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Purification and Characterization of a Prolidase from *Lactobacillus casei* subsp. *casei* IFPL 731

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A peptidase showing a high level of specificity towards dipeptides of the X-Pro type was purified to homogeneity from the cell extract of *Lactobacillus casei* subsp. *casei* IFPL 731. The enzyme was a monomer having a molecular mass of 41 kDa. The pH and temperature optima were 6.5 to 7.5 and 55°C, respectively. Metal chelating agents completely inhibited enzyme activity, indicating that the prolidase was a metalloenzyme. The Michaelis constant (K_m) and V_{max} for several proline-containing dipeptides were determined.

Because of the unique structure of proline, most microbial enzymes are not able to hydrolyze bonds in which this amino acid is involved. Degradation of proline-rich oligopeptides liberated from caseins by proteinases requires the activity of proline-specific peptidases (12). These types of enzymes may play a very important role in cheese ripening, either directly by degrading proline-containing peptides which are often bitter or indirectly by making peptides accessible to the action of other peptidases by removing proline (1).

Among proline-specific peptidases, X-prolyl-dipeptidyl aminopeptidase (dipeptidyl-peptidase IV; EC 3.4.14.5) and proline iminopeptidase (prolyl aminopeptidase; EC 3.4.11.5) have been purified from *Lactobacillus casei* (7, 9, 10). X-prolyl-dipeptidyl aminopeptidase (PepXP) catalyzes the hydrolytic removal of N-terminal dipeptidyl residues from peptides containing proline in the penultimate position, while proline iminopeptidase catalyzes the cleavage of unsubstituted N-terminal prolyl residues from dipeptides, tripeptides, and polypeptides of the form Pro-X. Although a cooperative interaction between PepXP and prolidase (imidodipeptidase; EC 3.4.13.9) has been suggested by various authors, prolidase has been purified so far only from *Lactococcus lactis* subsp. *cremoris* (3, 11). Recently, from a genomic library of *Lactobacillus delbrueckii* subsp. *lactis*, a recombinant clone which complemented a mutation in the prolidase gene (*pepQ*) of *Escherichia coli* was selected (16). Purification and characterization of the *pepQ* product was reported, but the broad substrate specificity of the gene product argues against the classification of *pepQ* as an X-Pro dipeptidase gene.

Lactobacillus casei subsp. *casei* IFPL 731 was isolated from artisanal goat's milk cheese. The organism was grown in 5 liters of MRS broth at 30°C. Cells were harvested during the late exponential growth period by centrifugation (10,000 × *g*, 15 min, 4°C). For disruption, the cells were resuspended in 70 ml of 20 mM Tris-HCl (pH 7.5), mixed with glass beads (1:1, vol/wt; 150 to 212 μm in diameter; Sigma Chemical Co., St. Louis, Mo.), and shaken in an Osterizer blender (Sunbeam-Oster)(4 times for 4 min each at 4°C). Glass beads, unbroken cells, and cell debris were removed by centrifugation (15,000 × *g*, 30 min, 4°C) and filtration (Whatman no. 1 filter paper). The clear supernatant (cell extract) was treated with DNase (8 μg/ml) and RNase (8 μg/ml) (Boehringer GmbH Mannheim, Germany) for 30 min at 20°C to hydrolyze DNA and RNA.

During purification, prolidase activity was determined with Leu-Pro as substrate by a coupled-enzyme reaction as described by Boven et al. (4). The reaction mixture contained (total volume, 1 ml) 0.5 mg of peroxidase, 0.1 mg of L-amino acid oxidase, 0.18 mg of *o*-dianisidine, 50 mM Tris-HCl (pH 7.5), 2 mM Leu-Pro, and the appropriate concentration of enzyme. The hydrolysis of amino acyl-*p*-nitroanilides (acyl-*p*NA) was determined by the method of El-Soda and Desmazeaud (8). The protein content was determined by the micromethod of Bradford (5) with a protein assay kit (Bio-Rad, Munich, Germany).

