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# Purification and Characterization of a Substrate-Size-Recognizing Metalloendopeptidase from *Streptococcus cremoris* H61

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**During the ripening of Gouda-type cheese, two kinds of endopeptidases were found to participate in the degradation of αs1-CN(f1-23), a specific product from αs1-casein hydrolyzed by chymosin. One of the endopeptidases, lactic acid bacteria endopeptidase (LEP-II), which can recognize the size of its substrates, has already been purified and characterized (T. R. Yan, N. Azuma, S. Kaminogawa, and K. Yamauchi, Eur. J. Biochem. 163:259–265, 1987). The other endopeptidase, LEP-I, was purified to homogeneity by conventional chromatographic techniques from *Streptococcus cremoris* H61. The enzyme appeared to be monomeric, with an apparent molecular weight of 98,000, and its isoelectric point was 5.1. For the hydrolysis of αs1-CN(f1-23), the enzyme had an optimum pH and temperature of 7.0 to 7.5 and 40°C, respectively. Its activity was inhibited by such chelating agents as EDTA and 1,10-phenanthrolin, and it could be fully reactivated by $Mn^{2+}$. Inhibitors specific for serine and thiol proteases had no effect on the protease activity. The enzyme showed a high affinity toward the Glu-Asn peptide bond of αs1-CN(f1-23) and αs1-CN(f91-100) but showed no hydrolysis activity toward αs1-CN(f1-52), αs1-CN(61-122), αs1-CN(136-196), αs1-casein, β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin. The $K_m$ and $V_{max}$ of LEP-I for αs1-CN(f1-23) were 14.2 pM and 139 U, respectively.**

The proteolytic enzymes of lactic streptococci used as a cheese starter play a central role in cheese ripening. These enzymes act synergistically with the coagulant, chymosin, and the indigenous proteinases in milk to break down milk caseins to achieve cheese ripening (8, 24, 27, 29, 34). Under acidic conditions, chymosin (EC 3.4.23.4) rapidly and specifically cleaves the peptide bond (Phe-105—Met-106) of κ-casein to destroy the stable casein micelles, which induces the milk to coagulate (7, 16). During the early stage of the ripening process, chymosin also specifically cleaves the Phe-23—Phe-24 bond of αs1-casein to form 23 N-terminal residues of αs1-casein [αs1-CN(f1-23); the other peptide fragments derived from αs1-casein were designated in the manner suggested by Eigle et al. (11)] and αs1-CN(f24-199) (4, 18). The latter constitutes the texture of mature cheese (5, 21), while the former is further hydrolyzed by the proteases of starters into several kinds of low-molecular-weight peptides (22), which are considered to be suitable substrates for such exopeptidases as aminopeptidases (10, 12), dipeptidases (12, 19, 30), tripeptidases (25), and prolidases (20) of the starters. Consequently, the flavor or the flavor precursor compounds are developed in ripened cheese (1, 35).

In the previous study, we isolated three major peptides from a water-soluble fraction of ripened Gouda-type cheese and found that these peptides are derived from the hydrolysis of αs1-CN(f1-23) by the endopeptidases of lactic streptococci (22). To illustrate the role of these endopeptidases during cheese ripening, we used a specific substrate, αs1-CN(f1-23), and high-performance liquid chromatography (HPLC) to monitor the hydrolyzing activity of the enzymes. We found two novel endopeptidases which can specifically hydrolyze αs1-CN(f1-23) and which possess some unique properties. One of these two endopeptidases, LEP-II, which has been purified and determined to possess a substrate size specificity, has already been described (37a). In the present study, the purification and characterization of the other endopeptidase, lactic acid bacteria endopeptidase-I (LEP-I), are described and the role of the enzyme in peptide degradation during cheese ripening is discussed.

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## MATERIALS AND METHODS

**Chemicals.** DEAE-Sephacel, Chromatofocusing Polybuffer 74, Polybuffer Exchanger PBE 94, and Sephacryl S-300 were purchased from Pharmacia, Uppsala, Sweden. Angiotensin, oxidized insulin B chain, glucagon, bradykinin, neurotensin, substance P, and β-casomorphin were purchased from the Peptide Institute Inc., Osaka, Japan. Pepsin (EC 3.4.23.1) and trypsin (EC 3.4.21.4) were purchased from Sigma Chemical Co., St. Louis, Mo., and all the other materials used were of analytical grade when available.

