Purification and Characterization of Proline Iminopeptidase from *Lactobacillus casei* ssp. *casei* LLG

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ABSTRACT

Proline iminopeptidase was purified 76-fold from crude cell-free extracts of *Lactobacillus casei* ssp. *casei* LLG by ion-exchange chromatography (preparative and analytical) and gel filtration chromatography using fast protein liquid chromatography. The purified enzyme appeared as a single band on native- and SDS-PAGE and had a molecular mass of 46 kDa. Enzyme activity was maximal at pH 7.5 and 40°C with proline aminomethyl coumarin as substrate. The activity was inhibited by Fe^{3+} and Hg^{2+} ions. This enzyme evidently was sulfhydryl; *p*-chloromercuribenzoate caused complete inhibition at 10 m*M*. The Michaelis-Menten constant and maximum velocity were .6 m*M* and 1.7 n*M*/mg per min respectively, using the same substrate. This enzyme showed the ability to cleave the Pro-Pro bond, which is of significant importance in cheese ripening.

(**Key words**: proline iminopeptidase, purification, cheese ripening, lactobacilli)

Abbreviation key: **AMC** = 7-amino-4-methylcoumarin, **β-NA** = β-naphthylamide.

INTRODUCTION

Proline-containing peptides at the N-terminal residue are less susceptible to the action of general aminopeptidases that remove most of the other amino acid residues. To contribute to flavor production during cheese ripening, the proline-rich milk protein, casein, should be degraded properly by the means of a balance of proteolytic and peptidolytic enzymes, such as proline-specific peptidases, which are thought to play a vital role in the debittering process. The presence of proline-specific peptidases from starter and nonstarter lactic acid bacteria has been reported first by Casey and Meyer (8), followed by a detailed study on proline-specific peptidases of *Streptococcus cremoris* AM2 by Booth et al. (6) and of *Lactobacillus casei* spp. (9). Several enzymes of this group from both starter and nonstarter lactic acid bacteria have been purified to homogeneity and characterized in detail (7, 10, 13).

An aminopeptidase from *Lactobacillus casei* ssp. *casei* LLG that has specificity for the proline residue has been detected, purified, and characterized (3). *Lactobacillus casei* ssp. *casei* LLG was chosen for this study because of superior peptidase activities in crude extracts (2, 3), which have been used successfully in the acceleration of cheese ripening (17) and enzyme-modified cheese (21) without bitterness. This enzyme is considered to play a significant role in removal of bitter peptides during cheese ripening, but the specificity and utility of proline iminopeptidase have not been studied.

Among microbial aminopeptidases, prolyl aminopeptidase (proline iminopeptidase EC 3.4.11.5) is of particular interest because of the capability of releasing proline from their substrates and may play a vital role in the process of proteolysis in ripening cheese. Because of the importance of free proline in the production of a specific type of cheese, in particular Swiss cheese, this enzyme from secondary starters such as propionibacteria has been studied (15, 16, 20). This report is the first describing the purification and characterization of a proline iminopeptidase from a cell extract of *Lactobacillus casei*. Additionally, the action of pure enzyme on tryptic digests of β-casein was studied to determine whether this enzyme

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could be used as a debittering agent in dairy and other protein-based products (unpublished data).

MATERIALS AND METHODS

Organism and Preparation of Cell Extracts

The strain used in this study was obtained from the Food Research and Development Center, Agriculture Canada (St. Hyacinthe, QC, Canada). The stock cultures were maintained at −30°C in skim milk solution diluted equally with growth medium. Working cultures were prepared by two successive transfers of stock cultures to MRS broth. The culture was grown in a laboratory fermenter (1.5 L) at 37°C and 70 rpm; pH was maintained at 6.0. The preparation of crude cell-free extracts was as previously described (9), using the French press for cell disintegration.

