Isolation and Characterization of a Prolidase from *Streptococcus cremoris* H61

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A prolidase with a molecular weight of 43,000 was purified to homogeneity from a cell-free extract of *Streptococcus cremoris* H61. The optimum pH of the enzyme was in the range of 6.5 to 7.5. The hydrolyzing activity was specific for dipeptides of the X-Pro type. Kinetic constants for 4 dipeptides (Leu-Pro, Phe-Pro, Val-Pro and Ala-Pro) were estimated. K_m values were not very different for these substrates, but V_{max} values were quite different (Leu-Pro > Phe-Pro, Val-Pro > Ala-Pro). The enzyme was activated by cobalt ion and inactivated by metal-chelating agents or with 2-mercaptoethanol.

The amino peptidase present in such bacteria as *Escherichia coli* and *Salmonella typhimurium* plays an important role in the peptide catabolism.¹⁾ The several kinds of aminopeptidases with different substrate specificities have been reported.¹⁾ The aminopeptidases of lactic streptococci may function in the peptide catabolism as the bacteria described above do.

In the case of dairy lactic bacteria, it is considered that their aminopeptidases may also have some significance in cheese ripening. The aminopeptidases released from autolyzed cells in cheese seem to contribute to the formation of amino acids from casein under the cooperation of proteinases present in cheese.

We have purified and characterized a dipeptidase^{2,3)} from *Streptococcus cremoris* H61. Thereafter, we have found the presence of aminopeptidase which was capable of hydrolyzing dipeptides of the X-Pro type in the same strain. This type of the enzyme had been found in porcine intestinal mucosa,⁴⁾ horse erythrocyte,⁵⁾ rat brain⁶⁾ and *Arthrobacter*,⁷⁾ and is designated as prolidase, proline dipeptidase and imidodipeptidase (EC 3.4.13.9). Prolidases from porcine^{8,9,10)} and bovine intestine¹¹⁾ have been purified and characterized.

The physiological significance of these enzymes is considered to be the cooperative hydrolysis with other proteases.¹²⁾ We purified and characterized the prolidase from *Str. cremoris* H61 and found that the enzyme had different molecular properties from those of mammalian intestine. The present paper describes these results.

MATERIALS AND METHODS

Reagents. DEAE-cellulose was purchased from Whatman Co. (DE-23), London, hydroxyapatite from Seikagaku-Kogyo Co., Tokyo, and Sephadex G-150 from Pharmacia Fine Chem., Uppsala. Gly-L-Ser and L-Ala-L-Tyr were obtained from Seikagaku-Kogyo Co., Tokyo, DL-Leu-L-Phe and DL-Leu-Gly-DL-Phe from Tokyo-Kasei Co., Tokyo. Cbz-Gly-L-Ala was obtained from Cyclo Chem. Corp., California, DL-His-DL-His from Nutritional Biochem. Co., Ohio, and the other peptides were purchased from Sigma. Bestatin was a kind gift from Dr. T. Aoyagi, the Institute of Microbial Chemistry, Tokyo. All other reagents were of guaranteed grade. Constituent amino acids of the peptide substrates used are of L-configuration, unless otherwise specified.

Organism and cell-free extract. *Streptococcus cremoris* H61 was a kind gift from Dr. T. Morichi, the National Institute of Animal Industry, Tsukuba, Japan. The organism was grown in a tomato medium.¹⁾ The cells were harvested by centrifugation at $5000 \times g$ for 30 min at 4°C

and washed three times with sterile saline solution (0.85% NaCl). The washed cells were resuspended in 50 mM sodium phosphate buffer at pH 6.0 and lyophilized. The cell-free extract was prepared by the method²⁾ described previously.

Assay of enzyme activity. Prolidase activity and the hydrolytic activity for peptides containing a proline residue were measured using the ninhydrin method of Yaron and Mlyner.¹³⁾ The reaction mixture containing 0.1 ml of 1 mM X-Pro dipeptide and the enzyme solution was made in 50 mM sodium phosphate buffer at pH 6.0 and the final volume was adjusted to 1 ml by adding the same buffer. After incubation at 37°C for 30 min, the reaction was stopped by the addition of 2.5 ml of glacial acetic acid. The proline released by the enzyme was estimated by measuring the extinction at 480 nm of the red color formed after adding 2.5 ml of ninhydrin reagent and heating in boiling water for 30 min.

Assay of protein. Protein concentration was measured by the method of Lowry *et al.*¹⁵⁾ using bovine serum albumin as the standard.

Polyacrylamide-gel electrophoresis (PAGE). Disc-PAGE was performed on 7.5% gel by the method of Davis.¹⁶⁾

Amino acid analysis. Amino acid composition of the enzyme was tentatively estimated in a Hitachi 835 amino acid analyzer after hydrolysis in 6N HCl for 24 hr at 110°C.

