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A STUDY OF AMINOPEPTIDASES FROM LACTIC STREPTOCOCCI

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

RICHARD JOHN LLOYD

1989

ABSTRACT

Two aminopeptidase enzymes from the proteolytic system of *Streptococcus lactis* 4760 have been studied.

An X-Prolyl dipeptidyl aminopeptidase has been purified and characterised. The enzyme has a native molecular weight of approximately 150 kDa determined by gel filtration, and a subunit molecular weight of 83 000, determined by denaturing polyacrylamide gel electrophoresis, showing the native enzyme to be a dimer. It is inhibited by phenyl methyl sulphonyl fluoride and is active over a pH range of 6 - 9. A range of X-Prolyl-amido methyl coumarin (X-Pro-AMC) derivatives with different aminoacyl residues in the X position have been used to define the steady state kinetic parameters. The Km and k_{cat} values obtained with all of the X-Pro-AMC substrates tested were similar, with the exception of Glu-Pro-AMC, which gave a somewhat higher Km value. The action of the enzyme in degrading small peptides has been studied. It was found to be capable of removing X-Proline residues from peptides, except where two proline residues are situated in consecutive positions.

A Lysyl-aminopeptidase has been partially purified and its characteristics studied. This enzyme has been shown to have a native molecular weight of approximately 78 000. It hydrolyses lysyl-, arginyl-, and leucyl-amido methyl coumarin derivatives, but has little or no activity with other aminoacyl-AMC substrates. It also catalyses the removal of lysine and arginine residues from the amino-terminus of short peptides. The partially purified aminopeptidase preparation also has endopeptidase activity which is probably due to contamination by a separate enzyme.

The individual and combined effects of these two enzymes on β-casein-derived oligopeptides (produced by proteolytic action of the *S.lactis* proteinase) have been studied. These results indicate that these enzymes may be important in degradation of some casein-derived peptides during cheese ripening, while other peptides are resistant to hydrolysis.

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LIST OF ABBREVIATIONS

AH-SEPHAROSE	aminohexyl-Sepharose
AMC	7-amino-4-methyl coumarin
BOC	t-butyl oxy carbonyl
CBZ	benzyl chloroformate
DEAE	diethylaminoethyl
EDC	1-ethyl-3-(3-dimethyl amino propyl) carbodiimide
EDTA	ethylenediaminetetra-acetic acid
FITC	fluorescein isothiocyanate
MES	2-(N-morpholino-) ethane sulphonic acid
NADH	nicotinamideadeninedinucleotide
PAGE	polyacrylamide gel electrophoresis
pNA	p-nitroanalide
RP HPLC	reverse phase- high performance liquid chromatography
RSM	reconstituted skim milk medium
SDS	sodium dodecyl sulphate
TEMED	NNN'N'-tetramethylethylene-diamine
TES	(N-tris[hydroxymethyl] methyl-2-amino ethane sulphonic acid)
TRIS	tris-(hydroxymethyl-) aminomethane
TCA	trichloroacetic acid
TFA	trifluoroacetic acid

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CHAPTER 1 - INTRODUCTION

1.1 IMPORTANCE OF PROTEOLYTIC ACTIVITY OF STARTER BACTERIA

The group N (or lactic) streptococci, consisting of the species *Streptococcus lactis, S. cremoris* and *S.diacetylactis* together with the closely related thermophilic bacteria *S.thermophilus, Lactobacillus bulgaris* and *L.helveticus* are of major importance to the dairy industry as cheese starter bacteria. Individual strains, or combinations of different strains, ferment the lactose in milk by the Embden-Meyerhof pathway to pyruvate, $NADH/H^+$ and ATP. This process provides the major source of ATP for bacterial growth. To regenerate NAD^+ the pyruvate is reduced to lactic acid, and consequently the milk pH is lowered to 4.5 to 5.0, preventing the growth of other undesirable microorganisms.

Lactic streptococci are nutritionally fastidious organisms being unable to synthesise most of the amino acids they require. They may grow to high cell densities of approximately 500μg (dry weight) bacteria per ml (or about 10^9 colony forming units per ml) in milk, requiring the synthesis of approximately 260μg bacterial protein per ml. The concentrations of free amino acids in milk are well below the minimum required for synthesis of this amount of protein (Thomas and Mills 1981). Therefore amino acids must be provided by degradation of milk proteins, primarily casein. (Table 1.1)

The process of casein hydrolysis during cheese manufacture has recently become of interest to the dairy industry since the texture and taste developed during cheese ripening is, at least in part, related to the size and composition of peptides resulting from proteolysis. Casein molecules contain a high proportion of hydrophobic residues (for example leucyl, prolyl, phenylalanyl residues). Bitterness in cheese has been attributed to peptides with a high proportion of these amino acids. (Visser *et al.*, 1983).

The degradation of milk proteins during milk fermentation and subsequent ripening of cheese is due to the combined action of rennet, a proteolytic enzyme added as a coagulant, and the proteolytic enzymes of the starter bacteria. Studies of the respective roles of these (O'Keeffe *et al.*, 1976, 1978) suggest that the main role of the starter enzymes is the slow degradation of the β-casein and of the polypeptides generated from α-casein hydrolysis by rennet.

The lactic streptococci possess two distinct groups of proteolytic enzymes which together degrade casein to small peptides and free amino acids.

a) Proteinases Located on or near the cell surface which degrade one or more of the casein components to large oligopeptides.

b) Peptidases Located in the cell wall, attached to the cell membrane and/or present within the cell which degrade the oligopeptides generated by the proteinases.

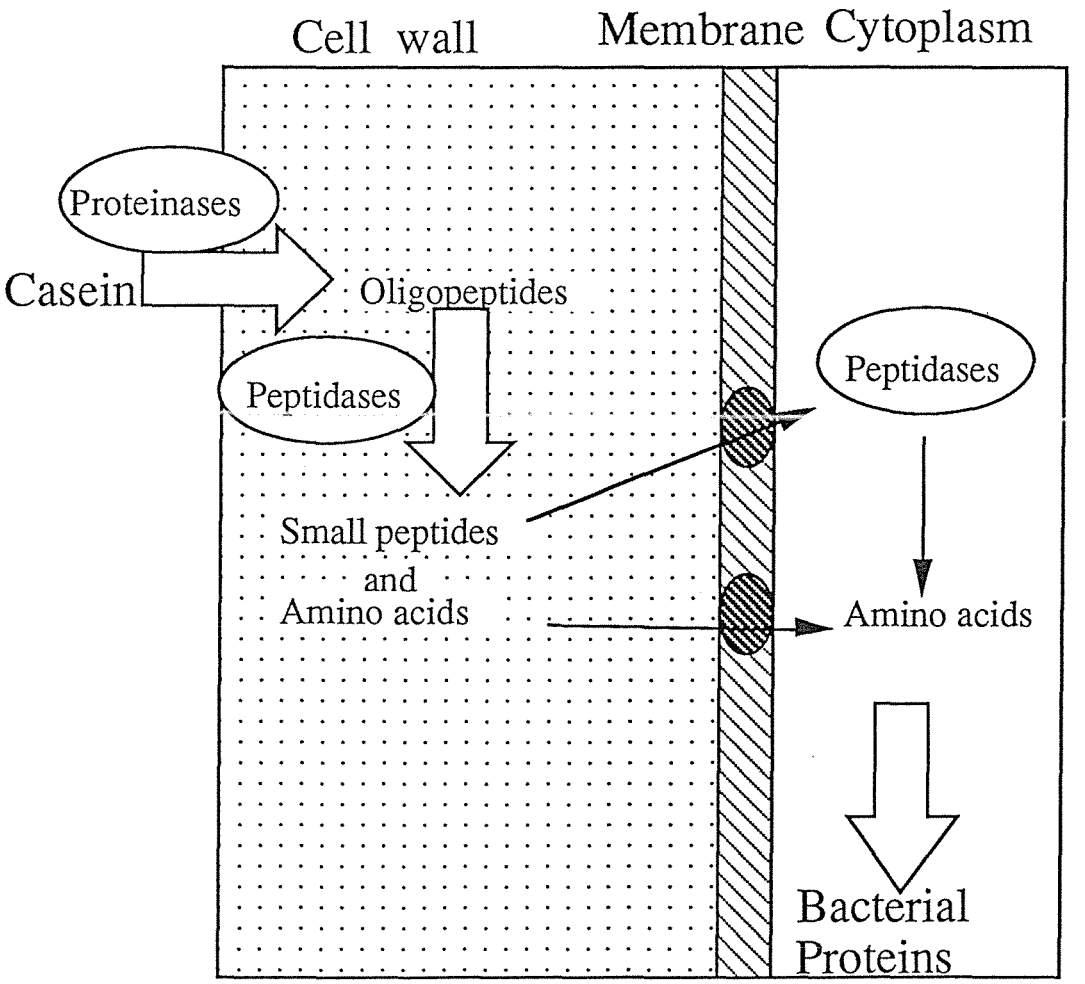


FIG 1.1 Proposed utilisation of casein for synthesis of bacterial proteins by lactic streptococci.

AMINO ACID	REQUIREMENT FOR GROWTH [a]		MINIMUM	CONCENTRATION
	S.cremoris	*S.lactis*	REQUIRED (µg/ml)	IN MILK (µg/ml)
Asp	-	-	29.4	5.0
Thr	-	-	14.9	1.3
Ser	+-	-	12.0	3.7
Glu	+	+	40.1	35.9
Pro	+	-	8.8	0.8
Gly	+-	-	11.9	5.3
Ala	+-	-	19.2	3.5
Cys	-	-	ND	nd
Val	+	+	14.7	2.6
Met	+	+	6.5	nd
Ile	+	+	12.5	0.8
Leu	+	+	21.5	1.2
Tyr	+-	-	10.4	ND
Phe	+	+-	15.8	ND
Lys	+-	-	23.1	4.1
His	+	+	5.9	2.8
Trp	+-	-	ND	ND
Arg	+-	+-	13.1	1.6

TABLE 1.1 *Amino acid requirements of lactic streptococci and the minimum concentrations required for bacterial protein synthesis compared with the levels of amino acids present in milk. (Adapted from Thomas and Pritchard, 1987)*

[a] + = required by all strains tested :+- = required by some strains tested : - = not required.
ND = Not determined
nd = not detectable

1.2 PROTEINASES

Proteinases have the ability to catalyse hydrolysis of intact proteins producing peptides. In the strains of lactic streptococci studied to date, proteinases primarily responsible for the degradation of extracellular proteins remain bound to the cell wall. (Thomas and Mills, 1981). The only recorded exception is the strain *S.cremoris* ML1 which grows in milk, but lacks any detectable cell bound proteinase activity, suggesting the possibility of proteinase liberation into the medium (Exterkate 1976).

The most widely studied strains of *S.cremoris* have a single cell wall proteinase encoded on a plasmid (the proteinase or lactose plasmid). The gene coding for the proteinase from *S.cremoris* Wg2 has been sequenced, and the amino acid sequence deduced from the nucleotide sequence (Kok *et al.*, 1988a). The sequence bears some homology to subtilisin around its active site but is a much larger protein. Several proteolytically active components of differing molecular weights have been reported (Hugenholz *et al.*, 1984; Cliffe and Law, 1985) but these are probably a result of proteinase autoproteolysis (Kok *et al.*, 1988b).

Two clearly distinct types of proteolytic activity have been identified by studying the patterns of degradation of the different caseins by *S.cremoris*. One type designated AM1 degrades both α_{s1} and β-casein, whereas the HP type of proteinase only degrades β-casein (Visser *et al.*, 1986). Whether the proteinases from all strains of lactic streptococci show similar specificity for α and β-casein is uncertain. However, marked preference for β-casein has been found with proteinases from several other *S.cremoris* and *S.lactis* strains, and preference for α and κ-caseins found with the proteinases of some lactobacilli.

The peptide products of β-casein hydrolysed by a purified cell wall proteinase from *S.lactis* 763 have recently been investigated (Monnet *et al.*, 1986). Figure 1. 2 identifies the cleavage points producing five oligopeptides from the C-terminal region which range in size from two to sixteen amino acids.

A largely identical cleavage pattern was found for β-casein hydrolysis by the proteolytic enzymes from *S.cremoris* HP (Visser *et al.*, 1988) although some minor differences in the cleavage pattern were found. A third study (Ng, 1989) of the β-casein cleavage pattern carried out in this laboratory using the protease from *S.lactis* 4760 (the strain used for much of the work reported in this thesis) confirmed the findings of Visser *et al.* (1988). Figure 1. 2 summarizes the results of these three studies which indicate a very consistant cleavage pattern for the proteinases from three different strains. It has yet to be established whether this cleavage pattern represents the process of β-casein hydrolysis during growth of these starter bacteria utilising micellar casein in milk. However these studies give an indication of the possible nature of the oligopeptide substrates for the second group of enzymes involved in proteolysis - the peptidase.

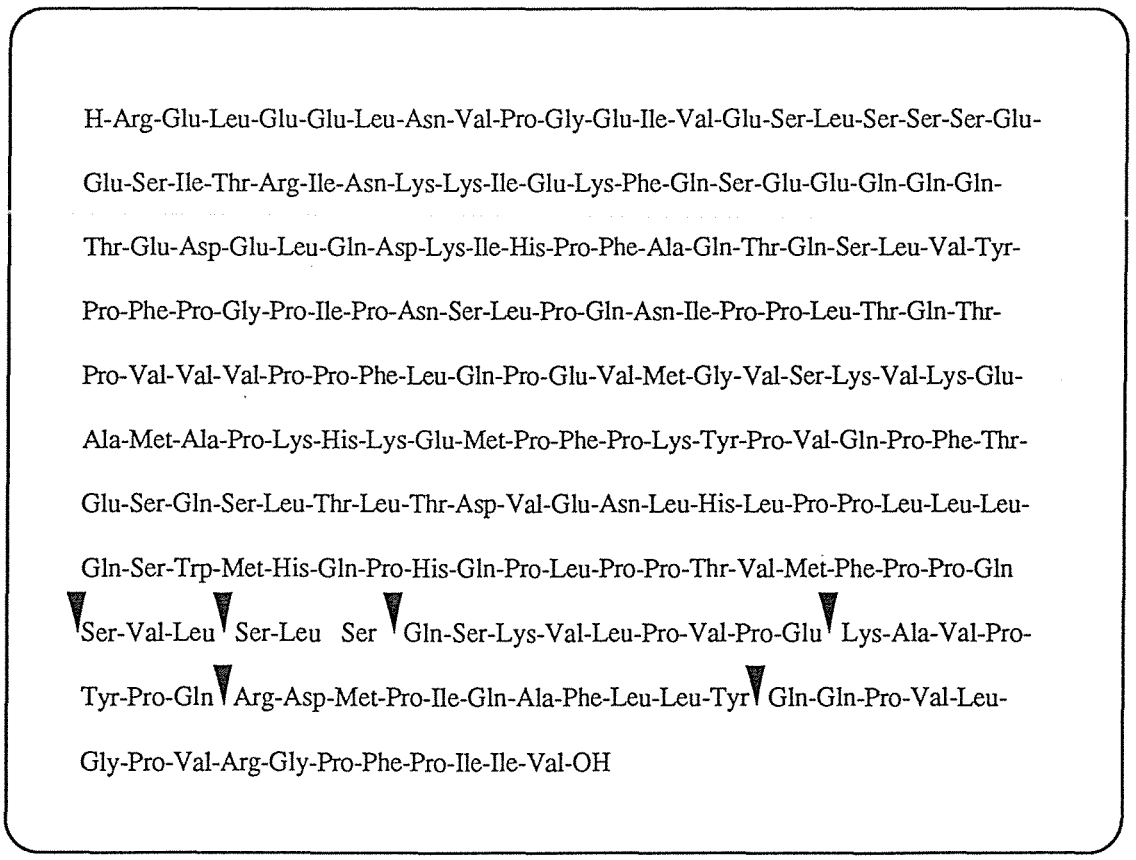


FIG.1.2 *Sequence of β-casein showing the peptides formed after incubation with proteinase enzyme.*

1.3 PEPTIDASES

These enzymes degrade oligopeptides to small peptides and amino acids (Fig 1.1). Although there have been numerous published studies on peptidases from lactic streptococci, there are no systematic studies of the complete peptidase complement from any one strain, and therefore the synergistic interactions between them leading to the hydrolysis of proteinase-generated oligopeptides is not understood.

1.3.1 NOMENCLATURE AND CLASSIFICATION

Peptidase nomenclature in the literature is quite variable. The following is a summary of the peptidase classification used in this thesis.

Peptidases may be classified as endopeptidases or exopeptidases. Endopeptidases cleave bonds distant from the ends of a polypeptide. In addition to the proteinases referred to above, other endopeptidases catalysing the hydrolysis of oligopeptide products of proteinase action may be present in lactic streptococci. Exopeptidases, on the other hand, cleave bonds only near (one or two residues from) the ends, and can be assigned, at least to their major classes, on the basis of substrate specificity. Exopeptidases may be further subclassed into the aminopeptidases (including di -and tri-peptidases) which hydrolyse amino acids from the N-terminal end of a peptide, and the carboxypeptidases which hydrolyse amino acids from the C-terminus. A survey of the published literature reveals the presence of several types of peptidase in lactic streptococci. No carboxypeptidase activity has been reported in any of the strains of lactic streptococci studied, hence the following types may be classified as aminopeptidases.

Peptidase	Bond specificity (*)
a) A"general" aminopeptidase	X--*--Y.....
b) A proline iminopeptidase	Pro--*--Y.....
c) Aminopeptidase P	X--*--Pro.....
d) X-Prolyl dipeptidyl peptidase	X-Pro--*--Y.....
e) A "general" dipeptidase	X--*--Y
f) An imino dipeptidase	Pro--*--X
g) An imido dipeptidase	X--*--Pro
h) A "general" tripeptidase *	X--*--Y-Z or X-Y--*--Z

*TABLE 1.2 A list of aminopeptidase types isolated from lactic streptococci. *The mechanism of action of the tripeptidase has not been studied so the bond specificity pattern is uncertain.*

1.3.2 THE "GENERAL" AMINOPEPTIDASE.

The designation "general" aminopeptidase is given to an enzyme which is active in cleaving aminoacyl derivatives of various chromogenic or fluorogenic substrates with relatively broad specificity for the amino acid. The most widely used substrates are the β-napthylamide and p-nitroanalide (pNA) derivatives, although more recently fluorogenic substrates such as 7-amido 4-methyl coumarin (AMC) derivatives have been introduced (Kato *et al.*, 1978). There are numerous reports of this enzyme in lactic streptococci. It was found to be present in DEAE column fractions distinct from those containing di- and tri-peptidases in a wide range of *S.lactis* and *S.cremoris* strains (Kaminogawa *et al.*, 1984). Aminopeptidases specific for leucyl, glycyl and glutamyl N-terminal amino acids have been found in the soluble fraction of *S.cremoris* after ultrasonic disruption of lysosyme-treated cells (Exterkate, 1984). These aminopeptidase activities, when isolated, appear to be associated with proteins which show different mobilities in gel electrophoresis. A membrane bound aminopeptidase with specificity for glutamyl or aspartyl N-terminal residues has also been reported (Exterkate *et al.*, 1986). In this case however, a free γ–carboxyl group rather than the amino group of the N-terminal amino acid appears to be essential for catalysis. The specific nature of these enzymes contrasts with the wide specificity of the "general" aminopeptidase reported by Kaminogawa *et al*. (1984) although it was not established in this work that the DEAE column fractions contained a single type of enzyme.

A peptidase of molecular weight 36 000 has been purified from the cell wall of *S.cremoris* AC1 which shows aminopeptidase activity, effectively hydrolysing lysyl- p-nitroanalide and, to a lesser extent, leucyl-, alanyl-, and alanyl-alanyl-p-nitroanalides (Geis *et al.,* 1985). This enzyme was found to be active over a pH range of 5.5 to 8 with a maximum at about 7, and was inhibited severely by 1mM EDTA.

"General" aminopeptidases have been purified from two other species of lactic acid bacteria, *L.acidophilus* R26 (Machuga and Ives 1984) and from *L.lactis* (Eggimann and Bachmann 1980).The peptidase from *L.acidophilaus* R26 was responsible for all of the N-terminal exopeptidase and amidase activities observed in crude extracts. The native enzyme, which was found to be a tetramer of molecular weight 156 000, contained four tightly bound Zn atoms. The catalytically inactive native Zn metalloenzyme was capable of being activated by either Zn^{2+}, Co^{2+}, Ni^{2+} or Mn^{2+}.This peptidase is however clearly distinct from the enzyme reported by Kaminogawa *et al,* since it is active against a wide variety of L-amino acid, peptide, amide and p-nitroanalide derivatives including di-, tri- and tetrapeptides.

During the preparation of this thesis, a paper describing the purification and characterisation of an aminopeptidase from *S.cremoris* AM2 was published (Neviani *et al.*, 1989). This intracellular enzyme is reported to be a hexamer of 300 000 molecular weight composed of identical 50 kDa subunits. The authors claim, using a range of β-napthalamide substituted amino acids and di- and tripeptides, that the enzyme had a broad specificity of aminopeptidase activity, but no endopeptidase activity. It was also shown that this enzyme was not a metallopeptidase, but activity was dependant on a thiol group. This distinguishes it from other aminopeptidases detected in mesophilic streptococci or lactobacilli, these generally being metalloenzymes.

1.3.3 "GENERAL" DIPEPTIDASE

There have been numerous studies on dipeptide utilisation and dipeptidase activity from lactic streptococci .

A highly purified dipeptidase from *S.cremoris* H61 has been shown to catalyse hydrolysis of a wide range of dipeptides except those containing proline or glycine as the N-terminal amino acid (Hwang *et al.,* 1981). The substrates of this enzyme can be classified into groups according to their kinetic properties. The Km of this enzyme for various dipeptides were divided into three groups. The first group comprised mainly neutral dipeptides such as Leu-Gly, Leu-Leu and Leu-Ala which had a low Km in the range 4.0 - 6.0mM. Group two consisted of dipeptides with aromatic amino acids at either N or C terminal positions, such as Leu-Phe, Phe-Ala and Leu-Tyr which had very low Km values of 1.0 - 2.4mM. The

third group comprised dipeptides with acidic or basic amino acids at the N terminal position, for example, His-Ala and Glu-Val. This group had high Km values of 10 - 20mM. The optimum pH for this enzyme was found to be pH 8.0.

A second highly purified dipeptidase purified from *S.cremoris* Wg2 has been reported (Van Boven *et al.,* 1988). This enzyme had a molecular weight of 49 000 and specifically hydrolysed a range of dipeptides. It clearly differs from the dipeptidase purified from *S.cremoris* H61, which had a molecular weight of 100 000 (Hwang *et al.,* 1981). Furthermore, the turnover numbers for substrates such as leucyl-leucine or alanyl-alanine were significantly different for the two enzymes. Moreover the metal dependence of both enzymes was distinctly different. For example, 1mM Co^{2+} inhibits the enzyme from *S.cremoris* Wg2, but activates the *S.cremoris* H61 enzyme. There are several clear differences between the types of substrate the two enzymes hydrolyse. There are however also certain similarities between the two enzymes, such as their broad specificity , the requirement of metal ions for activity and the inhibition of the enzymes by reducing agents.

Dipeptidases purified from *S.thermophilus* (Rabier *et al.,* 1973) and *S.diacetylactis* (Desmazeaud *et al.*., 1977) show some similarities to the enzyme from *S.cremoris* Wg2, having a molecular weight of 50 000 - 51 000 and being metalloenzymes with a broad substrate specificity.

Electrophoretic separation of the exopeptidases and the use of a range of dipeptide substrates for activity staining of the gels (Kolstad and Law, 1985) also suggests the presence of only a small number of dipeptidases of broad specificity.

1.3.4 PROLINE AMINOPEPTIDASES

The high proline content of casein has prompted several groups to look specifically for peptidases acting on proline-containing substrates. The five types listed in Table 1.2 have been demonstrated in various lactic streptococci, although there have been no studies demonstrating the presence of all five activities in a single strain. An imido dipeptidase (prolidase) which is highly specific for X-prolyl dipeptides, which may have been removed from the N terminus of a larger peptide, has been purified and characterised from *S. cremoris* H61 (Kaminogawa *et al.,* 1984). An aminopeptidase P which specifically cleaves the amino terminal amino acid when the penultimate residue is proline, and an imino dipeptidase which is specific for proline-X dipeptides have been distinguished in cell-free extracts in a range of lactobacilli and streptococci (Hickey *et al.,* 1983).

Casey and Meyer (1985) have studied the iminopeptidase and dipeptidyl peptidase activity from several lactic acid bacteria by disc electrophoresis. In species such as *L.casei* subsp. *rhamnosus*, the relative activities were found to be about equal and the relative mobilities of the two enzymes were quite separate, but in some species, for example, *S.cremoris* and *S.thermophilus*, the two activities were indistinguishable by electrophoresis . It is conceivable that the iminopeptidase activity in the latter examples is a result of a residual activity of the dipeptidyl peptidase for the iminopeptide. This is supported by the observation that in *S.thermophilus* the dipeptidyl peptidase activity is about five hundred fold greater than that of the proline iminopeptidase.

Meyer and Jordi (1987) have charactised the dipeptidyl peptidase from *L.lactis* and *S.thermophilus*. The enzyme, in both cases, is a serine-type peptidase which has specificity for a wide range of proline containing di- and tri-peptide pNA or AMC derivatives, but no activity with proline-AMC and therefore does not act as an iminopeptidase. It was shown, however, that the *S.thermophilus* enzyme had some endopeptidase activity with the substrate N-succ-gly-pro-AMC. Both enzymes had a molecular weight of 165 000 daltons, an isoelectric point near pH 4.5, and are dimers in their native form. The pH optimum of the enzyme isolated from *L .lactis* was 7.0, whereas the enzyme from *S.thermophilus* possessed a broad pH optimum between 6.5 and 8.2 using Gly-Pro-AMC as a substrate. At pH values lower than 5.0, both enzymes were found to be unstable. However, although of similar molecular weight, the structure of the two dipepeptidyl peptidases differs since the *L.lactis* enzyme is more sensitive to SH blocking agents such as iodoacetate and p-chloromercuribenzoate, while the *S.thermophilus* enzyme is more sensitive to PMSF, a serine protease inhibitor. Both enzymes were only slightly sensitive to EDTA. Even after

incubation with EDTA for 20 hours, activity was not totally lost, and re-addition of bivalent cations did not restore initial activity. To date, a similar enzyme has not been reported in *S.lactis* or *S.cremoris*.

During the course of writing this thesis, a paper describing the purification of an X-Prolyl dipeptidyl peptidase from the cell wall proteolytic system of *S.cremoris* has been published (Kiefer-Partsch *et al.,* 1989). This serine peptidase was reported to have a subunit molecular weight of about 90 000, a native molecular weight of 160 000 - 180 000 and a broad pH optimum between 6.5 and 8. However little work was done to characterise the enzyme further, the extent of testing its substrate specificity being limited to Gly-Pro-pNA and Ala-Pro-pNA.

1.3.5 TRIPEPTIDASES

Existence of tripeptidase activity, distinct from dipeptidase and aminopeptidase activity, has been clearly established by the studies of Kaminogawa *et al.,* (1984) and Kolstad and Law (1985) but to date no tripeptidase has been purified from lactic streptococci.

1.3.6 ENDOPEPTIDASES

Two distinct endopeptidases which may contribute to degradation of casein fragments have been found in the cell free extract of *S.cremoris* H61 (Yan *et al.,* 1987 a and b). One of the endopeptidases termed LEP 2 was purified to homogeneity and shown to be a metallo-endopeptidase of the serine protease type with a molecular weight of 80 000 and a specificity for the peptide bonds involving the amino groups of hydrophobic residues, and has been shown to cleave the N-terminal twenty three-residue oligopeptide from αs1 casein. The involvement of this enzyme in the degradation of β-casein oligopeptide fragments is as yet unclear. From its broad substrate specificity, and its ability to recognise the size of its substrate, it may play a role in providing peptides of reduced size. However its cytoplasmic location might suggest that LEP 2 participates in the cleavage of signal peptides which are highly hydrophobic and of the correct size to be substrates for the enzyme.

A second metallo-endopeptidase termed LEP1 was purified to homogeneity from the same strain (Yan *et al.,* 1987 b) and was found to be a monomeric enzyme with a molecular weight of 98 000. This enzyme showed no detectable hydrolysing activity for milk caseins. LEP1 showed affinity towards a range of peptide bonds found in casein derived peptides and some peptide hormones showing that the substrate specificity of LEP1 was dependent not only on the subsite sequence of substrates but also on the spatial construction of the

substrates. In these respects LEP1 is similar to LEP 2, however the maximum substrate size which could be hydrolysed by LEP1 was smaller than the largest substrate hydrolysed by LEP 2. Both enzymes are clearly different to the cell wall proteinase discussed in section 1.2.

1.4 CELLULAR LOCATION OF PEPTIDASES

There is an increasing body of evidence for the existence of distinct peptidases in different subcellular locations of lactic streptococci. Kolstad and Law (1985) have isolated electrophoretically distinct peptidases from the cell wall and compared them with intracellular fractions using di- and tri-peptide substrates. Contamination of the cell wall fraction by leakage of intracellular enzymes was assessed to be less than three percent by aldolase and lactate dehydrogenase activites. Peptidases located in the cell wall or associated with the cell membrane appeared to differ slightly in their substrate specificity from intracellular peptidases but it is arguable whether these differences are sufficient to substantiate the claim by Kolstad and Law that these are distinct enzymes (Thomas and Pritchard, 1987). However inhibition studies of the lysosyme-released cell wall fraction, and cytoplasmic fractions, has supported the view that the dipeptidases in the cell wall fraction are distinct from the intracellular enzymes (Law 1979). The cell wall-located peptides from *S.cremoris* 1196 and *S.lactis* 763, in contrast to intracellular enzymes were inhibited by mercaptoethanol,and inhibition of cell wall dipeptidases by EDTA was reversed by calcium but not cobalt or manganese, whereas with intracellular peptidases cobalt and manganese were specifically required.

No aminopeptidase activity was detected by Kolstad and Law (1985) in the cell wall fraction but since the dipeptidase recovered from the growth medium by Law (1979) appears to be the same as one of the intracellular dipeptidases, the absence of other peptidase activities, such as aminopeptidase activity, in the growth medium raises the question of the basis for the apparently selective "leakage" of specific peptidases (Kolstad and Law,1985). Exterkate (1984) found that intact cells do possess aminopeptidase activity, as assayed by amino acid-pNA derivatives. However the ability of the pNA derivatives to penetrate the cell membrane and therefore assay intracellular aminopeptidases has not been studied. In support of Exterkate though, an apparently cell wall-bound aminopeptidase of molecular weight 36 000 has been isolated from *S.cremoris* AC1 by repeated washing of intact cells with Tris buffer (Geis *et al.*, 1985). No lysis or protoplasting was observed by microscopy, and no significant contamination of the preparation with cytoplasmic material was detected, confirming that this enzyme is located at the outer boundary of the bacterial cell wall. The penetration of substrates in this instance was not an issue, since the enzyme was completely separated from the cell. Thus, although the existence of different intracellular and cell wall-bound peptidases has been implied, more work is needed to characterise these differences.

1.5 OBJECTIVES OF THE PRESENT PROJECT

The main aim of the present study was to purify as far as possible two aminopeptidases from lactic streptococci and to study their properties and possible role in the degradation of casein-derived oligopeptides.

1) An X-Pro dipeptidyl peptidase. This enzyme has been purified and characterised from *L. lactis* and *S.thermophilus* (Meyer and Jordi 1987) but not from mesophilic lactic streptococci. Their study of the specificity of the enzyme was restricted to X-proline dipeptide derivatives so the ability of this enzyme to hydrolyse proline-containing peptides has not been examined.

2) A "general" aminopeptidase. There are no published studies of a purified "general" aminopeptidase from lactic streptococci even though many studies have reported the presence of an enzyme (or enzymes) catalysing the hydrolysis of a range of aminoacyl-pNA or AMC derivatives. The ability of this enzyme to catalyse the sequential hydrolysis of amino acids from oligopeptides has not been studied nor is there any information on its substrate specificity.

The specific objectives are as follows:

a) The purification and study of a dipeptidyl peptidase and the partial purification of a "general" aminopeptidase from a strain of *S.lactis* * (*S.lactis* H1) . This will involve the assessment of potential affinity purification techniques and the development of gel electrophoresis activity staining procedures.

b) Synthesis of a range of fluorogenic substrates and a study of the specificity of the purified enzymes using these substrates .

c) An investigation of the ability of these enzymes to degrade large oligopeptides and a comparison of their bond specificity for oligopeptides and AMC derivatives.

d) A comparison of the dipeptidyl peptidase purified from *S.lactis* H1 with that from an *S.cremoris* strain.

*The lactic streptococci have recently been placed in a new genus *Lactococcus,* with the single species having two sub-species, *L.lactis* subsp.*lactis,* and *L.lactis* subsp.*cremoris*. While this new nomenclature is currently gaining wide acceptance in the literature, the old designations are still in use and are retained in this thesis.

CHAPTER 2 - MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 BACTERIAL GROWTH MEDIA

Yeast extract was obtained from Gibco laboratories. Beef extract and Trypticase peptone were from BBL USA. Lactose was from BDH Chemicals, England. Low heat skim milk powder was provided by the New Zealand Dairy Research Institute and the sodium β-glycerophosphate was from Sigma Chemical Co.USA.

2.1.2 SUBSTRATES FOR ENZYME ASSAY

Aminoacyl AMC compounds were either prepared from their BOC-amino acid derivatives (obtained from Sigma) or obtained as such from Sigma. AMC was prepared by the Separation Science Unit, Massey University. All other reagents used in substrate synthesis were, wherever possible, of analytical reagent grade, and supplied by BDH or May and Baker, England.

2.1.3 CHROMATOGRAPHIC RESINS

Diethylaminoethyl (DEAE) cellulose resin for anion exchange was from Whatman Biochemicals, England. Sephacryl S300 resin for gel permeation chromatography was obtained from Pharmacia fine chemicals, Sweden. Pharmacia also supplied the arginine Sepharose 4B used in some of the enzyme purification preparations. However, in the final purification procedure, a resin prepared by Dr. N.Haggarty of the Separation Science Unit, Massey University, was used (see Section 2.2.10).

2.2 METHODS

2.2.1 BACTERIAL STRAINS

The following strains of lactic streptococci were obtained from the New Zealand Dairy Research Institute Palmerston North :

S.cremoris H2 strain 4409

S.lactis strain 4125 (a plasmid-free strain of *S.lactis* H1)

S.lactis strain 4760 (a transconjugant strain derived from *S.lactis* 4125 into which the lactose/proteinase plasmid from *S.cremoris* strain H2 has been transferred by conjugation).

2.2.2 MEDIA

a) Lactose broth medium. This contained the following components per litre of deionised water:

Lactose 20g
Peptone 10g
Yeast extract 10g
Potassium dihydrogen phosphate 5g
Beef extract 2g
Magnesium sulphate 0.2g
Manganese chloride 0.05g
The complete medium was sterilised at 121° C

b) Reconstituted skim milk medium (RSM). This was prepared by thoroughly blending 10g of low heat skim milk powder in water using a homogeniser and making up to a final volume of 100ml.(Thomas and Turner 1977). In order to grow the bacteria to high cell densities, RSM was buffered by adding 3ml of 2.5M sodium β-glycerophosphate per 100ml of medium. This avoids the coagulation of the milk medium by the lactic acid produced during bacterial growth.

2.2.3 GROWTH OF BACTERIA.

Bacterial strains were maintained as milk cultures grown on RSM to the point of coagulation and stored either at 4° C (short term) or at - 80° C (long term). Short term stocks were subcultured at approximately monthly intervals. For *S.lactis* strain 4125 the RSM was supplemented with 0.2% (w/v) peptone and 0.5% (w/v) glucose.

For preliminary studies, cultures were grown either in flasks using RSM buffered with glycerophosphate, or in a 3 litre fermentor in broth. Milk cultures were prepared by inoculating 100ml of RSM medium with 3ml of starter culture, and incubating it for 18 hours at 24° C. Prior to harvesting the bacteria by centrifugation, the cultures were neutralised to pH 7.0 with 1M NaOH, and 6ml of 25% sodium citrate was added per 100 ml of culture to complex calcium and disperse protein micelles in the milk.

Broth-grown cells were grown in a 3 litre fermentor (LH Engineering UK.) at 30° C, gassed with 5% CO_2 in nitrogen and maintained at pH 6.0 to 6.5 by automatic alkali addition. For *S.lactis* strain 4125 which lacks the lactose plasmid, the broth medium contained 40g/l (w/v) of glucose replacing the lactose. Approximately 20ml of broth-grown cells (8 hour culture) was used to inoculate 2.5 litres of medium. Cultures were harvested either after 6 hours growth in batch culture or, when grown as continuous cultures, after overnight collection of the overflow on ice. Cells were harvested by centrifugation at 10 000xg for 15 minutes and washed twice by resuspension in cold 50mM Tris/HCl buffer pH7.5 containing 20% glycerol by volume (this will subsequently be referred to as Tris/glycerol buffer, other details and additions being specified in the appropriate places) and recentrifugation.

For most of the major enzyme purifications described in the Results section, cells were grown in 35 or 40 litre batches in a Fermacell fermentor (New Brunswick Scientific Co USA.) at the Biochemical Processing Centre DSIR, using the same growth conditions as for smaller cultures. Cells were harvested after 6 hours growth by continuous flow centrifuge and washed. The yield from broth cultures was approximately 9 to 10g wet packed weight of cells per litre of culture. Cells were stored frozen at -15° C until required.

2.2.4 PREPARATION OF CELL-FREE EXTRACT

The frozen pellet of cells from broth or milk cultures was slowly thawed. Milk-grown cells were resuspended in two volumes of 10mM phosphate buffer pH 7.0. Large batches of broth-grown cells used for dipeptidyl peptidase purification were resuspended in two volumes of 50mM Tris/glycerol buffer pH 7.5. Cells used for "general" aminopeptidase purification were resuspended in 50mM Tris/glycerol pH 7.0. The cells were disrupted by a single passage through a French pressure cell at 5500 psi (38 Mpa). Cellular debris and unbroken cells were removed by centrifugation at 27 000xg for 30 minutes and the clear supernatant collected. The pellet was resuspended in a small volume of buffer and recentrifuged, the clear supernatant being added to that collected previously. The combined supernatants are referred to as the cell-free extract.

2.2.5 GROWTH AND HARVESTING BACTERIA FOR PROTEINASE PREPARATION

A *S.lactis* 4760 culture was grown in 3x1litre batches of RSM as described in Section 2.2.3. Cultures were harvested by centrifugation at 10 000xg for 10 minutes at 4° C. Pelleted cells were rapidly washed twice by resuspending in ice-cold 50mM sodium acetate/phosphate buffer, pH 6.4, and recentrifuged.