Chemicals, Reagents, and Equipments

Unless otherwise specified, all amino acid and dipeptide derivatives and other chemicals were from Sigma Chemical Co. (St. Louis, MO) and Bachem Bioscience Inc. (King of Prussia, PA). A fast protein liquid chromatography system (Pharmacia, Uppsala, Sweden), for purification of the enzyme consisted of two P-500 pumps, two injection valves MV-7 with appropriate loops and superloops for sample injection, an LCC-501 Plus controller, UV monitor, UV M II (1-cm optical path) set at 280 nm, a fraction collector Frac-100, and a REC-102 dual pen recorder. Prepacked columns for chromatography, such as Mono Q HR 16/10 (preparative; 16 × 1 cm i.d), Mono Q HR 5/5 (analytical; 5 × .5 cm i.d.), and Superose 12 HR 10/30 (30 × 1.0 cm i.d.) from Pharmacia were used for enzyme purification. Gel electrophoresis was performed on Phast-System™ (Pharmacia) using native (8 to 25%), SDS (12.5%), and isoelectric focusing (pH 3.5 to 10) minigels. Gels, staining dye, and molecular mass markers for the electrophoresis were supplied by Pharmacia (Montreal, QC, Canada). All other chemicals were of analytical reagent grade.

Protein Determination

Protein was determined spectrophotometrically by the bicinchoninic acid assay reagent supplied with the system (Pierce Chemical Ltd., Rockford, IL (22). The method is based on the reaction of Cu^{2+} with peptides in an alkaline solution (yielding Cu^{+}) detected by a highly sensitive and selective reagent (bincinchoninic acid). Bovine serum albumin was used for the standard curve.

Enzyme Assay

The peptidolytic activities were measured using 7-amino-4-methylcoumarin (**AMC**) derivatives by the modified method of Kato et al. (12). The substrate used for proline iminopeptidase was L-prolyl-AMC. The fluorescence of AMC released by the action of enzyme on the specific fluorogenic substrate was measured as relative fluorescence units, using a spectrofluorimeter (Kontron Instrument SFM 25, Zurich, Switzerland). Excitation and emission wavelengths were 375 and 440 nm, respectively. The instrument was standardized daily with 1.35 μM solution of quinine sulfate in .1N H_2SO_4, which gave 1.0 RFU (relative fluorescence units). The extent of release of AMC was computed by reference to the standard graph relating AMC to the relative fluorescence units under the assay conditions.

For assay, 100 μl of the crude extract was added to 900 μl of .111 mM substrate in 50 mM Tris·HCl (pH 7.5) for 15 min at 30°C. The reaction was terminated by the addition of 2 ml of 1.5 M acetic acid. One unit of enzyme activity was defined as the amount of enzyme required to release 1 nmol of AMC/min under the conditions of the assay. Specific activity was expressed as units per milligram of protein.

Substrate Specificity

The hydrolysis of AMC derivatives of amino acids and peptides was followed by the standard enzyme assay procedure as previously described. The activities for substrates containing β-naphthylamide (β-**NA**) were assayed using the method of Abdus Satter et al. (1) with minor modification. To 800 μl of 20 mM Tris·HCl buffer (pH 7.5), 100 μl of enzyme solution were added, and the mixture was preincubated at 37°C for 3 min. The reaction was initiated by addition of 100 μl of β-NA substrate (5 mM). After 10 min of incubation, 500 μl of Fast Garnet GBC salt (1 mg/ml

containing 10% Triton-X100 in 1 *M* acetate buffer pH 4.0) were added, and the absorbance was measured at 550 nm. The enzyme activity toward peptides with blocked α-amino acids, such as N-succinyl-glycyl-L-prolyl-L-leucyl-L-glycyl-L-prolyl (N-Suc-Gly-Pro-Leu-Gly-Pro), N-benzyloxycarbonyl-prolyl (N-CBZ-Pro), and dipeptides (Pro-Pro, Pro-Gly, Pro-Leu, and Leu-Pro), was assayed in a reaction containing 900 μl of substrate (.1 m*M* in .05 *M* Tris-HCl buffer, pH 7.5) and 100 μl of purified enzyme incubated at 30°C. The amount of α-amino acids or peptides released was determined by the ninhydrin method, as described by Moore and Stein (19).

