Characterization of X-prolyl dipeptidyl aminopeptidase from *Lactococcus lactis* subsp. *lactis*

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A dipeptidyl aminopeptidase catalysing hydrolysis of X-prolyl amidomethylcoumarin (AMC) substrates has been purified from *Lactococcus lactis* subsp. *lactis* H1. The active enzyme has a molecular mass of approximately 150 kDa, a subunit molecular mass of 82 to 83 kDa and is inhibited by the serine protease inhibitor phenylmethylsulphonyl fluoride. The K_m and k_{cat} values for five different dipeptidyl AMC substrates (Gly-Pro-; Leu-Pro-; Lys-Pro-; Phe-Pro- and Glu-Pro-AMC) are similar except for the K_m value for Glu-Pro-AMC, which is about threefold higher than that for the other substrates. The enzyme also catalyses hydrolysis of X-Ala-AMC substrates but with much lower k_{cat} and higher K_m values than the corresponding X-Pro-AMC substrates. The β -casein-derived heptapeptides Lys-Ala-Val-Pro-Tyr-Pro-Gln and Tyr-Pro-Phe-Pro-Gly-Pro-Ile were hydrolysed, but bradykinins with N-terminal sequences Arg-Pro-Pro- and Lys-Pro-Pro- were not. Dipeptidyl aminopeptidase specific activity is the same in a plasmid-free strain of *L. lactis* subsp. *lactis* H1 and in the wild-type, indicating that the enzyme is chromosomally encoded.

Introduction

The proteolytic activity of lactococci is an essential biochemical property contributing to their role as starters in cheese manufacture, enabling them to use milk proteins as a source of amino acids for growth to high densities and influencing the flavour and texture of the product (Thomas & Mills, 1981). The first step in proteolysis has been shown to be catalysed by a cell-wall-located proteinase (Mills & Thomas, 1978; Exterkate, 1975; Hugenholz *et al.*, 1987) which, in most of the lactococcal strains studied to date, preferentially degrades the β -casein component of bovine milk. Certain peptide bonds near the C-terminal end of the β -casein molecule are particularly sensitive to hydrolysis, yielding a series of oligopeptides (Monnet *et al.*, 1986, 1989; Visser *et al.*, 1988). The further breakdown of these oligopeptides depends on the action of various peptidases (Law & Kolstad, 1983; Thomas & Pritchard, 1987). The proline-rich nature of β -casein (proline comprising 17% of the amino-acid residues) suggests that one or more peptidases capable of catalysing hydrolysis of

peptide bonds involving proline will be an essential part of the proteolytic enzyme system. One such enzyme is X-prolyl dipeptidyl aminopeptidase (dipeptidyl peptidase IV; EC 3.4.14.5), which catalyses the hydrolytic removal of N-terminal dipeptidyl residues from peptides containing proline in the penultimate position. This enzyme, extensively studied from mammalian sources (Kenny *et al.*, 1976; McDonald & Schwabe, 1977), has recently been purified from several lactic acid bacteria. Meyer & Jordi (1987) purified the enzyme from *Streptococcus thermophilus* and *Lactobacillus lactis* and demonstrated its activity towards X-prolyl *p*-nitroanilide (*p*-NA) and amidomethylcoumarin (AMC) derivatives. An enzyme with very similar properties has been purified from *Lactobacillus helveticus* CNRZ 32 (Khalid & Marth, 1990). Kiefer-Partsch *et al.* (1989) and Zevaco *et al.* (1990) have purified X-prolyl dipeptidyl aminopeptidase enzymes from *Lactococcus lactis* subsp. *cremoris* P8-2-47 and from *L. lactis* subsp. *lactis* NCDO 763, respectively, and have established the exopeptidase action of the enzyme in degrading the β -casein-derived oligopeptide Tyr-Pro-Phe-Pro-Gly-Pro-Ile (β -casomorphin). Booth *et al.* (1990b) carried out qualitative specificity studies on a post-proline dipeptidyl aminopeptidase from *L. lactis* subsp. *cremoris* AM2 showing that a range of peptides with proline or alanine as the penultimate N-terminal residue could act as substrates.

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Abbreviations: AMC, 7-amido-4-methylcoumarin; *p*-NA, *p*-nitroanilide; Boc, *t*-butoxycarbonyl; TFA, trifluoroacetic acid.

