Purification and Properties of Intracellular Proteinase from *Streptococcus cremoris*

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Proteolytic activity in the extract from the cells of *Streptococcus cremoris* increased in the presence of casein, lactose, glucose, and CaCl₂ in the media but was negligibly detectable in the extract of the cells harvested from the culture containing succinate or citrate. The intracellular proteinase from *S. cremoris* harvested from tomato medium was purified 150-fold in this experiment. The enzyme had a molecular weight of 140,000, optimum pH at 6.5 to 7.0, and maximum activity at 30°C. The proteinase was activated by Ca²⁺ and inhibited by Zn²⁺, Cu²⁺, Hg²⁺, Fe³⁺, ethylenediaminetetraacetate, and sodium lauryl sulfate. The *Kₘ* value of the enzyme towards each casein fraction was almost the same, and the *Vₘₐₓ* of the enzyme towards α₅-casein was smaller than those towards the other casein fractions.

At the early stage of milk fermentation, as well as at the ripening stage, proteolytic action of lactic acid bacteria on casein has been recognized to be very important for the development of the flavor and taste of fermented milk products.

Gutnauer and Zimmerman (7), Williamson et al. (28), and Sasaki and Nakae (21) have detected extracellular proteinases from lactic acid bacteria cultures. Baribo and Foster (3), Van der Zant and Nelson (24), and Cowman et al. (5), in addition to ourselves (22), revealed the existence of the proteolytic activity in the extract of the bacterial cells.

The importance of the intracellular proteinase was demonstrated in successive studies (17, 18), which indicate a lytic phenomenon of the cells when a marked increase in the proteolytic activity was observed in aseptic rennet curd containing cell pellets of *Streptococcus cremoris* or *Lactobacillus helveticus*.

The enzymatic properties of the intracellular proteinase from *S. cremoris*, *S. lactis*, *L. helveticus*, and *L. bulgaricus* in the crude state were presented elsewhere (15, 16). The present paper describes an attempt to purify the proteinase from the extract of *S. cremoris* and reveal some properties of the purified intracellular proteinase.

**MATERIALS AND METHODS**

Materials. Whole casein was prepared from fresh skim milk by precipitation at the isoelectric point, pH 4.7. α₅, β₅, and κ-caseins were fractionated from the whole casein by the methods of Hipp et al. (9) and Tsugo and Yamauchi (23). All casein fractions were stored at low temperature in the lyophilized state until used.

Diethylaminoethyl-cellulose (DE-23, Whatman) and Sephadex G-200 (Pharmacia Co.) were employed for the column chromatography.

All the reagents used in this experiment were of guaranteed grade.

Organism. *S. cremoris* (H-61) was a kind gift from the National Institute of Animal Industry, Chiba, Japan.

Growth conditions of *S. cremoris*. The organism was grown in the media, the components of which are listed in Table 1. The media were sterilized at 120°C for 15 min. The organism was activated by repeated subculture in the same medium used for the large-scale cultivation.

Enzyme assay. (i) Determination of proteolytic activity. Routine assay was performed with casein (Hammerstein casein) as the substrate at 30°C for 3 h. The activity was estimated by the increase in trichloroacetic acid-soluble materials resulting from the action of the proteinase on whole casein and fractionated caseins. One milliliter of the 0.5% solution of these casein fractions in tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer at pH 7.0 was incubated with 1 ml of enzyme solution in the same buffer. After the 3-h incubation, the reaction mixture was combined with an equal volume of 10% trichloroacetic acid solution to remove unhydrolyzed casein molecules. The amount of tyrosine liberated from casein in the deproteinized filtrate was determined from color intensity at 660 nm according to the Folin method (8). The enzymatic activity was described as micromoles of tyrosine released from casein during a 1-min reaction.

(ii) Determination of dipeptidase activity. Hydrolysis of dipeptidase substrates by the enzyme was assayed by the estimation of liberated amino acid using modified Matheson and Tattatreinhydrin reagent (13).
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Table 1. Composition of the liquid media* for S. cremoris