Purification of Enzyme

Ammonium Sulfate Fractionation. The prepared crude cell-free extracts were fractionated by salting out with solid ammonium sulfate to 80% saturation. The ammonium sulfate was added gradually to the crude extacts and stored with gentle stirring for 8 h. The precipitate formed was collected by centrifugation at 10,000 × *g* for 20 min, dissolved in a minimal amount of .02 *M* Tris·HCl buffer, pH 8.0, and then dialyzed overnight against the same buffer, using Spectrapor membrane (30,000 molecular weight cutoff, Spectrum Co., Houston, TX). The dialyzed fraction was analyzed for protein concentration and for proline iminopeptidase activity as mentioned previously in this paper.

First Ion-Exchange Chromatography. The ion-exchange (Preparative Mono Q) column was equilibrated with .02 *M* Tris·HCl buffer (pH 8.0). Approximately 100 mg of protein from the dialyzed ammonium sulfate fraction were applied to the column at a flow rate of 3 ml/min. After the column was washed with the equilibration buffer, the bound enzyme was eluted with a linear gradient of NaCl (.2 to .6 *M*) in the same buffer. Fractions (7 ml) were collected and tested for proline iminopeptidase activity. Fractions with the highest enzyme activities were pooled, concentrated, and desalted by ultrafiltration using Centriprep 30) (Amicon Corp., Toronto, ON, Canada).

Second Ion-Exchange Chromatography. The concentrated enzyme fraction from the previous step was applied to the second ion-exchange (analytical Mono Q) column that had been previously equilibrated with .02 *M* Tris·HCl buffer (pH 7.0). The column was washed with the same buffer, and the enzyme was eluted with a stepwise gradient of NaCl (.2 to .6 *M*) in the same buffer at a flow rate of .5 ml/min. The active fractions were pooled, concentrated, and desalted using Centricon 30 (Amicon Corp.).

Gel-Filtration Chromatography. The concentrated enzyme from the previous step was then applied to a column (30 × 1.0 cm) of Superose™ 12 (Pharmacia) that had been previously equilibrated with .02 *M* Tris·HCl buffer containing .6 *M* NaCl, pH 7.0. The enzyme was eluted with the same buffer at a flow rate of .5 ml/min, and 2-ml fractions were collected. The active fraction was concentrated and desalted using Centricon 30 for further studies.

PAGE. The purity of the enzyme preparation at each purification step was examined by native PAGE with 4% stacking and 8 to 25% gradient running gels. Mini gels (5 × 4 cm) were run on Phast electrophoresis system™ (Pharmacia) at 15°C and 30 V/cm. The purity of enzyme was confirmed on 12.5% acrylamide gel with a 4% stacking gel with SDS, using the method of Laemmli (14). The protein samples were mixed 1:1 (vol/vol) with sample buffer (20 m*M* Tris·HCl, pH 8.0; 2 m*M* EDTA, 5% SDS, 10% β-mercaptoethanol, and .001% bromophenol blue), boiled for 5 min, and applied to the gels. Tris-glycine·HCl buffer, pH 8.8, was used as running buffer. Protein bands were stained with Coomassie blue R-250 (Pharmacia).

Determination of Molecular Mass. The molecular mass of purified enzyme was estimated by SDS-PAGE using 4% stacking and 12.5% running gels. The proteins with low molecular mass [α-LA (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), BSA (67 kDa), and phosphorylase b (94 kDa)] were used as standards. The gels were stained with Coomassie blue-R 250 followed by flooding with the preserving solution according to the guidebook. The molecular mass of the enzyme was then determined from the standard curve.

Properties of Purified Enzyme

Determination of Isoelectric Point. Isoelectric focusing of the purified enzyme was carried out on 5% acrylamide slab gels at 2000 V,

5.0 mA, 3.5 W, and 15°C using the Phast System™ (Pharmacia). Pharmalytes in the pH range of 3.5 to 10 were used as carrier ampholytes, which formed a stable pH gradient across the gel between two electrodes. The isoelectric point of the pure protein was estimated from a migration profile of the reference proteins.