Limited activity of the enzyme in hydrolysing N-terminal X-alanyl residues was also shown by Meyer & Jordi (1987) and by Zevaco *et al.* (1990).

In view of the potential importance of X-prolyl dipeptidyl aminopeptidase in the hydrolysis of β -casein peptides by lactococci a further study was undertaken of the properties of this enzyme from *L. lactis* subsp. *lactis* with particular emphasis on a quantitative characterization of its specificity.

Methods

Micro-organisms and growth conditions. The following lactococcal strains were obtained from the New Zealand Dairy Research Institute, Palmerston North: *Lactococcus lactis* subsp. *cremoris* H2; *L. lactis* subsp. *lactis* H1; *L. lactis* subsp. *lactis* 4125, a plasmid-free derivative of the H1 strain (Crow *et al.*, 1983) and *L. lactis* subsp. *lactis* 4760, obtained by conjugal transfer of the lactose/proteinase plasmid from *L. lactis* subsp. *cremoris* H2 into the plasmid-free derivative (4125) of *L. lactis* subsp. *lactis* H1. This transconjugant strain was used for the large-scale purification of enzyme in preference to the wild-type which forms clumps in broth culture. The clumping characteristic is absent from both the transconjugant and plasmid-free strains. Bacteria were grown at 30 °C in either a 3 litre or a 40 litre fermenter. The culture was gassed with nitrogen and the pH maintained at approximately 6.2 by automatic addition of sodium hydroxide. The medium contained the following constituents (g l⁻¹): lactose 20; peptone 10; yeast extract 10; KH₂PO₄ 5; beef extract 2; MgSO₄ · 7H₂O 0.2; MnCl₂ · 4H₂O 0.05. For growth of the plasmid-free strain (4125), glucose (20 g l⁻¹) replaced the lactose in this medium. Bacteria were harvested at the end of the exponential phase (approximately 6 h after inoculation) by centrifugation at 8000 g for 15 min at 4 °C. A yield of 12–14 g l⁻¹ wet packed weight of cells was obtained. The cells were washed twice in cold 50 mM-Tris/HCl buffer (pH 7.5) and stored at –15 °C until required.

Enzyme purification. All steps were carried out at a temperature of 0–4 °C. The thawed cells were resuspended in Tris/glycerol buffer (50 mM-Tris/HCl pH 7.5 containing 10%, w/v, glycerol). Cells were disrupted by a single passage through a French pressure cell operated at 55 MPa. The resulting homogenate was centrifuged at 27000 g for 30 min and the supernatant collected. The cell-free extract from 100 g wet packed weight of cells (obtained from approximately 7.5 l of culture) was loaded onto a column (5 cm × 15 cm) of DEAE-cellulose (Whatman DE23) equilibrated with Tris/glycerol buffer. Protein was eluted with a linear 0–400 mM-NaCl gradient in Tris/glycerol buffer at a flow rate of 1.5 ml min⁻¹. Fractions containing dipeptidyl aminopeptidase activity were pooled and concentrated by passage through a PM30 Diaflo membrane (Amicon) to a volume of 10 ml. The concentrated sample was loaded onto a column (3 cm × 75 cm) of Sephacryl S300 equilibrated with 10 mM-Tris/glycerol buffer and eluted with the same buffer at a flow rate of 0.5 ml min⁻¹. Active fractions were pooled and loaded onto a column (2 cm × 12 cm) of arginine-Sepharose 4B equilibrated with 10 mM-Tris/glycerol buffer. Protein was eluted using a linear 0–200 mM-NaCl gradient at a flow rate of 0.5 ml min⁻¹. The pooled active fractions were applied to a DEAE-Sephadex column (3 cm × 15 cm) equilibrated with 10 mM-Tris/glycerol buffer and eluted with a linear 0–400 mM-NaCl gradient (flow rate 0.5 ml min⁻¹). Finally, active fractions from the DEAE-Sephadex column, after overnight dialysis against 10 mM-Tris/glycerol buffer to remove NaCl, were loaded onto and eluted from the arginine-Sepharose 4B column using the same procedure as described above for this column.