The cell extract was first applied to a DEAE-Sepharose fast-flow column (Pharmacia) (15 by 5 cm) equilibrated with 20 mM Tris-HCl (pH 7.5). Bound proteins were eluted with a linear NaCl gradient (0 to 0.5 M) in the same buffer. Fractions were assayed for Leu-Pro-, Arg-Pro-*p*NA-, and Pro-*p*NA-hydrolyzing activity. The fraction with activity towards Arg-Pro-*p*NA eluted as a separate peak at 0.18 M NaCl, whereas the fractions with Leu-Pro and Pro-*p*NA activities coeluted between 0.27 and 0.28 M NaCl (Fig. 1). Fractions active towards Leu-Pro were concentrated by ultrafiltration (Amicon YM 10 membranes; Danvers, Mass.), made 1.5 M by the addition of solid $(NH_4)_2SO_4$, and applied to a Phenyl-Superose HR 5/5 column (Pharmacia) equilibrated with 20 mM Tris-HCl (pH 7.5) containing 1.5 M $(NH_4)_2SO_4$. Bound proteins were eluted in a decreasing $(NH_4)_2SO_4$ gradient (1.5 to 0 M), with the fraction having prolidase activity eluting from the column at 0.75 M $(NH_4)_2SO_4$. By this chromatographic step prolidase activity was separated from Pro-*p*NA activity. Active Leu-Pro fractions were concentrated as described above and desalted by being passed through a Sephadex G-25M column (Pharmacia) previously equilibrated with 20 mM Tris-HCl (pH 7.5). Desalted fractions were applied to a Mono Q HR 5/5 column (Pharmacia) equilibrated with 20 mM Tris-HCl (pH 7.5) and eluted with a linear NaCl gradient (0.1 to 0.4 M) in the same buffer. Fractions containing enzyme activity eluted at 0.2 M NaCl. Further purification was achieved by gel filtration. The concentrated fraction from the Mono Q HR 5/5 column was applied on a Superose 12 HR 10/30 column (Pharmacia) previously equilibrated with 20 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl and eluted in the same buffer.

Results of the purification procedure are summarized in Table 1. Overall, 3% of the initial enzyme activity was recovered and the specific activity was increased 759-fold. The purity

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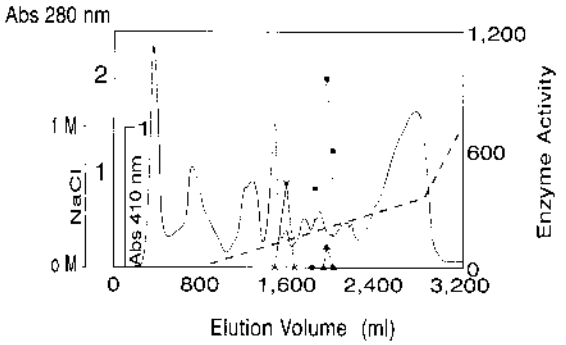

FIG. 1. Resolution of prolidase from the cell extract of *Lactobacillus casei* subsp. *casei* IFPL 731 on anion exchange chromatography (DEAE-Sepharose fast flow). Prolidase (■) activity was monitored by using Leu-Pro and was expressed as nanomoles per minute per milliliter. Proline iminopeptidase (▲) and PepXP(*) activities were measured by determining the absorbances (Abs) at 410 nm by using Pro-*p*NA and Arg-Pro-*p*NA, respectively. Protein (−) activity was measured by determining the absorbance at 280 nm. – – –, gradient.

of the enzyme was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Pharmacia 12.5% polyacrylamide gels) after silver staining. The molecular mass of the enzyme was estimated to be around 41 kDa by SDS-PAGE as well as by gel filtration chromatography, suggesting that the enzyme is a monomer. Similar results were found for the purified *Lactococcus* prolidases and the reported gene product of *pepQ* from *Lactobacillus delbrueckii* subsp. *lactis* (3, 11, 16). However, prolidases of pig, bovine, and guinea pig intestine have been found to be dimeric, with molecular masses of 108 to 113 kDa, by gel filtration (13, 15, 17).