2.2.6 RELEASE AND PARTIAL PURIFICATION OF CELL WALL-ASSOCIATED PROTEINASE

The procedure for the release of cell wall-associated proteinase was based on the studies of Mills and Thomas, (1978). Milk grown cells were washed and resuspended in 50mM phosphate buffer at pH 6.4. The cell suspension was incubated at 30° C for 1 hour and then centrifuged at 12 000xg for 5 minutes at 4° C. The supernatant containing the crude cell wall-associated proteinase was retained, and the incubation of the resuspended pellet repeated for a further hour after which the cell suspension was centrifuged and the supernatant added to that retained earlier.

The crude cell wall proteinase was equilibrated with 10mM Tris/HCl buffer pH 8.0 by extensive washing over a PM 30 ultrafiltration membrane in an Amicon Diaflo concentrator until the pH was about 8. The diafiltered enzyme was then applied to a 3.3cm x 12cm column of DEAE cellulose and subsequently eluted with a linear gradient of 0 - 1 M NaCl. The fractions were assayed for proteinase activity (see Section 2.2.8),

and the fractions containing high activities were pooled and concentrated using an Amicon Diaflo concentrator with a PM 30 membrane to a volume of about 6ml. The concentrated material was then loaded onto a Sephacryl S300 column (3cm x 75cm) previously equilibrated with 10mM Tris /HCl, at pH 8.0. Fractions with proteinase activity were pooled. This partially purified proteinase preparation was found to be free of Lys-AMCase and Gly-Pro-AMCase activity.

2.2.7 SYNTHESIS OF AMINOACYL AMC DERIVATIVES

Peptide amides of 7-amino-4-methyl coumarin (AMC) were prepared using a method provided by Dr. D.R.K. Harding of the Massey Separation Science Unit which was modified from Kato *et al.*, (1978).

A) Preparation of BOC-Pro-AMC

0.1 mole of α N-BOC -Proline was dissolved in 100ml of tetrahydrofuran, and the symmetrical anhydride was formed by the addition of 0.05 mole of dicyclohexyl carbodiimide with continuous stirring at 0° C. After 45 minutes, the dicyclohexyl urea formed was removed by filtration. A solution of AMC (0.05 moles) in 60ml of N, N-dimethyl formamide was added to the above filtrate at 0° C.

After overnight stirring at room temperature, the reaction mixture was concentrated to an oily residue under reduced pressure (rotary evaporator and high vacuum pump). The residue was dissolved in ethyl acetate, and the solution was washed twice with both 10% sulphuric acid and saturated sodium bicarbonate followed by a single wash with saturated sodium chloride. (The acid removes unreacted AMC while the bicarbonate removes unreacted BOC-Pro). The organic layer was dried over anhydrous magnesium sulphate and filtered through Whatman No. 40 filter paper. The filtrate was concentrated to a crystalline solid under reduced pressure.

B) Preparation of the HBr salt of Pro-AMC

The BOC-Pro was reacted for one hour with HBr in glacial acetic acid (20ml). A flocculent off-white solid was then precipitated by the addition of 100ml of anhydrous diethyl ether. The ether was decanted, and a second 100ml addition made. The solid was then filtered off under vacuum and the remaining HBr gas removed under high vacuum overnight. The product was dissolved in 20ml of methanol and re-precipitated as a white solid with ether. The solid was collected by filtration and dried under vacuum.

C) Preparation of BOC-Gly-Pro-AMC

1.4×10^{-3} mole BOC-glycine was dissolved in 20ml of p-dioxane and the active ester formed by the addition of 1 equivalent each of dicyclohexyl carbodiimide and hydroxybenzotriazole. After two hours the dicyclohexyl urea was removed by vacuum filtration, and 1 equivalent of HBr.Pro-AMC dissolved in 10ml p-dioxane plus 10 ml dimethyl formamide, was added at 0^{o} C, together with 1 equivalent of triethylamine.

After overnight stirring at room temperature the reaction mixture was concentrated to a oily residue under reduced pressure as before. The residue was dissolved in a two phase mixture of ethyl acetate and a saturated solution of sodium bicarbonate (the hydroxybenzotriazole was soluble in the aqueous bicarbonate phase which was discarded) and washed with 10% sulphuric acid, sodium bicarbonate and sodium chloride, followed by dessication using magnesium sulphate. The ethyl acetate was removed after filtration resulting in a solid crystalline product. The BOC group was removed with HBr in acetic acid as described above (2.2.7 B).
This procedure was followed for the synthesis of all the substrates listed below.

HBr.Lys-AMC
HBr.Leu-AMC
HBr.Gly-Pro-AMC
HBr.Glu-Pro-AMC
HBr.Phe-Pro-AMC
HBr.Leu-Pro-AMC
HBr.Lys-Pro-AMC

In the case of HBr.Glu-Pro-AMC, the benzyl ether protecting the γ carboxyl was removed by hydrogenation using a palladium on charcoal catalyst. However, since little hydrogen uptake occurred during the reaction, the benzyl group may have been previously removed during the procedure used to remove the BOC group. HBr.Gly-Ala-AMC was synthesised similarly, substituting proline with alanine and using a tenfold reduction in scale.

A sample of each substrate was subjected to acid hydrolysis to determine the amino acid composition. For each dipeptide substrate, a 1.0 : 1.0 ratio of the two amino acids was found.

In the case of HBr.Gly-Pro-AMC, which was used as the standard substrate in routine

assays, and HBr.Glu-Pro-AMC, the composition of which was in some doubt, an elemental analysis was carried out by the microanalytical laboratory, Chemistry Department, University of Otago. Their analysis showed these two substrates to have the following compositions (Henceforth the HBr will be omitted for convenience) :

	Gly-Pro-AMC		Glu-Pro-AMC	
Element	(theoretical %)	(determined %)	(theoretical %)	(determined %)
Carbon	49.6	47.4	49.6	46.6
Hydrogen	5.1	5.2	5.4	5.3
Nitrogen	10.2	9.6	8.7	8.0
Bromine	19.4	19.4	16.3	17.1

In both cases the carbon result is low, perhaps as a result of contamination by salts used in the work up procedure, or by water, since all substrates were hydroscopic. However, the reasonable agreement for the elements other than carbon indicates only low levels of contamination.

The purity of all X-Pro-AMC derivatives was determined by using the Gly-Pro-AMCase enzyme to completely remove the AMC from a known concentration of substrate. The final fluorescence value, determined relative to a 100mM AMC standard showed the substrates to have the following purity:

Substrate	% Purity
Gly-Pro-AMC	90.0
Glu-Pro-AMC	89.0
Leu-Pro-AMC	89.0
Lys-Pro-AMC	91.1
Phe-Pro-AMC	90.0

2.2.8 ENZYME ASSAYS

i) AMINOPEPTIDASE ASSAYS

Instead of the chromogenic p-nitroanilide group, other compounds can be attached to an oligopeptide to facilitate assay of peptidase activity. If these groups have specific fluorescent characteristics their liberation by amidolytic enzymes can be followed by using a spectrofluorimeter. Fluorimetry is known to be about ten-fold more sensitive than spectrophotometry. AMC derivatives were used to assay for both dipeptidyl peptidase activity (Gly-Pro-AMCase activity) and "general" aminopeptidase activity (Lys-AMCase activity).

A) Quantitative assay

Peptidase activity towards AMC substrates was quantitatively measured using the following assay mixture:

100mM MES buffer pH 6.8	1.5ml
Substrate 3.0mM	0.5ml
Sample	0.10ml

1 unit of enzyme activity is defined as that amount of enzyme producing 1μmole AMC per minute.

Analysis was carried out using an AMINCO SP500 Ratio Spectrofluorimeter. The relative fluorescence ratio of the reaction mixture was measured at an excitation wavelength of 385nm and an emission wavelength of 460nm (spectra shown in Fig. 2.3). To quantitate the liberation of AMC in the reaction mixture, the fluorimeter was accurately standardised using a solution of recrystallised AMC prior to the measurement of the rate of enzymatic hydrolysis of the substrate. AMC was recrystallised from boiling 80% isopropanol (melting point 225-229° C, theoretically 223-226° C). 17.5mg of the crystalline solid was dissolved in 1 litre of methanol to give a concentration of 100μM. This solution was measured using an Aminco DW 2a spectrophotometer, the calibration of which had been checked using 0.001M potassium dichromate in 1M sulphuric acid. An ε value of 2300 l/mol/cm was obtained for this dichromate solution (theoretical value = 2270 l/mol/cm). The methanolic AMC solution gave an absorbance of 1.70, (theoretically, 100μM AMC = 1.68, Ram Reddy *et al.*,1986) showing the solution to be exactly 100μM. 17.50mg of the crystalline AMC was accurately weighed and completely dissolved (overnight) in 1 litre of

deionised water. The absorbance spectrum for the aqueous solution was slightly different compared to that of the methanolic solution, the λmax for the aqueous solution being at 340nm rather than 350nm seen with the methanolic solution. Thus, the direct comparison of the two solutions was not possible, and no value for the λmax of AMC in aqueous solution could be found in the literature. The aqueous solution was found to have an absorbance of 1.03 at 360nm, and this was used as a standard for calibrating other AMC solutions, and to calibrate the fluorimeter, giving a maximum full scale deflection on the chart recorder equivalent to 100 μM AMC.

Linearity of quantitative assay procedure

To establish that the assay procedure used to determine enzyme activity gave a linear dependence of enzyme activity on enzyme concentration, activity measurements were made at several different concentrations of either purified X-Pro-dipeptidyl peptidase or partially purified Lys-aminopeptidase. The linear graph plotted from the data obtained using the X-Pro-dipeptidyl peptidase is shown in Fig 2.1.

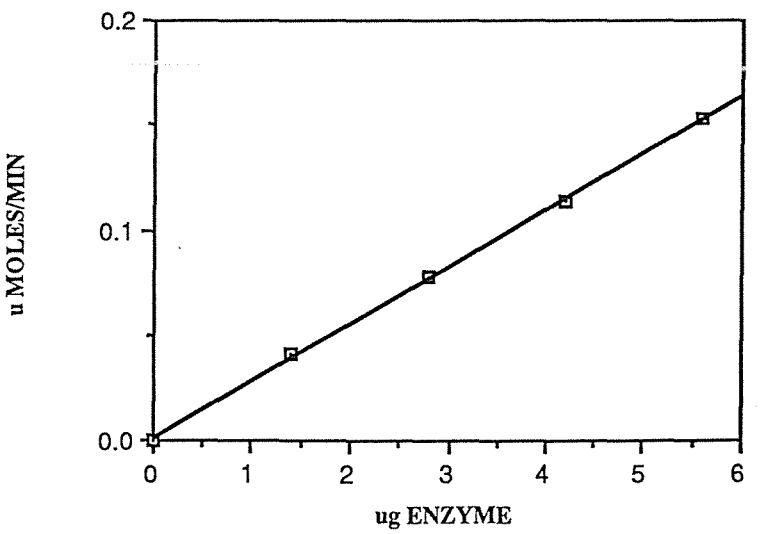


FIG.2.1. Linearity of the assay procedure. Activity of the dipeptidyl peptidase using Gly-Pro-AMC as a substrate, is plotted against enzyme concentration showing that activity is directly related to the amount of enzyme in the assay mixture. Similar results were obtained for activity verses enzyme concentration with the Lys-aminopeptidase.

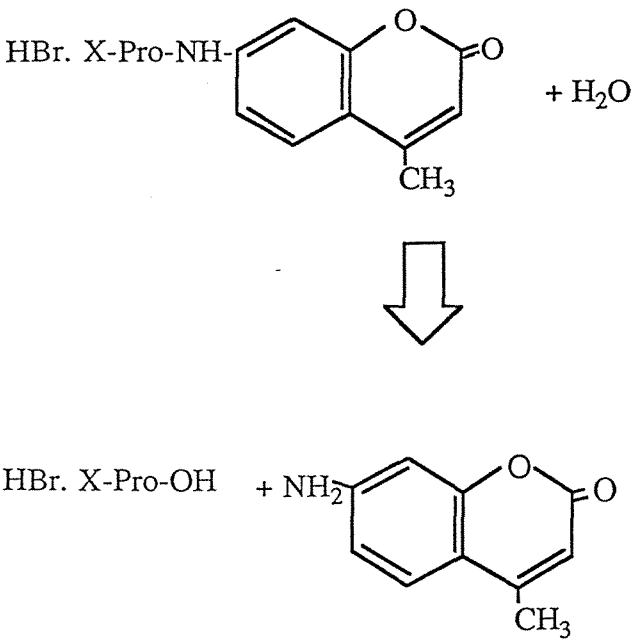


FIG.2.2. A peptidase with limited specificity will hydrolyse a peptide-AMC substrate, releasing 7-amino-4-methyl coumarin.

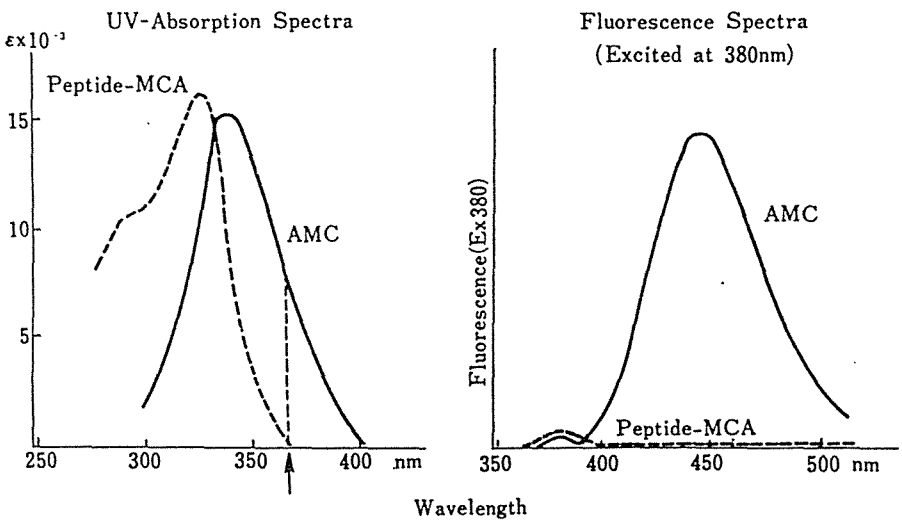


FIG.2.3. UV absorbtion spectra and fluorescence spectra for 7-amino-4-methyl coumarin

B) Semi quantitative assay

Fractions eluted from columns were assayed by a semi quantitative assay procedure to establish the relative rates of hydrolysis by each fraction by measuring the increase in the concentration of free AMC liberated in the following reaction mixture.

Deionised water	5ml
HBr.Gly-Pro-AMC	50µl
Column fraction	~20µl

Each mixture was incubated for a standard length of time (usually about 5 minutes) at room temperature and the AMC concentration measured using a fluorimeter as described above.

ii) OTHER PEPTIDASE AND PROTEINASE ASSAYS

ii a) Dipeptidase assay: Dipeptidase activity was determined by measurement of hydrolysis of alanyl-alanine. 0.4ml of diluted sample was added to a reaction mixture containing 0.4ml of 0.1M phosphate buffer pH 8.0, and 0.4ml of 1mM alanyl alanine. 200µl samples were removed after 2, 5, 10 and 20 minute intervals and added to 100µl of 0.1M acetic acid. To each 200µl sample was added 0.7ml demineralised water, 0.5ml of 0.2M citrate buffer pH 5.0 and 1.2ml of ninhydrin/KCN solution (see below).

Ninhydrin/KCN solution:
Solution α; 0.01M KCN diluted 5ml to 250 ml with methoxy ethanol.
Solution β; 5% ninhydrin in methoxy ethanol.
Mix 10 parts α to 2 parts β 10-30 minutes before use.

The samples were heated in a boiling water bath for exactly 7.5 minutes cooled and 1ml of water added. Enzyme activity was estimated from the rate in increase of absorbance at 570nm over the 20 minute period.

ii b) Tripeptidase assay: Tripeptidase activity was determined by measurement of hydrolysis of 1mM leucyl-glycyl-glycine. The procedure was the same as that used for the dipeptidase assay.

ii c) Proteinase assay. The method of Twining (1984) based of the rate of production of TFA soluble fluorescent peptides from fluorescein isothiocyanate (FITC) conjugated casein was used. Proteinase activity towards FITC-β-casein was measured using the following reaction mixture:

50mM $NaOAc/H_2PO_4$	100µl
FITC-β-casein	100µl
Enzyme extract	100µl

The reaction mixture was incubated at 22° C and 60ml aliquots were removed at various time intervals for up to two hours and then added to 150µl of 5% (w/v) trichloroacetic acid in Eppendorf tubes. After standing for 30 minutes at room temperature, the TCA precipitate was removed by centrifugation for 2 minutes at full speed in an Eppendorf Microcentrifuge. 100µl of the supernatant was added to 3ml of 500mM Tris/HCl buffer at pH 8.5 and the relative fluorescence ratio of the solution measure in an Amicon SP 500 ratio spectrofluorimeter using an emission wavelength of 525nm and an excitation wavelength of 490nm.

2.2.9 PROTEIN DETERMINATION

Protein was measured using bicinchoninic acid solution (Sigma Chemicals USA. procedure number TP RO 562). The assays were performed by adding 0.4ml of 4% copper sulphate to 25ml of the bicinchoninic acid solution and incubating 2ml of this mixture with 100µl of sample for 30 minutes at 37° C before measuring the intensity of absorbance at 562nm. A standard curve was constructed from 0.5mg/ml BSA to cover the range 0-50µg protein per tube. Although the bicinchoninic acid method is less sensitive than the Folin protein assay (Lowry *et al.*, 1951) the latter method is unsuitable since 10mM Tris used in the buffers interferes, giving erroneously high results.

2.2.10 SYNTHESIS OF ARGININE-SEPHAROSE 4B RESIN

Separations were initially carried out using arginine-Sepharose obtained from Pharmacia, Sweden. However the major purifications of both the X-Pro dipeptidyl peptidase and "general" aminopeptidase were achieved using resin prepared in this laboratory. In the Pharmacia resin L-arginine is coupled to Sepharose 4B after epoxy activation. The resulting product contains arginine attached to the matrix via a stable alkylamino linkage and a long hydrophobic spacer arm formed from 1,4-butanediol diglycidyl ether as shown in Fig 2.4.

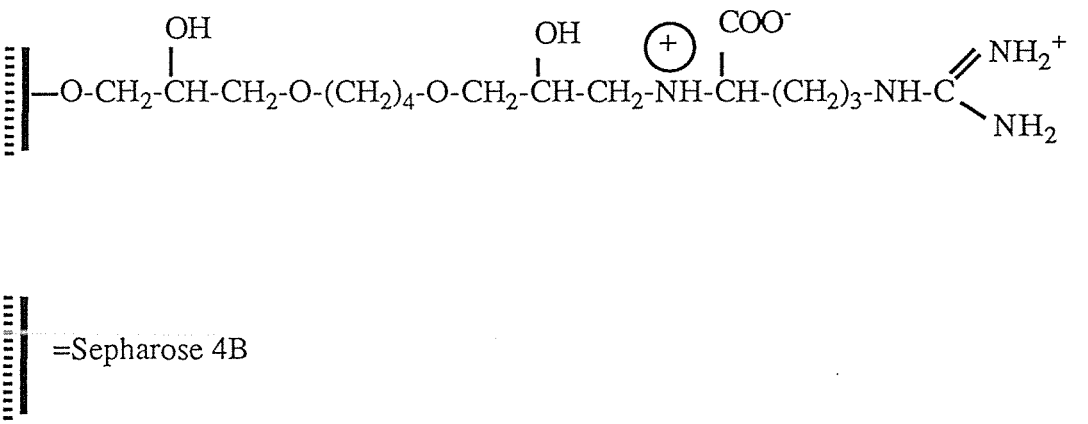


FIG.2.4. Arginine-Sepharose 4B produced by Pharmacia

Initial attempts to synthesise a resin which had the same separation properties as the Pharmacia arginine -Sepharose failed, although the same linker was used and titration of the activated resin revealed good substitution of 0.8 meq arginine. A second attempt using carbonyl diimidazole activation and a γ-amino-caproic acid spacer was more successful. The separation properties, namely, the conductivity at which specific proteins were eluted, was identical to that seen with the Phamacia resin. The method of synthesis of this resin is as follows.

Equivalent weights of Sepharose 4B and carbonyl diimidazole were reacted for 1 hour at 25° C. An 8M excess of γ-amino-caproic acid was added and the synthesis adjusted to pH 11.0. After reacting for 18 hours at 25° C with constant gentle agitation (on a rolling machine) the resin was filtered under vacuum and washed to remove the excess γ-amino-caproic acid. To check the substitution at this stage a small quantity of resin which had been washed several times, firstly with 0.1M HCl and then with demineralised water, was titrated with 0.1M NaOH. A 5 to 8 M excess of arginine, previously adjusted to pH 4.7 was then added to the resin. Finally, 1-ethyl-3-(3 dimethyl amino propyl)

carbodiimide in a similar excess and adjusted to pH 4.7 was added. The pH of the reaction mixture was monitored. An initial pH rise was followed by a decrease which was again adjusted to pH 4.7. The resin was left for 18 hours, and then filtered under vacuum, washed with demineralised water and stored as a slurry containing 0.9% NaCl, and 0.01% thiomersal to prevent bacterial decomposition. A substitution of approximately 95% was achieved.

2.2.11 SYNTHESIS OF GLYCINE-PROLINE-AH-SEPHAROSE RESIN

Glycine-Proline-AH-Sepharose has been used by other workers to purify dipeptidyl peptidases from both mammalian and bacterial sources (see Section 3.2.5).

t-BOC-glycyl-proline was prepared by adapting the method of Agarwal *et al.* (1969). L-proline (20m moles) was dissolved in 10ml of demineralised water and 20ml of dimethyl formamide (DMF). Triethanolamine (20mmoles) was added. The solution was cooled to 0° C, and t-BOC-glycine was added together with 20mmoles of hydroxy succinamide and 20 m moles dicyclohexyl carbodiimide. After an hour, the dicyclohexyl urea was removed by filtration and the reaction was left to proceed overnight. The product was worked up as described in Section 2.2.5, and crystallised from ethyl acetate, the crystals having a melting point of 141-144° C and a composition by amino acid analysis of glycine and proline in a 1 : 1 ratio.

Removal of the BOC protecting group was achieved by dissolving 0.57g of the crystalline BOC-glycyl-proline in 40ml of TFA and 40ml of dichloromethane. After 1 hour, the solvent was removed by rotary evaporation. The oil remaining was dissolved in water and freeze dried. However, after this procedure the product was still in the form of an oil. Thin layer chromatography revealed the reduced mobility of the glycyl-proline product (in butanol, acetic acid, water 4:1:1) when compared with the less polar BOC-protected dipeptide, confirming that the BOC group had been removed by the TFA.

Coupling to AH-Sepharose resin

Glycyl-proline (100mg) was dissolved in 40 ml of water and adjusted to pH 4.7. 2g of AH-Sepharose was added with stirring and 1mmole of 1-ethyl-3-(3-dimethyl amino propyl) carbodiimide (EDC) dissolved in 5ml of water was added dropwise. The pH was monitored for 1 hour after EDC addition and re-adjusted to pH 4.7. The coupling reaction was continued overnight after which excess reagents were removed by vacuum filtration and washing with water. Since such a small quantity of resin was produced, it was not

possible to check the degree of substitution.

2.2.12 POLYACRYLAMIDE GEL ELECTROPHORESIS

A) NATIVE GELS

The method used for polyacrylamide gel electrophoresis (PAGE) was modified from a protocol developed by the Separation Science Unit, Massey University. The following stock reagents were prepared and stored refrigerated for up to two months.

Resolving gel buffer	
Tris	22.7g
1M HCl	30.0ml
Demineralised water	up to 100ml
25% acrylamide	
Acrylamide	25.0g
Bis-acrylamide	0.65g
Demineralised water	up to 100ml
Stacking gel buffer	
Tris	5.98g
1M HCl	48ml
Demineralised water	up to 100ml
30% acrylamide	
Acrylamide	29g
Bis-acrylamide	1g
Demineralised water	up to 100ml

Running buffer concentrate	
Tris	15.125g
Glycine	72.1g
demineralised water	up to 500ml

A 7.5% gel was prepared as follows:

<u>Mixture A</u>	
25% acrylamide	9.0ml
Demineralised water	15.0ml
Resolving gel buffer	6.0ml

To prepare the sealing gel, 7.5ml of mixture A was taken, and to this 15µl of TEMED and 150µl of 10% ammonium persulphate was added. This solution was mixed and poured at once along the base of the glass plates on a casting stand. Setting time was approximately 30 minutes. To the remainder of mixture A, 15µl of TEMED and 150µl of 10% ammonium persulphate was added, and the solution mixed and quickly poured between the glass plates of the slab gel apparatus. A layer of water was gently poured on top of the resolving gel. Polymerisation time was approximately 1 hour.

Mixture B

30% acrylamide	1.0ml
Demineralised water	5.0ml
Stacking gel buffer	1.5ml

To prepare the stacking gel, 10µl of TEMED and 100µl of 10% ammonium persulphate was added to mixture B. This was quickly poured between the glass plates on top of the resolving gel (after removing the water from above the resolving gel) and a 10 tooth comb inserted so that 1cm of stacking gel was left below the wells.

50ml of running buffer concentrate was made up to a final volume of 500ml after adjusting the pH to 8.8 with 1M NaOH. The gel was run at room temperature at a constant current of 22mA.

B) DENATURING GELS

The protocol for running SDS PAGE was identical to that described for native gels with the exception of 2.5ml of 20% SDS added to resolving gel and stacking gel stock solutions and to the diluted running buffer.

Samples to be run on SDS gels were prepared by adding 100µl of 6M urea/10% SDS and 10µl of β-mercaptoethanol per 100µl of sample and boiling for 5 minutes. 5µl of bromophenol blue (0.5% in 5% glycerol) was added to samples for both native and denaturing gels, and 30-50µg of protein per well gave optimum resolution when stained with Coomassie blue.

C) LOW pH GELS

To maintain the activity of enzymes labile at high pH values usually used for electrophoresis, it was necessary to develop a low pH gel system. Native PAGE was carried out as described previously, however the buffers were substituted with those shown below (adapted from Davis, 1964)

Resolving gel buffer

35ml of 2.0M triethanolamine titrated to pH 7.4 with 50% HCl
Demineralised water up to 100ml

Stacking gel buffer

7.0ml of 3-picoline titrated to pH 5.4 with 50% HCl
Demineralised water up to 100ml

Running buffer

6.75g of TES titrated to pH 7.6 with 2.0M triethanolamine
Demineralised water up to 500ml

Low pH gels were run at 22mA at 4^0 C

2.2.13 AMINO ACID COMPOSITION AND SEQUENCE ANALYSIS

Freeze dried proteins and peptides were dissolved in 200µl of 5.9M glass distilled HCl and sealed in test tubes below 0.2mm pressure. Hydrolysis was typically carried out for 24 hours at 110°C. In the case of amino acid composition of the purified dipeptidyl peptidase, hydrolysis was carried out for 24, 48 and 72 hours. After hydrolysis, the tubes were opened and the contents desiccated using P_2O_5. The hydrolysed peptide was re-dissolved in a minimum volume of demineralised water and loaded onto a Beckman 119 BL amino acid analyser using a standard amino acid hydrolysis format. Amino acid composition was estimated by integration using a Specta-Physics 4290 integrator. The system was calibrated using Beckman 25nM amino acid standards. Amino acid sequence data were obtained by the automated Edman method using an Applied Biosystems 470 gas-phase protein sequencer.

2.2.14 ASSAYS USED TO CALIBRATE THE S300 GEL FILTRATION COLUMN

a) β-amylase: 100µl samples were incubated with 0.5ml of 0.1% starch for 15 minutes. 50µl saturated I_2 solution in KI was added and the samples diluted to 5ml. Enzyme activity was determined from the decrease in absorbance at 600nm.

b) β-galactosidase: 10µl samples were added to 1ml of o-nitrophenyl β D-galactopyranoside (3mg/ml) and 2ml of 0.1M Tris, buffered at pH 8.0. Enzyme activity was determined from the increase in absorbance at 400nm.

c) Carbonic anhydrase: Enzyme activity was determined by measurement of esterase activity. 100µl samples were incubated in a reaction mixture containing 0.5ml of 1mM p-nitrophenyl acetate and 2ml of Tris buffer, pH 8.0. Enzyme activity was determined from the rate of increase in absorbance at 400nm.

d) Alcohol dehydrogenase: 10µl samples were added to a reaction mixture containing 100µl of 0.1% (v/v) acetaldehyde in 100mM Tris (pH 7.0), 100µl of 1mg/ml NADH and 2ml of Tris, buffered at pH 7.0. Enzyme activity was determined from the rate of decrease in absorbance at 340 nm due to the oxidation of NADH.

2.2.15 REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC)

Separation of peptides and peptide hydrolysates was carried out on RP-HPLC using a C18 column. Initially, separations (including tetrapeptide, β-casomorphin and bradykinin) were performed using a Waters Associates C18 column with a radial compression module RCM-100 and a Waters HPLC pump and detection system. All other separations were performed using an Alltech vydac 218TP C18, 10 micron column of 250mm x 4.6mm dimensions and either a Philips PU 4100 liquid chromatograph or a Spectrophysics SP 8800 HPLC. The solvent gradients and running conditions are specified with the individual chromatograms.

CHAPTER 3 - RESULTS

3.1. PRELIMINARY STUDIES ON THE PURIFICATION OF PEPTIDASE ENZYMES

Prior to embarking on purification of specific peptidases, some preliminary investigations were carried out to compare peptidase activities in various strains and in bacteria grown on different media. The finding, during this work, that significant losses of peptidase activity occurred during the initial step of precipitating DNA from cell free extracts also prompted the investigation of the need to include this step in subsequent purifications.

3.1.1 SELECTION OF STRAINS FOR LARGE SCALE ENZYME PURIFICATION

In previous studies on proteolytic activity of lactic streptococci in this laboratory, the *S.lactis* strain 4760 was widely used. This strain grows rapidly on both milk and broth media and has good proteolytic activity. Furthermore the availability of large quantities of this strain for other studies in this department on enzymes of carbohydrate metabolism offered considerable advantages in using this same strain in the present work. Strain 4760 is a transconjugant strain. The parent strain from which it is derived (*S.lactis* H1) is not suitable for experimental work since it forms clumps in broth culture. This clumping characteristic is absent from a plasmid cured strain (4125) of H1, but this strain is unable to use lactose as an energy source or milk proteins as a nitrogen source since it has lost the lactose/proteinase plasmid. Introduction of the lactose/proteinase plasmid from *S.cremoris* H2 (4409) restores the ability to use lactose and utilise milk protein, but not the clumping characteristic.

In order to determine whether the presence of the *S.cremoris* plasmid influenced the type or level of the peptidases produced, a preliminary comparison of the three strains, *S.lactis* 4760, *S.lactis* 4125, (the plasmid free H1 strain) and *S.cremoris* 4409 (the source of the H2 plasmid) was carried out.

100ml batches of the three strains of lactic streptococci were grown in RSM medium as described in Section 2.2.3. For strain 4125, the RSM medium was supplemented with 0.2% (w/v) peptone and 0.5% glucose. Initial comparisons of specific activities using the substrates Gly-Pro-AMC to assay for dipeptidyl peptidase activity (subsequently referred to as Gly-Pro-AMCase) and Lys-AMC to assay for "general" aminopeptidase activity

(subsequently referred to as Lys-AMCase) were made using cell free extracts from the three strains. The results are summarised in Table 3.1. The comparison of the specific activities in the cell-free extracts (prepared as described in Section 2.2.4) suggested that the levels of Gly-Pro-AMCase and of Lys-AMCase were similar in the three strains. The level of Gly-Pro-AMCase was approximately 4 to 9 times higher than that of the Lys-AMCase.

The cell-free extracts of the three strains were fractionated on a DEAE cellulose column by elution with a 0 - 0.8M NaCl gradient. The elution profiles are compared in Fig 3.1.a,b and c. The pattern of elution of these activities, and of Leu-AMCase, dipeptidase and tripeptidase activities from the DEAE cellulose column was found to be similar when comparing the two *S.lactis* strains. However in the *S.cremoris* strain 4409 the Lys-AMCase activity was eluted in earlier fractions than in the *S.lactis* strains when compared with the Gly-Pro-AMCase peak, resulting in overlapping peaks of activity (Fig 3.1c). The dipeptidase and tripeptidase activities eluted in later fractions than the AMCase enzymes with broad elution peaks, although the peak profiles of both the dipeptidase and tripeptidase are narrower in the *S.cremoris* strain than in the *S.lactis* strains.

These preliminary results show that the peptidase profiles of *S.lactis* 4760 and 4125 are similar while that from *S.cremoris* 4409 is somewhat different. The results tentatively suggest that the peptidase complement is determined by chromosomal DNA rather than the plasmid DNA, ie. that the peptidase enzymes in *S.lactis* 4760 may be regarded as those of the *S.lactis* parent. Further evidence for this will be presented in later Sections.

	Lys-AMCase µmoles/min/mg	Gly-Pro-AMCase µmoles/min/mg
S.lactis 4125	0.0095	0.066
S.cremoris 4409	0.0169	0.063
S.lactis 4760	0.0118	0.108

TABLE 3.1 *Comparison of specific activities of Lys-AMCase ("general" aminopeptidase) and Gly-Pro-AMCase (X-Pro dipeptidyl peptidase) in cell-free extracts from three different strains of lactic streptococci grown on RSM medium.*

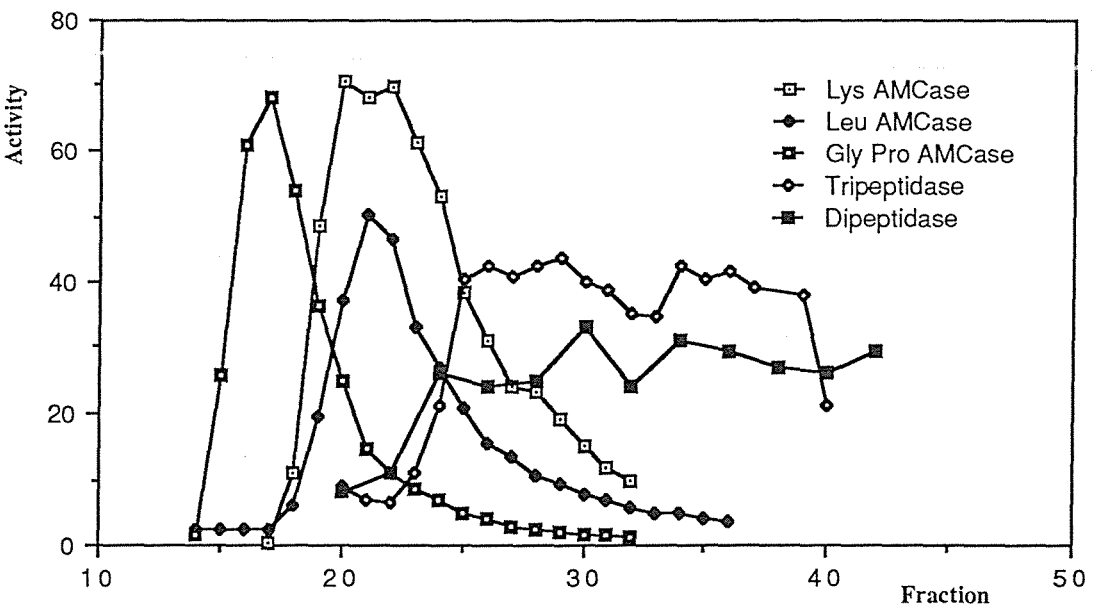


FIG. 3.1a *DEAE cellulose elution profile of peptidase activities from S.lactis 4760. Column was eluted with 0.1M phosphate buffer pH 6.8 containing 10% glycerol with a linear gradient of 0 - 0.8M NaCl. Column dimensions were approximately 2cm X 10cm. The enzyme assays are described in Section 2.2.8 B. The units of activity are arbitrary, and only indicate the relative positions of elution of the different peptidases.*

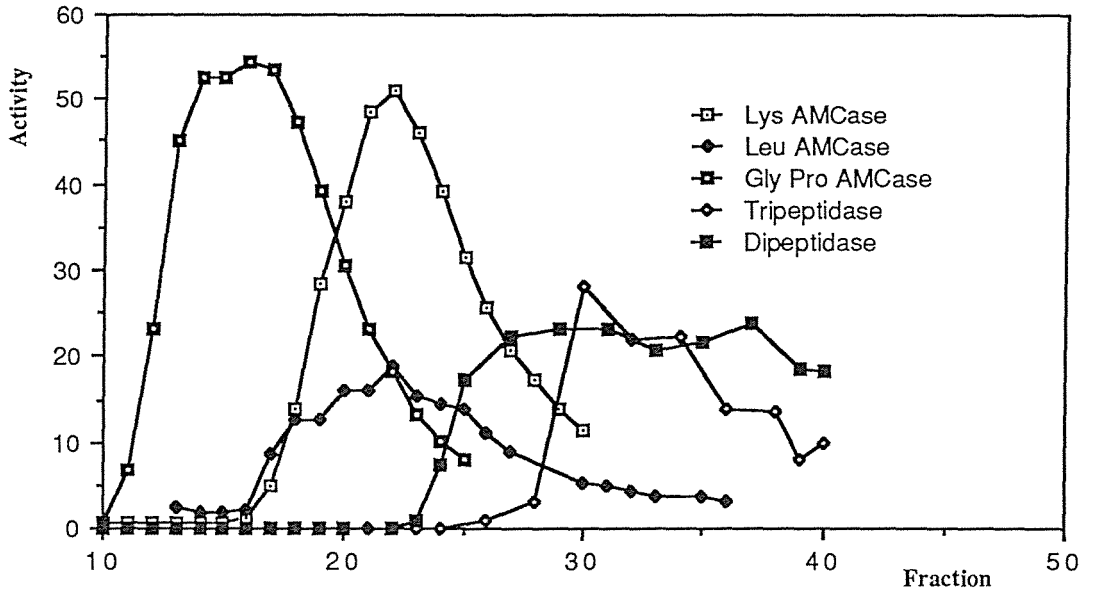


FIG. 3.1b DEAE cellulose elution profile of peptidase activities from S.lactis 4125 (using the same elution conditions as for 4760).