Enzyme assay. Dipeptidyl aminopeptidase activity was routinely determined using glycyl prolyl 7-amido-4-methylcoumarin (Gly-Pro-AMC) as substrate (Kato *et al.*, 1978). The routine assay mixture contained: 1.5 ml 100 mM-MES buffer (pH 6.8); 0.5 ml 3 mM-Gly-Pro-AMC; 0.1 ml enzyme solution. The rate of hydrolysis of Gly-Pro-AMC was determined by measuring the relative fluorescence of the released AMC in a spectrofluorimeter (SP500; American Instrument Co.) using an excitation wavelength of 385 nm and an emission wavelength of 460 nm. The fluorimeter was calibrated using an aqueous solution of AMC (100 μ mol l⁻¹). A unit of enzyme activity is defined as that amount of enzyme liberating 1 μ mol AMC min⁻¹.

Synthesis of dipeptidyl AMC substrates. Peptide amides of 7-amino-4-methylcoumarin (AMC) were prepared using a modification of the method of Kato *et al.* (1978). The modifications involved the following. (1) Starting the synthesis with Boc-proline to make Boc-Pro-AMC rather than with Boc-glycyl-proline but otherwise following the method and quantities described by Kato *et al.* (1978). This permitted the synthesis of a range of different X-Pro-AMC derivatives by coupling the desired N-terminal amino acid to the Pro-AMC. (2) Preparing the HBr salt rather than the *p*-toluene sulphonate (tosylate) salt since preliminary tests suggested some inhibition of the enzyme by the tosylate ion. The Boc-Pro-AMC was reacted for 1 h with 30% (w/w) HBr in glacial acetic acid. The HBr. Pro-AMC was precipitated with anhydrous diethyl ether, filtered and dried under vacuum overnight. It was recrystallized by dissolving in methanol and reprecipitating with diethyl ether. (3) Coupling of the different N-terminal amino acids to the HBr. Boc-Pro-AMC followed the procedure of Koenig & Geiger (1970) using dicyclohexylcarbodiimide and hydroxybenzotriazole in *p*-dioxane to form the active ester of the Boc-amino acid which was then reacted with the HBr. Pro-AMC and triethylamine in 1:1 mixture of *p*-dioxane and dimethylformamide. After washing and crystallizing, the Boc group was removed with HBr in acetic acid as described above. In the case of HBr. Glu-Pro-AMC, the γ -carboxyl of the glutamate was protected by a benzyl ether group which was subsequently removed by hydrogenation using a palladium on charcoal catalyst.

For each dipeptidyl-AMC compound prepared the product was subjected to acid hydrolysis to determine the amino acid composition. A 1:1 ratio of the expected amino acids was found in each case. The concentration of AMC removed by complete enzymic hydrolysis (using the purified dipeptidyl aminopeptidase) was determined fluorimetrically. This indicated, for the five different dipeptidyl AMC products, a purity of between 89 and 91%. The remaining 10% is probably due to residues of salts used in the work-up procedure and water since all solids were hygroscopic.

Polyacrylamide gel electrophoresis (PAGE). Non-denaturing PAGE was carried out according to the procedure of Laemmli (1970) using 7.5% (w/v) acrylamide plus 0.2% bisacrylamide in the running gel and 4% (w/v) acrylamide plus 0.13% bisacrylamide in the stacking gel. Protein bands were detected by staining in 0.125% Coomassie brilliant blue R250 in methanol/acetic acid/water (9:1:20, by vol.) followed by destaining in the same solvent. Dipeptidyl aminopeptidase activity was detected by covering the gel surface with 1 mM-Gly-Pro-AMC solution for not more than 5 min and examining under UV light. It was necessary to photograph the stained gel immediately to prevent diffusion of the fluorescent band. The procedure for SDS-PAGE was the same as for native gels except that the resolving and stacking gel solutions and the reservoir buffer contained 0.1% SDS. Samples to be run on SDS-polyacrylamide gels were prepared by adding 100 μ l 6 M-urea/10% (w/v) SDS and 10 μ l β -mercaptoethanol to 100 μ l of sample and boiling for 5 min. Bromophenol blue [5 μ l of a 0.5% solution in 50%, v/v, glycerol] was added to the samples before loading.

Determination of molecular masses. Subunit molecular mass was determined using the SDS-PAGE procedure described above, with the

following proteins as standards: lactate dehydrogenase (37 kDa), citrate synthase (50 kDa), bovine serum albumin (68 kDa), transferrin (80 kDa) and β -galactosidase (116 kDa). The molecular mass of the native purified enzyme was determined by its elution volume from a 3 cm $\times$ 75 cm column of Sephacryl S300 relative to that of the following standards: β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (68 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12 kDa).

