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# Some Properties of *Clostridium butyricum* ID-113 Autolytic enzyme

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# *Clostridium butyricum* ID의 자가분해 효소

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**Cellular autolytic enzyme was isolated from the supernatant fluid of exponentially growing culture of *Cl. butyricum* ID-113. The autolysin was partially pruified by ammonium sulfate fractionation, chromatography on DEAE-Sephadex A-50 and gel filtration through Sephadex G-200. This autolytic enzyme lysed SDS-treated cell wall fractions of *Cl. butyricum* ID, but not whole cells at all. Its optimum pH and temperature were 5.0 and 37°C, respectively. This enzyme was relatively stable at neutral pH, but sensitive to heat treatment. Enzyme activity was not influenced bythe addition of various divalent cation, but inhibited by $Cu^{++}$.**

Becterial autolytic enzyme represents a class of enzymes that are probably both essential and potentially detrimental to the cell. Autolytic enzymes are supposed to play important roles in such processes as cell elongation, cell division, cell separation, and cell wall biosynthesis (1-4).

Bacterial autolytic enzyme have been found in a variety of bacteria, and for some of them, the enzymatic properties were studied in detail (5-9). *Cl. butyricum,* as intestinal tonic, has been used in the treatment of abnormal-fermentation in intestine, diarrhea, indigestion, and constipation for a long time (10-12). Although autolysins have been studied on other *Clostridium* species (13-15), surprisingly nothing is known in *Cl. butyricum.*

In this paper a procedure for the purification of the autolytic enzyme of *Cl. butyricum* ID-113 was presented, and some of its properties were described. Characterization of properties of its autolytic enzyme may help to clarify how the autolytic enzyme takes part in the specific cellular autolysis phenomenon and cell growth of *Cl. butyricum* ID.

## Materials and Methods

### Microorganisms

*Cl. butyricum* ID-113 used throughout this work was isolated from infant feces and was a antagonistic strain to enteropathogenic *Cl. perfringens* (16). *Cl. sporogenes* ATCC 7955, was also used to prepare the substrate of autolytic enzyme.

### Media and culture

Microorganisms were cultured at 37°C under anaerobic condition (16). SM medium, consisting of 1.5% starch, 1.5% soybean extract, 1.0% molasses, 0.3% ammonium sulfate, and 1.2% $CaCO_3$, was used for the production of the autolytic enzyme.

### Growth measurement

Total bacterial counts and clostridial stage

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counts were carried out with a Thomas counting chamber (Weber Scientific International, Lancing, England) and a photomicroscope (Olympus BH-2) with phase contrast optics.

**Preparation of substrate of autolytic enzyme**

Sodium dodecyl sulfate-treated cell wall fractions (SDS-CWF) used as substrate for lytic enzyme was prepared by the method of Ogata *et al.*(17) and formalin-treated cells were prepared by the method of Hongo *et al.*(18). Unless otherwise stated, the SDS-CWF of *Cl. butyricum* ID-113 was used as a substrate for autolytic enzyme

**Assay of the lytic enzyme**

Lytic enzyme activity was assayed by measuring the decrease in turbidity of SDS-CWF. The assay mixture contained 2.4 m*l* of cell wall fractions in 0.05 M sodium acetate buffer (pH 5.0) and 0.1 m*l* of enzyme solution. The initial OD at 600 nm was adjusted to 1.0. Turbidity was measured at 10 min intervals for 60 min at 37 °C. One unit of enzyme activity was defined as the amount which caused a change in turbidity of 0.01 per min.

**Protein determination**

Protein concentration was determined by the method of Lowry *et al.*(10) using bovine serum albumin as the standard, or spectrophotometrically by measuring the absorbance at 280 nm.

**Partial purification of extracellular autolytic enzyme**

All purification steps were carried out at 4 °C. The buffer used throughout the purification was 67 mM potassium phosphate buffer (pH 6.0).

