Purification and Characterization of an Extracellular Proteinase Having Milk-Clotting Activity from *Enterococcus faecalis* TUA2495L

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Lactic acid bacteria (157 stock cultures) were screened for their ability to produce extracellular proteinase with milk-clotting activity. A strain identified as *Enterococcus faecalis* TUA2495L showed the highest ratio of milk-clotting activity (MCA) to proteinase activity (PA). The molecular weight of the purified enzyme from the strain was estimated to be 34–36 kDa by gel filtration and by SDS-PAGE. Its isoelectric point was about 5.4, and the K_m value on casein (Hammarsten) was 0.61% (w/v). The optimum temperature was 70°C for MCA and 50°C for PA. The MCA increased with a decrease in the pH from 7.8 to 5.8 but the PA was optimum at pH 8.0–9.0. Both MCA and PA were stable within the pH range of 5.5–10.0. The enzyme was inhibited by heavy metal ions (Fe^{2+}, Cd^{2+}, Ni^{2+}, Cu^{2+} and Al^{3+}), SDS and EDTA. Reactivation of the enzyme activity with Co^{2+}, Mn^{2+} or Zn^{2+} indicates the importance of these metals in the catalytic function of the enzyme. The enzyme was especially active on κ-casein, and SDS-PAGE analysis showed that the degradation patterns of κ-casein by *Ec. faecalis* enzyme and *Mucor miehei* milk-clotting enzyme were almost the same.

Keywords: milk-clotting enzyme, extracellular proteinase, *Enterococcus faecalis*

Cheese is one of the foods that has kept the human race nourished from ancient times and is produced in various ways all over the world. Cheese is diverse for two reasons: one is the many kinds of materials contained: milk, milk-clotting enzyme (MCE), and starter microorganism such as lactic acid bacteria (LAB), mold and so on. The other is the method used to make it. In the case of ripened cheese, the action of the LAB starter and MCE during the ripening are known to form its characteristic flavor and texture (Sousa *et al*., 2001). Proteolytic enzymes produced by LAB starter play an especially important part in the development of cheese flavor and texture (Takafuji, 1998). Therefore, there have been many investigations on the degradation of milk proteins by these proteinases and peptidases from LAB (Thomas & Prichard, 1987; Kunji *et al*., 1996). However, most of these reports are on cell-bound proteinase (Geis *et al*., 1985; Monnet *et al*., 1987; Exterkate & De Veer, 1987; Hugenholtz *et al*., 1987; Bockelmann *et al*., 1989; Nissen-Meyer & Sletten, 1991; Næs & Nissen-Meyer, 1992). There are only a few reports on extracellular proteinases from LAB.

On the other hand, it is also well known that MCE is calf rennet used for cheese-making from the fourth stomach of the calf, as well as microbial rennet from the microorganisms *Mucor pusillus* (Iwasaki *et al*., 1967a; Iwasaki *et al*., 1967b; Arima *et al*., 1968)*, Mucor miehei* (Sternberg, 1971)*, Endothia parasitica* (Hagemeyer *et al*., 1968; Larson & Whitaker, 1970; Sardinas, 1968), and *Irpex lacteus* (Kawai, 1971; Kobayashi *et al*., 1983; Kobayashi *et al*., 1985a; Kobayashi *et al*., 1985b). These have

been purified, and their enzymatic properties investigated in detail. However, reports on bacterial MCE are limited to those from *Bacillus* (Melachouris & Tuckey, 1968; Srinivasan *et al*., 1964; Rao & Mathur, 1979), and there is yet no report on the production of MCE by LAB.

We have screened for LAB producing extracellular proteinase having milk-clotting activity (MCA) in order to develop a new LAB starter and MCE for cheese making. In this paper, the results of the screening, the identification of an isolate, the purification and some properties of this enzyme are described.

Materials and Methods

Microorganisms and cultivation Various strains of LAB (*Lactobacillus* sp. 65 strains, *Carnobacterium* sp. 1 strain, *Wesslla* sp. 2 strains, *Leuconostoc* sp. 18 strains, *Pediococcus* sp. 23 strains, *Streptococcus* sp. 4 strains, *Enterococcus* sp. 15 strains, *Lactococcus* sp. 7 strains, and 22 unidentified strains) preserved in our laboratory were used for this study. Many LAB strains were obtained from the NODAI Culture Collection Center (Faculty of Applied Bio-Science, Tokyo University of Agriculture, Tokyo, Japan). The 22 unidentified were isolated from various fermented foods such as *miso*, soy sauce, and yogurt. For the pre-culture, MRS broth (OXOID, Ltd., Hampshire, England) was used, and each strain was pre-cultured statically at 30°C for 24 h. For the screening was used: litmus milk medium (skim milk (Snow Brand Milk Products Co., Ltd., Japan) 10 g, azolitmin 50 mg, D.W. 100 ml; pH 6.8) sterilized by autoclaving at 110°C for 10 min, and the screening medium (Glucose 10 g, Casein (Hammarsten) 10 g, $CH_3COONa \cdot 3H_2O$ 2 g, $MgSO_4 \cdot 7H_2O$ 200 mg, $MnSO_4 \cdot 4H_2O$ 10 mg, $FeSO_4 \cdot 7H_2O$ 10 mg, NaCl 10 mg, D.W. 1000 ml; at pH 6.8) sterilized by autoclaving at 121°C for 10 min. The enzyme production medium was the same as the

