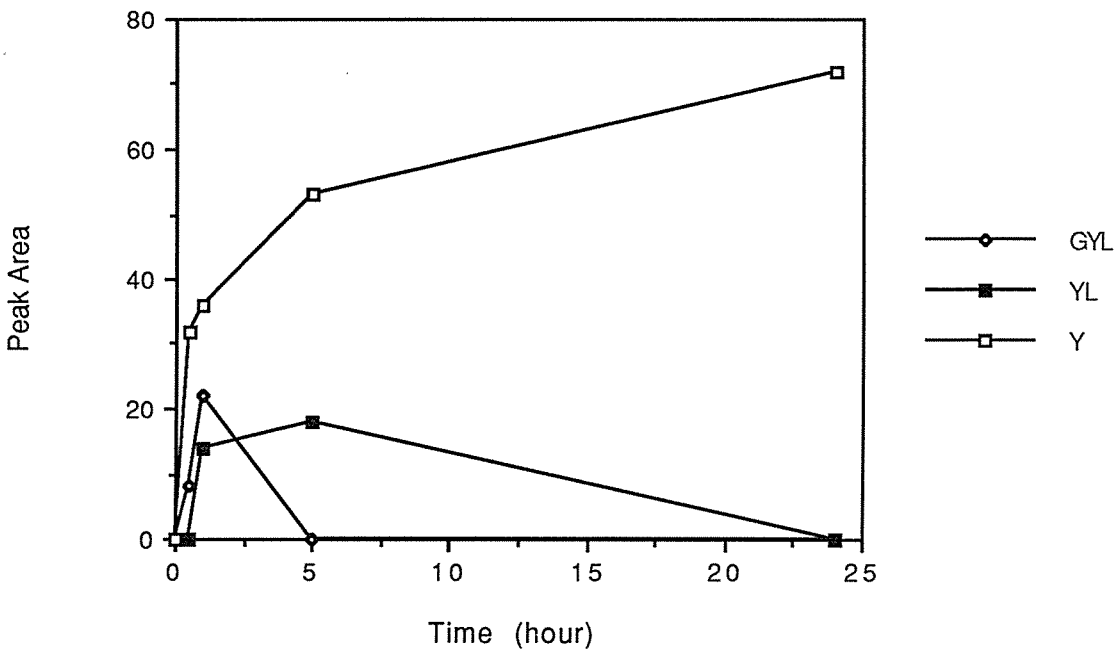


Figure 3.25. α-casein fragment (90-95) hydrolysis over 24 hours, by the lys-aminopeptidase.

Conditions as in Figure 3.24

3.3.5.4 Action of the aminopeptidase on lactococcal proteinase-generated fragments of β-casein:

Two of the major peptides obtained from a 4 and 24 hour digests of β-casein by the cell-wall proteinase from *L.cremoris* 4409 H2 were used as substrates.These had been previously purified by HPLC. The two peptides (isolated by Miss A.Williams) originated from cleavage of β-casein at sites close to the C-terminus (Figure 1.1).

β-casein: fragment (176-182) lys-ala-val-pro-tyr-pro-gln:

The peptide was dissolved in 200µl of water and was digested with 20µl of enzyme (1.4µg) at 25°C. 50µl samples were removed at 0, 1, 2, and 5 hours and stopped by adding to 25µl of 3% TFA. Each sample was analysed by HPLC using a gradient from 0-50% B (Solvent A water- 0.1% TFA solvent B acetonitrile 0.08% TFA) over 30 minutes.
This peptide was degraded by the aminopeptidase to a final product identified by sequence analysis as val-pro-tyr-pro-gln. Thus the aminopeptidase had cleaved the N-terminal lys and ala residues but not the val-pro bond (Figure 3.26). This is consistent with the result obtained with bradykinin indication that a proline residue in the penultimate position prevents cleavage of the N-terminal residue.

β-casein: fragment (183-193) arg-asp-met-pro-ile-gln-ala-phe-leu-leu-tyr:

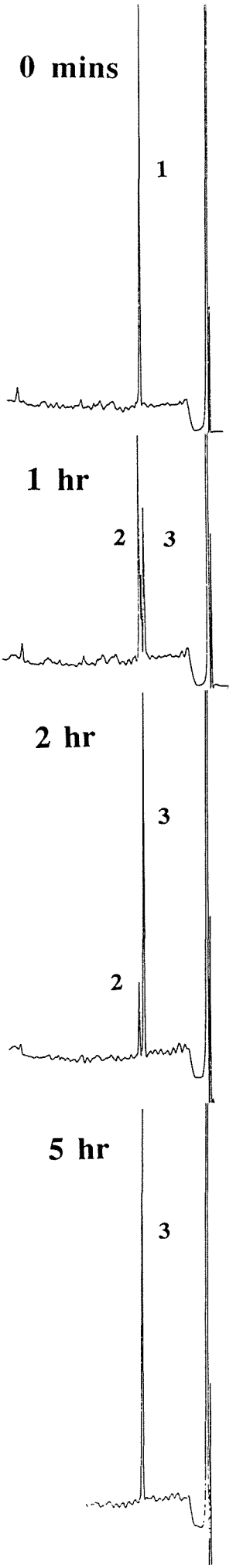
The peptide was dissolved in 200µl of water the reaction was initiated by the addition of 20µl of enzyme. The peptides were separated on HPLC with a gradient of 0-50% B over 30 minutes.
This peptide was not degraded even after a 24 hour incubation.

Figure 3.26 The hydrolysis of β-casein fragment (176-182) by the lys-aminopeptidase

Samples were run on a gradient of 0-50% B over 30 minutes.

Peak1 lys-ala-val-pro-tyr-pro-gln Peak 2 ala-val-pro-tyr-pro-gln

Peak 3 val pro tyr-pro-gln


0 mins
1
1 hr
2
3
2 hr
3
2
5 hr
3

3.4 Cellular Location of the lys-aminopeptidase:

As described in the introduction (Section 1.4) conflicting claims have been made for the cellular location of peptidase activity. To provide evidence for the location of the lys-aminopeptidase of *S. thermophilus* , cells harvested from RSM were separated into various fractions by following the protocol described in Section 2.2.10
At each stage of the fractionation procedure lactate dehydrogenase, an intracellular marker enzyme, was assayed to asses the extent of contamination by cytosolic protein (Table 3.6).

Location	Relative Lys pNA activity	Relative LDH activity
Loosely associated fraction	10%	0.3%
Cell Wall fraction	0.2%	0.4%
Cytosolic fraction	80%	90%
Pellet	10%	8.6%

Table 3.6 Relative proportion of aminopeptidase activity found in the different fractions of *S.thermophilus* cells.
Lactate dehydrogenase was used as the intracellular marker enzyme.
Assay procedures are described in section 2.2.7 (ii d). Fractionation protocol is described as in section 2.2.10

80% of the lys-aminopeptidase activity was found in the cytosolic fraction.Thus most of the aminopeptidase is apparently located intracellularly. It was also found that 10% of the lys activity was in the loosely associated cellular material released by incubating cells in phosphate buffer at 30^0C for 1 hour. Under these conditions only 0.3% of the lactate dehydrogenase was released indicating a low level of leakage of cytosolic protein. It is possible that there is another different peptidase associated with the cell surface with lower activity towards lys pNA. However this finding requires confirmation by repetition of the fractionation procedure since it is at variance with other results from this laboratory.

3.5 Comparative studies on the lys-aminopeptidase from two lactococcal strains:

One of the aims of this project was to compare the properties of the lys-aminopeptidase from the major groups of dairy starter bacteria, the thermophilic starter *S.thermophilus* and the two subspecies of the mesophilic starter *Lactococcus lactis* . The strain AM2 was chosen as representative of *L.lactis* subsp.*cremoris* since the properties of an aminopeptidase from this strain had already been reported (Neviani et al.,1989). Strain ML3 (otherwise known as NCDO 763) was selected as representative of the *lactis* subspecies since it has been previously used for studies of other proteolytic enzymes by the French group (Zevaco et al.,1990)

The lys-aminopeptidase from the lactococcal strains proved to be less stable than that from *S.thermophilus* and the arginine-Sepharose separation step did not give a satisfactory purification. The enzymes from both lactococcal strains have only been partially purified to a sufficient extent to obtain useful comparative information.

3.5.1 Partial Purification of lys-aminopeptidase from *L.cremoris* AM2:

The purification protocol previously used to purify the lys-aminopeptidase from *S.thermophilus* was also used to purify the aminopeptidase from *L.cremoris* AM2.

3.5.1.1 DEAE Cellulose Chromatography:

A culture of strain AM2 was grown in 2.5 litres of lactose broth inoculated with 25ml of a freshly grown 6 hour culture. The cells were grown and harvested using the conditions described in section 2.2.5 [7 hours at 30°C]. A cell-free extract (Section 2.2.6) was prepared from 31.7g of cells by a single pass through a cooled French press (at 38 Mpa). The resulting cell-free extract (25ml) was loaded directly onto a DEAE-23 cellulose column and eluted using identical conditions to those described for the *S.thermophilus* in section 3.2.1.The fractions were assayed for protein and lys-pNA hydrolysis.The elution profile of the aminopeptidase is shown in Figure 3.27
The profile of the aminopeptidase elution was different from that obtained for the *S.thermophilus* lys-aminopeptidase(Figure 3.1); the enzyme was eluted at a lower salt concentration (approximately 9 mmho in comparison with 12 mmho for the *S.thermophilus* aminopeptidase).The purification and recovery of the aminopeptidase was similar to that obtained for the *S.thermophilus* (Table 3.2 , 3.7).

Fractions containing the highest lys-pNA hydrolysing activity were pooled and further purified using gel permeation chromatography on a Sephacryl S-300.

3.5.1.2 Gel Permeation Chromatography on Sephacryl S-300:

The pooled fractions with high lys-aminopeptidase activity were concentrated to between 5-10ml by ultrafiltration using a PM30 membrane. Chromatography was carried out on a 2.6x90cm column as described in section 3.2.2.The elution profile is shown in Figure 3.28. The activity eluted as a single peak at an elution volume of 290 ml .The elution profile of the lys-aminopeptidase was very similar to that found for the aminopeptidase from *S.thermophilus* (Figure 3.2). As found with *S.thermophilus*, the lower molecular weight proteins were not fully removed from the enzyme possibly because the low ionic strength of the eluting buffer may have resulted in association with the low molecular weight proteins (Figure 3.31).

3.5.1.3 Chromatography on arginine-Sepharose 4B:

Fractions from the Sephacryl S-300 column containing lys-aminopeptidase were pooled and loaded directly onto an arginine-Sepharose 4B column. Unlike the*S.thermophilus* enzyme, the lys-aminopeptidase activity did not separate from the major protein peak. The lys-aminopeptidase did not bind to the column but eluted with the majority of the protein (Figure 3.29). Consequently this step accomplished little overall purification [2-fold purification in comparison to the 36-fold enrichment for the *S.thermophilus* enzyme].

3.5.1.4 FPLC on Mono Q:

The pooled active fractions from the arginine-Sepharose column were desalted on a G-25 Sephadex column, equilibrated in 20mM Bis-Tris-Propane (BTP) buffer. The eluted aminopeptidase activity was concentrated using a Centricon microconcentrator and further purified at room temperature on a Mono Q HR 5/5 FPLC column under the conditions described in section 3.2.4. The FPLC profile had one major peak, which contained several sub-peaks (Figure 3.30). Both peaks A and B showed lys-aminopeptidase activity and were collected separately and cooled immediately on ice.

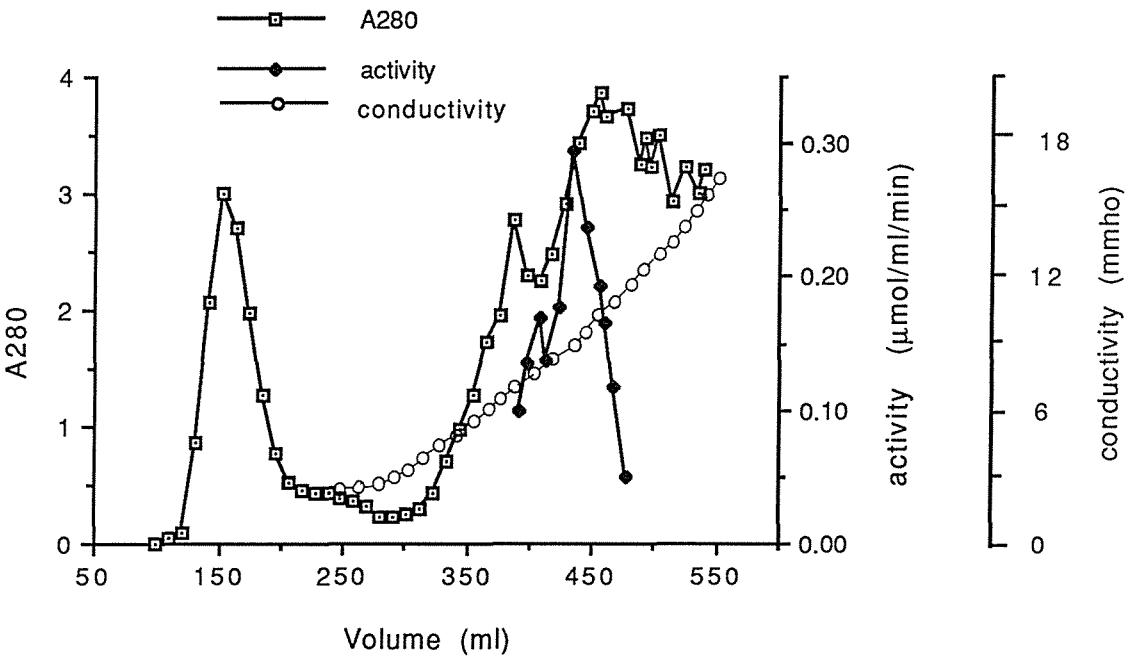


Figure 3.27 Elution profile of the aminopeptidase from *L.cremoris* AM2 from DEAE 23-cellulose chromatography.

Elution conditions: protein was eluted from the column (3x24cm) with a gradient of 0-0.8M NaCl in 20 mM phosphate buffer pH 7.0.

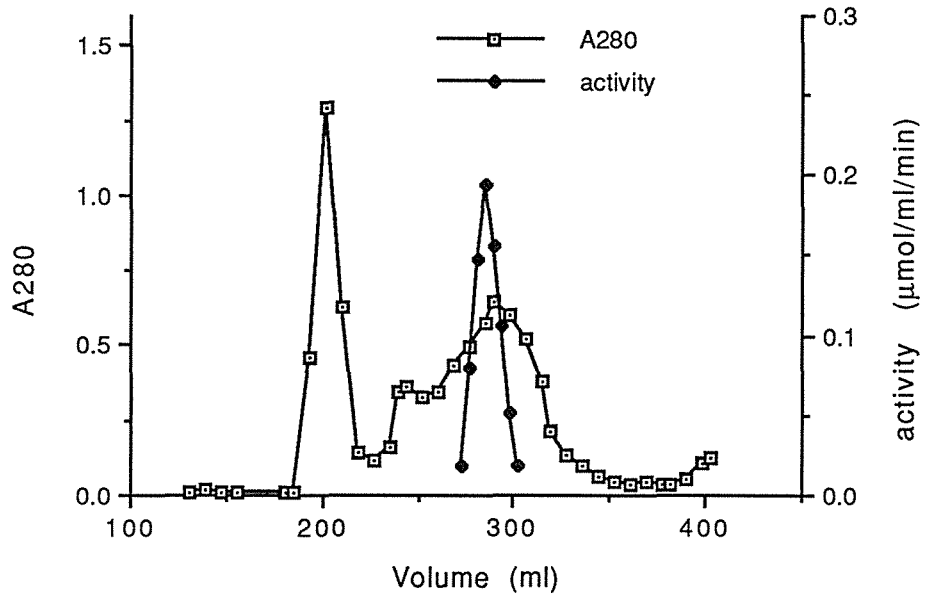


Figure 3.28 Elution profile of the lys-aminopeptidase from a Sephacryl S-300 gel filtration column.

