Purification and characterization of aminopeptidase P from *Lactococcus lactis* subsp. *cremoris*

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Summary. Aminopeptidase P was purified 65·3-fold from the cytoplasm of *Lactococcus lactis* subsp. *cremoris* AM2 with a 5·8% yield. The purified enzyme was found to consist of one polypeptide chain with a relative molecular mass of 41600. Metal chelating agents were found to be inhibitory and Mn^{2+} and Co^{2+} stimulated activity 7-fold and 6-fold respectively. The purified enzyme removed the N-terminal amino acid from peptides only where proline (and in one case alanine) was present in the penultimate position. No hydrolysis was observed either with dipeptides even when proline was present in the C-terminal position or when either N-terminal proline or pyroglutamate was present preceding a proline residue in the penultimate position of longer peptides. On the basis of this substrate specificity either aminopeptidase P or post-proline dipeptidyl aminopeptidase are necessary along with a broad specificity aminopeptidase to effect complete hydrolysis of casein-derived peptides containing a single internally placed proline residue. However, both aminopeptidase P and post-proline dipeptidyl aminopeptidase would be required together with a broad specificity aminopeptidase in order to completely hydrolyse casein-derived peptides that contain two internally placed consecutive proline residues. As bitter casein-derived peptides are likely to contain either single prolines or pairs of prolines, aminopeptidase P appears to be an important enzyme for debittering.

The proteolytic system of *Lactococcus* spp. has been shown to contain a variety of aminopeptidase enzymes having different substrate specificities. These include a dipeptidase (Van Boven *et al*. 1988), a tripeptidase (Bosman *et al*. 1990; Bacon *et al*. 1993), a number of broad specificity aminopeptidases (Neviani *et al*. 1989; Tan & Konings, 1990; Exterkate *et al*. 1992; Rul *et al*. 1994) and aminopeptidase A (Niven, 1991; Bacon *et al*. 1994). These enzymes have been shown to be incapable of hydrolysing bonds that immediately precede the amino acid proline. Specialized enzymes have evolved to hydrolyse bonds preceding and following proline residues. A number of these activities have been purified from lactococci and characterized. These include prolidase, a dipeptidase specific for dipeptides containing proline in the carboxy-terminus (Kaminogawa *et al*. 1984; Booth *et al*. 1990*b*), an aminopeptidase

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P from *Lactococcus lactis* that removes N-terminal amino acids from peptides containing proline in the penultimate position (Mars & Monnet, 1995) and two enzymes that cleave the bond following proline residues, a post-proline dipeptidyl aminopeptidase (PPDA) that removes a dipeptidyl moiety from the N-terminus of peptides where either proline or alanine is present in the penultimate position from the N-terminus (Booth *et al*. 1990*c*; Zevaco *et al*. 1990) and a peptidase that removes proline residues from the N-termini of dipeptides and tripeptides (Baankreis & Exterkate, 1991). Casein peptides are rich in proline and there are five instances where consecutive prolines occur in β-casein. Bitterness in cheese and in casein hydrolysates is thought to be associated with intermediate length casein-derived peptides. Broad specificity aminopeptidases such as aminopeptidase N will only hydrolyse these peptides up to the residue that precedes the proline. If only one proline residue is present, then either aminopeptidase P or PPDA can remove the blockage due to proline and allow aminopeptidase N to recommence hydrolysis (Booth *et al*. 1990*a*). When the peptide contains two consecutive proline residues PPDA cannot remove the X–Pro dipeptide (Booth *et al*. 1990*c*). This study was undertaken to establish whether the substrate specificity of an aminopeptidase P, purified from the cytoplasm of *Lc. lactis* subsp. *cremoris* AM2, would enable it to play a role in the hydrolysis of peptides containing consecutive prolines.

