Production and Some Properties of the Intracellular Protease of *Leuconostoc citrovorum**

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(Received November 17, 1975)

Abstract The present study was undertaken to provide some basic aspects of the intracellular protease of *Leuconostoc citrovorum* IAM 1087. *Leu. citrovorum* produced intracellular protease optimally at 30 C and production of intracellular protease reached a maximum in 24 hr. The optimum pH of the intracellular protease which was prepared from the cells grown at 30 C for 24 hr by sonication and ammonium sulfate precipitation was 7.5 in phosphate buffer. The optimum temperature was 45 C, and the inactivation of the protease was observed to begin already even at 40 C. The protease hydrolyzed more actively α -casein than the other casein fractions.

Production of proteases by homofermentative lactic acid bacteria is well known. These proteases usually play an important role in the ripening of various types of cheeses. With the purpose to investigate biochemical changes occurring during cheese making and ripening, production and properties of intracellular¹⁻⁴⁾ or extracellular^{5,6)} proteases from lactic streptococci and lactobacilli have been studied by many workers^{2-4, 7-9)}. Recently, OHMIYA and SATO^{7,10-12)} have also carried out extensive studies on the proteolytic actions of dairy lactic acid bacteria such as *Streptococcus lactis*, *S. cremoris* and *Lactobacillus bulgaricus*.

On the other hand, proteolytic actions of heterofermentative lactic acid bacteria which are commonly used as starter cultures for the production of dairy products have been studied by the limited number of investigators. DUTTA *et al.*¹³⁾ examined proteolytic properties of *Leuconostoc citrovorum* and *Leu. dextranicum* and recognized that only *Leu. citrovorum* exhibited appreciable proteolytic activity after 24 and 72 hrs' incubation in milk.

However, we have as yet little information as to intracellular proteases produced by heterofermentative lactic acid bacteria.

The present study was initiated to investigate the production of intracellular protease by *Leu. citrovorum* under various cultural conditions and some enzymatic properties of the intracellular protease produced were studied.

Materials and Methods

Culture: *Leu. citrovorum* IAM 1087 obtained from the stock culture collection in the Institute of Applied Microbiology, University of Tokyo, was used in this study.

Growth and harvesting of cells: The medium used in this study was composed of lactose 1%, peptone 0.7%, yeast extract 0.5%, Na₂HPO₄ 0.6% and NaH₂PO₄ 0.4% (pH 6.5). *Leu. citrovorum* was incubated at 30 C and transferred daily into the above medium. One liter of the medium was dispensed in 200 ml quantities in 500 ml flasks. After sterilization each was inoculated with

*Studies on the intracellular protease produced by *Leuconostoc citrovorum*. I.

1 ml of 24 hrs' culture and incubated at 30 C for given hours. At the end of incubation, the cells were harvested in a centrifuge operated at 3000 rpm for 20 min. The packed cells were suspended, washed in normal saline (0.9% NaCl) and centrifuged two or more times until a clean supernatant fluid was obtained.

Separation of the intracellular protease from the cells: The packed cells were stored at 4 C in 2 gram (wet weight) portions suspended in 20 ml of sterile deionized water. Twenty milliliters of the cell suspension was placed in a 50 ml beaker and was put in a mixture of ethanol, sodium chloride and ice sufficient to maintain the temperature below -10 C. The sonifer (Model-TA 4201, Kaijo Denki Co. Ltd., Tokyo) was operated at 40 kHz for 15 min. The unbroken cells and cellular debris were sedimented in a centrifuge at 3000 rpm for 20 min. The supernatant was made up to a volume of 30 ml by adding sterilized deionized water. To 25 ml of the enzyme extract adjusted to pH 7.0 was slowly added 38 ml of saturated ammonium sulfate solution. The resulting precipitate was collected by centrifugation and the intracellular protease was extracted with a minimum amount of 0.1 M calcium acetate solution. The extract was dialized against tap water for 20 hr. After dialysis, the dialized solution was messed up to 20 ml in whole with 0.1 M phosphate buffer (pH 7.0) and used as intracellular protease solution.