| Component          | Salt medium (g/liter) | Tomato medium (g/liter) |
|--------------------|----------------------|-------------------------|
|                    | I        | II   | A      | B      | C      | D      | E      |
| Casein             | 3.0      | -    | 20.0   | 20.0   | 20.0   | 20.0   | 20.0   |
| Peptone            | 10.0     | 10.0 | 10.0   | 10.0   | 10.0   | 10.0   | 10.0   |
| Yeast extract      | 1.5      | 1.5  | 5.0    | 5.0    | 5.0    | 5.0    | 5.0    |
| Meat extract       | 1.5      | 1.5  | -      | -      | -      | -      | -      |
| Glucose            | 15.0     | 15.0 | 30.0   | 20.0   | 20.0   | 20.0   | 20.0   |
| Lactose            | -        | -    | -      | -      | -      | -      | -      |
| Sodium succinate   | 10.0     | 10.0 | -      | -      | -      | -      | -      |
| Sodium citrate     | -        | -    | -      | -      | -      | -      | -      |
| NaCl               | 10.0     | 10.0 | -      | -      | -      | -      | -      |
| CaCl₂              | -        | -    | -      | -      | -      | -      | -      |
| Tomato juice       | -        | -    | 100¹  | 100    | 100    | 100    | 100    |

* The pH of each medium was adjusted to 7.0 with NaOH (salt medium) or NH₄OH (tomato medium) before sterilization at 120°C for 15 min.

†, Absence of the component in the media.

* Results for tomato juice are given in milliliters per liter.

**Protein determination.** The concentration of protein was estimated by the method of Lowry et al. (12) with bovine serum albumin as a standard.

**Molecular weight determination.** Molecular weight was determined by the method of Andrews (1) on Sephadex G-200 in 0.1 M Tris-hydrochloride buffer, pH 7.0. The same column, sample application, and flow rate were used as described in Results. The void volume was known to be 65 ml from a calibration run with blue dextran (molecular weight, 2,000,000). Horse ferritin (450,000), human gamma globulin (160,000), bovine serum albumin (67,000), and cytochrome c (12,400), provided from Mann Research Laboratories, were chosen as standards. The molecular weight of the proteinase was determined by interpolation.

**RESULTS**

**Production and preparation of the proteinase.** The organisms harvested from each medium shown in Table 1 were washed well with physiological saline (0.9% NaCl solution). For purification of the intracellular proteinase, harvested cells of S. cremoris were frozen rapidly in a Hughes press in the freezer at -80°C and then disrupted with high pressure. After observing the almost perfect disruption of the cells by microscope, the disrupted cells were suspended in the Tris-hydrochloride buffer (0.01 M) at pH 7.0 and stirred for 30 min to extract intracellular substances. Cell debris was removed by centrifugation at 10,000 rpm for 15 min. The clear supernatant fractions containing proteinase activity were dialyzed overnight against the same buffer and are referred to as the crude extract. The amounts of the protein (about 1.5 g from 10 liters of culture) in the crude extracts from seven kinds of culture closely resembled each other. The proteolytic activities of the cell extracts, however, differed. (One unit [U] is 1 μmol of tyrosine released from casein during a 1-min reaction.) The activity in the extract from salt medium I (21 × 10⁴ U/mg of protein) was remarkably greater than that from medium II (10⁵ U/mg of protein), and the value from the tomato medium A (10 × 10⁴ U/mg of protein) was also greater than those from media B and C (10⁴ U/mg of protein) but smaller than those from media D and E (16 × 10⁴ and 22 × 10⁴ U/mg of protein, respectively). The extract from medium E contained the same or greater activity than the extract from salt medium I.

The pH of the culture of S. cremoris (tomato medium E) was readjusted aseptically to pH 7 to 7.5 with NH₄OH after 24 h of cultivation, since the pH had dropped to about 4.6 or below as a result of lactic acid fermentation. Thereafter the cultivation was continued for an additional 24 h. At the end of the second cultivation, the pH of the culture had again been lowered to 4.6 to 4.9. Because of this pH readjustment, the amount of protein extracted from the bacterial cells increased about twofold, but the total activity was nearly the same as that of the cultivation for 48 h without the readjustment of pH. Therefore, the enzyme activity was decreased by a factor of 2. In the remainder of this paper, tomato medium E without pH readjustment was usually employed for the preparation of crude extract.

**Purification procedure of intracellular proteinase.** (i) Diethylaminoethyl-cellulose chromatography. This step and all subsequent steps for purification of intracellular proteinase were carried out at 0 to 5°C. The crude extract
containing about 500 mg of protein was applied to a diethylaminoethyl-cellulose column (3 by 50 cm) equilibrated with 0.01 M Tris-hydrochloride buffer (pH 7.0). The adsorbed protein was eluted with 2 liters of the same buffer, with increasing ionic strength of NaCl (0 to 1 M) linearly (Fig. 1, broken line). The flow rate was 22 ml/min and 15-ml fractions were collected. The elution pattern is shown in Fig. 1 (solid line). Only one peak of proteinase activity (Fig. 1, dotted line) was detected. It appeared at the middle of the large second peak of protein, which was eluted at about 0.25 M NaCl. The fractions having the highest proteinase activity were combined. The specific activity of the proteinase in the combined fraction was 0.033 U.

