Proline iminopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397: purification and characterization

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Proline iminopeptidase (PepIP) is a major peptidase in *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ397, encoded by the *pepIP* gene. Amplification and expression of this gene in *Escherichia coli* K12 resulted in a very high level of enzyme production. Moreover, export into the *E. coli* periplasm of 45 % of PepIP activity allowed us to purify the enzyme easily by a single ion-exchange chromatography step. PepIP is a trimer of M_r 100 000, composed of three identical subunits. In the presence of 0.1 % BSA, PepIP activity was optimal at pH 6–7 and stable at temperatures below 40 °C. The enzyme was strongly inhibited by 3,4-dichloroisocoumarin, a serine protease inhibitor, by bestatin and by heavy metal ions. It was also inactivated by *p*-chloromercuribenzoate, but was reactivated by adding dithiothreitol. PepIP is characterized by a high specificity towards di- or tripeptides with proline at the NH₂-terminal position, but is not able to hydrolyse longer peptides, or peptides with hydroxyproline at the NH₂-end. The NH₂-terminal amino acid sequence of the purified PepIP corresponds to the amino acid sequence deduced from the nucleotide sequence of the *pepIP* gene.

Keywords: *Lactobacillus delbrueckii* subsp. *bulgaricus*, proline iminopeptidase, casein hydrolysis, peptidases

INTRODUCTION

Like most species of lactic acid bacteria, *Lactobacillus delbrueckii* subsp. *bulgaricus* (referred to in this paper as *L. bulgaricus*) is auxotrophic for many amino acids (Mou *et al.*, 1975) and possesses a complex proteolytic system to degrade proteins, particularly caseins in milk (Atlan *et al.*, 1994). Recently, we have cloned in *Escherichia coli* and sequenced the *L. bulgaricus pepIP* gene, encoding a cell-envelope proline iminopeptidase (PepIP) (EC 3.4.11.5) (Atlan *et al.*, 1994) with a predicted M_r of 33 006. PepIPs are able to hydrolyse peptides with proline at the ultimate position. Two intracellular PepIPs have been previously described in lactic acid bacteria, *Lactobacillus casei* (Mäkinen, 1969) and *Lactococcus lactis* (Baankreis & Exterkate, 1991).

In this work, we report the purification and character-

ization of the PepIP from *L. bulgaricus* CNRZ397, after its overproduction and partial export into the periplasm of *E. coli* K12.

METHODS

Bacterial strains, plasmid and culture conditions. *L. delbrueckii* subsp. *bulgaricus* strain CNRZ397 was obtained from the Centre National de Recherches Zootechniques (INRA, Jouy-en-Josas, France). *E. coli* strain 1617 is a *pepN recA* derivative of strain JM105 (Atlan *et al.*, 1994; Yanisch-Perron *et al.*, 1985).

Plasmid pDA3 is a pUC18 derivative (Yanisch-Perron *et al.*, 1985) containing a 1.8 kb fragment of *L. bulgaricus* chromosomal DNA, carrying the *pepIP* gene (Atlan *et al.*, 1994).

Strain 1617 carrying pUC18 or pDA3 was routinely grown aerobically at 37 °C in LB medium (Miller, 1972) supplemented with 100 µg ampicillin ml⁻¹.

Growth was estimated by determining the increase in OD₆₀₀ with a spectrophotometer (SP320 Jouan, Saint-Nazaire, France). One unit of OD₆₀₀ corresponds to 500 µg of bacterial dry weight ml⁻¹.

Subcellular fractionation. Periplasmic proteins were extracted from *E. coli* using an osmotic shock procedure (Heppel, 1971).

Abbreviations: AP, aminopeptidase; APMF, (4-amidinophenyl)-methanesulphonyl fluoride; DFP, diisopropyl fluorophosphate; E64, *N*-(1-*L*-3-*trans*-carboxyoxirane-2-carbonyl)-*L*-leucyl-L-agmatine; PCMB, *p*-chloromercuribenzoate; PepIP, proline iminopeptidase; pNA, paranitroanilide; X-Pro-DPAP, X-prolyl-dipeptidyl aminopeptidase.