**1) Fractionation with ammonium sulfate**

3L of SM culture broth was centrifuged at 12,000 × g for 10 min and the supernatant was salted out with ammonium sulfate (30-60% saturation). After storage for 48 hr at 4 °C, the precipitate was collected by centrifugation at 10,000 × g for 10 min. The sediment was dissolved in a small amount of the buffer and dialyzed against the same buffer at 4 °C for 2 days.

**2) Column chromatography on DEAE-Sephadex A-50**

20 m*l* of dialyzed enzyme solution was applied to a column (2.5 × 24 cm) containing DEAE-Sephadex A-50 equilibrated with the buffer. This material was eluted with a linear gradient of 0.0 M to 0.5 M NaCl in the buffer. Five m*l* fractions were collected at the flow rate of 20 m*l* per hr.

**3) Colmn chromatography on Sephadex G-200**

Five m*l* of concentrated enzyme solution was applied on Sephadex G-200 equilibrated with the buffer and eluted with the same buffer at the flow rate of 6 m*l* per hr. And 2 m*l* elutes were collected. Fractions having cell wall lytic enzyme activity were collected and stored at -20 °C.

## Results and Discussion

**Accumulation of autolytic enzyme**

Autolytic enzyme activity was found in the supernatant fluid of exponentially growing culture in sporeforming SM medium at 37 °C as shown in Fig. 1. The autolytic enzyme activity began to increase when the growth was started, and continued to increase with the cell growth.

**Partial purification of extracellular autolytic enzyme**

Extracellular autolytic enzyme was purified by the procedure of 3 steps decribed in Materials and Methods. DEAE-Sephadex fraction was purified about 30.1 fold from first salted out fraction (Fig. 2) and was purified further by gel filtration (Fig. 3). The purifying procedures resulted in 47.2-fold purification with the overall yield of 12%, as shown in Table 1.

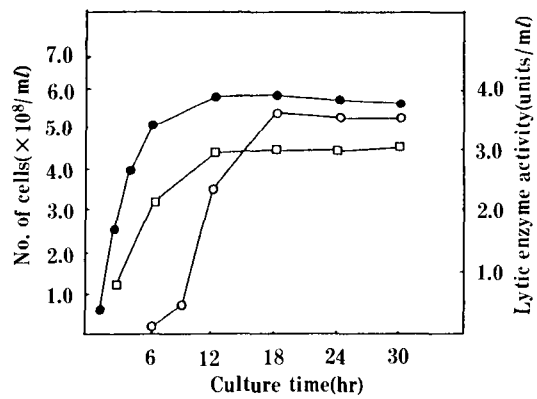


**Fig. 1. Growth and extracellular autolytic enzyme activity of *Cl. butyricum* ID-113 in SM medium.**

○ - ○ ; No. of total cells, ● - ● ; No. of spores, □ - □ ; lytic enzyme activity

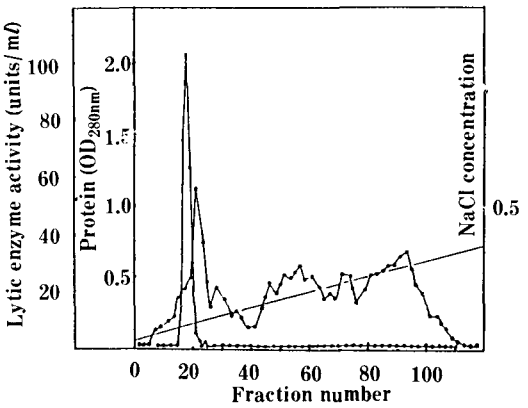


**Fig. 2. Ion exchange chromatography of autolytic enzyme on DEAE-Sephadex A-50.**

To column(2.5 × 24cm) of DEAE-Sephadex A-50 was loaded 20 m*l* of the dialyzed solution of autolytic enzyme preparation obtained from first ammonium sulfate fractionation. The column was eluted with a linear gradient of 0.0 M to 0.5 M NaCl in 67 mM potassium phosphate buffer (pH 6.0). Five m*l* elutes were collected at the rate of 20 m*l* per hr.

