Purification and characterization of a lysine-*p*-nitroanilide hydrolase, a broad specificity aminopeptidase, from the cytoplasm of *Lactococcus lactis* subsp. *cremoris* AM2

By MAEVE Mc DONNELL*, PAUL BOUCHIER†, RICHARD J. FITZGERALD† and GERARD O'CUINN‡§

**Biochemistry Department, National University of Ireland, Galway, Irish Republic*
†*National Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork, Irish Republic*
‡*Department of Life Sciences, Galway–Mayo Institute of Technology, Galway, Irish Republic*

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Summary. A hydrolase activity that cleaves lysyl-*p*-nitroanilide (Lys-pNA) has been purified from the cytoplasm of *Lactococcus lactis* subsp. *cremoris* AM2 by chromatography on DE52, DEAE Affi-Gel Blue Gel, Hydroxyapatite Bio-Gel HTP and Phenyl Sepharose. The purified aminopeptidase was found to have a native M_r of 50000–55000 by gel filtration chromatography and by FPLC gel filtration on Superose 12 and to be composed of a single polypeptide chain following SDS-PAGE. Enzyme activity was almost completely inhibited by EDTA, amastatin, puromycin and bestatin, while the sulphydryl-reactive agents *p*-chloromercuribenzoate and iodoacetamide were inhibitory. The enzyme was found to be very unstable during the purification procedures at 4 °C and its stability was greatly improved when 10 ml glycerol/l and 2 mM-dithiothreitol were included in the purification buffers. The purified enzyme was found to hydrolyse a wide range of dipeptides, tripeptides and longer peptides provided that proline was not present in the penultimate position from the N-terminus or that a pyroglutamyl residue was not present at the N-terminus. While neither Asp-pNA nor Pro-pNA was hydrolysed by the purified enzyme, the release of N-terminal acidic residues from peptides was observed in addition to the release of N-terminal proline from Pro–Leu–Gly–NH_2, Pro–Leu–Gly–Gly and Pro–His–Pro–Phe–His–Leu–Phe–Val–Tyr. This ability of Lys-pNA hydrolase to release N-terminal proline residues was employed in concert with a purified aminopeptidase P preparation to release alternate N-terminal amino acids from Tyr–Pro–Phe–Pro–Gly. The complementary action of these enzymes represents an alternative mechanism to that of post-proline dipeptidyl aminopeptidase for metabolism of proline-containing peptides.

The initial stages of casein breakdown during cheese manufacture are mediated by cell wall proteinases derived from the starter cell culture in conjunction with chymosin and endogenous plasmin. Many of the peptides produced are small enough to be transported into the starter cells together with the free amino acids that are

§ For correspondence.

also produced. It has recently been shown that the genes encoding some aminopeptidases in starter cultures do not contain signal sequences required for transport to the outside or to the surface of the cell (Van Alen-Boerrigter *et al*. 1991; Chapot-Chartier *et al*. 1993). This suggests that casein-derived peptides, which are not small enough to be transported into the starter cells, are not further degraded by aminopeptidase action at this stage. Those peptides that do gain access to the starter cells are then hydrolysed by intracellular aminopeptidases and dipeptidases to free amino acids, which are necessary for cell growth (Mills & Thomas, 1981).

It is now apparent that certain strains of starter cultures lyse during the ripening process, releasing their contents into the cheese matrix (Wilkinson *et al*. 1994). This brings the partly digested peptides of the cheese matrix into contact with the range of cytoplasmic aminopeptidases and dipeptidases. These include aminopeptidases A, C, N and P, prolyl aminopeptidase, post-proline dipeptidyl aminopeptidase, tripeptidase, dipeptidase and prolinase (Kunji *et al*. 1996). While the cheese matrix may not provide optimum conditions for the activity of the released cytoplasmic enzymes, they may be able to make a contribution to the hydrolysis of bitter peptides and to the provision of free amino acids which may be further metabolized to flavour-providing compounds. In the present study we examined one such cytoplasmic enzyme, a lysyl-*p*-nitroanilide (Lys-pNA) hydrolase and compared its properties with those of previously reported aminopeptidases.

