Purification and Characterization of a Novel Serine Aminopeptidase from *Lactobacillus casei* Ssp. *casei* IFPL 731

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An aminopeptidase showing broad specificity has been purified to homogeneity from the cell-free extract of *Lactobacillus casei* ssp. *casei* IFPL 731. Enzyme activity was inhibited by the serine protease inhibitor, phenylmethanesulfonyl fluoride, and reducing agents such as dithiothreitol and β-mercaptoethanol. The metal chelating agent, ethylenediamintetraacetic acid, also reduced enzyme activity. The molecular mass of the purified enzyme was estimated to be 67 kDa by gel filtration and sodium dodecyl sulfate−polyacrylamide gel electrophoresis, indicating that the enzyme exits as a monomer. The purified enzyme hydrolyzed *p*-nitroanilides of several amino acids and peptides as well as di- and tripeptides. The best substrates were Arg-Pro-*p*-nitroanilide, Ala-Pro-*p*-nitroanilide, Phe-Met, Leu-Gly, Phe-Ala, and Leu-Gly-Phe. K_m values for Arg-Pro-*p*-nitroanilide and Leu-Gly were 4.8 and 1.1 mM, respectively. The properties of the enzyme are compared with those of other aminopeptidases isolated from lactic acid bacteria.

Keywords: *Enzyme purification; aminopeptidase; mesophilic lactic acid bacteria; Lactobacillus casei*

INTRODUCTION

Lactobacilli constitute most of the nonstarter flora of many cheese varieties; they gain access to the cheese through pre- or postpasteurization contamination of milk and grow to high cell densities ($>10^7$/g of Cheddar cheese) during cheese ripening (Peterson and Marshall, 1990). Among lactic acid bacteria, lactobacilli have the most extensive amino acid requirements (Morishita *et al.*, 1974), and to meet these requirements, lactobacilli possess a very broad spectrum of peptidase activities.

In recent years, there has been an increasing interest in the use of lactobacilli as adjuncts to commercial starter lactococci to accelerate cheese ripening. Thus, *Lactobacillus casei* has been reported to enhance cheese flavor when added with the starter culture (Puchades *et al.*, 1989; Broome *et al.*, 1991; Trépanier *et al.*, 1991; Requena *et al.*, 1992). There has also been interest in employing *Lb. casei* to improve the flavor and texture of reduced-fat cheese (Czulak and Spieler, 1984; El-Neshawy *et al.*, 1986; Tempas and Morris, 1993; Merrill *et al.*, 1996). Whereas the proteolytic enzyme system of *Lactococcus* has been studied extensively, that of mesophilic *Lactobacillus* spp. is relatively unknown. Most of the work in *Lactobacillus* has concentrated on thermophilic species, e.g. *Lb. delbrueckii*.

Purification and characterization of several peptidases from *Lb. delbrueckii* have revealed that *Lactobacillus* possess a proteolytic system similar to that of *Lactococcus*. On the basis of biochemical and genetic aspects, four types of aminopeptidases have been demonstrated in *Lactococcus*: aminopeptidase A (PepA), pyrrolidonyl carboxylyl peptidase (PCP), aminopeptidase N (PepN), and aminopeptidase C (PepC). While PepA and PCP have a narrow substrate specificity (glutamyl and aspartyl peptides for PepA and pyroglutamyl for PCP), the other aminopeptidases isolated so far have a broad substrate specificity. PepN and PepA are metalloenzymes, whereas PepC is a thiolenzyme and PCP is a serine enzyme (Kok and De Vos, 1994).

Casein is rich in proline. Proline structure restricts the action of common endo- and exopeptidases. Therefore, proline-specific peptidases are thought to play a vital role in cheese ripening. By definition, these types of enzymes are able to hydrolyze only peptides containing proline. The purification of an X-prolyl-dipeptidyl aminopeptidase (PepX), an iminopeptidase, a prolidase, and PepN from *Lb. casei* has been reported (El-Abboudi *et al.*, 1992; Arora and Lee, 1992; Habibi-Najafi and Lee, 1994, 1995; Fernández-Esplá et al., 1996), and they seem to be similar to enzymes purified from other *Lactococcus* or *Lactobacillus* strains.