F1 extracts corresponded to the supernatant recovered from sucrose-treated cells. F2 extracts (osmotic fluids) contained periplasmic proteins released from sucrose-treated cells submitted to an osmotic shock. Shocked cells were broken using a French pressure cell and centrifuged to remove unbroken cells. The supernatants (F3 extracts) corresponded to inner membrane and cytoplasmic components.

Enzyme assays. Amino-peptidase activities were assayed as previously described (Atlan *et al.*, 1989, 1990, 1994) using prolyl- (40 mM), alanyl- (40 mM), leucyl- (15 mM), lysyl- (3 mM), arginyl- (10 mM), alanyl-prolyl- (6 mM) or glycyl-prolyl- (5 mM) *p*-nitroanilide (pNA) as the substrates at 30 °C in 0.2 M Tris/HCl buffer (pH 7.0), supplemented with 0.1 % bovine serum albumin (BSA, Sigma). All substrates were obtained from Bachem.

Standard published procedures were used for assaying β -lactamase (Sykes & Nordstrom, 1972) and glucose-6-phosphate dehydrogenase (Malamy & Horecker, 1961), using ampicillin (0.4 mM), or glucose 6-phosphate (1 mM) and NADP (0.4 mM), respectively, as the substrates.

One unit (U) of enzyme activity was defined as the amount of enzyme that hydrolysed 1 μ mol substrate min^{-1} . [In Table 3, U represents units per mg bacterial dry weight.]

Hydrolysis of non-chromogenic peptides (Bachem) was detected by thin-layer chromatography on precoated silica gel 60 plates with a gel layer thickness of 0.25 cm (E. Merck), as described by Tan & Konings (1990).

Electrophoretic analysis. SDS-PAGE was performed as described by Laemmli (1970), using 12 % (w/v) acrylamide slab gels at 4 W constant power. Before electrophoresis, samples were boiled for 3 min in 0.1 M β -mercaptoethanol in the presence of 1 % (w/v) SDS. After electrophoresis, gels were stained with 1 % (w/v) Coomassie Brilliant Blue R250 (Prolabo) in methanol/acetic acid/water (50:10:40, by vol.) at 50 °C, and diffusion-destained in successive baths of methanol/acetic acid/water (5:10:85, by vol.) at 37 °C.

Isoelectric focusing in polyacrylamide gel. Before electrophoresis, the purified PepIP solution was dialysed against distilled water at 4 °C for 3 h, using a 0.05 μ m membrane

(Millipore). Servalyt precoated gel pH 3–6 (Serva) was used with the electrophoresis conditions described by the manufacturer. Electrophoresis was performed at 10 °C for 150 min with the Multiphor II system (Pharmacia). After electrophoresis, the gel was fixed and stained with Coomassie Brilliant Blue R250.

The following proteins were used as pI markers: glucose oxidase from *Aspergillus niger* (Serva; pI 4.15); trypsin inhibitor from soybean (Sigma; pI 4.55); carbonic anhydrase A from bovine erythrocytes (Sigma; pI 5.4); and carbonic anhydrase B from bovine erythrocytes (Sigma; pI 5.85).

Determination of protein concentration. Protein contents were determined by the method of Bradford (1976) using bovine serum albumin (Sigma) as the standard.

Elution profiles of the proteins separated by ion-exchange chromatography were monitored by measuring A_{220} .

Purification of PepIP. An F2 fluid was applied to a column of Monobeads (MonoQ HR 5/5; Pharmacia) that had been equilibrated with 20 mM Tris/HCl buffer pH 7.5. The column was washed with 3.5 ml of the same buffer and a first linear gradient of NaCl (0–0.2 M) was run over 7 ml at a flow rate of 1 ml min^{-1} . Then, the PepIP activity was eluted with a 20 ml linear gradient of NaCl (0.2–0.4 M) in 20 mM Tris/HCl buffer pH 7.5, at a flow rate of 1 ml min^{-1} , and 1 ml fractions were collected. The fractions containing the PepIP activity were pooled (2 ml).