**Preparation of caseins and peptide fragments of αs1-casein.** αs1-Casein, β-casein, and κ-casein were prepared from fresh, raw skim milk by the methods of Zittle et al. (38), Fox and Guiney (15), and Doi et al. (9), respectively. α-Lactalbumin and β-lactoglobulin were prepared by the method of Aschaffenburg and Drewry (2) and then purified by DEAE-Sephacel chromatography. αs1-CN(f1-23) was prepared from the peptic hydrolysis of purified αs1-casein (31). αs1-CN(f91-100) was prepared from the tryptic hydrolysis of αs1-casein as follows. Trypsin was added to a 2.0% αs1-casein solution in a 0.05 M Tris buffer (at pH 8.0) to give an enzyme/substrate ratio of 1:500, and the reaction mixture was incubated at 30°C for 2 h. The pH of the solution was then adjusted to 4.6 with 0.1 N HCl, and the solution was centrifuged at 1,480 × *g* for 10 min. The supernatant was fractionated by HPLC (LC-6A; Shimadzu Corp., Kyoto, Japan) by use of a large-pore-size (30 nm) C-4 column (Senshu Pak, SSC-SC4; Senshu Kagaku, Tokyo, Japan) and a UV detector at a wavelength of 230 nm. Elution was with a linear gradient of acetonitrile (1.0%/min) in trifluoroacetic acid at a flow rate of 1.0 ml/min. The identity of the purified peptide as αs1-CN(f91-100) was confirmed from its amino

acid composition. αs1-CN(f1-54), αs1-CN(f61-123), and αs1-CN(f136-196) were prepared by chemically cleaving αs1-casein with cyanogen bromide in 70% formic acid at 25°C for 24 h (17). This hydrolysate was purified by HPLC in the manner described above, and the identity of each peptide was confirmed by its amino acid composition.

**Organism.** The stock culture of *Streptococcus cremoris* H61 was a kind gift from T. Morichi of the National Institute of Animal Industry, Chiba, Japan.

**Culture conditions.** The culture was transferred in a 10% skim milk medium three times and was then grown in a tomato medium by the method of Ohmiya and Sato (26). Cells were harvested after a 24-h static incubation at 30°C.

**Cell harvesting.** The cells were harvested by centrifugation at 4,000 × *g* for 30 min at 4°C and were washed three times with sterilized saline (0.85% NaCl). The washed cells were suspended in 0.05 M sodium phosphate buffer at pH 6.0 and were then lyophilized. The dry cells were stored at 4°C.

**Preparation of a cell extract.** The dried cells (1 g) were suspended in 200 ml of 0.05 M sodium phosphate buffer at pH 6.0 before being sonicated (100 W, 10 kV) for 60 min in a sonicator (type N-50-6; Toyoriko Corp., Tokyo, Japan). The temperature of the cell suspension solution was held below 8°C. The disrupted cells were centrifuged at 35,000 × *g* at 4°C for 20 min. After centrifugation, the supernatant (cell extract) was collected by decantation and dialyzed against the same buffer for 24 h. About 2.4 liters of the dialysate was obtained by repeating this procedure 10 times, and 590 ml was taken for fractionation of the endopeptidase.

**Ammonium sulfate fractionation.** The cell extract was first fractionated with ammonium sulfate. The precipitate between 40 and 100% ammonium sulfate saturation was collected, dissolved in a minimum amount of 0.05 M sodium phosphate buffer (at pH 6.0), and dialyzed against the same buffer at 4°C for 24 h.

**Chromatography on DEAE-Sephacel.** The ammonium sulfate-precipitated fraction was applied to a column of DEAE-Sephacel (2.0 by 50.0 cm) preequilibrated with 0.05 M sodium phosphate buffer (at pH 6.0). The column was washed with 200 ml of the same buffer, and the enzyme activity was eluted with a linear gradient of 0.0 to 0.6 M NaCl in 0.05 M sodium phosphate buffer at pH 6.0 (700 ml). The flow rate was 25 ml/h, and 7-ml fractions were collected. The fractions containing the enzyme activity (80 ml) were pooled and dialyzed against 0.01 M sodium phosphate buffer (pH 6.0) at 4°C for 24 h.

**Chromatography on chromatofocusing exchanger PBE 94.** The pooled and dialyzed fractions were then applied to a chromatofocusing column (1.0 by 15 cm) preequilibrated with 0.025 M histidine hydrochloride buffer (at pH 6.2). The column was washed with 10 ml of the same buffer, and the enzyme was eluted with Polybuffer 74 with the pH adjusted to 4.0 with HCl. Elution was performed by the method recommended by the manufacturer. The flow rate was 20 ml/h, and 2.0-ml fractions were collected. The fractions containing the enzyme activity were pooled, and the Polybuffer was removed by precipitating the enzyme proteins with saturated ammonium sulfate solution. The pooled solution was then dialyzed against 0.01 M sodium phosphate buffer (at pH 6.0) for further purification.

**Gel chromatography on Sephacryl S-300.** The enzyme fraction obtained from the chromatofocusing procedure was concentrated in a collodion bag (Ultra-Thimbles UH 100/25; Schleicher & Schuell, Dassel, Federal Republic of Germany) to a final volume of about 2.0 ml and then applied to a Sephacryl S-300 column equilibrated with 0.01 M sodium phosphate (at pH 6.0). Elution was carried out with the same buffer at a flow rate of 20 ml/h, and 2.5-ml portions were collected.