Materials and methods

Reagents

Lc. lactis subsp. *cremoris* AM2 and low-heat skim milk powder were provided by the National Dairy Products Research Centre, Moorepark, β-glycerophosphate, DNAase (bovine pancreas, type IV), RNAase (bovine pancreas, type III A), Tris(hydroxymethyl)aminomethane, fructose 1,6-diphosphate, NADH disodium salt, disodium ATP, pyruvic acid, iodoacetamide, 1,10-phenanthroline, 8-hydroxyquinoline, benzamidine, amastatin, bestatin, puromycin, bacitracin, phenylmethylsulphonylfluoride and *N*-ethylmaleimide were obtained from Sigma Chemical Co. (Poole BH17 7NH, UK). Diethylaminoethyl cellulose was supplied by Whatman (Maidstone ME14 2LE, UK). Ethylene glycol and precoated silica gel (type 60) thin layer chromatography plates were obtained from Merck (D-64293 Darmstadt 1, Germany). Hydroxyapatite Bio-Gel HTP was supplied by Bio-Rad Laboratories Ltd (Hemel Hempstead HP2 7TD, UK). Sephacryl S-200 high resolution gel and phenyl Sepharose CL-4B were obtained from Pharmacia Chemicals AB (S-751 28 Uppsala, Sweden). All peptides and peptide derivatives were obtained from Bachem Feinchemikalien (CH-4416 Bubendorf, Switzerland) with the exception of Leu–Pro–Pro, which was supplied by Peninsula Laboratories Europe Ltd (St Helens WA9 3AJ, UK).

Subcellular fractionation of Lactococcus lactis *subsp.* cremoris *AM2*

Lc. lactis subsp. *cremoris* AM2 was grown in reconstituted skim milk. The cells were harvested, washed and subjected to subcellular fractionation as described previously (Booth *et al*. 1989, 1990*b*). The presence of cell wall material in the fractions recovered was monitored by measurement of glucosamine by a modification (Aidoo *et al*. 1981) of the method of Rondle & Morgan (1955). The presence of cell membranes and cytoplasmic material was monitored by measurement of ATPase (Abrams, 1965) and lactate dehydrogenase activities (Wittenberger & Angelo, 1970) respectively.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) as modified by Hartree (1972), using bovine serum albumin as standard.

Determination of aminopeptidase P activity

Aminopeptidase P activity was determined using Leu–Pro–Pro as substrate. Enzyme preparation (50 μl) was preincubated with 50 mM-potassium phosphate buffer, pH 8·5 (30 μl) and 10 mM-$MnCl_2$ (10 μl) at 30 °C for 5 min. The reaction was initiated by addition of 5 mM-Leu–Pro–Pro (10 μl) and the leucine released after 20 min was determined by the ninhydrin method (Doi *et al.* 1981).

Purification of aminopeptidase P

The cytoplasmic fraction recovered during subcellular fractionation was dialysed at 4 °C against 50 mM-potassium phosphate buffer, pH 7·5 containing 10 ml glycerol/l (buffer A) and then applied to a DE52 anion-exchange column (60 × 22 mm) pre-equilibrated with buffer A. Aminopeptidase P activity was eluted with buffer A using a linear gradient of 0–0·25 M-NaCl at a flow rate of 15 ml/h. Fractions (2 ml) were collected and assayed for aminopeptidase activity as described above. Fractions containing activity were pooled, dialysed overnight at 4 °C against 1 mM-potassium phosphate–0·15 M-NaCl buffer, pH 7·2 containing 10 ml glycerol/l (buffer B) and applied to a hydroxyapatite (Biogel HTP) column (50 × 12 mm) pre-equilibrated with buffer B. The column was then washed with two column volumes of buffer B and enzyme activity was eluted in a linear gradient between buffer B and 100 mM-potassium phosphate–0·15 M-NaCl buffer, pH 7·2 containing 10 ml glycerol/l at a flow rate of 15 ml/h. Fractions (1 ml) were collected and assayed for aminopeptidase P activity. The active fractions were pooled and dialysed overnight at 4 °C against 50 mM-potassium phosphate–2 M-NaCl buffer, pH 7·0 containing 10 ml glycerol/l (buffer C) and applied to a phenyl Sepharose CL-4B column (10 × 22 mm) pre-equilibrated with buffer C. Two column volumes each of the equilibrating buffer and 50 mM-potassium phosphate buffer, pH 7·0 containing 10 ml glycerol/l (buffer D) were applied. The column was then washed with one column volume of buffer D with 300 ml ethylene glycol/l followed by two column volumes of buffer D with 500 ml ethylene glycol/l. Fractions (1 ml) were collected at a flow rate of 15 ml/h and activity was determined. The fractions containing activity were pooled, dialysed against buffer A at 4 °C overnight and stored at −20 °C.

