Inhibitor and activator requirements of the intracellular protease of *Leuconostoc citrovorum**

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Introduction

The limited data available indicated that *Leuconostoc citrovorum* (now called *Leuconostoc cremoris*[1]) exhibited appreciable proteolytic activity in milk[2]. In the preceding paper[3], the authors investigated the production of intracellular protease by *Leu. citrovorum* and showed that this strain produced intracellular protease optimally at 30C. In the same study, the authors also investigated on enzymatic properties of the intracellular protease which was partially purified. During the course of that investigation, the authors have obtained a result that the enzyme preparation hydrolyzed α_s-casein more easily than the other casein farctions. This fact was well consistent in the result obtained with the intracellular proteases of homofermentative lactic acid bacteria such as *Streptococcus cremoris*, *S. lactis*, *Lactobacillus bulgaricus*[4,5], and suggested that the substrate specificity of the intracellular protease of *Leu. citrovorum* have a well 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 intracellular protease of *Leu. citrovorum*, therefore, was needed.

In the present report inhitor and activator requirements of the intracellular protease of this strain were described.

Materials and Methods

Culture :

The same strain of *Leu. citrovorum* IAM 1087 as in the preceding paper[2] was used for the experiment. This strain was incubated at 30 C and transferred weekly in a medium (pH 7.0) consisting of lactose 1 %, peptone 0.7 %, yeast extract 0.5 %, Na_2HPO_4 0.6 % and NaH_2PO_4 0.4 %.

Cultivation for enzyme production :

One liter of the medium described above was dispensed in 200 ml quantities,

* (Studies on the intracellular protease produced by *Leuconostoc citrovorum*. II)
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into five shanking flasks (500 ml capacity), and inoculated 10 ml of preincubated culture of *Leu. citrovorum*. They were statically incubated at 30 C for 24 hr. After the incubation, the cells were harvested in a centrifuge operated at 3000 r. p. m. for 20 min. The packed cells were suspended, washed in normal saline (0.9 % NaCl) and centrifuged two or more times untill a clean supernatant fluid was obtained.

Purification of the enzyme :

Step 1. Preparation of cell free extract : The packed cells in 2 grams (wet weight) portions were suspended in 20 ml of sterile deionized water. This suspension was placed in a 50 ml beaker and the beaker was put in a mixture of ethanol, sodium chloride and ice sufficient to mantain the temperature below −10 C. The sonifer was operated at 40 KHz for 15 min. The unbroken cells and cellular debris were sedimented in a centrifuge at 3000 r. p. m. for 20 min. The supernatant was made up to a volume of 30 ml by adding sterilized deionized water.

Step 2. Ammonium sulfate fractionation : 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 amonunt of 0.1 N calcium acetate solution. The extract was dialized against tap water for 20 hr. After dialysis the solution was messed up to 20 ml in whole with 0.1 M phosphate buffer (pH 7.0).

Step 3. Sephadex column chromatography : The dialized solution was applied into a Sephadex G-100 column (2.5 × 45 cm) and eluted with 0.1 M phosphate buffer

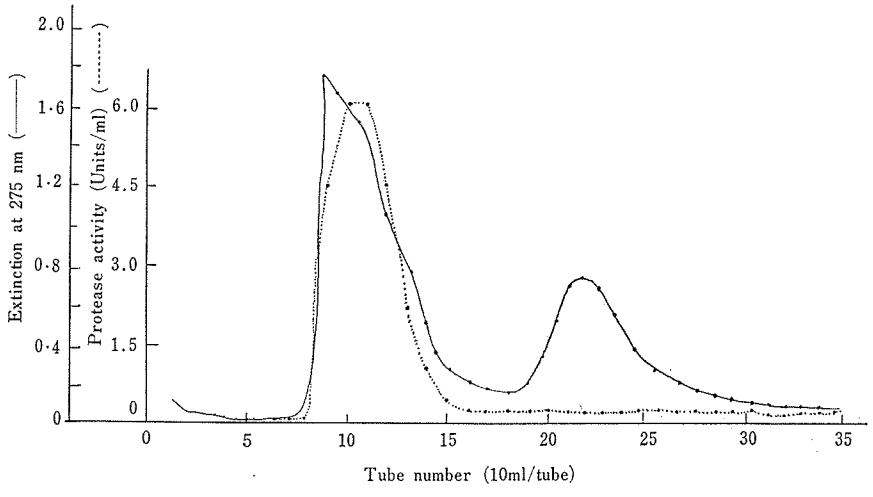

Fig. 1 Gel filtration of the cell free supernatant liquid on Sephadex G-100.