**Assay of endopeptidase.** The activity of the endopeptidase was measured by using αs1-CN(f1-23) as a substrate. The reaction mixture contained 50 μl of the substrate solution (0.1% [wt/vol] in 0.01 M sodium phosphate buffer at pH 8.0), 50 μl of the enzyme solution, and 400 μl of 0.05 M sodium phosphate buffer (at pH 6.0). After incubation at 37°C for 60 min, 0.1 ml of 0.1 M acetic acid was added to the mixture to stop the reaction. The hydrolysate was analyzed by HPLC with a Zorbax ODS column (4.6 by 150 mm; Du Pont Co., Wilmington, Del.). The mobile phase was of 0.1% trifluoroacetic acid and acetonitrile, which was increased linearly from 0 to 40% in 40 min at a flow rate of 1.0 ml/min. The effluent was monitored at 230 nm. The area of each hydrolysate peak was calculated with a Chromatopac (C-R3AX; Shimadzu). One unit of enzyme activity is defined as the decreased unit area of the peak of αs1-CN(f1-23) or the increased unit area of the peak corresponding to αs1-CN(f1-18) per 60 min at 37°C. Specific activity is expressed as units per milligram of protein.

**Determination of protein concentration.** Protein concentration was measured by the method of Bradford (3), with bovine serum albumin as the standard. In each chromatography procedure for enzyme purification, the protein concentration of each fraction was monitored by measuring its $A_{280}$.

**Determination of molecular weight.** The apparent molecular weight of the enzyme was determined by gel filtration on a Sephacryl S-300 column in the manner described above, by HPLC with a TSK-G3000SW column (Toyo Soda Manufacturing Co., Ltd., Tokyo, Japan) eluted with 0.1 M sodium phosphate buffer at pH 6.0 with a flow rate of 0.5 ml/min, and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). β-Galactosidase (molecular weight, 138,000), phosphorylase *a* (molecular weight, 94,000), bovine serum albumin (molecular weight, 68,000), and ovalbumin (molecular weight, 43,000) were used as standards.

**Electrophoresis.** Disc polyacryamide gel electrophoresis (disc-PAGE) was carried out by the procedure of Davis (6), with 7.5% gel at pH 8.4 and 4°C. SDS-PAGE was performed in 10% gel by the method of Weber and Osborn (37). The standard proteins used in gel filtration on Sephacryl S-300 were also used in SDS-PAGE.

**Amino acid analysis.** The amino acid compositions of the purified enzyme and of each peptide separated from the hydrolysate of each substrate were estimated in an amino acid analyzer (model 835; Hitachi, Ltd., Tokyo, Japan) after the samples were hydrolyzed for 24 h in 6 N HCl at 110°C.

## RESULTS

**Enzyme purification.** A summary of the purification procedure for the endopeptidase is given in Table 1. At the step of DEAE-Sephacel column chromatography (Fig. 1), the activity toward αs1-CN(f1-23) was divided into two peaks; one was eluted at 0.25 M NaCl, and one was eluted at 0.35 M NaCl. The former was termed LEP-I, and the latter was termed LEP-II. The purification and characterization of LEP-II has been described elsewhere (37a). LEP-I was further purified by chromatofocusing and gel filtration on Sephacryl S-300. The activity of LEP-I was eluted during chromatofocusing with Polybuffer 74 at pH 5.1 (Fig. 2) and was eluted from the Sephacryl S-300 column at 108 ml (Fig.

TABLE 1. Purification of LEP-I from *S. cremoris* H61

| Fractionation step | Vol (ml) | Total protein (mg) | Sp act[a] | Total activity | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|---|
| Crude extract | 590 | 370 | 1,080 | 400,000 | 1 | 100 |
| Ammonium sulfate (40–100%) | 30 | 111 | 3,060 | 340,000 | 2.8 | 85 |
| DEAE-Sephacel | 75 | 17.7 | 13,300 | 236,000 | 12.3 | 58.9 |
| Chromatofocusing | 85 | 2.13 | 51,500 | 109,000 | 47.7 | 27.4 |
| Sephacryl S-300 | 18.2 | 0.52 | 145,000 | 75,100 | 134 | 18.8 |

[a] The specific activity of LEP-I was calculated by the increased peak area of αs1-CN(f1-18) per 60 min per mg of enzyme protein.

3). Total recovery of the enzyme activity was about 12%. The enzyme was purified about 134-fold more than that from the cell extract.

The homogeneity of LEP-I was determined by disc-PAGE (Fig. 4) and further confirmed by HPLC filtration with a TSK-G3000SW column (data not shown).