Protein determination. Protein concentration was measured by the Lowry method except at low protein concentrations in Tris buffer, where the bicinchoninic acid method of Smith *et al.* (1985) gave more reliable estimates since it was not subject to interference by the Tris in the buffer.

Separation of peptides by high-performance liquid chromatography (HPLC). Peptides were separated by reversed-phase HPLC using either a Spectra Physics SP 8800 chromatograph with a Vydac 218TP C18 column (250 mm $\times$ 4.6 mm) or with a Waters M6000A high-pressure pump system and 660 programmer and a Waters 8 MBC18 cartridge column (120 mm $\times$ 8 mm) in a radial compression module. Peptides were eluted with acetonitrile in water containing 0.1% trifluoroacetic acid (TFA). Gradients used are specified in the legend to Fig. 1. Peptides were detected by their absorbance at 220 nm. Peptide-containing fractions were collected and dried under vacuum for analysis by amino acid composition and/or sequencing.

Isolation of fragment 176–182 from β -casein. Lactococcal proteinase was obtained from milk-grown cells of *L. lactis* subsp. *lactis* H1 (4760) by incubation of washed cell suspensions in Ca^{2+} -free 50 mM-sodium phosphate buffer (pH 6.4) according to the procedure of Mills & Thomas (1978). The proteinase was partially purified by chromatography on DEAE-cellulose (eluting with a 0–0.8 M-NaCl gradient in 50 mM-phosphate buffer, pH 6.4) and subsequent passage of the pooled, concentrated active fractions through a column of Sephacryl S300. Proteinase activity was monitored using the method of Twining (1984). The partially purified proteinase was incubated with β -casein for 22 h at room temperature. The incubation mixture contained 1 ml proteinase solution and 2 ml β -casein (10 mg ml⁻¹). The digest was passed through a Millipore centrifugal membrane filter (10 kDa cutoff) to remove high molecular mass oligopeptides. Oligopeptides in the filtrate were fractionated by HPLC as described above using a linear gradient, from 5% to 80% (v/v), of acetonitrile in 0.1% aqueous TFA at a flow rate of 1 ml min⁻¹ over 60 min. Peptide peaks were collected and analysed by sequencing and by amino acid analysis. The major low M_r oligopeptide products were identical to those reported by Visser *et al.* (1988) and by Monnet *et al.* (1989). Fragment 176–182 eluted at an acetonitrile concentration of 17% (v/v).

Amino acid composition and sequence analysis. Freeze-dried peptides were dissolved in 200 μ l 5.9 M-HCl in test-tubes sealed below 0.2 mmHg pressure. Hydrolysis was carried out for 24 h at 110 °C. After hydrolysis the tubes were opened and the contents dried over phosphorus pentoxide. The hydrolysed peptides were redissolved in a minimum volume of deionized water and loaded onto an amino acid analyser (Beckman 119B). Amino acid composition was estimated from peak areas determined using an integrator (Spectra-Physics 4290). Amino acid sequence data were obtained by the automated Edman method using a gas-phase protein sequencer (Applied Biosystems 470).

Materials. Yeast extract was obtained from Gibco Laboratories (NZ); beef extract and Trypticase peptone from BBL; lactose from BDH Chemicals. Low-heat skim-milk powder was obtained from the New Zealand Dairy Research Institute, Palmerston North, New Zealand. Aminoacyl AMC derivatives were prepared as described above from Boc-amino acids obtained from Sigma. AMC and hydroxybenzotriazole were synthesized by the Separation Science Unit, Massey University. HBr (30%, w/w) in glacial acetic acid was obtained from Aldrich Chemical Company. Buffer constituents (MES and Tris) and peptides were obtained from Sigma. DEAE-cellulose (DE 23) was obtained from Whatman Biochemicals, Sephacryl S300, DEAE-Sephadex and arginine-Sepharose 4B from Pharmacia. Acetonitrile was Hipsolv grade from BDH Chemicals. Bicinchoninic acid was from Sigma. All other chemicals were, wherever possible, of analytical grade and obtained from BDH or May and Baker, UK.