Effect of pH and Temperature. The effect of pH on the activity of purified enzyme was measured using buffers of .05 *M* acetate (pH 4.0 to 6.0), .05 *M* phosphate (pH 6.5 to 8), and .05 *M* glycine·NaOH (pH 8.5 to 10.5) at 30°C. The appropriate amounts of enzyme and substrate diluted at a specific pH were incubated for 15 min, and the release of AMC was measured. To estimate the stability of enzyme, proline iminopeptidase was incubated for 1, 12, and 24 h at 4°C and pH 5 to 10.5 before the residual activity was determined at 30°C. The effect of temperature on activity was measured in the range of 15 to 70°C. The .05 *M* Tris·HCl buffer (pH 7.5) was equilibrated for 15 min at the test temperature before the enzyme was added. The mixture was then incubated for 10 min, and the residual enzyme activities were measured at different temperatures.

Enzyme Kinetics. Portions (100 μl) of the enzyme were incubated with various concentrations of substrate (Pro-AMC), ranging from .1 to 1.0 m*M*. The hydrolysis of the substrate was determined as mentioned previously under the enzyme assay. The Lineweaver-Burk plot was constructed, and the Michaelis-Menten constant and maximal velocity were estimated from the slope and intercept of the regression line (18).

Effect of Metal Chelators and Other Inhibitors. The purified enzyme solution was incubated in the presence or absence of cations (Fe^{3+}, Fe^{2+}, Cu^{2+}, Co^{2+}, Ca^{2+}, Hg^{2+}, Pb^{2+}, and Zn^{2+}) and other inhibitors (EDTA, phenylmethylsulfonylfluoride diisopropylfluorophosphate, *o*-phenanthroline, dithiotheritol, iodoacetic acid and *p*-chloromercuribenzoate) for 5 min at 30°C at final concentrations of .1, 1.0, and 10 m*M* in .05 *M* sodium phosphate buffer, pH 7.1. The reaction was initiated by addition of 100 μl of enzyme solution, and the enzyme activities were measured. Inhibition was expressed as a percentage of the activity without effector (control).

RESULTS AND DISCUSSION

Purification of Proline Iminopeptidase

Purification of proline iminopeptidase is summarized in Table 1. The elution profiles of the enzyme during the purification process (ion-exchange chromatography and size exclusion chromatography) are shown in Figures 1 and 2, respectively. The enzyme was purified approximately 76-fold with a yield of 7%, and the preparation exhibited a single band by SDS-PAGE (Figure 3). The electrophoretic pattern of enzyme from different purification steps on native PAGE showed a single, sharp band after the third step (gel filtration chromatography). When the native gel was soaked in Pro-AMC solution for a few minutes and the gel was examined under UV light, a bright fluorescent band corresponding to the proline iminopeptidase activity was observed because of released AMC.

TABLE 1. Summary of the purification steps of proline iminopeptidase from *Lactobacillus casei* ssp. *casei* LLG.

Purification step	Total protein	Total activity[1]	Specific activity	Purification	Yield
	(mg)	(units)	(units/mg)	(-fold)	(%)
Crude extract	1378.5	2856.3	21	1	100
Ammonium sulfate	966.4	2781.5	2.9	1.3	97
Ion-exchange I (preparative)	36.9	1237.3	33.5	16.2	43
Ion-exchange II (analytical)	4.5	326.7	72.6	35.1	11
Gel filtration	1.2	191.1	159.3	75.9	7

[1]One unit of enzyme is defined as the amount of enzyme required to release 1 nmol of aminomethylcoumarin per minute under the conditions of assay.