screening medium except that Trypticase peptone (Becton Dickinson and Company, MD) was used instead of casein (Hammarsten) as nitrogen source. Four milliliters of the pre-culture broth was inoculated into a 500-ml Erlenmeyer flask containing 400 ml of the enzyme production medium with or without $CaCO_3$ 4 g sterilized by dry heating at 180°C for 2 h. When $CaCO_3$ was added, the culture broth was calmly agitated using a stirrer to mix the $CaCO_3$.

Screening of LAB for production of extracellular proteinase having MCA As the first step in the screening, the ability of each LAB to separate whey from skim milk was examined as follows. Each strain was pre-cultured, and this pre-culture broth (100 μl) was inoculated into a test tube containing 5 ml of litmus milk medium and cultured statically at 30°C for 7 days. After the cultivation, the strains which formed whey were selected for further study. As the second step in the screening, the selected strains were cultured at 30°C for 48 h with agitation by stirrer in the screening medium containing 1% (w/v) $CaCO_3$. After the cultivation, this culture broth was centrifuged at $9,000\times g$ for 20 min at 4°C, and this supernatant fraction was collected. The MCA and PA in the supernatant were measured, and the MCA/PA ratio was calculated.

Enzyme assay The MCA was assayed using the method described by Arima *et al*. (1967). A 10% solution of skim milk (Snow Brand Milk Products Co.) containing 10 mM $CaCl_2$ was used as the substrate. Substrate solution (5 ml) was added to the enzyme solution (0.5 ml) at 35°C. The time required for curd particles to form was measured with a stop watch. Under the above assay condition, 400 units of activity (Soxhlet Unit) was defined as the amount of enzyme that clotted the milk solution in 1 min.

The PA was measured using a 1.0% solution of Hammersten casein (M/10 Tris-HCl buffer, pH 7.5) as the substrate. Five milliliters of the substrate solution was incubated with 1 ml of enzyme solution at 45°C for 30 min and the enzyme reaction was stopped with 5 ml of trichloroacetic acid mixture solution (0.11 M CCl_3COOH, 0.22 M CH_3COONa, and 0.33 M CH_3COOH). After 30 min of incubation, the reaction mixture was filtered using filter paper (Advantec NO.4A, Toyo Roshi Kaisha, Ltd., Japan) and 2 ml of the filtrate was added to 5 ml of 0.55 M Na_2CO_3 and 1 ml of Folin's reagent (×3). This mixture was held at 30°C for 30 min and the optical density at 660 nm was measured. One unit of the activity was defined as the amount of enzyme which released 1 μg of amino acid expressed as the tyrosine concentration per min under the above condition.

Phenotypic properties of the strain Various phenotypic properties (morphological, cultural, and biochemical characteristics) of the strain were investigated according to Kozaki's manual (1992).

Sequencing of 16S rRNA gene Cloning and sequencing of 16S rRNA gene was carried out as described by Nakagawa *et al*. (2000). Oligonucleotides 5′-AGTTTGATCATGGCTCA-3′ (primer 20F) and 5′-AAGGAGGTGATCCAACCGCA-3′ (primer 1540R) were used as the primers for the amplification of 16S rRNA gene by the PCR. Amplified 16S rRNA gene fragment was used for sequencing template. A Dye Terminator Cycle-sequencing FS Ready Reaction kit (Perkin-Elmer) and a model ABI 373A automatic DNA sequencer (Perkin-Elmer) were used. The four oligonucleotides 5′-GTAGTCCACGCCGTAAACGA-

3′ (primer 800F), 5′-CGGCCGTACTCCCCAGGCGG-3′ (primer 900R), primer 20F and primer 1540R were used as primers for sequencing of 16S rRNA gene fragment. The nucleotide sequence of the strain has been deposited in the DNA Data Bank of Japan (DDBJ). The accession number of the deposite is AB-098122.