LEP-I had an apparent molecular weight of about 98,000 as estimated by Sephacryl S-300 (Fig. 3) and TSK-G3000SW HPLC (data not shown). On SDS-PAGE, LEP-I gave only one band, even in the presence of 2-mercaptoethanol, which coincided with a molecular weight of 98,000 (Fig. 5). LEP-I is therefore considered to be a monomer in its active state.

The amino acid composition of LEP-I is shown in Table 2. The values are expressed as the nearest integer of residues per molecule, assuming a molecular weight of 98,000 for LEP-I. LEP-I was rich in Glu or Gln residues and deficient in Tyr.

**General properties of purified LEP-I.** The effect of pH on LEP-I activity was examined at pH values ranging from 4.5 to 10.0. The maximum activity was obtained at pH 7.0 to 7.5 (Fig. 6). The pH stability of LEP-I was also determined by preincubation of the enzyme at different pH values at 4°C. The activity of LEP-I was irreversibly lost below pH 4.0 and was fairly stable within pH 6.0 to 9.0 (Fig. 6).

The effect of temperature on LEP-I was measured at several temperatures, the maximum activity being obtained at 40°C (Fig. 7). The thermal stability of LEP-I was estimated by preincubating the enzyme at different temperatures for 10 min. LEP-I was stable below 50°C, and even after being held at 55°C for 10 min, 50% of the activity remained.

The activity of LEP-I was not inhibited by inhibitors for

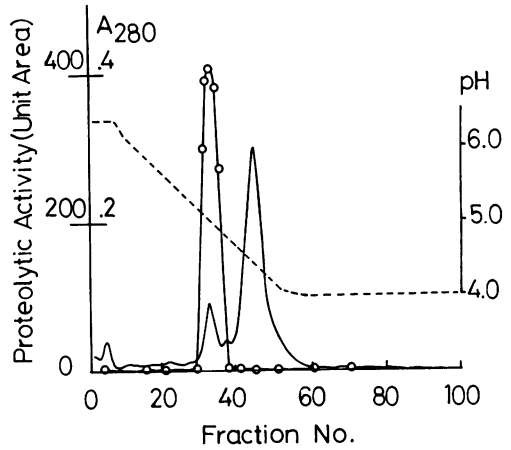


FIG. 2. Chromatofocusing of LEP-I. The pooled LEP-I activity fractions, obtained from DEAE-Sephacel chromatography, were applied to a column of PBE 94 (1 by 18 cm). LEP-I was eluted at a flow rate of 30 ml/h by using a pH gradient (- - - -) by 8 bed volumes of Polybuffer 74 (pH 6.2 to 4.0). Fractions of 2.0 ml were collected. Protein (——) was monitored at 280 nm. ○, Activity of LEP-I.

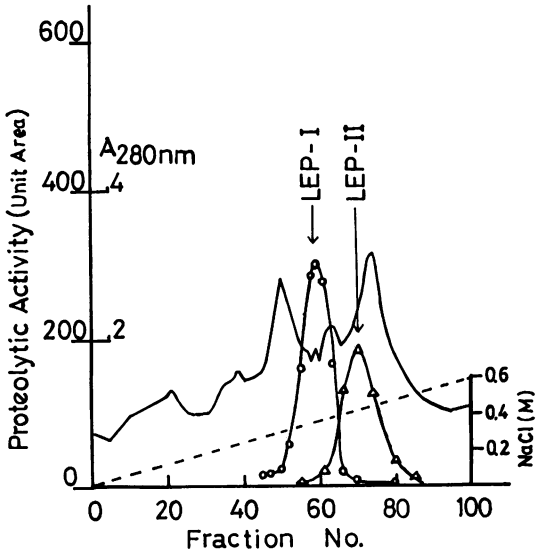


FIG. 1. DEAE-Sephacel chromatography of LEP-I. The desalted ammonium sulfate fraction was applied to a column of DEAE-Sephacel (2.0 by 50 cm). LEP-I activity was eluted at a flow rate of 25 ml/h with a linear gradient of NaCl (0 to 0.6 M) in 10 mM sodium phosphate buffer (at pH 6.0). Fractions of 7.0 ml were collected. Protein (——) was monitored at 280 nm. LEP-I activity (○) was assayed as described in Materials and Methods. - - - -, NaCl gradient; △, activity of LEP-II.