Lb. casei ssp. *casei* IFPL 731, isolated from an artisanal goat's milk cheese, was selected for its ability to produce high levels of amino acid nitrogen and debittering action in a model cheese system prepared from goats' milk (Parra *et al.*, 1996). In this paper, we describe the purification and characterization of a new aminopeptidase which, although it hydrolyzes peptides containing proline at the penultimate position, as do normal PepXs, was also able to hydrolyze tri- and dipeptides not containing proline. The biochemical properties of this enzyme indicate that a similar enzyme has so far not been described in lactic acid bacteria.

MATERIALS AND METHODS

Organism, Growth, and Preparation of Cell-Free Extract. *Lb. casei* ssp. *casei* IFPL 731 was grown at 30 °C in 5 L of MRS medium with an inoculum of 2% and without regulation of the pH. Cells were harvested by centrifugation (10000*g*, 15 min, 4 °C) at the late exponential growth period and subsequently washed twice in 50 mM phosphate buffer, pH 7.0.

For disruption, the cells were resuspended in 70 mL of 20 mM Tris-HCl, pH 7.5, mixed with glass beads (1:1, v/w; 150−

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212 μm; Sigma Chemical Co., St. Louis, MO) and subjected to the action of an Osterizer (4 × 4 min at 4 °C). Glass beads, unbroken cells, and cell debris were removed by centrifugation (15000*g*, 30 min, 4 °C), followed by filtration through Whatman No. 1 filter paper. The clear supernatant, which constituted the cell-free extract, was treated with DNase and RNase (8 μg/mL) (Boehringer Mannheim GmbH, Germany) for 30 min at 20 °C to hydrolyze DNA and RNA and used as the starting material for enzyme purification.

Enzyme Purification. All chromatographic steps during the purification process were carried out at 4 °C using a FPLC system (Pharmacia, Uppsala, Sweden).

First Anion-Exchange Chromatography. A DEAE-Sepharose fast flow column (Pharmacia) (15 cm × 5 cm) was equilibrated with 20 mM Tris-HCl buffer, pH 7.5. The cell-free extract was applied to the column, which was washed with 800 mL of equilibrating buffer. Bound proteins were eluted with a linear gradient of 0−0.5 M NaCl in the same buffer (2 L), at a flow rate of 10 mL/min. Fractions were assayed for Arg-Pro-*p*NA and Ala-Leu-Gly hydrolyzing activity.

Hydrophobic Interaction Chromatography (HIC). Fractions active on Ala-Leu-Gly were concentrated by ultrafiltration using Amicon YM 10 membranes (Amicon, Danvers, MA), and solid ammonium sulfate was added to a concentration of 1.5 M. The resulting fraction was then applied to a phenyl-Superose HR 5/5 column (Pharmacia) previously equilibrated with 20 mM Tris-HCl, pH 7.5, containing 1.5 M $(NH_4)_2SO_4$. Proteins were eluted in a decreasing gradient of $(NH_4)_2SO_4$, 1.5−0 M, at a flow rate of 0.3 mL/min.

Second Anion-Exchange Chromatography. Fractions from HIC with maximum Ala-Leu-Gly hydrolyzing activity were concentrated by ultrafiltration and applied to a Mono Q HR 5/5 column (Pharmacia), equilibrated with 20 mM Tris-HCl, pH 7.5. The column was washed with 1 mL of equilibrating buffer and then with a linear NaCl gradient, 0.1−0.3 M, in the same buffer, at a flow rate of 0.5 mL/min.

Gel Filtration. Active fractions obtained after the second anion-exchange chromatography were concentrated by ultrafiltration and applied to a Superose 12 HR 10/30 column (Pharmacia) previously equilibrated with 20 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl. Proteins were eluted with equilibrating buffer at a flow rate of 0.3 mL/min. Fractions with the highest activity were desalted using a Sephadex G-25 M column (Pharmacia), equilibrated with 20 mM Tris-HCl buffer, pH 7.5, and stored at −90 °C for further studies.

Enzyme Assay. X-prolyl-dipeptidyl aminopeptidase activity was measured using Arg-Pro-*p*-nitroanilide (Arg-Pro-*p*NA) as substrate (Sigma). The reaction mixture contained an appropriate concentration of enzyme diluted in 50 mM sodium phosphate buffer, pH 7.0, and Arg-Pro-*p*NA at a final assay concentration of 1 mM. The reaction mixture was incubated at 37 °C for 10 min. Hydrolysis of the substrate was monitored continuously by following the release of *p*-nitroaniline at 410 nm. A molar absorbance coefficient of 8100 M^{-1} cm^{-1} was used to calculate the specific enzyme activity (Kiefer-Partsch *et al.*, 1989). One unit of enzyme activity was defined as the amount of enzyme required to release 1 nmol of *p*-nitroaniline/min.