(ii) Membrane filtration. The smaller-molecular-weight substances were removed from the combined fraction by membrane filtration (Diaflo, PM 10, Amicon Co.) under nitrogen pressure of 4 atm. Before removing all of the low-molecular-weight materials and buffer solution from the enzyme solution, about 50 ml of Tris-hydrochloride buffer (0.1 M, pH 7.0) was added to the residual solution with enzymatic activity in the Diaflo cell, and sieving was continuously carried out at low temperature until the solution was concentrated to about 3 ml. This sieving procedure (instead of the dialyzing procedure) was repeated three times for the purpose of replacement of the buffer and concentration of the enzyme solution, in addition to the removal of low-molecular-weight materials (less than 10,000 molecular weight). Depending on this procedure the total amount of protein contained in enzyme solution decreased to half and total activity decreased to two-thirds.

(iii) Sephadex gel chromatography. The enzyme solution (containing about 20 mg of protein) concentrated from the preceding procedure was applied to a Sephadex G-200 column (2.6 by 40 cm) equilibrated with 0.1 M Tris-hydrochloride buffer (pH 7.0). The column was maintained at 5°C and eluted with an upward flow of 25 ml/h, and 6.5-ml fractions were collected.

The activity towards casein was located at the second peak of protein as monitored by the absorbance at 280 nm (Fig. 2). The fractions in the major active peak were combined and concentrated to about 3 ml within the Diaflo cell under the same conditions as described above. Thereafter it was rechromatographed on the same gel column. The chromatographic patterns are presented in Fig. 3. Two peaks of protein appeared in fractions number 10 (void volume) and 14 (1.42 of V/Vo value). Only the second peak retained the activity. The specific activity of the enzyme (0.333 U/mg) was the same as that obtained by the first gel filtration.

Fig. 1. Gradient elution of proteinase from S. cremoris on DE-23 cellulose. Enzymatic activity towards casein (dotted line) was determined by the Folin method as described in Materials and Methods. Protein concentration was monitored by the absorption at 280 nm (solid line). The volume of each fraction was 15 ml. The salt gradient (dashed line) was produced from 1 liter of stirring buffer and 1 liter of stirring buffer containing 1 M NaCl. Bar represents the fractions pooled.
(Table 2). The first peak in Fig. 3 might be an inactivated enzyme protein polymerized during the reconcentration procedure.

(iv) Polyacrylamide gel electrophoresis. Disc electrophoresis of the enzyme fraction obtained from the procedure of rechromatography on Sephadex G-200 was run by the method of Davis (6) on polyacrylamide gel at pH 8.9. The constant current was 3 mA/gel for 50 to 60 min at room temperature. Six milligrams of protein was applied and was detected by staining in amido black 10B. A single protein component appeared (Fig. 4), which had a mobility of one-half that of bromophenol blue. This purified

| Table 2. Purification of intracellular proteinase from S. cremoris |
|----------------|----------------|---------------|----------------|
| Procedure       | Total activity | Sp act (U x | Purification |
|                 | (U x 10^9)     | 10^9/mg)     | Activity yield |
| Cell extraction | 333            | 2             | 100            |
| DE-23 chromatography | 139         | 33            | 42             |
| PM 10 filtration | 92             | 66            | 30             | 28             |
| G-200 chromatography (I) | 45           | 333          | 150            | 13             |
| G-200 chromatography (II) | 23           | 333          | 150            | 7              |

* Enzymatic activity was determined as micromoles of tyrosine released during a 1-min reaction.

* Cell extract was prepared from 20 g of wet cells.
preparation was used in further studies on the enzyme.

These results of a typical purification are summarized in Table 2. The overall yield was 7%, and the specific activity of the purified enzyme against casein was 0.333 U/mg. In this way the enzyme was purified 150-fold.

Properties of the purified intracellular proteinase. (i) Absorption spectrum. The absorption spectrum of the purified enzyme at pH 7.0 revealed a maximum peak at 278 nm (Fig. 5). A typical spectrum for protein was presented.

(ii) Molecular weight. Molecular weight was determined by comparing its elution volume from the column with those of a series of proteins of known molecular weight. The molecular weight is 140,000 (Fig. 6).

(iii) Effect of pH on enzyme activity. With casein as the substrate the optimum pH of the enzyme was 6.5 to 7.0 at 30 C for the 3-h reaction (see Fig. 7).