RESULTS

Isolation of the enzyme

The cell-free extract was fractionated with ammonium sulfate. The precipitate at 80% of ammonium sulfate saturation was collected and dialyzed against 10 mM sodium phosphate buffer at pH 6.0 for 24 hr at 4°C. The dialyzed solution (73 ml) was applied to a column (2.0 × 50 cm) of DEAE-cellulose which had been equilibrated with 10 mM sodium phosphate buffer at pH 6.0. After being washed with a small volume of the same buffer, the enzyme adsorbed was eluted by a linear gradient of sodium chloride from 0.15 to 0.6 M in the same buffer and the eluent was collected in 10 ml portions. The fractions from No. 67 to 82 (NaCl concentration: 0.3~0.35 M) were found to contain dipeptidase having some activity for Leu-Pro. These fractions were combined and dialyzed against 10 mM sodium

phosphate buffer at pH 6.0.

The enzyme solution (160 ml) eluted from a DEAE-cellulose column was applied to a column of hydroxyapatite (1.8 × 9.0 cm), which had been equilibrated with 10 mM sodium phosphate buffer at pH 6.0. The enzyme was eluted in 5 ml portions by a linear gradient of sodium phosphate concentration from 0.01 to 0.4 M at pH 6.0. The prolidase activity was found in fractions from No. 75 to 98 (phosphate concentration: 0.16~0.3 M) and these were collected and dialyzed against sodium phosphate buffer at pH 6.0.

The enzyme solution (114 ml) obtained was concentrated to about 1 ml on a Collodion-Bag-SM 13200 (Sartrius Membrane Filter GmbH) and applied to Disc-PAGE. After electrophoresis at 4°C for 2 hr, the gels were sliced at intervals of 2 mm. The fractions possessing hydrolysis activity for Leu-Pro were collected and the enzyme was extracted with 10 mM phosphate buffer at pH 6.0. The extract was dialyzed against the phosphate buffer and then applied to a column of Sephadex G-150 (2.0 × 50 cm), which had been equilibrated with the same buffer. Elution was carried out at 4°C. The flow rate was 5 ml per hr and the eluate was collected in 5 ml portions. The fractions having a hydrolysis activity for Leu-Pro were pooled and used for the following experiments.

The purification process is summarized in Table I. The enzyme was purified about 490-fold over the cell-free extract and the yield of activity was about 30% in the case of using L-Leu-L-Pro as the substrate.

Figure 1 shows the Disc-PAGE pattern of the enzyme and the activity in the gel slice. In the Disc-PAGE pattern, a single band corresponding to the enzyme activity was observed with a relative mobility of 0.8 against the dye front.

Molecular weight estimation

The molecular weight of the enzyme was estimated by gel filtration on a column of Sephadex G-150 in the manner described above. Bovine milk α -lactalbumin, hen egg

TABLE I. PURIFICATION OF PROLIDASE

Step of purification	Total protein (mg)	Total activity* (units)	Specific activity* (units/mg)	Purification	Yield of activity* (%)
Cell-free extract	454.9	9862	21.7	1	100
(NH ₄) ₂ SO ₄ precipitate (80%)	320.2	7233	25.5	1.2	73.3
DEAE-cellulose chromatography	42.2	6925	164.0	7.6	70.2
Hydroxylapatite chromatography	11.5	5423	472.2	21.8	55.0
Disc-PAGE gel-slice extract	0.26	2759	10601.7	488.6	29.0

* Activity was measured by using Leu-Pro as a substrate.

FIG. 1. Disc-PAGE Pattern of Prolidase and the Hydrolyzing Activity in the Gel Slice.

Leucylproline was used as a substrate.

ovalbumin, bovine serum albumin and soy-bean trypsin inhibitor were used as standard proteins. The molecular weight of this enzyme was estimated to be 43,000 by a plot of the elution volume against the logarithm of molecular weight of each protein.

Amino acid composition

The amino acid composition of the enzyme is shown in Table II. The content of acidic amino acids was relatively high, which might cause fast migration of this enzyme on Disc-PAGE (relative mobility=0.8).

Substrate specificity

The hydrolytic activity of the enzyme on various peptides was investigated. Table III shows the relative rates of hydrolysis of various substrates by the enzyme (Leu-Pro as 1).