Results and Discussion

Purification

A typical purification is summarized in Table 1. After the first arginine-Sepharose column three contaminating proteins were found to be present on SDS-PAGE; these were removed by subsequent passage through DEAE-Sephadex and the second arginine-Sepharose step. The procedure described gave a reproducible purification of the dipeptidyl aminopeptidase to virtual homogeneity as verified by both denaturing and non-denaturing PAGE. The single protein band was shown to correspond to the dipeptidyl aminopeptidase activity by flooding the non-denaturing gel in Gly-Pro-AMC solution for a few

Table 1. Purification of X-prolyl dipeptidyl aminopeptidase from *L. lactis* subsp. *lactis* H1 (4760)

The results presented are for a purification from 100 g wet wt of cells.

Purification step	Protein concn (mg ml ⁻¹)	Total protein (mg)	Activity (units ml ⁻¹)	Total activity (units)	Specific activity (units mg ⁻¹)	Purification (-fold)	Yield (%)
Crude extract	14.8	3700	2.58	645	0.18	1	100
DEAE-cellulose	3.5	385	4.23	465	1.21	6.7	72
Sephacryl S300	1.6	150	3.87	365	2.42	13.4	57
Arginine-Sepharose 1	0.102	11.9	2.49	292	24.4	136	45
DEAE-Sephadex	0.080	7.8	1.92	188	24.0	133	29
Arginine-Sepharose 2	0.042	3.1	1.71	127	40.7	226	20

seconds and examining the gel under UV light to detect a bright fluorescent band due to released AMC.

General properties

The subunit molecular mass, estimated by SDS-PAGE, was 82–83 kDa (four separate gels). The molecular mass of the native enzyme determined from its elution volume from a calibrated Sephacryl S300 column was approximately 150 kDa. These values are similar to those found by Meyer & Jordi (1987) for the dipeptidyl aminopeptidase from *Streptococcus thermophilus* (80 kDa and 160 kDa, respectively), by Kiefer-Partsch *et al.* (1989) for the enzyme from *Lactococcus lactis* subsp. *cremoris* P8-2-47 (90 kDa and 160–180 kDa, respectively) and by Zevaco *et al.* (1990) for the enzyme from *L. lactis* subsp. *lactis* NCDO 763 (85 kDa and 190 kDa, respectively). Booth *et al.* (1990b) reported a lower molecular mass (117 kDa) for the active form of the post-proline dipeptidyl aminopeptidase from *L. lactis* subsp. *cremoris* AM2.

Enzyme activity showed little variation over a broad pH range from 6 to 9; above this range a time-dependent inactivation of the enzyme was evident from the rate of hydrolysis of substrate although initial activity remained high until pH 10.

Enzyme activity was strongly inhibited by phenyl-methylsulphonyl fluoride (1 mM final concentration) but not by EDTA, iodoacetate or *o*-phenanthroline (all at 1 mM). These inhibition characteristics, indicative of an active-site serine residue, resemble those found by Meyer & Jordi (1987) for the *S. thermophilus* dipeptidyl peptidase and by Kiefer-Partsch *et al.* (1989), Booth *et al.* (1990b) and Zevaco *et al.* (1990) for the enzyme from lactococci. On the other hand, the dipeptidyl aminopeptidases from *Lactobacillus lactis* (Meyer & Jordi, 1987) and from *Lb. helveticus* (Khalid & Marth, 1990) were strongly inhibited by the thiol protease inhibitor *p*-chloromercuribenzoate.

Kinetic parameters of the enzyme with different dipeptidyl AMC substrates

While the specificity of X-prolyl dipeptidyl aminopeptidase for cleaving dipeptidyl residues from substrates in which proline is the penultimate N-terminal residue has been well established (McDonald & Schwabe, 1977), the influence of the N-terminal residue on the activity of the enzyme has not been quantitatively investigated for this enzyme from bacterial sources. Five X-prolyl AMC substrates were synthesized with different N-terminal amino acids representing the various types of amino acid side chain (basic, acidic, aromatic, hydrophobic). Values

Table 2. Kinetic parameters of X-prolyl dipeptidyl aminopeptidase from *L. lactis* subsp. *lactis* H1

Assays were carried out in 100 mM-MES buffer (pH 6.8) at 20 °C using eight different substrate concentrations covering the range 5–100 µM. Each assay contained 0.042 µg purified enzyme in a total volume of 2.0 ml. Substrate concentrations were corrected for the percentage purity determined as described in Methods. A subunit molecular mass of 83 kDa was used to calculate the concentration of enzyme in µmol ml⁻¹. Non-linear regression analysis of experimental data was performed using the program ENZFITTER (Leatherbarrow, 1987). Values in parentheses are the standard errors of fit to the data.