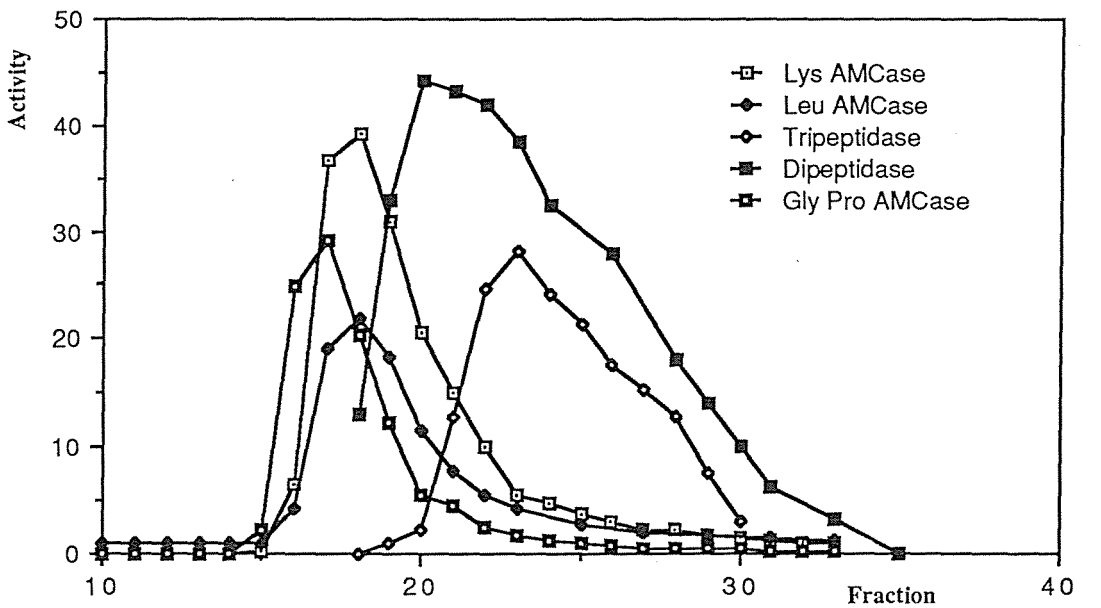


FIG. 3.1c DEAE cellulose elution profile of peptidase activities from S.cremoris 4409 (using the same elution conditions as for 4760).

3.1.2 SELECTION OF MEDIUM FOR LARGE SCALE ENZYME PURIFICATION

Small batches of *S lactis* strain 4760 were grown in RSM medium and in lactose broth medium as described in Section 2.2.3. The specific activities of both the Gly-Pro-AMCase and the Lys-AMCase in the cell free extract were compared to determine whether the yields of the two enzymes were similar in bacteria grown on the two different media, since large scale fermentor cultures would be more easily grown in broth than in milk. The specific activities for the two enzymes are shown in Table 3.2

It was concluded that broth- and milk-grown cells produced comparable yields of the two AMCase enzymes, and therefore that broth was a suitable growth medium.

3.1.3 INVESTIGATION OF THE NEED FOR DNAase AND STREPTOMYCIN SULPHATE TREATMENT OF THE CELL FREE EXTRACT

The initial step in purification of the cell-free extract involved treatment of the lysed bacterial suspension resulting from passage through the French pressure cell, with DNAase (2-3mg for 30min) to degrade the DNA. This reduced the viscosity of the solution to enable effective centrifugation at 27000xg to remove cellular debris. Following centrifugation, treatment of the CFE supernatant with streptomycin sulphate (1.5mg/mg protein for 1 hour) was employed to precipitate DNA, which was then removed by centrifugation at 27000xg. Treatment with streptomycin sulphate usually led to a significant loss of activity (Table 3.3). The Lys-AMCase activity dropped to 63% and the Gly-Pro-AMCase activity dropped to 79% of the original cell free extract activity in this particular preparation. It was found however that both DNAase and streptomycin sulphate treatments could be omitted without detriment to subsequent treatments. The cell debris pelleted cleanly without DNAase treatment to reduce viscosity and, provided a sufficiently large DEAE cellulose column was used, it was found that the CFE could be applied directly to this, thus removing the DNA and giving an initial fractionation of the peptidase in a single step.

	Lys-AMCase µmoles/min/mg	Gly-Pro-AMCase µmoles/min/mg
Broth	0.011	0.11
Milk	0.014	0.13

TABLE 3.2 Comparison of specific activities for Lys-AMCase and Gly-Pro-AMCase from S.lactis 4760 in cell-free extracts from broth- and milk-grown cells.

	Lys-AMCase µmoles/min/mg	Gly-Pro-AMCase µmoles/min/mg
CFE	0.011	0.078
STREP SO_4	0.007	0.062

TABLE 3.3 Effect of treatment of the cell-free extracts from S.lactis 4760 with streptomycin sulphate on the specific activity of peptidases.

3.2 PURIFICATION OF DIPEPTIDYL PEPTIDASE FROM *S.lactis* 4760

As discussed in the Introduction, (Section 1.5) the main aim of the present study was to purify two aminopeptidases and study their properties and possible role in the degradation of β-casein-derived oligopeptides. The dipeptidyl aminopeptidase was selected as the first enzyme for detailed study since preliminary purification trials indicated that it was relatively stable. Previous studies on similar enzymes from other bacteria suggested that it was an X-Pro dipeptidyl aminopeptidase so the substrate Gly-Pro-AMC was used routinely throughout development of a purification strategy. For this reason, as mentioned earlier, it will be referred to as a Gly-Pro-AMCase until shown to be a true dipeptidyl aminopeptidase.

For large scale purification (30-40 litres), cultures of *S.lactis* 4760 were grown in a Fermacell fermentor in lactose broth. Harvested cells were stored as a paste at -15°C in convenient sized batches. Cell free extracts were prepared as described in Section 2.2.4.

3.2.1 DEAE CELLULOSE CHROMATOGRAPHY

The cell free extract from 100g (wet packed weight) of cells was loaded directly onto a DEAE Cellulose (Whatman DE23) column of 300ml wet volume (5x15cm) previously equilibrated with 50mM Tris/glycerol buffer pH 7.5. Bound protein was eluted with a linear concentration gradient of 0 - 0.4M NaCl at a flow rate of 1.5ml/min. The elution profile is shown in Fig 3.2. The column fractions containing Gly-Pro-AMCase activity (assayed by the semi-quantitative procedure described in Section 2.2.8 B) were pooled and concentrated using an Amicon Diaflo concentrator with a PM 30 or XM 50 membrane to a volume of 10 - 20ml. Assay of filtrates showed that the peptidase was retained by both of these membranes.

3.2.2 SEPHACRYL S300 GEL PERMEATION CHROMATOGRAPHY.

A concentrated sample of no more than 8ml from the DEAE cellulose purification step was loaded onto a Sephacryl S300 column (3cm x 75cm) which had been equilibrated in 10mM Tris/glycerol buffer at pH 7.5. The column was eluted at 0.5ml per minute with the same buffer. The elution profile is shown in Fig. 3.3. The fractions containing Gly-Pro-AMCase activity were pooled. This step resulted in a small increase in specific

activity (see Table 3.4), the Gly-Pro-AMCase activity eluting slightly later than the majority of the protein. The 280nm absorbance profile shows two major peaks in Fig.3.3. The first peak was probably due to turbidity of the samples and not to protein explaining the relatively small (2 fold) increase in specific activity after passage through this column. However, if this step was omitted from the procedure, it was found in subsequent steps, particularly the first arginine-Sepharose step, that the effectiveness of the separation was reduced significantly perhaps as a result of the turbid material binding to, or clogging the resin. The gel permeation column also served to remove the NaCl which eliminated the need for a dialysis step before proceeding to the next, and most significant, step in the purification procedure.

3.2.3 CHROMATOGRAPHY ON ARGININE-SEPHAROSE 4B

Meyer and Jordi (1987) employed an "affinity" chromatography step (the use of the term "affinity" for this type of separation is discussed in Section 4.1.1) using an arginine-Sepharose resin buffered at pH 7.0, and eluted with a linear salt gradient to partially purify an X-Pro dipeptidyl peptidase from *Lactobacillus lactis*. They achieved a 7.5-fold purification in this single step. Initial experiments with this resin at pH 6.8 gave a 4-fold purification of the Gly-Pro-AMCase. Examination of the protein constituents of the Gly-Pro-AMCase fractions by PAGE revealed many contaminating bands. However, increasing the buffer pH to 7.5 resulted in a substantially better separation. A 10-fold purification was achieved in a single step with only minor contamination by other proteins evident on PAGE. Thus the higher pH buffer was employed in all subsequent purifications of this enzyme.

The total pooled fractions eluted from the Sephacryl S300 column were loaded on to an arginine-Sepharose 4B column of 25ml wet volume (2cm x 12cm) prepared as discussed in Section 2.2.10. The column had been previously equilibrated with 10mM Tris/glycerol at pH 7.5. Bound protein was eluted using a linear concentration gradient of 0 to 0.2M NaCl at a flow rate of 0.5ml/min. The elution profile is shown in Fig 3.4. This method allows separation of the Gly-Pro-AMCase from most of the protein eluted.

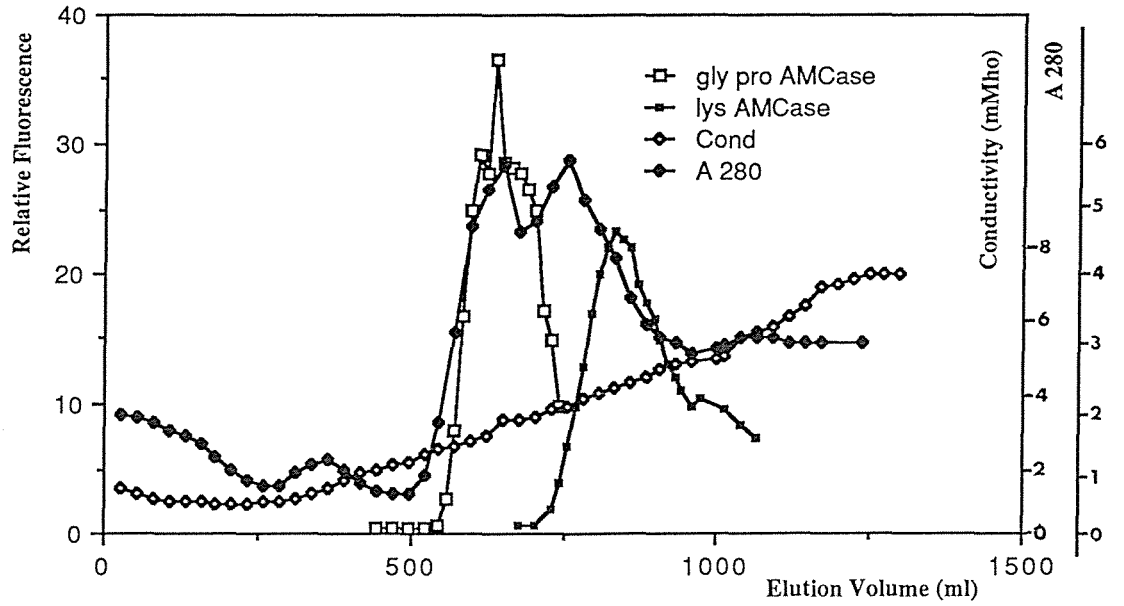


FIG. 3.2 Elution profile of Gly-Pro-AMCase and Lys-AMCase activities from DEAE Cellulose. Elution conditions are as described in Section 3.2.1. Relative fluorescence is in arbitrary units, being determined by the semi-quantitative method described in Section 2.2.8 (B)

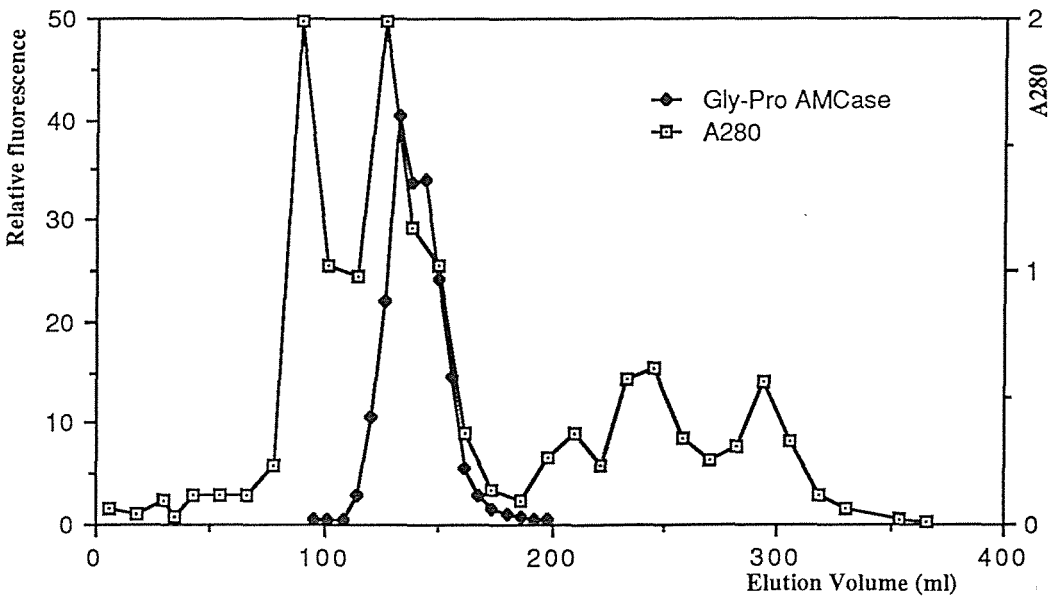


FIG. 3.3 Elution profile of Gly-Pro-AMCase activity from Sephacryl S300 gel permeation column. Elution conditions are as described in Section 3.2.2.

3.2.4 DEAE SEPHADEX ION EXCHANGE CHROMATOGRAPHY

Pooled fractions from the arginine-Sepharose column were shown by PAGE to contain at least three contaminating proteins as shown in the photograph of the SDS PAGE (Fig 3.6). DEAE Sephadex was found to be effective in the removal or reduction of several of these contaminating proteins, even though it did not result in a significant increase in specific activity. The pooled material from the first arginine-Sepharose step was loaded on to a DEAE Sephadex column (3cm x 15cm) equilibrated with 10mM Tris/glycerol pH 7.5, and eluted with a linear NaCl gradient of 0 - 0.4M at a flow rate of 0.5ml/min. The elution profile is shown in Fig 3.5.

3.2.5 SECOND ELUTION FROM ARGININE-SEPHAROSE 4B

Fractions with Gly-Pro-AMCase activity eluted from the DEAE Sephadex column were pooled and dialysed overnight against 500ml of 10mM Tris/glycerol pH 7.5. The pooled material was loaded on to the arginine-Sepharose column and eluted using the same conditions as described previously (Section 3.2.3). SDS PAGE revealed a single band of protein when stained with Coomassie blue (Fig.3.6).

In the procedures used to purify both bacterial dipeptidyl peptidase (Meyer and Jordi, 1987), and mammalian dipeptidyl peptidase IV (Gonschor and Schafer, 1985), a Gly-Pro-AH-Sepharose column was reported to give the most significant step in purification. A Gly-Pro-AH-Sepharose resin was prepared (as described in Section 2.2.11) and was used in the present study to further purify the material eluted from the first arginine-Sepharose column. However, although the Gly-Pro-AMCase bound, and could be eluted at a conductivity of 5.4mMho, no significant purification was achieved. When the eluant was analysed by silver stained SDS PAGE, none of the contaminating bands had been removed. One possibility to explain the failure of this column is that inadequate coupling of the Gly-Pro to the six carbon spacing arm occurred. It was impossible to check the degree of coupling in this case, and the preparation of the resin was only attempted once. Further study of the usefulness of this resin could be of value, but since the procedure described in Section 3.2.1-3.2.5 resulted in a single band on SDS PAGE, the Gly-Pro-AH-Sepharose column proved unnecessary, and was not used in subsequent purifications of this enzyme.

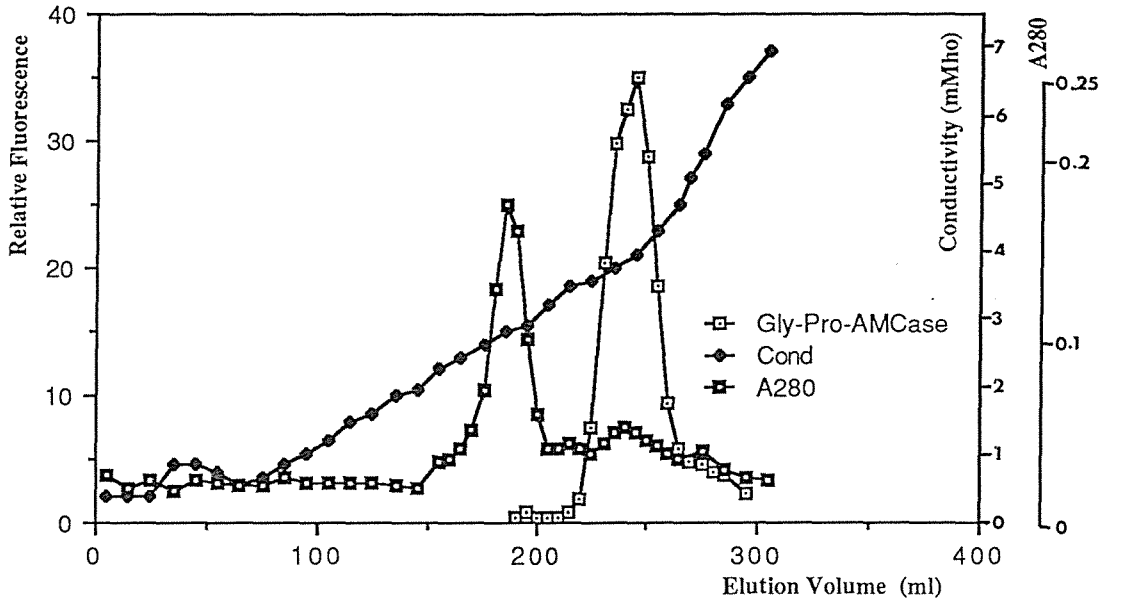


FIG. 3.4 Elution profile of Gly-Pro-AMCase activity from arginine-Sepharose. Elution conditions are as described in Section 3.2.3.

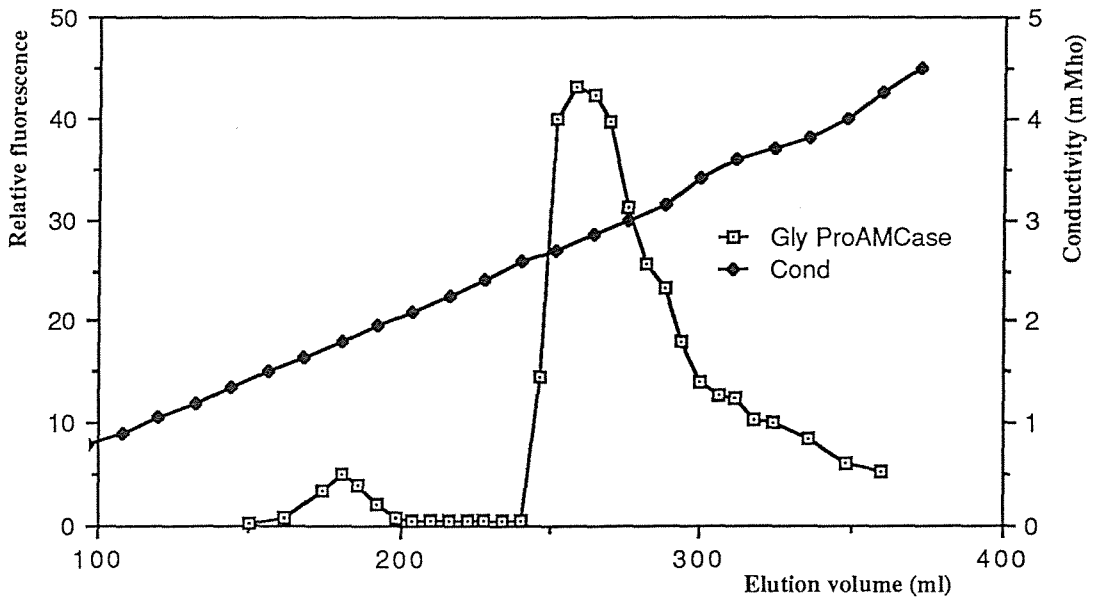


FIG. 3.5 Elution profile of Gly-Pro-AMCase activity from DEAE Sephadex. Elution conditions are as described in Section 3.2.4. The A280 was below the limits of detection.

3.2.6 SUMMARY OF PURIFICATION OF GLY-PRO-AMCase FROM 100g (WET WEIGHT) OF *S.lactis* 4760

The results of the purification procedure described in the preceding pages are shown in Table 3.4.

A total of 5.2mg of purified Gly-Pro-AMCase was obtained from 100g (wet packed weight) of *S.lactis* 4760 representing a 20% recovery of the original activity. Significant losses of activity occurred at each stage of the preparation. The loss at the S300 gel filtration stage also includes the loss of activity resulting from ultrafiltration. Ultrafiltration gave more severe losses of activity if attempted at later stages in the procedure, and was therefore omitted, being replaced by dialysis after the DEAE Sephadex step.

The reliability of the values for protein concentration and for specific activity was limited by the sensitivity of the bicinchoninic acid protein assay (Section 2.2.9). This assay was used in preference to the Folin assay because of Tris interference leading to erroneously high protein values. At concentrations of less than 0.1mg/ml the reliability of determinations by the bicinchoninic acid assay is questionable. To obtain more accurate measurements, samples of the purified enzyme were concentrated in a 2ml ultrafiltration centrifugal concentrator before determining protein concentration. The final specific activity shown in Table 3.4 was based on protein estimates with concentrated enzyme. A total purification of greater than 200 fold was achieved in all the large scale preparations.

An SDS polyacrylamide gel comparing each stage of the purification is shown in Fig 3.6. Fig 3.7 shows a polyacrylamide gel with the purified Gly-Pro-AMCase along with a range of molecular weight markers.

3.2.7 CONFIRMATION OF THE IDENTITY OF THE PURIFIED PROTEIN

To confirm that the single protein band seen by SDS PAGE was the Gly-Pro-AMCase, an assay based on the enzyme's ability to release AMC from Gly-Pro-AMC was developed. Since native PAGE allowed the Gly-Pro-AMCase to retain its activity within the gel, staining the gel with 0.5ml of 1mM Gly-Pro-AMC allowed visualisation of the enzyme's activity by the development of a fluorescent band under ultraviolet light. The gel was then immediately photographed and subsequently stained with Coomassie blue.

Photography of the fluorescent band proved difficult since the diffusion of the AMC band was rapid. Good results were obtained only within five minutes of staining with Gly-Pro-AMC. The relative mobility of the fluorescent band and the Coomassie blue protein band corresponded exactly as seen in Fig 3.8.

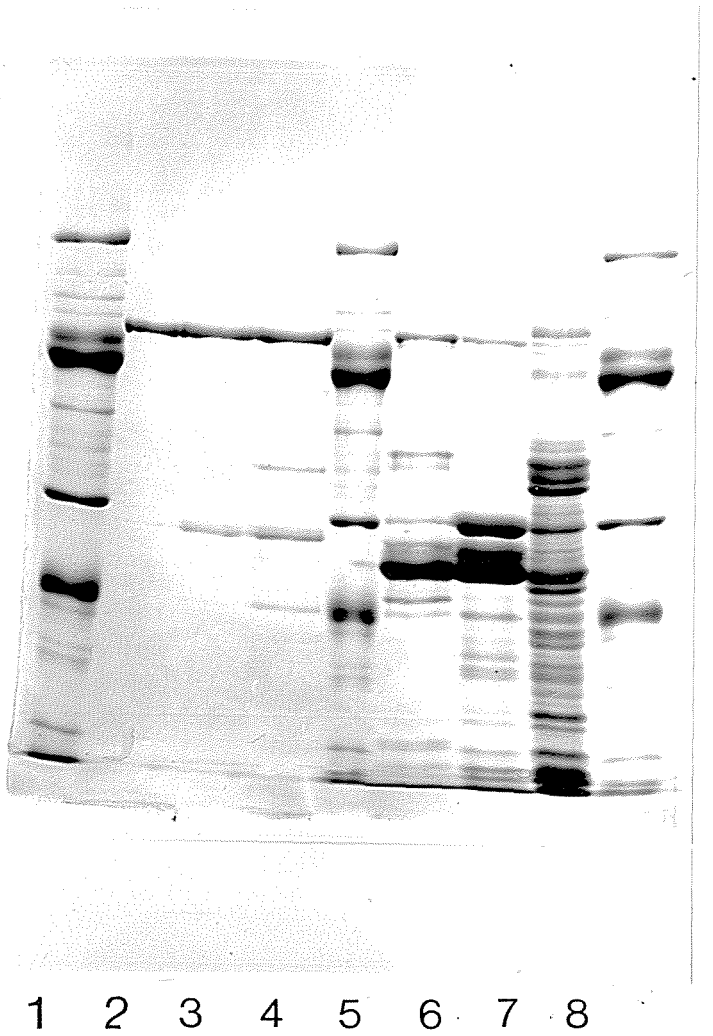


FIG. 3.6 *7.5% SDS PAGE showing the steps in the purification of the Gly-Pro-AMCase from S.lactis 4760.*

Track	*Identity*
1	*MW markers (refer to Table 3.5)*
2	*Arginine-Sepharose 2*
3	*DEAE Sephadex*
4	*Arginine-Sepharose 1*
5	*MW markers*
6	*Sephacryl S300*
7	*DEAE cellulose*
8	*Cell-free extract*

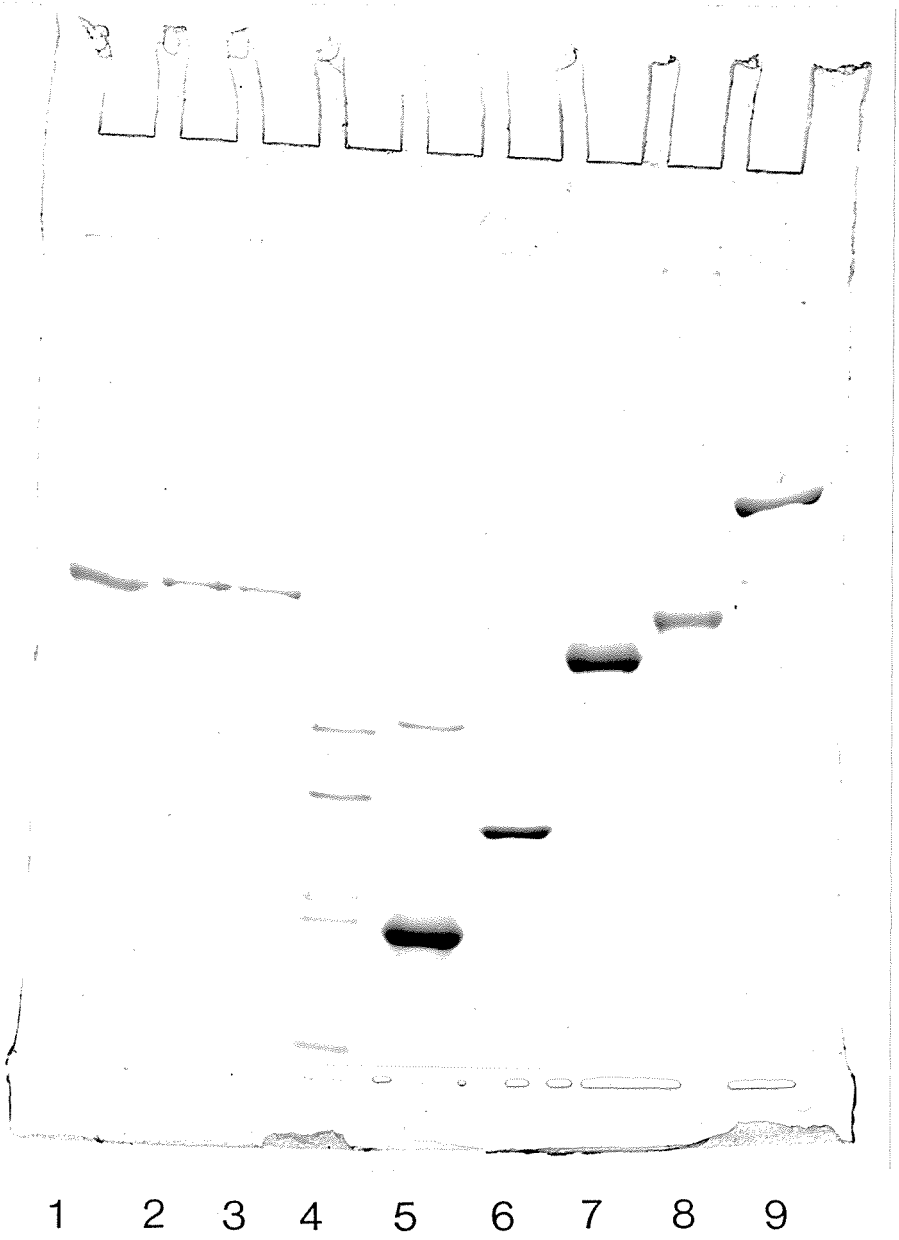


FIG. 3.7 *7.5% SDS PAGE showing the purified Gly-Pro-AMCase along with a range of molecular weight markers.*

Track	*Identity*
1, 2 and 3	*Purified Gly-Pro-AMCase*
5	*Lactate dehydrogenase monomer*
6	*Citrate synthase*
7	*Bovine serum albumin*
8	*Transferrin*
9	*β-galactosidase*

Purification Step	Protein (mg/ml)	Total Protein (mg)	Activity (units/ml)	Specific Activity (units/mg)	Total Activity (units)	Recovery (%)	Fold Purif.
CFE	14.8	3700	2.58	0.18	645	100	0
DEAE	3.5	385	4.23	1.21	465	72	6.7
S300	1.6	150	3.87	2.42	365	57	13.4
Arg-Seph	0.102	11.9	2.49	24.4	292	45	136
DEAE-Seph	0.080	7.8	1.92	24.0	188	29	133
Arg-Seph	0.042	5.2	1.71	40.7	127	20	226

TABLE 3.4 *Summary of the purification of the dipeptidyl peptidase from 100g (wet weight) of S.lactis 4760.The protein concentration of the purified enzyme was measured by concentrating a 2ml sample using a centrifugal concentrator and correcting the result obtained by multiplying by the concentration factor to give the protein concentration of the original sample.*

GEL A *GEL B*

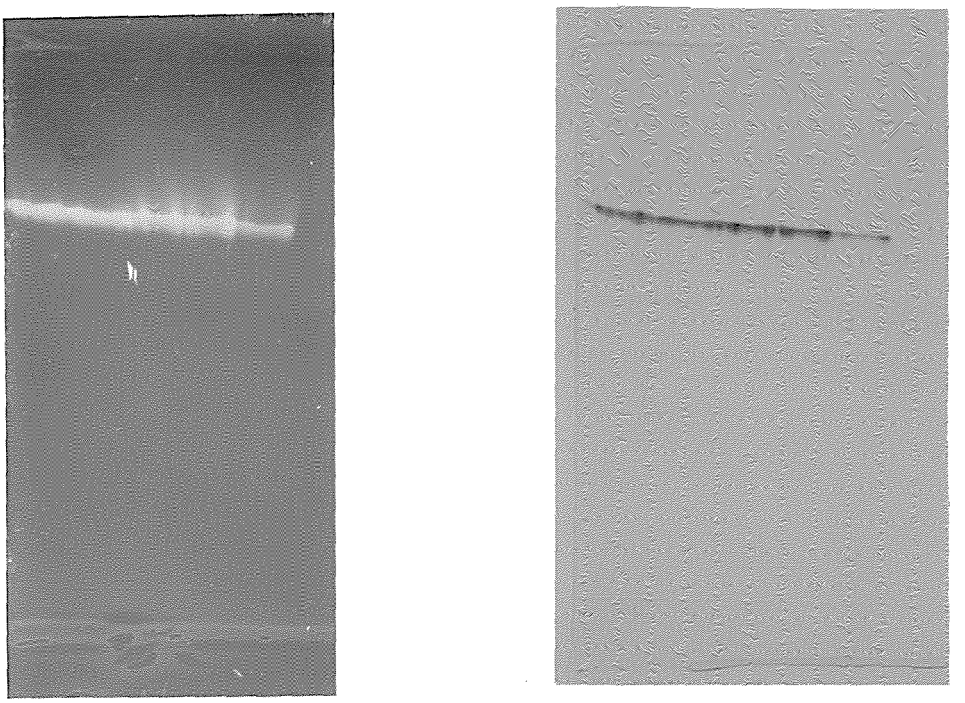


FIG. 3.8 *Native PAGE of purified Gly-Pro-AMCase (see Section 2.2.12). Gel A shows the relative mobility and intensity of the purified enzyme stained with 0.5ml of 1mM Gly-Pro-AMC and photographed under ultra violet light, as outlined in Section 3.2.7 . Gel B shows the same native gel stained with Coomassie blue highlighting the protein band.*

3.3 CHARACTERISATION OF THE GLY-PRO-AMCase

3.3.1 MOLECULAR WEIGHT DETERMINATION BY SDS PAGE

The subunit molecular weight of the purified Gly-Pro-AMCase was determined by SDS polyacrylamide gel electrophoresis as described in Section 2.2.12. The monomer was found to have a molecular weight of 83 000 by averaging the results obtained from four separate gels, two of which are shown in Figs 3.6 and 3.7. The curve obtained by plotting log of molecular weight against relative mobility of the six marker proteins is shown in Fig. 3.9. The marker proteins used are listed in Table 3.5 together with their relative mobility values.

3.3.2 MOLECULAR WEIGHT DETERMINATION BY GEL FILTRATION CHROMATOGRAPHY

The molecular weight of the native enzyme was determined by its elution volume from a calibrated Sephacryl S300 gel filtration column. Calibration was achieved using a series of protein standards which are listed in Table 3.6 together with the assay procedures used. Fig. 3.10 shows the elution volume plotted against molecular weight for the standards, and indicates the relative elution position of the Gly-Pro-AMCase. The molecular weight determined by this method was approximately 150 000, suggesting that this enzyme is a dimer in its native form.

Standard protein	Mol. Wt.	Relative mobility
Myosin	210 000	0.13
β-Galactosidase	130 000	0.28
Transferrin	80 000	0.43
BSA	68 000	0.50
Citrate synthase	50 000	0.70
LDH	37 000	0.84

TABLE 3.5 Molecular weight markers used in SDS PAGE determination of Gly-Pro-AMCase subunit molecular weight. The relative mobilities are measured from the top of the resolving gel relative to the bromophenol blue dye front.

Standard proteins	Mol. Wt.	Assay procedure
β-amylase	200 000	See Section 2.2.14
Alcohol dehydrogenase	150 000	See Section 2. 2.14
BSA	68 000	A280 absorbance
Carbonic anhydrase	29 000	See Section 2. 2.14
Cytochrome C	12 000	A 420 absorbance

TABLE 3.6 Molecular weight markers used in Sephacryl S300 Gel filtration determination of the native molecular weight of the Gly-Pro-AMCase.

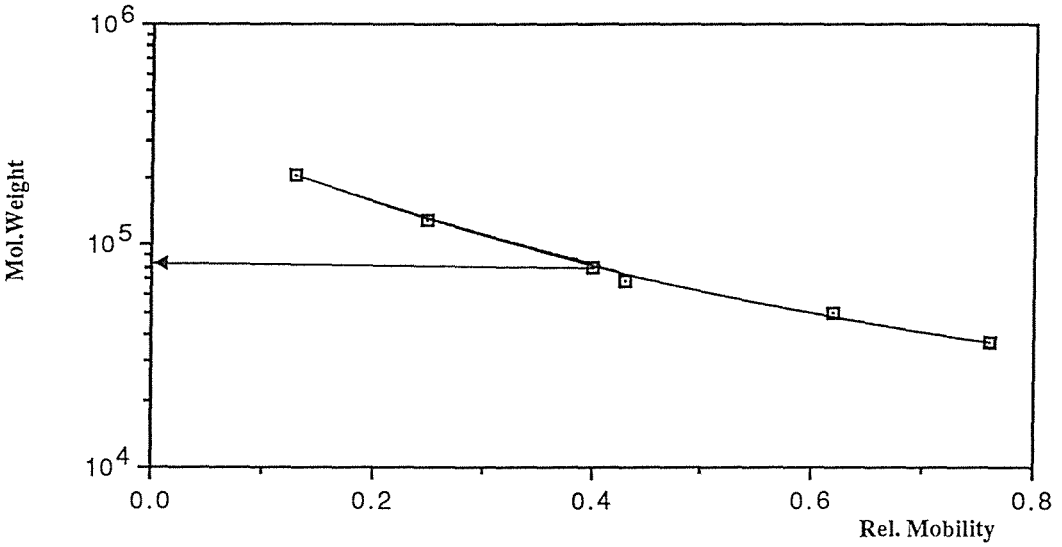


FIG. 3.9 Molecular weight calibration by SDS PAGE, using the standard molecular weight markers shown in Table 3.5. The subunit molecular weight of the Gly-Pro-AMCase is indicated with an arrow.

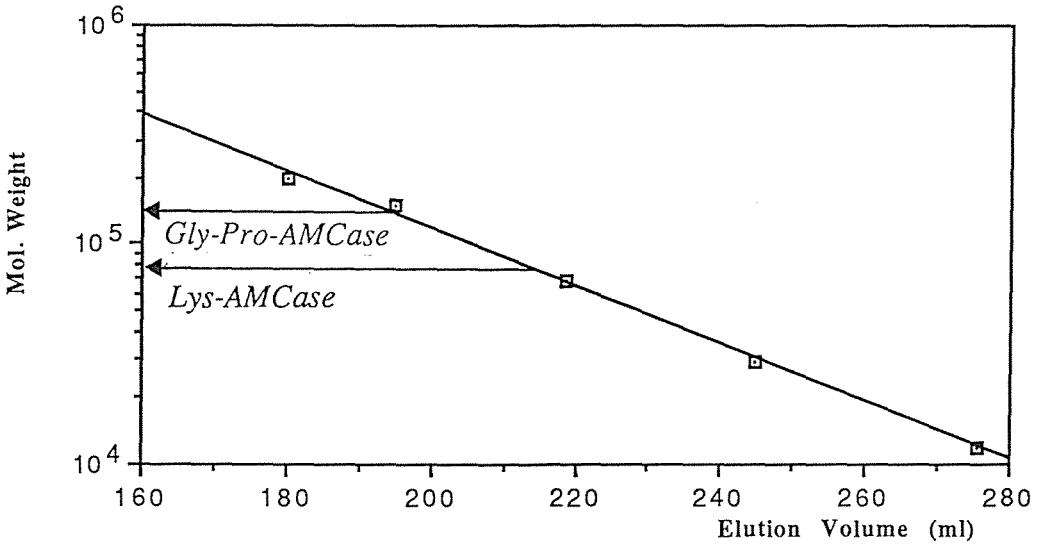


FIG. 3.10 Molecular weight calibration of Sephacryl S300 gel filtration column using the standard molecular weight markers shown in Table 3.6. The molecular weights of the native Gly-Pro-AMCase and the Lys-AMCase (Section 3.6.1) are indicated with an arrow.