TABLE II. AMINO ACID COMPOSITION OF PROLIDASE

Amino acid	Mol%
Aspartic acid	7.8
Threonine	4.4
Serine	13.6
Glutamic acid	9.4
Proline	2.9
Alanine	9.9
Half Cystine	2.3
Valine	5.3
Methionine	0.2
Isoleucine	4.0
Leucine	5.3
Tyrosine	2.5
Phenylalanine	2.6
Lysine	4.1
Histidine	5.3
Arginine	3.2
Tryptophan	n.d.*

The content of glycine was not determined because the glycine was contained in the enzyme preparation from the acrylamide gel slice.¹⁶⁾

*n.d. = not determined.

It was capable of hydrolyzing highly X-Pro type dipeptides such as Leu-Pro, Phe-Pro, Val-Pro and Ala-Pro. None of the other peptides or carbobenzoxy peptides were hydrolyzed.

Effect of pH on the enzyme activity

The optimum pH for enzyme activity was measured with Leu-Pro using the buffers in a pH range from 5.2 to 8.8. Buffers used were 0.1 M sodium phosphate buffer for pH 5.2 to 8.0 and 0.1 M Tris-HCl buffer from pH 7.2 to 8.8. The optimum pH of the enzyme was in the pH range of 6.5 to 7.5.

TABLE III. RELATIVE ACTIVITY OF THE ENZYME FOR VARIOUS SYNTHETIC PEPTIDES

	Peptide	Relative activity	Peptide	Relative activity	Peptide	Relative activity
(A)	Leu-Pro	1.00	Poly-Pro	0.00	Ala-Pro	0.30
	Gly-Pro	0.00	Phe-Pro	0.58	Val-Pro	0.52
	Gly-Pro-Ala	0.00	Gly-Pro-Glu	0.00	Gly-Pro-Leu	0.00
	Pro-β-Na	0.00				
(B)	Gly-Leu	0.00	Ala-Tyr	0.00	Pro-Gly	0.00
	Glu-Phe	0.00	Ala*-Gly	0.00	Pro-Val	0.00
	Gly-Val	0.00	Ala-Leu	0.00	Pro-Tyr	0.00
	Leu-Gly	0.00	Ala*-Val*	0.00	Pro-Leu	0.00
	Leu-Val	0.00	Ala*-Ser*	0.00	His-Leu	0.00
	Leu*-Tyr	0.00	Ala*-Met*	0.00	His-Gly	0.00
	Leu*-Phe*	0.00	Ala*-Phe*	0.00	His-Ser	0.00
	Val-Leu	0.00	Val-Gly	0.00	Glu-Val	0.00
	Gly-Gly-Gly	0.00	Ala*-Gly-Gly	0.00	Cbz**--Gly-Phe	0.00
	Gly-Gly-Val	0.00	Ala*-Leu*-Gly	0.00	Cbz**--Gly-Pro	0.00
	Gly-Gly-His	0.00	Leu-Leu-Leu	0.00	Cbz**--Gly-Val	0.00
	Gly-Phe-Ala	0.00	Leu*-Gly-Phe*	0.00	Cbz**--Gly-Ala	0.00
	Val-Gly-Gly	0.00	Leu-Gly-Gly	0.00	Cbz**--Gly-Gly	0.00
	Val-Tyr-Val	0.00	Pro-Gly-Gly	0.00	Cbz**--Gly-Ala	0.00
	Glu-Val-Phe	0.00				

* DL-Amino acid.

** Carbobenzoxy.

A) The enzyme activity was measured by the method of Yaron and Mlyner.¹³⁾B) The enzyme activity was measured by the method of Matherson and Tatridge.¹⁴⁾

The rate of Leu-Pro hydrolysis was taken as 1.

Effect of temperature on the enzyme activity and thermal stability

The optimum temperature for the enzyme was determined with Leu-Pro as a substrate by incubating at pH 7.0 for 30 min, and was found to be 40°C. The enzyme was stable up to 40°C for 30 min at pH 7.0, but rapidly lost its activity at temperatures higher than 40°C.

Effect of metal ions and reagents on the enzyme activity

The effect of some metal ions on the enzyme activity was checked using Leu-Pro as the substrate. The results are shown in Table IV. The activity was not affected by Co^{++} , Ca^{++} and Mg^{++} . It was weakly inhibited by Zn^{++} and completely inhibited by Hg^{++} , Cu^{++} and Fe^{++} . After removing the metal ions from the enzyme by treating with EDTA, the activity lost was only recovered by the addition of Co^{++} .

The effect of some reagents on the enzyme

TABLE IV. EFFECT OF METAL IONS ON THE ENZYME ACTION

After preincubation with 1 mM of each metal ion for 10 min, the enzyme solution was added to the reaction mixture and incubation was performed at 37°C and pH 7.0 for 30 min. Leu-Pro was used as the substrate at a concentration of 0.1 mM.