The molecular mass of the enzyme was estimated to be 46 kDa by SDS-PAGE. The purified proline iminopeptidase from *Lactobacillus casei* ssp. *casei* LLG showed a monomeric form of 46 kDa. Not many bacterial proline iminopeptidases have been purified compared with those from animal or plant sources, probably because of low activity. The enzyme purified in this study differed somewhat from those of other bacterial sources; although the purified enzyme from *Lactococcus lactis* ssp. *cremoris* HP was a dimer (150 kDa by gel filtration), enzymes from *Bacillus coagulanse, Bacillus megatrium,* and *Propionibacterium shermanii* were monomers of 40, 58, and 61 kDa, respectively (5, 20, 23, 24).

The isoelectric point of LLG proline iminopeptidase, as determined by isoelectric focusing gel electrophoresis, was 4.46 (data not shown), which agreed well with proline iminopeptidase from other bacterial sources (24).

Effect of pH and Temperature. The effect of pH on pure enzyme was examined at pH from 4 to 10. As shown in Figure 4, the optimal pH appeared to be 7.5. Although this enzyme showed a relatively broad pH range of activity, activities were generally higher at alkaline pH. The pH stability of this enzyme was determined at 4°C by preincubation of the enzyme at different pH (data not shown). Proline iminopeptidase was fairly stable over the range of pH 5 to 10. More than 60% of activity was retained after 24 h at pH 5.5, which is the pH of Cheddar cheese during ripening. This result may well be compared with the broad activity of enzymes from the same strain (3, 10) or iminopeptidases from other strains (20, 24). Figure 5 shows the effect of temperature from 15 to 70°C on enzyme activity. The temperature was optimal at 40°C, but around 70% of activity was retained at 45°C. However, activity was sharply decreased at 50°C. The thermal stability of this enzyme appeared to be similar to that of the aminopeptidase and x-prolyl dipeptidyl peptidase from the same strain (3, 10). Enzyme activity at 40°C is beneficial when used in high cooking temperature of cheeses and cheese slurries. This optimal temperature was also similar to that of the iminopeptidase isolated from propionibacteria (20).

Effect of Metal Chelators and Enzyme Inhibitors. The effects of several agents on the

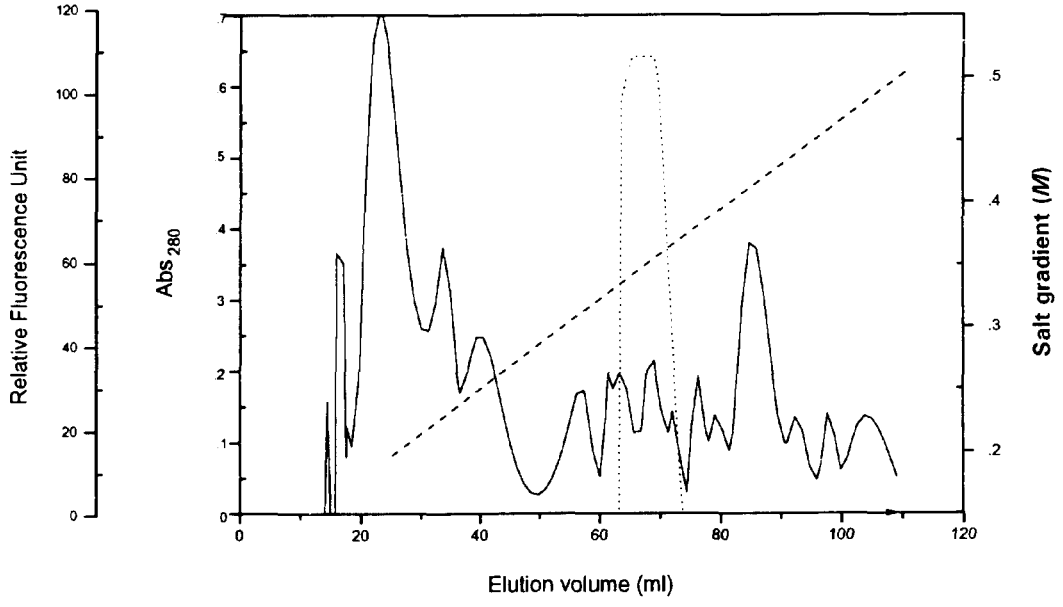

Figure 1. Preparative ion-exchange elution profile of crude extract of *Lactobacillus casei* ssp. *casei* LLG on Mono Q (16 × 1 cm). Enzyme activity at emission of 440 nm (---), protein at absorbance of 280 nm (—), and salt gradient (– – –).