3.3.3 EFFECT OF pH ON GLY-PRO-AMCase ACTIVITY

To determine the pH profile for the Gly-Pro-AMCase, activity was measured at several pH values over the range 3.8 to 10.25 using 100mM MES and Tris buffers. Although the pH values of the buffers at the lower end of this range were below the effective buffering range of MES, the reaction mixture pH was measured before and after addition of the enzyme and found to be unchanged. The profile (Fig. 3.11) shows a broad pH optimum, the activity of the Gly-Pro-AMCase remaining unchanged between pH 6 and 9. At pH 9.5 and above, a time-dependent inactivation of the enzyme was observed (Fig. 3.12). At pH 10 and above the steady state rate reached after the initial non-linear activity was due entirely to the spontaneous hydrolysis of the Gly-Pro-AMC substrate, the amide linkage being unstable at this pH.

3.3.4 EFFECT OF CATIONS AND PROTEASE INHIBITORS ON GLY-PRO-AMCase ACTIVITY

The effect of a range of different cations and proteinase inhibitors on the activity of the purified Gly-Pro-AMCase was investigated. For the metal ions, final concentrations of 1mM, and in some cases, 10mM were used when practicable. The effects of inhibitors were determined at 1mM final concentration as shown in Table 3.7.

Of the metal ions, only Cu^{2+} and Hg^{2+} had a significant inhibitory effect on activity. The high result obtained for Pb^{2+} was an artifact since a precipitate formed in the reaction mixture on addition of Gly-Pro-AMC which changed the fluorescent properties of the sample. The effect of $FeCl_2$ was also difficult to establish since the initial linear rate began to decrease after 10 minutes due to the formation of a yellow compound in the assay mixture. Little effect was seen using the chelating agent o-phenanthroline, which complexes iron and zinc, or with EDTA which binds divalent cations. 1mM PCMB (p-chloromercuribenzoic acid), an inhibitor of sulphydryl proteases, also had little effect on enzyme activity. However incubation of the enzyme with 1mM PMSF (phenyl methyl sulphonyl fluoride), a serine protease inhibitor, over an hour period, saw almost complete inhibition of enzyme activity suggesting that this enzyme is a serine-type peptidase.

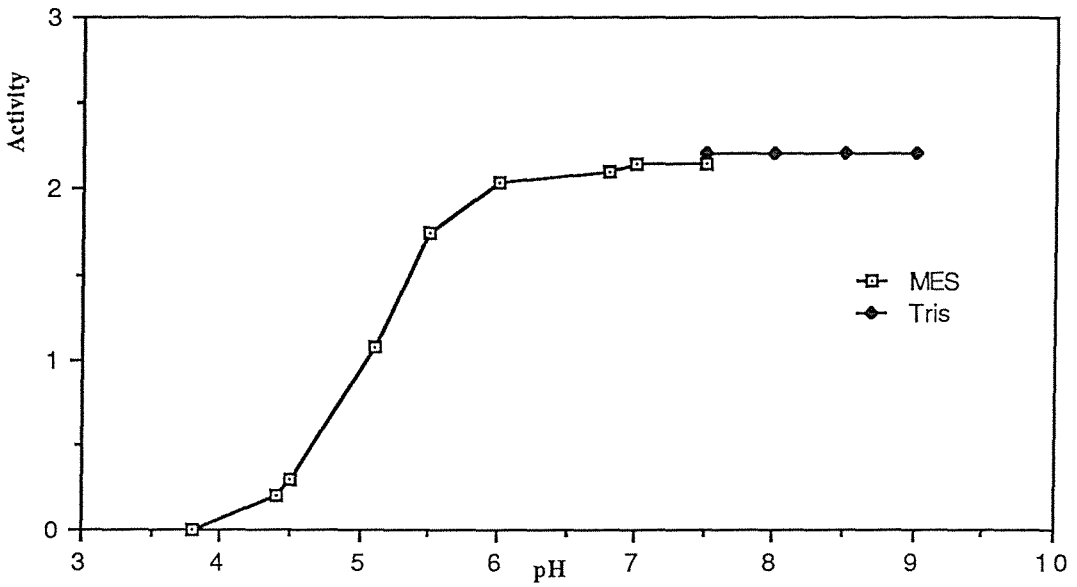


FIG. 3.11 pH profile for Gly-Pro-AMCase purified from S.lactis 4760. 100mM MES and Tris buffers were used to cover the pH range 3.8 to 9.0. Activity is in μmoles/min/ml, and 0.4μg of protein was used per assay. Gly-Pro-AMC concentration was 0.71mM (final concentration in assay mixture).

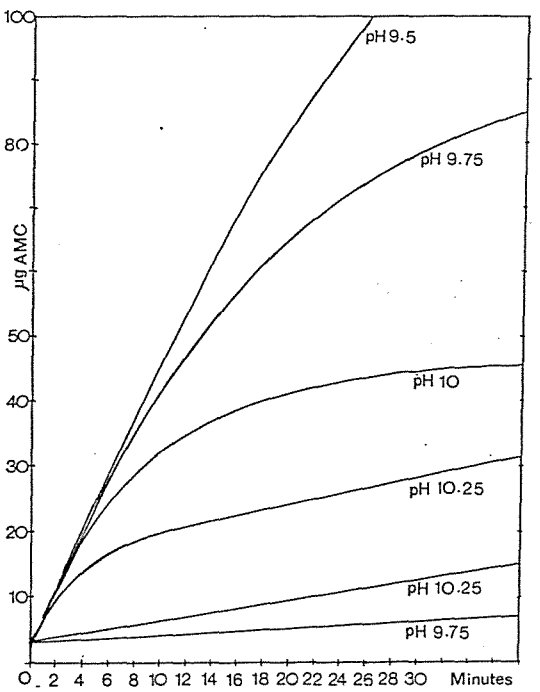


FIG. 3.12 Time-dependent inactivation of the Gly-Pro-AMCase at pH values of 9.5 and greater. The initial linear slopes becomes non-linear after a time which decreases with increasing pH. The two linear rates shown at the bottom of the Figure are the spontaneous rates for hydrolysis of the substrate at pH 9.75 and 10.25.

Agent	Activity with 1mM agent % control	Activity with 10mM agent % control
Mn++ (Chloride)	76	84
Ca++ "	92	90
Mg++ "	87	87
Co++ "	83	73
Zn++ "	98	93
Fe++ "	96	
Cu++ "	1	
Pb++ (Nitrate)	133	
Hg++ "	0	
o-Phenanthroline	81	
PMSF(0 min)	83	
PMSF(10 min)	59	
PMSF(30 min)	12	
PMSF(60 min)	3	
PCMB	76	
Iodoacetate	100	
EDTA (15 min)	107	

TABLE 3.7 *Effect of cations and other agents on the Gly-Pro-AMCase activity. Activity is expressed as a percentage of the activity of the Gly-Pro-AMCase without any additional agent. Enzyme activity was determined using 0.71mM Gly-Pro-AMC substrate (final concentration), 100mM MES pH 6.8 and 0.4 μg of purified enzyme per assay. The times indicated refer to a period of pre-incubation of agent and enzyme.*

PMSF=Phenylmethyl-Sulphonyl Fluoride
PCMB=p-Chloromercuribenzoic acid

3.3.5 RELATIVE ACTIVITY OF GLY-PRO-AMCase WITH VARIOUS DIPEPTIDYL-AMC SUBSTRATES

One important objective of the characterisation of the Gly-Pro-AMCase was to investigate its specificity using dipetidyl-AMC substrates, since this is relevant to its potential role in degradation of casein oligopeptides.

In a preliminary survey, (detailed results not shown), the activity of the enzyme was compared at a single concentration (0.71mM final concentration) of various X-Pro-AMC substrates, Gly-Ala-AMC and Pro-AMC. This study revealed that the enzyme showed high activity in hydrolysing the X-Pro-AMC substrates, that Pro-AMC was not hydrolysed at all, whilst the Gly-Ala-AMC was hydrolysed at 3.7% of the rate of hydrolysis of Gly-Pro-AMC. These results confirmed the assumption that the enzyme acts as an X-Pro arylamidase as indicated by a previous study on dipeptidyl peptidase activity in *S.thermophilus* and *Lactobacillus lactis*. (Meyer and Jordi, 1987). Accordingly, subsequent specificity studies with dipeptidyl-AMC derivatives were restricted to a comparison of activity with different X-Pro substrates to determine whether the nature of the N-terminal aminoacyl residue influenced activity.

Rates of hydrolysis of five different X-Pro-AMC substrates were compared at four concentrations (Table 3.8). The X-Pro-AMC derivatives used were chosen to represent different types of aminoacyl R-group (basic, aromatic, hydrophobic and acidic). At concentrations of 0.12 and 0.24mM the rates of hydrolysis were relatively similar with all five substrates, but at higher substrate concentrations, a marked inhibition of activity was seen in the case of Phe-Pro-AMC and Leu-Pro-AMC, and to a lesser extent, Lys-Pro-AMC. This may be due to true substrate inhibition of the enzyme or it may be due to contaminating compounds in the substrate since they were shown to be only about 90% pure by end point hydrolysis as described in Section 2.2.7. Further purification of substrates by column chromatography may be necessary to obtain greater purity since precipitation from methanol with diethyl ether did not yield crystals, but yielded a powder which was often slightly coloured. No significant inhibition was found at higher concentrations of Gly-Pro-AMC, so the 0.71mM substrate concentrations routinely used to determine activities during enzyme purification (Section 3.2.6) would not have been inhibitory.

	Activity (μmoles/min/ml) at 4 substrate concentrations			
Substrate	0.12mM	0.24mM	1.19mM	2.38mM
Gly-Pro-AMC	0.143	0.143	0.135	0.135
Lys-Pro-AMC	0.111	0.116	0.107	0.092
Leu-Pro-AMC	0.091	0.099	0.056	0.028
Glu-Pro-AMC	0.133	0.133	0.123	0.113
Phe-Pro-AMC	0.163	0.166	0.095	0.057

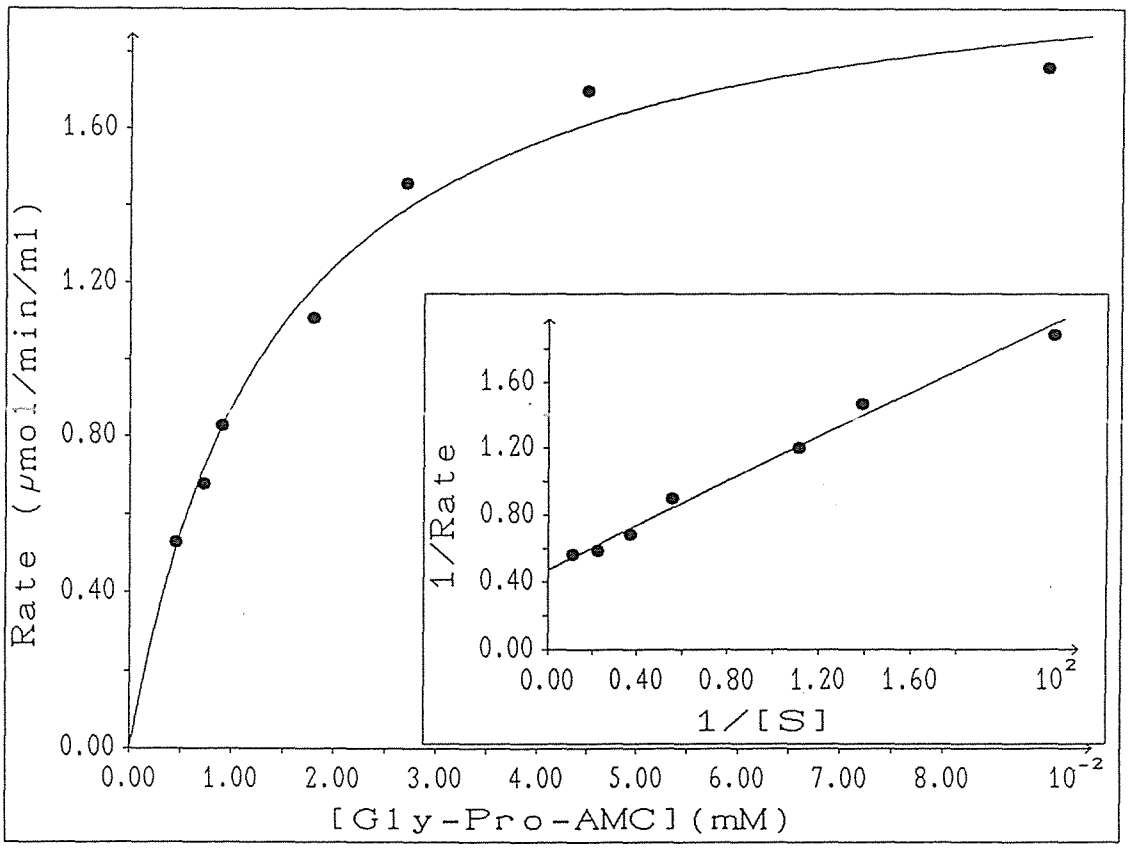
TABLE 3.8 *The activity (μmoles/min/ml) of Gly-Pro-AMCase with five X-Pro-AMC substrates at four different substrate concentrations. Each assay contained 0.042μg of purified enzyme in a total volume of 2.0ml.The substrate concentrations represent 10mM, 5mM, 1mM and 0.5mM solutions diluted in the reaction mixture as described in Section 2.2.8.*

3.3.6 KINETIC PARAMETERS OF THE GLY-PRO-AMCase FROM *S.lactis* 4760 WITH DIFFERENT X-PRO-AMC SUBSTRATES

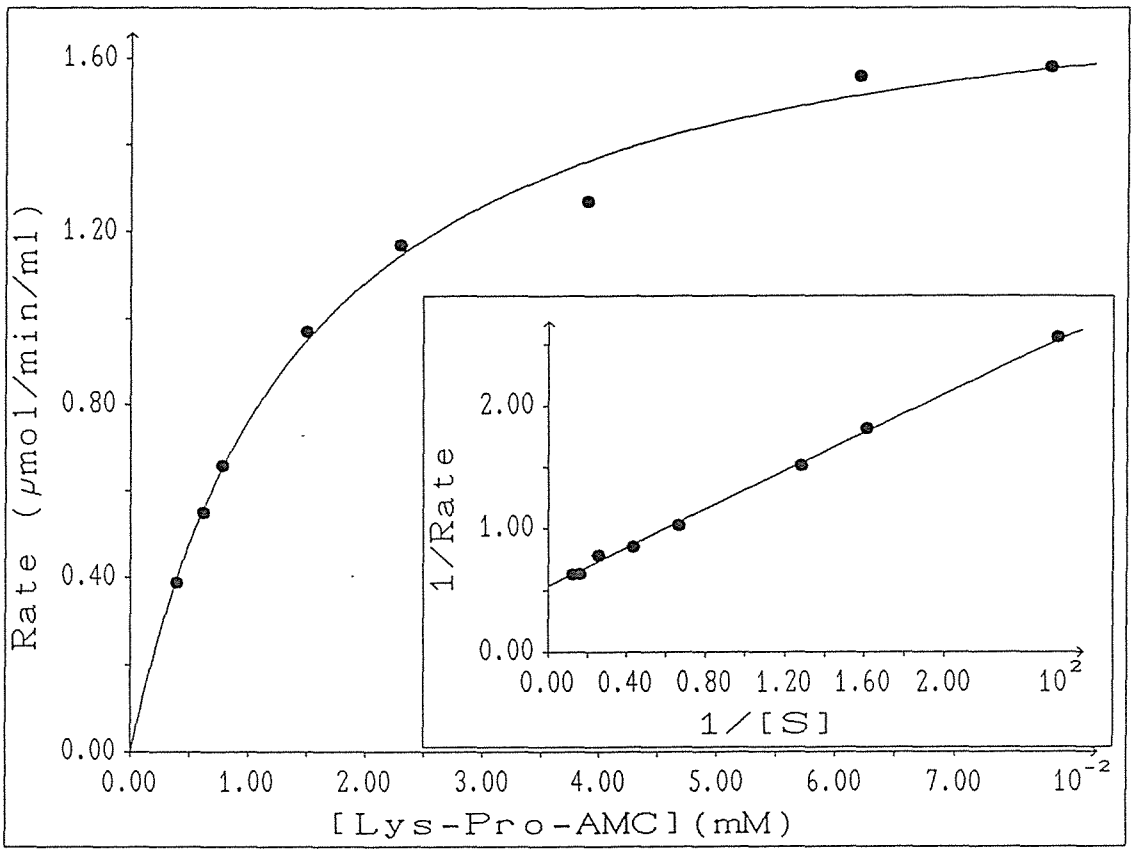
A more detailed study of the specificity towards different X-Pro-AMC substrates was carried out by determining the Km and k_{cat} values. In a preliminary investigation, Km and V_{max} determinations were carried out at two different pH values using Gly-Pro-AMC as a substrate. At pH values of 6.8 and 8.5, the V_{max} was identical, whereas the Km value of 15μM at pH 6.8 increased slightly to 20μM at pH 8.5, indicating that the kinetic parameters were not markedly dependant on pH over the optimal range found earlier (Section 3.3.3)

The Km, V_{max} and k_{cat} values for the enzyme with each substrate was determined for each of the five different X-Pro-AMC substrates used in the preliminary study. A substrate concentration range of 5μM to 100μM was found to give reproducible Km, V_{max} and k_{cat} values using two independent enzyme preparations. The accuracy of the values for protein concentration used in the calculation of the V_{max} and k_{cat} was limited by the sensitivity of the bicinchoninic acid protein assay as previously referred to in Section 3.2.6. Examples of rate verses substrate concentration and Lineweaver-Burk plots are given in Fig. 3.13 a, b, c, d and e, showing adherence to Michaelis Menten kinetics. Non-linear regression analysis of experimental data was performed by using the program Enzfitter (Leatherbarrow, 1987) with an IBM compatible PC XT computer. The results shown in Table 3.9 reveal similar Km values for all but Glu-Pro-AMC, this substrate having a significantly higher Km value. The high Km value for Glu-Pro-AMC could possibly be due to impurity of the substrate since the removal of the benzyl ether group during preparation may not have been complete (Section 2.2.7 C). The elemental analysis of the Glu-Pro-AMC preparation (Section 2.2.7 C), while showing a small discrepancy from the predicted composition in respect to carbon content, does not support the degree of contamination that would be necessary to account for the three-fold higher Km value found with this substrate. Also, the yield of AMC found on complete hydrolysis of the Glu-Pro-AMC indicates a purity of at least 90%. The k_{cat} and V_{max} values shown in Table 3.9 for Gly-Pro-AMC, Lys-Pro-AMC and Glu-Pro-AMC substrates were very similar, whilst Leu-Pro-AMC gave substantially lower values, and Phe-Pro-AMC gave slightly higher values.

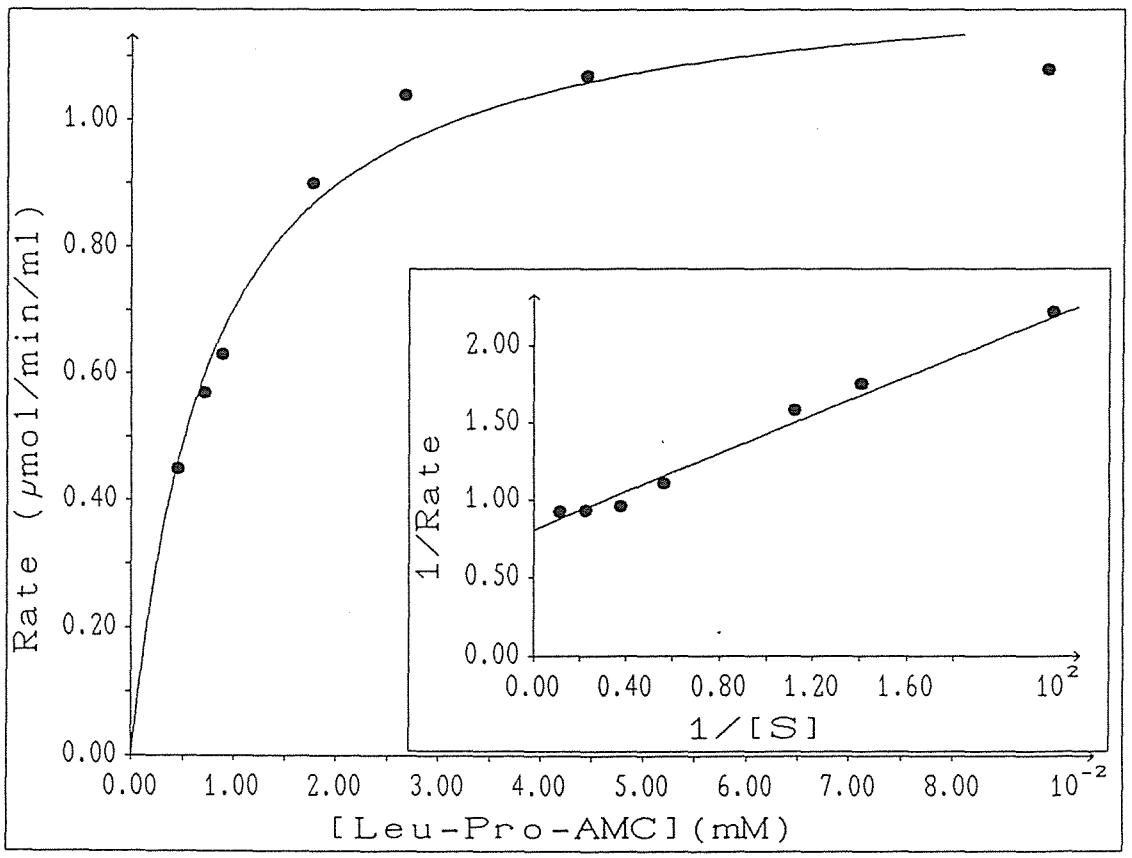
FIG. 3.13 *Rate verses substrate concentration plots and Lineweaver-Burk plots for the Gly-Pro-AMCase, using the substrates a) Gly-Pro-AMC, b) Lys-Pro-AMC, c) Leu-Pro-AMC, d) Glu-Pro-AMC, and e) Phe-Pro-AMC. Each assay contained 0.042µg of purified X-Pro-AMCase in a total volume of 2.0ml. Assays were carried out in 100mM MES buffer, at pH 6.8 using substrate concentrations covering the range 5 - 100µM*



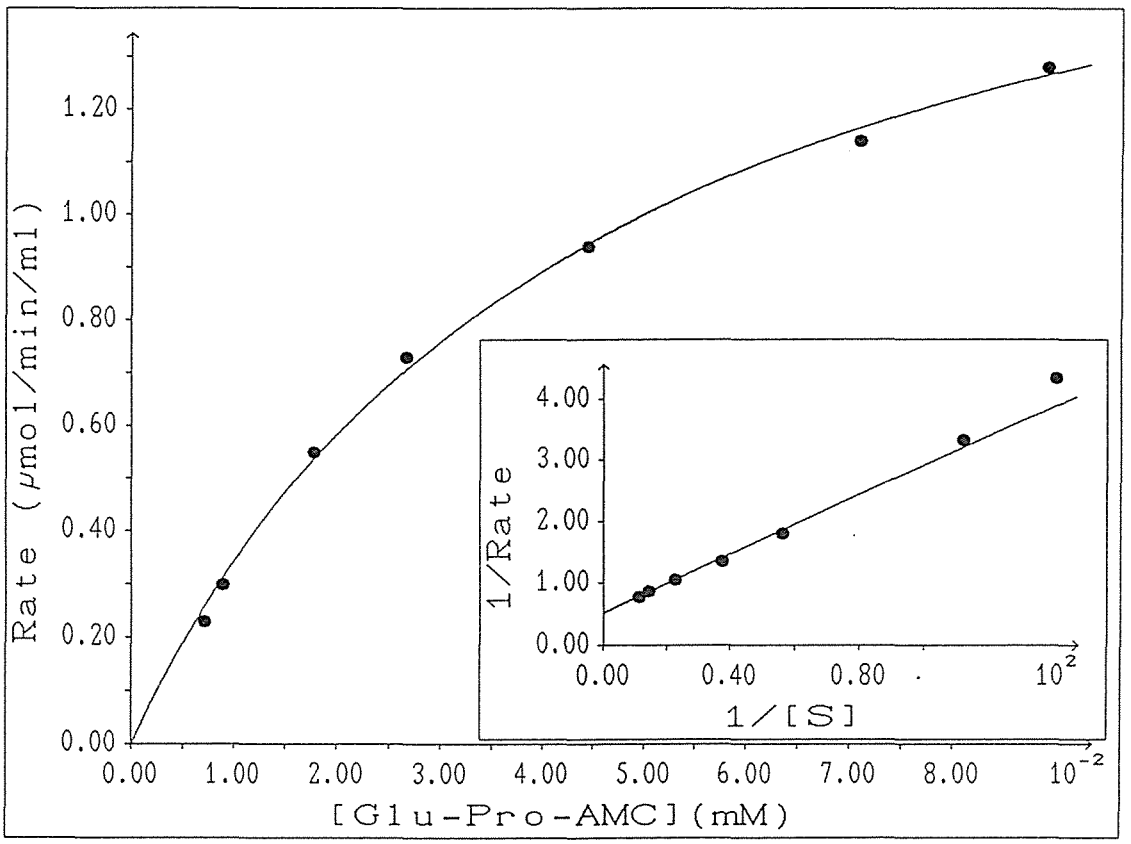
a


Rate (μmol/min/ml)
1.60
1.20
0.80
0.40
0.00
0.00 1.00 2.00 3.00 4.00 5.00 6.00 7.00 10⁻²
[Lys-Pro-AMC] (mM)
1/Rate
2.00
1.00
0.00
0.00 0.40 0.80 1.20 1.60 2.00 10²
1/[S]

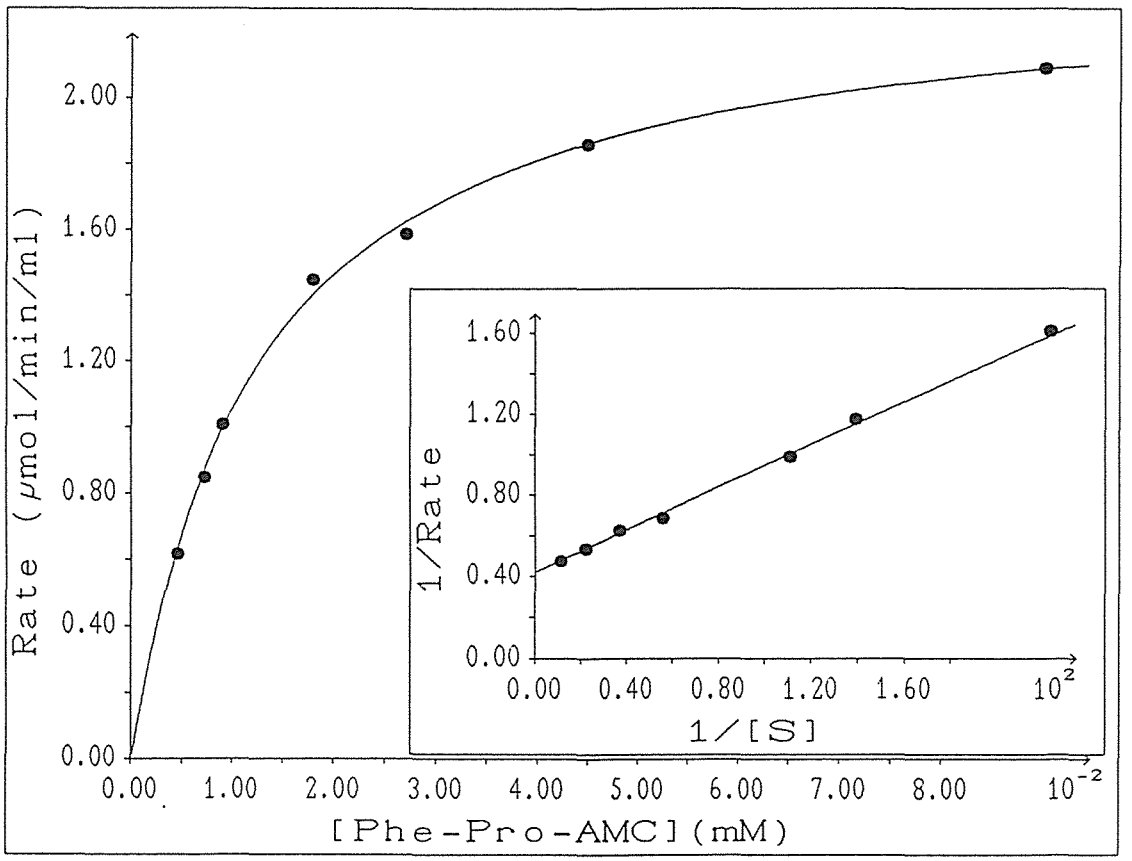
b


Rate (μmol/min/ml)
1.00
0.80
0.60
0.40
0.20
0.00
0.00 1.00 2.00 3.00 4.00 5.00 6.00 7.00 8.00 10⁻²
[Leu-Pro-AMC] (mM)
1/Rate
2.00
1.50
1.00
0.50
0.00
0.00 0.40 0.80 1.20 1.60 10²
1/[S]

c


Rate (μmol/min/ml)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.00 1.00 2.00 3.00 4.00 5.00 6.00 7.00 8.00 10⁻²
[Glu-Pro-AMC] (mM)
1/Rate
4.00
3.00
2.00
1.00
0.00
0.00 0.40 0.80 10²
1/[S]

d


Rate (μmol/min/ml)
2.00
1.60
1.20
0.80
0.40
0.00
0.00 1.00 2.00 3.00 4.00 5.00 6.00 7.00 8.00 10⁻²
[Phe-Pro-AMC] (mM)
1/Rate
1.60
1.20
0.80
0.40
0.00
0.00 0.40 0.80 1.20 1.60 10²
1/[S]

e

Substrate	Km (std err.) (μM)	V_{max} (μmoles/min/mg)	k_{cat} (/second)
Leu-Pro-AMC	9.2 (1.1)	31.7	43.8
Phe-Pro-AMC	12.4 (0.5)	54.1	78.4
Gly-Pro-AMC	14.1 (2.1)	50.5	69.8
Lys-Pro-AMC	14.6 (1.5)	44.5	61.6
Glu-Pro-AMC	45.7 (3.4)	43.4	62.9

TABLE 3.9 *Kinetic parameters obtained from measuring the rates of hydrolysis of X-Pro-AMC substrates by the purified Gly-Pro-AMCase from S.lactis 4760. The standard error for the Km is shown in brackets. The standard errors for the V_{max} and k_{cat} are not given since the error associated with the protein assay was unsure. Each assay contained 0.042μg of purified enzyme in a total volume of 2.0ml. Assays were carried out in 100mM MES buffer at pH 6.8 using substrate concentrations covering the range 5 - 100μM. The purity of each substrate (Section 2.2.7) was taken into account in the calculation of substrate concentration, and a molecular weight of 83,000 was used in the calculation of enzyme concentration in μmoles /ml.*

3.3.7 KINETIC STUDIES ON THE HYDROLYSIS OF THE TETRAPEPTIDE GLY-PRO-GLY-GLY TO GLY-PRO AND GLY-GLY

All the experimental evidence previously described shows the X-Pro-AMCase to be active as an arylamidase cleaving the bond between X-Pro and AMC. An experimental procedure to confirm that this enzyme acts as a true X-Pro dipeptidyl peptidase was developed using the tetrapeptide Gly-Pro-Gly-Gly as a substrate, and following the rate of its hydrolysis to two dipeptides, Gly-Pro and Gly-Gly by reverse phase HPLC.

A stock solution of 1mg/ml of tetrapeptide was diluted to concentrations spanning the range 0.02 to 0.5mg/ml. 15µl of enzyme solution containing 0.042mg/ml Gly-Pro-AMCase was added to 1ml of each tetrapeptide solution. The final pH of the incubation mixture was 7.0.

A 50µl sample of each incubation mixture was immediately injected onto an HPLC column, using an isocratic buffer system containing 1% acetonitrile and 0.1% trifluoro acetic acid in demineralised water to elute the peptide. Further injections were made every 10 minutes, and the rate of hydrolysis was calculated at each substrate concentration by measuring the decrease in tetrapeptide peak area (height x width at half height) assuming the tetrapeptide peak area at time zero to be equivalent to the initial concentration. An example of an HPLC profile showing the decrease in concentration of tetrapeptide with a corresponding increase in concentration of dipeptides is shown in Fig 3.14. The identity of the two peaks preceding the tetrapeptide peak was established as follows. The retention time for glycyl-glycine corresponded with the first and smaller of the two peaks. The second peak was collected, freeze dried, and analysed following a 24 hour acid hydrolysis as described in Section 2.2.13. The second peak had a 1:1 ratio of of glycine to proline and therefore corresponded to the Gly-Pro dipeptide.

From the rate of dipeptide hydrolysis, determined at several substrate concentrations, an initial rate verses concentration plot was constructed as shown in Fig 3.15. (At low initial substrate concentrations the rate was linear for the first ten minutes only). The Km calculated from the plot was 330µM, which is approximately 20 fold higher than that found using the Gly-Pro-AMC substrate. However the k_{cat} value (27.5/sec), was similar to that found with the Gly-Pro-AMC substrate (41.9/sec).

These results confirm that the purified enzyme is a true X-Pro dipeptidyl peptidase, and henceforth shall be referred to as such.

3.3.8 INVESTIGATION OF THE ACTION OF THE X-PRO DIPEPTIDYL PEPTIDASE ON OTHER PEPTIDES

Small peptides beginning with the sequence X-Pro were used as substrates for the dipeptidyl peptidase, to complement the results obtained with the tetrapeptide Gly-Pro-Gly-Gly and confirm its role as a proteolytic enzyme. The peptides chosen were the β-casein-derived, seven-residue peptide, β-casomorphin, and the nine-residue peptide bradykinin. Degradation of the peptides was followed using reverse phase HPLC (Section 2.2.15).

β-casomorphin has the sequence Tyr-Pro-Phe-Pro-Gly-Pro-Ile making it an excellent potential substrate for the dipeptidyl peptidase. It was shown by HPLC to be degraded rapidly by the dipeptidyl peptidase. To identify the products of degradation, peaks were collected and evaporated to dryness. The samples were then hydrolysed and the composition determined. The time course for the hydrolysis of β-casomorphin and the identity of the products formed is shown in Fig. 3.16. The HPLC profile shows that β-casomorphin was hydrolysed to three peptides: Tyr-Pro, Phe-Pro, and the tripeptide Gly-Pro-Ile. The fact that the C-terminal tripeptide was not broken down to Gly-Pro and isoleucine suggests that tripeptides might be too small to be substrates for the dipeptidyl peptidase. However this is discussed at more length in Section 4.1.3.

Both bradykinin (with the sequence Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and [Lys^1]-bradykinin (with N-terminal Lys-Pro) were incubated for varying periods with dipeptidyl peptidase. The N-terminal dipeptides were not removed from the peptide even after long periods of incubation with the dipeptidyl peptidase. The third residue in the bradykinin sequence is also a proline. The two consecutive prolines may impose a constraint (perhaps a severe kink) preventing access of the enzyme to cleave the Pro-Pro bond.

Two further peptides, the first, fragment 90-95 of α-casein with the sequence Arg-Tyr-Leu-Gly-Tyr-Leu, and the second, fragment 32-36 of thymopoietin II with the sequence Arg-Lys-Asp-Val-Tyr, were also resistant to hydrolysis by the dipeptidyl peptidase even after extended incubation of 72 hours.