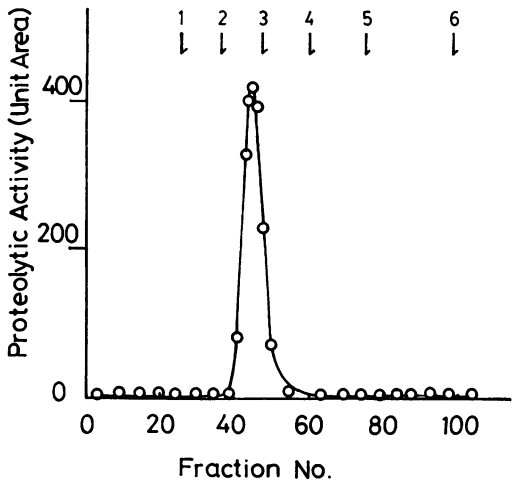


FIG. 3. Sephacryl S-300 gel filtration of LEP-I. The pooled LEP-I active fractions obtained from the chromatofocusing column were applied to a Sephacryl S-300 column (1.5 by 90 cm). LEP-I activity was eluted at a flow rate of 20 ml/h with 10 mM sodium phosphate buffer (at pH 6.0). Fractions of 2.5 ml were collected. Protein (——) was monitored at 280 nm. ○, Activity of LEP-I. The numbers indicate the eluted position of each standard protein: 1, blue dextran; 2, β-galactosidase (molecular weight, 130,000); 3, phosphorylase *a* (molecular weight, 94,000); 4, bovine serum albumin (molecular weight, 68,000); 5, ovalbumin (molecular weight, 43,000); 6, 2,4-dinitrophenol-alanine.

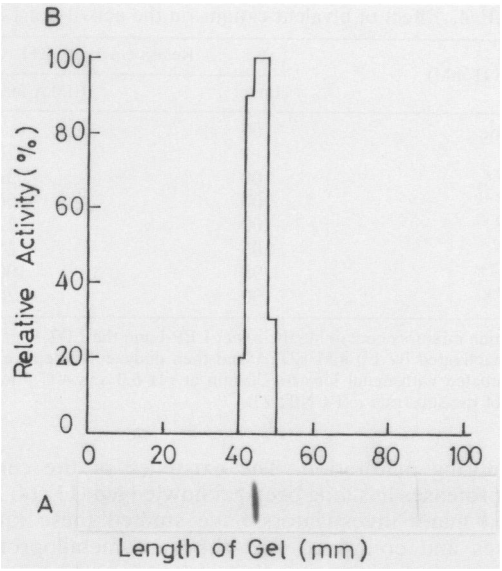


FIG. 4. Disc-PAGE of LEP-I under nondenaturing conditions. Purified LEP-I (15 μg) was applied to the top of the gel, and electrophoresis was carried out by the method of Davis (6). (A) One gel was stained for protein. (B) A second gel was cut into 2-mm slices, and each sliced gel was assayed for LEP-I activity.

serine and thiol protease (Table 3), which suggests that serine and a sulfhydryl residue do not participate in the active center of the enzyme, while the activity was inhibited by such chelating agents as EDTA and 1,10-phenanthrolin.

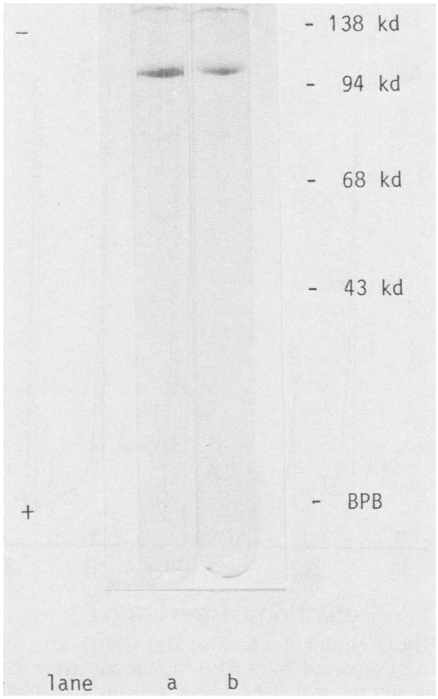


FIG. 5. SDS-PAGE of LEP-I. Purified LEP-I (7.6 μg) was denatured by 1.0% SDS with (lane a) and without (lane b) 2-mercaptoethanol and then applied to a 10% SDS-polyacrylamide gel. Electrophoresis was performed as described in Materials and Methods. The marker proteins and bromophenol blue (BPB) are shown (sizes are in kilodaltons).

TABLE 2. Amino acid composition of LEP-I

| Amino acid | Mole ratio (%) | Nearest integer[a] |
|---|---|---|
| Asp/Asn | 9.93 | 84 |
| Thr | 4.96 | 42 |
| Ser | 8.44 | 72 |
| Glu/Gln | 15.56 | 132 |
| Pro | 4.46 | 38 |
| Gly | 6.51 | 53 |
| Ala | 7.93 | 67 |
| Cys | 1.57 | 13 |
| Val | 4.29 | 37 |
| Met | 2.62 | 22 |
| Ile | 4.77 | 41 |
| Leu | 10.44 | 89 |
| Tyr | 0 | 0 |
| Phe | 3.40 | 29 |
| Lys | 8.55 | 73 |
| His | 2.19 | 19 |
| Arg | 2.96 | 25 |
| Trp | ND[b] | |

[a] The nearest integer is based on a molecular weight for LEP-I of 98,000.
[b] ND, Not determined.

