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Cellular Autolysis of *Clostridium butyricum* ID-113

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Clostridium butyricum ID의 자가분해

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The optimum conditions for cellular autolysis in *Clostridium butyricum* ID-113 have been investigated. Cellular autolysis was optimal at pH 7.0 in 0.05 M potassium phosphate buffer and at 37 °C. The rate of cellular autolysis depended on the age of culture. The most rapid cellular autolysis occurred in the cells of mid-exponentially growing cultures, but cellular autolysis decreased sharply when the cultures entered the stationary phase. A growing culture of *Cl. butyricum* ID-113 was induced to autolyze and lost its turbidity spontaneously in the hypertonic NaCl, sucrose, or glucose medium. The autolytic enzyme activity was found in the autolysate of cells and the supernatant of the culture.

Autolytic enzymes are supposed to play important roles in such processes as cell elongation, cell division, cell separation, and cell wall biosynthesis (1-4). However, under some abnormal culture conditions the autolytic enzyme is activated and lyses its own cell wall and causes cellular autolysis. Among the different factors influencing the triggering of bacterial autolysis, growth condition, osmotic enviornment, and pH have been recognized as being particularly important (5).

Cl. butyricum ID used in this study is a strain of sporeforming butyric acid bacteria, which counteracts with the intestinal harmful microflora, stimulates the growth of lactic acid bacteria, assists the re-establishment of the normal intestinal microflora, and prevents the production of ammonia in intestine (6-8). In industrial production of *Cl. butyricum* ID spores, vegetative cells in the mid-exponential phase are abnormally autolyzed with consequent reduction in viability before endospore formation. Therefore, it is necessary to inhibit the abnormal cellular autolysis in the fermentor so as to promote the production of spore.

For these reasons, this study was carried out to examine the experimental conditions which stimulate cellular autolysis. Investigation of the cellular autolysis could help to understand how changes in environmental and fermentational conditions trigger action of the autolytic enzyme.

Materials and Methods

Microorganism and culture condition:

Cl. butyricum ID-113 used throughout this work was isolated from infant feces and was a antagonistic strain to enteropathogenic *Cl. perfringens*(9). *Cl. butyricum* ID-113 was cultured at 37 °C under

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anaerobic condition(10) in TYG(11, 12) and TYA (13) medium. The TYG medium contaied 3% tryptone, 2% yeast extract, 0.4% glucose, and 0.1% cystein HCl. The TYA medium contained 6% glucose, 0.6% tryptone, 0.2% yeast extract, 0.3% ammonium acetate, and 0.05% KH_2PO_4.

Turbidity measurement

OD of the culture or cell suspension was measured at 600 nm with *spectrophotometer* (beckman QC50).

Cellular autolysis

The cells taken from different stages in the growth phase were harvested by centrifugation and washed with cold distilled water. The washed cells were suspended in 0.05 M potassium phosphate buffer (pH 7.0) or the same buffer containing 0.3 M NaCl. The initial OD at 600 nm was adjusted to 1.0 and cellular autolysis at 30 °C was monitored turbidimetrically. One unit of rate of cellular autolysis was defined as a decrease in turbidity of 0.01 per min. The initial rate constant of cellular autolysis was calculated as $K = 2.31\log(C_0/C_1) \times \min^{-1}$, where C_0 and C_1 were the turbidities as t_0 and t_1.

Induction of cellular autolysis in culture

The cells were harvested in exponential growth phase by centrifugation at room temperature, and inoculated in fresh hypertonic TYG medium containing NaCl, sucrose, or glucose. The incubation was carried out at 37 °C.

Preparation of substrate for the lytic enzyme

Sodium dodecyl sulfate-treated cell wall fraction (SDS-CWF) used as substrate for the lytic enzyme was prepared by the method of Ogata *et al.* (14).

Assay of the lytic enzyme

Lytic enzyme activity was assayed by 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.