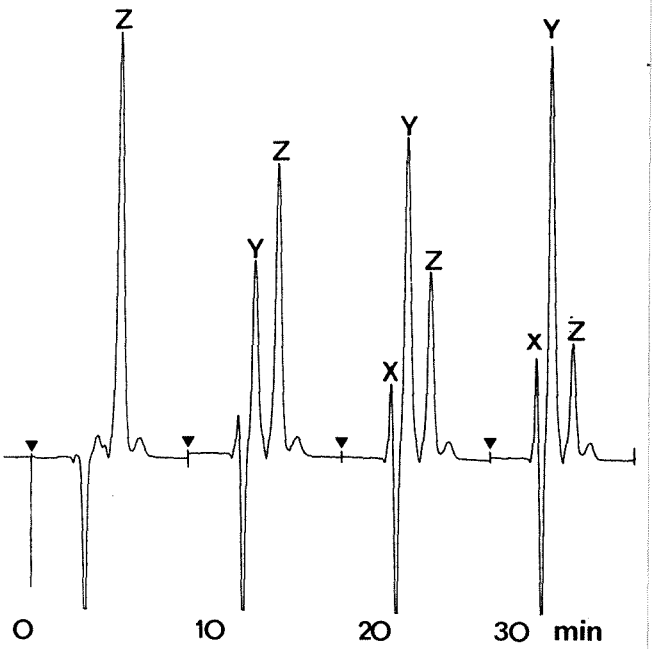


FIG. 3.14 *Time course showing an example of the hydrolysis of the tetrapeptide Gly-Pro-Gly-Gly by the dipeptidyl peptidase forming two dipeptides. Separation of peptides was performed by reverse phase HPLC on a C18 column, using an isocratic buffer system with 1% acetonitrile and 0.1% TFA eluting at a flow rate of 1ml/min. The rate of hydrolysis was calculated by measuring the decrease in tetrapeptide peak area as explained in Section 3.3.7. The peptides were detected by measuring the A220 of the eluate.*

Identity of peaks

X = Gly-Gly

Y = Gly-Pro

Z = Gly-Pro-Gly-Gly

▼ *indicates the point of sample injection.*

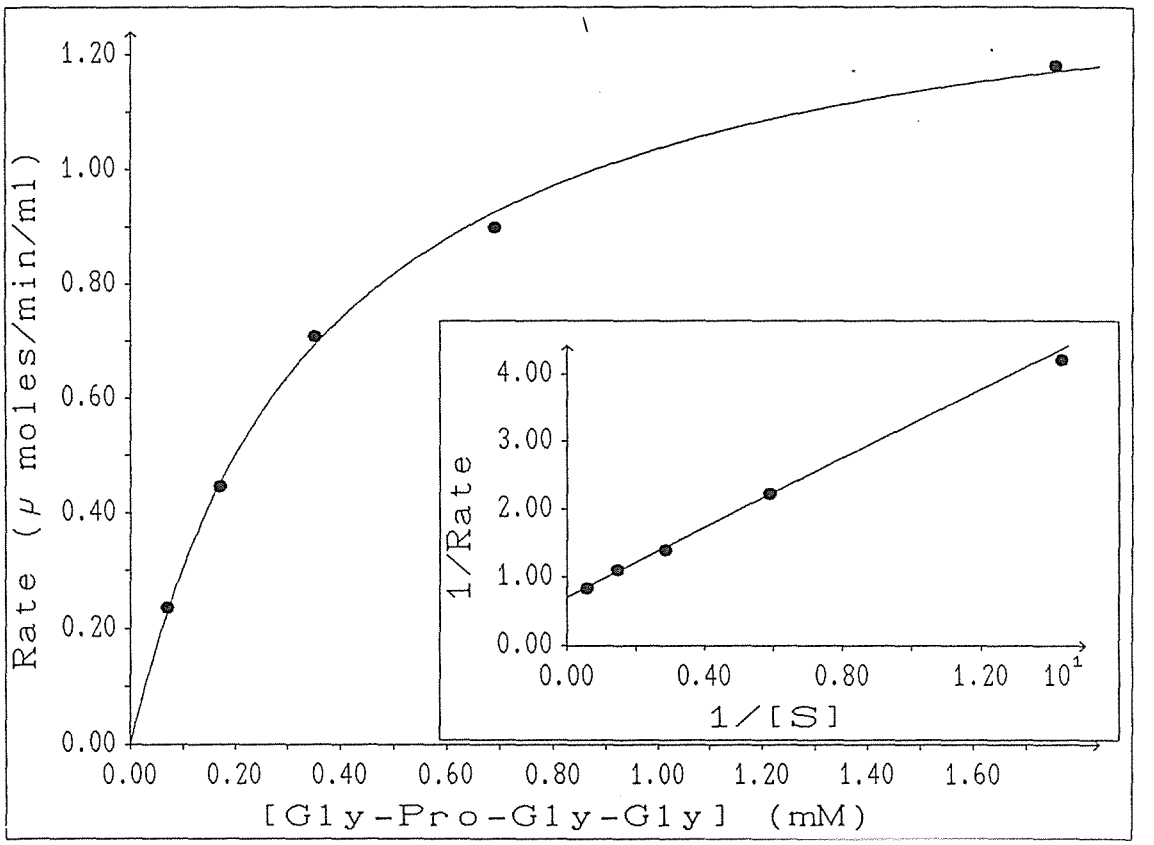


FIG. 3.15 *Rate verses substrate concentration and Lineweaver-Burk plots of Gly-Pro-Gly-Gly tetrapeptide degradation. 15 μl of 0.042mg/ml Gly-Pro-AMCase was added to 1ml of each tetrapeptide solution at pH 7.0. 50μl of each sample was injected onto the HPLC column, using an isocratic aqueous buffer system containing 0.1% TFA and 1% acetonitrile.*

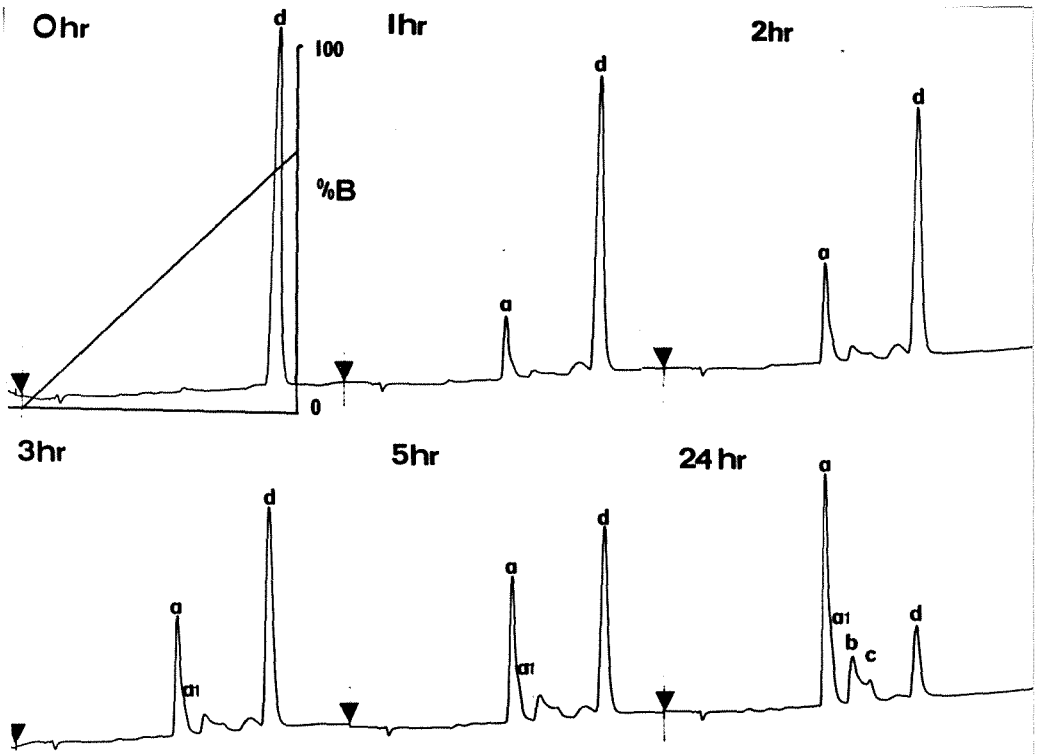


FIG 3.16 *Time course showing the degradation of β-casomorphin by the dipeptidyl peptidase over a 24 hour period using reverse phase HPLC on a C18 column. The peptides were eluted with a linear acetonitrile gradient of 0 - 80 % B as indicated. B contained 80% acetonitrile 20% water, and A contained 5% acetonitrile 95 % water. Both buffers contained 0.1% TFA and were eluted at 1ml/min. Peptides were detected by measuring the A220 of the eluate.*

Identity of peaks (compositional analysis)

a = *Tyr-Pro*

a1 = *Gly-Pro-Ile*

b and c = *Phe-Pro*

d = *Tyr-Pro-Phe-Pro-Gly-Pro-Ile*

▼ *indicates the point of sample injection.*

3.3.9 AMINO ACID COMPOSITION OF X-PRO DIPEPTIDYL PEPTIDASE.

A batch of dipeptidyl peptidase was purified from 100g of *S.lactis* 4760 as described in Section 3.2, giving very similar specific activities to those reported in Section 3.2.6 at each step of the procedure, and a single Coomassie blue band on SDS PAGE. The total purified peptidase was dialysed against 3x1 litre changes of demineralised water to remove glycerol and salt, and was freeze dried. The peptidase was then re-dissolved in 1ml of 0.02M ammonia and ten 100µl aliquots were transferred into test tubes and freeze-dried in preparation for hydrolysis as described in Section 2.2.13. Duplicate hydrolyses were carried out for 24, 48 and 72 hours. The amount of serine and threonine was plotted as a function of hydrolysis time and extrapolated to zero hydrolysis time to correct for decomposition. The composition of the hydrophobic amino acids valine, leucine and isoleucine increased slightly after the longer hydrolysis times, the results reported here being those obtained after 72 hour hydrolysis. The remaining results are averages of values obtained after the different hydrolysis times. A summary of these results is shown in Table 3.10.

Amino acid residue	Residue number per molecule
Asp and Asn	94.5
Thr	41.0
Ser	58.0
Glu and Gln	75.6
Pro	25.7
Gly	54.1
Ala	48.4
Val	47.0
Met	11.2
Ile	40.0
Leu	83.0
Tyr	29.6
Phe	37.6
His	18.6
Lys	58.6
Arg	31.8

TABLE 3.10 *Summary of the results of amino acid composition of X-Pro dipeptidyl peptidase. Results are, unless otherwise stated in the text, averages of duplicate 24, 48 and 72 hour acid hydrolyses. The protocol for acid hydrolysis is described in Section 2.2.13. Residue number per mole was calculated using the estimated molecular weight of 83 000, by the following expression:*

(1) nmoles X molecular weight of residue = weight in ng

(2) $\frac{\text{residue weight in ng}}{\text{total weight of residues}} \times 83\,000 = \text{residue number per mole}$

3.4 COMPARATIVE STUDY OF DIPEPTIDYL PEPTIDASE FROM THREE STRAINS OF LACTIC STREPTOCOCCI

The dipeptidyl peptidase discussed in the previous Sections was purified from two further strains, *S.cremoris* 4409 and *S.lactis* 4125 to clarify the findings of the preliminary studies (Section 3.1). The pH profiles, kinetic properties and relative mobilities on native and denaturing PAGE of the purified dipeptidyl peptidase were studied to compare the properties of the *S.lactis* 4760 enzyme with those of the enzyme from the *S.lactis* parent strain (4125) and the *S.cremoris* strain (4409) which was the source the prt plasmid of 4760.

3.4.1 PURIFICATION OF DIPEPTIDYL PEPTIDASE FROM *S.cremoris* 4409

30g (wet packed weight) of *S.cremoris* 4409 was harvested from a 3 litre lactose broth culture grown as described in Section 2.2.3. The enzyme was purified by the procedure described in Section 3.2. The results obtained for the purification of the dipeptidyl peptidase are shown in Table 3.11. An enrichment of 277 fold was achieved, giving a final specific activity of 30.5. This is somewhat lower than that achieved with the *S.lactis* 4760 enzyme, although the cell-free extract specific activity was also significantly lower. PAGE (Fig. 3.19) revealed several minor contaminating bands in the enzyme preparation. The enzyme from *S.cremoris* 4409 eluted differently at the DEAE cellulose step compared to the 4760 enzyme (see Fig. 3.1c), so additional contaminating proteins may have been carried through from this step.

3.4.2 EFFECT OF pH ON *S.cremoris* 4409 DIPEPTIDYL PEPTIDASE ACTIVITY

The pH profile was determined by measuring the activity using a 0.71mM (final concentration) Gly-Pro-AMC substrate at several pH values over the range 4.5 to 9.0 using 100mM MES and Tris buffers. The characteristic broad pH profile (similar to that for the *S.lactis* 4760 dipeptidyl peptidase, Fig. 3.11) is shown in Fig 3.17.

Purification Step	Protein (mg/ml)	Total Protein (mg)	Activity (units/ml)	Specific Activity (units/mg)	Total Activity (units)	Recovery (%)	Fold Purif.
CFE	14.0	1092	1.55	0.11	120.9	100	0
DEAE	1.35	164.7	0.66	0.49	80.6	66.7	4.5
S300	1.25	30.0	2.53	2.02	60.7	50.2	18.4
Arg-Seph	0.085	3.57	1.27	14.9	53.3	44.1	135.5
DEAE-Seph	0.075	3.60	0.83	11.1	40.3	33.3	100.9
Arg-Seph	0.0164	0.75	0.50	30.5	23.0	19.0	277.3

TABLE 3.11 Summary of the purification of the dipeptidyl peptidase from 30.0g (wet weight) of S.cremoris 4409. The protein concentration of the purified enzyme was measured after concentration using a 2ml centrifugal concentrator and correcting the result obtained by multiplying by the concentration factor to give the protein concentration of the original sample.

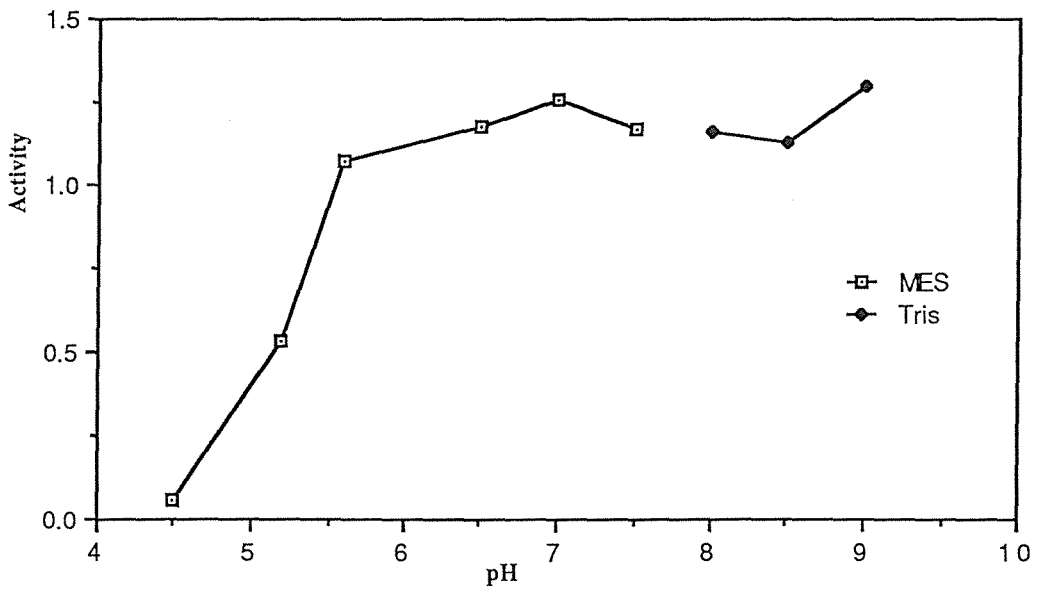


FIG.3.17 pH profile for the dipeptidyl peptidase from S.cremoris 4409. Activity is in µmoles/min/ml. 100mM MES and Tris buffers were used to cover the range 4.5 to 9.0. The concentration of substrate in the reaction mixture was 0.71mM, and 0.164µg protein was used per assay.

3.4.3 KINETIC PARAMETERS OF THE DIPEPTIDYL PEPTIDASE FROM *S.cremoris* 4409 WITH DIFFERENT X-PRO-AMC SUBSTRATES

The rate of hydrolysis of five synthetic X-Pro-AMC substrates was measured over a range of substrate concentrations as described in Section 3.3.6. The results for Km, V_{max} and k_{cat} are given in Table 3.13, and the rate verses substrate concentration and Lineweaver-Burke plots are shown in Fig. 3.20 a, b, c, d and e.

The Km values agree closely with those determined for the *S.lactis* 4760 enzyme, Glu-Pro-AMC again giving a significantly higher Km than the other substrates tested. The V_{max} and k_{cat} results for *S.cremoris* 4409 are lower than the corresponding results for *S.lactis* 4760. This may be due to different properties of the enzyme, but more probably is a result of protein contamination and inaccuracies associated with the measurement of the very low protein concentration. The V_{max} and k_{cat} results, however, show the same relative relationship, Leu-Pro-AMC giving lower and Phe-Pro-AMC slightly higher values.

3.4.4 PURIFICATION OF DIPEPTIDYL PEPTIDASE FROM *S.lactis* 4125

35.5g wet packed weight of *S.lactis* 4125 was harvested from a 3 litre broth culture supplemented with 40g/l glucose as described in Section 2.2.3. The results obtained for each stage of purification are shown in Table 3.12. A purification of 183 fold was achieved, giving a final specific activity of 36.6- very similar to that obtained with *S.lactis* 4760. PAGE (Fig. 3.19) reveals a single protein band with very low levels of contamination.

3.4.5 KINETIC PARAMETERS OF THE DIPEPTIDYL PEPTIDASE FROM *S.lactis* 4125 WITH DIFFERENT X-PRO-AMC SUBSTRATES

The rates of hydrolysis of three X-Pro-AMC substrates were measured over the same concentration range as described in Section 3.3.6. Table 3.14 summarises the results for Km, V_{max} and k_{cat}. The rate verses substrate concentration and Lineweaver-Burk plots are shown in Fig. 3.21 a, b and c.

The Km values are higher than those found with the enzyme from *S.lactis* 4760. However when the errors associated with the measurement of rates and initial weighing

of the substrate are taken into account, the significance of the difference is questionable. The relative values, with the enzymes from all three strains having a significantly higher Km for Glu-Pro-AMC are similar. The V_{max} and k_{cat} values are very similar to those obtained for *S.lactis* 4760.

3.4.6 COMPARISON OF THE RELATIVE MOBILITY OF PURIFIED DIPEPTIDYL PEPTIDASE FROM THE THREE STRAINS ON PAGE

The relative mobility of the purified enzymes from *S.lactis* 4760, 4125 and *S.cremoris* 4409 was compared on 7.5% native PAGE and 10% SDS PAGE. Native PAGE was performed as described in Section 2.2.12. The identity of the Coomassie blue-protein complex was confirmed as the dipeptidyl peptidase by activity staining, as described in Section 3.2.7. The mobility of the dipeptidyl peptidase from the two *S.lactis* strains was identical, whereas the enzyme from *S.cremoris* had a higher relative mobility. The photographs of these gels are shown in Fig. 3.18. SDS PAGE (Fig. 3.19) confirmed the identical size of the enzymes from the two *S.lactis* strains and the dissimilarity of the *S.cremoris* enzyme, which had a molecular weight of about 3 000 greater than the *S.lactis* enzymes.

The findings presented here therefore confirm that the dipeptidyl peptidase purified from *S.lactis* 4760 and 4125 are identical and the dipeptidyl peptidase from *S.cremoris*, although similar in many of its properties, has slightly different column fractionation and gel mobility characteristics.

Purification step	Protein (mg/ml)	Total Protein (mg)	Activity (units/ml)	Specific Activity (units/mg)	Total Activity (units)	Recovery (%)	Fold Purif.
CFE	14.5	1015	2.89	0.20	202.3	100	0
DEAE	0.9	90	1.06	1.18	106.0	52.3	5.9
S300	1.10	38.5	2.59	2.35	90.7	44.8	11.8
Arg-Seph	0.075	2.85	2.0	26.7	75.8	37.5	133.5
DEAE-Seph	0.055	1.13	1.26	22.9	51.7	25.6	114.5
Arg-Seph	0.0172	0.507	0.63	36.6	37.2	18.3	186.0

TABLE 3.12 *Summary of the purification of the dipeptidyl peptidase from 35.5g (wet weight) of S.lactis 4125. The protein concentration of the purified enzyme was measured after concentration using a 2ml centrifugal concentrator and correcting the result obtained by multiplying by the concentration factor to give the concentration of the original sample.*

Substrate	Km (std err.) (μM)	V_{max} (μmoles/min/mg)	k_{cat} (/second)
Leu-Pro-AMC	12.5 (0.3)	17.6	24.3
Phe-Pro-AMC	14.1 (1.6)	31.7	43.9
Gly-Pro-AMC	13.7 (1.6)	25.6	35.4
Lys-Pro-AMC	9.8 (1.6)	20.7	28.7
Glu-Pro-AMC	45.7 (7.9)	23.8	32.9

TABLE 3.13 *Kinetic parameters obtained by measuring the rates of hydrolysis of X-Pro-AMC substrates by the purified dipeptidyl peptidase from S.cremoris 4409. The standard error for the Km is shown in brackets. Each assay contained 0.0328μg of purified enzyme in a total volume of 2.0ml. Assays were carried out in 100mM MES buffer at pH 6.8 using substrate concentrations covering the range 5 - 100μM. The purity of each substrate (Section 2.2.7) was taken into account in the calculation of substrate concentration, and a molecular weight of 83 000 was assumed in the calculation of enzyme concentration in μmoles/ml.*

Substrate	Km (std.err) (μM)	V_{max} (μmoles/min/mg)	k_{cat} (/second)
Leu-Pro-AMC	15.5 (2.3)	30.8	42.6
Gly-Pro-AMC	21.7 (2.8)	54.2	75.0
Glu-Pro-AMC	74.0 (8.9)	52.9	73.2

TABLE 3.14 *Kinetic parameters obtained from measuring the rates of hydrolysis of X-Pro-AMC substrates by the purified dipeptidyl peptidase from S.lactis 4125. Each assay contained 0.0344μg of purified enzyme in a total volume of 2.0ml. Assays were carried out in 100mM MES buffer at pH 6.8 using substrate concentrations covering the range 5 - 100μM. The purity of each substrate (Section 2.2.7) was taken into account in the calculation of substrate concentration, and a molecular weight of 83 000 was assumed in the calculation of enzyme concentration in μmoles/ml.*

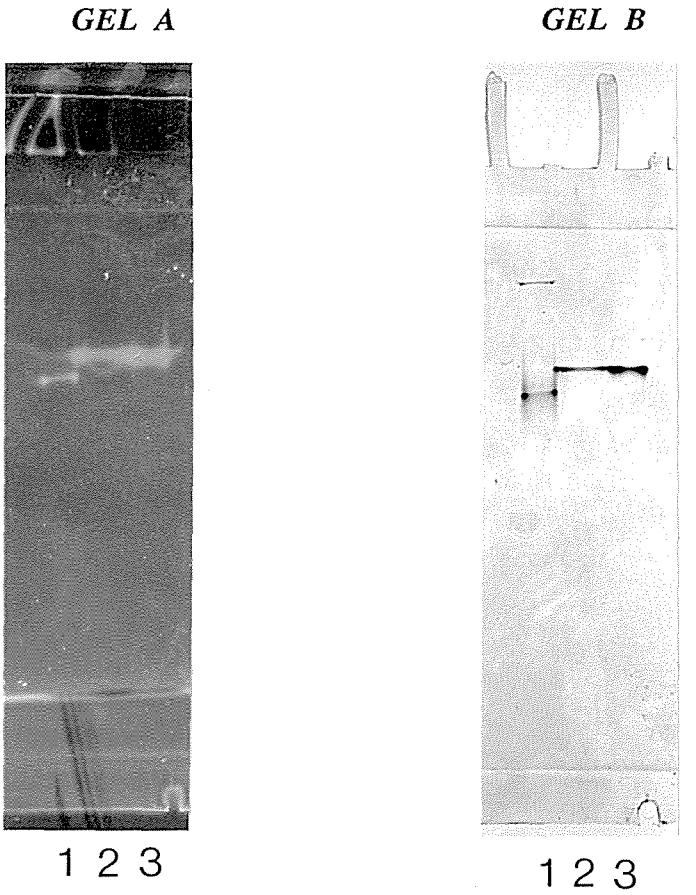


FIG. 3.18 *Native PAGE of the purified dipeptidyl peptidase from the three strains of lactic streptococci studied. Track 1 shows the dipeptidyl peptidase from S.cremoris , tracks 2 and 3 show the dipeptidyl peptidase from S.lactis 4125 and 4760 respectively. Gel A shows the relative mobility of the purified enzymes stained with 0.5ml of 1mM Gly-Pro-AMC and photographed under ultraviolet light. Gel B shows the same native gel stained with Coomassie blue highlighting the protein band.*

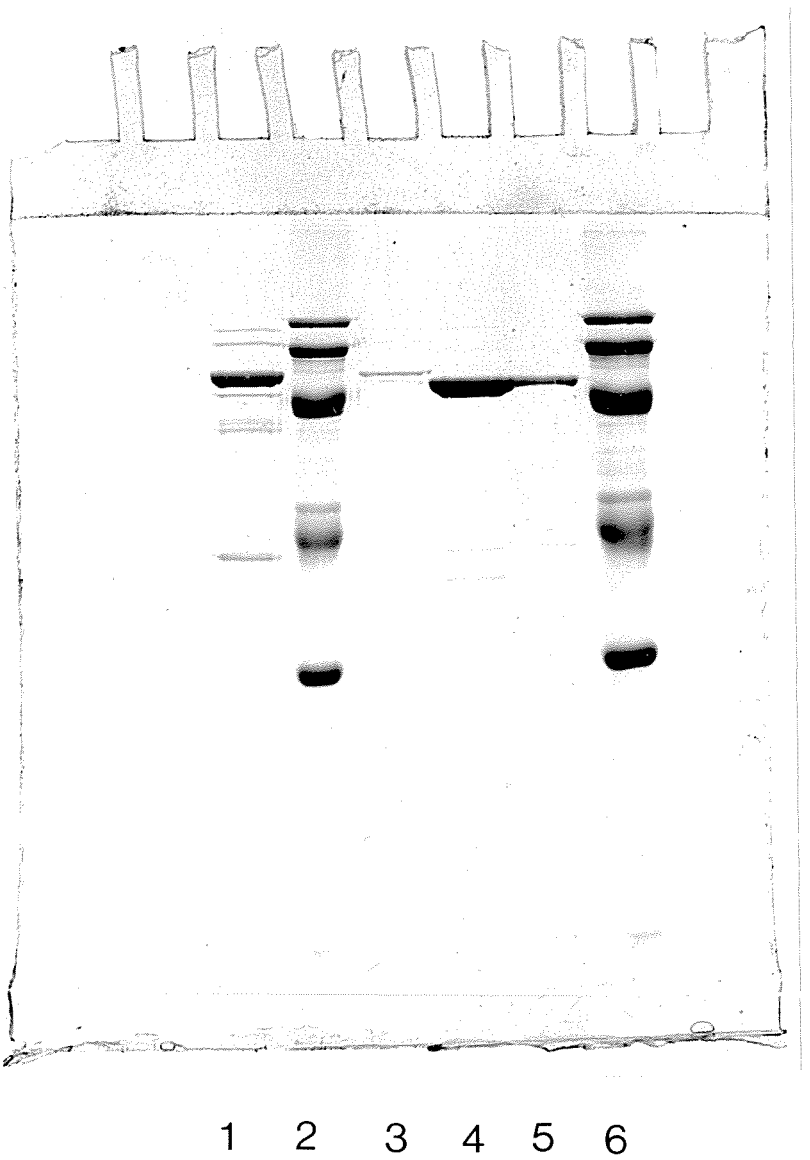
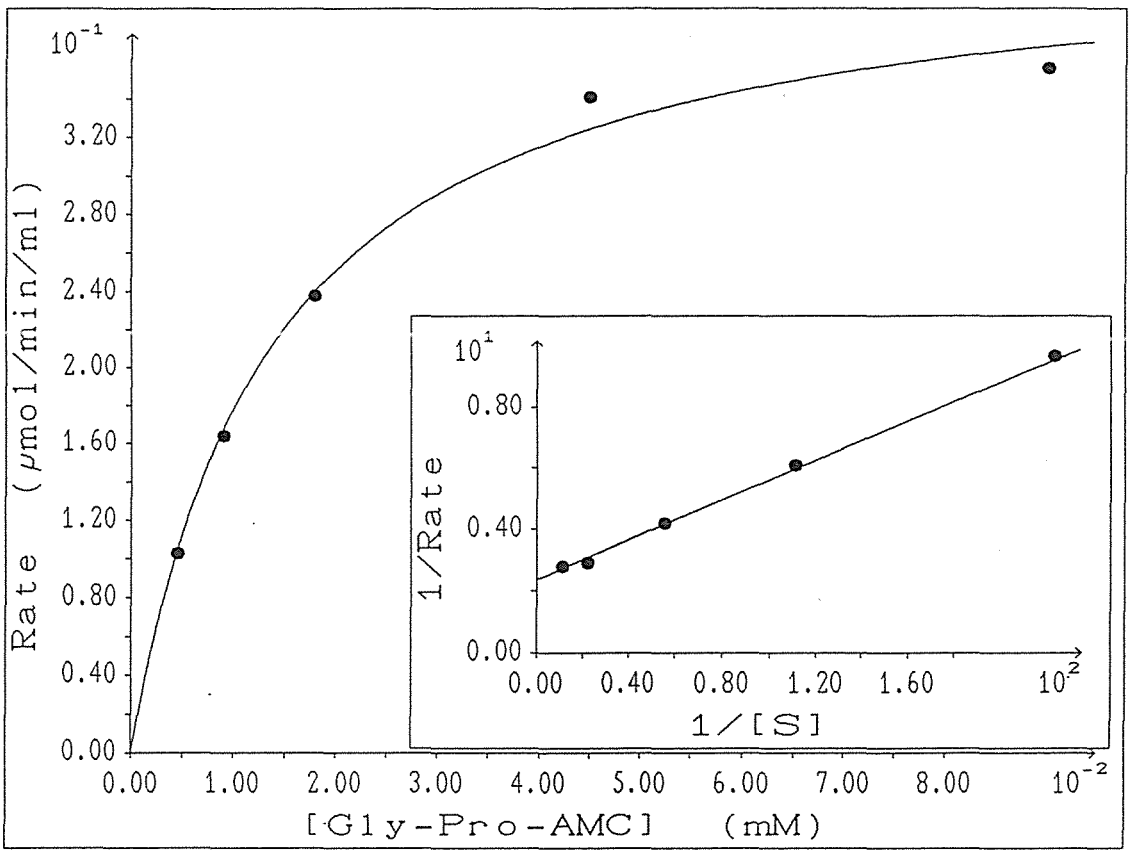


FIG. 3.19 *10% SDS PAGE showing the mobility of the dipeptidyl peptidase purified from the three strains studied. Track 1 shows a heavy loading of the enzyme from S.cremoris while track 3 shows a lighter loading. Tracks 4 and 5 show the enzymes purified from S.lactis 4125 and 4760 respectively, and tracks 2 and 6 show the molecular weight markers.*

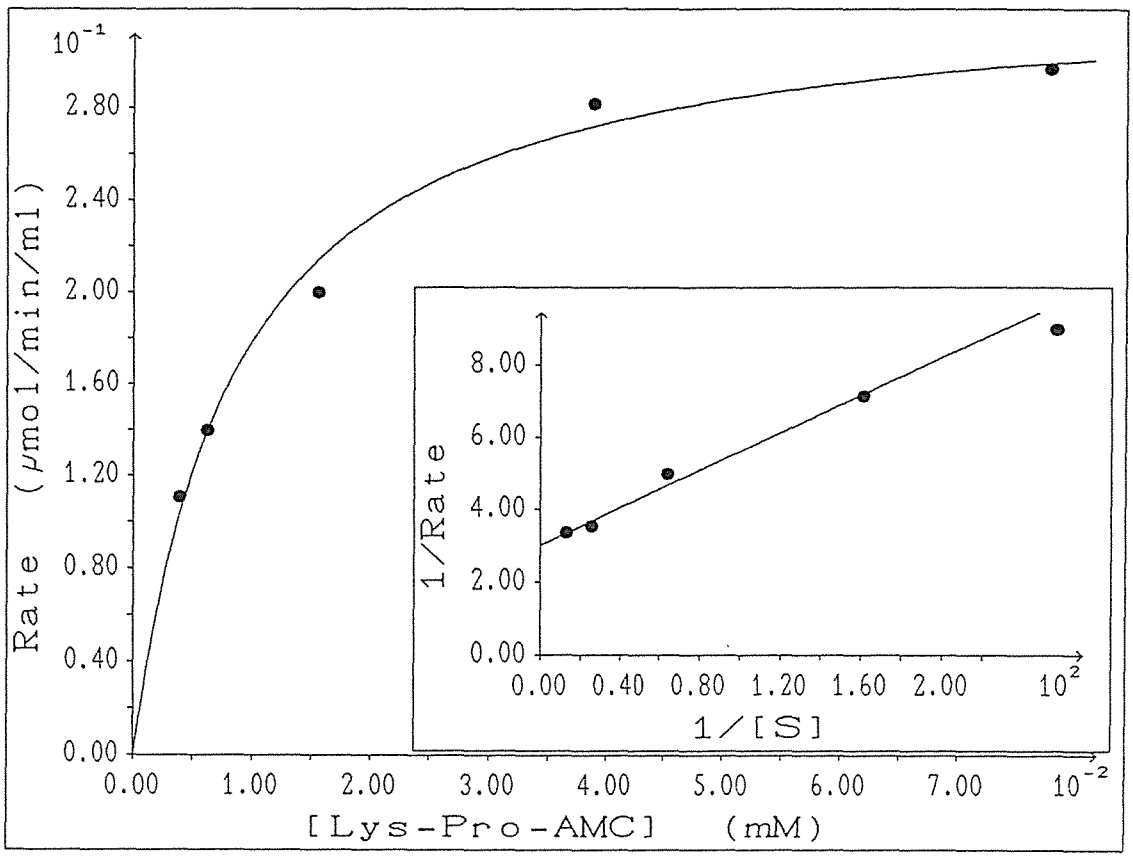
Identity and molecular weight of markers (in descending order)

β-galactosidase	*116 000*
Phosphorylase B	*97 000*
Bovine serum albumin	*66 000*
Egg albumin	*45 000*
Carbonic anhydrase	*29 000*

FIG. 3.20 Rate verses substrate concentration plots and Lineweaver-Burk plots for the dipeptidyl peptidase from S.cremoris 4409, using the substrates a) Gly-Pro-AMC, b) Lys-Pro-AMC, c) Leu-Pro-AMC, d) Glu-Pro-AMC, and e) Phe-Pro-AMC. Each assay contained 0.0328µg of purified dipeptidyl peptidase in a total volume of 2.0ml. Assays were carried out in 100ml MES buffer, at pH 6.8 using substrate concentrations covering the range 5 - 100µM.



a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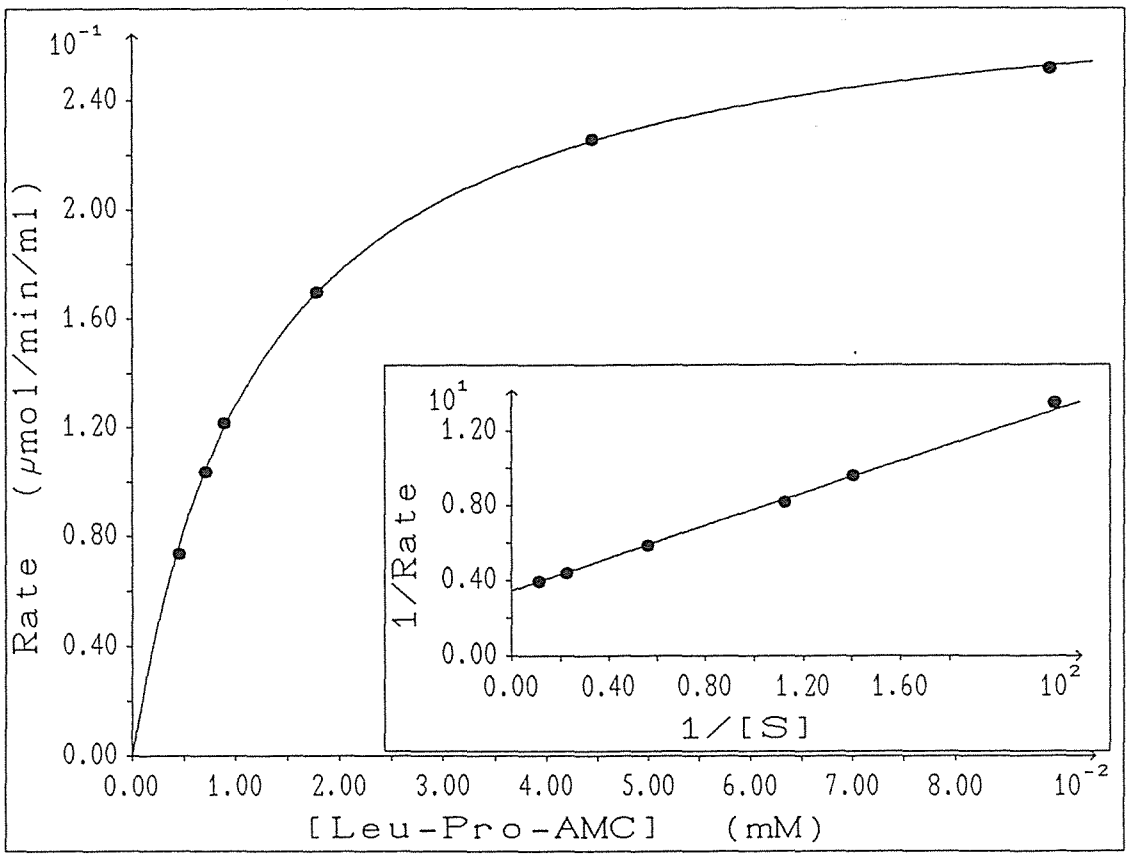
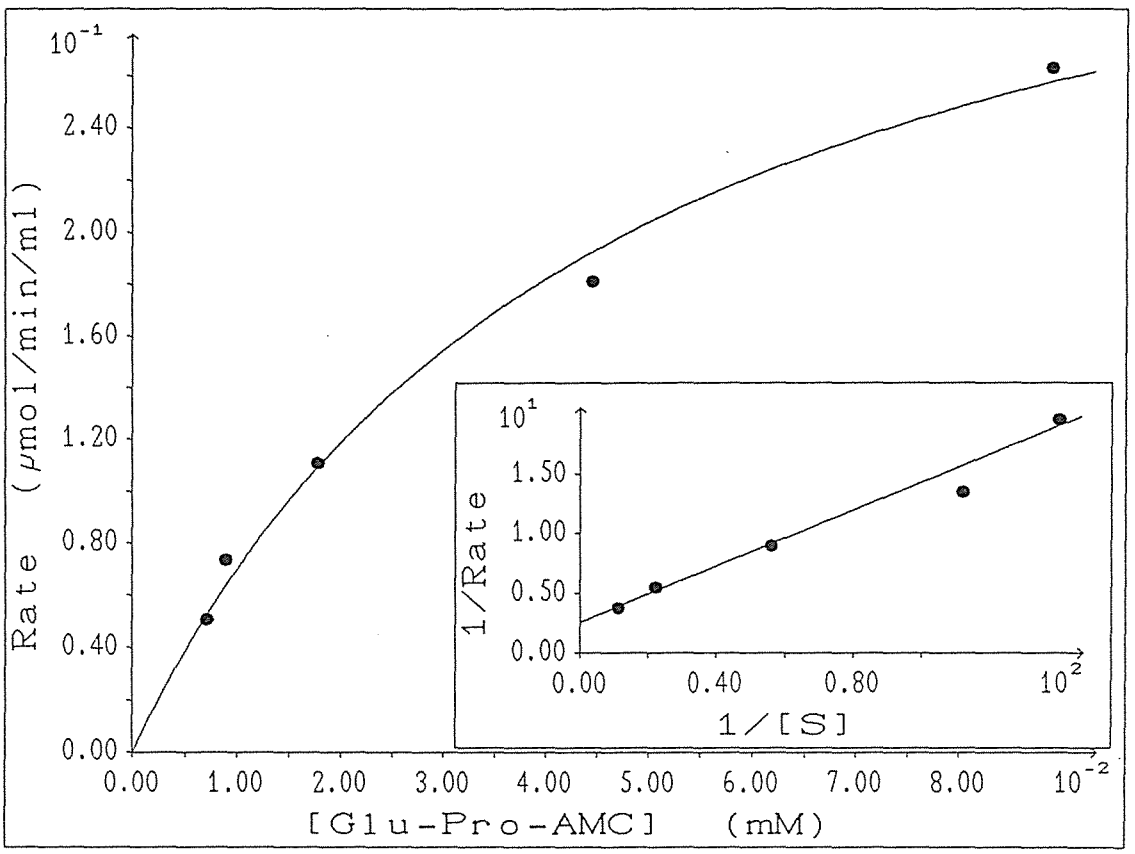
2.80
2.40
2.00
1.60
1.20
0.80
0.40
0.00
Rate (μmol/min/ml)
0.00 1.00 2.00 3.00 4.00 5.00 6.00 7.00 10^{-2}
[Lys-Pro-AMC] (mM)
1/Rate
8.00
6.00
4.00
2.00
0.00
0.00 0.40 0.80 1.20 1.60 2.00 10^{2}
1/[S]