activity of pure enzyme are summarized in Table 2. The rate of hydrolysis of Pro-AMC in the absence of any inhibitor, reducing agent, or chelator was taken as 100%. Inhibition of enzyme activity was almost complete with 10 m*M* *p*-chloromercuribenzoic acid. The partial inhibition with another sulfhydryl inhibitor, iodoacetic acid, indicates the possible involvement of functional sulfhydryl group at or near the active site. Inhibition was similar for other bacterial proline iminopeptidases (24). Incubation of this enzyme with metal effectors, such as EDTA and *o*-phenanthroline, had no effect on the enzyme activity. This finding revealed that divalent cations do not seem to be involved in the catalytic mechanism. The enzyme was partially inhibited by phenylmethyl sulfonylfluoride and diisopropylfluorophosphate, specific inhibitors for serine proteases, which suggests the presence of serine residue near the active site and its involvement in catalysis. Inhibition also was strong for the incubation of pure enzyme with Fe^{3+} and partial for $HgCl_2$. No inhibition was observed with other metal-chelating agents. The inhibitory studies on this enzyme in crude cell-free extracts showed approximately the same results (9).

Enzyme Kinetics and Substrate Specificity. The apparent Michaelis constant and maximum velocity of the pure enzyme for Pro-AMC under assay conditions, calculated from Lineweaver-Burk plots were .6 m*M* and 1.7 m*M*/mg per min, respectively. Comparison of the kinetics of the other purified proline iminopeptidases is difficult because of the use of different substrates. The purified enzyme was incubated with AMC and β-NA derivatives of amino acids, di- and tripeptides, and peptides with free α-amino acids. (Table 3). Although