Results and Discussion

Effect of pH and buffer concentration on cellular autolysis

The concentration of potassium phosphate and

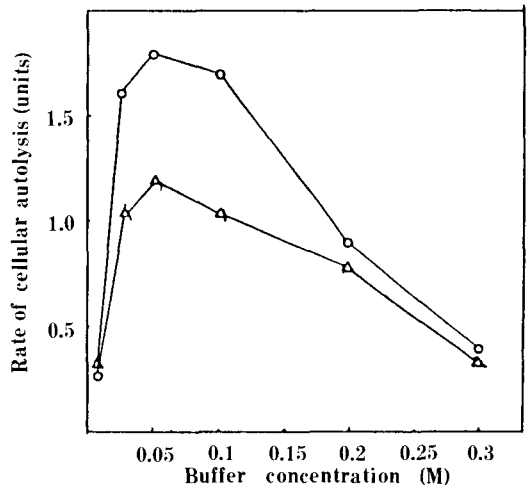

Fig. 1. Effect of buffer concentration on the rate of cellular autolysis of *Cl. butyricum* ID-113.

Exponential phase cells grown at 37 °C in TYA medium were suspended in different concentration of the following solutions.

Cellular autolysis was monitored at 30 °C
○ – ○ : Potassium phosphate buffer(pH 7.0)
△ – △ : Sodium acetate buffer (pH 6.5)

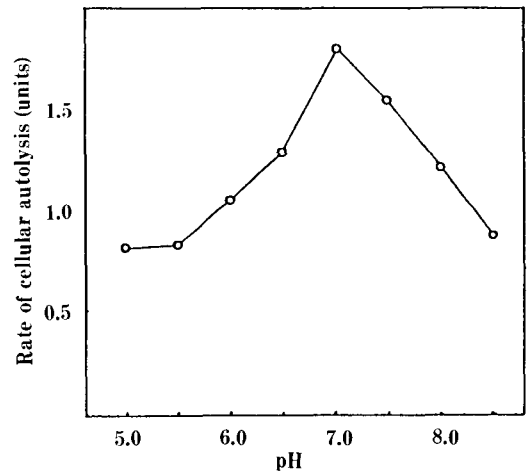

Fig. 2. Effect of pH on the rate of cellular autolysis of *Cl. butyricum* ID-113.

Exponential phase cells grown at 37 °C in TYG medium were suspended in 0.05 M potassium phosphate buffer of different pH values. Cellular autolysis was monitored at 30 °C.

sodium acetate buffers also affected autolysis of exponential-phase cells and its optimum concentration was 0.05 M (Fig. 1). Because ammonium acetate is not a good buffer at pH values near neutrality, potassium phosphate buffer was used in all further experiments. Autolysis of exponential-phase cells in 0.05 M potassium phosphate buffer was optimal at pH 7.0(Fig. 2). At above and below pH 7.0, cellular autolysis rate decreased markedly. These effects were identical to that observed by Allock *et al.*(15) with *Clostridium acetobutylicum*.

Effect of temperature on cellular autolysis

Initial rate of cellular autolysis was clearly dependent on the temperature at which cells were autolyzed (Fig. 3). Initial rate of cellular autolysis in 0.05 M potassium phosphate buffer (pH 7.0) was optimal at 37 °C (Fig. 4). At 23 ° and 30 °C, plots of OD versus time were linear, whereas at 37 ° and 45 °C, the plots were not(Fig. 3). This result seemed to indicate that at the higher temperature a competition must exist between cellular autolysis and a certain inactivation of the autolytic system. A similar situation has been described at 37 °C and above with *E. coli* (16).