The column (2.6x90cm) was eluted with 20mM phosphate pH 7.0 at 0.3mlmin^{-1}

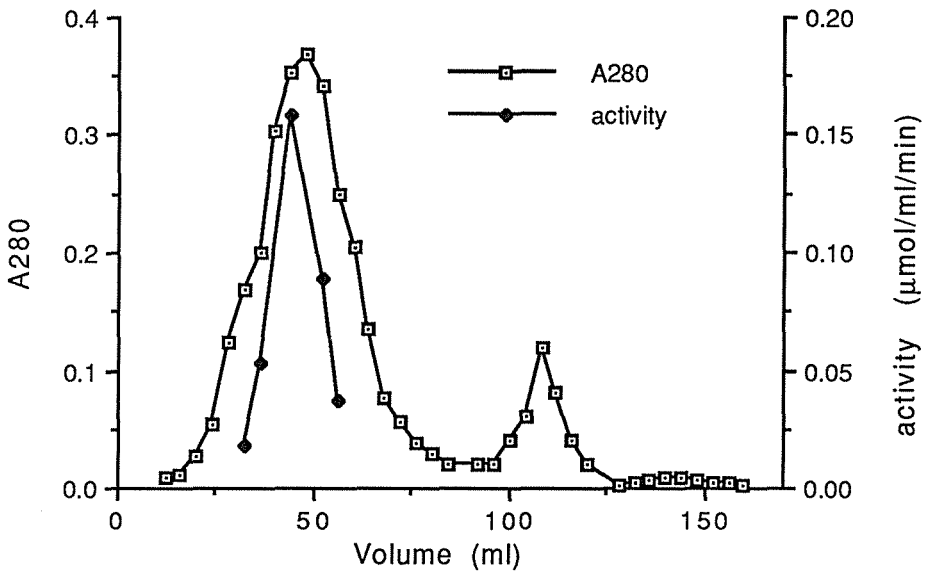


Figure 3.29: Elution profile of the lys-aminopeptidase from Arginine-Sepharose 4B
The column (2x10cm)was eluted with a gradient of 0.2-0.6M NaCl in 20mM phosphate buffer pH 7.0

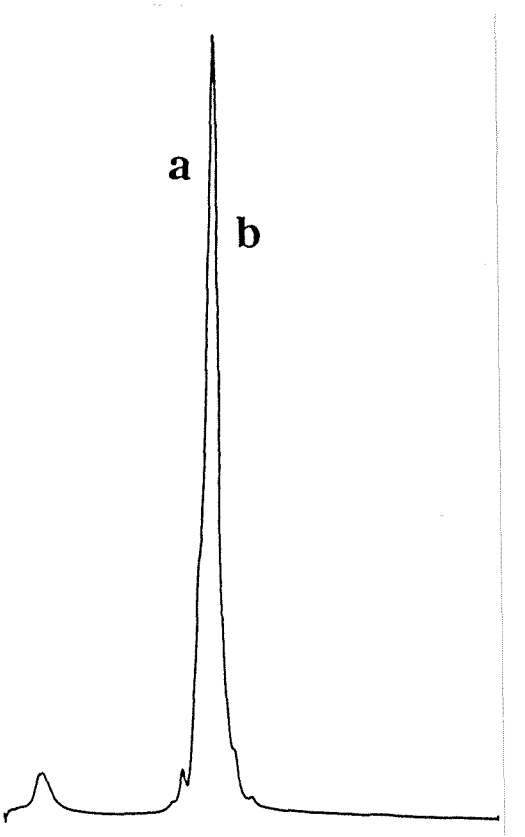


Figure 3.30 FPLC trace of the aminopeptidase from *L.cremoris* AM2
Two peaks were collected that showed lys-aminopeptidase activity.(a,b)

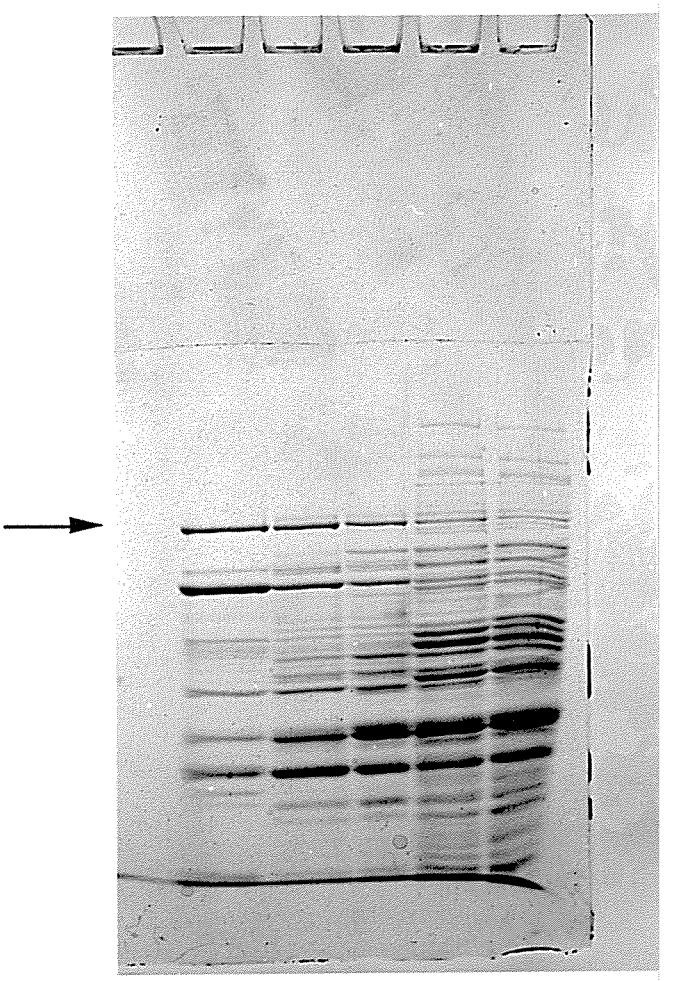
3.5.1.5 Assessment of purification:

The final recovery of activity from Peaks A plus B for lys-aminopeptidase from strain AM2 was approximately 3% compared to 7% obtained with *S.thermophilus* (Table 3.2). The purification was only 12 to 15-fold compared to the 500-fold for *S.thermophilus* mainly as a result of the low efficiency obtained with the arginine-Sepharose step.

	Total Protein mg	Total Activity $\mu molmin^{-1}$	Specific activity	Recovery (%)	Purification Fold
CFE	552	17.53	0.032	100	1
DEAE	63	9.9	0.157	56	4.9
S-300	18.7	5.72	0.305	32.6	10.5
arg-Sepharose	8.51	4.6	0.54	26.2	17
FPLC Peak A	1.075	0.4	0.37	2.3	11.6
PeakB	0.36	0.168	0.47	1	15

Table 3.7: Summary of the purification of lys-aminopeptidase from *L.cremoris* AM2
Quantitities specified in this table are those from 31.3 g wet packed weight of cells.

Each step of the purification protocol was monitored using SDS-PAGE. This showed that there was a major contaminant in the enzyme obtained from the FPLC step (Figure 3.31). All the subsequent characterisation experiments were carried out using peak A although the enzyme in this peak had a lower specific activity.



1 2 3 4 5

Figure 3.31 SDS-PAGE of the purification steps for the aminopeptidase from *L.cremoris* AM2

Lane 1 Mono Q (peak`A)
Lane 2 Arginine-Sepharose
Lane 3 Sephacryl S-300
Lane 4 DEAE-23
Lane 5 Cell-free extract.

3.5.2 Characterisation of the lys-aminopeptidase from *L.cremoris* AM2:

3.5.2.1 Molecular Weight Determination by SDS-PAGE:

A 7.5% SDS gel was used to determine the molecular weight of the lys-aminopeptidase from *L.cremoris* AM2. Because of the difficulty in purifying this enzyme unequivocal identification of the enzyme on the SDS-gel was not possible. Hence the aminopeptidase was assumed to be the protein band which increased in intensity on SDS-PAGE throughout the purification. (figure 3.31). Thus the monomeric molecular weight of the lys-aminopeptidase was estimated from duplicate gels run with standards to be approximately 104,000 Daltons ± 5000 (Figure 3.32 & 3.34).

3.5.2.2 Native molecular weight determination by S-300 gel chromatography:

The native molecular weight of the lys-aminopeptidase was determined by gel-filtration on a Sephacryl S-300 (2.6x90cm) column. The enzyme peak was detected by a lys-pNA assay and the molecular weight of the enzyme was determined from the peak elution volume which was 290ml. This gave an approximate molecular weight of 100,000 Daltons (Figure 3.33).

This indicates that the lys-aminopeptidase from the *L.cremoris* AM2 is a monomer with a molecular weight of approximately 100-104 kDa.

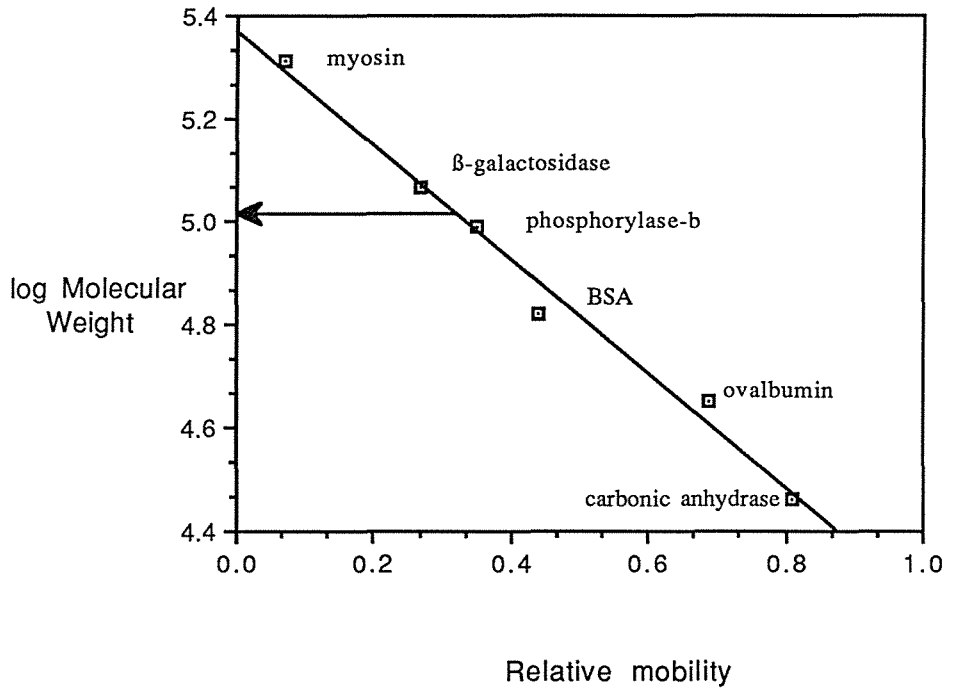


Figure 3.32 Molecular weight determination of lys-aminopeptidase run on a 7.5% SDS-PAGE.

Subunit molecular weight of the aminopeptidase is indicated with an arrow.The molecular weight markers used are as in Figure 3.34.

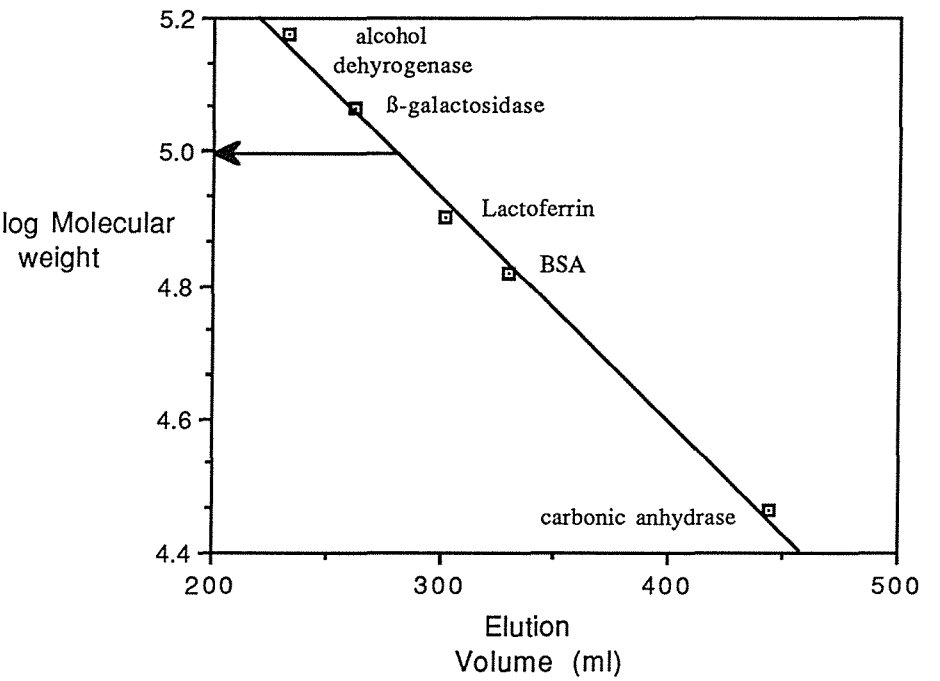


Figure 3.33. Native molecular weight determination of lys-aminopeptidase by S-300 gel chromatography.

The aminopeptidase molecular weight is indicated by an arrow.

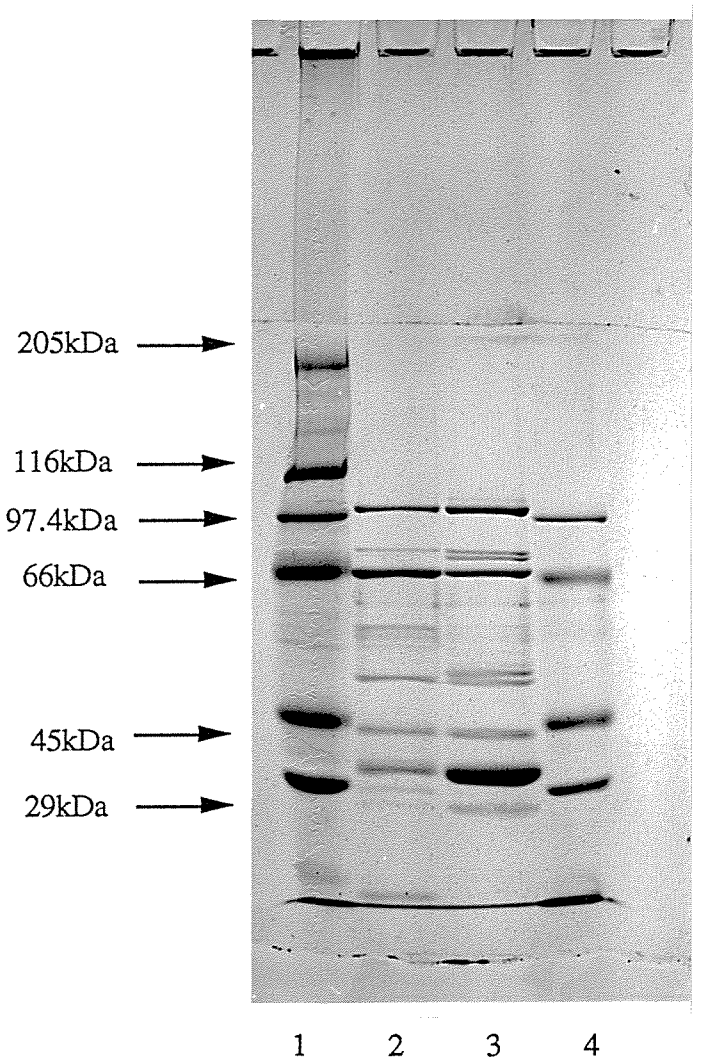


Figure 3.34 SDS-PAGE of the molecular weight of the lys-aminopeptidase from *L.cremoris* AM2

Molecular weight standards used were: Myosin, 205kDa, β-Galactosidase 116kDa, Phosphorylase b 97.4kDa, Bovine serum albumin 66kDa, Ovalbumin 45kDa and Carbonic anhydrase 29kDa.

Lane 1 molecular markers Lane 2 Peak A

Lane 3 Peak B Lane 4 molecular markers

3.5.2.3 The effect of pH and temperature on the activity of the lys-aminopeptidase:

The optimal pH for aminopeptidase activity was determined by measuring the hydrolysis of lys-pNA over a pH range from 4.5-8.5 in 100mM MES and BTP buffers using a pH meter calibrated at two pHs with freshly prepared pH standards. The pH of the buffers was checked before and after the addition of the enzyme and was found to be unchanged. The assay was carried out as described in section 2.2.7.
The profile (Figure 3.35) shows a pH optimum of 6.9-7.1, with a rapid fall off in activity on either side of this pH.

The optimal temperature for the enzyme activity was determined by incubating the enzyme in the assay buffer (pH 6.8) at a set temperature for 10 minutes in a Gilford 260 Spectrophotometer before initiating the reaction by the addition of the substrate (lys-pNA). The optimal temperature for the lys-pNA hydrolysing activity was found to be 45-50°C (Figure 3.36)

3.5.2.4 Effect of divalent cations on aminopeptidase activity:

The effect of several divalent cations on the activity of the enzyme was also studied. Inhibition of the enzyme occurred at concentrations of 0.5mM Zn^{2+} and Co^{2+}, total inhibition of the enzyme occurred in the presence of Cu^{2+}, while the metal ions Mn^{2+} and Mg^{2+} had little or no inhibitory effect (Figure 3.37 & Table 3.8).

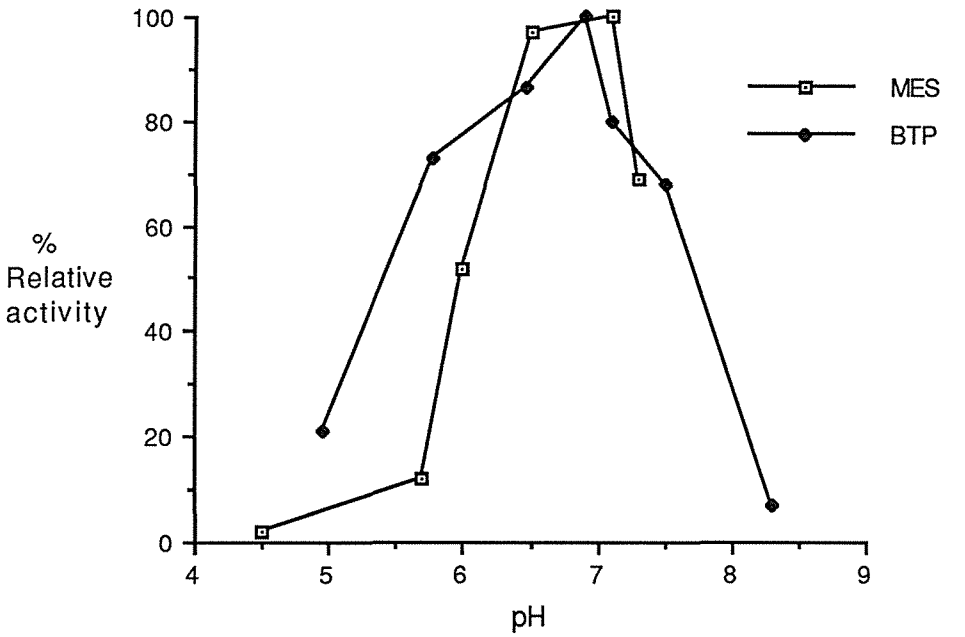


Figure 3.35. pH profile of activity obtained for lys-aminopeptidase from *L.cremoris* AM2.

Over the pH range of 4.5-8.5 in 100mM MES or Bis-Tris-Propane. The enzyme was incubated at the pH before the reaction was initiated by the addition of 0.5mM lys-pNA.