(pH 7.0). The elution porifile was presented in Fig. 1. Each aliquot sample showing protease activity was pooled.

Step 4. DEAE cellulose column chromatography : The active fraction collected was loaded into a DEAE cellulose column (3.0 × 30 cm) equilibrated with the same buffer. After the column was successively washed with the 0.1 M phosphate buffer (pH 7.0) containing 0.1 M NaCl, the protease was eluted with a linear gradient increase in NaCl at flow rate of 50 ml/hr. Five milliliter fractions were collected and each fraction was assayed.

Preparation of the substrate :

Whole casein was prepared from raw fresh skim milk according to the general method[6] of acid casein preparation. A 2 % solution of each casein, which was prepared and adjusted to pH 7.0 was used as substrate.

Assay of protease activity :

One milliliter of 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, the amount of free tyrosine was determined by the method of LOWRY *et al*[5] as described previously[2]. The activity of the enzyme was measured in term of optical density (O.D.) difference at 0 and 3 hr. During these period, increase in O.D. was linear. Under these condition of assay, 1 unit of activity was defined as an increase of 0.1 O.D. after 3 hr.

Blanks, wherein 3 ml of 5 % trichloroactic acid solution was added to the enzyme solution before the addition of the substrate were run with all tests.

Results

Results of purification :

Results of enzyme purification were shown in Table 1. The proteolytic activity of the cell free extract was generally very dilute and a large portion of the enzyme was lost during purfication, that is, fractionation with ammonium sulfate and chromatography on Sephadex G-100 and on DEAE cellulose resulted in 21.7

Table 1. Purification of intracellular protease of *Leu. citrovorvm.*

Step	Volume (ml)	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg protein)	Purification fold
Crude extract	100	192.4	256	1.33	1
$(NH_4)_2SO_4$	68	14.8	136	9.18	6.9
Sephadex G-100	176	12.8	140	10.93	8.2
DEAE cellulose	120	2.8	82	28.90	21.7

Table 2. Effect of inhibitors on proteolytic activity.

Inhibitors	Residual activity (%)
None	100
o-Phenanthroline	100
Iodoacetate	50
PCMB	0
Diisopropyl-fluorophosphate	92

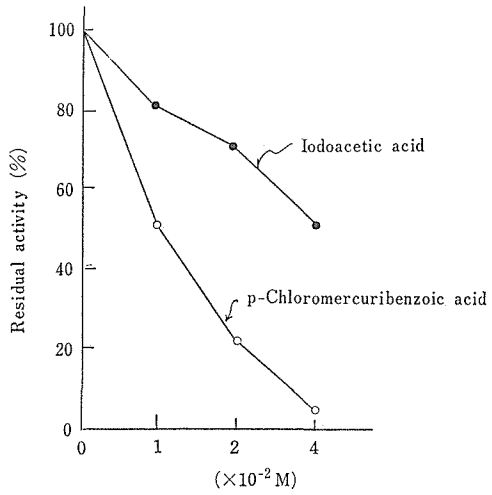

Fig. 2. Effect of PCMB and iodoacetate on the activity of the intracellular protease.

fold purification with 32 % recovery.

Inhibition of enzyme activity :

Effect of several chemical reagents such as o-phenathroline, iodoacetate, p-chloromarcuribanzoate (PCMB) and diisopropylfluorophosphate on protease activity was examined. Each reagent was dissolved in 0.5 ml of distilled water and added to 0.5 ml of enzyme solution in 0.1 M phosphate buffer (pH 7.0) to attain the final concentration of 10^{-2} M, and the mixture was preincubated for 10 min at 30 C, then the residual activity was determined by the standard method. From the results shown in Table 2, it was found that the enzyme activity was specifically inhibited by PCMB and iodoacetate and not by other reagents tested. Effect of concentrations of PCMB and iodoacotate on activity of the intracellular protease were examined and compared with each other. As seen in Fig. 2. activity of the enzyme decreased linealy with the increase of the concentration of either PCMB and iodoacetate, and the concentration of PCMB and iodoacetate at 50 %

inhibition for the enzyme were almost 4×10^{-2} M, and 1×10^{-2} M, respectively.