Protein Determination. Protein content was determined according to the micromethod of Bradford (1976), using the Bio-Rad protein assay (Bio-Rad, Munich, Germany) with bovine serum albumin as standard.

Determination of Molecular Mass. The molecular mass of the enzyme was estimated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and by gel filtration on a Superose 12 HR 10/30 column. SDS−PAGE was performed using the Phast electrophoresis system (Pharmacia) with 12.5% polyacrylamide gels, following the manufacturer's instructions. Proteins were silver stained, and the molecular mass of the purified enzyme was estimated by reference to the migration of marker proteins (LMW standards; Pharmacia). For determination of molecular mass by gel filtration, the column was calibrated using GF-200 Kit molecular weight markers (Sigma).

Effect of pH and Temperature. The effect of pH on the activity of the purified enzyme was measured at 37 °C using Arg-Pro-*p*NA as substrate. The appropriate amount of enzyme was diluted in sodium acetate (pH 4.0−5.5), sodium phosphate (pH 6.0−7.0), Tris-HCl (pH 7.5−8.5), and sodium carbonate (pH 9.0−10.0) buffers, each at 50 mM. For determination of the temperature optimum, enzyme activity was assayed in the range 30−60 °C using 50 mM sodium phosphate buffer, pH 7.0, and Arg-Pro-*p*NA as substrate.

Substrate Specificity and Enzyme Kinetics. Hydrolysis of *p*-nitroanilide derivatives of amino acids and peptides was measured according to the standard assay procedure described above. Enzyme activity on dipeptides and tripeptides was determined by measuring the release of α-amino acids according to the modified cadmium−ninhydrin method described by Doi *et al.* (1981). Ten microliters of the substrate (10 mM in H_2O) was added to an appropriate amount of enzyme diluted in 50 mM sodium phosphate buffer, pH 7.0, and the final assay volume adjusted to 100 μL. After incubation at 37 °C for 60 min, 900 μL of the ninhydrin reagent was added. Samples were then heated for 5 min at 84 °C and cooled, and the absorbance was measured at 507 nm.

For kinetics studies, the enzyme solution was incubated with various concentrations of substrate (Arg-Pro-*p*NA or Leu-Gly), ranging from 0 to 4 mM. A Lineweaver−Burk plot was constructed, and the Michaelis−Menten constant (K_m) was calculated from the slope of the regression line.

Effect of Chemical Reagents and Metal Ions. A mixture (90 μL) containing 20 μL of purified enzyme solution and the indicated concentration of each reagent (Sigma) in 50 mM sodium phosphate buffer, pH 7.0, was preincubated for 15 min at 25 °C. Residual enzyme activity was assayed by adding 10 μL of substrate solution (10 mM Phe-Ala in H_2O). Hydrolysis was estimated indirectly according to the modified cadmium−ninhydrin method described before. For metal ions the same procedure was used but with 50 mM Bis-Tris buffer, pH 7.0, instead of sodium phosphate buffer. Metal ions were added as chlorides.

RESULTS

Purification of a Novel Aminopeptidase. When the cell-free supernatant was applied to the first ion-exchange chromatography (DEAE-Sepharose fast flow), two peaks active on Arg-Pro-*p*NA were eluted at 0.18 and 0.24 M NaCl (Figure 1A). The second peak, eluting at 0.24 M NaCl, contained 5% of total Arg-Pro-*p*NA activity, but showed high activity on the tripeptide Ala-Leu-Gly. Due to this particular characteristic, this peak was selected for further purification. The first peak was also studied, and it will be discussed latter.

Fractions active on Ala-Leu-Gly were separated from other proteins by hydrophobic interaction chromatography. The enzyme showed a high degree of hydrophobicity, as it eluted as a single peak at the end of the ammonium sulfate gradient (Figure 1B). Further purification of the enzyme was achieved by a second ion-exchange chromatography (Mono Q); the enzyme eluted as a single peak at 0.24 M NaCl, the same salt concentration as for the first anion-exchange chromatography. Finally, an electrophoretically pure enzyme was obtained by gel filtration on a Superose 12 column.