Effect of divalent cations and chemical reagents on PepIP activity. The purified enzyme was preincubated in the assay buffer with or without various ions or inhibitors for 30 min at 4 °C. After addition of the substrate (prolyl-pNA), enzyme activity was determined at 30 °C in 0.2 M Tris/HCl pH 7.0, supplemented with 0.1 % BSA.

N-terminal sequencing. The sample was prepared by applying the purified enzyme solution to a C_4 column and eluting at 46 % (v/v) acetonitrile/trifluoroacetic acid buffer by reverse-phase chromatography. The polypeptide sequence was determined by the Edman degradation method using a pulse-liquid sequencer (model 470.1; Applied Biosystems). The sequencing was carried out by the Service Central d'Analyse CNRS (Vernaison, France).

Table 1. Enzyme activities assayed in different cellular extracts from *E. coli* strain 1617 containing plasmids pUC18 or pDA3

Fractions F1, F2 and F3 are defined in Methods. U, units per mg bacterial dry weight.

Strain	Fraction	PepIP			β -Lactamase			Glucose-6-phosphate dehydrogenase		
		Activity (U)	%	Ratio*	Activity (U)	%	Ratio	Activity (U)	%	Ratio
1617(pUC18)	F1	0.02	2.6		0.046	6.6		< 0.001	< 0.3	
	F2	0.02	2.6		0.620	90.4		0.008	2.2	
	F3	0.73	94.8		0.020	3.0		0.359	97.5	
	Total	0.77	100.0	1	0.686	100.0	1.00	0.368	100.0	1.00
1617(pDA3)	F1	1450	7.1		0.022	17.7		< 0.001	< 0.4	
	F2	7750	38.0		0.090	73.5		0.015	5.6	
	F3	11200	54.9		0.011	8.8		0.254	94.0	
	Total	20400	100.0	26500	0.123	100.0	0.18	0.270	100.0	0.73

* Ratio: activity measured in the strain divided by the activity present in strain 1617(pUC18).

RESULTS

Cell localization of the PepIP activity overproduced in *E. coli* strain 1617

E. coli strain 1617 transformed with pUC18 synthesized PepIP at a very low level. The PepIP activity present in *E. coli* 1617(pUC18) was mainly recovered from F3 cytoplasmic extracts. In contrast, strain 1617 transformed with pDA3 exhibited a PepIP activity more than 26000-fold higher (Table 1; Atlan *et al.*, 1994). Forty and ninety percent of the PepIP and β -lactamase activities, respectively, were recovered from the periplasmic extracts (F1 and F2) under limited cell lysis (as estimated by assaying cytoplasmic glucose-6-phosphate dehydrogenase activity).

The protein composition of F1 and F2 periplasmic extracts from strain 1617 carrying either pUC18 or pDA3 was analysed by SDS-PAGE (Fig. 1). Only one additional major polypeptide, of M_r about 34000, was overproduced by strain 1617(pDA3) and released into periplasmic extracts.

Fig. 1. SDS-PAGE of periplasmic proteins (F1 and F2 extracts) from transformed *E. coli* strains grown in LB medium: lanes A and C, F1 and F2 extracts, respectively, from *E. coli* 1617(pUC18); lanes B and D, F1 and F2 extracts, respectively, from *E. coli* 1617(pDA3). Samples prepared from 5×10^8 cells were loaded on the gel. Lane E, 0.5 μ g purified PepIP after chromatography on MonoQ HR 5/5.

Purification of PepIP

F2 fluids from strain 1617(pDA3) were used as crude extracts for purifying the PepIP of *L. bulgaricus* CNRZ 397. A single purification step by ion-exchange chromatography (MonoQ HR 5/5) was sufficient to remove all contaminating proteins from F2 extracts and to recover 39% of the PepIP activity (Table 2). PepIP activity, assayed with prolyl-pNA as the substrate, was eluted at 0.32 M NaCl (Fig. 2). Only one protein band, of M_r approximately 34000, was detected in the PepIP fraction by SDS-PAGE (Fig. 1 lane E); this fraction was used as purified PepIP. No protein contaminant was detected during the N-terminal sequencing of PepIP from this purified preparation (data not shown).