Inactivation by storage or iodine oxidation, and reactivity by gluthione :

The studies with the inhibitors indicated SH groups were involved in the activity of the enzyme. Therefore, the possible involvement of this functional group in the protease activity was detemined by treating the unstored and stored enzmes with iodine and/or with glutathione. As shown in Table 3, iodine oxidation of the unstored enzyme resulted in a 47 % reduction in activity. Activity of the enzyme stored at 3 C for 3 days was decreased by 15.8 and iodine oxidation of the stored enzyme reduced activity to 5.7%.

Table 3. Inactivation of protease activity by storage or iodine oxidation and reactivation by glutathione.

Treatment	Time stored at 3 C	
	0 days	3 days
	—— Residual activity (%)——	
Control	100	15.8
Control + Iodine*	52.6	5.6
Control + GSH**	113.2	47.3
Iodine treated enzyme + GSH after incubation for 2 hrs at 37 C.	73.7	26.3

* Added as 0.5 ml of 0.01 % iodine solution per 3 ml reaction mixture.
** Added as 0.2 ml of 10^{-2}M reduced glutathione per 3 ml reaction mixture.

On the other hand, glutathione added to the unstored enzyme alone increased activity nominally. Storage for 3 days at 3 C lowered residual protease activity as observed previously, but activity of the stored enzyme was reactivated considerably by glutathione. Addition of glutathione to the iodine oxidized unstored enzyme resulted in 73 % reactivity, whereas 47 % reactivation was observed with the oxidized stored enzyme.

Discussion

Ammonium sulfate precipitation is usually used in the first step to concentrate intra- or extracellular proteases of lactic acid bacteria, in case that proteolytic activities are very dilute[7,8]. In the present study ammonium sulfate precipitation was also used at the initial step of purification. Because of too weak protease activity of the crude cell free extract of *Leu. citrovorum*, about 47 % of the proteolytic activity was lost in the first step.

In this experiment, the authors recognized the intracellular protease of *Leu.*

citrovorum was not inhibited by both diisophenylfluorophosphate and o-phenantholine. According to HARTLEY's description, this fact indicates that the intracellular protease of *Leu. citrovorum* is neither serine protease nor metalo protease[9]. In contrast, the intracellular protease of *Leu. citrovorum* was inhibited by PCMB, iodine and iodoacetate, and reactivated by glutathione. This fact strongly suggests that a free sulfhydryl group(s) is required for activity. SPECK *et al.*[7,10] studied about proteinase enzyme system of Str. lactic and found that the intracellular protease of this strain was inhibited progressively with increasing concentrations of PCMB. They also recognized that the oxidied intracellular protease by iodine was reactivated by the addition of glutathione. There is, therefore, definite resemblance on functional group(s) for activities between the intracellular proteases of *Str. lactis* and *Leu. citrovorum*. This fact has an interesting bearing on the mechanism of the action of these protease on milk, because the two lactic acid bacteria are the predominant species in dairy starter cultures.

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Summary

In the present study, the intracellular protease of *Leuconostoc citrovorum* was purified by ammonium precipitation and by Sephadex column and DEAE-cellulose column chromatography. Activity of the purified enzyme was progrssively inhibited with increasing of p-chloromercuribenzoate and iodoacetate, but not by o-phenanthroline and diisopropylfluorophosphate. This fact strongly suggest that a free sulfhydyl group(s) is required for activity. The possible involvement of this functional group, then, determined by treating the enzyme with iodine and/or glutathione. As results, activity of the enzyme was reactivated considerably by glutathione, and addition of glutathione to iodine oxidized enzyme resulted in 73.7% reactivity, whereas iodine oxidation of the enzyme resulted in 52.6% reduction in activity.

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Leuconostoc citrovorum の菌体内プロテアーゼの活性阻害剤ならびに賦活剤

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要　　約

Leuconostoc citrovorum の生産する菌体内プロテアーゼを硫安沈殿ならびにセファデックス，DEAE セルロースカラムにより精製した。

精製プロテアーゼの活性は PCMB ならびに iodoacetate に対し，強い阻害を受けるのに対し，o-phenanthroline ならびに diisopropylfluorophosphate では殆んどまたは全く阻害を受けなかった。このことから，この酵素の活性部位にＳＨ基が関与していることが推定される。更に，この酵素を0.1Mリン酸緩衝液中で3°Ｃで72時間放置した場合，残存活性がもとの活性の15.8%になるのに対し，glutathione の存在下では47.3%にとどまり，またヨウ素の存在下では5.6%に低下した。また，ヨウ素により活性阻害を受けた酵素は glutathione により活性が回復することも同時に認めた。