Preparation of the substrate: Whole casein was prepared from raw fresh skim milk according to the general method of acid casein preparation. α_s -, crude κ - and β -caseins were prepared by the methods of HIPP *et al.*¹⁴⁾ and WAUGH *et al.*¹⁵⁾

A 2% solution of each casein, which was prepared and adjusted to pH 7.0 was used as substrate. One milliliter of the enzyme solution and 1 ml of 0.1 M phosphate buffer (pH 7.0) were added to 1 ml of the substrate. The digestion mixture was incubated at 30 C for 3 hr. After incubation 3 ml of 5% trichloroacetic acid (TCA) was added. The mixture, after standing for 30 minutes, was filtered. Absorbance at 280 nm of the filtrate was measured and the amount of tyrosine was determined by the LOWRY *et al.* modification¹⁶⁾ of the FOLIN-CIocalTEAU method¹⁷⁾. The color developed with the FOLIN-CIocalTEAU reagent was measured spectrophotometrically at 660 nm. The activity of the enzyme was measured in terms of the difference of optical density (O.D.) at 0 and 3 hr. During this period, increase in O.D. showed linear relation with time. Under these conditions of assay, an increase of 0.1 O.D. after 3 hr was defined as 1 unit of activity.

Blanks, wherein TCA was added to the enzyme extract before the addition of the substrate were run with all tests.

Results

Production of intracellular protease:

Production of intracellular protease by *Leu. citrovorum* is shown in Fig. 1. Enzyme production rapidly increased in the logarithmic phase of growth and reached the maximum at 24 hr of incubation, then gradually decreased.

Fig. 2 shows the relation between temperature of incubation and production of intracellular protease. Production of the intracellular protease by this strain was greatly affected by temperature of incubation, and the optimum temperature was 30 C.

Enzymological properties of the intracellular protease:

The intracellular protease which was prepared from the culture incubated for 24 hr at 30 C was used for this experiment.

Intracellular Protease of *Leu. citrovorum*.

Fig. 1. Production of intracellular protease by *Leu. citrovorum*.

Fig. 2. Effect of incubation temperature on the production of intracellular protease by *Leu. citrovorum*.

Fig. 3. Effect of pH on the activity of the intracellular protease.

Fig. 4. Effect of temperature on the activity of the intracellular protease.

Fig. 5. Effect of temperature and pH on the stability of the protease activity.

The optimum pH of the activity of the intracellular protease was determined with McILVAINE's and ATKINS-PANTIN's buffer solutions of which pH levels ranged from 5.0 to 10.0.

As shown in Fig. 3, it was observed that the enzyme has its optimum activity in the neutral pH range from pH 7.0 to 7.5.

The activity of the enzyme preparation at various temperatures from 20 C to 45 C was determined under the standard assay conditions except for the reaction temperature. As Fig. 4 shows, the enzyme preparation was found to have an optimal temperature of 45 C, and its activity fell off rapidly above 45 C.

Fig. 5 shows stability of the enzyme at various pH. The enzyme preparation of this strain was mixed with 0.1 M phosphate buffer with pH levels ranging from pH 5.0 to 9.0 and the solution was incubated at either 5 C or 30 C for 24 hr. After the incubation, the residual enzyme activities were compared to the initial activity. As can be seen from this figure, the enzyme preparation was found to be most stable at pH 9.0 when incubated at 5 C.

The thermal stability of the intracellular protease was measured. Samples of enzyme solutions were stood for 10 min at 30, 35, 40, 45, 50, 55 or 60 C in 0.1 M phosphate buffer (pH 7.0), and the activities remaining after the incubation were expressed as percentages of the initial activity. As shown in Fig. 6, the protease was found to be remarkably sensitive to heat, *i. e.*, inactivation began already at 40 C, and 10 minutes heating at 60 C caused 80% loss of the original activity.

Fig. 6. Thermostability of the intracellular protease.

Fig. 7. Proteolysis of casein fractions by the intracellular protease of *Leu. citrovorum*.