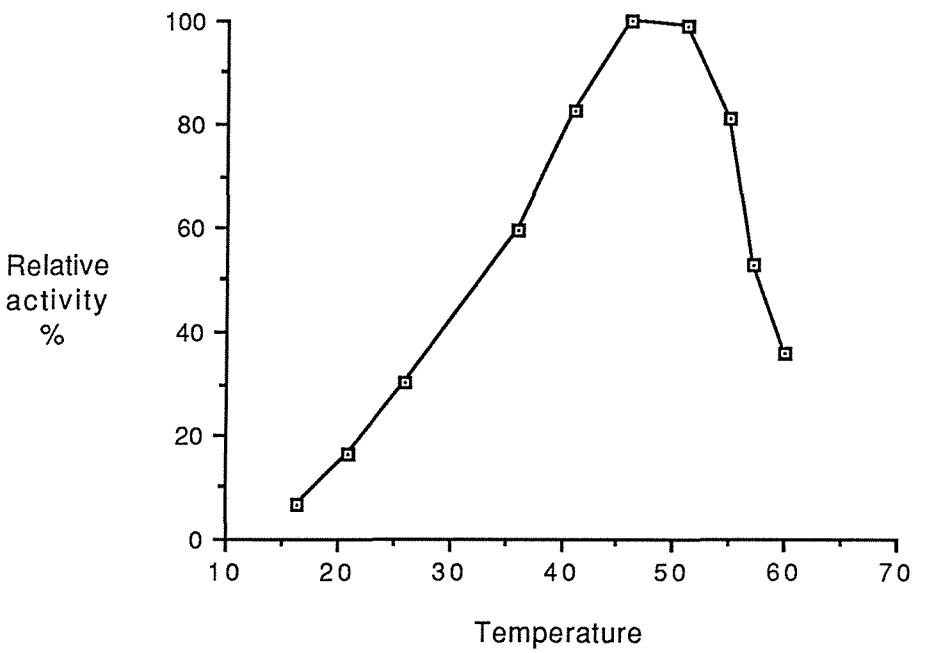


Figure 3.36. Temperature activity curve showing the optimal temperature for lys-aminopeptidase activity.

The enzyme was incubated for 10 minutes in 100mM MES (pH 6.8) at a set temperature the reaction was initiated by the addition of 0.5mM lys-pNA.

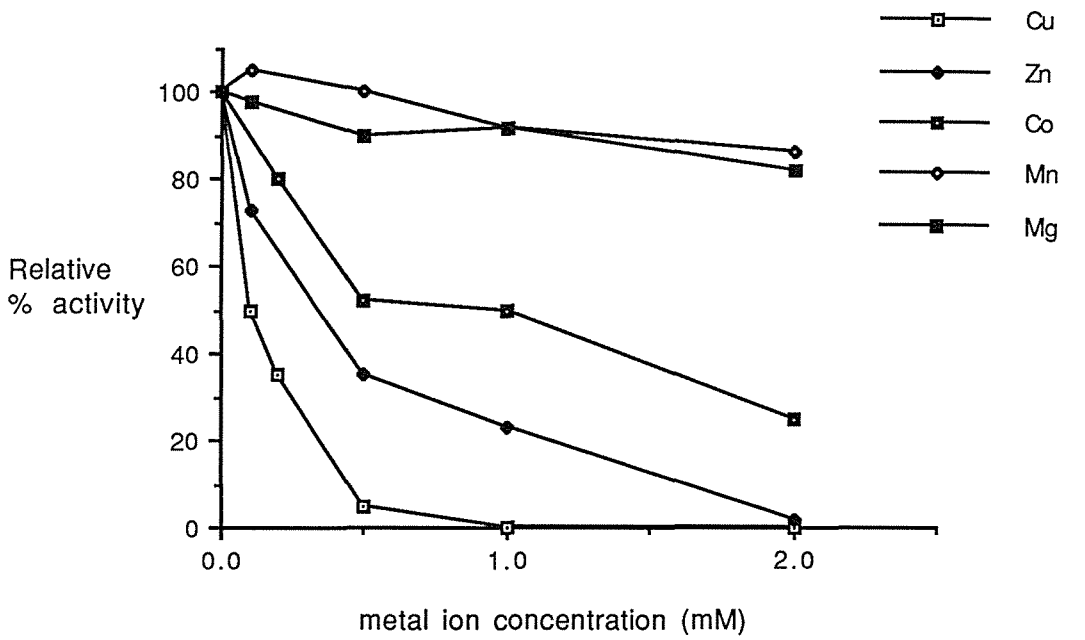


Figure 3.37 Inhibition of the aminopeptidase with various metal ions over a range of concentrations.

The enzyme (0.01mg) was incubated for 10 minutes with various metal ions in pH 6.8 buffer at 21°C. The assay was initiated with the addition of 0.5mM lys-pNA (final concentration).

Reagents	% activity at 1mM
no addition	100
Zn	23
Cu	0
Co	50
Mn	88
Mg	91

Table 3.8: The effect of cations on lys-aminopeptidase activity at 1mM concentration.

Activity is expressed as a percent of the activity with no inhibitor present.

Enzyme activity was determined under the standard conditions at pH6.8. Each assay was incubated with the appropriate metal ion (as the chloride) for 10 minutes at 21°C before initiating the reaction by the addition of 0.5mM (final concentration) lys-pNA

3.5.2.5 Effect of inhibitors on activity of the lys-aminopeptidase:

The effect of inhibitors on the activity of the aminopeptidase was investigated. Enzyme from Peak A (0.01mg) was incubated with various concentrations of inhibitor. After equilibration of the enzyme in 100mM MES buffer in the presence of the inhibitor for 10 minutes at room temperature the enzyme activity was determined using the standard assay. Results are shown in Table 3.9 and in Figure 3.38.

Aminopeptidase activity was completely inhibited by the sulphydryl-blocking reagents pCMB and iodoacetic acid at 1mM (Figure 3.38).

PMSF a serine protease inhibitor, produced no inactivation of the enzyme at 1mM and even produced a slight (12%) increase in activity (Table 3.9). The protease inhibitor, TLCK (an inhibitor of serine and some cysteine proteases) at 1mM strongly inhibited the enzyme; only 13% of the original activity being left after a 10 minute incubation with the enzyme.

Treatment of the enzyme with the metal chelating agents EDTA and 1,10-phenanthroline inactivated the enzyme. In the presence of (0.2mM) 1,10-phenanthroline the enzyme was completely inactivated and 80% inactivated in the presence of 1mM EDTA (Figure 3.38).

Activity of the 1,10-phenanthroline (0.5mM) treated enzyme could be restored to 28% of its original activity in the presence of 1mM $CoCl_2$ and to 4.5% by 1mM $MnCl_2$ with no effect produced by 1mM Zn^{2+},Cu^{2+} or Mg^{2+} (Table 3.10). EDTA inactivation could not be reversed by any of the metal ions at the concentration used.

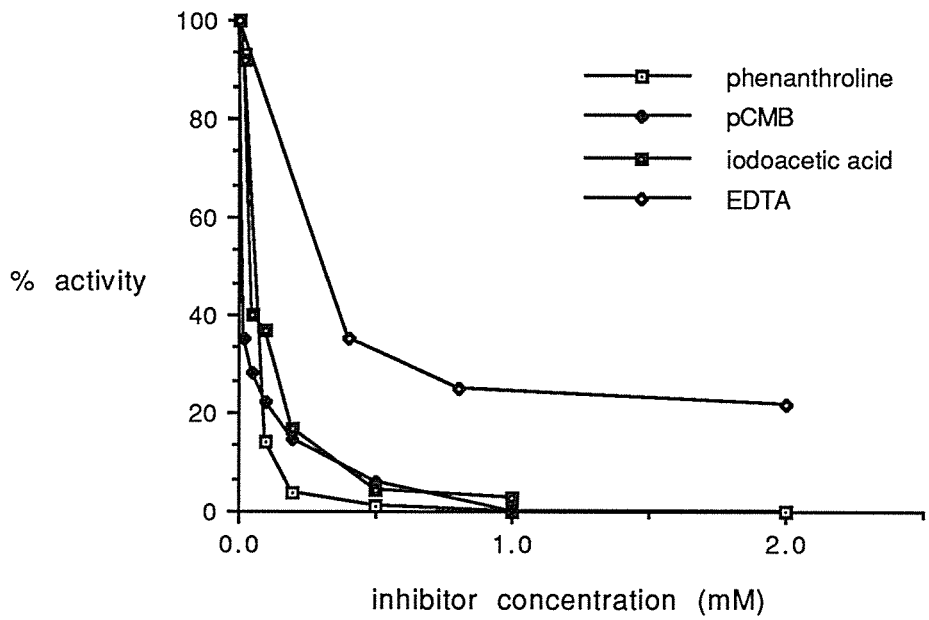


Figure 3.38 Inhibition of the aminopeptidase in the presence of inhibitors.
The enzyme (0.01mg) was incubated with an inhibitor for 10 minutes and the reaction initiated by the addition of 0.5mM lys-pNA (final concentration).

Reagents		% activity at 1mM inhibitor concentration
no addition		100
Iodoacetic		2.6
pCMB		0.2
EDTA		21
1,10-phenanthroline		0
PMSF	10(min) incubation	112
	2(hours) incubation	112
TLCK	10(min) incubation	13
	30(min) incubation	15
	2(hours) incubation	14.6

Table 3.9. The effect of inhibitors on lys-aminopeptidase activity.
Activity is expressed as a percent of the activity with no inhibitor present. The enzyme was incubated for 10 minutes with each reagent and then the activity of the enzyme was measured by the addition of 0.5mM (final concentration) lys-pNA.

Reagent	% Activity
No reagents	100
0.5mM 1,10,phenanthroline	0
0.5mM 1,10 phen + 1mM Zn	1.7
0.5mM 1,10, phen + 1mM Mn	4.5
0.5mM 1,10, phen + 1mM Co	28
0.5mM 1,10, phen + 1mM Cu	0
0.5mM 1,10, phen + 1mM Mg	2

Table 3.10: Reactivation of lys-aminopeptidase inhibited by 0.5mM 1,10,phenanthroline with several metal ions at 1mM concentration.

3.5.2.6. Specificity of the aminopeptidase:

The specificity of the lys-aminopeptidase was investigated using several amino-acyl pNA substrates. The activity of the enzyme from Peak A was determined with a range of substrates at a single concentration (0.5mM final concentration) (Table 3.11). As was the case with*S.thermophilus*, enzyme activity was greatest with lys-pNA and exhibited activity with arg-pNA and leu-pNA. Activity with phe- ala- and gly-pNA was low and no activity was detected with pro-pNA.

Substrate	Relative activity (%)
lys-pNA	100
arg-pNA	49
leu-pNA	16
ala-pNA	6
phe-pNA	4
gly-pNA	<1
pro-pNA	0

Table 3.11 Activity of the lys-aminopeptidase towards several pNA.
All pNA were at 0.5mM final concentrations. Activities are expressed relative to that with lys-pNA. Assays were carried out as in section 2.2.7

The "general" aminopeptidases so far purified (Neviani et al. 1989; Tan & Konings 1990) have been found to have dipeptidase activity. During purification in the present study the major dipeptidase peak was partially separated from the lys-aminopeptidase peak (Lloyd,1989). The possibility that the partly purified aminopeptidase was contaminated with residual activity from a separate dipeptidase made it difficult to establish whether the lys-aminopeptidase had any intrinsic dipeptidase activity as claimed by other workers.

Peak A and Peak B from the final FPLC step (Figure 3.30) were tested separately with several dipeptide substrates. A comparison of the relative activities of these two fractions towards dipeptides could give some indication as to whether dipeptide activity was due to a contaminant enzyme or not.

Substrate (1.7mM)	Dipeptidase activity (average fluorescence units 30 min^{-1}) Peak A	Peak B	Ratio A/B
leu-gly	106	38	2.79
leu-ala	173	57	3.03
ala-ala	28	32	0.87
leu-gly-gly	6.5	213	0.03

Table 3.12 Activity of peak A and peak B towards several dipeptides.
The dipeptidase assay was carried out as described in section 2.2.7 (ii).over a 30 minute interval, with each dipeptide at a final concentration of 1.7mM. The values shown are the dipeptidase activity for peak A and B relative to the aminopeptidase activity assayed at 0.5mM lys-pNA of the preparation.

By comparing the relative activities of peak A and B towards the tripeptide leu-gly-gly it can be seen that the tripeptidase activity seen in the lys-aminopeptidase is almost certainly due to contamination from a separate tripeptidase rather than intrinsic aminopeptidase activity acting on the tripeptide. It appears that peak A has dipeptide hydrolysing activity.
However these data are insufficient to permit any clear conclusion on the activity of the aminopeptidase towards dipeptides since it is unclear if the activity is due to dipeptidase contamination or to low dipeptide hydrolysing activity intrinsic to the aminopeptidase. It would need a pure enzyme preparation to resolve this matter.

3.5.2.7 Kinetic parameters of the lys-aminopeptidase from *L.cremoris* AM2:

The rate of hydrolysis of lys-p-nitroanilide over a concentration range of 0.02-1mM was assayed to determine the Km value of the enzyme for this substrate (Figure 3.39). The kcat was not determined as the true concentration of the lys-aminopeptidase in its partially pure state was unknown. The Km value was 0.34mM towards the substrate lys-pNA and the Vmax was 0.68 $\mu molml^{-1}$ min^{-1}. The Km value is similar to that of the *S.thermophilus* aminopeptidase for this substrate. The much lower Vmax value reflects the impure state of the enzyme and its low stability during purification. In view of the impurity of the enzyme further kinetic characteristics were not undertaken.

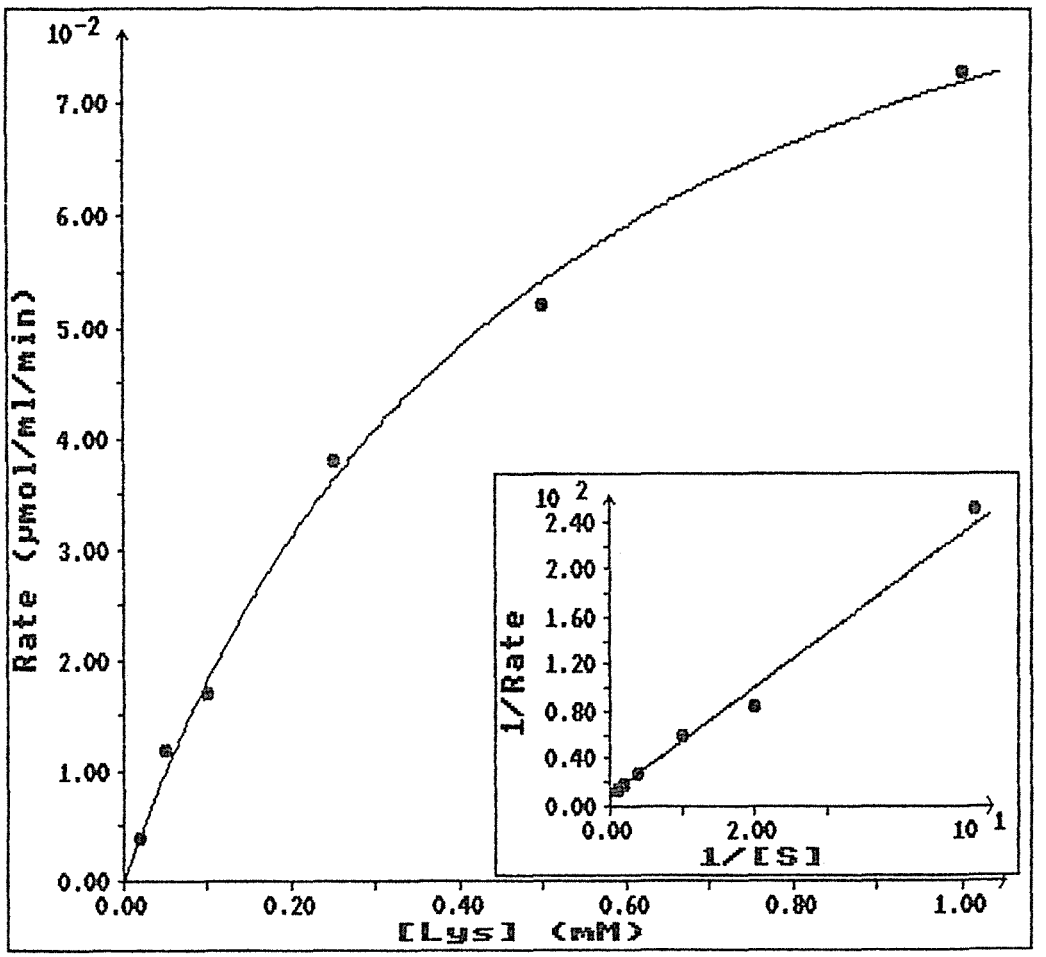


Figure 3.39 Rate versus concentration plot and Lineweaver-Burke plot for the lys-aminopeptidase from *L.cremoris* AM2 using the substrate lys-pNA.

3.5.3.Partial Purification of the lys-aminopeptidase from *L.lactis* ML3:

The protocol used to purify the aminopeptidase from *L.lactis* ML3 was identical to that used to purify the lys-aminopeptidase from *S.thermophilus* 5109.

3.5.3.1 DEAE Cellulose Chromatography:

A cell-free extract [40ml] from 62g wet weight of *L.lactis* ML3 grown in lactose broth was loaded onto DEAE cellulose column (3x24cm) and eluted with a gradient from 0-0.8M NaCl in 20mM phosphate buffer pH 7.0 in an identical manner to that used for the purification of the lys-aminopeptidase from *S.thermophilus* 5109 (Section 3.2.1). The elution profile of lys-aminopeptidase activity from the DEAE cellulose column was different from that obtained for either the *S.thermophilus* or the *L.cremoris* AM2 aminopeptidase. The enzyme was eluted at a lower salt concentration of approximately 5mmho (Figure 3.40) in contrast to the 9 and 12 mmho salt concentration required to elute the aminopeptidases from *L.cremoris* AM2 and*S.thermophilus* under identical conditions.
The purification of the lys-aminopeptidase at this step (about 3-fold) was similar to that obtained for the *S.thermophilus* and the *L.cremoris* enzyme (Table 3.12). The recovery was high with 78% of the original activity being recovered in contrast with the 56% recovery for the AM2 enzyme.

3.5.3.2 Gel Permeation Chromatography on Sephacryl S-300:

The activity peak from the DEAE step was concentrated to a volume of 8.0 ml on a Diaflo PM30 membrane and loaded onto a Sephacryl S-300 gel chromatography column [2.6x90cm]. The column was run at a flow rate of 0.3ml min^{-1}. The lys-aminopeptidase peak eluted at approximately 260ml (Figure 3.41). The activity elution profile closely resembled those found for the other aminopeptidases purified in this study (Figure 3.2 & 3.27).