Determination of relative molecular masses of the purified enzyme and subunits

The M_r of aminopeptidase P was determined by gel filtration of the purified enzyme on a Sephacryl S-200 column (913 × 27 mm). The running buffer was 50 mM-Tris-HCl–0·1 M-NaCl buffer, pH 7·5. The column was calibrated using (M_r in parentheses) ferritin (400000), aldolase (158000), bovine serum albumin (67000), ovalbumin (43000), ribonuclease A (13700) and cytochrome c (12700). Aminopeptidase P was eluted from the column in 3 ml fractions at a flow rate of 16 ml/h and the M_r computed.

To determine the M_r of subunits, SDS-PAGE was performed as described by Laemmli (1970) using acrylamide gels (100 g/l). Coomassie blue R-250 was used to visualize the protein bands and the M_r of aminopeptidase P was estimated by reference to the migration of protein standards (M_r in parentheses) phosphorylase b (94000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100) and α-lactalbumin (14000).

Effect of inhibitors and divalent metal ions

Purified enzyme (50 μl) was preincubated with inhibitor (10 mM, 10 μl) or metal ion (10 mM, 10 μl) and 50 mM-potassium phosphate buffer, pH 8·5 (30 μl) at 30 °C for 15 min. The reaction was initiated by the addition of 5 mM-Leu–Pro–Pro (10 μl) and terminated after 20 min. Appropriate controls were set up with 10 μl water, acetone or ethanol replacing inhibitor or metal ion.

Effect of pH

The effect of pH on aminopeptidase P activity was tested using 100 mM-citric acid buffer (pH 7·0–7·6), 100 mM-Tris-HCl buffer (pH 7·1–8·9) and 100 mM-sodium borate buffer (pH 8·6–10·6) instead of the normal assay buffer. The experiment was repeated with the same buffers containing 50 g NaCl/l.

Substrate specificity of aminopeptidase P and determination of kinetic constants

Purified aminopeptidase P in 50 mM-potassium phosphate buffer, pH 8·5 (50 μl) was incubated with 50 μl of each peptide to be tested (4 mM in water) at 30 °C for 16 h. Then 30 μl was applied to a thin layer plate precoated with silica gel G 60. The chromatograms were developed in vapour-saturated tanks using either butan-1-ol–formic acid–water (20:6:5 by vol.) or chloroform–methanol–ammonia (350 g/l)–water (125:75:18:7 by vol.) Appropriate standards were cochromatographed. Both standards and reaction products were visualized using ninhydrin (10 g/l) in acetone containing trichloroacetic acid (170 g/l) followed by heating to 105 °C for 5 min.

Kinetic constants for aminopeptidase P were investigated using a range of peptides diluted to give a final concentration range of 0·25–5·0 mM. Standard incubations in triplicate were set up together with appropriate enzyme and substrate blanks. Leucine release was determined as described previously and K_m and V_{max} values were determined by Eadie–Hofstee analysis (Eadie, 1942; Hofstee, 1952).

RESULTS

Cultures of *Lc. lactis* subsp. *cremoris* AM2 were harvested and resolved into cell wall, cell membrane and cytoplasmic fractions. Aminopeptidase P activity was found to be predominantly associated with the soluble cytoplasmic fraction. The enzyme was purified using a three step procedure involving chromatography on DE52, hydroxyapatite HTP and phenyl Sepharose (Table 1). One protein band was observed when the purified enzyme was subjected to electrophoresis on polyacrylamide gels under native conditions. From chromatography of the purified enzyme on a calibrated Sephacryl S-200 column the M_r was 41600. When the purified enzyme was subjected to SDS-PAGE under reducing conditions, one protein band was observed with a mobility corresponding to an M_r of 40000. This value was similar to that reported for the enzyme from *Lc. lactis* (Mars & Monnet, 1995).