Properties of purified PepIP

Subunit composition, NH_2 -terminal amino acid sequence and isoelectric point. The M_r of native PepIP was estimated to be between 95000 and 105000 by high-performance liquid chromatography on a Superose 12 column (size-exclusion chromatography; data not shown). SDS-PAGE of purified PepIP fractions resulted in a single protein band corresponding to an M_r of 34000 (Fig. 1). These results strongly suggested that PepIP was a trimer composed of three identical subunits, each of M_r about 34000.

Purified PepIP was used to determine the NH_2 -terminal amino acid sequence of the enzyme. The first 11 amino acids were identified as follows: Met, Met, Gln, Ile, Thr, Glu, Lys, Tyr, Leu, Pro and Phe, respectively.

The pI of PepIP was estimated to be pH 4.5 by isoelectric focusing (data not shown).

Effects of temperature and pH. PepIP activity was unstable when measured at low protein concentration. The addition of 0.1% BSA in Tris/HCl buffer (0.2 M, pH 7.0) significantly stabilized the purified enzyme. The effect of temperature on enzyme activity was determined in the range 5–60 °C. Above 35 °C, initial reaction rates still increased but enzyme kinetics were not linear (data not shown) and the determination of enzyme activities was not very reliable. Therefore, PepIP activity was routinely assayed at 30 °C.

The effect of pH on PepIP activity was determined between pH 4 and 9.5 by using a polybuffer composed of malic acid, MES, HEPES and boric acid (each at 20 mM).

Table 2. Purification of the *L. bulgaricus* CNRZ 397 PepIP from periplasmic extracts of *E. coli*

Purification step	$10^{-3} \times$ Total activity (U)	Protein (mg)	$10^{-3} \times$ Specific activity [U (mg protein) $^{-1}$]	Purification (-fold)	Yield (%)
F2 (periplasmic extract)	1245	11.0	113	1.0	100
Anion-exchange chromatography (MonoQ HR 5/5)	490	0.9	530	4.7	39

Table 3. Substrate specificity of PepIP

Assays were performed at 30 °C for 2 h. Peptide hydrolysis was analysed by thin-layer chromatography. + + +, Complete hydrolysis; + +, partial hydrolysis; +, weak hydrolysis; —, no hydrolysis.

Substrate	Activity
Dipeptides	
Alanyl-glycine	+
Alanyl-histidine	+
Alanyl-leucine	++
Alanyl-methionine	++
Alanyl-phenylalanine	++
Alanyl-proline	+
Glycyl-alanine	+
Glycyl-phenylalanine	+
Glycyl-proline	—
Glycyl-tryptophan	+
Glycyl-tyrosine	+
Glycyl-valine	+
Histidyl-serine	—
Hydroxyprolyl-proline	—
Leucyl-alanine	+
Leucyl-glycine	+
Leucyl-proline	—
Lysyl-tyrosine	—
Phenylalanyl-proline	—
Prolyl-alanine	+++
Prolyl-glycine	+++
Prolyl-hydroxyproline	+++
Prolyl-leucine	+++
Prolyl-phenylalanine	+++
Prolyl-proline	+++
Valyl-glycine	—
Valyl-leucine	—
Valyl-proline	—
Z-glycyl-leucine	—
NCBZ-glycyl-phenylalanine	—
Tripeptides	
Glycyl-prolyl-leucine	—
Leucyl-alanyl-proline	—
Leucyl-leucyl-leucine	—
Prolyl-glycyl-glycine	+++*
Valyl-glycyl-glycine	—
Tetrapeptide	
Prolyl-phenylalanyl-glycyl-lysine	—
Pentapeptide	
Prolyl-glycyl-lysyl-alanyl-arginine	—

* Proline and glycyl-glycine were the products of hydrolysis.

supplemented with 0.1% BSA and adjusted to the appropriate pH value. PepIP activity was optimal between pH 6.0 and pH 7.0. Below pH 5.5 and above pH 7.5, PepIP activity decreased rapidly (data not shown).