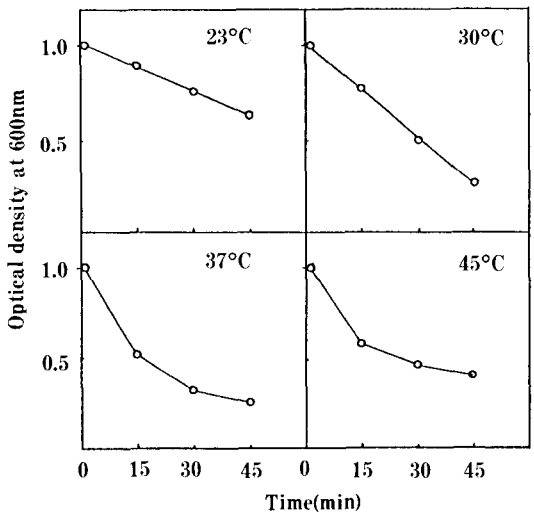

Fig. 3. Effect of temperature on cellular autolysis of *Cl. butyricum* ID-113.
Exponential phase cells were suspended in 0.05 M potassium phosphate buffer (pH 7.0). Cellular autolysis was monitored at different temperatures and was expressed as a plot of OD versus time.

Effect of growth phase on cellular autolysis and NaCl-induced lysis

It was observed that rate of cellular autolysis was influenced by the phase of growth at which cells were harvested. The rate of cellular autolysis was maximum at 6 hr of cultivation when the growth reached the mid-exponential phase and decreased sharply at the end of the exponential phase (Fig. 5), independently of the medium in which they were grown. The nutritional composition of medium appeared to influence the rate of cellular autolysis. The rate of cellular autolysis in TYG medium was

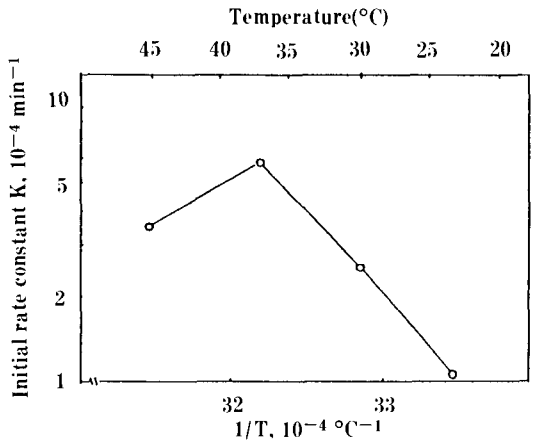

Fig. 4. Arrhenius plot of the initial rate of cellular autolysis.
The conditions are those of Fig. 3.

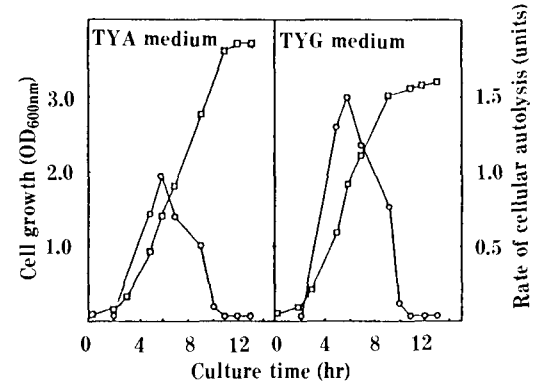

Fig. 5. Rate of cellular autolysis of *Cl. butyricum* ID-113 during different stages in growth phase.
Exponential phase cells were harvested in TYG and TYA cultures, and subjected to cellular autolysis in 0.05 M potassium phosphate buffer (pH 7.0). Cellular autolysis was monitored at 30 °C.
□ – □ ; cell growth, ○ – ○ ; rate of cellular autolysis