Substrate	K_m (µM)	k_{cat} (s ⁻¹)
Leu-Pro-AMC	9.2 (± 1.1)	43.8 (± 1.6)
Phe-Pro-AMC	12.4 (± 0.5)	78.4 (± 1.3)
Gly-Pro-AMC	14.1 (± 2.1)	69.8 (± 2.9)
Lys-Pro-AMC	14.6 (± 1.5)	61.6 (± 2.0)
Glu-Pro-AMC	45.7 (± 3.4)	62.9 (± 2.3)
Gly-Pro-pNA	107 (± 6.2)	62.1 (± 1.2)
Gly-Ala-AMC	385 (± 35)	2.1 (± 0.08)
Lys-Ala-AMC	231 (± 14)	17.1 (± 0.35)

of K_m and k_{cat} were determined using substrate concentrations covering the range 5–100 µM (Table 2). The rate of hydrolysis showed hyperbolic dependence on substrate concentration with all five substrates. The K_m values were very similar with the different substrates with the exception of Glu-Pro-AMC, which had a K_m value approximately three times that for the other substrates. The k_{cat} values were also very similar for four of the five substrates, that for Leu-Pro-AMC being somewhat lower than the values for the other substrates. Thus the nature of the N-terminal residue has relatively little influence on the rate of hydrolysis of X-Pro-AMC substrates. Comparison of the kinetic parameters for Gly-Pro-AMC and Gly-Pro-*p*-nitroanilide (*p*-NA) showed that the nature of the C-terminal leaving group has virtually no effect on maximum catalytic activity (k_{cat} values) but does affect binding (the K_m value for Gly-Pro-*p*-NA being significantly higher).

In a similar study of the influence of differing N-terminal amino-acyl residues of X-Pro-*p*-NAs on the kinetic parameters of dipeptidyl peptidase IV from pig kidney, Harada *et al.* (1985) found k_{cat} values ranging from 30 to 80 s⁻¹, i.e. of the same order as those found in the present study for the lactococcal enzyme (Table 2). The K_m values of the pig kidney enzyme (about 10 times those for lactococcal enzyme) were again very similar for the Lys-Pro-, Phe-Pro- and Gly-Pro-*p*-NAs, while that for Glu-Pro-*p*-NA was significantly lower (cf. the higher K_m for Glu-Pro-AMC relative to that for the other dipeptidyl AMC substrates found in the present study).

Fig. 1. Reversed-phase HPLC analysis of products of hydrolysis of oligopeptides by X-prolyl dipeptidyl aminopeptidase from *L. lactis* subsp. *lactis* H1. Peptides were detected by measuring the A_{220} of the eluate. The separations in (a) and (b) were obtained using a Waters 8 MBC 18 column and a 2 ml injection loop, those in (c) with a Vydac 218 TP C18 column and a 0.2 ml injection loop. (a) Time course of hydrolysis of the tetrapeptide Gly-Pro-Gly-Gly. Separation was performed using an isocratic solvent system containing 1% (v/v) acetonitrile and 0.1% TFA in water. Peak identity was established by amino acid analysis and co-elution with known dipeptides. Peak 1, Gly-Gly; peak 2, Gly-Pro; peak 3, Gly-Pro-Gly-Gly. (b) Time course of hydrolysis of the β -casein-derived heptapeptide Tyr-Pro-Phe-Pro-Gly-Pro-Ile. Buffer system: buffer A, 5% acetonitrile/95% water; buffer B, 80% acetonitrile/20% water. Both buffers contained 0.1% TFA. Separation of products was obtained using a linear gradient of 0% to 80% B over 60 min at 1 ml min⁻¹. Identity of peaks was established by

The kinetic parameters of the lactococcal dipeptidyl aminopeptidase for the hydrolysis of two X-alanyl-AMC substrates were also determined since other studies (Meyer & Jordi, 1987; Booth *et al.*, 1990b; Zevaco *et al.*, 1990) have shown that it has some activity with X-alanyl substrates as well as X-prolyl substrates. As seen from Table 2, the k_{cat} value with Gly-Ala-AMC was only 3% of that for Gly-Pro-AMC; the K_m value for Gly-Ala-AMC was 25 times that for Gly-Pro-AMC. However, with Lys-Ala-AMC the enzyme showed much higher activity, the k_{cat} value being 26% of that with Lys-Pro-AMC although the K_m value was still higher (15 times). Thus, unlike the situation with the X-Pro-AMC substrates, the nature of the N-terminal amino acid residues does have a marked effect on the kinetic parameters with X-Ala substrates.