(iv) Effect of temperature on enzymatic activity. The enzymatic activity was determined at pH 7.0 at various temperatures. The highest activity appeared at 30 C (Fig. 8A). The thermostability is depicted in Fig. 8B. The enzyme solution in 0.1 M Tris-hydrochloride buffer (pH 7.0) without any substrate was incubated at various temperatures for 1 h, and the remaining activity was assayed at 30 C. Some loss of activity was recognized even at 30 C.

(v) Effects of ions and other compounds on
enzymatic activity. The effects of various ions and other compounds on the activity of the enzyme were investigated by adding them at a given concentration to the enzyme and casein solutions. The results are summarized in Tables 3 and 4. Zn$^{2+}$, Cu$^{2+}$, Hg$^{2+}$, and Fe$^{2+}$ caused 60 to 80% inhibition at a concentration of 1 mM, and Co$^{2+}$ and Mg$^{2+}$ did not affect the activity. Ca$^{2+}$ caused 37% activation (Table 3). The activity was, however, inhibited by ethylenediaminetetraacetate as a result of the chelation of Ca$^{2+}$.

There is no effect of monoiodoacetic acid, N-ethylmaleimide, p-chloromercuribenzoate, or cysteine on the enzymatic activity within the range of experimental error. Thus a thio- or disulfide group does not seem to be essential for enzymatic activity. This characteristic is in conformity with that of an intracellular proteinase from a mutant of S. lactis revealed by Westhoff et al. (27).

(vi) Substrate specificity. Proteolytic activity of the enzyme was determined at a given substrate concentration, and their reciprocal numbers were plotted by drawing Lineweaver-Burk plots (Fig. 9) against the reciprocal values of substrate concentration (percentage). The apparent $K_m$ values of the enzyme towards these

| Metal salts$^a$ | Relative activity (%) |
|-----------------|----------------------|
| None            | 100                  |
| ZnSO$_4$        | 25                   |
| CuSO$_4$        | 21                   |
| CoCl$_2$        | 117                  |
| HgCl$_2$        | 43                   |
| CaCl$_2$        | 137                  |
| FeSO$_4$        | 29                   |
| MgSO$_4$        | 110                  |
| MgCl$_2$        | 111                  |

$^a$ The concentration added was 1 mM.

| Reagents$^a$ | Relative activity (%) |
|--------------|-----------------------|
| None         | 100                   |
| Ethylenediaminetetraacetate | 54                  |
| Monoiodoacetic acid        | 106                  |
| N-ethylmaleimide           | 92                   |
| Sodium lauryl sulfate      | 5                    |
| p-Chloromercuribenzoate    | 87                   |
| Cysteine                 | 113                  |

$^a$ The concentration was 0.5 mM.

Fig. 8. Effects of temperature on the proteolytic activity (A) and stability (B) of the intracellular proteinase. Stability was presented by the remaining activity of the enzyme, which was incubated in 0.05 M Tris-hydrochloride buffer solution (pH 7.0) for 1 h at a given temperature.

The effects of various reagents on intracellular proteinase activity were summarized in Table 4. The effects of metal ions on the intracellular proteinase activity are presented in Table 3. The results of the experiments are shown in Fig. 9. The intracellular proteinase against each casein fraction was examined. The line plots of the intracellular proteinase (Fig. 9) were obtained by plotting the reciprocal of the % activity against the reciprocal of the [V].

Fig. 9. Lineweaver-Burk plot of the intracellular proteinase against each casein fraction. Symbols: (●) $\alpha_c$-casein; (○) $\beta$-casein; (□) $\kappa$-casein; (●, dotted line) whole casein.

The results of the experiments are shown in Table 4. The effects of metal ions on the intracellular proteinase activity are presented in Table 3. The results of the experiments are shown in Fig. 9. The intracellular proteinase against each casein fraction was examined. The line plots of the intracellular proteinase (Fig. 9) were obtained by plotting the reciprocal of the % activity against the reciprocal of the [V].

casein fractions and whole casein were found to be almost the same, 0.1%. This value was 1/20 that of trypsin (2). The $V_{max}$ of $\alpha_c$-casein was...
smaller than the other casein fractions.

(vii) Dipeptidase activity. Dipeptidase activity of this purified intracellular proteinase was examined at 30 C for 3 h. The activities towards alanine-glycine, alanyl-alanine, and glycyl-leucine were almost negligible.