b


10^{-1}
2.40
2.00
1.60
1.20
0.80
0.40
0.00
Rate (μmol/min/ml)
0.00 1.00 2.00 3.00 4.00 5.00 6.00 7.00 8.00 10^{-2}
[Leu-Pro-AMC] (mM)
10^{1}
1.20
0.80
0.40
0.00
1/Rate
0.00 0.40 0.80 1.20 1.60 10^{2}
1/[S]

c


10^{-1}
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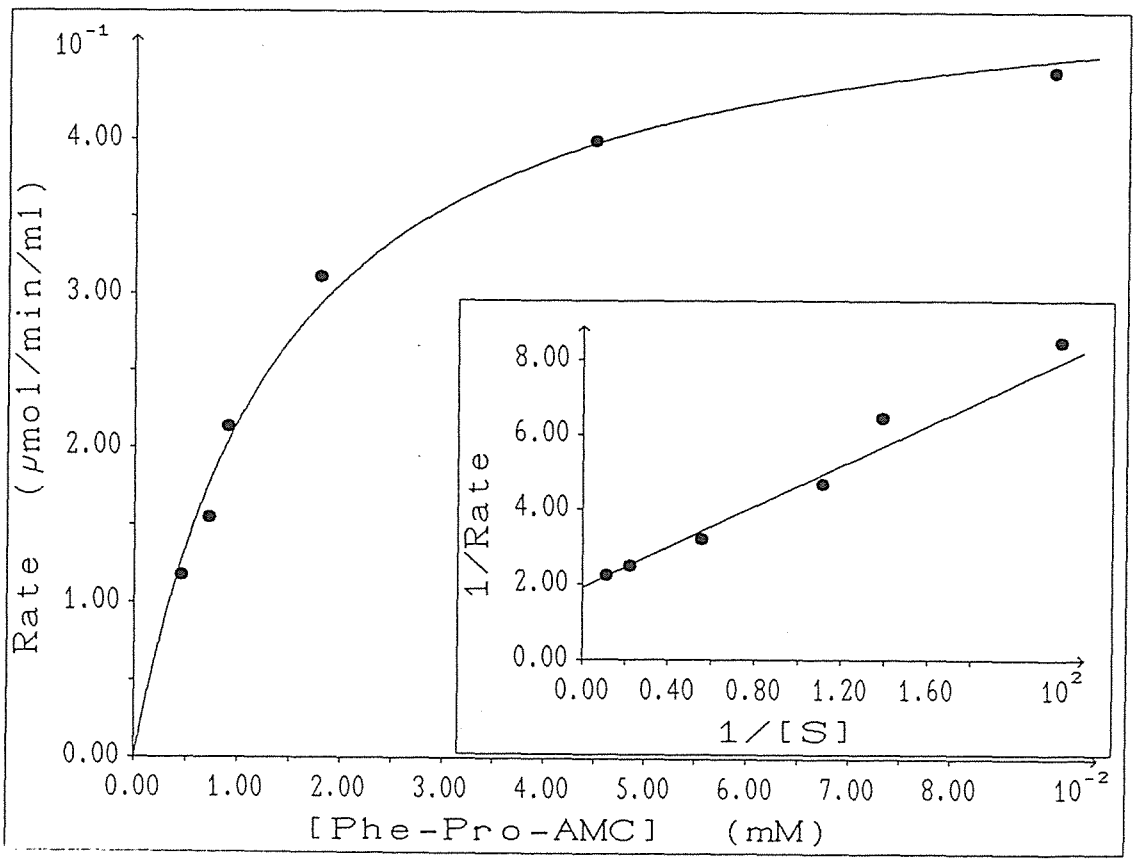
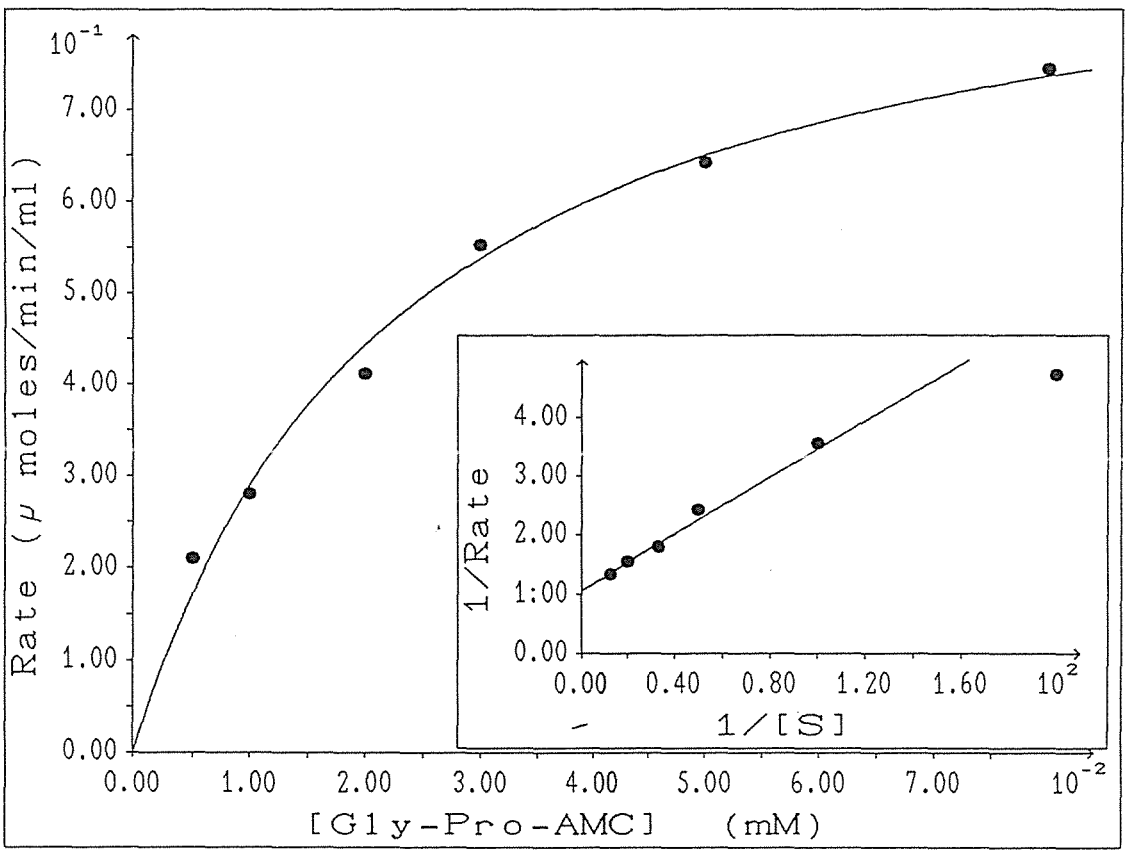
1.60
1.20
0.80
0.40
0.00
Rate (μmol/min/ml)
0.00 1.00 2.00 3.00 4.00 5.00 6.00 7.00 8.00 10^{-2}
[Glu-Pro-AMC] (mM)
10^{1}
1.50
1.00
0.50
0.00
1/Rate
0.00 0.40 0.80 10^{2}
1/[S]

d

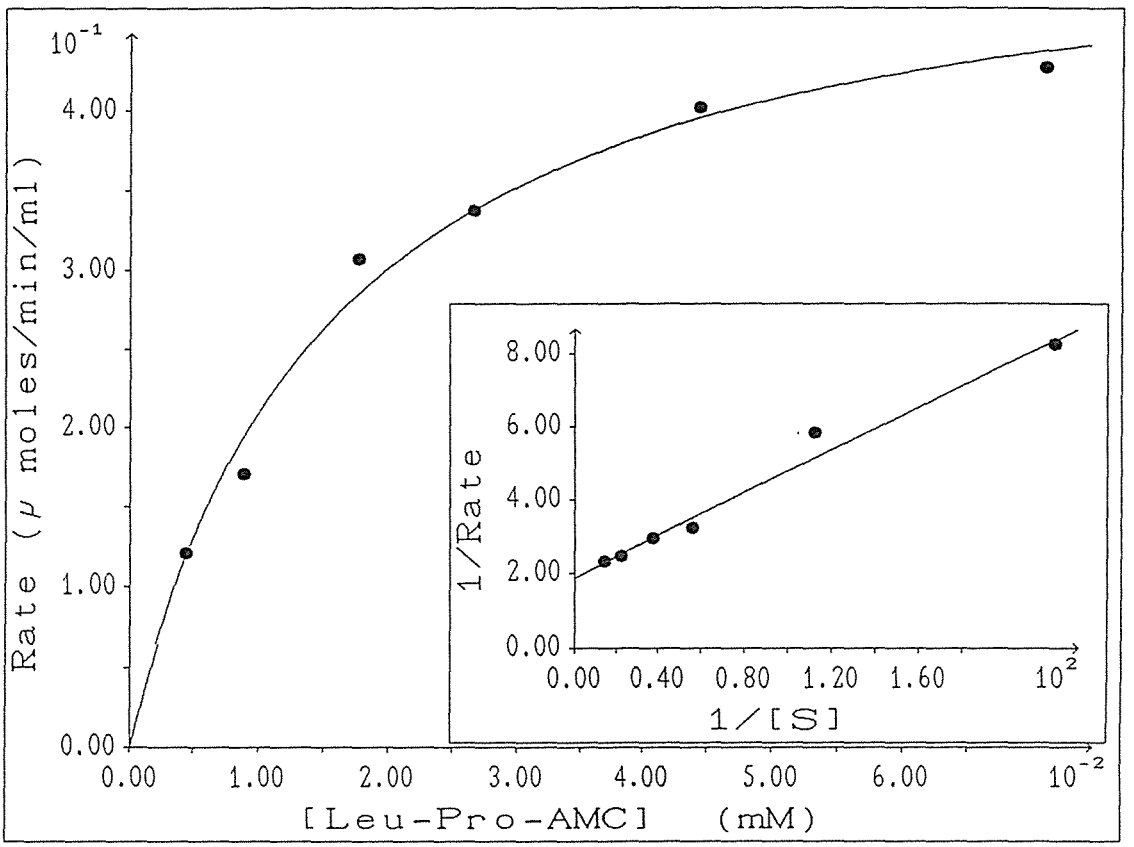

10^{-1}
4.00
3.00
2.00
1.00
0.00
Rate (μmol/min/ml)
0.00 1.00 2.00 3.00 4.00 5.00 6.00 7.00 8.00 10^{-2}
[Phe-Pro-AMC] (mM)
8.00
6.00
4.00
2.00
0.00
1/Rate
0.00 0.40 0.80 1.20 1.60 10^{2}
1/[S]

e

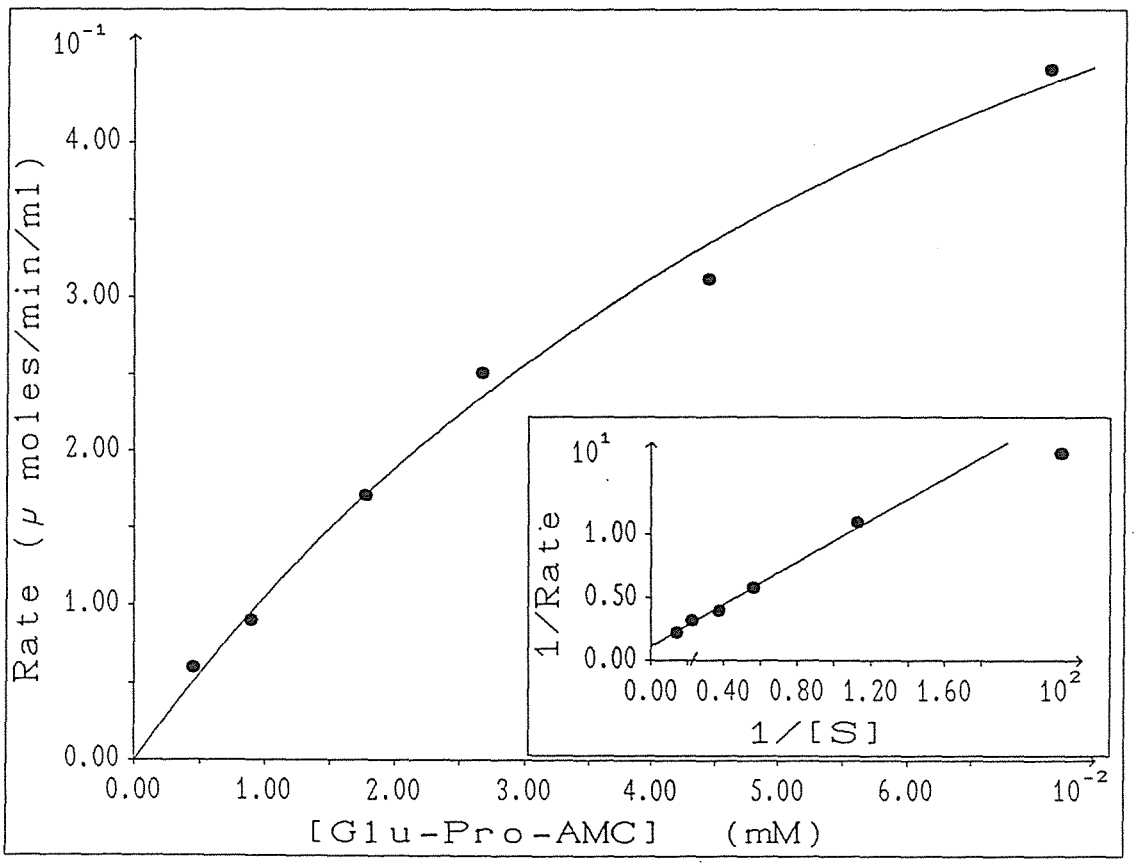
FIG. 3.21 Rate verses substrate concentration plots and Lineweaver-Burk plots for the dipeptidyl peptidase from S.lactis 4125, using the substrates a) Gly-Pro-AMC, b) Leu-Pro-AMC, c) Glu-Pro-AMC, Each assay contained 0.0344µg of purified X-Pro-AMCase in a total volume of 2.0ml. Assays were carried out in 100ml MES buffer, at pH 6.8 using substrate concentrations covering the range 5 - 100µM



a


10^{-1}
4.00
3.00
2.00
1.00
0.00
Rate (μ moles/min/ml)
0.00 1.00 2.00 3.00 4.00 5.00 6.00 10^{-2}
[Leu-Pro-AMC] (mM)
8.00
6.00
4.00
2.00
0.00
1/Rate
0.00 0.40 0.80 1.20 1.60 10^{2}
1/[S]

b


10^{-1}
4.00
3.00
2.00
1.00
0.00
Rate (μ moles/min/ml)
0.00 1.00 2.00 3.00 4.00 5.00 6.00 10^{-2}
[Glu-Pro-AMC] (mM)
10^{1}
1.00
0.50
0.00
1/Rate
0.00 0.40 0.80 1.20 1.60 10^{2}
1/[S]

C

3.5 PARTIAL PURIFICATION OF A "GENERAL" AMINOPEPTIDASE FROM *S.lactis* 4760

Preliminary studies on aminopeptidase activity in crude cell-extracts fractionated on DEAE cellulose (see Section 3.1) revealed an enzyme catalysing the hydrolysis of lysyl-AMC and leucyl-AMC, the activity profile with these two substrates being identical. Previous studies (Kaminogawa *et al.*, 1984, and Geis *et al.*, 1985) have reported aminopeptidase activity in extracts from lactococci using aminoacyl p-nitroanalide substrates, and on the basis of activity towards several different aminoacyl p-nitroanalides have referred to the enzyme as a general aminopeptidase. Such an aminopeptidase could complement the activity of the X-Pro dipeptidyl peptidase in the degradation of oligopeptides and was accordingly selected as the second enzyme for study in the present project. Since it was assayed during purification using the substrate lysyl-AMC, it will be referred to as the Lys-AMCase until its specificity characteristics have been defined.

Large scale lactose broth grown cultures of *S.lactis* 4760 were grown and cell-free extracts prepared as described in Section 2.2.3 and 2.2.4

3.5.1 DEAE CELLULOSE CHROMATOGRAPHY

The cell-free extract from 100g (wet packed weight) of cells was loaded onto a DEAE cellulose column as described in Section 3.2.1, previously equilibrated with 50mM Tris/glycerol buffer pH 7.0. Bound protein was eluted with a linear concentration gradient of 0 - 0.4M NaCl at a flow rate of 1.5ml/min. The elution profile is shown in Fig. 3.2. The column fractions containing Lys-AMCase activity were pooled and concentrated, using an Amicon Diaflo concentrator with a PM30 membrane, to a volume of less than 10ml (XM50 membrane gave significant losses of enzyme activity).

3.5.2 SEPHACRYL S300 GEL PERMEATION CHROMATOGRAPHY

A concentrated sample of no more than 8ml from the DEAE cellulose purification step was loaded onto the S300 column which had been previously equilibrated with 10mM Tris/glycerol buffer at pH 7.0. The column was eluted at 0.5ml/min with the same buffer. The elution profile is shown in Fig. 3.22. The fractions containing Lys-AMCase activity were pooled. This step resulted in a slight increase in specific activity when compared with the material eluted from the DEAE column, although if the material eluted from the

S300 column is compared with the concentrated fraction loaded on to this column a decrease in specific activity is seen together with a substantial loss of total activity. Nevertheless, omission of this step led to a serious loss of effectiveness of the arginine-Sepharose column as discussed in Section 3.2.2.

3.5.3 PURIFICATION OF LYS-AMCase BY AFFINITY CHROMATOGRAPHY

Several attempts were made to find a suitable system for affinity purification of the Lys-AMCase, without success. The systems tried are outlined below:

a) Affinity elution from arginine-Sepharose with lysine.HCl. It was established in Section 3.2.3 that this resin separates dipeptidyl peptidase activity from the majority of other proteins. It was also shown that Lys-AMCase activity eluted at a higher ionic strength. Since the Lys-AMCase bound tightly, it appeared reasonable that lysine might elute the Lys-AMCase specifically. The arginine-Sepharose column was equilibrated with 10mM Tris/glycerol pH 7.0, and a sample (which had already been salt-eluted once from the same column and dialysed as described in Section 3.5.5) loaded. Elution with a linear gradient of 0 - 100mM lysine.HCl failed to separate Lys-AMCase activity from dipeptidyl peptidase activity, both enzymes eluting at approximately 40 to 50 mM lysine.HCl. Elution of the enzymes appeared to be on the basis of ionic strength rather than a specific affinity process.

b) Glycine-proline-AH-Sepharose. This resin was prepared by the method described in Section 2.2.11. 5ml of pooled sample from the S300 column was loaded onto a column containing 8ml of the resin, previously equilibrated with 50mM Tris/glycerol. Elution with a 0 - 1.0M NaCl gradient failed to elute detectable Lys-AMCase activity.

c) CBZ-phenylalanyl-AH-Sepharose. This resin was prepared by the Separation Science Unit, Massey University and although it was unlikely to be successful in purifying an aminopeptidase (its N-terminus is blocked with a CBZ group), since it was available, it was tried. CBZ-phenylalanyl-triethylene-tetramine-Sepharose has been employed successfully to separate α-chymotrypsin, subtilisin and neutral metalloendopeptidases at pH 5.0 - 7.0, while a range of acid proteases showed affinity to the adsorbant at pH 3.0-5.0. Chymotrypsin was eluted with 0.5M NaCl at pH 9.0 (too high to retain Lys-AMCase activity), and subtilisin and thermolysin were eluted with 30% ethylene glycol at pH 7.0. Other elution procedures were also used but were too harsh to be used with the Lys-AMCase and still retain activity. A sample (2ml) of pooled

material from the S300 column was loaded onto a column containing 5ml of this resin, equilibrated with 10mM Tris/glycerol pH 7.0. The column eluant contained no Lys-AMCase activity. Elution of the enzyme with 0.5M NaCl and 30% ethylene glycol was unsuccessful.

The failure of b) and c) above may be due to tight binding of the enzyme to the resin, making elution at the salt concentrations used impossible. Alternatively, elution of an inactive form of the enzyme may have occurred. In the case of c), since such a small column and sample size was used it proved difficult to detect protein elution at 280nm. The A280 was however measured for b), the majority of the protein eluting from the column in the equilibration buffer (conductivity 0.2mMho) with a second small A280 peak at a conductivity of 4.3mMho, but no significant activity was detected in the eluted fractions.

3.5.4 ATTEMPTS TO MINIMISE ACTIVITY LOSSES DURING PURIFICATION

Major losses of Lys-AMCase activity were found to occur at each purification step. However, the enzyme was quite stable in its partially purified form at 4° C. One possible explanation of the loss of activity is that during purification a co-factor or ion associated with the enzyme was being lost. The Lys-AMCase was completely inactivated by 1,10-phenanthroline, further suggesting a metal ion dependence. Addition of the chloride salts of Fe^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+} and Co^{2+} had no effect on the activity of the enzyme. To overcome the possible interference by the chloride ion, the acetate salts of each cation were also tested, again with negative results.

3.5.5 SEPARATION OF LYS-AMCase ACTIVITY FROM DIPEPTIDYL PEPTIDASE ACTIVITY USING ARGININE-SEPHAROSE 4B

The ultimate aim of studying the aminopeptidase was to investigate its activity towards oligopeptides and synthetic aminoacyl derivatives. This requires the separation of the aminopeptidase from other enzymes with peptidase activity. However, separation from other non-peptidase proteins, although desirable, is less important. Since large losses of activity made the task of purifying the Lys-AMCase difficult, it was decided to concentrate on separating the dipeptidyl peptidase and Lys-AMCase activities using the arginine-Sepharose column.

The total pooled material from the Sephacryl S300 column was loaded onto the arginine-Sepharose 4B column (as described in Section 3.2.3) previously equilibrated with 10mM Tris/glycerol pH 7.0. Bound protein was eluted with a linear concentration gradient of 0 - 0.2M NaCl at a flow rate of 0.5ml/min. Two peaks of Lys-AMCase activity eluted from the column, the first being associated with a large A280 peak and thus having a low specific activity (see Fig. 3.23). Fractions containing Lys-AMCase activity from the second peak were pooled and dialysed against 250ml of 10mM Tris/glycerol for 3 - 4 hours to reduce the salt concentration. The column was re-equilibrated with 10mM Tris/glycerol buffer pH 7.0, and the dialysed material loaded. A second elution with a 0 - 0.2M NaCl gradient resulted in complete separation of the Lys-AMCase and dipeptidyl peptidase activities as shown in Table 3.15 a and b.

3.5.6 DIPEPTIDASE AND TRIPEPTIDASE ACTVITY OF PARTIALLY PURIFIED LYS-AMCase

The dipeptidase and tripeptidase activity of the partially purified Lys-AMCase was measured by a semi-quantitative method described in Section 2.2.8 ii). Dipeptidase activity was determined to be 0.19% of that found in the cell-free extract while the Lys-AMCase activity of the partially purified enzyme was 5.85% of the activity found in the cell-free extract. Thus the specific activity of the aminopeptidase had been substantially enhanced when compared with that of the dipeptidase. No tripeptidase activity was detected in the partially purified Lys-AMCase preparation.

3.5.7 SUMMARY OF THE PURIFICATION OF LYS-AMCase FROM *S.lactis* 4760

The partial purification of the Lys-AMCase from *S.lactis* 4760 is summarised in Table 3.15a. The specific activity of the Lys-AMCase after the second arginine-Sepharose column represents a purification of about 25 fold. However the reliability of the specific activity is influenced by the difficulty in accurately measuring the protein concentration at such low levels. The actual value for specific activity may be higher than this. The extent of removal of dipeptidyl peptidase activity from the Lys-AMCase is shown by the data in Table 3.15b. A low pH native PAGE (see Section 2.2.12c) showing the reduction in contaminating bands at each step of the procedure is shown in Fig. 3.24. The activity stained gel (Gel A) shows poor resolution and low intensity since even at the low pH used for running the gel, the majority of the Lys-AMCase was inactivated. It is clearly evident from the gel stained with Coomassie blue (Gel. B) that the partially purified enzyme is contaminated by many other proteins. However, as discussed in Section 3.5.5, removal of contaminating peptidases was of more importance than absolute purity of the enzyme. The contamination of the preparation by peptidases other than the peptidases actually assayed may pose a problem in characterising the activity associated directly with the Lys-AMCase.

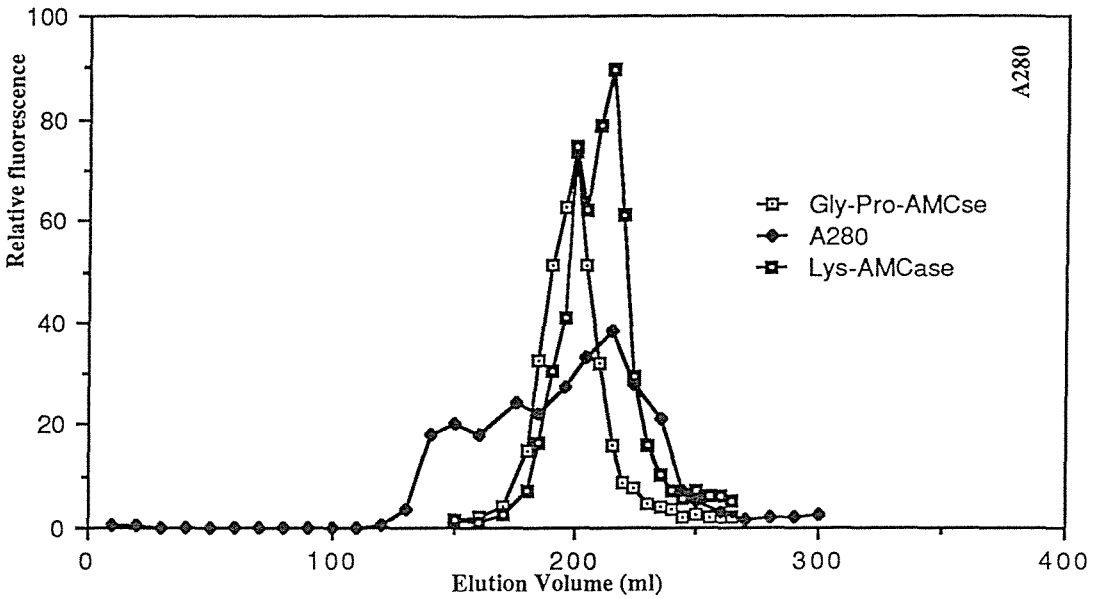


FIG. 3.22 Elution profile of Lys-AMCase and Gly-Pro-AMCase activities from Sephacryl S300 gel filtration column. Elution conditions are as described in Section 3.5.2.

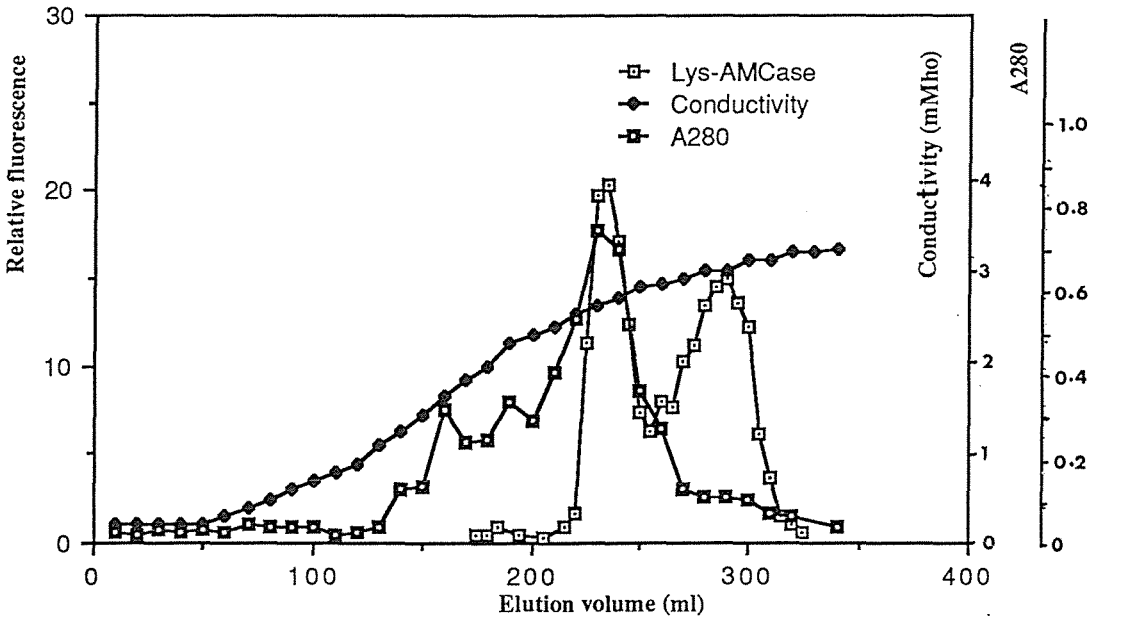


FIG. 3.23 Elution profile of Lys-AMCase activity from Arginine-Sepharose column . Elution conditions are as described in Section 3.5.5.

Purification step	Protein (mg/ml)	Total Protein (mg)	Activity (units/ml)	Specific Activity (units/mg)	Total Activity (units)	Recovery (%)	Fold Purif.
CFE	13	2444	0.118	0.009	22.1	100	0
DEAE	2.25	445	0.075	0.033	14.8	67.0	3.7
PM 30	11.0	126.5	0.924	0.084	10.6	48.0	9.3
S300	4.1	82.0	0.241	0.059	4.8	21.7	6.6
Arg-Seph	0.17	6.63	0.055	0.32	2.15	9.8	35.6
Arg-Seph	0.085	5.53	0.0197	0.23	1.28	5.8	25.6

TABLE 3.15 a) Summary of the purification of the Lys-AMCase from 100g (wet packed weight) of S.lactis 4760. PM 30 refers to the purification achieved after concentration of the sample using a PM 30 ultrafiltration membrane.

Purification step	Protein (mg/ml)	Total protein (mg)	Activity (units/ml)	Specific activity (units/mg)	Total activity (units)
PM 30	11.0	126.5	4.13	0.375	47.5
S300	4.1	82.0	0.59	0.144	11.81
Arg-Seph	0.17	6.63	0.0019	0.011	0.074
Arg-Seph	0.085	5.53	<0.0004	<0.005	<0.026

TABLE 3.15 b) Summary of the elimination of the dipeptidyl peptidase during the purification of the Lys-AMCase. The very low levels of dipeptidyl peptidase activity were barely measurable above the slow baseline hydrolysis of the substrate caused by the excitation wavelength of 385nm.

GEL A *GEL B*

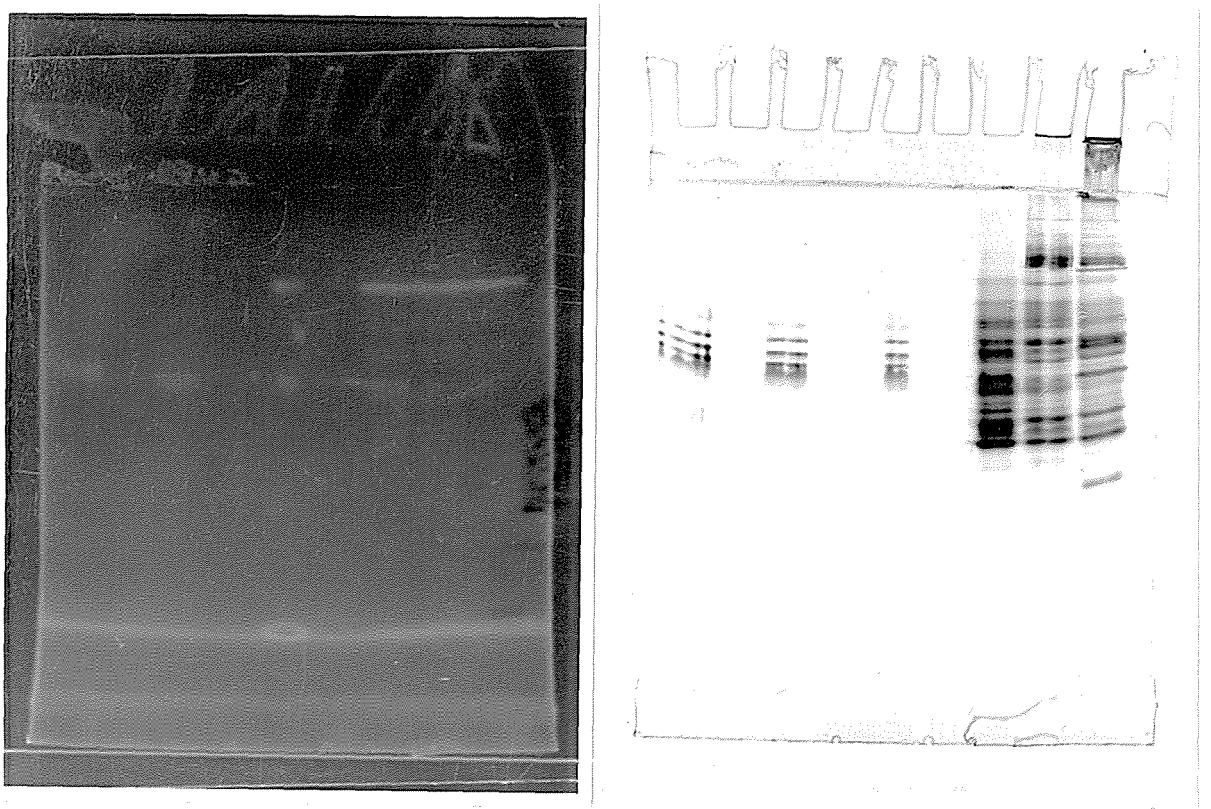


FIG. 3.24 *Low pH native PAGE (resolving gel pH 7.4, stacking gel pH 5.4, as described in Section 2.2.12c) showing the steps in the partial purification of the Lys-AMCase. Gel A shows the relative mobility and intensity of the Lys-AMCase activity stained with 0.5ml of 1mM Lys-AMC (lower fluorescent band) and the contaminating Gly-Pro-AMCase activity stained with 0.5ml of 1mM Gly-Pro-AMC (upper fluorescent band) photographed under ultraviolet light, as outlined in Section 3.2.7. Gel B shows the same native gel stained with Coomassie blue.*

Track	*Identity*
1	*Arginine-Sepharose 2*
2	*Arginine-Sepharose 2*
3	*Arginine-Sepharose 1*
4	*Sephacryl S300*
5	*DEAE Cellulose*
6	*Cell-free extract*

3.6 CHARACTERISATION OF THE LYS-AMCase

3.6.1 MOLECULAR WEIGHT DETERMINATION BY GEL FILTRATION CHOMATOGRAPHY

Native enzyme molecular weight was determined by the position of elution of Lys-AMCase activity from a calibrated Sephacryl S300 gel filtration column as described in Section 3.5.2. The molecular weight of the native Lys-AMCase was determined to be approximately 78 000. The relative elution position of the Lys-AMCase is shown in Fig.3.10. Since the enzyme was not purified to homogeneity, it was not possible to determine the molecular weight of the monomer by SDS PAGE.

3.6.2 EFFECT OF pH ON LYS-AMCase ACTIVITY

To determine the pH profile for the Lys-AMCase, activity was measured at several pH values over the range 4.5 to 8.5 using 100mM MES and Tris buffers. Maximum Lys-AMCase activity was seen at pH 6.5, with a rapid fall off either side of this pH (see Fig. 3.25). This is in contrast with the dipeptidyl peptidase which showed a broad pH optimum with the activity dropping off only below pH 6 and above pH 9. Although the stability at elevated pH was not studied, it became evident that enzyme activity was rapidly and irreversibly lost at pH values much above 7.5. For this reason, pH 7.0 buffer was used throughout the purification procedure.

3.6.3 EFFECT OF CATIONS AND PROTEASE INHIBITORS ON LYS -AMCase ACTIVITY

The effect of a range of different cations and proteinase inhibitors on the activity of the partially purified Lys-AMCase was investigated. Activity was measured with 1mM of each agent.

Of the metal ions, Zn^{2+}, Pb^{2+}, Hg^{2+}, Cu^{2+} and Fe^{2+} had inhibitory effects on activity and no cation studied had any stimulatory effects. The effect of $FeCl_2$ was difficult to establish since (as previously indicated in Section 3.3.4) the initial linear rate began to decrease after a short period of time due to the formation of a yellow precipitate in the reaction mixture.

The Lys-AMCase activity was rapidly and completely inhibited by the metal chelator o-phenanthroline. EDTA (1mM) also showed an inhibitory effect on the enzyme, although it was not as marked as that seen with o-phenanthroline. The sulphydryl protease inhibitors PCMB and iodoacetate gave conflicting results, 1mM PCMB causing almost complete inhibition while iodoacetate gave only slight inhibition. The effect of these inhibitors would have to be validated over a range of concentrations to resolve this contradiction. The serine protease inhibitor PMSF showed a significant inhibitory effect. However, data are insufficient to draw any clear conclusions on the nature of the active site residue involved.

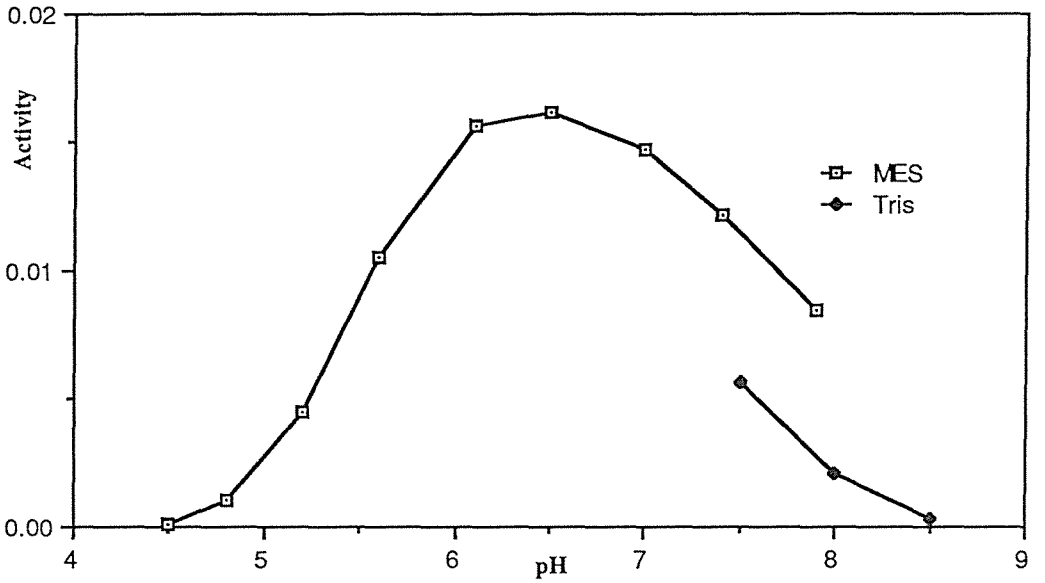


FIG. 3.25 pH profile for Lys-AMCase from S.lactis 4760 using 100mM MES and Tris buffers and 8.5µg protein per assay. Activity is in µmoles/min/ml and 0.71mM Lys-AMC (final concentration in assay mixture) was used per assay.