Purification of an extracellular proteinase having MCA

Step 1: Concentration The *Ec. faecalis* TUA2495L was cultivated at 37°C for 12 h. After the cultivation, this culture broth was centrifuged at $9,000\times g$ for 20 min at 4°C, and this supernatant fraction was collected. The supernatant (2300 ml) was adjusted to pH 8.0 with 1 N NaOH, and concentrated about 20-fold using an ultrafiltration unit with a molecular weight cut-off membrane of approximately 5,000 (Minitan, Millipore Co., USA). Thereafter, solid ammonium sulphate was added to the concentrate (105 ml) to reach 80% saturation at 4°C and kept overnight. Then, the precipitate was centrifuged for 20 min at $9,000\times g$ (4°C) and decanted. The pellet was dissolved in 25 mM Tris-HCl buffer (pH 8.0) 15 ml, and subjected to column chromatography as described below.

Step 2: Gel filtration The sample (15 ml) was put on a Sephacryl S-200HR (Amersham Pharmacia Biotech., Uppsala, Sweden) gel filtration column (26 mm×940 mm) equilibrated with 25 mM Tris-HCl buffer (pH 8.0). The enzyme was eluted with the same buffer at a flow rate of 0.4 ml/min. The active fractions were combined.

Step 3: Anion-exchange chromatography The enzyme solution (82 ml) was put on a DEAE-TOYOPEARL 650M (Tosoh Co., Tokyo) anion-exchange column (16 mm×220 mm) equilibrated with 25 mM Tris-HCl buffer (pH 8.0). The enzyme was eluted with a linear gradient of 0–0.4 M NaCl in the same buffer (400 ml) at a flow rate of 2.0 ml/min. The active fractions were collected.

Step 4: Hydrophobic interaction chromatography The enzyme solution (67 ml) was brought to 30% saturated $(NH_4)_2SO_4$, and it was put on an Ether-TOYOPEARL 650M (Tosoh Co.) column (16 mm×110 mm) equilibrated with 25 mM Tris-HCl buffer (pH 8.0) containing 30% saturated $(NH_4)_2SO_4$. The enzyme was eluted with a linear gradient of 30–0% saturated $(NH_4)_2SO_4$ in the same buffer (200 ml) at a flow rate of 4.0 ml/min.

Determination of protein concentration Protein concentrations were calculated from the absorbance at 280 nm. It was assumed that the absorbance of 1 mg/ml protein is 1.0.

Polyacrylamide gel electrophoresis PAGE was carried out with 7.5% polyacrylamide slab gel with or without a 4.5 M urea at pH 8.8. SDS-PAGE was done in a 10% polyacrylamide slab gel with a Tris/glycine buffer system and SDS-urea PAGE in a 16% polyacrylamide slab gel containing a 4.5 M urea (Laemmli, 1970). The low-molecular weight calibration kit for SDS-PAGE (Amersham Pharmacia Biotech.) was used. The protein bands were stained with Coomassie Brilliant Blue R-250.

Measurement of molecular weight The molecular weight of the enzyme was measured by gel filtration and SDS-PAGE. The purified enzyme solution was passed through a high-pressure gel permeation liquid chromatography on a TSK-gel G-$2000SW_{XL}$ column (7.8×300 mm; Tosoh Co.) at room temperature. The mobile phase was 50 mM KH_2PO_4-Na_2HPO_4 buffer (pH 7.0) containing 0.1 M Na_2SO_4 and the flow rate was 1.0 ml/

min. The absorbance of the eluent was automatically monitored at 210 nm with a data module. The relative molecular weight of the enzyme was calculated from its mobility relative to those of the standard proteins (gel filtration molecular weight markers, SIGMA).

Isoelectric point determination Isoelectric point of the enzyme was measured by IEF-PAGE mini (pH 3.0–7.0, TEFCO Co., Tokyo) slab gel. The purified enzyme solution was charged and the isoelectric focusing was carried out at 100 V for 60 min, 200 V for 60 min, and then 500 V for 30 min according to the TEFCO manual of isoelectric point analysis system. Trypsin inhibitor (4.6), β-Lactoglobulin (5.1), and Carbonic anhydrase II (5.4 and 5.9) obtained from SIGMA were used as the pI marker proteins.

Action of MCE on various casein fractions Degradation of various casein fractions by the enzyme was examined as described by Kobayashi *et al.* (1985a, b). A solution (1.5 ml, 0.1%) of α-casein, β-casein, and κ-casein obtained from SIGMA in 20 mM phosphate buffer (pH 6.0) was mixed with 0.03 ml of enzyme solution (approximately 500 Soxhlet Unit/ml) and incubated at 35°C. The samples were then used for PAGE or SDS-urea PAGE analysis containing a 4.5 M urea.

Results

Screening of LAB for production of an extracellular proteinase having MCA LAB capable of producing extracellular proteinase having MCA were screened from 157 stock cultures in our laboratory. Only 4 strains separated whey from litmus milk medium (data not shown). *Ec. faecalis* IAM10065 and *Ec. faecalis* No.156 strains separated large amounts of whey. They were red in color, indicating acidification by azolitmin (litmus). The amount of coagulate was very small and white in color. These observations indicated that skim milk protein was liquefied by the enzymes produced by these strains. On the other hand, the amount of whey separated by the isolated strains No. 408L and No. 2495L was very small and clear. Strain No. 2495L separated the least amount of whey. The amount of coagulate was very large and it was red in color, indicating acidification.