Action of dipeptidyl peptidase on oligopeptides

The activity of the purified dipeptidyl aminopeptidase towards oligopeptides was studied by incubating it with the following peptides, separating the hydrolysis products using reversed-phase HPLC and identifying the products by amino acid analysis or sequencing.

The tetrapeptide Gly-Pro-Gly-Gly. The HPLC profile (Fig. 1a) shows a time course for the decrease in the tetrapeptide peak and the increase in the dipeptide products – Gly-Gly and Gly-Pro. The rate of hydrolysis was determined quantitatively by measuring the area of the tetrapeptide peak at different times relative to that at zero time (produced by the known initial concentration of tetrapeptide in the incubation mixture). By measuring the rate of hydrolysis at several different substrate concentrations the K_m value for the tetrapeptide was found to be 362 (± 24) $\mu\text{mol l}^{-1}$ (about 20 times higher than that for Gly-Pro-AMC); the k_{cat} value was 46.4 (± 1.0) s⁻¹ (approximately 65% of that for Gly-Pro-AMC). Thus, as noted above for Gly-Pro-*p*-NA, while the nature of the C-terminal leaving group markedly affects the affinity for the substrate it has relatively little effect on the maximum catalytic activity (k_{cat}).

amino acid composition. Peak 1, Tyr-Pro; peak 1a (shoulder on peak 1), Gly-Pro-Ile; peaks 2 and 2a, Phe-Pro; peak 3, heptapeptide substrate. (c) Time course of hydrolysis of the β -casein-derived heptapeptide Lys-Ala-Val-Pro-Tyr-Pro-Gln. Buffer system: buffer A, 0.1% TFA in water; buffer B, 0.1% TFA in acetonitrile. Separation of products was obtained using a linear gradient from 0% to 25% B over 25 min at 1 ml min⁻¹. Identity of peaks was established by amino acid sequence analysis. Peak 1, Val-Pro; peak 2, Tyr-Pro; peak 3, unidentified contaminant in starting material; peak 4, heptapeptide substrate.

The heptapeptide Tyr-Pro-Phe-Pro-Gly-Pro-Ile (fragment 60–66 of β -casein or β -casomorphin). This peptide occurs as part of one of the low molecular mass oligopeptides resulting from the cleavage of β -casein by the lactococcal cell wall proteinase (Monnet *et al.*, 1989). HPLC analysis (Fig. 1*b*) established progressive degradation of this peptide in a manner consistent with the X-prolyl aminopeptidase specificity of the enzyme. The products (identified by analysis of the collected peak fractions) were Tyr-Pro, Phe-Pro and Gly-Pro-Ile. Kiefer-Partsch *et al.* (1989) and Zevaco *et al.* (1990) also reported the degradation of β -casomorphin by a dipeptidyl aminopeptidase from lactococci. In both of these studies the C-terminal tripeptide, Gly-Pro-Ile, was found to be cleaved only slowly to Gly-Pro and Ile so the persistence of the tripeptide in the products found in the present study may have been due simply to insufficient time for complete digestion.