Agent	Activity with 1mM agent	Activity with 1mM agent % control
Mn^{2+} (Chloride)	0.0092	82.1
Ca^{2+} "	0.0090	80.4
Mg^{2+} "	0.0103	92.0
Co^{2+} "	0.0095	84.8
Zn^{2+} "	0.0036	32.1
Fe^{2+} "	0.0055	49.1
Cu^{2+} "	0	0
Pb^{2+} (Nitrate)	0	0
Hg^{2+} "	0	0
o-Phenanthroline	0	0
Iodo acetate	0.0092	82.1
EDTA	0.0048	42.8
PCMB	0.00095	8.5
PMSF	0.0027	24.1

TABLE 3.16 *Effect of 1mM cations and other agents on the Lys-AMCase activity. Activity is expressed as a % relative to the activity of the Lys-AMCase using 0.71mM Lys-AMC substrate at pH 6.8 without any additional agent. Each assay was performed with 8.5µg protein.*

3.6.4 KINETIC PARAMETERS OF THE LYS-AMCase FROM *S.lactis* 4760

The rate of hydrolysis of several aminoacyl AMC derivatives over a concentration range from 5μM to 100μM was assayed to determine the Km values for the enzyme with each substrate. The k_{cat} was not determined since the true concentration of the Lys-AMCase in its partially purified state was unknown. The results are shown in Table 3.17 and examples of rate verses substrate concentration and Lineweaver-Burk plots are shown in Fig. 3.26.a, b and c. Lys-AMC and Leu-AMC were prepared by the method described in Section 2.2.7. Arg-AMC, Gly-AMC, Ala-AMC, Phe-AMC, Tyr-AMC and Ser-AMC were obtained from Sigma. No activity was found with Gly-AMC, Ala-AMC, Phe-AMC, Ser-AMC and Tyr-AMC as substrates (Tyr-AMC was only slightly soluble in water making a quantitative measurement difficult). Lys-AMC and Arg-AMC were hydrolysed readily, giving Km values of 147 and 114 μM respectively, and V_{max} results of 0.0104 and 0.0063 μmoles/min/ml. The enzyme had a somewhat lower Km of 50 μM for Leu-AMC and a very much lower V_{max} of 0.00089μmoles/min/ml.

To confirm this specificity of substrate hydrolysis by the Lys-AMCase, the relative rates of hydrolysis of four p-nitroanalide derivatives were measured. No hydrolysis was seen with Ala-pNA and Pro-pNA. A rate of 0.00093μmoles/min/ml was determined using 1mM leu-pNA, compared with 0.0076μmoles/min/ml for Lys-pNA. This represents approximately an 8 fold difference for Lys-pNA and Leu-pNA, similar to the relative V_{max} values obtained with Lys-AMC and Leu-AMC shown in Table 3.17.

The rate of hydrolysis of Lys-pNA was determined at several different concentrations to determine the kinetic parameters (Fig. 3.27). A Km value of 424.8 μM and a V_{max} value of 0.0106 μmoles/min/ml were obtained. The Km using the pNA substrate is higher than that for the corresponding AMC substrate while the V_{max} is exactly the same.

Substrate	Km (std err.) (μM)	V_{max} (std err.) (μmoles/min/ml)
Lys-AMC	147 (14)	0.0104 (0.0004)
Arg-AMC	114 (28)	0.0063 (0.0009)
Leu-AMC	50 (8.0)	0.00089 (0.00007)
Lys-pNA	424.5 (50.6)	0.0106 (0.0006)

TABLE 3.17 *Kinetic parameters obtained from measuring the rates of hydrolysis of X-AMC and Lys-pNA substrates by the partially purified Lys-AMCase from S.lactis 4760. Each assay,using AMC substrates, contained 8.5μg of protein in a total volume of 2.1ml. Assays were carried out in 100mM MES buffer at pH 6.8 using AMC substrate concentrations covering the range 5 - 100μM. The purity of each AMC substrate (Section 2.2.7) was taken into account in the calculation of substrate concentration. Assays using Lys-pNA substrate were carried out with 8.5 μg of protein in a total volume of 3.0ml using 100mM MES buffer at pH 6.8. Lys-pNA substrate concentrations covered the range 20 - 1000μM.*

3.6.5 INVESTIGATION OF THE ACTION OF THE LYS-AMCase ON PEPTIDES

To establish whether the Lys-AMCase was a true aminopeptidase and to investigate further its apparent specificities, the activity of the Lys-AMCase towards different peptides was investigated. The peptides were incubated with the partially purified Lys-AMCase and samples removed after various times and analysed using reverse phase HPLC for possible hydrolysis products. The ability of the Lys-AMCase to catalyse hydrolysis of the following peptides was tested:

Leu-Gly-Gly. No hydrolysis occurred after a 24 hour incubation period.

Gly-Pro-Gly-Gly. No hydrolysis occurred after a 24 hour incubation period.

β-casomorphin (Tyr-Pro-Phe-Pro-Gly-Pro-Ile). Since the studies with amino acyl-AMC derivatives had shown that the enzyme was specific for N-terminal lysine, arginine and leucine residues, no hydrolysis was expected. After overnight incubation however, it was found that limited hydrolysis of the peptide had occurred (shown in Fig. 3.28). Amino acid composition analysis of the peaks revealed that the sequence Tyr-Pro-Phe-Pro-Gly-Pro-Ile had been split, resulting in a tetrapeptide Tyr-Pro-Phe-Pro, and a tripeptide Gly-Pro-Ile product. This suggests that endopeptidase activity is present in the Lys-AMCase preparation.

Bradykinin and [Lys[1]]-bradykinin (Arg-(or Lys-)Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg). After incubation, two hydrolysis products were found (Fig. 3.28). One of these was Arg-(Lys-) Pro-Pro-Gly-Phe, and the other was Ser-Pro-Phe-Arg. Thus endopeptidase activity was again exhibited, hydrolysis occurring between the phenylalanine and the serine. On the other hand, the N-terminal lysine or arginine was not removed by hydrolysis raising the possibility that the Lys-AMCase was not an exopeptidase. However presence of the penultimate proline residue may impose a constraint on the ability of the Lys-AMCase to hydrolyse the Lys-Pro or Arg-Pro bond.

Fragment 90 - 95 of α-casein (Arg-Lys-Asp-Val-Tyr). After incubation with the Lys-AMCase, only a single peptidase peak was found, but this eluted at a lower acetonitrile concentration (Fig. 3.29). To confirm that this change corresponded with a change in peptide composition the digested sample was spiked with undigested pentapeptide. Two peaks resulted, clearly indicating that the original pentapeptide had been hydrolysed by

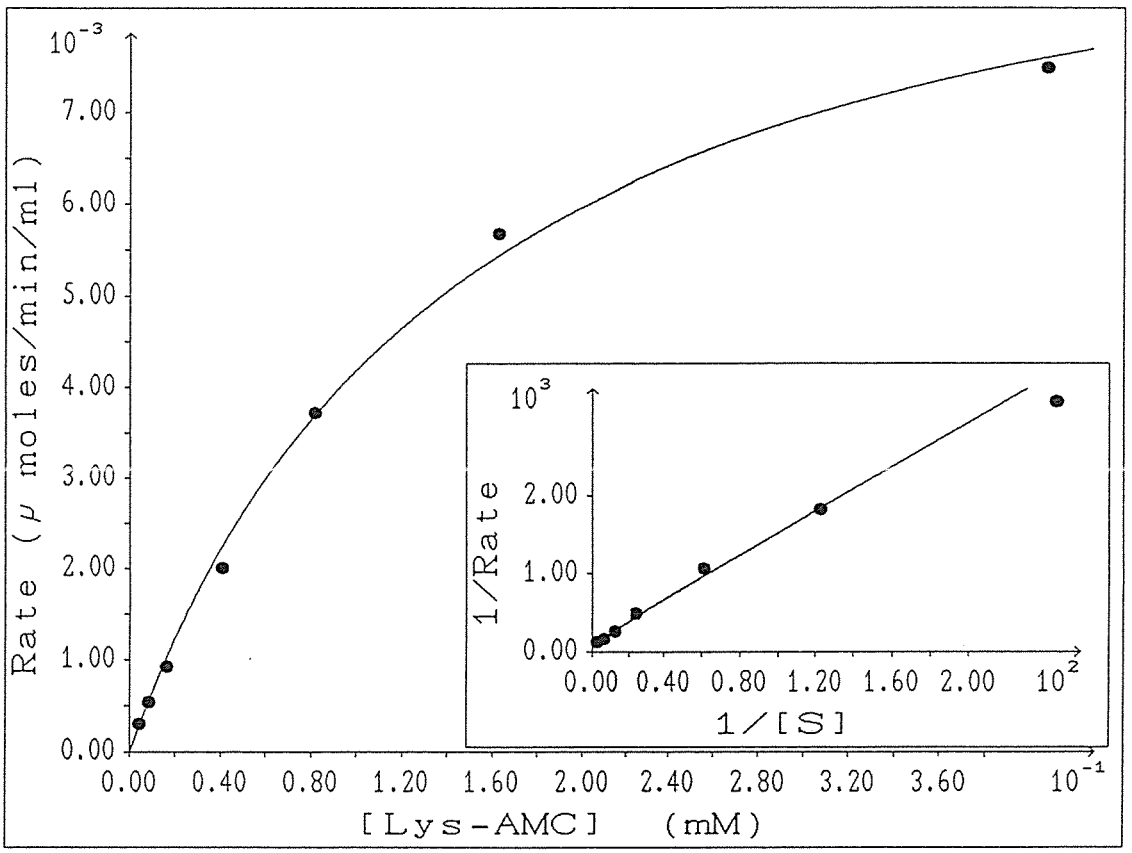
the Lys-AMCase. To establish the identity of the hydrolysis product, the peak was collected, and its composition determined by amino acid analysis. The results clearly showed that the product was comprised of the tripeptide Asp-Val-Tyr. Arginine and lysine were not detected as separate peaks since they would have a negligible absorbance at 220nm. Therefore, the N-terminal arginine and lysine residues had been removed by the Lys-AMCase, confirming that in this case it can act as a true aminopeptidase.

Fragment 32 - 36 of Thymopoietin (Arg-Tyr-Leu-Gly-Tyr-Leu) The results of digestion of this hexapeptide are shown in Fig. 3.29b. After incubation for 5 hours, a sample analysed by HPLC showed the digest to contain three compounds absorbing at 220nm. The amino acid composition of these compounds was determined as before. The largest product of digestion (peak I) had the composition Tyr-Leu-Gly-Tyr-Leu, the N-terminal arginine having been rapidly and completely removed by the Lys-AMCase. Peak J in Fig.3.29b, was found to consist of glycine, tyrosine and leucine, and peak K, just tyrosine. Thus as well as its amino peptidase activity, the Lys-AMCase preparation exhibited endopeptidase activity. Two alternative cleavage sites within the peptide may account for these results. Cleavage may have occurred between the leucine and glycine resulting in the tripeptide Gly-Tyr-Leu and the dipeptide Tyr-Leu, the latter being broken down, or cleavage may have occurred between the glycine and tyrosine yielding the tripeptide Tyr-Leu-Gly and the C-terminal dipeptide Tyr-Leu. Unfortunately time did not permit sequencing of the tripeptide.

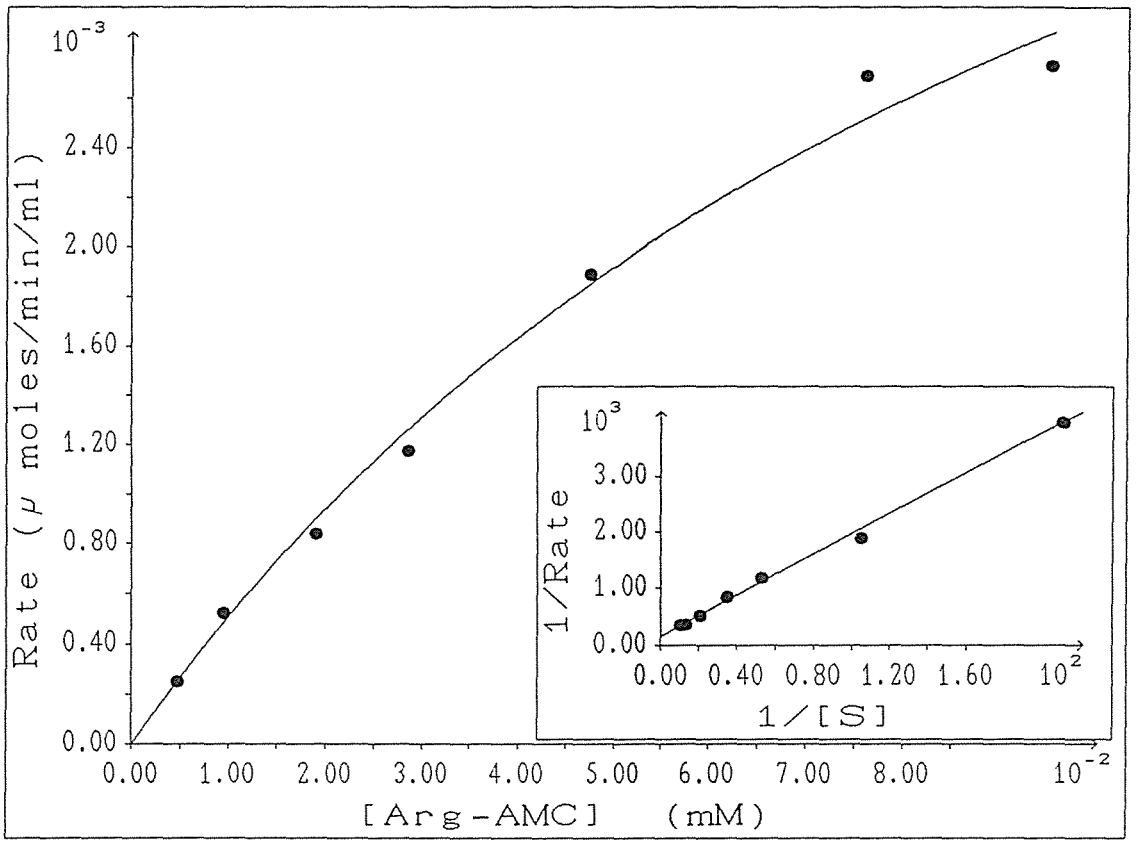
The hydrolysis products found with the last two of the above substrates shows that the Lys-AMCase does act as a true aminopeptidase with specificity towards basic amino acids, consistent with the the results obtained with aminoacyl-AMC and pNA substrates. Its peptidase activity towards leucine as the N-terminal residue has not been demonstrated. The cleavage of peptides within a sequence suggests that either:
a) the Lys-AMCase has endopeptidase activity itself or
b) the Lys-AMCase is contaminated with a separate endopeptidase.
The second of these two options seems more likely as substantial contamination of the Lys-AMCase preparation was evident from the PAGE analysis of the partially purified enzyme (Fig. 3.24).

Since it has now been established that the Lys-AMCase acts as a true aminopeptidase, it will subsequently be referred to as the Lys-aminopeptidase.

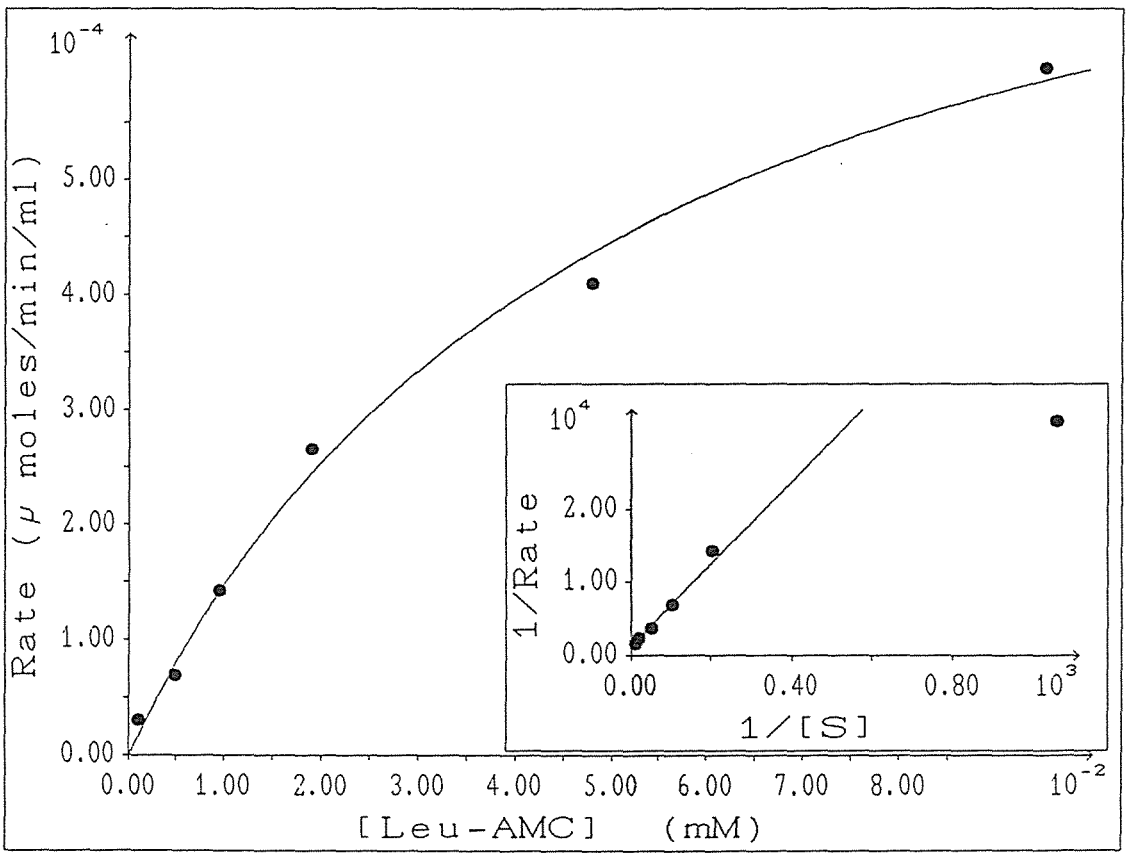
FIG. 3.26 *Rate verses substrate concentration plots and Lineweaver-Burk plots for the Lys-AMCase from S.lactis 4760, using the substrates a) Lys-AMC , b) Arg-AMC and c) Leu-AMC, Each assay contained 8.5µg of protein in a total volume of 2.1ml. Assays were carried out in 100mM MES buffer, at pH 6.8 using substrate concentrations covering the range 5 - 100µM.*



a


Rate (μ moles/min/ml)
10^{-3}
2.40
2.00
1.60
1.20
0.80
0.40
0.00
0.00 1.00 2.00 3.00 4.00 5.00 6.00 7.00 8.00
10^{-2}
[Arg-AMC] (mM)
1/Rate
10^{3}
3.00
2.00
1.00
0.00
0.00 0.40 0.80 1.20 1.60
10^{2}
1/[S]

b


Rate (μ moles/min/ml)
10^{-4}
5.00
4.00
3.00
2.00
1.00
0.00
0.00 1.00 2.00 3.00 4.00 5.00 6.00 7.00 8.00
10^{-2}
[Leu-AMC] (mM)
1/Rate
10^{4}
2.00
1.00
0.00
0.00 0.40 0.80
10^{3}
1/[S]

c

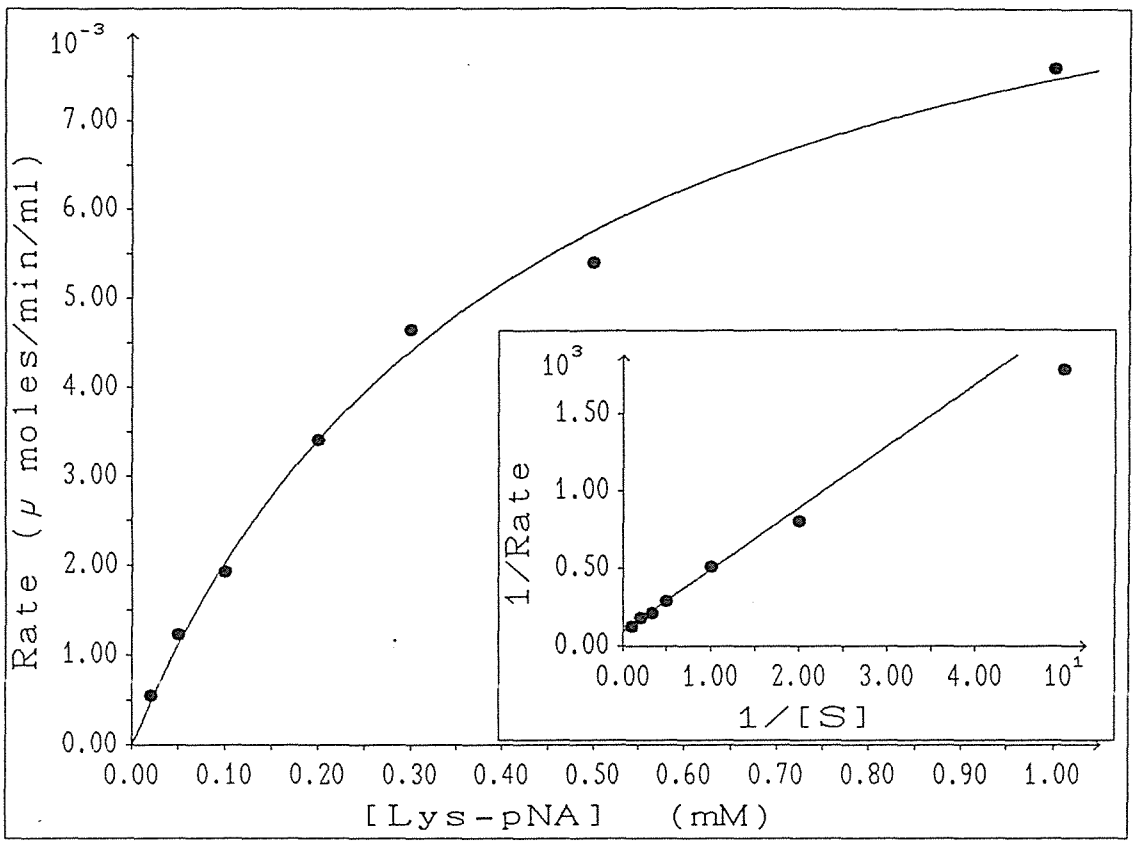


FIG. 3.27 *Rate verses substrate concentration plots and Lineweaver-Burk plots for the Lys-AMCase from S.lactis 4760, using the substrate Lys-pNA. Each assay contained 17.0μg of protein in a total volume of 3.0ml. Assays were carried out in 100mM MES buffer, at pH 6.8 using substrate concentrations covering the range 20 - 1000μM, and activity was detected by the release of p-NA measured at 405nm.*

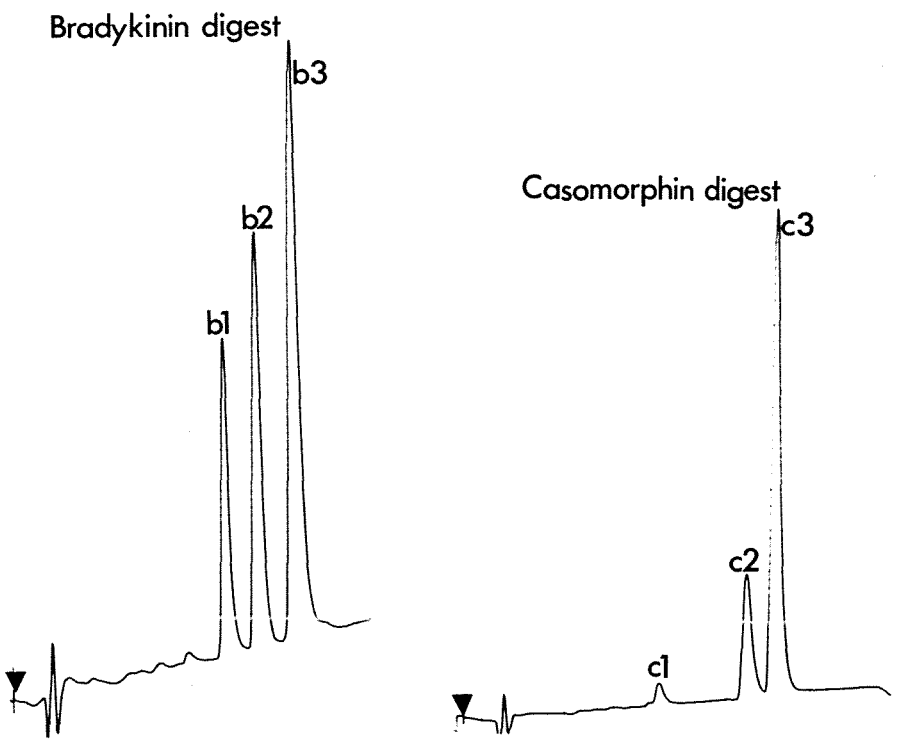


FIG. 3.28 *Result of incubation of a) β-casomorphin and b) [Lys[1]]-bradykinin with Lys-AMCase. Each peptide (1mg/ml)was incubated with 200µl of enzyme for a 24 hour period and the products of digestion separated by reverse phase HPLC with a C18 column. Peptides were eluted with a linear gradient of 0% B to 60% B over 30 minutes at a flow rate of 1ml/min. Buffer A contained 5% acetonitrile and 0.1% TFA, and buffer B contained 80% acetonitrile and 0.1% TFA. Peptides were detected at 220nm.*

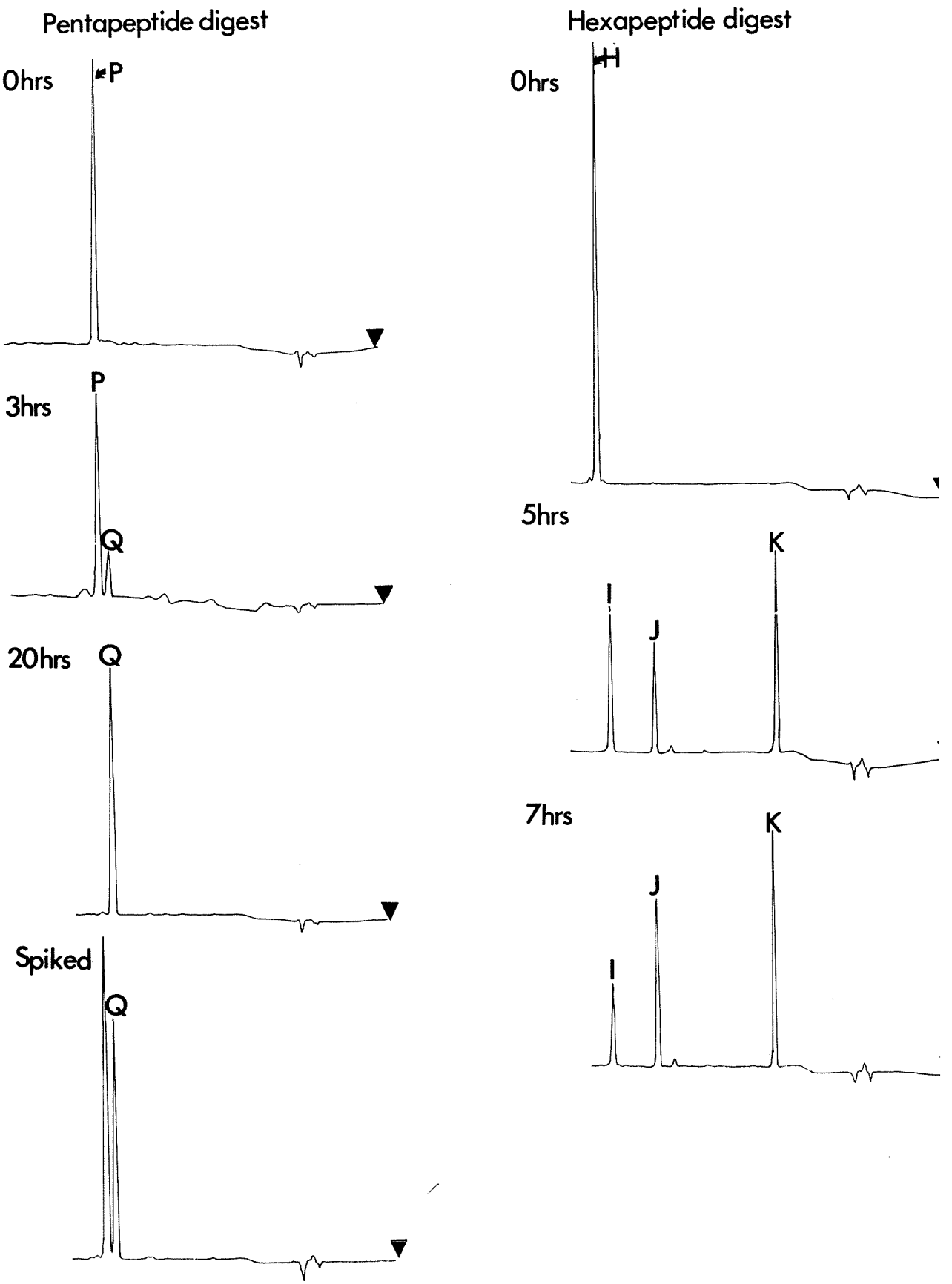
Identity of peaks	*[Lys[1]]-bradykinin*	*β-casomorphin*
	b1=Ser-Pro-Phe-Arg	*c1=Gly-Pro-Ile*
	b2=Lys-Pro-Pro-Gly-Phe	*c2=Tyr-Pro-Phe-Pro*
	b3= bradykinin	*c3=β-casomorphin*

FIG. 3.29. a) Result of incubation of the pentapeptide Arg-Lys-Asp-Val-Tyr with the Lys-AMCase. 200µl of 1mg/ml pentapeptide was incubated with 20µl of the enzyme preparation (buffered at pH 7.0). Peptides were eluted from a C18 HPLC column with a linear gradient of 0% B to 40% B over 30 minutes at a flow rate of 0.5ml /min.

***b)** Result of incubation of the hexapeptide Arg-Tyr-Leu-Gly-Tyr-Leu with the Lys-AMCase. 200µl of 1mg/ml hexapeptide was incubated with 20µl of the enzyme preparation (buffered at pH 7.0). Peptides were eluted with a linear gradient of 0% B to 70% B over 30 minutes at a flow rate of 0.5ml/min.*

Buffer A contained 5% acetonitrile and 0.1% TFA, buffer B contained 80% acetonitrile and 0.1% TFA.

Identity of peaks	*a)Pentapeptide*	*b) Hexapeptide*
	Q= Asp-Val-Tyr	*K= Tyr*
	P= Pentapeptide	*J= Gly-Tyr-Leu*
		I= Tyr-Leu-Gly-Tyr-Leu
		H= Hexapeptide


Pentapeptide digest
0hrs
P
3hrs
P
Q
20hrs
Q
Spiked
Q
Hexapeptide digest
0hrs
H
5hrs
I
J
K
7hrs
I
J
K

3.7 ACTION OF PEPTIDASES ON A PROTEINASE-GENERATED FRAGMENT (176 - 182) FROM β-CASEIN - (PEPTIDE B)

Previous sections describe the action of dipeptidyl peptidase and Lys-aminopeptidase on a variety of aminoacyl-AMC and -pNA derivatives, and these enzymes have been shown to have peptidase activity towards a variety of short peptides. A further aim of this study was to investigate the possible role of these peptidases in the degradation of proteinase-generated β-casein peptides.

β-casein has a high proportion of proline residues and several of the peptides reported to be produced by proteinase action (Fig. 1.2) are potential substrates for the dipeptidyl peptidase acting in conjunction with an aminopeptidase to expose the sequence X-Pro- at the N-terminus of a peptide.

The β-casein-derived substrate chosen for study was fragment 176 - 182, with the sequence Lys-Ala-Val-Pro-Tyr-Pro-Gln. This peptide is generated by incubation of β-casein with the cell wall-associated proteinase from *S.lactis* 4760 (Ng, MSc. thesis 1988), and is relatively easily separated from contaminating peptides using reverse phase HPLC and a C18 column.

3.7.1 GENERATION OF β-CASEIN DERIVED PEPTIDES BY THE ACTION OF THE CELL WALL-ASSOCIATED PROTEINASE

To generate β-casein-derived peptides, a cell wall-associated proteinase was partially purified from 3x1litre cultures of *S.lactis* 4760 as described in Section 2.2.5. No Lys-aminopeptidase activity was detected in the partially purified enzyme preparation.

A solution of β-casein (10mg/ml) was incubated with proteinase (0.5ml of the proteinase preparation per ml of β-casein solution) for 22 hours at room temperature. To remove undigested β-casein, and the proteinase enzyme, the incubation mixture was filtered using a Millipore Centrifugal Ultrafree polysulphone membrane filter, with a 10 000 molecular weight exclusion.

The filtered peptide mixture was separated by reverse phase HPLC using a C18 column, giving a reproducible pattern shown in Fig. 3.30. The peptides designated B and D in Fig. 3.30 were collected and sequenced as described in Section 2.2.13. Peptide B was

shown to have the sequence Lys-Ala-Val-Pro-Tyr-Pro-Gln, with no major contaminants. Peptide D, although fractionating as a single peak on the C18 column, proved to be a mixture of peptides. Sequence analysis suggested that the constituents of this peak were peptides 183 - 193, 194 - 209 and 133 - 141 all of which were found in a recent study of cell wall proteinase cleavage of β-casein (Monet *et al.*, 1989).

3.7.2 ACTION OF PEPTIDASES ON PEPTIDE B

Peptide B was re-dissolved in 200µl of deionised water and incubated with 20 µl of Lys-aminopeptidase. The time course showing the digestion of peptide B by this enzyme is shown in Fig. 3.31a. The products of digestion (peaks 1, 2 and 4) were collected and sequenced. Peak 1 was found to have the sequence Val-Pro-Tyr-Pro-Gln, the N-terminal lysine and alanine having been removed by the Lys-aminopeptidase. Removal of the lysine was anticipated from the known specificity of the enzyme. However, the removal of the alanine was an unexpected result in light of the findings with Ala-pNA and Ala-AMC, since no hydrolysis occurred with either of these two substrates. Peak 2 was shown to have the sequence Tyr-Pro-Gln, suggesting that the Lys-aminopeptidase preparation possessed endopeptidase activity, cleaving between the proline and tyrosine. Peak 3 was not collected due to its small size. In hindsight, its size may have been due to its lack of tyrosine, resulting in a low absorbance of the peptide at 220nm. Its amino acid composition may have been significant in interpreting the cleavage pattern. After 24 hours, a fourth peak (Peak 4) was evident, identified as the dipeptide Tyr-Pro. This again is an unexpected result as the dipeptide must have arisen by the removal of the C-terminal glutamine from the tripeptide Tyr-Pro-Gln.

Fig. 3.31b shows a time course digestion of peptide B with a combination of the Lys-aminopeptidase and dipeptidyl peptidase. After 2 hours of incubation two products were evident, peak 1 with the sequence Val-Pro-Tyr-Pro-Gln, and peak 4 with the sequence Tyr-Pro. However, after longer periods of incubation, peak 1 did not increase (and remained a very minor product), whereas peak 4 increased greatly. The very small quantity of the pentapeptide product (Val-Pro-Tyr-Pro-Gln) in this digestion, (in contrast with that with the Lys-aminopeptidase alone), can be accounted for by the action of the dipeptidyl peptidase on the pentapeptide. The fact that only the dipeptide Tyr-Pro was detected is due to the high absorbance of tyrosine at 220nm. The other expected dipeptide, Val-Pro, may not have been detected.

When peptide B was digested with the dipeptidyl peptidase alone (as shown in Fig. 3.32), peak 4 was again the main product. This suggests that the dipeptidyl peptidase

must be able to remove the N-terminal Lys-Ala dipeptide without the aid of the Lys-aminopeptidase, thus degrading the hexapeptide, presumably to Lys-Ala, Val-Pro, Tyr-Pro and glutamine. It was noted in Section 3.3.5 that Gly-Ala-AMC was hydrolysed at a slow rate relative to the X-Pro-AMC substrates. This activity towards X-alanine must therefore be responsible for removal of the N-terminal Lys-Ala from peptide B.

3.7.3 ACTION OF PEPTIDASES ON "PEPTIDE D"

"Peptide D" was shown to contain a mixture of peptides (Section 3.7.1). This mixture was incubated with the Lys-aminopeptidase and a combination of Lys-aminopeptidase and dipeptidyl peptidase for periods of up to 72 hours. During this time "peptide D" was completely resistant to hydrolysis by these two enzymes, singly or in combination. Furthermore, incubation of "peptide D" with cell-free extract from *S.lactis* 4760 was tried, again without any detectable change in the elution profile from the C 18 column. Further purification of the components of "peptide D" and the study of their apparent resistance to digestion by peptidases would be of considerable interest.