Materials and methods

Reagents

Lactococcus lactis subsp. *cremoris* AM2 and low heat skim-milk powder were provided by the National Dairy Products Research Centre (Moorepark, Fermoy, Co. Cork, Irish Republic). 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, *N*-ethylmaleimide, *p*-chloromercuribenzoate, dithiothreitol (DTT) and phenylmethylsulphonylfluoride were obtained from Sigma Chemical Company (Poole BH17 7NH, UK). Diethylaminoethyl (DEAE) cellulose anion exchanger and 0·2 μm syringe filters were supplied by Whatman (Maidstone ME14 2LE, UK). Precoated silica gel type 60 plates and ethylene glycol were obtained from Merck (D-64293 Darmstadt 1, Germany). Bio-Rad Laboratories (Hemel Hempstead HP2 7TD, UK) supplied Hydroxyapatite Bio-Gel HTP and DEAE Affi-Gel Blue Gel. Sephacryl S-100 high resolution gel, Superose 12 (HR 10/30), Phenyl Sepharose CL4B, and gel electrophoresis and gel filtration molecular mass markers were obtained from Pharmacia Chemicals AB (S-751 82 Uppsala, Sweden). Immobilon-P membrane and 0·45 μm HA (low protein binding) filter were supplied by Millipore (UK) Ltd (Watford WDY 8YW, UK). 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). Coomassie protein assay reagent was supplied by Pierce & Warringer (UK) Ltd (Chester CH1 4EF, UK).

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

Lc. lactis subsp. *cremoris* AM2 was grown in reconstituted skim milk, harvested, washed and subjected to subcellular fractionation as described previously (Booth *et al*. 1990*a*).

Protein determination

Protein was determined by a modification of the Bradford (1976) method, using the Pierce Coomassie protein assay reagent.

Determination of enzyme activity

Lys-pNA hydrolysing activity was measured by a modification of the method of Exterkate (1975) using Lys-pNA as substrate. Enzyme preparation (50 μl) was incubated with 50 mM-potassium phosphate buffer, pH 7·5 (400 μl) at 30 °C; after 5 min preincubation the reaction was initiated by the addition of 50 μl 20 mM-Lys-pNA. The reaction was terminated after 30 min at 30 °C by the addition of 1 ml 1·5 M-acetic acid. The absorbance of the liberated *p*-nitroaniline was measured at 410 nm and its concentration calculated using a molar extinction coefficient of 8800 M^{-1} cm^{-1} (Niven *et al.* 1995). The release of *p*-nitroaniline from Lys-pNA was found to be linear with respect to time over 30 min. Post-proline dipeptidyl aminopeptidase was assayed by incubating 50 μl sample with 450 μl 0·11 mM-Gly–Pro-7-amido-4-methylcoumarin in 0·1 M-potassium phosphate buffer, pH 7·5 at 30 °C. After 15 min the reaction was terminated by addition of 1 ml 1·5 M-acetic acid and the liberated 7-aminocoumarin was determined fluorimetrically using excitation and emission wavelengths of 370 and 440 nm respectively.

Stabilization of enzyme activity

As Lys-pNA hydrolase proved to be highly unstable the effect of various agents on its stability was investigated. A cytoplasmic fraction was dialysed against 50 mM-potassium phosphate, pH 7·5 (buffer 1) and applied to a DE52 (27 × 27 mm) anion-exchange column pre-equilibrated with buffer 1. The activity was eluted in a linear salt gradient and fractions containing Lys-pNA hydrolase activity were pooled and divided into aliquots. These aliquots were diluted 1:2 with buffer 1 and with buffer 1 containing each of the following: 20 g bovine serum albumin/l, 4 mM-DTT alone, 600 ml glycerol/l alone, and 4 mM-DTT containing glycerol at 600, 300, 150, 70 and 20 ml/l. These mixtures were stored at −20, 4 and 18 °C and assayed on days 0, 2, 4 and 6.

Purification of enzyme

The cytoplasmic fraction recovered from the subcellular fractionation of *Lc. lactis* subsp. *cremoris* AM2 was dialysed at 4 °C against 2 mM-DTT–50 mM-potassium phosphate, pH 7·5 containing 10 ml glycerol/l (buffer A) and then applied to a DE52 anion-exchange column (27 × 27 mm) pre-equilibrated with buffer A. Lys-pNA hydrolase activity was eluted with a linear gradient established between buffer A and buffer A containing 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 2 mM-DTT–50 mM-potassium phosphate, pH 6·8 containing 10 ml glycerol/l (buffer B) and applied to a DEAE Affi-Gel Blue Gel (a bifunctional affinity–ion-exchange chromatography matrix) column (35 × 17 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 buffer B containing 0·25 M-NaCl at a flow rate of 15 ml/h. Fractions (2 ml) were collected and assayed for aminopeptidase activity. The fractions containing activity were pooled and dialysed overnight at 4 °C against 2 mM-DTT–1 mM-potassium phosphate–0·15 M-KCl, pH 7·2