The activity of metal-depleted LEP-I was fully reactivated by the addition of $Mn^{2+}$ after EDTA had been removed by dialysis (Table 4). However, the addition of several kinds of divalent cations, including $Mn^{2+}$, to the intact LEP-I had no effect on its proteolytic activity (Table 4).

**Substrate specificity of LEP-I.** The HPLC profiles of the hydrolysates of αs1-CN(f1-23) and αs1-CN(f91-100), which were hydrolyzed by LEP-I, are shown in Fig. 8A and B, respectively. Both peptides were cleaved only at the Glu-Asn peptide bond, which was confirmed by an amino acid analysis of each peptide. However, the peptides that contained the same subsite sequence as αs1-CN(f1-23) or αs1-CN(f91-100), such as αs1-CN(f1-54), αs1-CN(f61-123), and

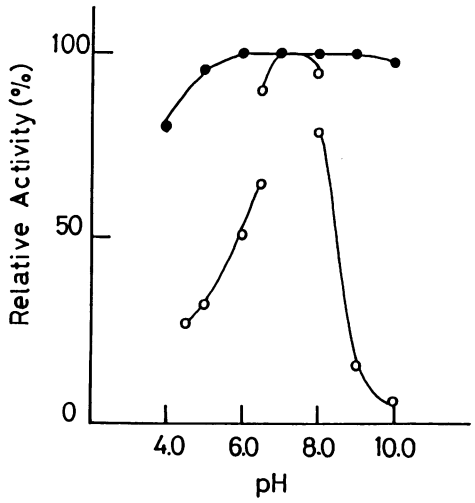


FIG. 6. Effect of pH on the activity and stability of LEP-I. The optimal pH of LEP-I (○) was measured with the following buffer systems: pH 4.5 to 6.5, 50 mM sodium citrate-citric acid; pH 6.0 to 8.0, 50 mM sodium phosphate buffer; and pH 8.0 to 10.0, 50 mM boric acid-NaOH. The stability of LEP-I (●) was measured by preincubating the enzyme at the pH range at 4°C for 30 min, after adjusting each solution to pH 6.0, and then adding the substrate αs1-CN(f1-23) to assay the remaining activity as described in Materials and Methods.

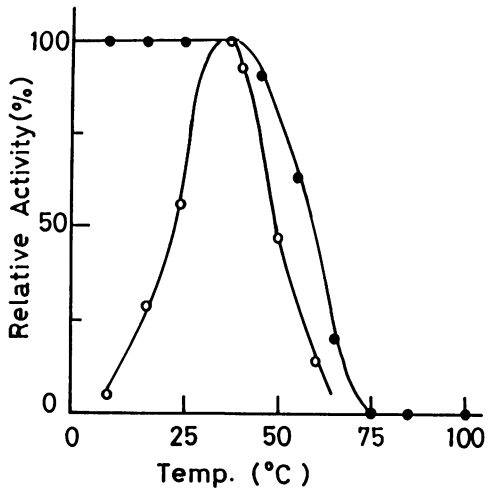


FIG. 7. Effect of temperature on the activity and stability of LEP-I. The optimal temperature of LEP-I (○) was measured by incubating the enzyme at the temperature range. The stability of LEP-I (●) was measured by preincubating the enzyme at the temperature range for 10 min, before the substrate αs1-CN(f1-23) was added to assay the remaining activity as described in Materials and Methods.

αs1-casein itself, were not hydrolyzed. The enzyme showed no activity toward such larger peptides or proteins as αs1-CN(f136-196), β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin (Table 5).

However, some lower-molecular-weight peptide hormones were hydrolyzed by LEP-I, and the cleavage point of each hormone peptide was found to be other than Glu-Asn (Table 5).

The Michaelis-Menten constant ($K_m$) and maximum velocity ($V_{max}$; expressed in units per milligram of proteins) of LEP-I for the hydrolysis of αs1-CN(f1-23) at pH 6.0 and 37°C were estimated to be 14.2 pM and 139, respectively (Fig. 9).