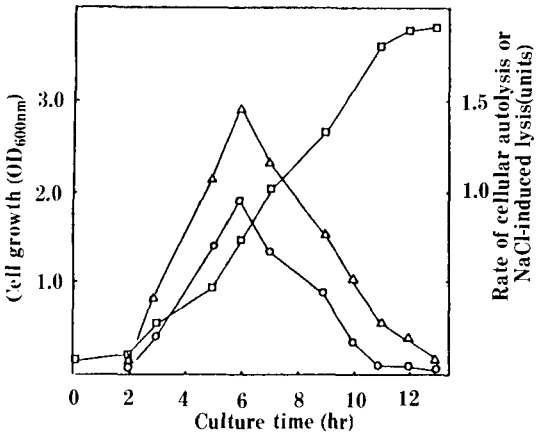

Fig. 6. Rate of cellular autolysis and NaCl-induced lysis of *Cl. butyricum* ID-113 during different stages in growth phase.

Cells harvested from TYA culture were suspended in 0.05 M potassium phosphate buffer (pH 7.0). Cellular autolysis was monitored at 30 °C.

□ - □ : cell growth, ○ - ○ : rate of cellular autolysis

△ - △ : rate of NaCl-induced lysis

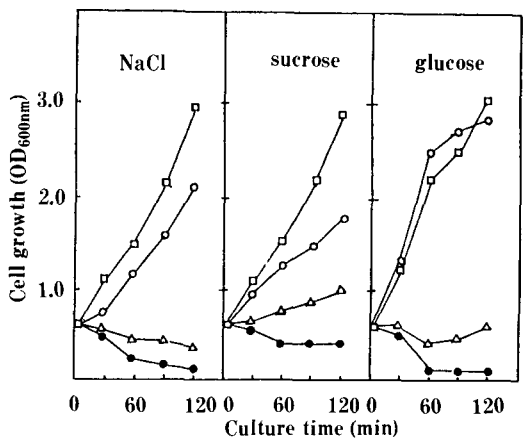

Fig. 7. Induced autolysis of *Cl. butyricum* ID-113 at various concentration of NaCl, sucrose, or glucose.

Exponential phase cells were suspended in fresh medium containing designated concentration of NaCl, sucrose, or glucsoe. Incubation was carried out at 37 °C.

□ - □ : 0.0 M, ○ - ○ : 0.1 M, △ - △ : 0.3 M, ● - ● : 0.5 M

higher than that in TYA medium.

Portions of TYA culture were withdrawn at intervals to compare the cell's sensitivity to NaCl-induced lysis and their tendency to autolyze (Fig. 6). Actively growing cells from mid-exponential phase were highly sensitive to NaCl-induced lysis and cellular atuolysis. The rate of NaCl-induced lysis was minimal at the late of stationary phase. The rate of NaCl-induced lysis was higher than that of cellular autolysis. At the peak, the ratio of NaCl-induced lysis to cellular autolysis was 3:2.

Cellular autolysis in presence of NaCl, sucrose, or glucose in culture

The harvested mid-exponential cells were inoculated in hypertonic TYG medium (pH 6.8) which contained NaCl, sucrose, or glucose. The lysis began at about 30 min of incubation at 37 °C with reduction of turbidity (Fig. 7). In medium containing glucose, sucrose, or NaCl conentration above 0.3 M, the growing cells induced autolysis. The cellular lysis may be induced indirectly by cellular plasmolysis under the hypertonic condition. In the NaCl and glucose-induced lysis, the cells were destroyed. However, in the sucrose-induced lysis, the original rods of *Cl. butyricum* were converted morphologically to spheres similar to protoplasts which were fragile to osmotic shock. It seems that sucrose acts as an inducer of cellular autolysis and at the same time a stabilizer for the developed protoplast-like cells (17). Since no lytic enzyme other than autolysin are known to exist in normal growing cells, these results suggest that the induced lysis is catalyzed by autolytic enzyme.