containing 10 ml glycerol/l (buffer C) and applied to a hydroxyapatite column (30 × 13 mm). The column was then washed with two column volumes of buffer C and enzyme activity was eluted in a linear gradient between buffer C and 2 mM-DTT–300 mM-potassium phosphate–0·15 M-KCl, pH 7·2 containing 10 ml glycerol/l. Fractions (1 ml) were collected and assayed for Lys-pNA hydrolase activity. The active fractions were pooled and dialysed overnight at 4 °C against 2 mM-DTT–50 mM-potassium phosphate–2 M-NaCl, pH 7·0 containing 10 ml glycerol/l (buffer D) and then applied to a Phenyl Sepharose CL4B (a hydrophobic interaction chromatography matrix) column (22 × 12 mm) pre-equilibrated with buffer D. This was washed with two column volumes each of the equilibrating buffer and 2 mM-DTT–50 mM-potassium phosphate, pH 7·0 containing 10 ml glycerol/l (buffer E). The column was then washed with two column volumes of buffer E containing 500 ml ethylene glycol/l followed by two column volumes of buffer E containing 700 ml ethylene glycol/l. Fractions (1 ml) were collected at a flow rate of 15 ml/h and Lys-pNA hydrolase activity was determined. The fractions containing activity were pooled, dialysed against buffer A at 4 °C overnight and stored at −20 °C.

Purification of aminopeptidase P

Aminopeptidase P was purified from *Lc. lactis* subsp. *cremoris* AM2 as described previously (Mc Donnell *et al.* 1997).

Determination of relative molecular mass

The M_r of Lys-pNA hydrolase was determined by gel filtration on a Sephacryl S-100 column (913 × 27 mm); the running buffer was 2 mM-DTT–50 mM-potassium phosphate–0·1 M-NaCl, pH 7·5 containing 10 ml glycerol/l. The column was calibrated using albumin (67000), ovalbumin (43000), chymotrypsinogen A (25000) and cytochrome C (12700). Lys-pNA hydrolase activity was eluted from the column in 3 ml fractions at a flow rate of 16 ml/h and the M_r computed.

The M_r was also determined on a calibrated Superose 12 FPLC gel permeation column. The Lys-pNA hydrolase was brought through the first three chromatography steps of the purification procedure, dialysed against 2 mM-DTT–50 mM-potassium phosphate, pH 7·5 containing 10 ml glycerol/l and stored at −20 °C until applied to the Superose 12 column. The sample was diluted 1:2 (total volume 600 μl) and filtered through a 0·2 μm Whatman filter; then 200 μl was applied to the column, which had been pre-equilibrated with 0·1 M-Tris-HCl–0·1 M-NaCl, pH 7·0 containing 100 ml methanol/l which had been filtered through a 0·45 μm Millipore filter. The Lys-pNA hydrolase was eluted from the column in 1 ml fractions and the eluted fractions were assayed for Lys-pNA hydrolase activity and the M_r computed.

To determine the M_r of subunits, SDS-PAGE was performed as described by Laemmli (1970) using acrylamide gels of 100 g/l. Coomassie blue R-250 was used to visualize the protein bands and the M_r was estimated by reference to the migration of protein standards: thyroglobulin (300000), ferritin half unit (220000), albumin (67000), catalase (60000), lactate dehydrogenase (30000) and ferritin (18700).

Electroblotting and N-terminal protein sequencing

Purified Lys-pNA hydrolase (1 ml after Phenyl Sepharose, containing 45 μg protein) was dialysed overnight at 4 °C against Millipore water, freeze dried and stored at −70 °C until SDS-PAGE was performed. Following the electrophoresis run, the protein was electroblotted using an Immobilon-P membrane. The N-terminal sequence was determined on an Applied Biosystems model 477 A protein

sequencer (Applied Biosystems, Foster City, CA94404, USA) at 50 °C with detection at 269 nm and analysed using an Applied Biosystems analyser (model 120 A).

Effect of pH

The effect of pH on Lys-pNA hydrolase was tested using 100 mM-sodium citrate (pH 4·6–7·6), 100 mM-Tris-HCl (pH 7·0–8·8) and 100 mM-sodium borate (pH 8·8–9·7) in place of the normal assay buffer.