The heptapeptide Lys-Ala-Val-Pro-Tyr-Pro-Gln. This peptide occurs between residues 176 and 182 of β -casein and is one of the major low molecular mass peptides released in the early stages of digestion of β -casein by the cell wall proteinase from lactococci (Monnet *et al.*, 1986, 1989; Visser *et al.*, 1988). Incubation of this peptide with the purified dipeptidyl aminopeptidase resulted in disappearance of the peptide over a 24 h period (Fig. 1*c*). The dipeptides Tyr-Pro (peak 2, Fig. 1*c*) and Val-Pro (peak 1, Fig. 1*c*) were identified by sequence analysis. (The large size of the Tyr-Pro peak is simply a consequence of the high A_{220} of tyrosine.) The remaining products, Lys-Ala and the single glutamine, were presumably present in the large initial peak but were not identified. No pentapeptide or tripeptide intermediates were detected. The disappearance of the heptapeptide and accumulation of Tyr-Pro and Val-Pro indicates that the dipeptidyl aminopeptidase must have catalysed cleavage of the initial Lys-Ala dipeptide as predicted from the specificity studies with dipeptidyl AMC substrates (see Table 2) and from other studies (Meyer & Jordi, 1987; Booth *et al.*, 1990*b*; Zevaco *et al.*, 1990). Thus X-prolyl dipeptidyl aminopeptidase could be involved in the degradation of this oligopeptide derived from the action of the cell wall proteinase on β -casein. However, current evidence suggests an intracellular location for X-prolyl dipeptidyl aminopeptidase (Meyer *et al.*, 1989; Booth *et al.*, 1990*a*). Since peptides larger than four or five amino acid residues are not readily transported into lactococcal cells (Law, 1978; Rice *et al.*, 1978) it is unlikely that X-prolyl dipeptidyl aminopeptidase contributes to the initial cleavage of this heptapeptide unless there is significant leakage of the enzyme into the cell wall.

The nonapeptides Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (bradykinin) and its *Lys-Pro-Pro*-N-terminal analogue

(*Lys*¹-bradykinin). The N-terminal Arg-Pro (or Lys-Pro) dipeptide was not removed even after 72 h incubation with the dipeptidyl peptidase. A similar finding was reported by Booth *et al.* (1990*b*). The two consecutive prolines may impose a steric constraint on the ability of the enzyme to cleave the Pro-Pro bond.

The pentapeptide Arg-Lys-Asp-Val-Tyr (fragment 32–36 of thymopoietin II) and the hexapeptide *Arg-Tyr-Leu-Gly-Tyr-Leu* (fragment 90–95 of α -casein). These were not hydrolysed even after 72 h incubation, consistent with the specificity of the enzyme for peptide bonds involving only proline or alanine in the penultimate N-terminal position.

These studies confirm that the lactococcal enzyme catalyses hydrolysis of N-terminal X-prolyl and X-alanyl dipeptides from oligopeptides and not just from synthetic dipeptidyl amide substrates. Where the third, as well as the second, residue is also a proline, cleavage does not occur.

Comparison of dipeptidyl aminopeptidases from L. lactis subsp. lactis and L. lactis subsp. cremoris

Dipeptidyl aminopeptidase was purified from *L. lactis* subsp. *cremoris* H2 using the same procedure as that described for *L. lactis* subsp. *lactis* H1. A 277-fold purification was achieved, yielding an essentially pure product (a few minor contaminating bands were evident on polyacrylamide gels at high loadings) with a specific activity of 30.5 units (mg protein)⁻¹. The pH profile of the purified enzyme was the same as that for *L. lactis* subsp. *lactis* H1, as were the K_m values for the five different X-Pro AMC substrates. The '*cremoris*' enzyme migrated slightly more slowly on SDS-PAGE corresponding to an approximate subunit molecular mass of 86 kDa (cf. 82–83 kDa for the '*lactis* H1' enzyme).

The dipeptidyl aminopeptidase specific activities in the plasmid-free strain (4125) of *L. lactis* subsp. *lactis* H1, in the wild-type strain and in the transconjugant strain (4760) containing the H2 '*cremoris*' lactose/proteinase plasmid in the H1 '*lactis*' background were virtually identical (all three strains yielding a specific activity value of 0.11 units mg⁻¹ when grown in overnight culture in the M17 medium of Terzaghi & Sandine, 1975). This indicates that the dipeptidyl aminopeptidase is chromosomally encoded. The enzyme was also purified from the plasmid-free strain (to a specific activity of 36.6 units mg⁻¹) and shown to have identical properties (including kinetic parameters for the different X-prolyl-AMC substrates and mobility on SDS and non-denaturing gels) to that from the transconjugant strain. The specific

activity is very similar in cultures grown on reconstituted skim-milk medium and on M17 medium (0.13 and 0.11 units mg^{-1} , respectively). Thus dipeptidyl aminopeptidase activity is apparently not subject to regulation by the same factors which affect the synthesis of cell wall proteinase (Exterkate, 1979).

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