When the purified aminopeptidase P was incubated with a wide range of inhibitors (all at 1 mM) only the chelating agents were found to be strongly inhibitory (Table 2), indicating that the enzyme was a metalloenzyme. No inhibition was found with 1 mM-dithiothreitol, whereas it almost completely inhibited the enzyme from *Lc. lactis* (Mars & Monnet, 1995). The activity of aminopeptidase P was measured in the presence and absence of various metal ions. Ba^{2+} was inhibitory and Mn^{2+} and Co^{2+} stimulated activity 7-fold and 6-fold respectively (Table 2). A pH optimum of pH 8·5 was found for the enzyme and this value was unaffected by the presence of

Table 1. *Purification of aminopeptidase P from* Lactococcus lactis *subsp.* cremoris *AM2*

Sample	Total activity, nmol/min	Total protein, mg	Specific activity, nmol/min per mg	Yield, %	Purification
Cytoplasm	1620·0	78·7	20·7	100·0	1·0
DE52 chromatography	522·0	3·0	176	32·2	8·4
Hydroxyapatite chromatography	256·0	1·3	197	15·8	9·6
Phenyl Sepharose chromatography	94·0	0·1	1343	5·8	65·3

Table 2. *Effect of inhibitors and divalent metal ions on activity of purified aminopeptidase P from* Lactococcus lactis *subsp.* cremoris *AM2*

Inhibitor or metal ion, 1 mM	Residual activity, %
Untreated control	100
N-ethylmaleimide	96
Iodoacetamide	94
EDTA	54
8-Hydroxyquinoline	7
1,10-Phenanthroline	7
Bacitracin	100
Puromycin	100
Benzamidine	96
Phenylmethylsulphonylfluoride	92
Bestatin	58
Amastatin	60
Dithiothreitol	100
$BaCl_2$	27
$CoCl_2$	600
$MgCl_2$	88
$MnCl_2$	680
$NiCl_2$	126
$ZnCl_2$	205

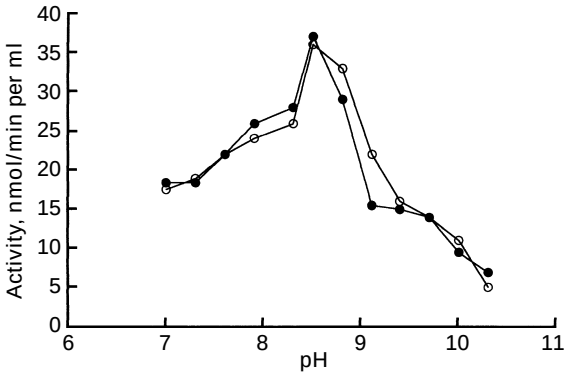

Fig. 1. pH profile of aminopeptidase P activity in the ○, presence and ●, absence of 50 g NaCl/l using Leu–Pro–Pro as substrate. The buffers used are given in the text.

50 g NaCl/l (Fig. 1). When portions of purified aminopeptidase P that had been incubated at 8 °C in 100 mM-sodium acetate buffer, pH 5·2 containing 50 g NaCl/l were assayed at intervals, a half life of 5 d was found for aminopeptidase P activity.

The purified enzyme was tested for its ability to remove the N-terminal amino

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Table 3. *Substrate specificity of aminopeptidase P from* Lactococcus lactis *subsp.* cremoris *AM2*