The selected strains were cultured at 30°C for 48 h in a screening medium containing 1% (w/v) CaCO₃, and the PA and MCA in each supernatant were measured as shown in Table 1. The PA and MCA of crude enzyme from *Ec. faecalis* IAM10065 was higher than those of the other strains, while the MCA/PA ratio of strain No. 2495L was the highest (4.9). In strain No. 408L, MCA and PA were not detected in the supernatant. Higher MCA/PA ratio is desirable for milk-clotting enzyme and it generally indicates the restricted degradation of casein. Therefore, strain No. 2495L was selected for further experiments because of its high MCA/PA ratio.

Identification of strain No. 2495L Various phenotypic properties of this strain were investigated. Strain No. 2495L was Gram-positive, catalase-negative, short-chain-forming, motility-negative, homofermentative cocci, and formed L-lactate. This strain grew at 10–45°C, and the optimum growth temperature was between 30 and 37°C. It was able to grow in a GYP medium with NaCl concentration up to 75 g/*l* (data not shown). Based on these properties (except for the result of litmus milk test), the strain was assigned to the genus *Enterococcus*, using Bergey's manual (Mundt, 1986) and Kozaki's manual (1992). The sequence of 16S rRNA from this strain was also analyzed, and the phylogenetic position of the strain based on 16S rRNA sequence was investigated. The complete sequence of 1,466 bases of the 16S rRNA gene was determined. The homologous sequences had the highest 16S rRNA gene sequence similarity (99.9%) to *Ec. faecalis*. Therefore, this strain was designated *Ec. faecalis* TUA2495L.

Production of an extracellular proteinase having MCA by Ec. faecalis TUA2495L The strain was cultured at 30°C for 24 h with agitation by stirrer in an enzyme production medium with or without 1% (w/v) CaCO₃, and the MCA in each supernatant was measured. Inhibiting the pH decrease of the broth by addition of 1% (w/v) CaCO₃ resulted in higher MCA (data not shown), and final pH of the medium with and without 1% (w/v) CaCO₃ was 5.5 and 4.6, respectively. MCA was inactivated by the pH decrease of the broth because MCA was stable within the pH 5.5–10.0, as described below. Therefore, controlling the pH of the broth was very important for MCE production. In investigating the effect of cultivation temperature on the MCE production, the strain was cultured at various temperatures (25, 30, 35, 37, and 40°C) in an enzyme production medium containing 1% (w/v) CaCO₃ with agitation by stirrer, and MCA was measured. It was found that the maximum MCA was obtained at 37°C (data not shown). The effect of nitrogen source on MCE production is shown in Table 2. In comparison with casein, casein acid hydrolysate inhibited, while casein enzymatic hydrolysate stimulated MCE production. It can thus be concluded that the extracellu-

Table 1. MCA/PA ratio of crude enzymes from various strains.

Strain	PA (U/ml)	MCA (Soxhlet Unit)	MCA/PA
IAM10065	7.4	28.3	3.8
156	6.0	22.7	3.8
2495L (isolate)	4.1	20.0	4.9

Each strain was cultured at 30°C for 48 h in a screening medium with CaCO₃, and then the PA and MCA were measured. The PA was assayed under the standard condition except that the reaction temperature was 35°C. The MCA was assayed as described in the text.

Table 2. Effect of nitrogen source on enzyme production by *Ec. faecalis* TUA2495L.

	Medium							
	(Casein)	(Casein enzymatic hydrolysate)				(Casein acid hydrolysate)		
	C	PP	TP	T	P	CA	A	HC-SF
Maximum MCA (Soxhlet Unit)	21	62	85	83	43	0	0	0