3.5.3.3 Chromatography on Arginine-Sepharose 4B:

The peak from the gel permeation step was brought to 0.2 M NaCl and loaded directly onto an arginine-Sepharose 4B column (2x20cm). After washing, the column was eluted with a linear gradient of 0.2-0.6 M NaCl in 20mM phosphate buffer pH 7.0. The lys-aminopeptidase did not bind to the column and eluted in the protein break-through peak (Figure 3.42). Little purification was achieved by this step and the recovery of the enzyme was low (Table 3.12)

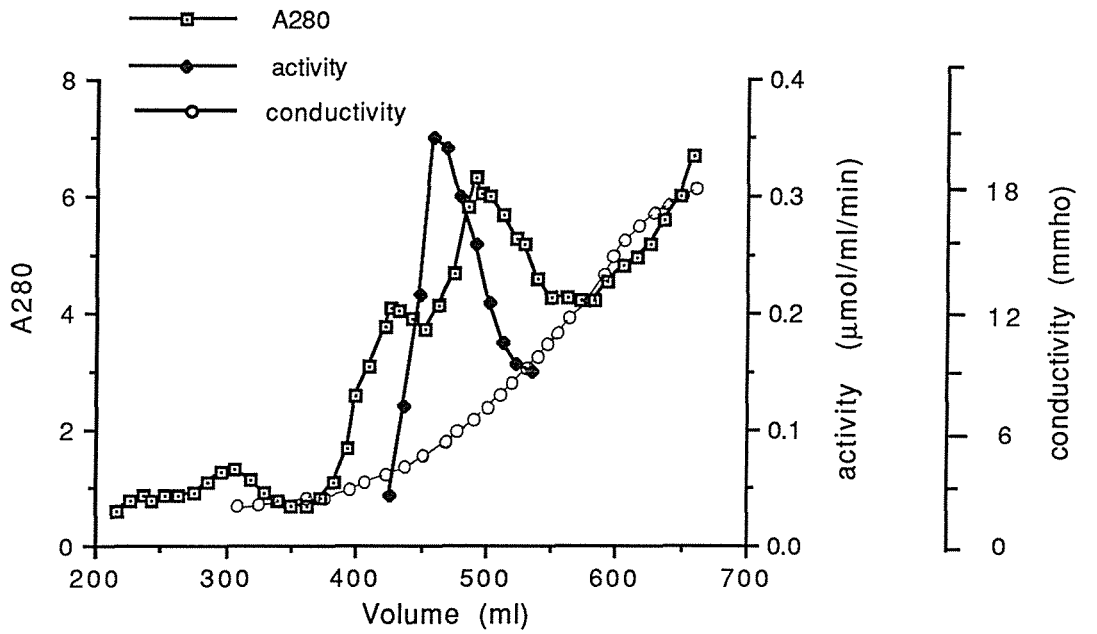


Figure 3.40 Elution profile of the lys-aminopeptidase from *L.lactis* ML3 on DEAE 23 cellulose

A gradient of 0-0.8M NaCl in 20mM phosphate buffer pH 7.0, was used to elute the aminopeptidase. Other conditions are described in section 3.2.1

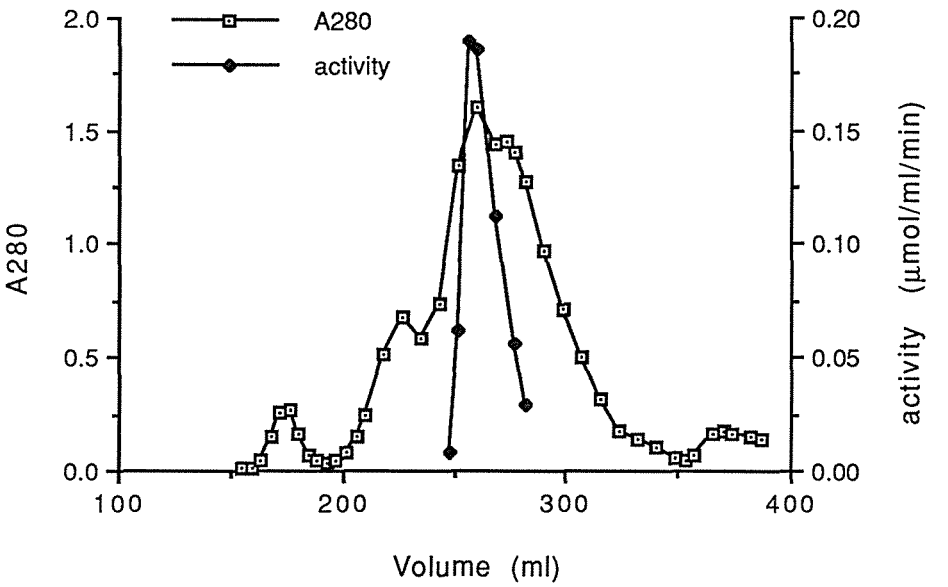


Figure 3.41 Elution profile of the lys-aminopeptidase from a Sephacryl S-300 gel filtration column.

Elution conditions were 20mM phosphate pH 7.0, at a flow rate of 0.3 ml min^{-1}.

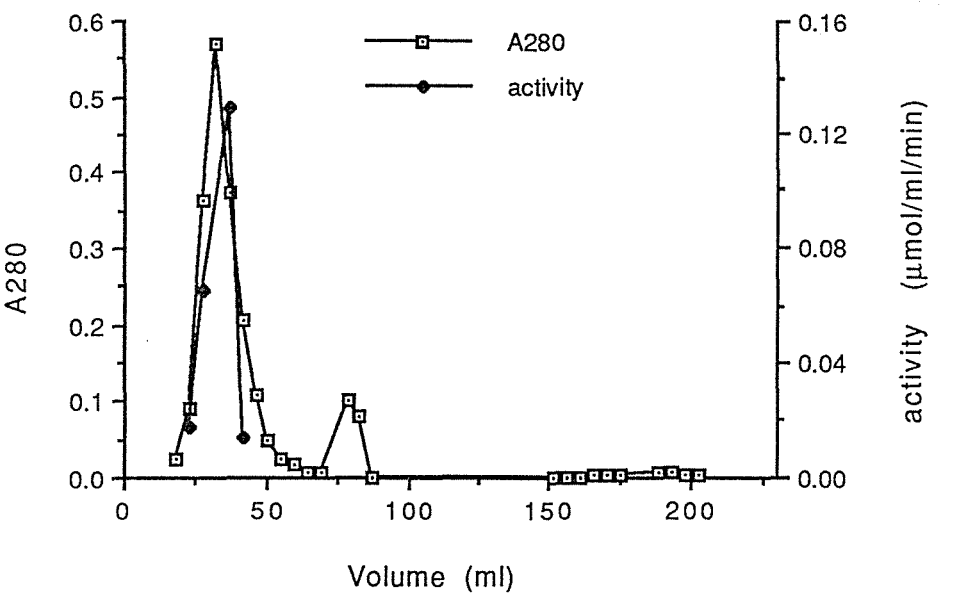
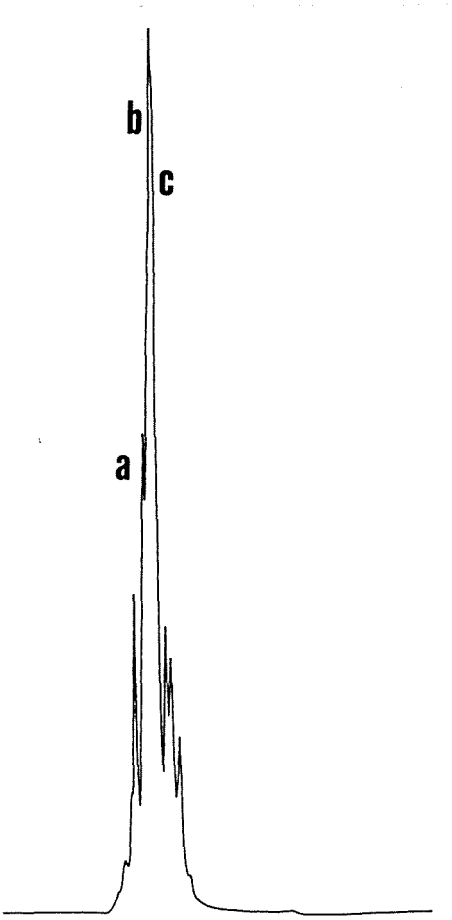
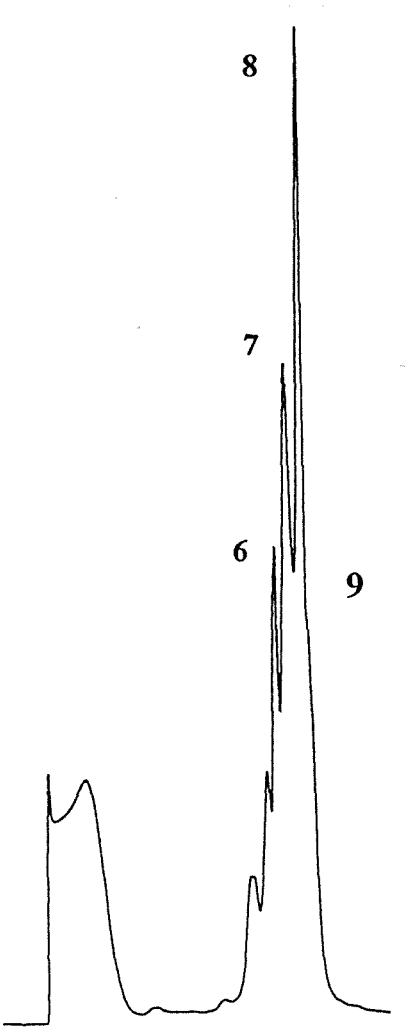


Figure 3.42 Elution profile of the aminopeptidase from an arginine-Sepharose column. Elution conditions are described in section 3.2.3

Figure 3.43 FPLC trace of the aminopeptidase from *L.lactis* ML3
Protein was run on a Mono Q anionic exchanger with a gradient of 20-100% B over 30 minutes.
Solvent A 20mM Bis-Tris-Propane pH 7.0.
Solvent B 20 mM Bis-Tris-Propane pH 7.0 with 1M NaCl
Three peaks were collected that had aminopeptidase activity (a,b,c) these were pooled.

Figure 3.44 FPLC of the aminopeptidaseusing a gradient of 20-60% B
The pooled samples from Figure 3.43 were run on a Mono Q with a gradient of 20-60% B over 30 minutes.
Peaks with aminopeptidase activity were collected separately (peaks 6,7,8 and 9).


b
c
a


8
7
6
9

3.5.3.4 FPLC on Mono Q:

The pooled fraction from the arginine-Sepharose column was desalted into 20mM Bis-Tris-Propane pH 7.0 on a Sephadex G-25 column (Section 3.2.4) and concentrated to approximately 10ml with a Centricon concentrator. 400µl aliquots of the concentrate were injected onto a Mono Q HR 5/5 anion exchange column and run using the standard program described previously. The protein profile for the lys-aminopeptidase eluting from the column was very different to that of the other two aminopeptidases purified in this study. The enzyme eluted in several fractions (Figure 3.43). The enzyme activity from 6 consecutive injections was pooled (peaks a, b and c). These pooled fractions gave a six-fold increase in purification (Table 3.12).

In an attempt to further purify the lys-aminopeptidase a second FPLC step was undertaken using a shallower gradient in the hope that this would remove some of the contaminating proteins. The major aminopeptidase fraction from the previous FPLC run was desalted on a G-25 Sephadex column and applied to a Mono Q FPLC column. A shallower gradient 20-60% B was used over 30 minutes. [In the initial purification the gradient was 2.66 % B per minute whereas the second gradient was 1.33% B per minute]. In the resulting elution profile (Figure 3.44) one peak contained a major part of the lys-aminopeptidase activity while several peaks on either side of this had smaller amounts of enzyme (Figure 3.48). The peaks were collected separately.

	Total Protein mg	Total Activity (µmol min^{-1})	Specific Activity	Recovery %	Purification Fold
CFE	1271	31.16	0.025	100	1
DEAE	307.4	24.38	0.079	78	3.2
S-300	39	6.2	0.159	20	6.4
arg-Sepharose	9.5	1.76	0.186	6	7.44
FPLC1 (peak fraction)	1.1	1.1	1	3.5	40
FPLC2 (peak fraction)	0.46	0.276	0.6	0.9	25

Table 3.12. Purification of lys-aminopeptidase from *L.lactis* ML3.
The quantities specified in this table are from 62g of wet packed weight of cells.

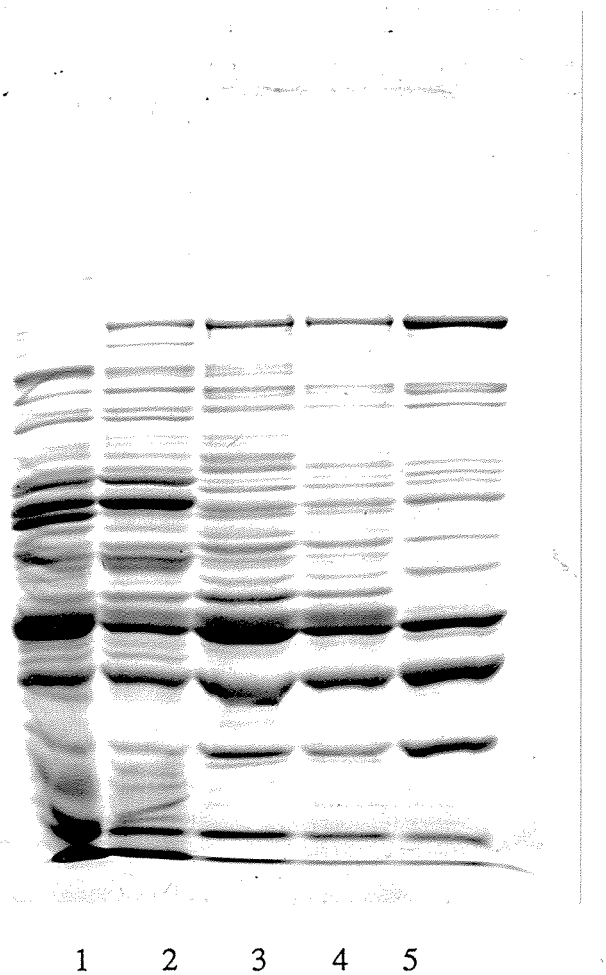


Figure 3.45 Photo of 7.5% SDS-PAGE showing the protein at each step from the purification of the lys-aminopeptidase for *L.lactis* ML3

Lane 1 Cell-free extract Lane 2 DEAE

Lane 3 Sephacryl S-300 Lane 4 Arginine-Sepharose

Lane 5 Mono Q

3.5.4. Characterisation of the lys-aminopeptidase:

3.5.4.1.Molecular weight determination .

The native molecular weight of the ML3 lys-aminopeptidase was determined by gel filtration on a Sephacryl S-300 gel permeation column. The molecular weight of the enzyme was calculated from the elution volume (260ml) against a series of molecular weight standards. This produced an approximate native molecular weight of 105,000 Daltons ± 2,000.

A 7.5% SDS gel was used to determine the monomeric molecular weight of the lys-aminopeptidase from *L.lactis* ML3 (Figure 3.47). Assuming that the lys-aminopeptidase was the major protein band on this gel, the monomeric molecular weight of approximately 100kDA was estimated from duplicate gels with a series of molecular weight standards (Figure 3.48). This suggests that the lys-aminopeptidase from *L.lactis* ML3 is a monomer with a native molecular weight of approximately 100-105kDa (Figure 3.46).

3.5.4.2 Effect of pH and temperature on the activity of the lys-aminopeptidase from *L.lactis* ML3:

The optimal pH for lys-pNA hydrolysing activity of the lys-aminopeptidase from *L.lactis* ML3 was investigated over a pH range of 4.5-9.0 in MES and BTP buffers. The assay was carried out as described in section 2.6.1. The pH of each buffer was checked with a calibrated pH meter using freshly prepared buffer standards.
The optimum pH of the lys-aminopeptidase was found to be 7.0 (Figure 3.49).

The optimum temperature for lys-pNA hydrolysis by the aminopeptidase was determined by incubating the enzyme in the assay buffer at a given temperature for 10 minutes before initiating the reaction by the addition of the substrate (see section 3.3.3 for details). The optimum temperature was found to be 40°C (Figure 3.50). With a rapid fall off in activity on either side of the optimum.

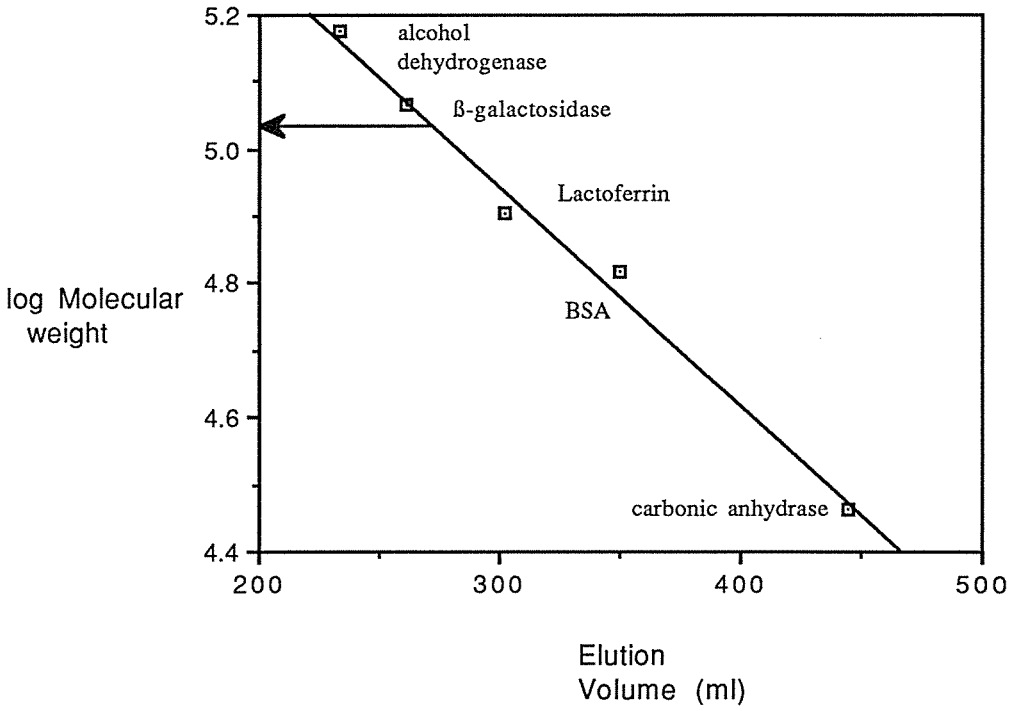


Figure 3.46 Native molecular weight determination of lys-aminopeptidase by S-300 gel chromatography.