Substrate specificity. The hydrolytic action of PepIP on various peptides was analysed by thin-layer chromat-

Fig. 2. Ion-exchange chromatography of PepIP. Proteins (2.7 mg) of F2 extracts were loaded on a MonoQ HR 5/5 column. The enzyme was eluted at a flow rate of 1 ml min⁻¹ with linear gradients of NaCl (-----) in 20 mM Tris/HCl buffer (pH 7.5). Fractions of 1.0 ml were collected. Protein concentration (—) was monitored by measuring A₂₂₀. PepIP activity (---) was assayed with prolyl-pNA as the substrate (as described in Methods).

Table 4. Hydrolysis of various pNA substrates by the purified PepIP

See Methods for details.

Substrate	Activity (%)
Prolyl-pNA	100.00
Alanyl-pNA	1.30
Leucyl-pNA	0.40
Lysyl-pNA	0.09
Arginyl-pNA	0.04
Alanyl-prolyl-pNA	0.03
Glycyl-prolyl-pNA	0.04

ography (Table 3). All di- and tri-peptides but not larger peptides containing proline at the NH₂-terminal end were hydrolysed. PepIP also partially hydrolysed peptides with alanine, glycine or leucine, but not hydroxyproline, at the NH₂-terminal position. Using chromogenic peptides (pNA derivatives) as substrates, the highest activity was obtained with prolyl-pNA (Table 4); the levels of PepIP activity measured with other peptides were very low, indicating that PepIP is a highly specific enzyme. The kinetic parameters of PepIP were determined with prolyl-pNA as the substrate. At 30 °C in Tris/HCl buffer (0.2 M, pH 7.0) supplemented with 0.1% BSA, PepIP hydrolysed prolyl-pNA with a *K_m* of 0.93 mM and a *V_{max}* of 738 mmol min⁻¹ (mg protein)⁻¹.

Table 5. Effect of various inhibitors and metal ions on PepIP activity

Assays were performed at 30 °C in 0.2 M Tris/HCl pH 7.0 supplemented with 0.1 % BSA, using prolyl-pNA as the substrate.

Cation or inhibitor	Concn (mM)	Activity (%)
No addition		100
Ca ²⁺	0.5	96
	1	97
	5	92
Mn ²⁺	5	100
Mg ²⁺	5	100
Co ²⁺	0.5	100
	1	79
	5	63
Cu ²⁺	0.5	41
	1	11
	5	2
Zn ²⁺	0.5	0
Fe ²⁺	0.5	65
	1	44
	5	7
EDTA	0.1	100
	1	95
1,10-Phenanthroline	0.1	90
	1	95
	10	75
Bestatin	0.033	65
	0.33	14
3,4-Dichloroisocoumarin	0.1	20
	1	5
DFP	0.1	96
	1	87
PMSF	0.1	100
	1	90
APMSF	0.1	95
	1	90
β -Mercaptoethanol	1	100
DTT	1	100
PCMB	0.1	35
	1	15
E64	1	100
Iodoacetic acid	1	100

Effects of chemical reagents and metal ions. PepIP activity was completely inhibited by Zn²⁺ (0.5 mM) whereas Co²⁺, Cu²⁺ and Fe²⁺ produced only a partial inhibition of the enzyme (Table 5). Inhibitors of metallo-enzymes (EDTA, 1,10-phenanthroline), compounds acting on cysteine proteases (E64, iodoacetic acid) and reducing agents (β -mercaptoethanol, DTT) had no significant effect on PepIP activity. Weak inhibition of PepIP activity was observed in the presence of PMSF (1 mM), APMSF (1 mM) and DFP (1 mM). On the other hand, only a small amount of activity was recovered after incubation with PCMB (1 mM), bestatin (0.33 mM) and

3,4-dichloroisocoumarin (1 mM), compounds known to inhibit thiol groups, aminopeptidases and serine proteases, respectively. The enzyme inactivated with PCMB was reactivated by adding DTT (10 mM).