Results of the purification procedure are summarized in Table 1. Enzyme purity was achieved after four chromatographic steps; the low yield obtained was due to the presence in the starting material (cell-free extract) of more than one enzyme active on Arg-Pro-*p*NA.

Properties of the Enzyme. The molecular mass of the purified enzyme was estimated to be 67 kDa by gel filtration chromatography as well as by SDS−PAGE (Figure 2). These results suggest that the native enzyme exits as a monomer.

The enzyme was active over a very narrow pH range, from 6.0 to 7.0. Maximum activity occurred at pH 7.0; enzyme activity was reduced by 47% at pH 6.0. The

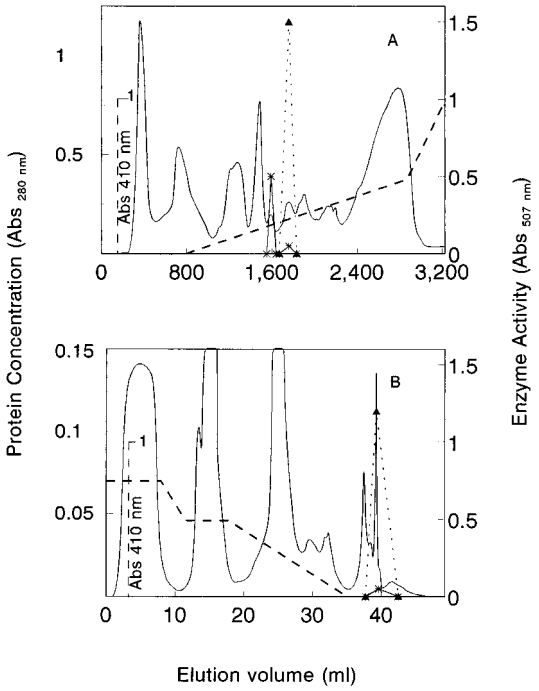

Figure 1. Chromatographic elution profiles of the novel serine aminopeptidase from *Lb. casei* ssp. *casei* IFPL 731: (A) first anion-exchange chromatography (DEAE-Sepharose fast flow column); (B) hydrophobic interaction chromatography (phenyl-Superose HR 5/5 column). Absorbance at 280 nm (−), salt gradient (− −); Arg-Pro-*p*NA activity was measured at 410 nm (*), and Ala-Leu-Gly activity was measured at 507 nm (▲).

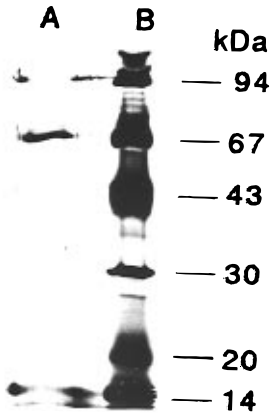

Figure 2. SDS−PAGE of the purified enzyme: (lane A) gel filtration fraction; (lane B) molecular mass markers.

Table 1. Purification of a Novel Serine Aminopeptidase from *Lb. casei* Ssp. *casei* IFPL 731

purifn step	total protein (mg)	total act. (nmol/min)	sp act. (nmol min^{-1} mg^{-1})	yield (%)	purifn (fold)
cell-free extract	643	26 054	40	100	1
DEAE-Sepharose	12	1 286	107	4.9	2.6
phenyl-Superose	0.40	240	600	0.9	14.8
Mono Q	0.02	82	2 562	0.3	63.2
Superose 12		62		0.2	

optimum temperature (at pH 7.0) was found to be 40 °C. At 60 °C, the enzyme still had 50% of the activity shown at 40 °C.

Table 2 shows the effect of different classes of inhibitors on the activity of the purified enzyme. As general aminopeptidases show high activity on dipeptides, in this experiment, Phe-Ala was used as substrate; the rate of hydrolysis of this dipeptide in the absence of any inhibitor, reducing agent, or metal chelator was taken as 100%. Enzyme activity was strongly inhibited by the serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF) and reducing agents such as dithiothreitol and β-mercaptoethanol (all at 1 mM). At a concentration of 1 mM, iodoactic acid and *p*-hydroxymercuribenzoic acid caused slight inhibition. The enzyme was partially inhibited by the metal-chelating agent ethylenediaminetetraacetic acid (EDTA) but not by phenanthroline (both at 1 mM). Among the divalent ions studied, Ca^{2+} and Co^{2+} enhanced enzyme activity slightly, whereas Mg^{2+}, Cu^{2+}, and Zn^{2+} had a moderate inhibitory effect (all at 1 mM) (Table 2). Na^{+} at 1.5 M also caused some inhibition (Table 2).