For characterization studies, enzyme activity was determined at 37°C by measuring the release of α-amino acids by the modified cadmium-ninhydrin method described by Doi et al. (6). The optimum pH was between 6.5 and 7.5 with sodium acetate (pH 4 to 5), sodium phosphate (pH 6 to 7), Tris-HCl (pH 7 to 8.5), and sodium carbonate (pH 9 to 10) buffers. At pH 6, 65% of the maximum activity was found, whereas at pH 9, only 32% of the activity remained. The optimum temperature (at pH 6.5) after incubation for 20 min in phosphate buffer (pH 6.5) at the assay temperature was found to be 55°C. Activity decreased sharply at 70°C. Temperature stability studies showed that preincubation of the enzyme at 30°C for 30 min reduced enzyme activity to 36%. These results may indicate that the presence of the substrate in the assay solution stabilized the enzyme.

The effect of various chemical reagents on Leu-Pro activity is shown in Table 2. Like *Lactococcus* prolidases and *pepQ* product from *Lactobacillus delbrueckii* subsp. *lactis* (3, 11, 16), the purified enzyme is a metal-dependent peptidase since 1,10-phenanthroline or EDTA (1 mM) inhibited enzyme activity completely. Strong inhibition of enzyme activity was also observed with the reducing reagent dithiothreitol. Sulfhydryl group reagents such as *p*-hydroximercuribenzoate and iodoacetic acid caused a moderate inhibition of the enzyme. In contrast, intestinal prolidases are inhibited by sulfhydryl group reagents but not by EDTA or 1,10-phenanthroline (13, 17), which suggests a different catalytic mechanism.

TABLE 1. Purification of a prolidase from *Lactobacillus casei* subsp. *casei* IFPL 731

Purification step	Total protein (mg)	Total activity (nmol/min)	Sp act (nmol/min/mg)	Yield (%)	Purification (fold)
Cell extract	531	8,710	16	100	1
DEAE-Sepharose	48	4,029	85	46	5
Phenyl-Superose	3.33	1,963	599	23	36
Mono Q	0.06	674	11,240	8	685
Superose 12	0.02	249	12,445	3	759

[a] DEAE-Sepharose, elution from a DEAE-Sepharose fast-flow column; Phenyl-Superose, elution from a Phenyl-Superose HR 5/5 column; Mono Q, elution from a Mono Q HR 5/5 column; Superose 12, elution from a Superose 12 HR 10/30 column. All columns were supplied by Pharmacia.

TABLE 2. Effect of inhibitors and metal ions on the hydrolysis of Leu-Pro by prolidase

Chemical reagent or ion	Concn (mM)	Relative activity[a] (%)
EDTA	1	0
1,10-Phenanthroline	1	0
Dithiothreitol	1	25
β-Mercaptoethanol	1	84
PMSF[b]	1	123
Iodoacetic acid	1	72
p-Hydroximercuribenzoate	1	62
E-64[c]	0.01	101
Ca^{2+}	1	96
Mg^{2+}	1	134
Cu^{2+}	1	11
Mn^{2+}	1	552
Zn^{2+}	0.1	49
Co^{2+}	1	269
Fe^{3+}	1	2
Na^{+}	1,000	117
	250	70

[a] Rate of hydrolysis of Leu-Pro (0.6 nmol/min) in the absence of any inhibitor, reducing agent, or metal chelator was taken as 100%.
[b] PMSF, phenylmethylsulfonyl fluoride.
[c] E-64, cysteine protease inhibitor.

Table 2 shows the residual enzyme activity after preincubation with various metal ions in *N*,*N*-methylenebisacrylamide-Tris buffer (pH 6.5). Co^{2+} and Mn^{2+} were the most effective stimulatory metal ions. After inhibition of the enyzme with 1 mM EDTA followed by diafiltration, full enzyme activity was restored by the addition of Co^{2+} and Mn^{2+}. *Lactococcus* and *Lactobacillus* prolidases showed different requirements for metal ions. Although the deduced amino acid sequence of PepQ from *Lactobacillus delbrueckii* subsp. *lactis* (16) contained a potential zinc-binding site, an inhibitory effect was observed with Zn^{2+} at 0.1 mM concentration for the purified enzyme.