The strain was cultured at 37°C for 24 h in each medium containing 1% each of the various nitrogen sources with CaCO₃. The MCA was measured as described in the text. These scores are the average of two independent experiments. C, Casein from milk (Wako); PP, Polypepton (Nihon Pharmaceutical); TP, Trypticase peptone (BBL); T, Tryptone (Difco); P, Pepticase (Sigma); CA, Casamino acid (Difco); A, Amicase (Sigma); HC-SF, Hy-case SF (Sigma).
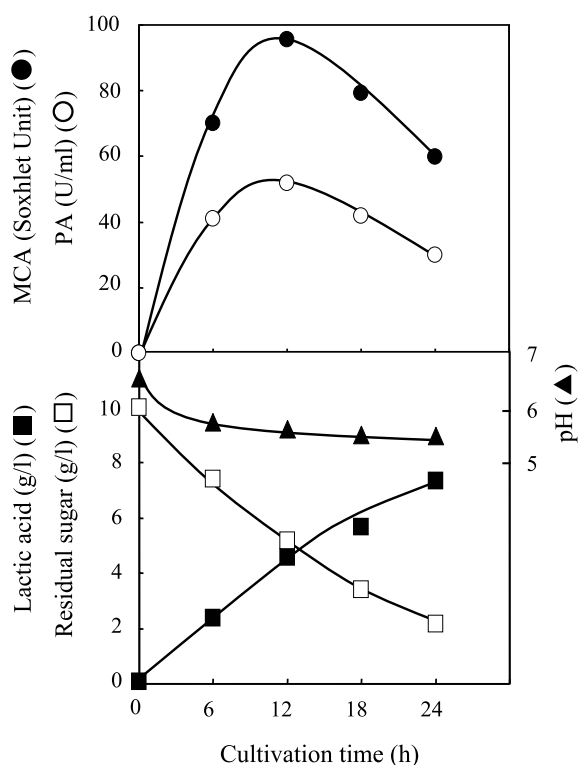
Fig. 1. Time course of enzyme production by *Ec. faecalis* TUA2495L. The culture conditions are given under Materials and Methods. The MCA (●) and PA (○) were measured as described in the text. Lactic acid (■) was assayed by HPLC. Residual sugar (□) was estimated by a modification of Somogyi's method. The pH of the medium (▲) was monitored by pH-meter. These values are the average of two independent experiments.

lar proteinase production by *Ec. faecalis* is induced by casein and its enzymatic hydrolysate. Among the nitrogen sources tested, the maximum MCA was obtained with Trypticase peptone. A typical time course of *Ec. faecalis* TUA2495L cultivation is shown in Fig. 1. The maximum MCA was about 95 Soxhlet Units; this maximum was obtained after 12 h of cultivation, and more prolonged cultivation resulted in a decrease in MCA.

Purification of the enzyme Purification of an extracellular proteinase having MCA from the supernatant of culture broth was performed by ultrafiltration, ammonium sulphate precipitation, gel filtration, anion-exchange column chromatography and hydrophobic interaction column chromatography as summarized in Table 3. The active fraction was demonstrated to be a single band on SDS-PAGE and PAGE analysis. The enzyme was purified about 1,000-fold with an overall recovery of 10%.

Some properties of the enzyme The molecular weight of the enzyme was estimated to be 36,000 by SDS-PAGE, but 34,000 by high-pressure gel permeation liquid chromatography on TSK G-2000SW$_{XL}$. The isoelectric point of the enzyme was determined to be about pI 5.4. The Michaelis-Menten constant (K_m) for casein (Hammarsten) was determined from a Lineweaver-Burk plot to be 0.61% (w/v).

The optimum temperature for MCA and PA were 70°C and 50°C, respectively (Fig. 2A). The enzyme was separately maintained at various temperatures (20–65°C) for 60 min, and then the residual activity was measured. The enzyme was stable up to 40°C. The MCA of the enzyme was measured at different pH values ranging from 5.8 to 7.8. The MCA test could not be done below pH 5.8, since the protein in skim milk coagulated at low pH values even in the absence of the enzyme. Figure 2B shows the effect of pH on MCA and PA. The MCA of the enzyme decreased as the pH increased from 5.8 to 6.7, and the optimum

Table 3. Purification of an extracellular proteinase having MCA from *Ec. faecalis* TUA2495L.

| Step	Volume (ml)	Total MCA (Soxhlet Unit)	Total protein (mg)	Specific activity (Soxhlet Unit/mg protein)	Fold	Yield (%)
Supernatant	2300	9.9×10^4	1.29×10^4	7.7	1	100
Ultrafiltration	105	9.8×10^4	1.25×10^3	78	10	99
80% saturated $(NH_4)_2SO_4$ ppt.	15	1.1×10^5	0.61×10^3	175	23	108
Sephacryl S-200HR	82	9.2×10^4	0.16×10^3	570	74	93
DEAE-TOYOPEARL 650M	67	4.8×10^4	0.54×10^2	884	115	48
Ether-TOYOPEARL 650M	18	1.0×10^4	1.28	7782	1011	10
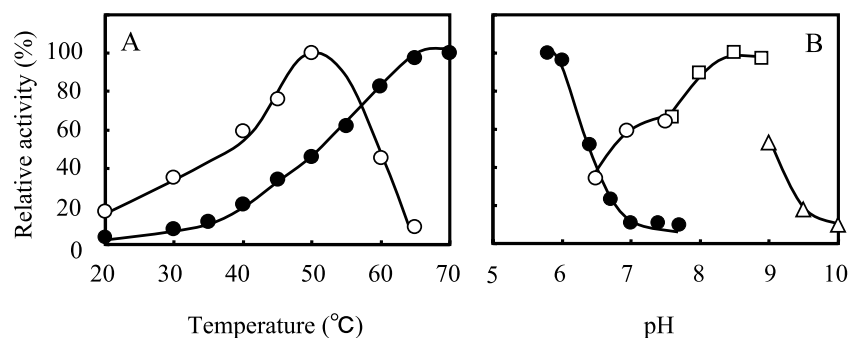
Fig. 2. Some properties of the purified proteinase. (A) Optimum temperature. The MCA (●) and PA (○) were measured at various temperatures under the standard condition. (B) Optimum pH. The MCA (●) was measured at various pHs using substrate adjusted by 1 N NaOH. The PA (○, □, △) was measured under the condition given in the text except that the following 0.1 M buffers were used to adjust the pH: KH$_2$PO$_4$-Na$_2$HPO$_4$ buffer (○), Tris-HCl buffer (□), and glycine-NaOH buffer (△).