## DISCUSSION

The proteolytic enzyme system of lactic streptococci plays an important role not only in the physiological functions of the cell itself but also in the breakdown of milk casein during the ripening of fermented dairy products. The cell-wall-bound proteases are considered to degrade milk proteins to provide the bacteria with indispensable peptides and amino acids which are not present in milk (23, 24, 34). The products also contribute to the development of cheese flavor during maturation. The existence of the cell-wall-bound proteases has long been acknowledged (13, 14, 32, 33, 36), and many investigators have studied these kinds of proteases and confirmed that they are metalloproteases, since they are inhibited by chelating agents (14, 32). However, there have been no recent studies on the purification of the cell-wall-bound proteases. This may be due to the difficulty of detecting the weak activity of the enzymes by the usual assay method. Therefore, in this study, we chose a proper substrate, αs1-CN(f1-23), and used high-sensitivity HPLC to survey and monitor cell-wall-bound LEP-I (most of the LEP-I activity was identified in the cell wall fraction of *S. cremoris* H61 [data not shown]) from *S. cremoris* H61. LEP-I was shown to have apparent homogeneity by disc-PAGE and TSK-G3000SW HPLC filtration.

The characteristics of LEP-I suggest classification of this enzyme as a neutral microbial metalloendopeptidase. This is

TABLE 3. Effect of various reagents on the activity of LEP-I[a]

| Reagent | Concn (mM or %) | Relative activity (%) |
|---|---|---|
| Control | | 100 |
| EDTA | 1.0 | 0 |
| 1,10-Phenanthrolin | 1.0 | 37 |
| Monoiodoacetic acid | 1.0 | 88 |
| *N*-Ethylmaleimide | 1.0 | 84 |
| Phenylmethanesulfonyl fluoride | 0.1 | 100 |
| Diisopropyl fluorophosphate | 1% | 100 |
| 2-Mercaptoethanol | 1% | 112 |
| Cysteine | 1.0 | 100 |
| Glutathione | 1.0 | 93 |

[a] Reaction mixtures lacking a substrate were incubated with inhibitors for 30 min at pH 6.0 and 4°C prior to the addition of the substrate αs1-CN(f1-23).

TABLE 4. Effect of bivalent cations on the activity of LEP-I[a]

| Metal ion (1 mM) | Relative activity (%) | |
|---|---|---|
| | Intact | EDTA inactivated |
| None | 100 | 16 |
| $Ca^{2+}$ | 100 | 30 |
| $Co^{2+}$ | 100 | 56 |
| $Cu^{2+}$ | 100 | 22 |
| $Mg^{2+}$ | 105 | 25 |
| $Mn^{2+}$ | 98 | 100 |
| $Zn^{2+}$ | 90 | 26 |

[a] Reaction mixtures containing the intact LEP-I and the EDTA-inactivated LEP-I (inactivated by 1.0 mM EDTA and then dialyzed to deplete EDTA) were incubated with metal ions for 30 min at pH 6.0 and 4°C prior to the addition of the substrate αs1-CN(f1-23).

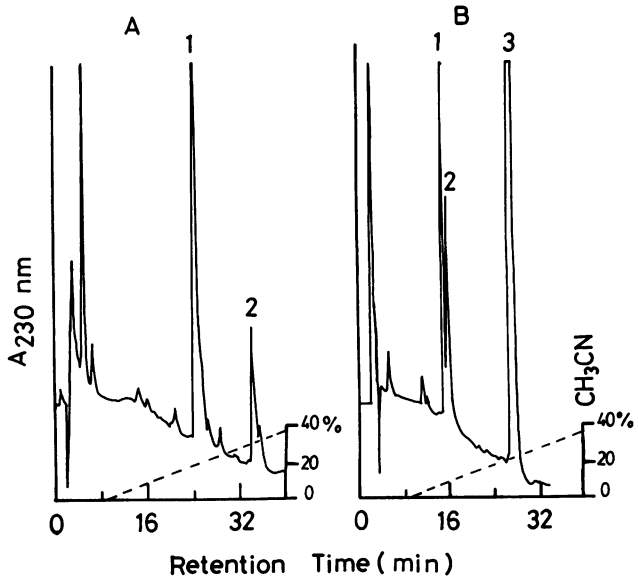


FIG. 8. HPLC elution profiles of αs1-CN(f1-23) (A) and αs1-CN(f91-100) (B) digested by LEP-I. Each substrate (50 μg) was incubated for 30 min with LEP-I (3.8 μg) in 500 μl of the assay buffer. The hydrolysates were analyzed by reverse-phase HPLC as described in the text. Each peak fraction was isolated and hydrolyzed for amino acid analysis. Peaks 1 and 2 in panel A show αs1-CN(f1-18) and αs1-CN(f1-23). Peaks 1, 2, and 3 in panel B show αs1-CN(f97-100), αs1-CN(f91-96), and αs1-CN(f91-100), respectively.