○ – ○ ; absorbance at 280 nm, ● – ● ; autolytic enzyme activity

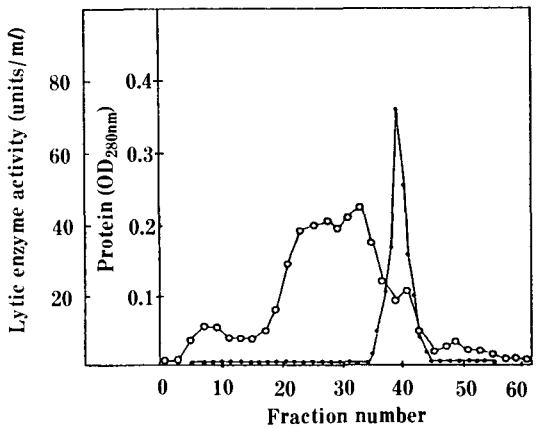


**Fig. 3. Purification of autolytic enzyme by gel filtration.**

To column(1.6 × 46 cm) of Sephadex G-20 was loaded 5 m*l* of the concentrated autolytic enzyme obtained from the DEAE Sephadex column after second ammonium sulfate fractionation. The column was eluted with 0.05 M potassium phosphate buffer solution (pH 6.0). Flow rate was 6 m*l* per hr. Two m*l* elutes were collected.

○ – ○ ; absorbance at 280 nm,

● – ● ; autolytic enzyme activity

## Properties of extracellular autolytic enzyme

### 1) Optimum pH and temperature

The optimum pH of extracellular autolytic enzyme on SDS-CWF was about pH 5.0, as shown in Fig. 4. The optimum temperature was about 37 °C, as shown in Fig. 5. And extracellular autolytic enzyme sharply decreased at 45 °C or above.

**Table 1. Purification of extracellular autolytic enzyme**

| Fraction | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Recovery (%) | Purification (fold) |
|---|---|---|---|---|---|
| Supernatant fluid of culture | 3200 | 6300 | 2.0 | 100 | 1.0 |
| 30-60% ammonium sulfate saturation | 825 | 3200 | 3.9 | 51 | 1.9 |
| DEAE-Sephadex A50 | 25 | 1405 | 60.1 | 22 | 30.1 |
| 60% Ammonium Fractionation | 21 | 1384 | 65.9 | 22 | 33.0 |
| Sephadex G-200 | 8 | 754 | 94.3 | 12 | 47.2 |

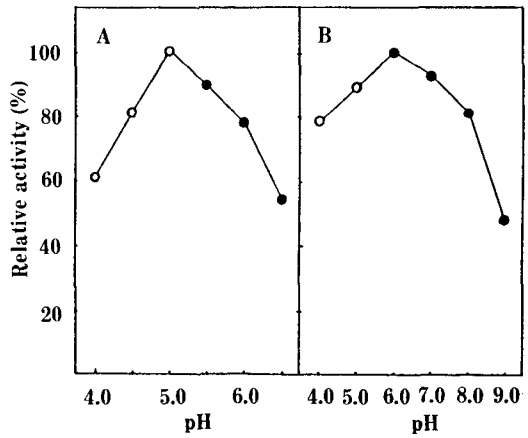


**Fig. 4. Optimum pH and pH stability of extracellular autolytic enzyme.**

A; Optimum pH, B; pH stability. The reaction mixture contained, in a total volume of 2.5 m*l*, SDS-CWF(1.0 OD at 600nm) and extracellular autolytic enzyme(7.0 units). The reaction mixtures were incubated at 37 °C for 30 min. The activity was tested in the following buffers.