The specificity of the enzyme is shown in Table 3. As observed for the prolidase of *Lactococcus lactis* subsp. *cremoris* H61 (11), the enzyme hydrolyzed all dipeptides containing proline at the carboxy terminal, with the exception of Gly-Pro and Pro-Pro. Of the other peptides tested, only Ala-Ala and Ala-Phe were hydrolyzed, but at very low rates, by the prolidase being studied. The prolidase from *Lactococcus lactis* subsp. *cremoris* AM2 (3) and PepQ from *Lactobacillus delbruekii* subsp. *lactis* (16) were active towards various dipeptides and tripeptides. Comparisons of the activities of other enzymes with that of the prolidase from strain AM2 are difficult because a pure enzyme was not used and the activity found towards dipeptides and tripeptides might be due to contamination of the prolidase with other enzymes. On the other hand, all intestinal prolidases were able to hydrolyze Gly-Pro, and even the dipeptide Pro-Pro was hydrolyzed by the prolidase from bovine intestine (13, 15, 17).

TABLE 3. Relative activity of prolidase on different substrates

Substrate	Relative activity[a]
Ala-Ala	0.11
Ala-Phe	0.05
His-Ala	0
Leu-Gly	0
Leu-Leu	0
Lys-Ala	0
Phe-Ala	0
Phe-Met	0
Leu-*p*NA	0
Lys-*p*NA	0
Arg-Pro-*p*NA	0
Arg-Pro-Lys-Pro	0
CBZ-Ala-Pro[b]	0
Val-Pro	0.08
Ile-Pro	0.64
Ala-Pro	0.20
Gly-Pro	0
Leu-Pro	1
Phe-Pro	0.50
Pro-Pro	0
Pro-Leu	0
Pro-Phe	0
Pro-Gly-Gly	0
Gly-Pro-Ala	0
Ala-Pro-Gly	0
Ala-Leu-Gly	0
Leu-Gly-Phe	0
Ala-Ala-Ala	0

[a] Activity relative to that towards Leu-Pro (0.6 nmol/min).
[b] CBZ, carbobenzoxy.

Kinetic parameters for the hydrolysis of Leu-Pro and Phe-Pro were calculated from the slopes and intercepts of the regression lines of Lineweaver-Burk plots by using the coupled enzyme reaction. K_m values were 0.2 mM for Leu-Pro and 25 mM for Phe-Pro. The enzyme had greater affinity for Leu-Pro than for Phe-Pro. Similar V_{max} values, 82 nmol/min/μg of protein, were calculated for both substrates. However, at high Leu-Pro concentrations (above 2.5 mM) strong inhibition of enzyme activity was observed and the measured V_{max} (66 nmol/min/μg of protein) was lower than the calculated value from the intercept of the regression line (Fig. 2). No such inhibitory effect was observed with Phe-Pro. These results are in agreement with the findings of Booth et al. (3) for the prolidase of *Lactococcus lactis* subsp. *cremoris* AM2. In contrast, prolidase from *Lactococcus lactis* subsp. *cremoris* H61 (11) was not inhibited by high Leu-Pro concentrations, and similar K_m values were reported for Leu-Pro and Phe-Pro.

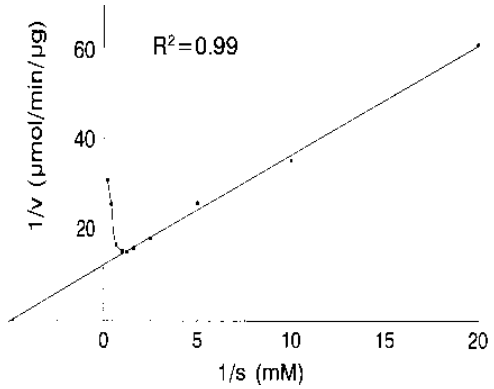

FIG. 2. Lineweaver-Burk plots of prolidase at various concentrations of Leu-Pro. R^2, index of determination.

Prolidase may have an important role in the metabolism of amino acids by lactobacilli. Proline-containing peptides of the X-Pro-Y type are rapidly degraded by PepXP, yielding X-Pro dipeptides which are suitable substrates for the prolidase. Dipeptides containing proline have been reported to be bitter (14), whereas free proline imparts a sweet flavor to cheese (2). Since the temperature, the pH, and the salt content of cheese during ripening are suitable for the *Lactobacillus* prolidase, this enzyme may contribute to cheese flavor development.

We have detected in the cell extract of *Lactobacillus casei* subsp. *casei* IFPL 731 the presence of various proline-specific peptidases. A possible role for proline-specific peptidases as debittering agents of dairy products is being investigated.

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