The aminopeptidase molecular weight is indicated with an arrow.

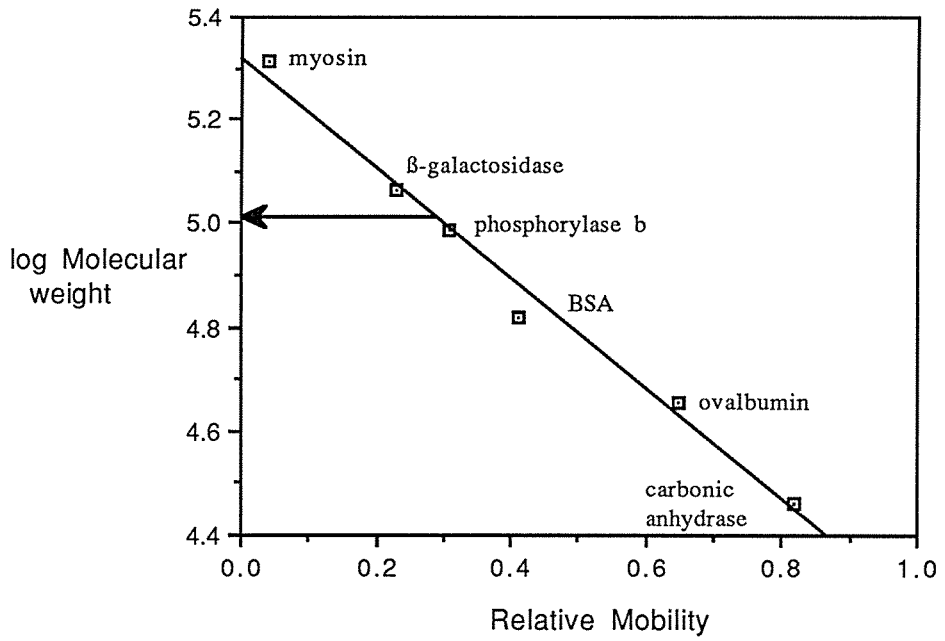


Figure 3.47 Subunit molecular weight determination for the aminopeptidase from *L.lactis* ML3

The aminopeptidase molecular weight is indicated by the arrow, the molecular weight markers are as in Figure 3.9

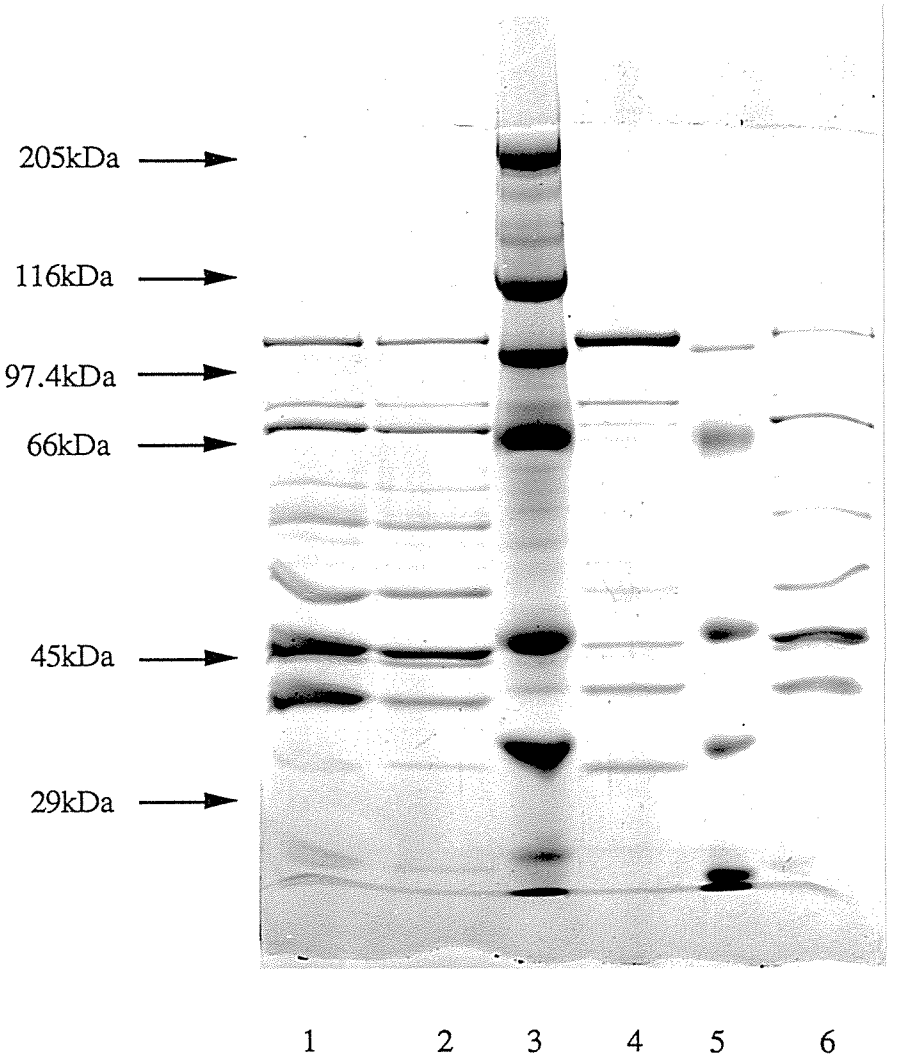


Figure 3.48 7.5% SDS gel of the molecular weight of the lys-aminopeptidase from *L.lactis* ML3

(see Figure 3.43 for FPLC trace)

Lane 1 peak 6
Lane 2 Peak 7
Lane 3 High molecular weights
Lane 4 peak 8 (aminopeptidase)
Lane 5 Low molecular weights
Lane 6 Peak 9

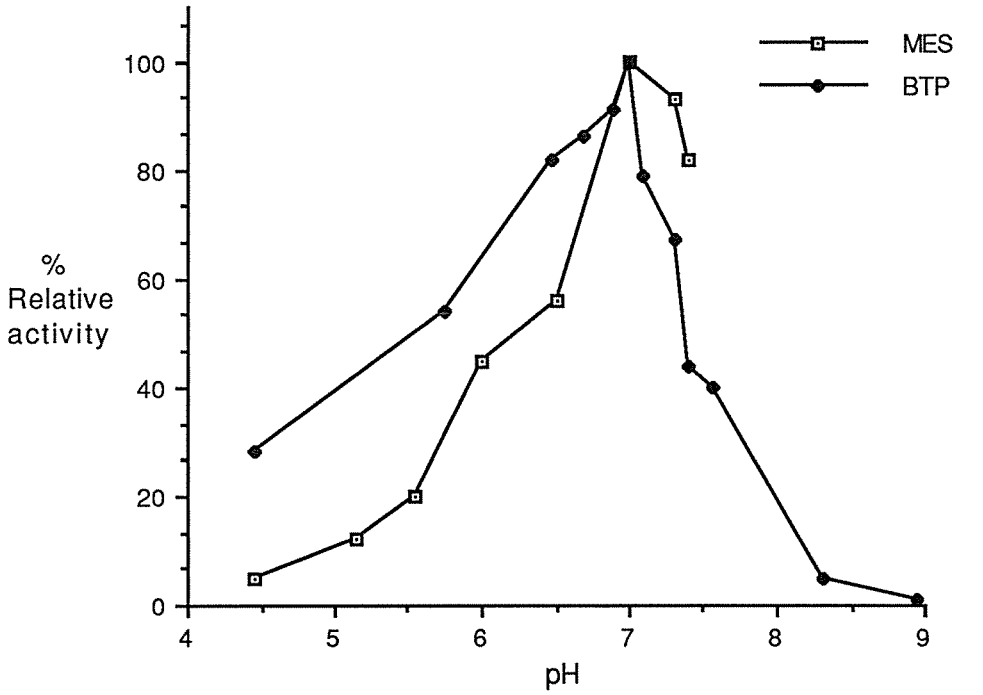


Figure 3.49 pH profile of activity for lys-aminopeptidase from *L.lactis* ML3 over the pH range of 4.5-9 in 100mM MES and Bis-Tris-Propane.

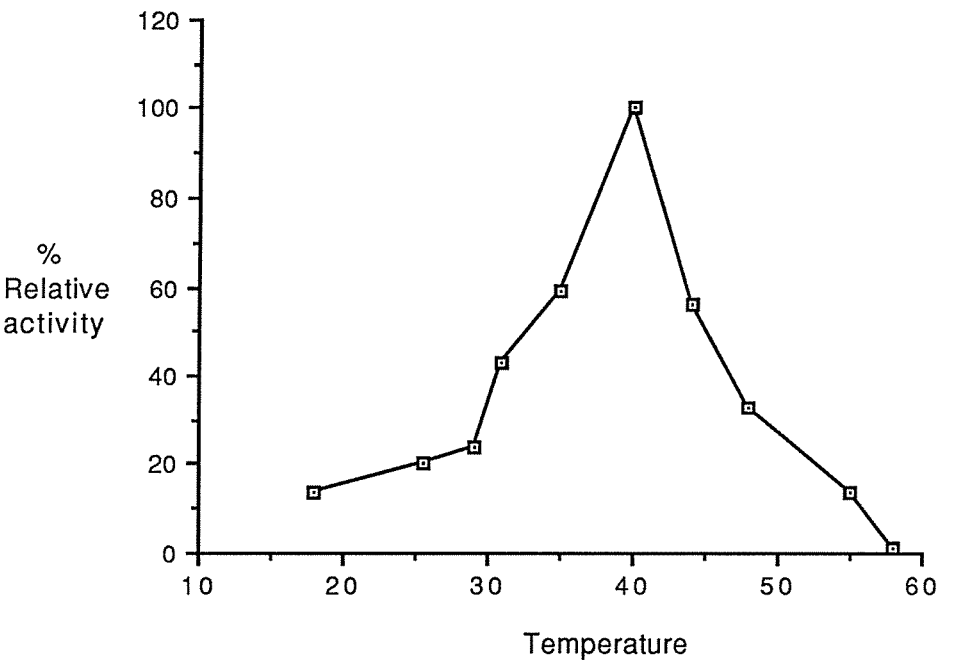


Figure 3.50 Temperature activity curve showing the optimal temperature for lys-aminopeptidase activity from *L.lactis* ML3.

3.5.4.3.Effect of cations and inhibitors on the activity of the lys-aminopeptidase from *L.lactis* ML3:

The effect of several inhibitors on lys-aminopeptidase activity was investigated. The enzyme from the first FPLC stage was used for this investigation.
The assay procedures used were those described in section 3.3.4. and the results are shown in Table 3.13 and Figure 3.51.
Aminopeptidase activity was severely inhibited by the sulphydryl-blocking reagents such as pCMB and iodoacetic acid at a concentration of 1mM (Table 3.13).
PMSF a serine protease inhibitor produced no inhibition of the enzyme at 1mM. However the protease inhibitor TLCK (an inhibitor of serine and some cysteine proteases) significantly inhibited the aminopeptidase; only 23% of the original activity being left after a 10 minute incubation with the enzyme.Treatment of the enzyme with the metal chelating agents EDTA and 1,10-phenanthroline produced almost complete inhibition.
These observations suggest that the lys-aminopeptidase from *L.lactis* ML3 was a metallo-enzyme that required a sulphydryl group for activity.

The effect of several divalent cations on the aminopeptidases activity was also studied. Significant inhibition of the enzyme ocurred at concentrations of 0.05mM Cu^{2+} and 1mM Zn^{2+} and Co^{2+} (Table 3.14 and Figure 3.52).

Reagents	% activity at 1mM Inhibitor
No addition	100
Iodoacetic acid	2
pCMB	12
PMSF	100
TLCK	23
EDTA	9
1,10-phenanthroline	0

Table 3.13: The effect of inhibitors on lys-aminopeptidase activity.
Activity is expressed as a percent of the activity of a control with no inhibitor present.

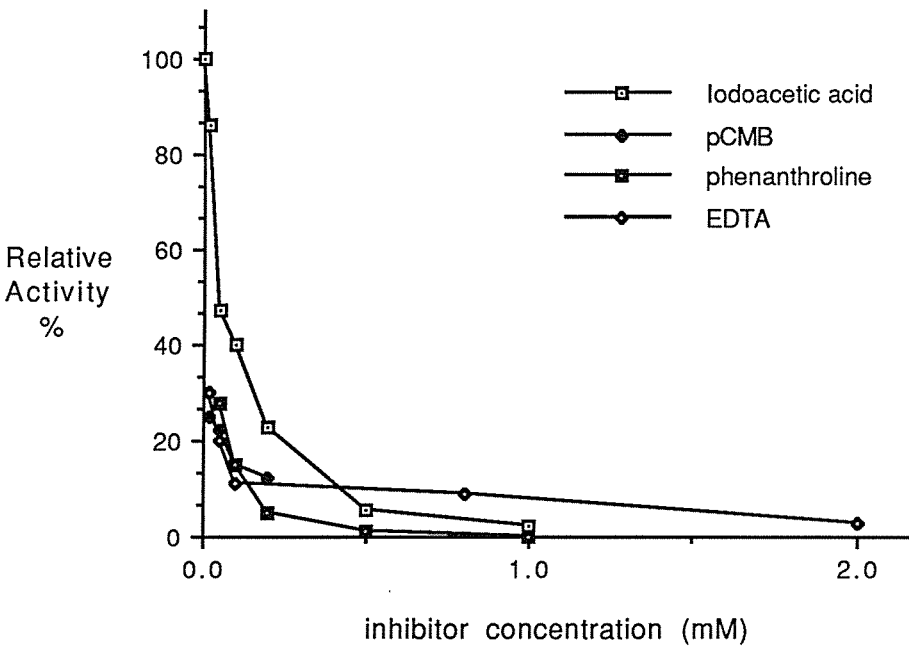


Figure 3.51: Investigation of the effect of various inhibitors on activity of the aminopeptidase.
Enzyme was incubated with various concentrations of inhibitor for 10 minutes before the addition of lys-pNA (final concentration of 0.5mM) to initiate the reaction.

Metal cation	% Activity at 1mM of metal ion
No addition	100
Zn^{2+}	18
Cu^{2+}	0.6
Co^{2+}	25
Mn^{2+}	82
Mg^{2+}	69

Table 3.14. The effect of various cations on the lys-aminopeptidase activity.
Activity is expressed as a percentage of the activity with no inhibitor present. All metals were added as their chlorides.

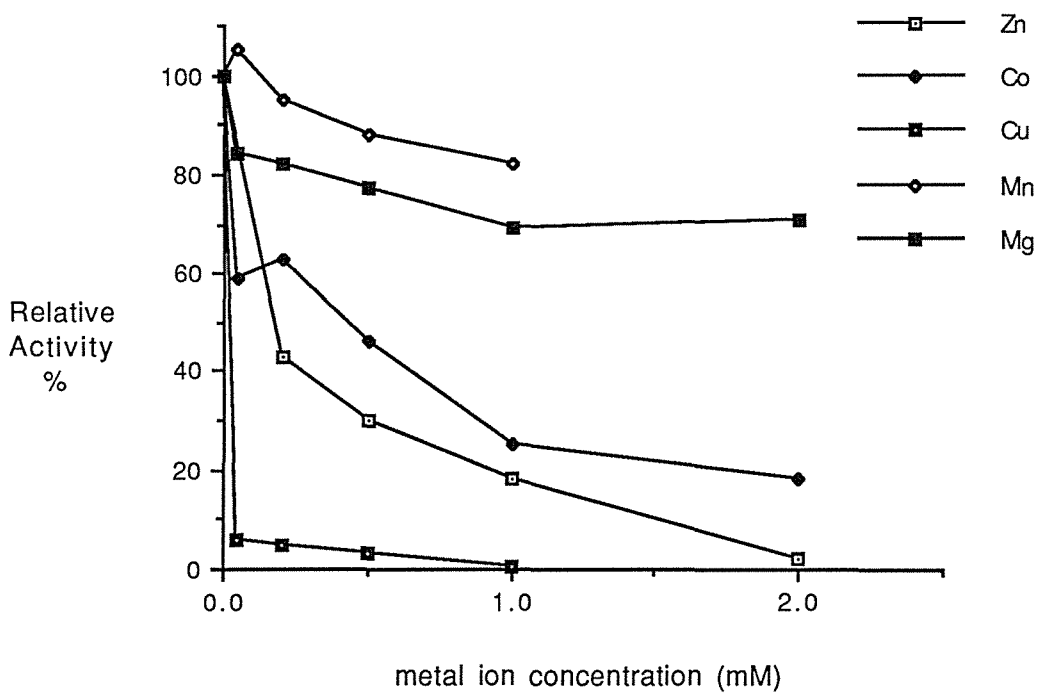


Figure 3.52: Effect of various cations over a concentration range from 0-2mM on the activity of lys-aminopeptidase
Assay conditions were as in section 3,3,4, where the enzyme was incubated for 10 minutes in MES buffer pH 6.8 with the appropriate metal ion before the reaction was initiated by the addition of 0.5mM lys-pNA (final concentration).

3.5.4.4 Specificity of the lys-aminopeptidase from *L.lactis* ML3:

The specificity of the lys-aminopeptidase towards various substrates was studied.

Activity with various amino-acyl pNA substrates

The activity of the aminopeptidase was determined with a range of amino-acyl pNA substrates at a single concentration (0.5mM final concentration) Table 3.15.