**DISCUSSION**

**Effect of medium components on proteolytic activity of S. cremoris.** Seven kinds of media were used for cell production (Table 1). Enhancement of proteolytic activity in the extract from the cells of *S. cremoris* was observed in tomato media; i.e., fairly high activity towards casein appeared when succinate (medium C) and citrate (medium B) were replaced by lactose (medium A) and by glucose (medium D) (see Results). The activity increased further by adding CaCl₂ (medium E). These results suggest that intracellular proteinase is increased by lactose and glucose and also by casein and CaCl₂. On the other hand, enhancement of activity was inhibited by the presence of citrate and succinate in the tomato media. This might depend on the property of succinate and citrate, which chelate Ca²⁺ contained in milk or yeast extract. In the salt medium, however, chelation by succinate seemed to be released by casein, which could absorb Ca²⁺ selectively. Since these media components, lactose, glucose, casein, and calcium ion, are the constituents of milk, milk is able to provide the best conditions for the production of intracellular proteinase of lactic acid bacteria during the fermentation of milk. This indicates that the starter lactic acid bacteria seem to have higher proteolytic activity during growth in milk than in any other synthetic media. The decrease in specific activity of the enzyme by the readjustment of the culture pH seems to assert that the proteinase could be enhanced in the acidic condition of a suitable culture containing salt medium I or tomato medium E. This phenomenon is consistent with the results reported by Sato and Nakajima (22), in which the activities of intracellular proteinases of lactic acid bacteria were the highest after cultivation for 48 h.

For enhancement of the proteinase activity, there might be a possibility of proteinase induction by casein. If induction by casein is essential for the appearance of the enzymatic activity of the lactic acid bacteria, casein particles have to penetrate or to be carried across the cell membrane into the cells, or the proteinase must be located at the cell membrane for the formation of the complex between the enzyme and casein particle. Reasonable results about this problem will be presented in further studies.

The protease activity usually appeared as a single peak (Fig. 1–3). According to this method of enzyme extraction and the purification technique, only one protease could be detected from *S. cremoris*.

**Hydrolytic properties of the proteinase towards casein fractions.** Optimum pH of this intracellular proteinase from *S. cremoris* was 6.5 to 7.0. This value was also presented by Sato and Nakajima (22) and by Krishna and Dutta (11). Similar results are reported for the enzymes from *S. lactis* (3, 10, 11, 22, 25, 26), *L. casei* (5, 22), and *L. bulgaricus* (22). This optimum pH range for intracellular proteinase was slightly on the acidic side from those of extracellular proteinases from *S. lactis* (21), *S. zymogenes* (28), and *S. faecalis* var. *liquefaciens* (7). However, the value by Westhoff et al. (27) was slightly on the alkaline side from those of many other intracellular proteinase. Relative activity of this enzyme on casein at pH values encountered in cheese (pH 5.3 to 5.8; mean value, pH 5.5) was about 70% (Fig. 7).

The *Vₘₐₓ* of the purified intracellular proteinase towards αₐ-casein was smaller than those towards the other casein fractions and whole casein (Fig. 9). The hydrolytic reactions against these casein fractions were also studied by using crude extracts of *S. cremoris* (H-61) harvested from salt medium I and tomato medium E. Proteolytic properties of the crude extracts against these casein fractions were almost the same as that of purified intracellular proteinase from tomato medium E in this paper, as described above. Those results are, however, different from those presented in the other papers (15, 16), where the same organism was grown in the same salt medium I. The conditions for the enzymatic reaction were also the same as those employed in this paper. In those papers, αₐ-casein was hydrolyzed by the crude extract more than the other casein fractions were. One of the reasons for these differences was caused by the changing properties of this organism by unknown factors during its storage for a long time at low temperature. More details, however, about these differences have to be studied further.

The same *Kₐ* values were evaluated from Fig. 9, in which reciprocal values of the concentration (grams per 100 ml) of each casein fraction were plotted on the abscissa. The evaluation of these values depends on the limited hydrolysis of casein by the enzyme, since one casein molecule contains many peptide bonds that can be hydrolyzed by the proteinase. When the molecular weights of each casein fraction determined by Mercier et al. (14) and Ribadeau-Dumas et al. (19, 20) are taken into considera-
tion for the evaluation of these $K_w$ values, the value against $\kappa$-casein would be larger than those against $\alpha_s$- and $\beta$-casein.

In this paper, we tried to carry out the enzymatic reactions without any infectious factors and established the experimental conditions as described above. The reaction time, 3 h, is rather long compared with the usual enzymatic reactions; however, it could be curtailed to one-fourth that employed by Westhoff et al. (27) and Krishna and Dutta (11).

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