Table 1. Specipic activity of autolytic enzyme

Source of autolytic enzyme	Specific activity (units/mg)
cytoplasm[a]	—[c]
autolysate of cell wall fraction[b]	2.9

Mid-exponentially growing cells in TYG medium were used. a; Cytoplasm was obtained after disrupting the cells by sonication. b; Preparation of cell wall fractions was the same as that of SDS-CWF. CWF were suspended in 0.05M potassium phosphate buffer (pH 7.0) and autolyzed at 37 °C for 60 min. The activity of the autolytic enzyme released into the supernatant was measured with SDS-CWF. c; Below limit of detection.

Distribution of autolytic enzyme

To investigate the distribution of autolytic enzyme in expoentially growing cells, the specific activity of autolytic enzyme in cytoplasm and autolysate of cell wall fractions were examined (Table 1). The lytic enzyme activity was found in the auto-

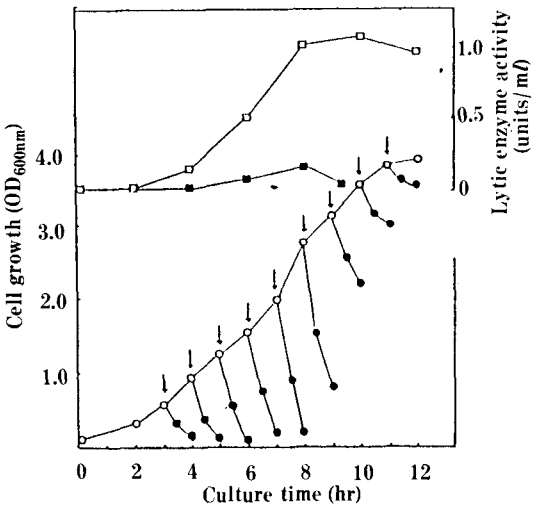

Fig. 8. Time course of cell growth and NaCl-induced cellular autolysis.

At the indicated time, aliquot of the culture was withdrawn and the turbidity was measured. NaCl-induced autolysis was induced by addition of 0.5 M NaCl, final concentration, to the withdrawn culture. Arrows indicate the addition of NaCl.

○ - ○ ; cell growth, ● - ● ; NaCl-induced autolysis

□ - □ ; extracellular autolysin activity

■ - ■ ; cell wall-bound autolysin activity

lysate of cell wall fractions but not in cytoplasm. This results indicated that the autolytc enzyme was cell wall-bound enzyme. In *Bacillus subtilis*(18), *Lactobacillus acidophilus*(19), and *Cl. saccharoperbutylacetonicum*(11), the autolysins were known to be bound to cell in exponentially growing cells.

Accumulation of extracellular lytic enzyme

A lytic activity was found in the supernatant fluid of a culture in TYA at 37 ℃, as shown in Fig. 8. The lytic enzyme activity began to increase when the growth was started, and continued to increase with the cell growth. The maximum activity was observed at 8 hr of cultivation when the growth had reached the stationary phase. Therefore, it seemed that the lytic enzyme was synthesized only when the cells were growing and dividing vigorously. The organisms in exponential phase were easily autolyzed by the addition of hypertonic concentrations of NaCl, an inducer of the autolytic enzyme.

The autolysate of cells in culture contained about 15% more lytic activity than that accumulated in the supernatant fluid of the culture. The excess of lytic activity found in the autolysate originated from cell wall-bound autolytic enzyme.

요 약

낙산균 *Cl. butyricum* ID의 자가분해 최적 조건과 특징을 조사하였다. 영양세포의 자가분해 최적 pH는 0.05M 완충용액에서 7.0 이었으며, 최적 온도는 37℃였다. 자가분해 속도는 대수기의 영양세포가 가장 높았으며, 대수기 이후의 영양세포에서는 급격히 감소하였다. 대수기의 영양세포는 0.3M 이상의 NaCl, sucrose, glucose를 포함하는 배양액에서는 자가분해가 일어나 정상적인 증식을 할 수 없었다. 자가분해 효소는 균체내에서 세포벽에 결합된 상태로 존재하였고 배양중 대수 증식기에 체외로 배출되어 배양액에도 존재하였다.

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