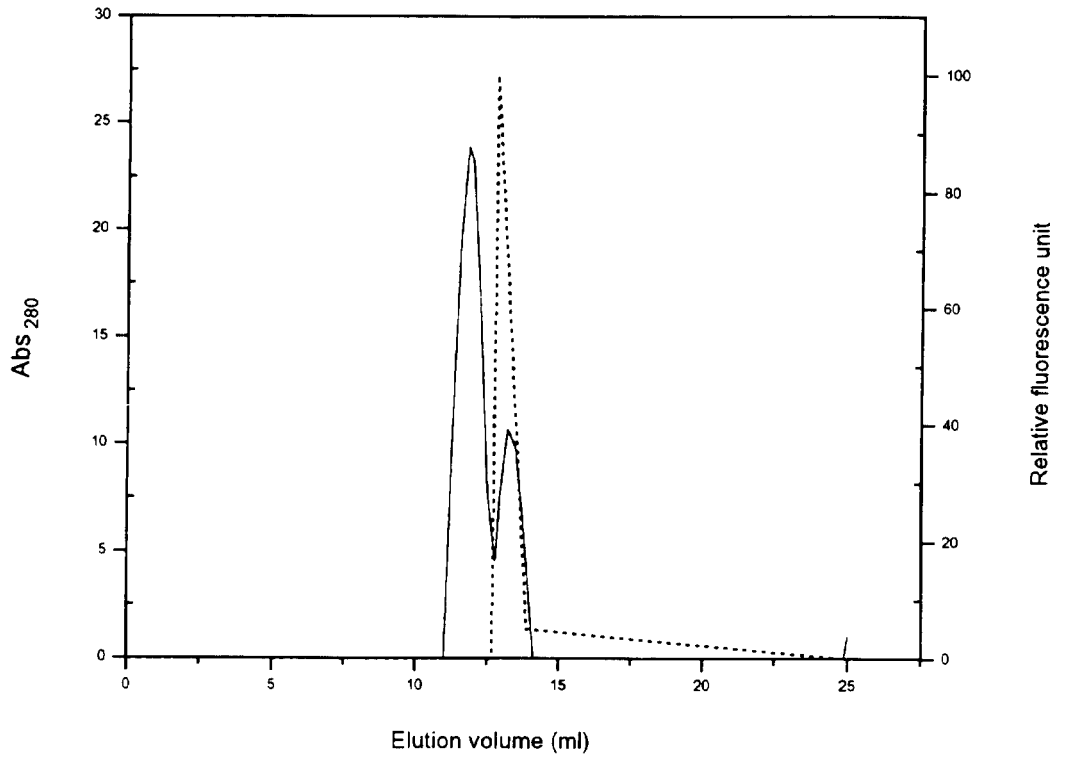

Figure 2. Elution profile of active proline iminopeptidase fractions of *Lactobacillus casei* ssp. *casei* LLG on gel filtration column. Enzyme activity at emission of 440 nm (---); protein at absorbance (Abs) of 280 nm (—).

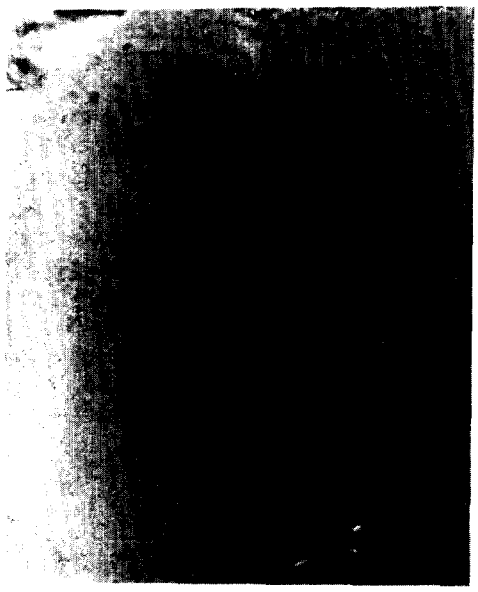

Figure 3. The SDS-PAGE of purified proline iminopeptidase from *Lactobacillus casei* ssp. *casei* LLG on 12.5% acrylamide gels. Lane 1, marker proteins; lane 2, purified enzyme. Molecular mass marker (top to bottom): α-LA (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), BSA (67 kDa), and phosphorylase b (94 kDa).

the enzyme exhibited a preference for substrates possessing proline as the N-terminal amino acid, it also hydrolyzed other substrates with N-terminal amino acids, such as leucine, alanine, and phenylalanine. Activity was also higher toward Leu-β-NA, but its specificity

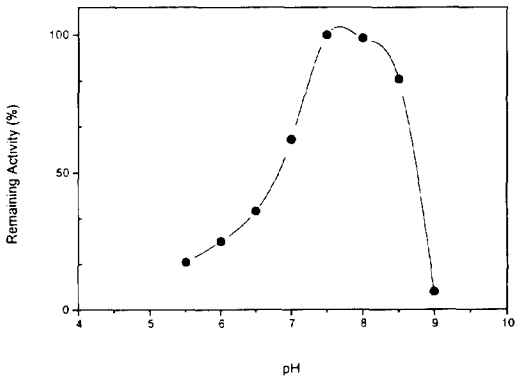

Figure 4. Effect of pH on proline iminopeptidase activity of *Lactobacillus casei* ssp. *casei* LLG.