Substrate	Relative Activity %
lys-pNA	100
arg-pNA	48
leu-pNA	11.3
ala-pNA	2
phe-pNA	2
gly-pNA	0
pro-pNA	0

Table 3.15: Activity of the lys-aminopeptidase towards several pNA substrates
All substrates were at a concentration of 0.5mM Activities are expressed relative to that with lys-pNA.

As with the aminopeptidase from *S.thermophilus* and *L.cremoris* AM2 the enzyme was most active with lys-pNA. The aminopeptidase also showed activity with arg-pNA and leu-pNA. Activity towards phe- and ala-pNA was very low and no activity was detected with pro- or gly-pNA.

Activity with dipeptides

As the lys-aminopeptidase was not fully separated from other contaminating proteins it was difficult to establish whether the lys-aminopeptidase had any dipeptidase activity. The separately collected fractions (peak 6 7,8,9 Figure 3.43 & 3.44) from the second FPLC step were assayed for dipeptidase and lys-pNA activity to assess whether the ratio of the two activities remained the same across the elution profile.

Substrates	Peak 6* ratio	Peak 7 ratio	Peak 8 ratio	Peak 9 ratio
leu-gly	222.9	147	24	172
ala-ala	388	256	38.6	288

Table 3.16. The ratio of dipeptidase activity to lys-aminopeptidase in four FPLC fractions
*Activity is expressed as a ratio of average fluorescence units per 30 minute digestion relative to lys pNA hydrolysing activity of the aminopeptidase, i.e. as dipeptidase activity in the fraction (fluorescence units min^{-1}) divided by lys pNA hydrolysing activity in the fraction ($\mu molesml^{-1}min^{-1}$)

A constant ratio of dipeptidase to lys-aminopeptidase activity would suggest that the dipeptide hydrolysing activity was an intrinsic property of the aminopeptidase. However it is clear from Table 3.16 that this ratio is not constant indicating that the dipeptidase activity was, at least in part if not entirely due to a contaminating enzyme.

Bradykinin hydrolysis: (arg-pro-pro-gly-phe-ser-pro-phe-arg)

The lys-aminopeptidase from *L.cremoris* strains AM2 and Wg2 (Neviani et al.1989 and Tan & Konings, 1990) were both found to attack bradykinin. In this study it was found that the aminopeptidase from *S.thermophilus* 5109 was inactive towards bradykinin (Section 3.3.5.3). To determine whether the aminopeptidase from the ML3 could degrade bradykinin, each fraction from the second FPLC step (6,7,8 and 9) was incubated with bradykinin (0.1mg).

The enzyme (30µl) containing approximately 6µg of protein was incubated with 80µl of bradykinin for 5 minutes, the reaction was stopped by the addition of 15µl of 3% TFA to 30µl of the mixture. The sample (30µl) was injected onto an HPLC column (Vydac 218 TP C18 column) and the peaks were eluted with a gradient of water-0.1% TFA to acetonitrile-0.08% TFA (0-40%B) over 24 minutes. Each fraction contained two peaks after 5 minutes incubation with the bradykinin. By sequencing the peptides from the HPLC it was found that these two products were arg-pro-pro-gly-phe-ser-pro and phe-arg indicating endopeptidase activity. The relative bradykinin degradation activity to lys-aminopeptidase activity was different for the four FPLC peaks suggesting an endopeptidase contamination rather than intrinsic endopeptidase activity of the lys-aminopeptidase.

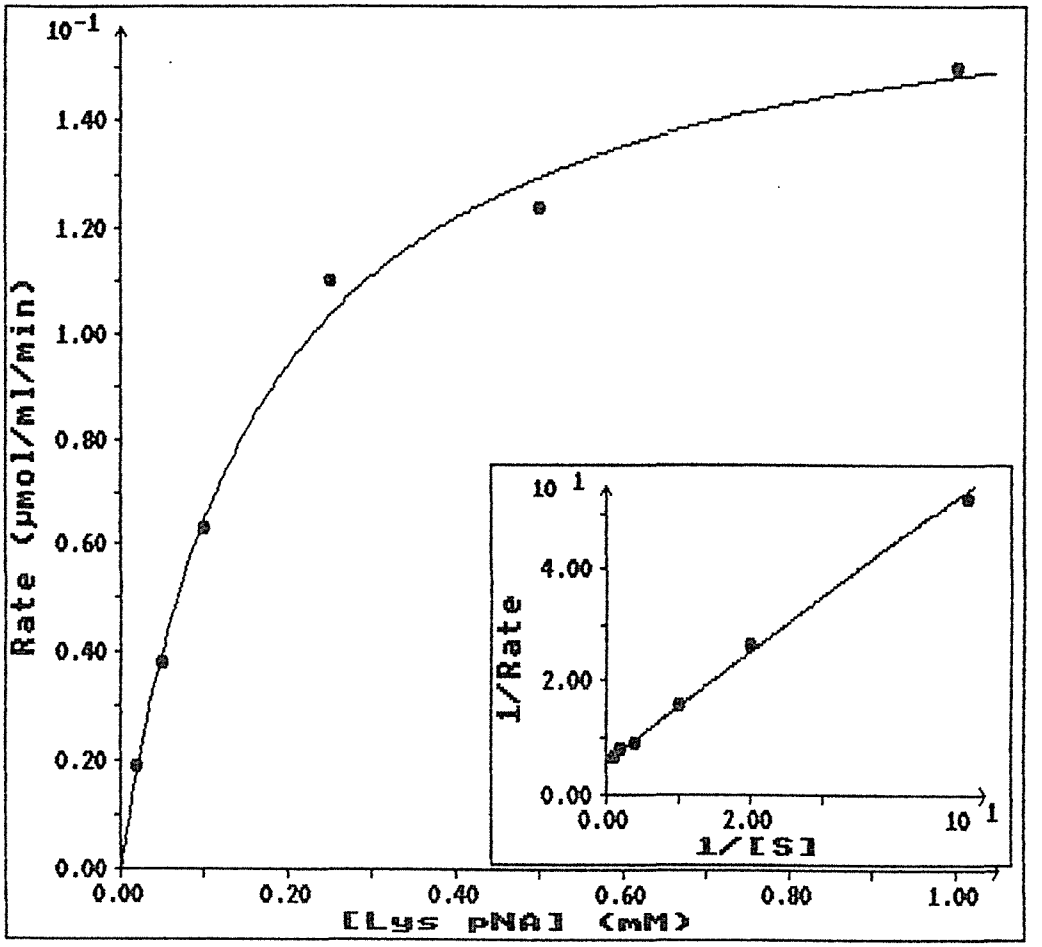
3.5.4.5 Kinetic parameters of the lys-aminopeptidase from *L.lactis* ML3:

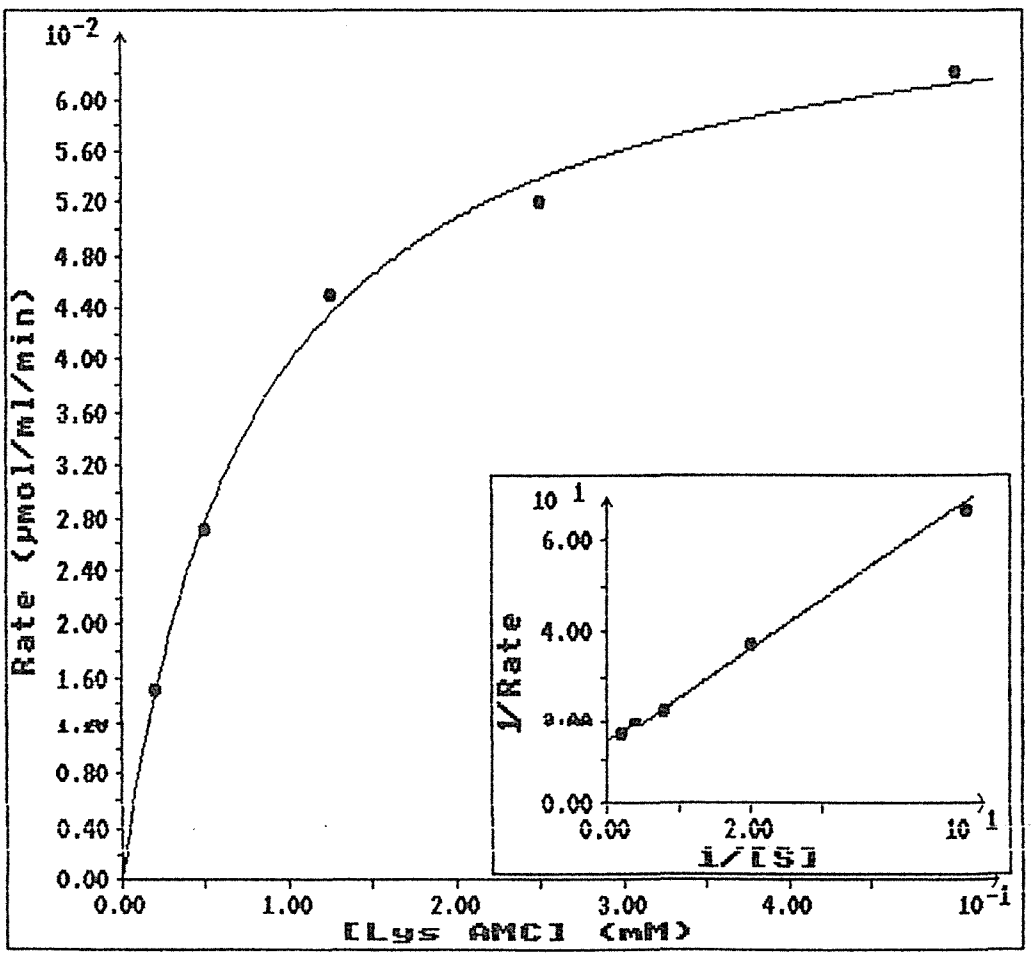
The rate of hydrolysis of lys-pNA and lys AMC were assayed over a concentration range of 0.02-1mM to determine the Km and Vmax values of the enzyme for these substrates (Table 3.17 Figure 3.53). Each assay was carried out in triplicate. The kcat could not be determined as the true concentration of the lys-aminopeptidase in the partially pure enzyme preparation was unknown.

Substrates	Km (mM)	Vmax $\mu molml^{-1}min^{-1}$
lys-pNA	0.17 ±0.02	1.94±0.1
lys AMC	0.083	0.73 ±0.04

Table:3.17 Kinetic values for the lys-aminopeptidase from *L.lactis* ML3
Standard errors are in brackets. Assay procedure is described in Section 2.2.7.

As found for the*S.thermophilus* aminopeptidase the Km of the enzyme for lys AMC was considerably lower than that for lys pNA while the Vmax for the lys-pNA was higher then for that for lys AMC.


10^{-1}
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Rate (µmol/ml/min)
0.00
0.20
0.40
0.60
0.80
1.00
[Lys pNA] (mM)
10^{1}
4.00
2.00
0.00
1/Rate
0.00
2.00
10^{1}
1/[S]


10^{-2}
6.00
5.60
5.20
4.80
4.40
4.00
3.60
3.20
2.80
2.40
2.00
1.60
0.80
0.40
0.00
Rate (µmol/ml/min)
0.00
1.00
2.00
3.00
4.00
10^{-1}
[Lys AMC] (mM)
10^{1}
6.00
4.00
0.00
1/Rate
0.00
2.00
10^{1}
1/[S]

3.6 Relative Substrate Specificities of the Aminopeptidases in Crude Extracts from a Range of Starter Strains:

The comparative studies of substrate specificity reported in the previous section for the aminopeptidase from the thermophilic starter *S.thermophilus* and the mesophilic *cremoris* and *lactis* starters indicate certain differences in relative activity towards amino-acyl substrates. Thus while the aminopeptidase from all these strains showed highest activity with lysyl derivatives the relative activity towards leucyl and alanyl derivatives was considerably higher with the *S.thermophilus* aminopeptidase than with those from the lactococcal starters.
In order to asses whether these differences are a general characteristic of the aminopeptidases from the three different groups of starters a brief study of these substrates was extended to a wider range of strains from the three groups.

Cell- free extracts from a range of lactococcal and*S.thermophilus* strains were obtained from the Dairy Research Institute. The extracts were obtained by lysis of spheroplasts following removal of the cell-wall by lysozyme and mutanolysin. The extracts were assayed for aminopeptidase activity with lys-,leu-,ala- and arg-pNA to ascertain whether there were significant differences between the enzymes from the different strains.The aminopeptidase from different strains all showed much higher activity towards lys-pNA hence the results shown in Table 3.18 are expressed as activity relative to the activity found when using the lysine substrate.(It was not feasible to determine specific activity since carry-over of lytic enzymes would influence the protein content of the samples.)

The relative activity towards leucyl, alanyl and arginyl substrates was significantly higher for *S.thermophilus* than for the lactococcal strains. No significant differences in activity were apparent between the *lactis* and *cremoris* groups of lactococcal starters.

The results presented in Table 3 18 show that the specificity differences found for the aminopeptidase from *S.thermophilus* 5109, *L.cremoris* AM2 and *L.lactis* ML3 apply generally to all other strains of each species or sub-species.

Strain	Substrate Ratio of Rates of activity for		
Strain	leu-pNA/lys-pNA [a]	ala-pNA/lys-pNA	arg-pNA/lys-pNA
S.thermophilus			
MC	0.594	0.15	0.495
11F	0.58	0.21	0.49
368	0.63	0.177	0.51
TS2	0.675	0.23	0.49
L.cremoris			
C5	0.24	0.129	0.29
H2	0.22	0.07	0.31
104	0.17	0.05	0.27
157	0.16	0.12	0.34
L.lactis			
121	0.19	0.06	0.30
SK2	0.14	0.04	0.30
BA1	0.175	0.06	0.38
MG1363	0.17	0.07	0.32

Table 3.18. Substrate specificities for the aminopeptidase from a range of starter strains

[a] the ratio of activity ($\mu molesml^{-1}min^{-1}$) with leu-,ala- or arg-pNA divided by the activity found for lys-pNA.

Chapter 4 Discussion:

4.1 Purification of lys-aminopeptidase:

A protein with lysyl-aminopeptidase activity was purified from *S.thermophilus* 5109, *L.cremoris* AM2 and *L.lactis* ML3 using the purification system described in Section 3.2. An advantage of using a single purification protocol for the enzyme was that any differences in the physical characteristics of the enzyme would perhaps become apparent during the purification.

The stability of the enzyme was poor for all three aminopeptidases purified; enzyme losses increased as the enzyme was purified. Loss of a metal ion, denaturation and autolysis have all been suggested to account for these losses in enzyme activity. Attempts to stabilize the aminopeptidase from *S.thermophilus* 5109 by the addition of glycerol and the addition of various metal ions (Section 3.2) were not successful. As the instability of the aminopeptidase appears to be a common problem in other lactic acid bacteria, a more systematic study of this problem could be worthwhile.

It soon became apparent that the entire purification had to be carried out as rapidly as possible. To this end several chromatographic techniques were investigated. Immobilised metal affinity chromatography (IMAC), which has been used to purify an aminopeptidase from *Aspergillus niger* , phenyl-Sepharose, which was used to purify an aminopeptidase from *L.cremoris* Wg2 (Tan & Konings ,1990) and a variety of dye columns were all investigated (Section 3.2.6).In our hands non of these provided any significant advantage over the standard methods of purification that were used. A further investigation of the available selective binding methods suitable for rapid purification might well be worthwhile. The most promising technique still appears to be an affinity binding method using a specific ligand.

Arginine-Sepharose 4B has been the most useful chromatographic medium investigated so far. It has been used for the purification of X-pro dipeptidyl peptidase (Lloyd, 1989 and Meyer & Jordi, 1987) and for similar enzymes from mammalian sources. Pharmacia claim that arginine-Sepharose 4B has been used to remove a number of different proteins from a wide range of starting materials; mainly for serine protease-type enzymes.

When used to purify the aminopeptidase from *S.thermophilus* 5109 the arginine-Sepharose produced up to a 30-fold purification with only minor loss of activity. The first indication that the aminopeptidases from *L.cremoris* AM2 and *L.lactis* ML3 might be different from the enzyme purified from 5109 came from the inability of the arginine-Sepharose to either bind the aminopeptidase (in the case of the AM2 enzyme) or, in the case of the enzyme from ML3,

to show a different binding pattern. While these results may be due to the Sepharose slowly losing arginine and changing its ability to bind aminopeptidase, a more likely reason is that the column had become loaded with adsorbed protein that was not removed during regeneration. This could be tested with a series of small-scale experiments using new arginine-Sepharose.

While the preparation of *S.thermophilus* achieved a 7% recovery with a 500 fold overall purification the enzymes from *L.cremoris* AM2 and *L.lactis* ML3 were only partially purified 15 and 40 fold respectively and were still heavily contaminated with other proteins.

An aminopeptidase from *L.cremoris* AM2 has been purified to homogeneity (Neviani et al., 1989) using two successive FPLC ion-exchange steps. The enzyme from burst spheroplasts was applied to an ion exchange column (Mono Q HR 10/10) and eluted with a NaCl gradient; the step was then repeated with a shallower gradient. This achieved a 136-fold increase in the aminopeptidase specific activity, with a recovery of 9% (comparable with the recovery in the present study of the aminopeptidase from *S.thermophilus* , of 6%). Purification of an aminopeptidase from *S.thermophilus* 160, the only other thermophilus strain studied, produced a purified aminopeptidase (Rabier & Desmazeaud, 1973) after ammonium sulphate precipitation, gel chromatography and several anionic exchange chromatography steps (Sepharose 6B, DEAE 23, DEAE 32). This enzyme appeared to be more stable then any of the other aminopeptidases with a 51.3% recovery.

4.2 General Properties of the lys-aminopeptidase:

A comparison of the general properties of the lys-aminopeptidase from the three strains studied in this investigation with those recently published from other strains indicates that they have many properties in common. The main points of similarity and their differences are discussed in the following section.

4.2.1 Molecular weight :

With two exceptions there is agreement between the molecular weights determined for the "general" aminopeptidase from the lactic acid bacteria listed in Table 4.1.
The enzymes appear to be monomers with a molecular weight in the range of 80-105kDa. This agrees with that predicted (95kDa) from the DNA sequence in a recent study of the aminopeptidase from *L.cremoris* HP (van Alen-Boerrigter et al., 1991). This degree of similarity between the molecular weights of the aminopeptidases is strikingly highlighted in Figure 4.1 where the aminopeptidases from this study,from three different genera, are shown on the same polyacrylamide gel.

