Note

Immunochemical Differences between Low- and Ordinary-temperature-active Proteinases from *Streptococcus lactis*

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Intracellular proteinases from lactic acid bacteria are considered to take part in the ripening of cheese. Only one type of proteinase that shows maximum activity at 30~40°C has been reported from species of these bacteria.^{1,2)} In previous investigations,^{3,4)} we isolated two types of intracellular proteinase from some lactic acid bacteria and indicated that the two proteinase fractions from *S. lactis*, *L. bulgaricus*, and *L. casei* showed maximum proteolytic activities at ordinary temperature (30~40°C) and low temperature (5~10°C), respectively. The authors also tried to purify and crystallize the low-temperature-active proteinase from *S. lactis*⁵⁾ for examination of some of its properties.

This investigation was carried out to verify the immunochemical difference between the ordinary- and low-temperature-active proteinases (OTAP and LTAP) from *S. lactis* using rabbit antiserum against crystalline LTAP.

Streptococcus lactis (IAM 1198) was used. It had been supplied from The Institute of Applied Microbiology, University of Tokyo, Tokyo. The microorganism was cultivated in the medium of Amundstad *et al.*⁶⁾ at 30°C for 12 hr. The cells were harvested by centrifugation and washed three times with 0.01 M phosphate buffer (pH 6.0). The cells were freeze-dried and stored at -30°C until needed. Cell extracts and crystalline LTAP were prepared according to the method described in a previous paper.⁵⁾ The methods are summarized as follows. Five grams of dry cells were suspended in 25 ml of 0.01 M phosphate buffer (pH 6.0), and disrupted in a porcelain mortar with 25 g of sea sand for 30 min. It was then centrifuged at 2000 × g for 10 min. The resulting aqueous layer and the middle layer of the cell debris which contained the proteinase activity were collected. They were treated together by ultrasonic oscillation at 20 kHz for 10 min at 2 to 15°C to completely disrupt the cells which had escaped the previous disruption in a porcelain mortar, and also to obtain a clear liquid. The clear liquid was centrifuged at

5000 × g for 60 min. Crystalline LTAP was prepared using the cell extract. The procedure resulting in 45.4-fold purification of the proteinase included protamine sulfate fractionation and DEAE-52-cellulose and Sephadex G-50 column chromatography. The purified fraction was freeze-dried, and then dispersed in deionized water containing ethanol (H₂O:EtOH = 1:1) to make a proteinase suspension (1 mg/0.1 ml), which was gradually cooled. The enzyme crystallized after storage at 2°C for 20 hr. Rabbit antiserum against crystalline LTAP from *S. lactis* was prepared by emulsifying half a milliliter (1 mg) of crystalline LTAP with an equal volume of Freund's complete adjuvant (Difco Co.) and injecting this intramuscularly in to rabbits at six sites. After three weeks, 0.5 ml (1 mg) of the enzyme emulsified with an equal volume of Freund's incomplete adjuvant (Difco Co.) were injected intramuscularly. First and second boosters were given another two and three weeks later respectively, and 0.5 ml (1 mg) of the enzyme with 0.86% sodium chloride was injected into the ear vein. Blood was obtained from the heart of immunized rabbits six days after the final booster injection, and the serum was separated. The proteolytic activity was determined according to the following procedure. A reaction mixture containing 1 ml of 0.5% casein solution (casein sol.: 0.1% thimerosal sol. = 100:1) buffered with 0.01 M phosphate buffer at pH 6.0 and 1 ml of enzyme solution was incubated at 30°C for 15 hr. After the end of the in-

FIG. 1. Elution Pattern of Intracellular Extract from *S. lactis*.

Fifty milligrams of protein of extract from *S. lactis* were loaded on a Sephadex G-200 column (1.5 × 90 cm) equilibrated with 0.01 M phosphate buffer (pH 6.0). Five-gram fractions were collected at the flow rate of 16 ml/hr. —; ultraviolet absorption at 280 nm;; proteinase activity.