Effect of chemical reagents and divalent metals

Purified enzyme (50 μl) was preincubated with chemical or metal ion (10 mM, 50 μl) and 50 mM-potassium phosphate, pH 7·5 (350 μl) for 15 min at 30 °C. The reaction was initiated by the addition of 20 mM-Lys-pNA (50 μl) and terminated after 30 min. Appropriate controls were set up in which 50 μl water, acetone or ethanol replaced the material being investigated. Dilutions of selected inhibitors were incubated with Lys-pNA hydrolase and inhibitor concentrations giving 50 % inhibition of activity (IC_{50}) values were calculated.

*Substrate specificity of lysyl-*p*-nitroaniline hydrolase*

Purified Lys-pNA hydrolase in 50 μl 2 mM-DTT–50 mM-potassium phosphate, pH 7·5 containing 10 ml glycerol/l was incubated with 50 μl of each peptide to be tested (4 mM in water) for 16 h at 30 °C. Then 30 μl of each incubate was applied to a thin layer chromatography plate precoated with silica gel G60. The chromatograms were developed in vapour-saturated tanks using butanol–formic acid–water (20:6:5 by vol.), chloroform–methanol–ammonia (350 g/l)–water (125:75:18:7 or 120:75:10:7 by vol.), butanol–acetic acid–water (200:100:100 by vol.) or isopropanol–ethyl acetate–acetic acid (50 ml/l) (100:100:50 by vol.). Appropriate amino acid and peptide standards were co-chromatographed. Both standards and reaction products were visualized by spraying the thin layer chromatography plate using ninhydrin (10 g/l in acetone) containing 170 g trichloroacetic acid/l followed by heating to 105 °C for 5 min.

Enzyme activity on various p*-nitroanilide substrates*

Purified Lys-pNA hydrolase (50 μl) was preincubated with 50 mM-potassium phosphate, pH 7·5 (350 μl) at 30 °C for 5 min. The reaction was initiated by the addition of various *p*-nitroanilide substrates (50 μl) and terminated after 30 min by addition of 1·5 M-acetic acid. Controls were carried out in which 50 μl enzyme was added after adding 1·5 M-acetic acid or 50 μl deionized water replaced the enzyme. In the latter case, no spontaneous release of *p*-nitroanilide could be detected after 30 min.

*Sequential hydrolysis of Tyr–Pro–Phe–Pro–Gly with purified aminopeptidase P and purified lysyl-*p*-nitroaniline hydrolase*

Purified aminopeptidase P (100 μl) was incubated with Tyr–Pro–Phe–Pro–Gly (100 μl, 4 mM in water) for 16 h at 30 °C. The reaction was stopped by heating at 95 °C for 1 min and 30 μl incubate (product A) was stored at −20 °C. Product A (50 μl) was then incubated with purified Lys-pNA hydrolase (50 μl) for 16 h at 30 °C; the reaction was terminated by heating at 95 °C for 1 min and 30 μl incubate (product B) was stored at −20 °C. Product B (50 μl) was then incubated with purified aminopeptidase P (50 μl) for 16 h at 30 °C; the reaction was terminated by heating at 95 °C for 1 min and 30 μl incubate (product C) was stored at −20 °C. Portions (30 μl) of products A, B and C were applied to a thin layer chromatography

plate precoated with silica gel G60. The chromatograms were developed in vapour-saturated tanks using chloroform–methanol–ammonia (350 g/l)–water (120:75:10:7 by vol.) and appropriate standards were co-chromatographed. Both standards and reaction products were visualized by spraying the plates with ninhydrin (10 g/l in acetone containing 170 g trichloroacetic acid/l) followed by heating at 105 °C for 5 min.

Stability of purified enzyme at low temperatures and acidic pH

Purified Lys-pNA hydrolase was dialysed overnight at 4 °C against 100 mM-sodium acetate, pH 5·2 containing 50 g NaCl/l. The dialysed Lys-pNA hydrolase preparation was stored at 8 °C and samples were withdrawn at 6 h and 1, 2, 3 and 7 d and assayed for residual aminopeptidase activity.