The molecular weight of the aminopeptidase from *L.cremoris* AM2 reported in the study by Neviani et al. (1989) is clearly different with respect to both its subunit and native molecular weight. It is difficult to reconcile their value with the results of the present study since the same strain of organism was used in both investigations. In our study no evidence of a second aminopeptidase was found in strain AM2 and a second enzyme with a similar specificity would appear to be unnecessary. The other aminopeptidase with a different molecular weight was that reported by Geis et al. (1985) from *L.cremoris* AC1. This had a subunit molecular weight of 36kDa although no native molecular weight was reported. This aminopeptidase was claimed to be extracellular which may account for its lower molecular weight.

Organism	Subunit molecular weight	Native molecular weight	Source
S.thermophilus 5109	98,000	96,000	This Study
S.thermophilus 160		62,000	Rabier &Desmazeaud (1973)
L.cremoris Wg2	95,000	95,000	Tan &Konings (1990)
L.cremoris AM2	50,000	300,000	Neviani et al.(1989)
L.cremoris AM2	104,000	100,000	This Study
L.cremoris AC1	36,000		Geis et al.(1985)
L.lactis ML3	100,000	105,000	This Study
L.diacetylactis 267		85,000	Desmazeaud& Zevaco (1979)
Lb.helveticus CNRZ 32		97,000	Khalid& Marth (1990)
Lb.lactis 1183	78,000	81,000	Eggimann& Bachmann (1980)

Table 4.1: Molecular weights of aminopeptidases from various lactic acid bacteria.
Determination of the subunit molecular weight was carried out by SDS-PAGE. The native molecular weight estimates were determined by gel filtration chromatography

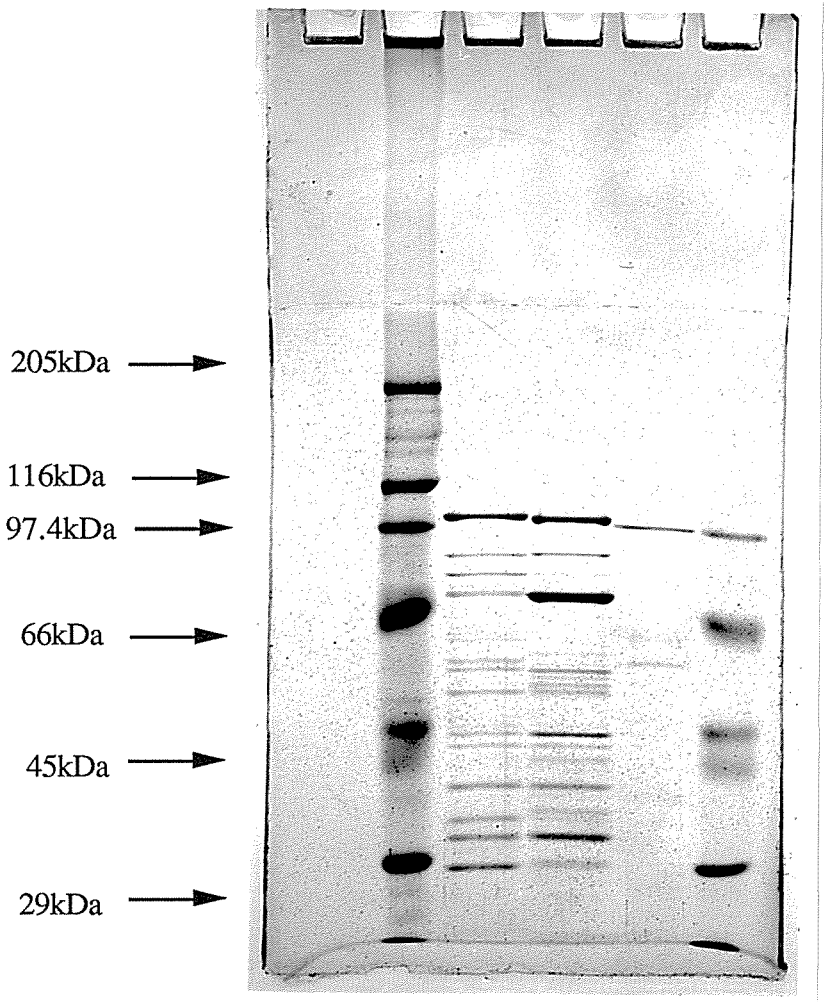


Figure 4.1:SDS-PAGE of lys-aminopeptidase from *S.thermophilus* 5109, *L.lactis* subsp.*lactis* and *L.lactis* subsp.*cremoris*

Lane 1 Molecular markers
Lane 2 *L.lactis* ML3
Lane 3 *L.cremoris* AM2
Lane 4 *S.thermophilus* 5109
Lane 5 Molecular markers

4.2.2 pH profiles:

A common feature of all the aminopeptidases currently characterised from the lactococci, lactobacilli and *S.thermophilus.* is a relatively narrow optimum pH of 6.5-7.0 (Table 4.2) The three aminopeptidases characterised in this present study all had optimal activity over this narrow pH range with an optimum at pH 7.0. However the pH optimum for the*S.thermophilus* 5109 aminopeptidase was slightly higher than the optimum pH of 6.4 found for an aminopeptidase from *S.thermophilus* 160, the only other *S.thermophilus* aminopeptidase currently characterised. It is interesting to note that while these enzymes all have an optimum pH of approximately 7.0 cheese maturation is carried out at around a pH of 4.5-5.0, at this pH the lys-aminopeptidase has very low activity. However as cheese maturation occurs over a number of months the aminopeptidase activity could still be significant.

4.2.3 Temperature optima:

The aminopeptidases previously isolated from lactococci were found to have optimal temperatures of 40°C (Tan & Konings,1990; Neviani et al.,1989; Geis et al.,1985),(Table 4.2). This was also the temperature optimum found for the aminopeptidase from *L.lactis* ML3 in this study. The*S.thermophilus* 160 and *L.diacetylactis* enzyme (Rabier & Desmazeaud, 1973, Desmazeaud &Zevaco, 1979) had optimal temperatures of 35°C as did that from*S.thermophilus* 5109 in this study. The lactobacilli have aminopeptidases with higher optimal temperatures of 45-47.5°C (Khalid& Marth 1990; Eggimann& Bachmann 1980) The *L.cremoris* AM2 enzyme, partially purified in this investigation, was also found to have a high temperature optima of 45°C. This was unexpected as all other lactococcal aminopeptidases had optima of 40°C including the enzyme from *L.cremoris* AM2 characterised by Neviani et al, (1989).

Again this seems to imply that the aminopeptidase purified by Neviani et al. is a different enzyme to that characterised by our investigation.

Organism	pH optima	Temperature optima	Source
S.thermophilus 5109	6.8-7.0	35	This study
S.thermophilus 160	6.4	35	Rabier & Desmazeaud (1973)
L.cremoris Wg2	7.0	40	Tan &Konings (1990)
L.cremoris AM2	7.0	40	Neviani et al. (1989)
L.cremoris AM2	6.9-7.1	45	This study
L.cremoris AC1	7.0	40	Geis et al.(1985)
L.lactis ML3	7.0	40	This study
L.diacetylactis 267	6.5	35	Desmazeaud & Zevaco (1979)
Lb.helveticus CNRZ 32	6.5	45	Khalid & Marth (1990)
Lb.lactis 1183	6.2-7.2	47.5	Eggimann & Bachmann (1980)
Lb.casei NCDO 151	6.5	45	El Soda et al. (1978)

Table 4.2: Comparison of optimum temperature and pH of the aminopeptidases from various lactic acid bacteria.

4.2.4 Effect of metal ions and protease inhibitors:

The data on the effect of metal ions and protease inhibitors on aminopeptidase activity for the enzymes purified in the present study are compared with those found for aminopeptidases purified by other workers in Table 4.3.
Inhibition by Cu^{2+} and usually Zn^{2+} appears to be common to all aminopeptidases studied so far. Strong inhibition by Cu^{2+} and Zn^{2+} precluded the use of immobilised ion chromatography (IMAC) to purify the enzyme (Section 3.2.6).
Several studies report activation of the aminopeptidase by metal ions particularly by Co^{2+} and Mn^{2+} (several of these reports showed the metal ions to reactivate the aminopeptidase after inhibition by EDTA or 1,10-phenanthroline). None of the three aminopeptidases purified in the present study were activated to any significant extent by metal ions. In fact Co^{2+} was inhibitory for all three enzymes, although the percentage inhibition at a concentration of 1mM (50% for AM2, 75% for ML3 and 85% for 5109) was lower then that for Cu^{2+} and Zn^{2+}. Interestingly an inhibitory effect of Co^{2+} was also found by Neviani et al.(1989) for the aminopeptidase from *L.cremoris* AM2, one of the few points of agreement between the findings of these workers and those of the present study.

All the aminopeptidases including those characterised in this study were strongly inhibited by the metal chelators, EDTA and 1,10-phenanthroline.This inhibition clearly indicates a metal

ion requirement for activity even though, in the present study,no activation by metal ions could be detected. The lack of activation by added metal ions suggests that the essential metal ion must be tightly bound. In view of the limited quantity and poor stability of the purified enzyme, removal of the essential metal ion by dialysis following EDTA treatment was not systematically attempted. Addition of metal ions to the EDTA-inhibited aminopeptidase from *L.cremoris* AM2 did restore some activity (Section 3.5.2.5). Surprisingly Co^{2+} was the most effective ion in restoring activity yet this ion led to significant inhibition (50% inhibition) when added to the untreated enzyme. A more complete study of the metal ion requirement of the aminopeptidase is clearly needed.

The one exception to the general finding of inhibition by metal-chelators is the aminopeptidase from *L.cremoris* AM2 (Neviani et al., 1989) for which the metal-chelators had no effect on activity of the peptidase. This enzyme therefore appears to be unique amongst the aminopeptidases of lactic acid bacteria.

Inhibition of the lys-aminopeptidase by sulphydryl and thiol group reagents was also studied (Table 4.3).The *L.cremoris* Wg2 (Tan & Konings, 1990) enzyme was completely inhibited by sulphydryl blocking reagents, while DTT and β-mercaptoethanol, disulphide reducing agents had no inhibitory effect on the enzyme. The aminopeptidases from *L.cremoris* AM2 (Neviani et al., 1989), *Lb. helveticus* CNRZ 32 (Khalid & Marth, 1990),and in the present study, from *S.thermophilus* 5109 *L.cremoris* AM2 and *L.lactis* ML3 were also found to be inhibited by SH-group reagents. From these results it is clear that a cysteine residue(s) in the protein appears to play an important role in the activity of the enzyme either in the active site or as a structurally important component. The aminopeptidases from lactobacilli were not affected by sulphydryl inhibitors (Eggimann & Bachmann 1980, and El Soda et al.,1978) nor was the enzyme from *S.thermophilus* 160 (Rabier & Desmazeaud 1973). This latter finding is surprising as the *S.thermophilus* 5109 aminopeptidase was strongly affected by sulphydryl inhibitors. In the light of these findings the use of thiol protecting reagents such as dithiothreitol would possibly have improved stability during purification.

The three aminopeptidases purified in this study, in common with nearly all other aminopeptidase purified from lactic acid bacteria, were unaffected by the serine protease inhibitor phenyl methyl sulphonyl fluoride (PMSF). In fact this inhibitor consistently gave a slight increase in activity in the present study, although the extent of activation (10-20%) may not be significant. The only report of an inhibition of aminopeptidase activity by a serine protease inhibitor (in this case diisopropyl fluorophosphate, DFP) was that from *S.thermophilus* 160 [the enzyme was inhibited 65% at 1mM and 97% at 10mM] (Rabier & Desmazeaud 1973). The other inhibitor used in the present study was TLCK (N-tosyl-L-lysine chloromethyl ketone) an inhibitor of both trypsin and papain. TLCK inhibits trypsin by alkylation of a histidine group at the active site (Shaw et al., 1965). However TLCK inhibits

other trypsin-like enzymes by forming covalent bonds with both sulphydryl and imidazole side chains at the active site. This inhibitor was shown to be a strong inhibitor of the *L.cremoris* AM2 aminopeptidase by Neviani et al. (1989). It also strongly inhibited the two lactococcal aminopeptidases in the present study (3.9 &3.13) but produced only limited inhibition of the *S.thermophilus* enzyme (Table 3.3). Thus it was the only inhibitor that differentiated between the thermophilus and lactococcal enzymes.

In summary the data in this study supports the finding by Tan & Konings (1990) that the lys-aminopeptidases from lactic acid bacteria are metallo-enzymes. A thiol group also appears to be required for activity and on the basis of the finding with TLCK the aminopeptidase from *L.cremoris* AM2 and *L.lactis* ML3 may also have a histidine at the active site.

Organism	Metal Chelators		Sulphydryl (1mM)		Serine	Metal ions		Source
	EDTA	phenanthroline	pCMB	iodoacetic	PMSF DIPF	inhibited	activated	
S.thermophilus 5109	32%	3%	0%	17%	no	Co Zn Cu		This study
S.thermophilus 160	0% [a]	0%	no effect		inhibition	Zn	Co Mn [e]	Rabier & Desmazeaud (1973)
L.cremoris Wg2	0%	0%	10%		no	Cu Cd Fe (Zn)[d]	Co Zn [f]	Tan & Konings (1990)
L.cremoris AM2	100% [b]	90%	0%	0%	no	Co Zn Cu		Neviani et al. (1989)
L.cremoris AM2	21%	0%	0.2%	2.6%	no	Co Zn Cu		This study
L.cremoris AC1	10%		inhibition	inhibition				Geis et al. (1985)
L.lactis ML3	9%	0%	12%	2%	no	Co Zn Cu		This study
L.diacetylactis 267	17%		0%		no		Mn Co [e]	Desmazeaud & Zevaco (1979)
Lb helveticus CNRZ32	0%	0%	2%	8%	no	Zn Hg Cu	Co Ca Mg Mn	Khalid & Marth (1990)
Lb.lactis 1183	0% [a]	0%	no effect		no	Fe HgCu	Zn Co	Eggimann & Bachmann (1980)
Lb.casei NCDO151	6% [c]	11% [c]	no effect		no		Co Mn [e]	El Soda et al. (1978)

Table 4.3: Effect of metal chelators, sulphydryl inhibitors, serine protease inhibitors such as DIP and PMSF, and metal ions on activity of the aminopeptidase from lactic acid bacteria.

The percentage values represent the residual activity in the presence of inhibitor (unless otherwise stated all inhibitor concentrations are at 1mM) .

[a] concentration of inhibitor is at 0.01mM [b] 10mM [c] 5mM.

[d] Brackets indicate that the enzyme is inhibited by this metal ion only at high concentrations.[e] the enzyme is reactivated by these metals after inhibition by EDTA or [f] 1,10-phenanthroline.

4.3 Specificity:

4.3.1 Specificity towards amino-acyl derivatives:

A major objective of this study was to define the specificity of the aminopeptidase more fully than has been done in other studies of this enzyme from lactic acid bacteria. As mentioned in the introduction this enzyme has frequently been refered to as a "general" aminopeptidase implying a very broad specificity without defining this in quantitative terms. Knowledge of the specificity of the enzyme is of importance in understanding its possible contribution to the degradation of oligopeptides derived from casein by the action of proteinase. Previous specificity studies of the aminopeptidases from lactococci and other lactic acid bacteria have mainly used amino-acyl pNA or AMC derivatives. Little systematic investigation of the ability of the aminopeptidase to degrade oligopeptides has been carried out.

In the present study the relative activity of the aminopeptidase with a range of amino-acyl pNA and AMC substrates was investigated, using substrates that were available commercially or from stocks previously synthesized in the Department (Lloyd,1989).
Activity towards dipeptides, tripeptides and several commercially available oligopeptides was also studied. Limited studies were also carried out with two oligopeptides derived from ß-casein by the action of the lactococcal proteinases.

A common feature of all the aminopeptidases from lactic acid bacteria studied so far using amino-acyl derivatives as the substrate, is that highest activity is always found for lysyl derivatives. This has been confirmed for the three aminopeptidases studied in the present work. In order to compare the specificities of the enzymes studied to date the activity against other substrates, expressed as a percentage of that with the lysyl derivatives, is shown in Table 4.4 . Such a comparison has limited value in that it is made at a single substrate concentration and the concentrations used by different workers are not necessarily the same.In the absence of detailed kinetic studies it is however the only information currently available. Nevertheless certain generalizations can be made. The enzyme shows relatively high activity towards arginyl derivatives; 40-50% of that of lysine pNA in this study and 78% for the aminopeptidase from *L.helveticus* studied by Khalid and Marth (1990). Similarly the enzyme from all species and strains show moderate activity with leucine derivatives, the variability between the enzyme from different sources is probably in part due to the use of different substrate concentrations.

Activity with alanyl and phenylalanyl derivatives was usually considerably lower. As with its other properties (see above) the aminopeptidase described from *L.cremoris* AM2 (Neviani et al. 1989) was exceptional in that it showed comparable levels of activity with lysyl, leucyl, alanyl and phenylalanyl derivatives. It is worth noting that Neviani et al. (1989) used

ß-naphylamide derivatives rather than pNA or AMC derivatives which may in part explain the differences in relative activity.

In this study the aminopeptidases from *S.thermophilus* 5109 *L.cremoris* AM2 and *L.lactis* ML3 had very low activity with the amino-acyl derivatives of tyrosine, serine and valine and no activity towards gly-pNA, glu AMC, pro pNA and pro AMC (Table 3.4, 3.11 & 3.15). The inability of all of the aminopeptidases to catalyse the removal of N-terminal proline is a common characteristic. Although the "general" aminopeptidases are frequently refered to as having a broad spectrum of activity it can be seen from this investigation that they in fact exhibit a very narrow range of specificity.