○ – ○ ; 0.05M sodium acetate buffer,

● – ● ; 0.05M potassium phosphate buffer

### 2) Stability of extracellular autolytic enzyme

Stability of extracellular autolytic enzyme stored

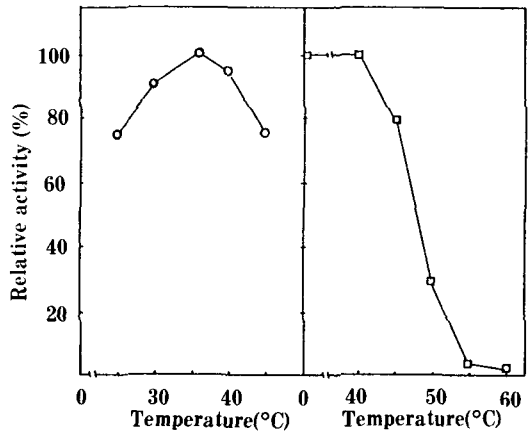


**Fig. 5. Optimum temperature and heat stability of extracellular autolytic enzyme.**

A; Optimum temperature, B; Heat stability. The reaction mixture contained, in a total volume of 2.5 *ml*, SDS-CWF(1.0 OD at 600 nm), extracellular autolytic enzyme (7.0 units) and 0.05M sodium acetate buffer (pH 5.0). The reaction mixtures were incubated at 37 °C for 30 *min*.

**Table 2. Effects of divalent cations and EDTA-Na on the extracellular autolytic enzyme activity**

| Reagents | Concentration (M) | Relative activity (%) |
|---|---|---|
| None | $1 \times 10^{-3}$ | 100 |
| $MgSO_4$ | $1 \times 10^{-3}$ | 98 |
| $CaCl_2$ | $1 \times 10^{-3}$ | 100 |
| $MnSO_4$ | $1 \times 10^{-3}$ | 103 |
| $FeSO_4$ | $1 \times 10^{-3}$ | 100 |
| $CuSO_4$ | $1 \times 10^{-3}$ | 34 |
| EDTA-Na | $1 \times 10^{-3}$ | 99 |
| $ZnSO_4$ | $1 \times 10^{-3}$ | 88 |

The reaction mixture contained, in a total bolume of 2.5 m*l*, SDS-CWF (1.0 OD at 600 nm), extracellular autolytic enzyme (7 units), metal ions and 0.05M sodium acetate buffer (pH 5.0). The mixture was incubated at 37 °C for 30 min.

at 4 °C for 24 hr was stable between pH 4.0 and 8.0, as shown in Fig. 4. The extracellular autolytic enzyme was completely denatured with incubation at 55 °C for 5 min, and 20% loss of activity was found at 45 °C for 5 min as shown in Fig. 5. These results indicated that the extracellular autolytic enzyme was relatively unstable to heat treatment.

**3) Effects of divalent cations and EDTA-Na**

The activity of the extracellular autolytic enzyme was not inhibited or stimulated by divalent cations tested except $Cu^{++}$ and $Zn^{++}$. $Cu^{++}$ was a strong enzyme inhibitor (Table 2).

**Table 3. Substrate specificity of extracellular autolytic enzyme**

| Substrate | |
|---|---|
| *Clostridium butyricum* ID | |
| whole cells | - |
| heated cell wall fractions | - |
| formalin-treated cells | - |
| SDS-treated cell wall fractions | + |
| *Clostridium sporogenes* ATCC 7955 | |
| whole cells | - |
| heated cell wall fractions | - |
| formalin-treated cells | - |
| SDS-treated cell wall fractions | + |

* +: positive, -: negative

**4) Substrate specificity of extracellular autolytic enzyme**

The extracellular autolysin degested SDS-CWF prepared from two kinds of *Clostridium* species. Whole cells and heated cell wall fractions were hardly lysed by the extracellular autolytic enzyme as shown in Table III. It might also be said that the intact and membrane-bound cell wall were not attacked from the outside by the autolysin. Properties of extracellular autolytic enzyme in *Cl. butyricum* ID-113 was different from that of the phage-induced lytic enzyme (endolysin) in its substrate specificity, for endolysins of other clostridia had been known to be active against formalin-treated whole cells as well as isolated cell wall fractions (17,18).