Table 2. Effect of Cations and Chemical Reagents on the Hydrolysis of Phe-Ala by the Purified Aminopeptidase

chemical reagent	concn (mM)	rel act.[a] (%)
EDTA	1	54
1,10-phenanthroline	1	117
dithiothreitol	1	0
β-mercaptoethanol	1	19
PMSF	1	13
iodoacetic acid	1	88
p-hydroxymercuribenzoate	1	75
Ca^{2+}	1	125
Mg^{2+}	1	61
Co^{2+}	1	126
Mn^{2+}	1	85
Cu^{2+}	1	63
Zn^{2+}	0.1	68
Na^{+}	1500	60

[a] Rate of hydrolysis of Phe-Ala in the absence of any metal ion, inhibitor, reducing agent, or metal chelator was taken as 100%.

Table 3. Relative Activity of the Purified Aminopeptidase on Different Substrates

substrate	rel act.[a] (%)	substrate	rel act.[b] (%)
Phe-Met	315	Arg-Pro-*p*NA	100
Leu-Gly	100	Ala-Pro-*p*NA	56
Phe-Ala	95	Glu-Phe-*p*NA	23
Asp-Gly	59	Gly-Pro-*p*NA	19
Lys-Ala	43	Ala-Ala-*p*NA	11
Leu-Leu	36		
Ala-Phe	27	Leu-*p*NA	21
Ala-Ala	22	Ala-*p*NA	6
Met-Ala	14	Lys-*p*NA	4
Ala-Val	0	Pro-*p*NA	2
Leu-Pro	0		
Ala-Pro	0		
Pro-Leu	0		
Pro-Phe	0		
Leu-Gly-Phe	75		
Ala-Leu-Gly	21		
Ala-Pro-Gly	0		
Ala-Ala-Ala-Ala	0		

[a] For peptides, activity on Leu-Gly was assigned a value of 100%. [b] For *p*-nitroanilides, activity on Arg-Pro-*p*NA was assigned a value of 100%.

Substrate Specificity and Enzyme Kinetics. The specificity of the enzyme is shown in Table 3. When the purified enzyme was incubated with *p*-nitroanilide derivatives of amino acids and dipeptides, activity was highest on Arg-Pro-*p*NA followed by Ala-Pro-*p*NA. The enzyme hydrolyzed other dipeptide *p*-nitroanilides and amino acid *p*-nitroanilides but at a very slow rate.

Among the dipeptides tested, Phe-Met was hydrolyzed at the highest rate, followed by Leu-Gly and Phe-Ala. Both amino acid residues in these dipeptides appeared to be of importance for the dipeptidase activity of the enzyme. Dipeptides containing proline at either the N or C terminus were not good substrates. Neither was

the tripeptide Ala-Pro-Gly hydrolyzed. However, the enzyme was active on Ala-Leu-Gly and Leu-Gly-Phe. These results indicate that the enzyme specificity is not limited to X-Pro-Y type substrates.

The K_m values for the hydrolysis of Arg-Pro-*p*NA and Leu-Gly at pH 7.0 and 37 °C were 4.8 and 1.1 mM, respectively. The enzyme showed higher affinity for Leu-Gly than for Arg-Pro-*p*NA.

Evidence for the Presence of Two X-prolyl-dipeptidyl Aminopeptidase Activities. As described, activity toward Arg-Pro-*p*NA of the cell-free extract of *Lb. casei* ssp. *casei* IFPL 731 was resolved into two peaks by anion-exchange chromatography on DEAE-Sepharose. Interestingly, both peaks showed different substrate specificity. Peak 1 eluted at 0.18 M NaCl and resembled an authentic PepX, whereas the second peak eluted at 0.24 M NaCl and was active on Ala-Leu-Gly, Leu-Gly, and Phe-Ala. The presence of two separate enzymes was confirmed by hydrophobic interaction chromatography and gel filtration. Using the same chromatographic conditions employed for the purification of the novel aminopeptidase, the first active peak obtained after DEAE-Sepharose chromatography was applied to a phenyl-Superose column. The activity toward Arg-Pro-*p*NA was found in a single peak that eluted at 0.7 M ammonium sulfate. This fraction was applied to a calibrated Superose 12 column, and on the basis of its elution volume was calculated to have a molecular mass of about 90 kDa.