RESULTS

Cultures of *Lc. lactis* subsp. *cremoris* AM2 were harvested and resolved into cell wall, cell membrane and cytoplasmic fractions. Lys-pNA hydrolase activity was found predominantly in the soluble cytoplasmic fraction. Lys-pNA hydrolase was highly unstable and could only be carried through the four chromatography steps in the presence of stabilizing agents. The results of stability studies indicated that the inclusion of both 10 ml glycerol/l and 2 mM-DTT in the running buffers maintained the stability of the enzyme during purification procedures carried out at 4 °C. Lys-pNA hydrolase was stable for up to 6 d at 4 °C in the presence of the buffer with 10 ml glycerol/l and 2 mM-DTT. In the first purification step, dialysed cytoplasm was chromatographed on a DE52 anion-exchange column. The major peak of Lys-pNA hydrolase eluted in 2 mM-DTT–50 mM-potassium phosphate–0·15 M-NaCl, pH 7·5 containing 10 ml glycerol/l, whereas a second minor peak of Lys-pNA hydrolase activity (~ 25% of the total recovered Lys-pNA hydrolase) eluted from the DE52 column in 2 mM-DTT–50 mM-potassium phosphate–0·25 M-NaCl, pH 7·5. The major peak of Lys-pNA hydrolase was selected for further purification and the overall purification scheme involved chromatography on DE52, DEAE Affi-Gel Blue Gel, Hydroxyapatite HTP and Phenyl Sepharose CL4B (Table 1). The Lys-pNA hydrolase eluted from the hydroxyapatite column at 0·09 M-potassium phosphate whereas post-proline dipeptidyl aminopeptidase eluted at 0·23 M-potassium phosphate. Chromatography of the purified enzyme on Sephacryl S-100 revealed an M_r of 55000 while chromatography on a Superose 12 FPLC gave Lys-pNA hydrolase an M_r of 50 000. When the purified activity was subjected to SDS-PAGE a single band was observed with an M_r of 50000 (Fig. 1). Further chromatography of the minor peak of Lys-pNA hydrolase, recovered from the DE52 column, on a calibrated Superose 12 column revealed an M_r of 100000. The N-terminal sequencing of the purified Lys-pNA hydrolase showed that the sequence of the first six amino acids was Ala–Val–Phe–Arg–Leu–Phe. Optimal activity of Lys-pNA hydrolase was observed at pH 7·0.

Effect of chemical reagents and metal ions

When the purified enzyme was incubated with a wide range of chemical reagents (all at 1 mM), amastatin, puromycin and bestatin were found to be strongly inhibitory (Table 2). The chelating agents EDTA and 1,10-phenanthroline were also inhibitory. The sulphydryl reagents *p*-chloromercuribenzoate and iodoacetamide were less inhibitory, but *N*-ethylmaleimide gave an IC_{50} value comparable to that

Table 1. *Purification of lysyl*-p-*nitroanilide hydrolase from* Lactococcus lactis *subsp.* cremoris *AM2*†

Sample	Total activity, nmol/min	Total protein, mg	Specific activity, nmol/min per mg	Yield, %	Purification
Cytoplasm	3·54	57·6	0·06	100·0	1·0
DE52 chromatography	2·83	14·7	0·19	79·9	3·2
DEAE Affi-Gel Blue Gel chromatography	2·36	6·2	0·38	66·7	6·3
Hydroxyapatite chromatography	1·17	2·5	0·47	33·0	7·8
Phenyl Sepharose CL4B chromatography	0·70	0·13	5·18	19·9	86·3

† See text for experimental details.

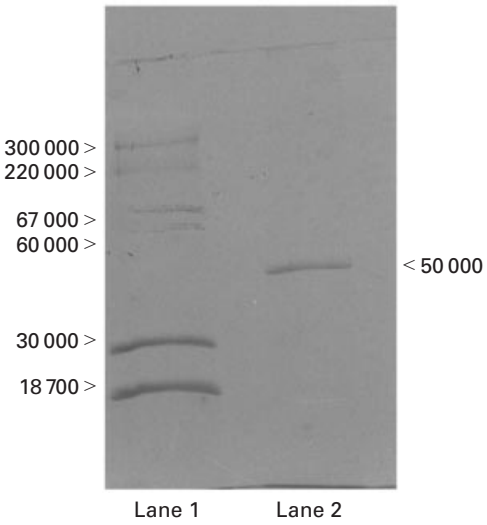

Fig. 1. SDS-PAGE of soluble lysyl-*p*-nitroanilide hydrolase purified from *Lactococcus lactis* subsp. *cremoris* AM2. Samples were separated by SDS-PAGE under reducing and denaturing conditions and protein was visualized by Coomassie blue R-250 staining. Lane 1 (M_r in parentheses), thyroglobulin (300000), ferritin half unit (220000), bovine serum albumin (67000), catalase (60000), lactate dehydrogenase (30000) and ferritin (18700); lane 2, purified enzyme (fractions eluted after the four step chromatographic procedure described in the text), loading 15 μg protein.