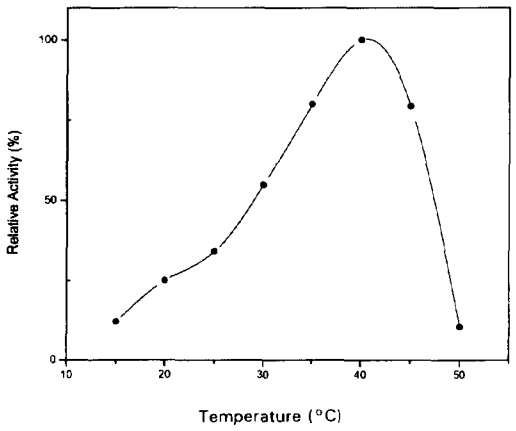

Figure 5. Effect of temperature on proline iminopeptidase activity of *Lactobacillus casei* ssp. *casei* LLG.

was not as broad as general aminopeptidase from the same strain and showed a weak affinity toward proline (3). This enzyme is markedly different from the general aminopeptidase in molecular mass, isoelectric point, and other characteristics (optimal pH and temperature, inhibitory reaction).

TABLE 2. Effect of cations and other agents on proline iminopeptidase of *Lactobacillus casei* ssp. *casei* LLG.

	Relative activity (%)[1]		
Agent	.1 m*M*	1 m*M*	10 m*M*
Cu^{2+}	87	57	ND[2]
Ca^{2+}	93	92	ND
Zn^{2+}	100	90	ND
Co^{2+}	88	79	ND
Fe^{2+}	89	76	ND
Fe^{3+}	14	4	ND
Pb^{2+}	92	90	ND
Hg^{2+}	80	15	ND
Iodoacetic acid	100	87	78
EDTA	100	90	80
Dithiothreitol	110	100	100
o-Phenanthroline	100	94	90
PMSF[3]	100	88	28
DIFP[4]	100	89	88
PCMB[5]	100	100	6
Control	100	100	100

[1]Means of duplicates (SE ± 5%).

[2]Not determined.

[3]Phenylmethylsulfonylfluoride.

[4]Diisopropylfluorophosphate.

[5]*p*-Chloromercuribenzoic acid.

Unlike proline iminopeptidase from *Lactococcus lactis* ssp. *cremoris* HP (5), this enzyme showed very weak reactions with di- and tripeptides containing proline at the amino terminal amino acid. Among the dipeptidase substrates tested, the enzyme had maximum activity with Pro-Leu (100%), followed by Leu-Pro (80%), Pro-Pro (30%), and Pro-Gly (5%). It is noteworthy that the hydrolysis of Leu-Pro is due to the ability of proline iminopeptidase to react highly with leucine at the N-terminal position, and this ability should not be mistakenly considered to be prolidase activity. Prolidase was specific for aminoacyl proline dipeptides. Preliminary results indicated the presence of prolidase in comparatively low amounts in this strain (unpublished data). The ability of the enzyme to cleave Pro-Pro is of significant importance in this study because neither general aminopeptidase nor previously purified microbial proline iminopeptidases could hydrolyze this dipeptide.

TABLE 3. Substrate specificity of proline iminopeptidase from *Lactobacillus casei* ssp. *casei* LLG.

Substrate	Relative activity[1]
	(%)
A AMC[2] Derivatives of imino and amino acids	
Pro-AMC	100
Phe-AMC	34
Ala-AMC	23
Gly-AMC	0
Arg-AMC	0
Glu-AMC	0
B AMC Derivatives of di- and tripeptides	
Pro-Arg-AMC	0
Pro-Phe-Arg-AMC	0
C β-NA Derivatives of imino and amino acids	
Pro-β-NA	100
Leu-β-NA	70
Ser-β-NA	20
Lys-β-NA	19
Pro-4-methoxy-β-NA	0
Try-β-NA	0
D Dipeptides with free N terminal	
Pro-Leu	100
Leu-Pro	80
Pro-Pro	30
Pro-Gly	5

[1]Means of duplicates (SE ± 5%).

[2]AMC = 7-amino-4-methylcoumarin; β-NA = β-naphthylamide.

CONCLUSIONS

Bitter tasting peptides consistently contain a high proportion of hydrophobic amino acid residues. Bitterness developed in cheese is partly due to the accumulation of proline-containing peptides (11). This enzyme, in conjunction with other proline-specific peptide hydrolases, could thus play a significant role in the debittering process in cheese, particularly during accelerated ripening of cheese and production of enzyme-modified cheese [(21); unpublished data].

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