DISCUSSION

Two X-prolyl-dipeptidyl aminopeptidase activities were found in the cell-free extract of *Lb. casei* ssp. *casei* IFPL 731. The enzymes differed with regard to substrate specificity, molecular mass, and hydrophobicity. Until now, only *Lb. brevis* has been shown to possess two PepX activities, using histochemical staining of gel electrophoregrams (Casey and Meyer, 1985).

In this paper, an aminopeptidase that showed high activity on Arg-Pro-*p*NA was purified to homogeneity and characterized. PepX has been purified to homogeneity from a wide range of lactic acid bacteria, and all purified enzymes had rather similar properties: they specifically hydrolyze peptides with the structure X-Pro-Y (X and Y can be any amino acid) and are not or are only slightly inhibited by EDTA but are strongly inhibited by serine proteinase inhibitors such as PMSF and diisopropyl fluorophosphate (Kok and De Vos, 1994). We consider the aminopeptidase isolated in this study to be different from normal PepX because it hydrolyzed dipeptides, tripeptides, and amino acid *p*-nitroanilides. Furthermore, various properties of the enzyme, e.g., the molecular mass, kinetics parameters, and hydrophobicity differ from those reported for PepXs.

To check that the enzyme was not contaminated with traces of other peptidases, which could be responsible for this broad substrate specificity, inhibition studies were performed using the dipeptide Phe-Ala. Dipeptidase activity was completely inhibited by PMSF, which confirmed the purity of the enzyme and the involvement of a serine residue in the hydrolysis of peptides. Because none of the general aminopeptidases purified so far was inhibited by serine protease inhibitors and only PepX is inhibited by this class of inhibitors (Kok and de Vos, 1994), the purified enzyme was considered to be unique. Apart from being inhibited by PMSF, the enzyme was completely inhibited by reducing agents, such as dithiothreitol and β-mercaptoethanol, indicating that disulfide bridges are important in maintaining an active conformation. A decrease in enzyme activity was observed with EDTA, which suggests a requirement for a metal ion.

The studied enzyme was found to be monomeric, with a molecular mass of about 67 kDa. Purified PepXs have been reported to be dimeric or trimeric, with a monomeric molecular mass in the range of 80−90 kDa (Meyer and Jordi, 1987; Kiefer-Partsch *et al.*, 1989; Zevaco *et al.*, 1990; Lloyd and Pritchard, 1991; Bockelmann *et al.*, 1991; Miyakawa *et al.*, 1991). This monomeric molecular mass is in agreement with the deduced amino acid sequence of the cloned PepXs (88−91 kDa) (Mayo *et al.*, 1991; Nardi *et al.*, 1991; Meyer-Barton *et al.*, 1993; Vesanto *et al.*, 1995). Only the PepX of *Lb. helveticus* (Khalid and Marth, 1990) and *Lb. casei* ssp. *casei* LLG (Habibi-Najafi and Lee, 1994) were reported to be monomeric, but these had a higher molecular mass than the purified enzyme, 72 and 79 kDa, respectively.

Under our experimental conditions, Arg-Pro-*p*NA was the best substrate among *p*-nitroanilides, but kinetics studies with this substrate revealed a low affinity (K_m 4.8 mM). High affinity for peptides of the X-Pro-Y type has been observed for PepX from *Lactococcus*, *Lactobacillus*, or *Streptococcus*.

One of the functions of peptidases is to supply the organism with essential amino acids. The role of the purified enzyme in amino acid metabolism could be more important than that of a strict PepX due to its broad substrate specificity and its capacity to produce free amino acids. Reported PepXs need the concerted action of prolidases to release free amino acids from X-Pro dipeptides. Although enzyme activity was observed over a very narrow pH range (6.0−7.0), cells of *Lb. casei* are acid tolerant, being able to maintain an intracellular pH near 6.0 even when the external pH is <5.0 (Nannen and Hutkins, 1991). Because lactobacilli are quite resistant to cell lysis, the enzyme characterized in this study may play a role in cheese ripening providing that lactobacilli cells are metabolically active.

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