Table 2. *Effect of chemicals and divalent metals on the activity of purified lysyl-*p*-nitroanilide hydrolase from* Lactococcus lactis *subsp.* cremoris *AM2*

	Residual activity, %	IC_{50}, mM
No chemical or metal	100·0	—
Chemical, 1 mM		
N-ethylmaleimide	39·0	0·11
Iodoacetamide	83·7	> 5·00
p-Chloromercuribenzoate	50·0	1·00
EDTA	0	0·01
8-Hydroxyquinoline	18·5	ND
1,10-Phenanthroline	0·0	0·18
Bacitracin	18·0	ND
Puromycin	9·5	0·115
Benzamidine	100·0	ND
Phenylmethylsulphonylfluoride	88·0	ND
Dithiothreitol	100·0	ND
Bestatin	12·5	0·125
Amastatin	1·1	0·003
Metal, 1 mM		
$MgCl_2$	88·0	ND
$BaCl_2$	54·0	ND
$MnCl_2$	50·0	ND
$CoCl_2$	20·0	ND
$ZnCl_2$	16·0	ND
$CuCl_2$	0	ND
$NiCl_2$	0	ND

ND, not determined; IC_{50}, concentration of inhibitor giving 50 % inhibition of activity.

obtained with 1,10-phenanthroline. Bacitracin was also strongly inhibitory. All other materials tested were non-inhibitory. The activity of Lys-pNA hydrolase was also measured in the presence and absence of various metal ions (Table 2). While all ions inhibited activity, Mg^{2+} caused least inhibition.

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Table 3. *Substrate specificity of lysyl*-p-*nitroanilide hydrolase purified from* Lactococcus lactis *subsp.* cremoris *AM2*

Substrates hydrolysed		
Dipeptides	Tripeptides	Longer peptides
Asp–Leu	Leu–Trp–Met	Leu–Trp–Met–Arg
Asp–Lys	Leu–Leu–Leu	Leu–Trp–Met–Arg–Phe
Glu–Val	Leu–Gly–Gly	Asp–Arg–Val–Tyr–Ile–His–Pro
Glu–Tyr	Pro–Leu–Gly–NH_2	Pro–Leu–Gly–Gly
Val–Tyr	Ala–Gly–Gly	Pro–Phe–Pro–Gly
Gly–Tyr	Gly–Gly–Leu	Ala–Gly–Gly–Gly
Gly–Leu	Gly–Gly–Lys	Ala–Gly–Gly–Gly–Gly
Gly–Phe		Arg–Gly–Asp–Ser
Gly–Lys		Asp–Tyr–Met–Gly
Leu–Leu		Asp–Tyr–Met–Gly–Trp–Met
Met–Gly		Lys–Phe–Gly–Lys
Ala–Leu		His–His–Leu–Gly–Gly–Ala–Lys–Gln–Ala–Gly–Asp–Val
Ala–Gly		Asp–Arg–Val–Tyr–Ile–His–Pro–Phe
		Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu–Leu–Val–Tyr–Ser
		Pro–His–Pro–Phe–His–Leu–Phe–Val–Tyr
Substrates not hydrolysed		
Ala–Pro	Arg–Pro–Pro	Arg–Pro–Lys–Pro (Substance P(1–4))
Arg–Pro	Leu–Pro–Pro	Arg–Pro–Gly–Pro
Lys–Pro	Lys–Pro–Arg	Arg–Pro–Pro–Gly–Phe–Ser (Bradykinin (1–6))
Pro–Leu	Pro–Gly–Gly	Pro–Pro–Gly–Phe–Ser (Bradykinin (2–6))
Pro–Gly	Gly–Gly–Gly	Gly–Pro–Gly–Gly
Gly–Gly		Tyr–Pro–Phe–Pro–Gly
Gly–Gly-NH_2		Glp–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly-NH_2 (Luliberin)
		Glp–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro–Tyr–Ile–Leu (Neurotensin)

Table 4. *Activity of a lysyl*-p-*nitroanilide hydrolase purified from* Lactococcus lactis *subsp.* cremoris *AM2 towards aminoacyl* p-*nitroanilides*

Substrate	Activity, μmol/min
Lys-pNA	0·050
Arg-pNA	0·011
Leu-pNA	0·009
Ala-pNA	0·001
Asp-pNA	0
Pro-pNA	0