DISCUSSION

Cloning of the *pepIP* gene from *L. bulgaricus* CNRZ 397 in the high-copy-number vector pUC18 and expression in *E. coli* K12 led to the overexpression of its product, PepIP, and allowed us to purify this enzyme. Overexpression of the *L. bulgaricus* PepIP protein in *E. coli* cells was shown to be associated with the export of 45 % of enzyme into the periplasmic space.

The PepIP purified from periplasmic extracts of *E. coli* cells harbouring plasmid pDA3 is a trimer of approximate M_r 100 000 (three identical subunits of M_r 34 000). The 11 NH₂-terminal amino acids of the purified PepIP corresponded to the translated sequence of the *pepIP* gene (Atlan *et al.*, 1994), which confirmed that this open reading frame, composed of 295 codons, constituted the structural gene encoding the PepIP enzyme from *L. bulgaricus*. Moreover, the translated protein had a calculated M_r of 33 006, which agrees very well with the apparent M_r of the purified PepIP monomer as determined by SDS-PAGE.

Several PepIPs or prolinases have been previously identified as multimers, e.g. those from bovine kidney (300 000; hexamer) (Khilji & Bailey, 1978) or apricot seeds (220 000; tetramer) (Ninomiya *et al.*, 1982), but others have been described as monomers, such as the PepIP from *Bacillus megaterium* (56 000) (Yoshimoto *et al.*, 1983), *Bacillus coagulans* (33 000) (Yoshimoto *et al.*, 1985; Kitazono *et al.*, 1992), and *Propionibacterium shermanii* (61 000) (Panon, 1990).

L. bulgaricus PepIP is a proline-specific peptidase able to hydrolyse di- and tripeptides with proline at the NH₂-terminal position, but it cannot hydrolyse tetra- or pentapeptides. This narrow substrate specificity is a property shared with a lactococcal tripeptidase, except for the ability to degrade prolyl-proline (Baankreis & Exterkate, 1991). However, this lactococcal tripeptidase is a metalloenzyme, whereas our protease inhibitor studies suggest that a serine residue is essential for the PepIP activity of *L. bulgaricus*. Analysis of the amino acid sequence deduced from the *pepIP* nucleotide sequence revealed the presence of a region exhibiting some homology with a catalytic site region of known serine hydrolases (Atlan *et al.*, 1994). The inhibition of PepIP activity observed in the presence of PCMB might be due to the alteration of PepIP conformational stability by oxidation of a thiol group directly or indirectly involved in the catalytic site.

Except for the PepIP purified from *Propionibacterium*, which is a serine protease (Panon, 1990), most PepIPs were previously described as thiol enzymes (Akrawi & Bailey, 1976; Mayer & Nordwig, 1973; Ninomiya *et al.*, 1982; Sarid *et al.*, 1962; Yoshimoto *et al.*, 1983). Their activities are optimal in a pH range from 8 to 9. In contrast, the optimal pH of PepIP from *L. bulgaricus*, as

well as those from *Bacillus megaterium* (Yoshimoto *et al.*, 1983) and *Bacillus coagulans* (Yoshimoto & Tsuru, 1985; Kitazono *et al.*, 1992), is near 7. The enzymes from bacilli also display a pI of 4.6, which is very similar to that of *L. bulgaricus* peptidase (4.5). However, these PepIPs display a marked difference in their substrate specificity: only *Bacillus* PepIPs hydrolyse tetra- or pentapeptides with a proline at the NH₂-terminal position.

Thus, the *L. bulgaricus* PepIP differs from the other PepIPs already identified in its biochemical properties and also in its probable cellular location (Atlan *et al.*, 1994; Baankreis & Exterkate, 1991; Mäkinen, 1969; Panon, 1990). Up to now, in most strains only one X-prolyl dipeptidyl aminopeptidase has been shown to hydrolyse peptides containing proline (Atlan *et al.*, 1990; Casey & Meyer, 1985; Khalid & Marth, 1990; Kiefer-Partsch *et al.*, 1989; Meyer & Jordi, 1987; Zevaco *et al.*, 1990). The *L. bulgaricus* PepIP reveals a new way for peptides containing proline as the NH₂-terminal residue to be utilized.

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