Peptides hydrolysed	
Leu–Ala–Pro	Gly–Pro–Gly–Gly
Leu–Pro–Pro	Gly–Pro–Arg–Pro
Ala–Pro–Gly	Tyr–Pro–Phe
Lys–Pro–Arg	Tyr–Pro–Phe–Pro
Arg–Pro–Pro	Tyr–Pro–Phe–Pro–Gly
Arg–Pro–Pro–Gly–Phe–Ser	Tyr–Pro–Phe–Pro–Gly–Pro–Ile
Arg–Pro–Pro–Gly–Phe–Ser–Pro	Asp–Pro–Gly–Phe–Tyr
Arg–Pro–Lys–Pro	
Arg–Pro–Lys–Pro–Gln–Gln–Phe–Phe–Leu–Gly–Met-NH_2	
Peptides not hydrolysed	
Ala–Leu	Leu–Trp–Met
Leu–Ser	Leu–Leu–Leu
Leu–Trp	Lys–Ala–Phe
Gly–Leu	Glu–Val–Phe
Gly–Ala	Glu–Thr–Tyr
Gly–Pro	Glu–His–Gly
Ala–Pro	Gly–Ala–Phe
Lys–Pro	Tyr–Lys–Thr
Pro–Pro	Asp–Arg–Val–Tyr
Phe–Leu	Pro–Leu–Gly–NH_2
Pro–Ser	Lys–Phe–Gly–Lys
Pro–Pro–Pro	Gly–Arg–Gly–Asp
Leu–Gly–Gly	Pro–Pro–Gly–Phe–Ser–Pro
Phe–Gly–Gly	Glp–Pro–Ser–Lys–Asp–Ala–Phe–Ile–Gly–Leu–Met-NH_2

Table 4. *Kinetic constants derived from the interaction of various peptides with aminopeptidase P from* Lactococcus lactis *subsp.* cremoris *AM2*

Peptide	K_m, mM	V_{max}, nmol/min per mg
Leu–Pro–Pro	0·9	102
Arg–Pro–Pro	1·4	124
Tyr–Pro–Phe	1·2	124
Tyr–Pro–Phe–Pro–Gly	1·6	140
Tyr–Pro–Phe–Pro–Gly–Pro–Ile	1·4	127

acid from a wide range of peptides. The results presented in Table 3 indicate that the purified enzyme removed the N-terminal amino acid only from peptides that contained proline (and in one case alanine) in the penultimate position from the N-terminus. Provided that this requirement was met the enzyme was found to act on peptides that contained between three and eleven amino acids. Table 3 also lists the peptides that were not hydrolysed by aminopeptidase P and includes peptides that did not contain proline in the penultimate position from the N-terminus. Longer peptides that commenced with either proline or pyroglutamate and where proline was in the penultimate position from the N-terminus were not hydrolysed. Dipeptides were not hydrolysed even if proline was present in the second position from the N-terminus. Two peptides that contained alanine in the penultimate position from the N-terminus were also not hydrolysed by the purified aminopeptidase P.

Table 4 presents K_m and V_{max} values obtained from the action of purified aminopeptidase P on a number of peptides. Neither value varied appreciably when the enzyme was presented with members of the β-caseomorphin series of peptides of increasing length.

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DISCUSSION

In mammalian systems aminopeptidase P activity is present in both soluble (Harbeck & Mentlein, 1991) and particulate (Hooper *et al*. 1990) forms. The present enzyme was largely cytoplasmic, as is the case for the other reported bacterial enzymes (Yoshimoto *et al*. 1988; Mars & Monnet, 1995). The M_r of 40000 for the present enzyme agrees well with the value of 43000 reported for the enzyme from *Lc. lactis* (Mars & Monnet, 1995) but is much less than that reported for *Escherichia coli* (Yoshimoto *et al*. 1988) or mammalian enzymes (Harbeck & Mentlein, 1991). The present enzyme appeared to be a metalloenzyme, stimulated by Mn^{2+} and Co^{2+} like the enzyme from *Lc. lactis* (Mars & Monnet, 1995) and those from other sources (Chen & Buchanan, 1980; Hooper *et al*. 1990). Unlike the *Lc. lactis* enzyme (Mars & Monnet, 1995), the present one was not inhibited by dithiothreitol, which may be because a different substrate was used. Aminopeptidase P activity from *Neisseria gonorrhoeae* (Chen & Buchanan, 1980) had a pH optimum of 7·5, while that from *Lc. lactis* had an optimum pH of 8·0 (Mars & Monnet, 1995) and the *Esch. coli* activity (Yaron & Mylnar, 1968) displays optimum activity at pH 8·6. It is possible that these differences reflect the different substrates and assay conditions used.