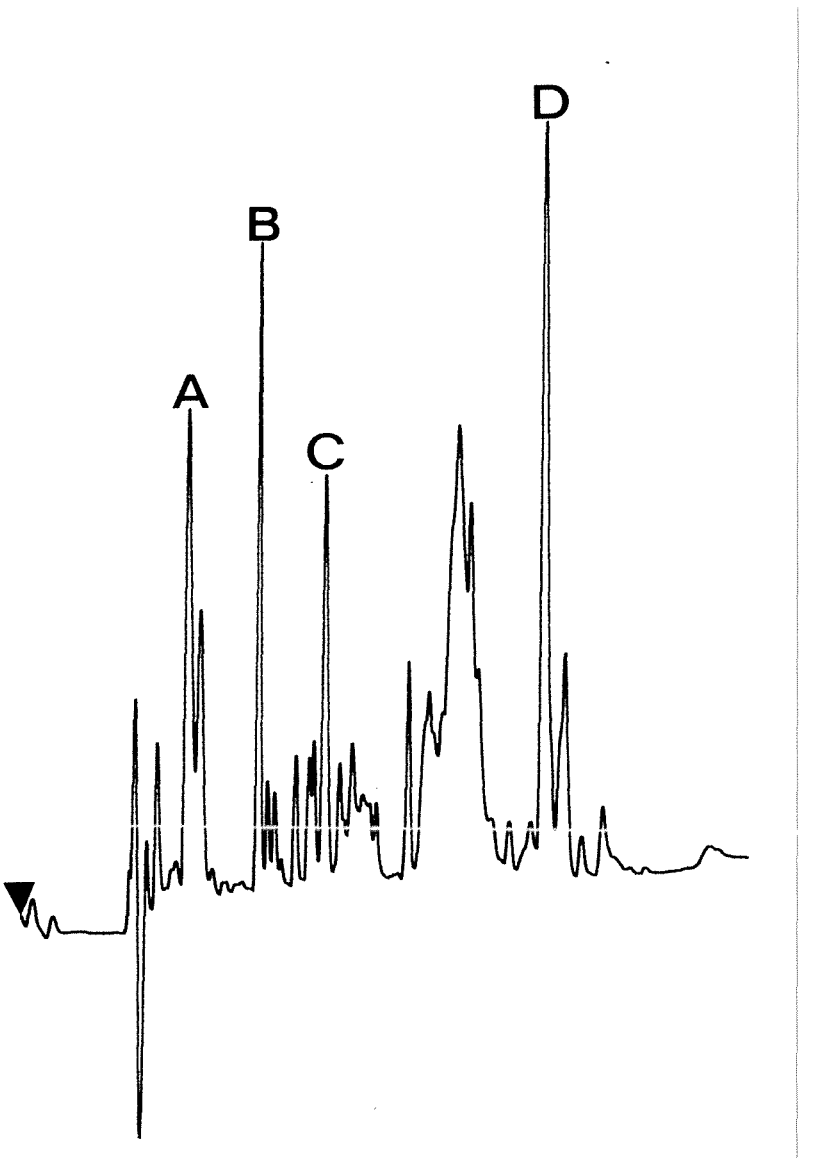


FIG. 3.30 *Reverse phase HPLC separation of proteinase-generated peptides using a C18 column. The peptides were produced by a 22 hour digestion of β-casein with the partially purified proteinase and were separated from undigested β-casein and proteinase enzyme using a Millipore centrifugal filter as described in the text. The peptides were eluted with a linear gradient of 5% B to 80% B over 1 hour. Buffer A contained 100% deionised water with 0.1% TFA, and buffer B contained 100% acetonitrile with 0.1% TFA. 200μl of an incubation mixture originally containing 10mg of β-casein and 0.5ml of proteinase preparation in a total volume of 1.5ml, was injected onto the column with the detector set at minimum sensitivity at 220nm.*

FIG.3.31 *a) Result of incubation of Peptide B with the Lys-AMCase. Peptides were eluted with a linear gradient of 5% B to 40% B over 40 minutes at a flow rate of 0.5ml /min.*

b) Result of incubation of Peptide B with equal ratios of the Lys-AMCase and dipeptidyl peptidase. The same elution procedure was used.

Buffer A contained 100% deionised water with 0.1% TFA, buffer B contained 100% acetonitrile with 0.1% TFA.

Identity of peaks

B=Lys-Ala-Val-Pro-Tyr-Pro-Gln (peptide B)

1=Val-Pro-Tyr-Pro-Gln

2=Tyr-Pro-Gln

4=Tyr-Pro

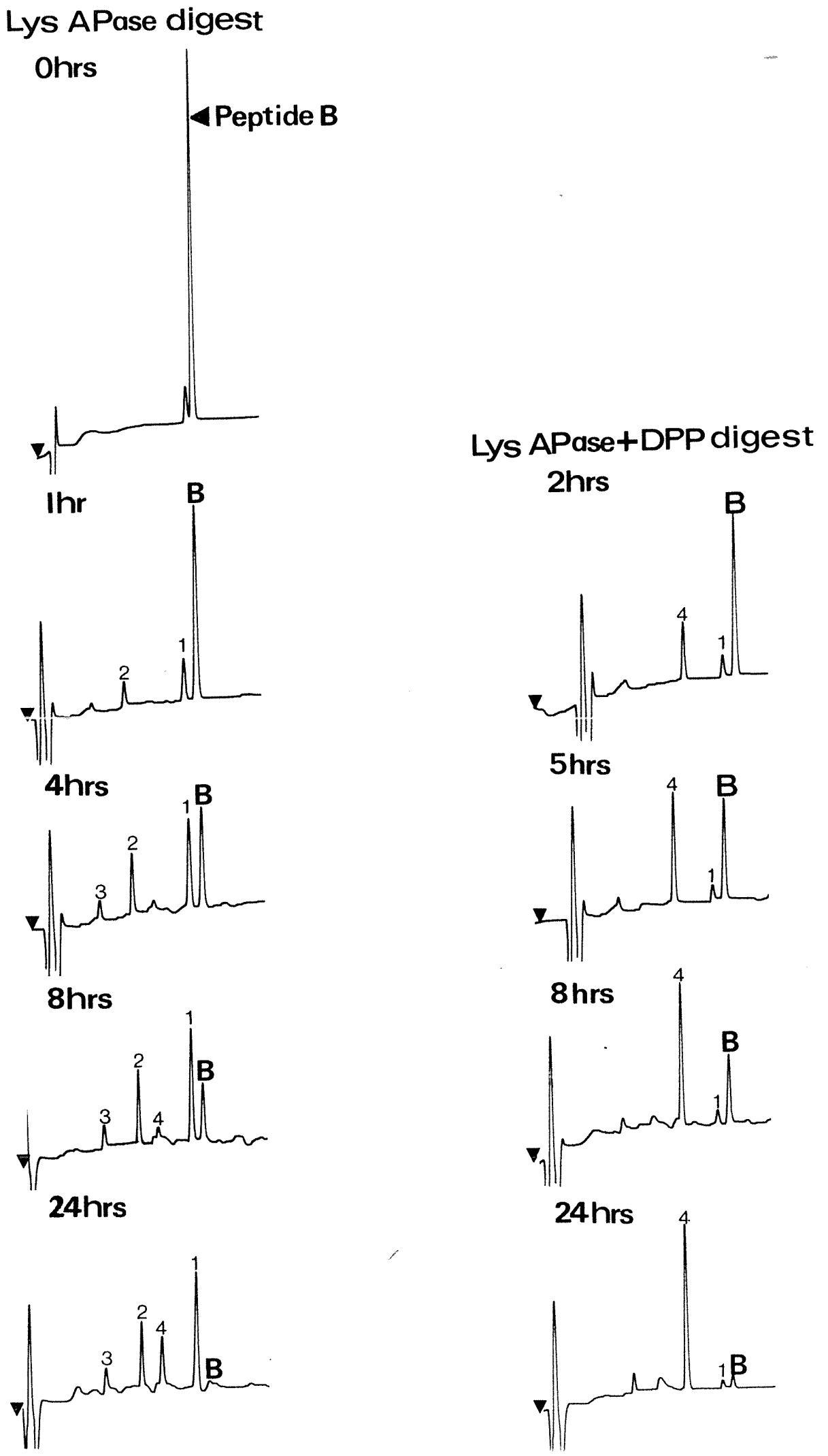
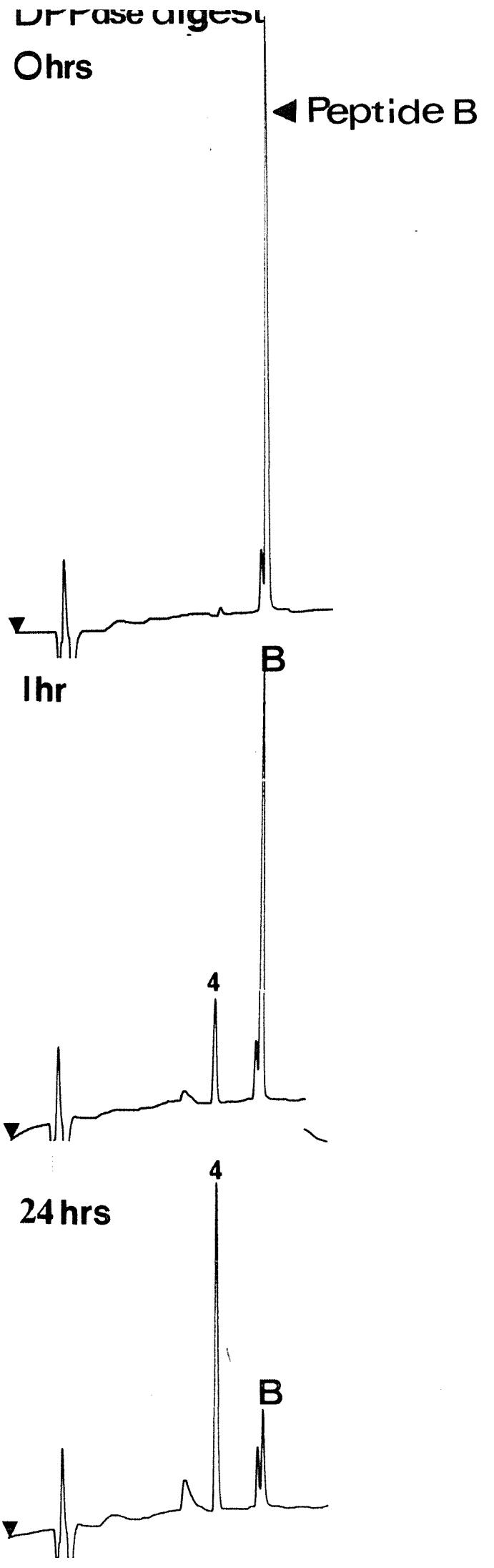

Lys APase digest
0hrs
Peptide B
1hr
B
1
2
4hrs
B
1
2
3
8hrs
1
B
2
3
4
24hrs
1
2
4
3
B
Lys APase+DPP digest
2hrs
B
4
1
5hrs
4
B
1
8hrs
4
B
1
24hrs
4
1
B

FIG. 3.32. *Result of incubation of peptide B with purified dipeptidyl peptidase. Peptides were eluted with a linear gradient of 5% B to 40% B over 40 minutes at a flow rate of 0.5ml/min.*

Identity of peaks
B=peptide B
4=Tyr-Pro


DPPase digest
Ohrs
Peptide B
B
1hr
4
4
24 hrs
B

CHAPTER 4 - DISCUSSION

The progress in research on the proteolytic system of the lactic streptococci has advanced rapidly in the past decade. The importance of the proteolytic system both for starter growth and for the production of cheese with desirable flavour and texture has provided the impetus for further work in this area. During the course of preparing this thesis, several reports on work of direct relevance to the aims of this investigation have been published. In the present study, a dipeptidyl peptidase has been purified, and its ability to hydrolyse X-Pro-AMC substrates and proline-containing peptides has been investigated. A Lys-aminopeptidase has been partially purified, and its specificity towards a range of aminoacyl-AMC and peptide substrates has been investigated. The results obtained are discussed in light of published findings.

4.1 THE DIPEPTIDYL PEPTIDASE

4.1.1 PURIFICATION OF THE X-PRO DIPEPTIDYL PEPTIDASE

A homogeneous protein with X-Pro-AMCase activity was obtained after purification by the method described in Section 3.2, adapted from the method of Meyer and Jordi (1987). The most significant step in the procedure was the use of arginine-Sepharose 4B. Meyer and Jordi refer to this step as affinity elution, and several groups including Gonschor and Schafer (1985) who have purified similar enzymes from mammalian systems, also refer to "affinity chromatography on arginine-Sepharose 4B". Pharmacia claim that arginine-Sepharose 4B has been used to isolate or remove a number of different proteins from a wide range of starting materials. Typically, affinity has been demonstrated for enzymes which contain serine at their active sites and which cleave proteins at the carboxyl group of an arginine residue. Applications reported include the isolation of prekallikrein, clostripain, plasminogen activator and prothrombin.

Section 2.2.8 describes the synthesis of arginine-Sepharose by carbodiimidazole activation using a γ-amino-caproic acid-derived spacer. The separation properties of this resin were similar to those of the Pharmacia resin, although the method of preparation was different. Earlier attempts to synthesise a resin identical to the Pharmacia product (in which the arginine is coupled via a 1,4-butanediol diglycidyl ether-derived spacer) resulted in a resin that had a poor binding capacity and poor affinity, the dipeptidyl peptidase eluting at a low NaCl concentration. It is possible that the resins produced by

the two different methods might have different pKa values for their α-amino groups coupled to their respective spacers. However, since the commercial Pharmacia resin bound the dipeptidyl peptidase this seems an unlikely explanation for the failure of the poor capacity resin. A more probable explanation is that the coupling of arginine to the 1,4-butanediol diglycidyl ether-derived spacer was unsuccessful.

A much more effective purification of the dipeptidyl peptidase was obtained at pH 7.5 rather than pH 7.0 (the pH used by Meyer and Jordi, 1987). Arginine has an α-amino group pKa of 8.99. Since the γ-amino-caproic acid spacer acts as an electron-withdrawing group, the pKa of the arginine's α amino group may be expected to be lowered by 1.0 to 1.5 pH units to 7.5 to 8.0. This may explain the more effective separation of the dipeptidyl peptidase at pH 7.5 if the loss of positive charge on the α amino group is important for binding. This also might suggest that the "affinity" of the dipeptidyl peptidase for the ligand is a subtle form of ion exchange rather than true affinity chromatography.

Gly-Pro-AH-Sepharose has been used by Meyer and Jordi (1987) and Gonschor and Schafer (1985), as an affinity resin for purification of an X-Pro-dipeptidyl peptidase. Gly-Pro-AH-Sepharose resin was synthesised in the present study, but did not give any improvement in specific activity. This may have been a result of poor substitution during the synthesis of the resin. In any case, use of this resin proved unnecessary in the purification procedure described in this thesis. Both groups refer to the binding of enzymes to this column as affinity chromatography, although, in the case of the purification procedure used by Gonschor and Schafer, the desired dipeptidyl peptidase IV did not even bind to the column. The enzyme was eluted in the equilibration buffer before the application of a NaCl gradient to remove bound protein. Thus again, a procedure has been referred to as affinity chromatography when apparently this is not the case.

An X-Pro dipeptidyl peptidase has been purified from *S.cremoris* by Kiefer-Partsch *et al.*, (1989) using anion exchange chromatography (Q-Sepharose column), chromatofocusing (Mono-P HR5/20 column) and finally, anion exchange chromatography (Mono-Q HR 5/5 column). However, although they achieved a 300 fold enhancement of dipeptidyl peptidase activity, their recovery was only 7% (compared with 20% for the method described in this thesis). Kiefer-Partsch *et al.* used, as a source of their enzyme, the supernatant resulting from incubation of cells in Ca^{2+}-free buffer on the assumption that the enzyme was localised in the cell wall. However, results obtained in this laboratory (G.G.Pritchard, personal communication) show that release of peptidase is proportional to the leakage of the intracellular marker enzyme, lactate dehydrogenase,

indicating that the dipeptidyl peptidase is intracellular. In the purification procedure described by Kiefer-Partsch *et al.*, 20g (wet packed weight) of cells yielded 0.04mg of purified dipeptidyl peptidase. The method described in this thesis results in a yield of about 5mg from 100g (wet packed weight) of cells. This represents a 25-fold greater yield of peptidase from the same weight of cells.

4.1.2 GENERAL PROPERTIES OF THE X-PRO DIPEPTIDYL PEPTIDASE

An X-Pro dipeptidyl peptidase has been purified from several bacterial sources. Meyer and Jordi (1987) isolated a dipeptidyl peptidase from *Streptococcus thermophilus* and *Lactobacillus lactis*, and Kiefer-Partsch *et al.* (1989) purified a similar enzyme from *S.cremoris*. A consistent feature of the *S.cremoris* and *S.thermophilus* enzymes is a broad pH optimum between pH 6.5 and 8.0 with Gly-Pro-AMC as substrate. This was also seen with the dipeptidyl peptidase from *S.lactis* (Section 3.3.3), however the pH optimum in this case was even broader, spanning the range pH 6.0 to 9.0. The dipeptidyl peptidase purified from *Lactobacillus lactis* showed a much narrower pH optimum. Meyer and Jordi point out "a broad pH optimum has been said to be a characteristic of enzymes of bacterial origin, but to us it seems rather to be a characteristic for enzymes from streptococci". The findings reported here support this statement.

In a study of the effects of metal ions and other reagents on the activity of the X-Pro dipeptidyl peptidase, Meyer and Jordi found that the enzymes purified from *S.thermophilus* and *L.lactis* were unaffected by Ca^{2+}, Mg^{2+}, Zn^{2+}, Se^{2+}, and Cd^{2+}. Co^{2+} and Fe^{2+} had some inhibitory effect, whereas Hg^{2+} totally inhibited both enzymes. Cu^{2+} and Pb^{2+} inhibited the *Lactobacillus* enzyme, but not the *S.thermophilus* enzyme. Furthermore the dipeptidyl peptidase from *S.thermophilus* was totally inhibited by PMSF and DFP (diisopropyl fluoro phosphate) and slightly inhibited by EDTA, o-phenanthroline, iodoacetic acid and PCMB (p-chloromercuribenzoate). The dipeptidyl peptidase purified from *Lactobacillus* was not inhibited by o-phenanthroline, only partially inhibited by PMSF, and totally inhibited by iodoacetic acid, PCMB and DFP. These findings suggest that in the case of the *Lactobacillus* dipeptidyl peptidase, sulphydryl groups are important for activity, but complete inhibition of both enzymes by DFP indicates that they are probably serine-peptidases. The dipeptidyl peptidase purified from *S.lactis* in the present study shows similar inhibition characteristics to the *S.thermophilus* enzyme, being inhibited by PMSF, and to a lesser extent by PCMB. However, unlike *S.thermophilus* , Cu^{2+} and Pb^{2+} rapidly inhibit the *S.lactis* enzyme. Iodoacetic acid and o-phenanthroline had little effect on the *S.lactis* dipeptidyl peptidase.

These data support the findings of Meyer and Jordi, and indicate that the dipeptidyl peptidase from *S.lactis* is probably a serine-peptidase. Kiefer-Partsch *et al.* have reached the same conclusions, the *S.cremoris* enzyme showing the same inhibition characteristics with PMSF and DFP.

The molecular weight value for the dipeptidyl peptidase reported by Meyer and Jordi and by Kiefer-Partsch *et al.* are compared with the results obtained in this study for the dipeptidyl peptidase from *S.lactis* 4760 and *S.cremoris* 4409 in Table 4.1. A consistent native molecular weight, about double that of the subunit molecular weight, was found with all the dipeptidyl peptidase enzymes shown, all evidence suggesting that the enzyme is a dimer in its native form.

	Native Mol. Wt.	Subunit Mol. Wt.
Meyer and Jordi (1987)		
Streptococcus thermophilus	165 000 ± 15 000	80 000 ± 5 000
Lactobacillus lactis	165 000 ± 15 000	90 000 ± 5 000
Kiefer-Partsch (1989)		
Streptococcus cremoris	160 000 - 180 000	90 000
Present study		
Streptococcus lactis 4760	150 000	83 000
Streptococcus cremoris 4409		86 000

TABLE 4.1. *Comparison of the molecular weights of dipeptidyl peptidase enzymes purified from several lactic streptococci.*

Several similarities between the mammalian and bacterial dipeptidyl peptidase may also be drawn. The dipeptidyl peptidase IV from pig kidney has a molecular weight of 90 000, and is a dimer of two identical subunits in its native form (Harada *et al.*, 1985). The pig kidney enzyme also shows inhibitor characteristics of a serine peptidase. However, little similarity is evident between the amino acid composition of the mammalian and *S.lactis* enzymes. The mammalian dipeptidyl peptidase IV has 9 cysteine residues, but none are evident in the *S.lactis* derived enzyme.

The substrate specificity of the dipeptidyl peptidase has been examined by Meyer and Jordi using aminoacyl p-nitroanilide (pNA) and AMC derivatives. The concentration of

the substrate was not given in their study, and thus relative activities may have been influenced by substrate inhibition (see Section 3.3.5). The enzyme was shown to have a higher relative activity with Gly-Pro-pNA than with Ala-Pro-pNA or Arg-Pro-pNA. The relative activity with Gly-Pro-pNA was also higher than with Gly-Pro-AMC. A low relative activity was also found with Gly-Ala-AMC (5.6% of the Ala-Pro-pNA activity), and with N-succ-Gly-Pro-AMC (3.2% of the Ala-Pro-pNA activity), a substrate with a blocked N-terminus. No activity was shown towards Gly-Phe-AMC, Gly-Arg-AMC or any single amino acyl-AMC derivative. The dipeptidyl peptidase purified from *S.lactis* 4760 in this study was shown to have activity towards a range of X-Pro-AMC derivatives, and low activity with Gly-Ala-AMC. Activity with other penultimate residues was not tested in light of the findings by Meyer and Jordi. The ability to hydrolyse X-Ala-Y substrates seems to be a general characteristic of X-Pro dipeptidyl peptidases, because dipeptidyl peptidase IV from pig kidney also shows this specificity. (Kenny *et al.*, 1976; Harada *et al.*, 1986). In the case of the dipeptidyl peptidase from *Lactobacillus lactis*, a rather different pattern of relative activities was found. Gly-Pro-pNA and Arg-Pro-pNA were less readily hydrolysed than Ala-Pro-pNA and a very much reduced activity was seen with the substrates Gly-Ala-AMC and N-succ-Gly-Pro-AMC. In no instance with any of the dipeptidyl peptidases tested was any iminopeptidase activity towards Pro-AMC or Pro-pNA detected.

The Km value for hydrolysis of Gly-Pro-AMC by the dipeptidyl peptidase purified from*S.lactis* 4760 was determined to be 14.1 μM (Section 3.3.6) which compares with the apparent Km found (using the same substrate and at the same pH) of 98μM for the *Lactobacillus* enzyme, and 60μM for the enzyme from *S.thermophilus* (Meyer and Jordi, 1987), i.e. about 4 to 5 fold higher than that found with the dipeptidyl peptidase in the present study.

The kinetics of hydrolysis of X-Pro-pNA substrates by dipeptidyl peptidase IV from pig kidney has been studied by Harada *et al.* (1985). The Km for hydrolysis of Gly-Pro-pNA was found to be 190μM (compared with 14.1μM for *S.lactis* 4760 with Gly-Pro-AMC). However, the k_{cat} determined for a range of X-Pro substrates spanned the range of 25.7 to 81.0 per second, very similar to the results obtained with the X-Pro-AMC substrates for the *S.lactis* dipeptidyl peptidase which spanned the range 43.8 to 78.4 per second (see Section 3.3.6).

The Km value for hydrolysis of a range of X-Pro-AMC substrates was determined using the purified dipeptidyl peptidase from *S.lactis* (Section 3.3.6). Little difference in Km was seen using the substrates Leu-Pro-AMC, Phe-Pro-AMC, Gly-Pro-AMC and Lys-

Pro-AMC. However, the enzyme showed a three fold greater Km value with Glu-Pro-AMC. The possibility that this higher Km may have resulted from contamination of the Glu-Pro-AMC substrate during synthesis was discussed in Section 3.3.6, but it was concluded that this result was probably not an artifact caused by contamination, since all evidence suggested that the Glu-Pro-AMC was about 90% pure, similar to the purity of the other AMC derivatives synthesised. A study of the hydrolysis of proline substrates by dipeptidyl peptidase IV (Heins *et al.*, 1988) has shown, using a large range of X-Pro-pNA substrates, that the Km and k_{cat} values are quite similar, no marked preference for any particular X-Pro-pNA being evident.

4.1.3 SPECIFICITY OF THE X-PRO DIPEPTIDYL PEPTIDASE TOWARDS PEPTIDES

The dipeptidyl peptidase purified from *S.lactis* has been shown (Section 3.5.5) to hydrolyse a wide range of X-Pro-AMC derivatives. The ability of the enzyme to hydrolyse short peptides was investigated using reverse phase HPLC. Time course digestions showed that substrates with the N-terminal sequence X-Pro-Y- were cleaved between the proline and Y, but peptides without this sequence were resistant to hydrolysis. The peptide β-casomorphin, an opioid peptide from β-casein, has the sequence Tyr-Pro-Phe-Pro-Gly-Pro-Ile and proved to be an excellent substrate for the dipeptidyl peptidase, being degraded into two dipeptides and a tripeptide (Section 3.5.8). Bradykinin, a mammalian bioactive peptide, has the N-terminal sequence Lys-Pro-Pro- or Arg-Pro-Pro-. This peptide is completely resistant to hydrolysis by the dipeptidyl peptidase, suggesting that the consecutive prolines impose a constraint on the ability of the dipeptidyl peptidase to cleave after the first proline, perhaps by inducing a kink in the peptide chain. A study of the digestion of β-casein by the mammalian dipeptidyl peptidase IV (Heymann and Mentlein, 1986) assumes that X-Pro-Pro sequences are resistant to the mammalian dipeptidyl peptidase, further supporting the similarity between the streptococcal and mammalian enzymes. A further two peptides, the first a fragment (90-95) of α-casein with the sequence Arg-Tyr-Leu-Gly-Tyr-Leu, and the second, a fragment (32-36) of thymopoietin II with the sequence Arg-Lys-Asp-Val-Tyr, were also completely resistant to hydrolysis by the dipeptidyl peptidase even after extended incubation periods of 72 hours.

Kiefer-Partsch *et al.* have also studied the effects of the dipeptidyl peptidase on β-casomorphin, and state "β-casomorphin is of special interest since it appears to be the first demonstration of a peptidase in the cell wall proteolytic system of *S.cremoris* which is able to degrade the proline-rich peptides which are abundant in β-casein". The products of

hydrolysis were found by Kiefer-Partsch to be Tyr-Pro, Phe-Pro, Gly-Pro and Ile. In the present study it was found that the *S.lactis* enzyme appears to be unable to hydrolyse the Gly-Pro-Ile tripeptide. However, since the peak containing the Gly-Pro-Ile was only analysed for amino acid composition and was not sequenced, the compositional analysis alone may lead to ambiguous conclusions because, although a 1:1:1 ratio of the three amino acids was found, there is no evidence that the three residues were in the form of a tripeptide. The isoleucine may possibly have co-eluted with the Gly-Pro dipeptide.

4.2 THE LYS-AMINOPEPTIDASE

4.2.1 PURIFICATION OF THE LYS-AMINOPEPTIDASE

Over the past decade many studies have been carried out on the aminopeptidase activity of a variety of lactic acid bacteria, including *Lactobacillus lactis, L.acidophilus* and several strains of *S.cremoris*. To date, there have been no reports discussing the properties of purified aminopeptidase from *S.lactis*.

The most recently published purification and characterisation of an aminopeptidase from lactic streptococci was carried out by Neviani *et al.* (1989) using *S.cremoris* AM2. This group employed ion exchange chromatography (using a Mono Q HR 10/10 column) to purify a 300 kDa hexameric aminopeptidase. After two ion exchange steps, they claim to have purified the enzyme to homogeneity with a 9% yield, achieving a 136 fold purification. Geis *et al.* (1985) purified an aminopeptidase from *S.cremoris* AC1 by a two step procedure using DEAE Sephacel ion exchange chromatography followed by gel filtration on a Sephadex G100 column. They claimed a purity for their enzyme preparation of 95% and found it had a molecular weight of a 36 kDa. The aminopeptidase activity was released from cells by incubation with Ca^{2+}-free buffer. They described it as a cell-wall located enzyme but did not give any data on the percentage total enzyme activity released. The initial DEAE cellulose and Sephacryl S300 steps employed in the present study for the partial purification of the Lys-aminopeptidase from *S.lactis* gave little enhancement of specific activity and led to a significant loss of total activity, even though the enzyme was reasonably stable on storage. As discussed in Section 3.5.4, attempts were made to reduce these losses, by the addition to the enzyme preparation of various cations that may have been lost during the purification procedure. However, no enhancement of activity was observed after the addition of any cation. Evidence from the PAGE of the purification of the Lys-aminopeptidase (Fig. 3.24) shows that the most successful step in the purification was salt elution from arginine-Sepharose. However, many contaminating

bands were still present after two elution steps from this resin. Since it proved so difficult to maintain AMCase activity during native gel electrophoresis, the identification of a particular protein band on the Coomassie blue stained gel with the diffuse band of activity on the Lys-AMC stained gel was uncertain, so it is not clear whether the Lys-AMCase was a major or minor component of the partially purified preparation.

Clearly then, to achieve greater purification of the Lys-aminopeptidase, a systematic study of the nature of the activity losses is necessary. The development of a true affinity chromatography procedure would be desirable, since the steps employed in the present study have succeeded only in separating dipeptidyl peptidase activity from the partially purified Lys-aminopeptidase.

4.2.2 CHARACTERISTICS OF THE LYS-AMINOPEPTIDASE

It is apparent from a comparison of recently published literature on aminopeptidases from lactic streptococci, that a variety of aminopeptidases with widely differing characteristics exist. The aminopeptidase purified from *S.cremoris* AM2 by Neviani *et al.*, (1989) is a hexameric protein with a molecular weight of 300 kDa composed of identical 50 kDa subunits. The effect of inhibitors showed that this enzyme was not a metallopeptidase, was inhibited by thiol protease inhibitors, but not inhibitors of serine proteases. It was shown to have broad specificity, hydrolysing a range of naphthylamide-substituted amino acids, dipeptides and tripeptides. However, substrates with a N-terminal proline residue were resistant to hydrolysis, and no endopeptidase activity was observed using amino acyl compounds with their amino groups blocked with a CBZ group. The aminopeptidase purified from *S.lactis* 4760 shows little similarity to the *S.cremoris* AM2 aminopeptidase since it has a native molecular weight of about 78 kDa and shows completely different inhibition characteristics, being inhibited rapidly by 1,10-phenanthroline. The two enzymes also differ markedly in their substrate specificity since the *S.lactis* enzyme is able to hydrolyse lysine, arginine and leucine AMC-derivatives but not glycine, alanine, phenylalanine, tyrosine or serine AMC-derivatives. The inability of these aminopeptidases to catalyse the removal of N-terminal proline residues appears to be a common characteristic, not only of bacterial aminopeptidases such as the*S.lactis* and*S.cremoris* enzymes, but also of enzymes purified from the mammalian system such as aminopeptidase M (Heymann and Mentlein, 1986).

An aminopeptidase with a molecular weight of about 80 kDa has been purified from *Lactobacillus lactis* (Eggimann and Bachmann, 1980). This enzyme, like the aminopeptidase purified from *S.lactis* 4760, was inhibited by 1,10-phenanthroline and

EDTA . However, the *Lactobacillus* enzyme had a broad substrate specificity, hydrolysing a number of amino acids arylamides and many peptides with unsubstituted amino-terminal amino acids. In contrast to the broad specificity of the aminopeptidase from *L.lactis* , a cell surface-bound aminopeptidase from *S.cremoris* (Exterkate, 1987), designated aminopeptidase A, had specificity for peptide bonds in which an amino-terminal L-α-glutamyl (aspartyl) residue was involved.

An aminopeptidase purified from *L.acidophilus* R26 (Machuga and Ives, 1984) was shown to be a tetramer of molecular weight 156 000 with 4 tightly bound zinc atoms per molecule. The authors claim that this enzyme was responsible for all the N-terminal exopeptidase and amidase activities in crude extracts, and also had detectable endopeptidase or esterase activity.

A similar substrate specificity to that shown by the *S.lactis* 4760 aminopeptidase has been reported to be shown by an aminopeptidase purified from *S.cremoris* AC1 (Geis *et al.,* 1985). This enzyme showed a marked preference for lysyl-pNA as a substrate but was also capable of hydrolysing leucyl-pNA. However, unlike the aminopeptidase purified from *S.lactis* 4760, the *S.cremoris* AC1 enzyme was also able to hydrolyse alanyl-pNA and alanyl-alanyl-pNA substrates.

4.2.3 SPECIFICITY OF THE LYS-AMINOPEPTIDASE TOWARDS PEPTIDES

Specificity studies on the aminopeptidases discussed above have mostly used aminoacyl-pNA or AMC derivatives. Little systematic study of the ability to degrade oligopeptides has been carried out. Neviani (1989) showed that as well as activity towards aminoacyl-β-naphthylamide derivatives, the aminopeptidase from *S.cremoris* AM2 was capable of hydrolysing dipeptides and tripeptides including Leu-Gly-Gly, but no study was made of the hydrolysis of larger oligopeptides. The partially purified enzyme from *S.lactis* 4760 apparently does not have this ability since Leu-Gly-Gly is completely resistant to hydrolysis (Section 3.6.5). This result is somewhat surprising since the Lys-aminopeptidase showed a low but detectable rate of hydrolysis of Leu-AMC (approximately 12 fold lower than that seen with Lys-AMC).

An aminopeptidase purified from *Lactobacillus lactis* (Eggimann and Bachmann, 1980) was shown to hydrolyse some tetra- and pentapeptides as well as di- and tripeptides although little systematic characterisation of its substrate specificity was done. It was shown in the present study (Section 3.6.5) that the aminopeptidase from *S lactis* is able

to remove N-terminal lysine and arginine residues from short peptides. However, if the penultimate amino acid is proline, as in the case of bradykinin, the aminopeptidase is unable to catalyse the removal of either lysine or arginine.

The hydrolysis products formed after incubation of various peptides with the Lys-aminopeptidase have suggested that endopeptidase activity is associated with the aminopeptidase preparation. β-casomorphin was cleaved between a proline and a glycine, and bradykinin was cleaved between a phenylalanine and a serine. Hydrolysis of the hexapeptide from thymopoietin provided further evidence to support the existence of an endopeptidase contaminant in the aminopeptidase preparation, although the site of cleavage was ambiguous in this case. Two alternative cleavage sites within the peptide, between leucine and glycine, or between glycine and tyrosine, could account for the degradation products found after hydrolysis. Thus, the recognition of cleavage sites does not seem to be based simply on amino acid side-chain functional groups. It is interesting to compare these results with those obtained with the cell wall-associated proteinase which shows a consistent cleavage pattern but little correlation between cleavage sites and amino acid functional groups.

4.3 INDIVIDUAL AND COMBINED EFFECTS OF PEPTIDASES ON HYDROLYSIS OF β–CASEIN-DERIVED PEPTIDES

One of the aims of the overall programme on starter peptidases in this laboratory is to elucidate the pathway by which oligopeptides derived from milk proteins are degraded to small peptides and amino acids. An interesting parallel to the present study was a detailed study of the digestion of β-casein by the mammalian proteolytic system carried out by Heymann and Mentlein (1986). This study focuses on the complementary action of dipeptidyl peptidase IV (already shown to bear similarities to its streptococcal equivalent in Section 4.1.2), and an aminopeptidase designated aminopeptidase M in degrading tryptic peptides from β-casein.

Relatively large amounts of dipeptidyl peptidase IV cover the intestinal microvilli, and it is assumed by Heymann and Mentlein that its role is in the degradation of proline-rich proteins. Aminopeptidase M also has a restricted specificity, being unable to hydrolyse proline amino groups and acidic residues. A third aminopeptidase designated aminopeptidase A is able to hydrolyse acidic groups and therefore a combination of these three peptidases should complete sequential degradation of almost all peptides from their N-terminus. In testing this hypothesis, it was found that without dipeptidyl peptidase IV almost no proline was released from β–casein peptides. After incubation with dipeptidyl

peptidase IV the release of proline increased to the value that was predicted, assuming that X-Pro sequences were resistant to aminopeptidase M, and that X-Pro-Pro sequences can not be cleaved by either enzyme. Dipeptidyl peptidase addition also dramatically increased the production rate of glycine, valine, isoleucine, leucine, tyrosine and phenylalanine. Thus, Heymann and Mentlein conclude that these two enzymes complement one another in the N-terminal degradation of peptides derived from β-casein.

It was hoped to make a similar study of the potential of the aminopeptidase and dipeptidyl peptidase to degrade oligopeptides, derived from β-casein by the action of the cell wall-associated proteinase. Given the limited specificity of the Lys-aminopeptidase, none of the peptides shown to be generated by the action of the proteinase on β-casein (Fig.1.2) were likely to be completely degraded by a combination of Lys-aminopeptidase and dipeptidyl peptidase. However, incubation of peptide B (shown to have the sequence Lys-Ala-Val-Pro-Tyr-Pro-Gln) with the Lys-aminopeptidase resulted in the removal of both lysine and alanine from the amino terminus, resulting in the pentapeptide Val-Pro-Tyr-Pro-Gln. Furthermore, a combination of the Lys-aminopeptidase and the dipeptidyl peptidase resulted in the formation of a minor product, the pentapeptide, and a major product, Tyr-Pro. In light of the result obtained with the Lys-aminopeptidase alone, the initial formation of the pentapeptide by the Lys-aminopeptidase and its subsequent degradation by the dipeptidyl peptidase is not surprising. However, when peptide B was digested with dipeptidyl peptidase alone, Tyr-Pro was again the main product. This suggests that the dipeptidyl peptidase must be able to remove the N-terminal Lys-Ala without the aid of the Lys-aminopeptidase. Thus peptide B may be degraded by two routes. Firstly the sequential removal of lysine and alanine, followed by the hydrolysis of the resulting pentapeptide by the dipeptidyl peptidase, or secondly, solely by the action of the dipeptidyl peptidase removing Lys-Ala, Val-Pro and Tyr-Pro. Hydrolysis of Gly-Ala-AMC was shown to occur at 3.7% of the rate of Gly-Pro-AMC hydrolysis in Section 3.3.5, thus supporting the findings with peptide B.

The limited specificity shown by what was originally thought to be a "general" aminopeptidase, complicates the picture of proteolysis by enzymes from *S.lactis*. To achieve complete sequential hydrolysis of a number of peptides, an enzyme with the ability to hydrolyse dipeptides formed by the action of the dipeptidyl peptidase (an imido-dipeptidase or prolidase), and one or more aminopeptidases with the ability to hydrolyse amino acids other than those removed by the Lys-aminopeptidase, would be required to provide the full range of amino acids needed for bacterial protein synthesis. The situation with *S.lactis* contrasts with that in *Lactobacillus acidophilus* R26 (Machuga and Ives,

1984) where a single aminopeptidase of broader specificity was shown to be responsible for all of the N-terminal exopeptidase activity observed in crude extracts of the bacteria.

A most interesting finding was that a group of three peptides eluting as a single HPLC peak (designated peptide D in Section 3.3.7) were completely resistant to further hydrolysis by any combination of enzymes from the proteolytic system of *S.lactis* 4760. These peptides, and others that may be found to be resistant to hydrolysis, may play an important role in determining the bitter flavour in cheese produced by the action of certain starter strains. One of the peptides found in this peak is the C-terminal peptide of β-casein (fragment 194-209) which has been reported to be a major contributor to the bitter flavour defect (Visser *et al.*, 1986).

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