pH for PA of the enzyme was 8.0–9.0. The enzyme was treated at different pH values (3–10) for 60 min at 30°C, and the residual activities were measured. About 50–100% of the initial enzyme activity remained after treatment in the pH range of 5.5–10.

Effects of calcium ion on the MCA   Calcium chloride was added to reconstituted skim milk at various concentrations (1, 5, 10, 30, 50, 70, 90, and 100 mM) and the MCA was assayed in each sample. Maximum activity of the enzyme was obtained when 50 mM $CaCl_2$ was added to the skim milk.

Effects of metal ions on the MCA   In investigations on the effects of metal ions on the MCA of the enzyme, the enzyme was treated with each metal ion in 0.1 M Tris-HCl buffer (pH 7.5) at 30°C for 30 min and residual MCA was assayed. Significant inhibition was observed in the case of $NiCl_2$, $CuCl_2$, $CdCl_2$, and $AlCl_3$, while $FeCl_2$ completely inhibited the enzyme activity at a concentration of 1 mM.

Effects of enzyme inhibitors   As shown in Table 4, the enzyme was inhibited by SDS and EDTA. The inhibition by EDTA suggests that the enzyme belongs to the metalloproteinase group.

Cofactor of the enzyme   Various divalent metal ions are known to act as enzyme cofactors. To determine whether EDTA-inactivated enzyme could be reactivated by metal ion, the effects of metal ions on MCA were examined. The purified enzyme was treated in 10 mM EDTA solution (M/10 Tris-HCl buffer, pH 7.5) at 30°C for 30 min and added to 10 mM of each of the metal ions, then incubated at 30°C for 30 min. The MCA in each mixture was assayed. It was found that Na^+, K^+, Ca^{2+}, Cu^{2+}, and Fe^{3+} did not reactivate, but Co^{2+}, Mn^{2+}, and Zn^{2+} reactivated the enzyme OR by: 70–80%. This suggests that the enzyme is a metalloproteinase which needs Co^{2+}, Mn^{2+}, and Zn^{2+} to express its activity.

Substrate specificity   Table 5 shows the relative enzymatic activities toward various proteins under the optimum conditions. The highest rates of proteolysis were observed with κ-casein. The rate of κ-casein hydrolysis was about two times higher than that of whole casein (Hammarsten). Although the enzyme also hydrolyzed other proteins such as gelatin and hemoglobin, their rates of hydrolysis were lower than that of casein. Albumin (from bovine serum) and elastin were not hydrolyzed at all.

Degradation of various casein fractions   Figure 3 shows the degradation patterns of κ-casein by enzymes from *Ec. faecalis* and *M. miehei*. In both cases, κ-casein was almost completely degraded within 5 min and a new product (MW 15,000) indicated by the arrow was formed. The degradation pattern of κ-casein

Table 4.   Effect of various inhibitors on MCA.

Reagent	Concentration (mM)	Remaining activity (%)
Control		100
EDTA	10	26
SDS	10	0
L-Cysteine	10	73
DFP	1	100
Iodoacetic acid	1	100
Antipain	0.1	97
Leupeptin	0.1	97
Phosphoramidone	0.1	69

The enzyme was treated with each reagent in 0.1 M Tris-HCl buffer (pH 7.5) at 30°C for 10 min and the residual activity was assayed. The activity was expressed as a percentage of the activity in the absence of the reagent (control). These scores are the average of two independent experiments.

Table 5.   Relative activity toward various proteins.