Substrate specificity

The purified enzyme was tested for its ability to remove the N-terminal amino acid from a wide range of peptides. The results presented in Table 3 indicate that the purified enzyme removed the N-terminal amino acid from a wide range of dipeptides, tripeptides and longer peptides, provided that proline was not the penultimate residue from the N-terminus. Lys-pNA hydrolase could act on peptides containing up to 14 amino acids. While it did not hydrolyse Pro–Leu or Pro–Gly–Gly (Table 3), Lys-pNA hydrolase had the ability to remove N-terminal proline residues from longer peptides. In addition, while it did not hydrolyse Gly–Gly, Gly–Gly–NH_2 or Gly–Gly–Gly, it did hydrolyse Gly–Gly–Lys and Gly–Gly–Leu. Table 3 also lists the peptides that were not hydrolysed by Lys-pNA hydrolase and includes those containing N-terminal pyroglutamyl residues and peptides that contain proline in the penultimate position from the N-terminus. Purified Lys-pNA hydrolase was incubated with a number of aminoacyl *p*-nitroanilides (2 mM) and Lys-pNA was hydrolysed most rapidly (Table 4). Pro-pNA and Asp-pNA were not hydrolysed, and the rate of hydrolysis of Leu-pNA was only a fifth that of Lys-pNA.

Cooperation between lysyl-p-*nitroanilide and aminopeptidase P in the hydrolysis of peptides containing proline*

When Tyr–Pro–Phe–Pro–Gly was incubated with purified aminopeptidase P followed by heat inactivation an examination of the products from thin layer chromatography showed that tyrosine was cleaved. When these products were then incubated with purified Lys-pNA hydrolase followed by heat inactivation, both tyrosine and proline were observed as cleaved products on thin layer chromatography. Further addition of purified aminopeptidase P to the second heat inactivated product resulted in the additional appearance of phenylalanine.

Stability of lysyl-p-*nitroanilide hydrolase at low temperatures and low pH*

Purified Lys-pNA hydrolase was highly unstable when stored at 8 °C after dialysis into 100 mM-sodium acetate, pH 5·2 containing 50 g NaCl/l, and had a half life of 24 h.

Discussion

Broad specificity aminopeptidases have previously been purified and characterized from a number of strains of lactococci and lactobacilli. Two of the classes into which they can be grouped are aminopeptidases N and aminopeptidases C (Tan *et al.* 1993). Aminopeptidases that consist of one polypeptide chain with M_r in the range 87000–95000 and that are inhibited by chelating agents are placed within the aminopeptidase N grouping (Tan & Konings, 1990; Rul *et al.* 1994), whereas

aminopeptidases that consist of either tetramers or hexamers with subunit M_r of 50000–54000 and that are inhibited by sulphydryl reactive agents but not by chelating reagents are placed within the aminopeptidase C grouping (Neviani *et al.* 1989; Wohlrab & Bockelmann, 1993). The native M_r value obtained for Lys-pNA hydrolase in this study suggested a resemblance between Lys-pNA hydrolase and the subunit M_r of aminopeptidase C. However, the amino acid sequence for the first six N-terminal amino acids (Ala–Val–Phe–Arg–Leu–Phe) of the purified Lys-pNA hydrolase revealed a homology in four positions with the sequence reported for aminopeptidase N from *Lc. lactis* subsp. *cremoris* Wg2 (Tan & Konings, 1990). The second, minor, peak of Lys-pNA hydrolase activity recovered from the DE52 column was found to possess an M_r of 100000, suggesting that this might be the classic aminopeptidase N activity described by a number of workers (Tan & Konings, 1990; Arora & Lee, 1992; Bockelmann *et al.* 1992; Exterkate *et al.* 1992; Miyakawa *et al.* 1992).

While aminopeptidase N preparations from *Lc. lactis* subsp. *cremoris* Wg2 (Tan & Konings, 1990), *Lc. lactis* subsp. *cremoris* HP (Exterkate *et al.* 1992), *Streptococcus cremoris* AC1 (Geis *et al.* 1985) and *Lactobacillus helveticus* CNRZ 32 (Khalid & Marth, 1990) are all inhibited by chelating reagents, each activity is also partly inhibited by sulphydryl-reactive agents, as we also found for the activity in the present study. The aminopeptidase N preparations from *Str. thermophilus* CNRZ 302 (Rul *et al.* 1994) and *Lc. lactis* subsp. *cremoris* HP (Exterkate *et al.* 1992) are also inhibited by bestatin, in common with the enzyme studied here. Our results were in agreement with those obtained with the purified enzyme from *Str. thermophilus* CNRZ 302, which also shows that hydrolysis of peptides containing an internal proline proceeds only as far as the residue immediately preceding the proline residue (Rul *et al.* 1994). The capacity of the present enzyme to release N-terminal proline from peptides containing more than one or two peptide bonds was a property not previously reported for aminopeptidase N or aminopeptidase C preparations, but it has been reported for a novel broad specificity aminopeptidase from *Lb. helveticus* SBT 217 (Sasaki *et al.* 1996). *Lc. lactis* subsp. *cremoris* HP contains an aminopeptidase capable of removing an N-terminal proline residue from dipeptides and tripeptides (Baankreis & Exterkate, 1991).