Metal ions (1 mM)	Relative activity
CoCl_2	131.0
CaCl_2	127.0
MgCl_2	126.0
MnCl_2	16.4
ZnCl_2	75.3
CuSO_4	0.0
FeCl_2	0.0
HgCl_2	0.0
None	100

activity was also examined, the results being shown in Table V. The enzyme was completely inhibited by EDTA and 1,10-phenanthroline, considerably inhibited by *p*-chloromercuri-

TABLE V. EFFECT OF VARIOUS CHEMICALS ON THE ENZYME ACTIVITY

Incubation was performed in the same way as described in Table IV, after preincubation with each chemical.

Reagents (final conc. mM)	Relative activity
EDTA (1)	0
1,10-Phenanthroline (1)	0
<i>p</i> -Chloromercuribenzoate (1)	67
<i>N</i> -Ethylmaleimide (1)	65
Monoiodoacetic acid (1)	84
2-Mercaptoethanol (1)	30
Cysteine (0.5)	94
Bestatin (0.0016)	84
None	100

FIG. 2. Kinetics of Prolidase for X-Pro Type Dipeptides. —○—, Leu-Pro; —●—, Phe-Pro; —△—, Val-Pro; —▲—, Ala-Pro. Incubation was performed at 37°C and pH 6.0 for 15 min.

benzoate or *N*-ethylmaleimide, and was strongly inhibited by 2-mercaptoethanol (in the presence of 10 mM 2-mercaptoethanol, inhibited completely). It was, however, slightly affected by other sulfhydryl reagents, S-S dissociating agents and bestatin.

Kinetics of the enzyme

The kinetic constants for the hydrolysis of X-Pro peptides were determined by measuring the amount of proline released. Mixture solutions of various concentrations of substrates containing 40 μ l of the enzyme solution were prepared in 10 mM phosphate buffer at pH 6.0 to make a final volume of 1 ml. After in-

cubation for 15 min in the same manner as described above, the amount proline released was measured and the constants of K_m and V_{max} were determined by Lineweaver-Burk plotting. As substrates, Leu-Pro, Phe-Pro, Ala-Pro and Val-Pro were chosen. The results are shown in Fig. 2. K_m values were calculated to be 0.82, 0.76, 0.41 and 0.61 mM for Leu-Pro, Phe-Pro, Val-Pro and Ala-Pro, respectively. The K_m values were not much different for these four substrates but the V_{max} values were quite different from each other—0.67, 0.29, 0.17 and 0.08 μ mol/ml·min, respectively.

DISCUSSION

A prolidase that is capable of hydrolyzing dipeptides of the X-Pro type was isolated from a cell-free extract of *Str. cremoris* H61. The purified enzyme appeared homogeneous from the results of Disc-PAGE (Fig. 1). This is the first report to describe the purification and characterization of the prolidase from the microorganisms including lactic streptococci.

The molecular weight of the enzyme was estimated to be 43,000 by gel filtration. The molecular weight is much smaller than those from pig and bovine intestines, which were 113,000¹⁰⁾ and 116,000,¹¹⁾ respectively. The amino acid composition of the prolidase from *Str. cremoris* H61 was quite different from that from intestines (Table II). These results suggest that the prolidase from *Str. cremoris* H61 has different molecular properties from those of intestines. However, the substrate specificity shows the preference of the enzyme for X-Pro type peptides (Table III) as well as those of the enzymes in intestines. These results seem to provide an interesting problem as to why two types of prolidasases with different molecular properties exhibit a similar substrate specificity.

The prolidase was completely inhibited by chemical chelators such as EDTA and 1, 10-phenanthroline and also by 2-mercaptoethanol (Table V). It is likely that the metal ion locates in the active center of the enzyme. On the other hand, the enzyme from bovine intestine was

not inhibited by EDTA and markedly inhibited by PCMB.¹¹⁾ The mechanism of catalysis seems to be different from that of the prolidase described here.

The physiological significance of prolidase has been discussed on the enzyme from intestines. Yoshimoto *et al.*¹²⁾ reported that the prolidase acted cooperatively with aminopeptidase M and dipeptidyl aminopeptidase in complete hydrolysis of the peptides in intestine. Therefore, the possibility of contribution of the prolidase described here to the degradation of intracellular protein of *Str. cremoris* can be considered.

We found that the prolidase reported here hydrolyzed the peptides, derived from α_{s1} -casein by chymosin, cooperatively with other proteases in *Str. cremoris* H61 (data not shown). These results suggest that the prolidase contributes to the hydrolysis of X-Pro type dipeptides which might be released by proteinases from milk and lactic streptococci in cheese ripening. Studies on the mechanism of degradation are in progress.

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