FIG. 2. Immunological Relationship between Crystalline LTAP and OTAP (F-I), and between Crystalline LTAP and LTAP (F-II).

Double immunodiffusion by the method of Ouchterlony was carried out with 1.2% agarose (agar noble, Difco Co.) in 0.86% sodium chloride. Well 4 was filled with the antiserum to crystalline LTAP. Wells 1, 2, and 3 contained OTAP (F-I), LTAP (F-II), and crystalline LTAP from *S. lactis*, respectively.

FIG. 3. Immuno-electrophoresis of OTAP (F-I), LTAP (F-II) and Crystalline LTAP from *S. lactis*.

Immunoagarose in barbital buffer (pH 8.6, ionic strength 0.05) at 3 mA/cm for 60 min. Wells 1, 2, and 3 contained OTAP (F-I), LTAP (F-II) and crystalline LTAP respectively. After these samples had been subjected to electrophoresis, the antiserum to crystalline LTAP was added to the troughs and then this plate was stored for 48 hr at room temperature. The anode is on the left in this illustration.

cubation period, an equal volume of 7.5% trichloroacetic acid was added to stop the reaction. The precipitated proteins were filtered through a Toyo No. 2 filter paper. The amount of tyrosine liberated from casein in the deproteinized filtrate was determined from the color intensity at 660 nm according to the Casein-Folin method.⁷⁾ The enzymatic activity was defined in terms of the micrograms of tyrosine released from casein during the reaction period. The concentration of protein was estimated by the method of Lowry *et al.*⁸⁾ using bovine serum albumin (Sigma Chemical Co.) as a standard.

Two types of intracellular proteinase were fractionated from *S. lactis* as shown in Fig. 1. The total proteinase activity of F-I and F-II obtained from 1 liter of media were 37.14 and 3.83 U respectively. The fractions were collected

and freeze-dried for immunochemical analysis. In an attempt to elucidate the immunological relationship between OTAP and LTAP, double diffusion analysis was also performed. In Ouchterlony gel diffusion⁹⁾ (Fig. 2), the antiserum gave a weak precipitin line against LTAP (F-II) and crystalline LTAP. No precipitin line was, however, formed between OTAP (F-I) and the antiserum. Immuno-electrophoresis¹⁰⁾ (Fig. 3) produced a single precipitin line formed with crystalline LTAP and two precipitin lines (A and B) formed with LTAP (F-II), differing in mobility. The precipitin line (B), which ran faster than the other (A), showed the same mobility as crystalline LTAP. The proteinase activities of fraction A and B, which were fractionated from 3 mg of protein of LTAP (F-II), were determined immediately after agar gel electrophoresis. The part from the origin to 7 mm for fraction A and the one from 8 mm to 20 mm for fraction B were cut. The fractions were crushed in a small amount of 0.01 M phosphate buffer (pH 6.0) with sea sand. They were then centrifuged at 5000 $\times g$ for 10 min. The supernatant was concentrated under vacuum at 30°C and then filled up to 2.5 ml as a sample for determination of the proteinase activity. The proteinase activity was determined at pH 5.5 for 15 hr at 6°C. Both the fractions had proteinase activity. The ratios of the proteinase activity in fractions A and B were 24 and 76%, respectively.

The above analysis confirmed that OTAP (F-I) had no antigenicity to the antiserum and was a protein immunologically different from LTAP (F-II) which had a weak antigenicity. From the difference in their intracellular location,¹¹⁾ these two types of immunologically different enzymes, OTAP (F-I) and LTAP (F-II), seemed to differ in biosynthetic origin, suggesting that these proteinases were formed separately in the cell. In addition, LTAP (F-II) was subfractionated by electrophoresis into two fractions (A and B), both having proteinase activity. This finding suggests that LTAP (F-II) was composed of two isozymes (A and B) having the same antibody-binding site.

Apart from the differences between OTAP and LTAP, the isoenzymes of LTAP (F-II) shown in this paper needed further detailed studies.

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