While neither the present enzyme nor aminopeptidase N preparations from *Str. thermophilus* CNRZ 302 (Rul *et al.* 1994) and from *Lc. lactis* subsp. *cremoris* Wg2 (Tan & Konings, 1990) could hydrolyse Asp-pNA, the present enzyme was found to release aspartate from a range of peptides of different lengths. In contrast, the aminopeptidase N preparation from *Str. thermophilus* CNRZ 302 can hydrolyse the peptide glucagon, which contains 29 amino acids, until an aspartyl residue is exposed, at which point hydrolysis ceases (Rul *et al.* 1994). In its capacity to hydrolyse aspartyl peptides the specificity of the present enzyme overlaps with that of an Asp-pNA-hydrolysing aminopeptidase A that has been purified from *Lc. lactis* subsp. *cremoris* AM2 (Bacon *et al.* 1994) with specificity restricted to peptides containing N-terminal aspartyl, glutamyl and seryl residues. In Table 5 the properties of Lys-pNA hydrolase are compared with those of preparations of PepN and an aminopeptidase from *Lb. helveticus*. While the present Lys-pNA hydrolase was a metalloenzyme and exhibited a broad specificity, it is discriminated from previously reported aminopeptidase N preparations by its smaller M_r and by its ability to release N-terminal proline and acidic amino acid residues.

Two mechanisms may exist in the cytoplasm of *Lc. lactis* subsp. *cremoris* AM2 to circumvent the blockage to further Lys-pNA hydrolase activity caused by proline.

Table 5. *Comparison of properties of a lysyl-*p*-nitroanilide hydrolase purified from* Lactococcus lactis *subsp.* cremoris *AM2 and those of aminopeptidases from other lactic acid bacteria*

		Aminopeptidase N		
	Lysyl-*p*-nitroanilide hydrolase	Tan & Konings (1990)	Rul *et al.* (1994)	Aminopeptidase Sasaki *et al.* (1996)
Native M_r	50000	95000	97000	95000
Subunit M_r	50000	95000	97000	95000
Inhibition by metal chelators	Strong	Strong	Strong	Strong
Inhibition by SH-reactive agents	Intermediate	Strong	Absent	Intermediate
Inhibition by 1 mM-bestatin	Strong	ND	Strong	Intermediate
Hydrolyses aminoacyl prolines	No	ND	No	No
Hydrolyses longer peptides with Pro in second position	No	Yes	No	No
Hydrolyses prolyl dipeptides	No	No	No	Some
Hydrolyses Pro–Gly–Gly	No	No	No	No
Hydrolyses longer prolyl peptides	Yes	ND	ND	Yes
Hydrolyses aspartyl and glutamyl peptides	Yes	Some	No	Some
Hydrolyses neurotensin	No	Yes	No	No

ND, not determined.

Post-proline dipeptidyl aminopeptidase can remove the N-terminal aminoacyl proline in the form of a dipeptide (Booth *et al*. 1990*c*) and the proline residue can then be hydrolysed from the dipeptide by prolidase (Booth *et al*. 1990*b*). The original peptide would be susceptible to further hydrolysis by Lys-pNA hydrolase. Alternatively, our previous results show that aminopeptidase P from *Lc. lactis* subsp. *cremoris* AM2 can remove Tyr from Tyr–Pro–Phe–Pro–Gly and so remove an N-terminal residue that precedes a proline residue (Mc Donnell *et al*. 1997). This exposes an N-terminal proline, and the present study showed that the Lys-pNA hydrolase removes the N-terminal proline, exposing the Phe residue preceding the second proline to further aminopeptidase P action. These results indicated that aminopeptidase P and Lys-pNA hydrolase may together represent an alternative mechanism for circumventing the blockage to aminopeptidase action represented by proline residues.

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