In comparing the substrate specificities of previously reported preparations of aminopeptidase P with that of the present enzyme, a number of features need to be considered. Firstly, in common with particulate activities from rat and bovine lung (Yoshimoto *et al*. 1994) and with a soluble activity from *Lc. lactis* (Mars & Monnet, 1995) the present enzyme did not hydrolyse dipeptides with proline in the C-terminal position. Other preparations from mammalian and bacterial sources are found to hydrolyse aminoacyl proline dipeptides (Chen & Buchanan, 1980; Harbeck & Mentlein, 1991; Maruyama *et al*. 1994; Yoshimoto *et al*. 1994). Secondly, peptides containing alanine in the second position from the N-terminus are hydrolysed but at a much slower rate than peptides where proline replaces alanine (Harbeck & Mentlein, 1991). Aminopeptidase P preparations from *Esch. coli* and from rat and bovine lung (Yoshimoto *et al*. 1994) also show little or no ability to hydrolyse X–Ala–Y peptides. Thirdly, tolerance was shown towards the residue at the carboxy side of the proline residue in the present study and in studies with preparations from cytoplasm of rat brain (Harbeck & Mentlein, 1991) and from *Esch. coli* (Yoshimoto *et al*. 1994). Mars & Monnet (1995) suggest that lactococcal aminopeptidase P attacks peptides commencing with X–Pro–Pro sequences in preference to those commencing with X–Pro–Y sequences. In the present study, amino acid release was only measured qualitatively to construct Table 3, so it was not possible to present an order of reactivity for substrates that were hydrolysed. It was also impossible to make any assessment as to whether X–Pro–Pro peptides were more favoured substrates relative to X–Pro–Y peptides. Fourthly, the aminopeptidase P from *Lc. lactis* subsp. *cremoris* AM2 could not release N-terminal Pro from Pro–Pro–X– sequences. However, a particulate activity from bovine brain can release N-terminal Pro from sequences where Pro is also present in the penultimate position (Maruyama *et al*. 1994), and this property has also been reported for the soluble activity from rat brain (Harbeck & Mentlein, 1991), albeit at a much slower rate than with peptides that contain other amino acids in the N-terminal position.

Bitterness in cheese and in proteinase-induced casein hydrolysates is thought to be due to certain casein-derived peptides (Stadhouders & Hup, 1975). Caseins are known to be enriched in proline and it has been shown that many bitter peptides also contain proline (Shinoda *et al*. 1986). The mechanism for reducing bitterness will

depend on the presence of proline-specific peptidases as most peptidases reported for lactococci are unable to hydrolyse the imido bond. Hydrolysis of proline-containing peptides by broad specificity aminopeptidases will therefore continue only to the residue that precedes the proline. PPDA can remove the N-terminal aminoacyl moiety as a dipeptide (Booth *et al.* 1990*c*). Alternatively, aminopeptidase P can remove the N-terminal amino acid before the proline. After either event a broad specificity aminopeptidase can then hydrolyse the resultant peptide. When the peptide to be hydrolysed contains two consecutive proline residues, broad specificity aminopeptidases will only hydrolyse the peptide to the residue preceding the two consecutive prolines. The resultant peptide now contains an X–Pro–Pro– sequence at its N-terminus. PPDA cannot cleave the imido bond linking the two prolines. Aminopeptidase P could remove the N-terminal (X) residue but, as reported in this paper, it could not continue the hydrolysis by removing the first proline even with proline in the second position. PPDA can however remove the Pro–Pro moiety as a dipeptide and allow a broad specificity aminopeptidase to continue the hydrolysis of the peptide. Thus, restrictions in the specificities of both PPDA and aminopeptidase P appear to require that both these enzymes are present for peptides containing two internally sited consecutive proline residues to be hydrolysed.

It has recently been shown that a number of lactococcal strains that are used as starters in Cheddar cheesemaking can lyse early in the ripening process and release their cytoplasmic enzymes into the cheese matrix (Wilkinson *et al.* 1994). While aminopeptidase P from *Lc. lactis* subsp. *cremoris* AM2 has a high pH optimum it nevertheless expressed activity ($\sim 26\%$ of that recorded at pH 8·5) under the conditions found in ripening cheese. In addition, the activity was found to survive several days when incubated under conditions that mirrored those of ripening cheese. It is therefore possible that this enzyme may participate in peptide degradation following release into the cheese matrix and may thus contribute to the abolition of bitterness and to the ripening of cheese.

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