TABLE 5. Substrate specificity of LEP-I[a]

| Substrate | Structure and cleavage site | Identified fragment(s) |
|---|---|---|
| αs1-Casein hydrolysate | | |
| αs1-CN(f1-23) | $R^1$—P—K—H—P—I—K—H—Q—G—L—P—E↑—Q—V—L—N—E—N—L—L—R—$F^{23}$ | $R^1$-$E^{18}$ |
| αs1-CN(f91-100) | $Y^{91}$—L—G—Y—L↑—E—N—L—L—$R^{100}$ | $Y^{91}$-$E^{96}$ |
| αs1-CN(f1-54) | | NH |
| αs1-CN(f61-123) | | NH |
| αs1-CN(f136-196) | | NH |
| Hormone peptides | | |
| Bradykinin | $R^1$—P—P—G—F↑—S—P—F—$R^9$ | $R^1$-$F^5$, $S^6$-$R^9$ |
| Angiotensin I | $D^1$—R—V—Y—I—H—P↑—F—H—$L^{10}$ | $D^1$-$P^7$ |
| Substance P | $R^1$—P—K—P—Q—Q—F—F↑—G—L—$M^{11}$ | $R^1$-$F^8$ |
| Neurotensin | $Pyr^1$—L—Y—E—N—K—P—R↑—R—P—Y—I—$L^{13}$ | $Pyr^1$-$R^8$, $R^9$-$L^{13}$ |
| β-Casomorphin | $Y^1$—P—F—P—G—P—$I^7$ | NH |
| Glugacon | | NH |
| Oxidized insulin B chain | | NH |
| Milk proteins | | |
| αs1-Casein | | NH |
| β-Casein | | NH |
| κ-Casein | | NH |
| α-Lactalbumin | | NH |
| β-Lactoglobulin | | NH |

[a] Each substrate (1.0 mM in the reaction mixture) was hydrolyzed by the purified LEP-I (3.8 μg/0.5 ml of reaction mixture) as described in Materials and Methods. The hydrolysate of each substrate was fractionated by HPLC and identified by its amino acid composition. The arrows show the cleft point of each substrate by LEP-I. NH, Not hydrolyzed.

coincident with the metal ion dependence of the cell-wall-bound proteinase which has been previously reported (14, 32). However, LEP-I was reactivated more effectively by $Mn^{2+}$ than by $Ca^{2+}$, and this reactivation was achieved only with ion-depleted LEP-I, the enzyme showing no detectable hydrolyzing activity for milk caseins. These properties reveal that LEP-I is significantly different from the previously reported cell-wall-bound proteinase.

LEP-I showed a high affinity toward the peptide bond of Glu-Asn of αs1-CN(f1-23) and αs1-CN(f91-100) but showed no hydrolysis activity for αs1-CN(f1-54), αs1-CN(61-123), and αs1-casein, which have the same splitting bond as αs1-CN(f1-23) and αs1-CN(f91-100). Furthermore, the larger proteins, such as β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin, were not hydrolyzed by the enzyme, although some of the lower-molecular-weight peptide hormones were cleaved at peptide bonds other than the Glu-Asn bond. These results show that the substrate specificity of LEP-I was dependent not only on the subsite sequence of substrates, but also on the spatial construction of substrates. In this respect, the substrate-size-recognizing specificity of LEP-I was similar to that of LEP-II. However, the maximum substrate size which could be hydrolyzed by LEP-I was αs1-CN(f1-23), that is, smaller than oxidized insulin B chain, which was the largest substrate that could be hydrolyzed by LEP-II. This result shows that LEP-I possesses a much greater small-size-recognizing specificity than LEP-II does.

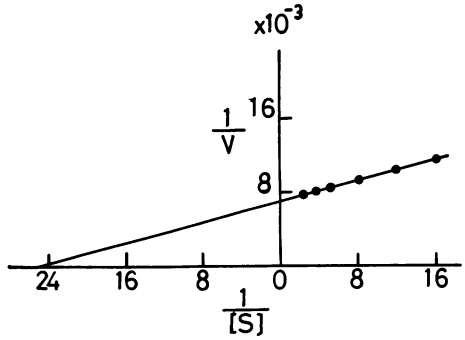


FIG. 9. Lineweaver-Burk plots for the hydrolysis of αs1-CN(f1-23) by LEP-I. The activity of LEP-I was calculated as the decreased peak area of αs1-CN(f1-23) as described in Materials and Methods.

LEP-I could specifically hydrolyze αs1-CN(f1-23) into αs1-CN(f1-18) and αs1-CN(f19-23). These two products are not major components of the water-soluble fraction of ripened Gouda-type cheese (22). This means that these two peptides were further hydrolyzed by the actions of the other endopeptidases and exopeptidases from the starters to develop the flavor compounds of the ripened cheese.

Although the physiological function of LEP-I in the lactic streptococci is not precisely understood, the enzyme is significantly different from the cell-wall-bound proteinases (14, 32). It is reasonable to believe that LEP-I may play a nutritional role by synergistically hydrolyzing milk caseins with the other extracellular proteinases to amino acids or peptides that are small enough to enter the cell (28). These peptides would then be further hydrolyzed by the intracel-

lular peptidases to release their constituent amino acids, permitting bacteria to utilize the milk proteins for growth.

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