To determine the substrate specificity of the intracellular protease, the abilities of the intracellular protease to hydrolyze various caseins were examined. As shown in Fig. 7, it is noted that α -casein was more easily hydrolyzed than β -, whole- and crude κ -caseins. Hydrolysis of crude κ -casein seemed to proceed during initial 1 hr but the hydrolysis was not so significant.

Discussion

The present study was undertaken to provide some basic aspects of the intracellular protease of *Leu. citrovorum* which is one of the most representative heterofermentative dairy lactic acid bacteria. Proteolytic enzymes of homofermentative lactic streptococci and lactobacilli are generally known to exist in the cells^{8,18-20} with a few exceptions^{5,9}.

In the preliminary experiment of the present study we recognized that proteolytic activity was mainly found in the cells, and neither culture supernatants nor buffers used to wash intact cells exhibited detectable protease activity. However, no attempt was made to concentrate any protein in the culture fluid, so that the possibility of presence of an extracellular protease has not been excluded.

FOSTER *et al.*²¹ described that *Leuconostoc citrovorum* strains grow best at 25 C to 30 C and are unable to ferment lactose to any degree. It was observed that *Leu. citrovorum* used in this experiment grew best at 30 C but very slowly at 25 C, and that pH of the media did not fall down below 4.7 throughout incubation period (Fig. 1).

Optimum pH of intracellular protease of *Leu. citrovorum* is in slightly alkaline range compared to those of homofermentative lactic acid bacteria, namely pH of intracellular proteases of *L. casei* and *S. cremoris* is 6.0 to 7.0 and that intracellular proteases of *L. bulgaricus* and *S. lactis* have their optima pH at 7.0 and 6.0, respectively⁸.

As shown in Fig. 1 the protease production was not generally occurred after 24 hr, when the culture was in the stationary or death phase. We tentatively investigated the properties of the intracellular proteases produced after 24 hr of growth and after 12 hr of growth, when the culture was in the early logarithmic growth phase. However, definite differences were not observed between these protease preparations.

The present observation that α_1 -casein was more easily hydrolyzed by the intracellular protease of *Leu. citrovorum* than the other casein fractions is consistent to the results of OHMIYA *et al.*^{10,11}, who used the intracellular proteases from several strains of homofermentative lactic acid bacteria such as *L. bulgaricus*, *L. helveticus*, *S. lactis* and *S. cremoris*. This fact suggests that substrate specificity of the intracellular protease of *Leu. citrovorum* has a strong resemblance to those of the intracellular proteases of such homofermentative dairy lactic acid bacteria. For the purpose to gain more detail knowledge of this point, further investigation on the enzymological properties of the intracellular protease of *Leu. citrovorum* is needed.

Detail investigation on inhibitor and activator requirements of the intracellular protease of *Leu. citrovorum* will be described in a subsequent report.

The authors express their gratitude to the Institute of Applied Microbiology, University of Tokyo for supplying lactic acid bacteria.

This work is supported in part by a grant-in-aid for Scientific Research from the Ministry of Education.

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Leuconostoc citrovorum による菌体内プロテアーゼの 生産とその性質

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ホモ型発酵乳酸菌による菌体内プロテアーゼの生産ならびにその性質については既に詳細な研究がなされているが、ヘテロ型発酵乳酸菌についてはそのプロテアーゼ活性が微弱であることもあってほとんど詳しい研究がなされていない。筆者らはヘテロ型発酵乳酸菌のうち、乳製品製造上重要な *Leuconostoc citrovorum* の菌体内プロテアーゼの生産と、産生酵素の抽出およびその性質について検討した。30℃で培養開始後、増殖が対数期に入った時点で菌体内プロテアーゼが最も顕著に生産され、

培養24時間目で最高に達し、以後培養時間の経過とともに漸減した。30℃で24時間培養の菌体を超音波処理後、硫酸分画をして得た酵素液の性質を全カゼインを基質にして調べた。その結果、活性至適pHおよび温度がそれぞれ7.5付近および45℃にあり、40℃で10分の加熱により活性の低下が始まることを認めた。また、 α_s -カゼイン、 β -カゼイン、粗 κ -カゼインのうち、本酵素は α_s -カゼインを最も顕著に分解することを認めた。