## 요 약

자가분해 효소는 *Cl. butyricum*의 포자형성 배지에서 배양할 때 체외로 배출되어 배양액에도 존재하였다. 배양액으로부터 약 50배로 부분정제된 자가분해 효소를 사용하여 효소의 성질을 조사하였다. 자가분해 효소의 최적 pH와 온도는 각각 5.0과 37℃였으며 중성 pH에서는 안정하나, 열에는 비교적 불안정하여 50℃에서 5분간 열처리한 후 효소활성의 70%가 소실되었다. 또한 Cu ion$^{++}$에 의해서 효소활성이 저하되었으나 그밖의 금속이온에 의하여서는

큰 영향을 받지않았다. 또한 자가분해 효소는 기질로서 영양세포에는 직접 활성을 나타내지 못하나, 세포벽 fraction에는 활성을 가지고 있었다.

## References


1. Forsberg, C., and H. Roger: *Nature*, **229**, 272 (1970).
2. Strominger, J., and J.M. Ghuysen: *Science*, **156**, 213 (1967).
3. Schokman, G.D., and J. Barret: *Ann. Rev. Microbiol.* **37**, 501 (1983).
4. Rogers, H., and W. Wiedel; *Bacterial Rev.*, **34**, 194 (1965).
5. Cornett, J.B., B.E. Rednam, and G.D. Schockman: *J. Bacteriol.*, **133**(2), 631 (1978).
6. Shugu, D.L., J.B. Cornett, and G.D. Schockman: *J. Bacteriol.*, **138**(2), 598 (1979).
7. Shugu, D.L., J.B. Cornett, and G.D. Schockman: *J. Bacteriol.*, **142**(3), 741 (1980).
8. Fein, J.E., H.J. Rogers: *J. Bacteriol.* **127**, 1427 (1976).
9. Brown, W.C.: *Appl. Microbiol.*, **25**(2), 292 (1973).
10. Han, I.K., S.C. Lee, J.H. Lee, J.D. Kim, P.K. Jung, and J.C. Lee: *Korean J. Animal Sci.*, **26**(2). 158 (1984).
11. Han, I.K., J.D. Kim, J.H. Lee, S.C. Lee, T.H. Kim, and J.H. Kwag: *Korean J. Animal Sci.*, **26**(2), 166 (1984).
12. 吉岡一, 田坂芳郎, 田坂重元: 臨床小兒醫學, **14**, 281(1967).
13. Kawata, T., and K. Takumi: *Jpn. J. Microbiol.*, **15**(1), 1 (1971).
14. Webster, J.R., S.J. Reid, D.D.T. Johnes, and D.R. Woods: *Appl. Environ. Microbiol.*, **41**(2), 371 (1981).
15. Sadazo, Y., S. Ogata, and S. Mayashida: *Agri. Biol. Chem.*, **46**(5), 1243 (1982).
16. Kwag, J.H., T.H. Kim, J.C. Lee, P.K. Jung, and K.K. Lee: *Kor. J. Appl. Microbiol. Bioeng.* (in press)
17. Willis, A.T.: *Methods in microbilolgy*, Academic press, New York, Volume 6A, 79 (1969).
18. Ogata, S., Y. Tehara, and M. Hong: *Agri. Biol. Cyem.*, **38**(4), 763 (1974).
19. Hongo, M., Y. Teharea, and S. Ogata: *Agri. Biol. Chem.*, **38**(4), 755 (1974).
20. Lowry, O.H., N.J., Rosenbrough, A.L. Farr, and R.J. Randall: *J. Biol. Chem.*, **193**, 265 (1951).


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