Substrate	Relative activity (%)
Casein (Hammarsten)	100
α-Casein	107
β-Casein	134
κ-Casein	194
Gelatin (from bovine bone)	43
Hemoglobin (from bovine)	37
Egg albumin	14
Soy bean flower	13
Albumin (from bovine serum)	0
Elastin	0

The enzymatic reaction was done at 45°C for 30 min in 0.1 M Tris-HCl buffer (pH 7.5). These scores are the average of two independent experiments.
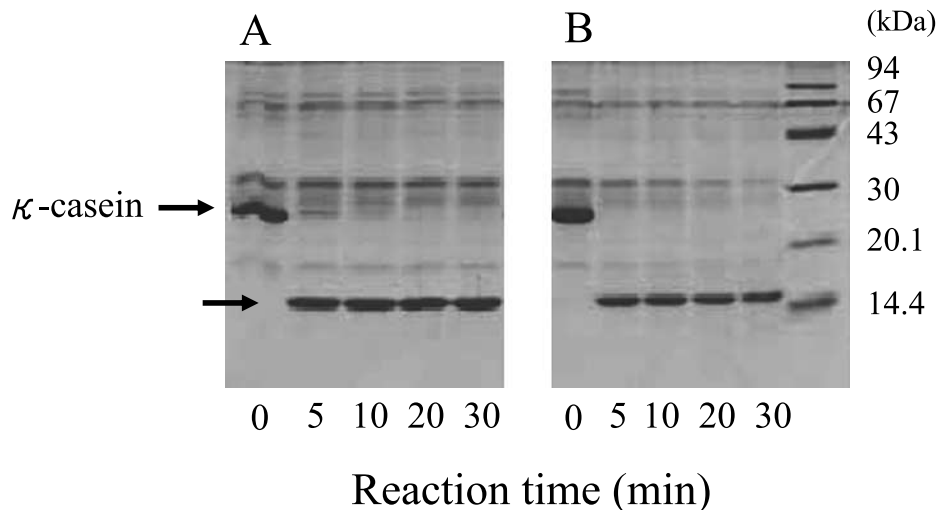

Fig. 3.   SDS-urea PAGE pattern of κ-casein hydrolysis products by milk-clotting enzymes. The enzymatic reaction was done as described in the text. (A) *Mucor miehei* Milk-clotting enzyme obtained from SIGMA, (B) Purified enzyme from *Ec. faecalis*. κ-Casein and its major hydrolysis products indicated by the arrows.
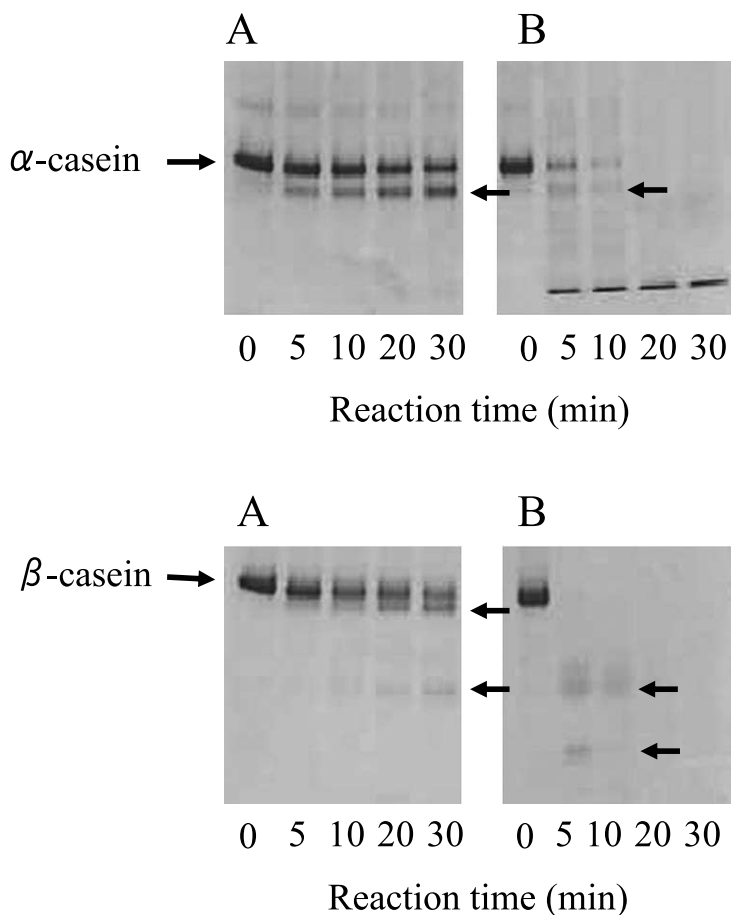
Fig. 4. Urea-PAGE pattern of α-casein and β-casein hydrolysis products by milk-clotting enzymes. The legends are the same as those for Fig. 3.

by *Ec. faecalis* enzyme was similar to that of *M. miehei* enzyme. Although, in comparison with *M. miehei* enzyme, *Ec. faecalis* enzyme hydrolyzed α- and β-casein rapidly (Fig. 4), the degradation patterns of α-casein were similar during the early reaction stage. The rate of β-casein hydrolysis by *Ec. faecalis* enzyme was very high.