Organism	Lys	Arg	Leu	Ala	Phe	Derivative used	Source
S.thermophilus 5109	100	39	38	6.3	5.1	pNA	This study
S.thermophilus 5109	100	-	22	36	-	AMC	This study
L.cremoris Wg2	100	-	24	4	4	pNA*	Tan & Konings (1990)
L.cremoris AM2	100	-	70	100	75	ß-NA**	Neviani et al. (1989)
L.cremoris AM2	100	49	16	6	4	pNA	This study
L.cremoris AC1	100	-	36	20	-	pNA	Geis et al. (1985)
L.lactis ML3	100	48	11.3	2	2	pNA	This study
L.diacetylactis 267	100	-	52	-	-	-	Desmazeaud & Zevaco (1979)
Lb.helveticus CNRZ 32	100	78	41	22	-	pNA***	Khalid & Marth (1990)
Lb.lactis 1183	100	-	13	-	-	pNA #	Eggimann & Bachmann (1980)

Table 4.4: Relative activity of the aminopeptidase towards synthetic substrates.
Activity is expressed as a percentage of that with lysyl derivatives. For the enzymes studied in the present work a substrate concentration of 1mM was used. For published studies concentrations were 1mM or * 2mM ** 0.4mM ***16.4mM
Various peptides were also used as substrates.

As well as showing activity towards amino-acyl derivatives, several of the aminopeptidases previously studied were reported to be able to hydrolyse dipeptides and tripeptides. Neviani et al.(1989) found that *L.cremoris* AM2 aminopeptidase could degrade a wide variety of di- and tripeptides except those containing proline, while the *L.cremoris* Wg2 enzyme, Tan & Konings (1990) degraded di- and tripeptides except those with alanine or a proline at the

N-terminal. In this study the purified *S.thermophilus* 5109 aminopeptidase showed no activity towards any dipeptides and very low activity towards the tripeptides leu-gly-gly and ala-ala-ala. The partially purified aminopeptidase from *L.cremoris* AM2 and *L.lactis* ML3 showed some activity towards dipeptides but in view of the likely presence of other peptidases in these preparations no clear conclusions can be drawn from these latter experiments.

4.3.2 Kinetic Properties:

Apart from the reported Km and Vmax values for the aminopeptidase from *L.cremoris* Wg2 using lys-pNA as the substrate (Tan & Konings 1990) none of the other published studies contain any kinetic information.

	lys-pNA		
Organism	Km (mM)	Vmax $\mu molmin^{-1}mg^{-1}$	Reference
L.cremoris Wg2	0.55	30	Tan & Konings 1990
L.cremoris AM2	0.38	-	This study
L.lactis ML3	0.17	-	This study
S.thermophilus 5109	0.2	15	This study

Table 4.5 Km and Vmax values of the aminopeptidase for lys-pNA.
Vmax values for the aminopeptidases from *L.cremoris* AM2 and *L.lactis* ML3 are not given because the preparation was not pure.

The Km for the hydrolysis of lys-pNA by the aminopeptidase from *S.thermophilus* 5109 *L.lactis* ML3 and *L.cremoris* AM2 found in this study were similar. The only other aminopeptidase with comparable data was that from *L.cremoris* Wg2 (Tan & Konings, 1990) which had a 2-fold higher Km and V max (Table 4.5).

	lys-pNA	leu-pNA	phe-pNA	arg pNA	ala-pNA	lys AMC	leu AMC
Km (mM)	0.2	0.17	0.11	0.094	1.4	0.06	0.02
Vmax $\mu molmin^{-1}\ mg^{-1}$	15	9.7	1.6	4.8	8.4	5.7	4.0

Table 4.6: Km and Vmax for the aminopeptidase from *S.thermophilus* 5109 for several pNA substrates.

Km values for the lys AMC and leu AMC for the aminopeptidase from *S.thermophilus* were considerably lower than that found for the corresponding pNA derivatives (Table 4.6). While the aminopeptidase had a higher affinity for the AMC substrates the maximal rate of hydrolysis was one third that found for the lys-pNA. The aminopeptidase from *S.thermophilus* showed very low affinity for ala-pNA with a 7-fold difference between the Km for ala-pNA and lys-pNA. However its Vmax was comparable to that for all of the other pNA substrates tested.
This low affinity found towards the ala-pNA may be a result of the small size of the methyl side chain; all of the other substrates tested had much larger amino-acyl side chains. The small size of the alanyl side chain may mean that it does not fit very well in the active site of the aminopeptidase. this work emphasised the importance of selecting the correct derivative if the results from different laboratories are to be compared.

4.3.3 Activity towards oligopeptides:

In previously published studies of aminopeptidases from lactic acid bacteria there is little or no information on the ability of the enzyme to act on oligopeptides. As well as the limited range of amino-acyl substrates that are rapidly hydrolysed, the inability of the purified *S.thermophilus* enzyme to catalyse hydrolysis of dipeptides and its minimal activity with the tripeptides leu-gly-gly and ala-ala-ala raised the question as to whether the enzyme may require a minimum peptide chain length in order to hydrolyse the N-terminal peptide bond. An investigation using HPLC to test this was carried out with a series of alanyl peptides from $(ala)_2$ to $(ala)_6$. (Alanyl peptides were used because they were the only readily available homo-polymer series.)
The peptides $(ala)_4$ and $(ala)_5$ were rapidly degraded to $(ala)_3$ which accumulated. This was consistent with the observation that $(ala)_3$ was only slowly hydrolysed.The peptide $(ala)_6$ was more slowly degraded possibly due to the fact that $(ala)_6$ would be able to form an α-helix which could make it less accessible to the active site of the aminopeptidase.

From these results it appears that the lys-aminopeptidase from *S.thermophilus* has a "size constraint" on its activity which requires a peptide of at least 4 residues for degradation to occur. The lys-aminopeptidase from *L.cremoris* Wg2 (Tan & Konings 1990) was also suggested as having a greater affinity for oligopeptides than for dipeptides although no quantitative data on the rate of hydrolysis was given. It is not known whether the limitation observed with the poly-alanine series would occur with polymers that did not readily form α-helices.

The ability of the aminopeptidase from *S.thermophilus* to catalyse the hydrolysis of several commercially available oligopeptides was also investigated.
The four oligopeptides studied (section 3.3.5.3) were

Thymopoeitin fragment 32-36	arg-lys-asp-val-tyr
ß-lipotropin fragment (88-91)	lys-lys-gly-glu
α-casein fragment 90-95	arg-tyr-leu-gly-tyr-leu
Bradykinin	arg-pro-pro-gly-phe-ser-pro-phe-arg

Of these bradykinin was not degraded, presumably because the presence of a proline in the second position applied a constraint on the action of the aminopeptidase. The cyclic structure of proline, which is an imino acid prevents free rotation about the peptide bond.
With thymopoeiten (32-36) and lipotropin (88-91) the N-terminal amino acids were cleaved leaving the tripeptides asp-val-tyr and lys-gly-glu respectively as undegraded residual products. The inability to further degrade these tripeptides is consistent with the finding discussed earlier that the purified enzyme showed little or no activity with the tripeptides $(ala)_3$ and leu-gly-gly.
The complete degradation of α-casein (90-95) to free amino acids was unexpected. The N-terminal glycine residue of the C-terminal tripeptide gly-tyr-leu was completely removed after 5 hours and even more surprisingly the remaining dipeptide tyr-leu was slowly degraded to free tyr and leu after 24 hours. However the rate of removal of the N-terminal residues of the tri-and dipeptide was much slower than that of larger peptide consistent with findings with the poly alanine series. It is clear that certain tripeptides and even dipeptides can be slowly degraded.
The total degradation of a peptide is probably related to the hydrophobicity of its component amino acids. This observation could be confirmed by further study using a series of carefully selected synthetic peptides.

4.3.4 Hydrolysis of β-casein derived peptides:

It has been proposed that a major function of the lys-aminopeptidase from lactococci is to degrade the oligopeptides produced by the action of the cell-wall proteinase on ß-casein (Tan & Konings, 1990, Smid et al., 1991). To date no direct demonstration of the degradation of these ß-casein oligopeptides by the aminopeptidase has been reported. An earlier attempt in this laboratory (Lloyd, 1989) to investigate this was unsuccessful due to endopeptidase contamination of the partially purified aminopeptidase. The difficulties in obtaining homogeneous preparations of the lactococcal aminopeptidase precluded their use for this purpose in the present study. However the purified aminopeptidase from *S.thermophilus* was free from contamination by other peptidases and was therefore used to investigate the ability

of the aminopeptidase to degrade two of the three C-terminal oligopeptides produced as primary products of lactococcal proteinase action (Figure 1.2 and 4.2).

In view of the similar specificities of the *S.thermophilus* and lactococcal aminopeptidases the results with the thermophilic enzyme will give some indication of the potential of the lactococcal aminopeptidase to degrade ß-casein oligopeptides.

S.thermophilus aminopeptidase degraded the hexapeptide (176-182) lys-ala-val-pro-tyr-pro-gln- by removing the N-terminal lys and ala residues leaving the C-terminal pentapeptide undegraded (Section 3.3.5.4). This is consistent with the prediction from the earlier specificity studies, that the lys and ala residues will be cleaved while the presence of a pro residue will prevent further cleavage of the N-terminal val.

The ß-casein fragment 183-193 [arg-asp-met-pro-ile-gln-ala-phe-leu-leu-tyr] was not degraded by the lys-aminopeptidase from *S.thermophilus* . This is surprising in view of the known ability of the enzyme to rapidly cleave N-terminal arginyl residues. The presence of the adjacent asp should not hinder the removal of the arg since both the arg and lys residues were rapidly removed from Thymopoeiten (32-36) [arg-lys-asp-val-tyr]. The overall conformation of the peptide may be a contributing factor.
Peptide 183-193 has a number of helix-directing amino acids so that formation of an α-helix may possibly prevent access of the peptide to the active site. The hypothesis that secondary conformation may limit access is corroborated by the earlier observed much slower rate of hydrolysis of $(ala)_6$ relative to $(ala)_5$ and $(ala)_4$. Unfortunately insufficient quantities of the pure C-terminal ß-casein fragment (194-209) were available for a more complete study using these peptides.

In summary the studies on oligopeptide degradation by the *S.thermophilus* aminopeptidase have demonstrated the ability of this enzyme to cleave N-terminal lys, arg, ala, tyr and leu except where these residues comprise the N-terminal of a tri- or dipeptide or are followed by a pro residue as in bradykinin.
The results with the polyalanyl series suggest that peptides with fewer than 4 residues are not readily hydrolysed and that an increase in size enabling the development of secondary structure may limit access of the substrate to the active site. One apparent inconsistency is the slow hydrolysis of the N-terminal tripeptide gly-tyr-leu from α-casein during the digestion of fragment 90-96 (Section 3.3.5.3). Not only is this a case of a tripeptide and dipeptide being hydrolysed but furthermore studies using synthetic substrates indicate that the enzyme shows no activity towards gly-pNA. Once again this experiment demonstrates the difficulty of using synthetic substrates to predict activity against naturally occuring peptides.

4.4 Location:

Establishing the cellular location of the lys-aminopeptidase is vital for understanding its role during microbiol growth in milk and for its possible contribution to the ripening and maturation of cheese. Very few of the aminopeptidases characterised have been unequivocally assigned a cellular location.

Geis et al.(1985) and Tan & Konings (1990) proposed an extracellular location for the aminopeptidase from *L.cremoris* strains HP and Wg2. However the authors gave no quantitative data to support their proposal. Neviani et al.(1989) found that 15% of total aminopeptidase activity from *L.cremoris* AM2 was released during spheroplast formation when mutanolysin was used to remove the cell wall, the remaining activity being released only when the spheroplasts were burst. (As discussed previously it seems unlikely that the aminopeptidase studied by these workers is the same as that partially purified from the same strain in this study).

Use of a comparable fractionation procedure in the present study showed that at least 90% of the total aminopeptidase activity of the *S.thermophilus* 5109 was intracellular. Fractionation studies of peptidase location in a wide range of other strains using this cell fractionation procedure give even higher values of 93-97% in the cytoplasmic fraction, with the activity lost during spheroplast formation being largely due to leakage or lysis (Crow, Coolbear and Pritchard, personal communication).

Independent confirmation of an intracellular location of the aminopeptidase from *L.cremoris* HP has recently been provided by van Alen-Boerrigter et al.(1991). These workers showed first, that the DNA sequence of the enzyme revealed no evidence of a signal sequence and second that immunogold staining of thin sections with an aminopeptidase specific antibody clearly indicated an intracellular location.

Given that the upper size limit for peptide uptake by lactococcal peptide transport systems is 6 residues (Rice et al., 1978 and Smid, 1991) and that the degradation of oligopeptides longer than this by the aminopeptidases may be subjected to conformational constraints an intracellular role in degradation of small peptides (3-6 residues) seems the most probable assignment consistent with the available data.

4.5 Possible role of the aminopeptidase in cheese manufacture:

It is not known which enzymes from the lactic acid bacteria are important for the formation of dairy product flavour. Hence it is important to know what flavours are generated by a particular set of enzymes. Investigations can take two approaches
- to isolate the actual flavour components and determine what enzymes are likely to have led to their formation or,
- isolate the proteinases and peptidases from the lactic acid bacteria and determine their action on the milk proteins, both individually and sequentially (Mulholland, 1991).

While isolation of the components that give cheeses their flavours is still at the early stages, the present study exemplifies the second approach. The eventual aim of this study was to purifiy and characterise an aminopeptidase from lactic acid starter bacteria and to elucidate its possible role in the proteolytic pathway of casein degradation.

It is likely that the peptidases from lactic acid bacteria act by breaking down the hydrophobic bitter peptides produced by proteinase action and by producing many of the small dipeptides and amino acids which contribute flavour to the cheese. Defining these activities is likely to show which peptidases are important in the formation of desirable flavour peptides.

Peptidases fulfil two different roles in the production of cheese. They are important during the initial growth of the starter culture to provide amino acids required for bacterial protein synthesis. Because of the low content of free amino acids in milk the starter must obtain the amino acids necessary for growth by degradation of milk proteins (Mills & Thomas, 1978). They are also important at a later stage during cheese ripening when they continue the degradation of milk proteins and thereby play a major part in determining the texture and flavour of the final product.

The aminopeptidase investigated in this study, because of its intracellular location, would appear to only play a role in the proteolytic sequence during growth of intact cells in milk. Its major function would be in degrading the small peptides that cross the membrane into amino acids that can be used in bacterial protein synthesis.

It has been proposed that intracellular peptidases are released by cell lysis during maturation of the cheese and in this way contribute to the breakdown of larger peptides and thus the final taste and aroma of the cheese. The aminopeptidase investigated in this study because of its intracellular location, appears to become important in flavour development in cheese only after bacterial lysis.
The time of bacterial lysis in itself is important to cheese flavour development as aminopeptidases released early during cheese ripening will presumably have greater

proteolytic effect on the cheese than if they are released later. Microscopic studies of 5 month Cheddar cheese found that the cell membranes were largely intact (Umemoto et al., 1978). Bitterness development may therefore be favoured, and flavour development reduced when starter bacteria remain intact in cheeses. However as cheese maturation occurs over a long period minor bacterial lysis and the release of the intracellular enzymes while slow would be cumulative.

Even if the aminopeptidase is released and has access to the large peptides produced by proteinase action its role would be limited since the high proline content of many of the peptides would severely restrict the lys-aminopeptidase action. Inspection of the major peptide products released by proteinase action from the C-terminal of ß-casein (Figure 4.2) indicates that only limited degradation of these would occur as a result of the lys-aminopeptidase action.

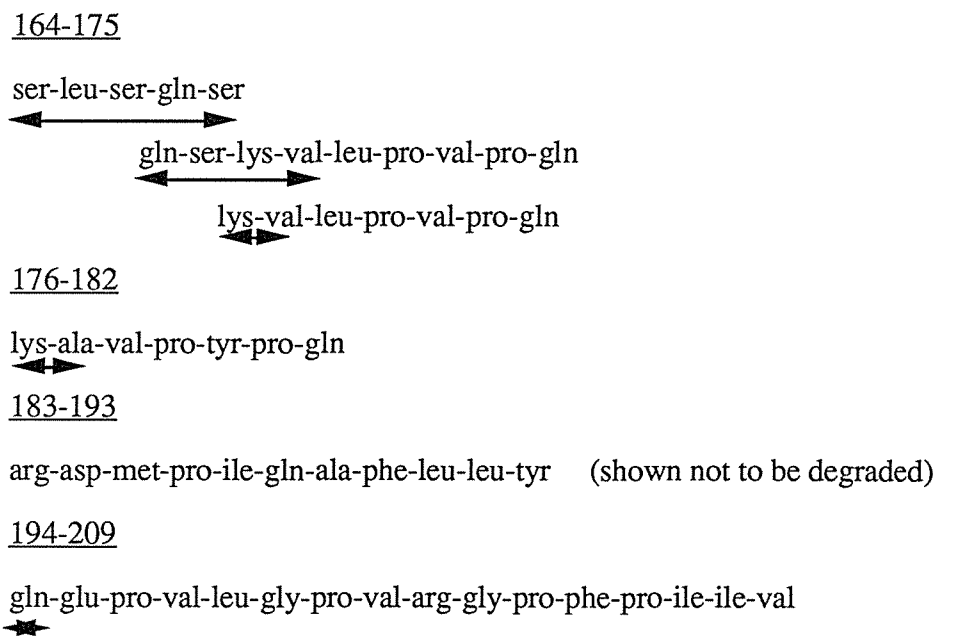


Figure 4.2: Possible degradation by the aminopeptidase from *S.thermophilus* 5109 of the major oligopeptides released from ß-casein.by lactococcal proteinases.
Arrows indicate where the lys-aminopeptidase might degrade the oligopeptides.

There is some suggestion that genetic manipulation of proteinases and peptidases to produce large amounts of selected enzymes could increase the rate of removal of bitter peptides. However the proteolytic system of the lactic acid bacteria is carefully balanced. A change in the activity of a single step in the proteolytic system is likely to cause severe inhibition of bacterial growth due to an imbalance in the peptides supplied as was found by Smid et al. (1989).

However with the growing number of characterised peptides, selection of strains for particular enzyme specificities to produce a particular flavour in a dairy product would be possible rather than the "hit and miss" method currently used in starter selection. With a fuller understanding of the specificities of the proteinases and peptidases in starter strains the possibility now exists for the systematic selection of cultures to produce a defined taste and aroma in the end products.

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