Discussion

Over the past several decades, many MCE have been reported but there has yet been no report on bacterial MCE except that from *Bacillus*.

The molecular weight of the *Ec. faecalis* enzyme was about 34,000–36,000, which is similar to those of MCE from *Irepex lacteus* (34,000–36,000) (Kawai, 1971; Kobayashi *et al.*, 1983), *E. parasitica* (37,500) (Hagemeyer *et al.*, 1968), *M. miehei* (34,000–39,000) (Sternberg, 1971), and *M. pusillus* (29,000–30,600) (Arima, 1968), but different from that from *B. subtilis* K-26 (27,000) (Rao & Mathur, 1979). The isoelectric point of the *Ec. faecalis* enzyme (pI 5.4) was higher than those of the MCE from *M. pusillus* (pI 3.5) (Iwasaki *et al.*, 1967a), *M. miehei* (pI 4.2) (Ottesen & Rickert, 1970), *E. parasitica* (pI 4.6) (Hagemeyer *et al.*, 1968), and *I. lacteus* (pI 4.9–5.3) (Kawai, 1971; Kobayashi, 1983). Furthermore, the K_m value for casein (Hammarsten) of the *Ec. faecalis* enzyme (0.61%) was higher than that of the MCE from *I. lacteus* (0.07%) (Kawai, 1971), but similar to those

of the MCE from *M. pusillus* (0.36%) and *Aspergillus ustus* (0.27%) (Takahashi & Kikuchi, 1993). There is yet no report on the pI and K_m values of the MCE from *B. subtilis* K-26. The optimum pH for proteolysis by *Ec. faecalis* enzyme (pH 8.0–9.0) was higher than those of the MCE from *M. miehei* (Sternberg, 1971), *M. pusillus* (Arima *et al.*, 1968), and *I. lacteus* (Kawai, 1971; Kobayashi, 1983) (4.0, 4.0, and 2.5–4.0, respectively). In addition, the pH stability of the *Ec. faecalis* enzyme (pH 5.5–10) was also different from those of the MCE from *M. miehei* (pH 4–8) (Sternberg, 1971), *M. pusillus* (pH 4–8) (Iwasaki *et al.*, 1967a), *I. lacteus* (pH 3–6) (Kawai, 1971; Kobayashi, 1983), and *B. subtilis* K-26 (most stable at pH 7.5) (Rao & Mathur, 1979). As in the case of MCE from *B. subtilis*, the *Ec. faecalis* enzyme was inhibited by EDTA and was therefore classified as metalloproteinase. On the other hand, the MCE from *I. lacteus, M. miehei,* and *M. pusillus* were inhibited by pepstatin and these were therefore classified as carboxyl proteinase.

The specificity of *Ec. faecalis* enzyme on hemoglobin and bovine serum albumin was different from that of the MCE from *I. lacteus* (Kobayashi *et al.*, 1983) (their rates of casein hydrolysis were 263–264% and 121–122%, respectively). While *Ec. faecalis* enzyme was especially active on κ-casein, its degradation patterns were very similar to those of *M. miehei* enzyme (Fig. 4). Thus, the cleavage sites of κ-casein by *Ec. faecalis* enzyme and *M. miehei* enzyme are almost the same, hence both

enzymes coagulate milk rapidly. Detailed study of the degradation of each casein fraction is currently in progress.

Ec. faecalis enzyme was also compared with common cell-bound proteinases from LAB used for dairy products. These enzymes appear to be higher molecular weight proteins (Geis *et al.*, 1985; Monnet *et al.*, 1987; Exterkate & De Veer, 1987; Hugenholtz *et al.*, 1987; Bockelmann *et al.*, 1989; Nissen-Meyer & Sletten, 1991; Næs & Nissen-Meyer, 1992) (the published molecular weight size ranges from 80–145 kDa), and are inhibited by phenylmethylsulphonylfluoride or DFP. They are thus classified as serine proteases. Thus, *Ec. faecalis* enzyme is different from cell-bound proteinase from LAB. We are now cloning the gene for this enzyme.

There is a poor kind of MCE used as an enzyme instead of calf rennet. Moreover, among MCE there are few enzymes which have unique enzymatic properties. As described above, most of MCE are classified as carboxyl proteinase, and these enzymes were low in activity at the neutral pH range, while the *Ec. faecalis* enzyme was low in activity at the acid pH range because it was unstable below pH 5.5. In addition, this enzyme as well as MCE acted to restrict the degradation of κ-casein. Thus, the *Ec. faecalis* enzyme acts at the neutral pH range as MCE was superior to the usual MCE which acts in the acid pH range because milk-clotting in cheese making is carried out in the neutral pH range. To evaluate the *Ec. faecalis* enzyme as rennet, we made camembert cheese and found that this cheese could be made with the *Ec. faecalis* rennet. Further detailed work is in progress on making camembert cheese with LAB rennet.

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