Some Properties of Autolysin of *Clostridium saccharoperbutylacetonicum*[†]

Sadazo YOSHINO, Seiya OGATA and Shinsaku HAYASHIDA

Laboratory of Applied Microbiology, Department of Agricultural
Chemistry, Kyushu University, Fukuoka 812, Japan

Received September 21, 1981

A lytic enzyme activity (*N*-acetylmuramidase activity) was found in the supernatant fluid of an exponentially growing culture of *Clostridium saccharoperbutylacetonicum* (ATCC 13564). The same enzyme activity was also found in the autolysate of the cells and the isolated crude cell walls. This enzyme was considered to be autolysin of *C. saccharoperbutylacetonicum*. This autolysin lysed the cell walls of *C. saccharoperbutylacetonicum* and some other clostridia, but whole cells and their glycan moiety hardly at all. Its optimum pH was about 4.5 and optimum temperature was about 35°C. Its molecular weight was 44,000.

Bacterial autolysins have been found in a variety of bacteria, and for some of them, the enzymatic properties were studied in detail.^{1~4)} Autolysin is supposed to play important roles in cell elongation, septum formation and cell separation.⁵⁾ However, under some abnormal cultural conditions the autolysin is activated and lyses its own cell wall and causes cellular autolysis.^{6,7)}

The autolysis of growing cells of *Clostridium saccharoperbutylacetonicum*, which had been used in butanol production,⁸⁾ could be induced by hypertonic NaCl or sucrose as described in previous papers.^{9,10)} The plasmolysing action of hypertonic NaCl (or sucrose) and cell wall digesting action of autolysin may induce this specific autolysis.¹¹⁾

In this work, we isolated the autolysin of *C. saccharoperbutylacetonicum*, and investigated its properties, because the results may help to clarify how the autolysin takes part in the specific autolysis phenomenon and cell growth of *C. saccharoperbutylacetonicum*.

MATERIALS AND METHODS

Organisms and cultural conditions. Clostridium

saccharoperbutylacetonicum N1-4 (ATCC 13564)⁸⁾ was used throughout this work, and two other strains, *C. sporogenes* and *C. kaiman* were also used.

Strain N1-4 was incubated at 30°C under reduced atmospheric pressure (5 mmHg) in TYA medium. TYA medium contained (per liter): glucose, 40 g; Bactotryptone (Difco), 6 g; yeast extract (Daigo Eiyo Kagaku Co.), 2 g; ammonium acetate, 3 g; KH₂PO₄, 0.5 g; MgSO₄·7H₂O, 0.3 g; FeSO₄·7H₂O, 10 mg. The pH was adjusted at 6.5 with *N* NaOH. For the preparation of exponentially growing cells, cells at the stationary phase were inoculated into fresh TYA medium to an optical density of 0.1 at 660 nm, and incubated at 30°C for the desired period.

Turbidity measurement. Optical density of the culture and cell wall suspension were measured at 660 nm with a photoelectric colorimeter (model 7A, Tokyo Koden Co.) or a spectrophotometer (Type 124, Hitachi Co.).

Preparation of cell walls and peptidoglycan. Cell walls and peptidoglycan of strain N1-4 were prepared as previously reported.¹²⁾ Cell walls of two other clostridia were prepared in a similar way to the method of cell wall preparation for strain N1-4.

Assay of lytic activity. Lytic activity was assayed by measuring the decrease of cell wall turbidity. The reaction mixture contained: 100 mM acetate buffer (pH 4.5), 0.9 ml; lytic enzyme solution, 0.1 ml; cell wall, 1 mg. The calculation formula for lytic activity was as follows; unit = decrease of OD₆₆₀ × 10³/incubation time (min).

[†] Lytic Enzyme Active on *Clostridium* Species. Part IV.

Purification of extracellular lytic enzyme. Fifty liter of culture broth (OD_{660} of 0.8) was centrifuged at $12,000 \times g$ and the supernatant was salted out with $(NH_4)_2SO_4$ at 60% saturation. After storage for 48 hr at $4^\circ C$, the precipitate was collected by decantation and centrifugation at $10,000 \times g$ for 30 min. The sediment was resuspended in a one-hundredth volume of 67 mM phosphate buffer (pH 6.0), and dialyzed against 150-fold of the same buffer for 48 hr. The dialysate was centrifuged at $15,000 \times g$ for 30 min and the supernatant was fractionated by 20% and 40% $(NH_4)_2SO_4$ saturation. The salted-out fraction was dialyzed as described above. The resulting dialysate was applied on a column of Sephadex G-75 (4.5×100 cm) equilibrated with the same buffer. The fractions having cell wall lytic activity were collected and condensed with a collodion bag (MS Co. Ltd.). This condensed enzyme solution was stocked at $-20^\circ C$ and used throughout the subsequent work as extracellular lytic enzyme.

Preparation of cell wall-bound (cellular) autolysin. Harvested log phase cells were washed twice with cold deionized water and sonicated with a Insonator 200M (Kubota Co. Ltd.) at 9,000 Hz for 20 min. Disrupted cells were collected by centrifugation at $15,000 \times g$ for 30 min, and suspended in chilled 67 mM phosphate buffer (pH 6.0). The suspension was centrifuged at $1,000 \times g$ for 30 min to remove whole cells and cell debris. The supernatant containing crude cell walls was centrifuged at $15,000 \times g$ for 30 min and washed twice with the same buffer. The washed precipitate was used as crude cell wall.

The crude cell wall suspended in 67 mM phosphate buffer (pH 6.0) was autolyzed at $30^\circ C$ for 4 hr, and then centrifuged at $15,000 \times g$ for 30 min. The supernatant was salted out with $(NH_4)_2SO_4$ at 60% saturation. The precipitate was suspended in a one-twentieth volume of chilled 67 mM phosphate buffer (pH 6.0) and dialyzed in the same way as for the extracellular lytic enzyme. The resulting dialysate was applied on a column of Sephadex G-75, and the fractions having cell wall lytic activity were condensed with a collodion bag. The condensed enzyme solution was stocked at $-20^\circ C$ and used as cellular autolysin.

Preparation of autolysate. NaCl was added to the log phase culture at 0.3 M, and the culture broth was incubated at $30^\circ C$ for 40 min to autolyse the cells completely as described previously.⁹⁾ The resulting autolysate was centrifuged at $15,000 \times g$ for 30 min at $4^\circ C$ to remove cell debris. This autolysate contained both cellular autolysin and extracellular lytic enzyme.

RESULTS

Accumulation of extracellular lytic enzyme

A lytic activity was found in the supernatant fluid of a culture of *C. saccharoperbutylacetonicum* incubated in TYA medium at $30^\circ C$, as

shown in Fig. 1. The lytic activity began to increase when the growth was started, and increased 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 cells in the log phase culture were easily autolysed by addition of hypertonic NaCl, an inducer of the autolysis of this *Clostridium*, as shown in Fig. 1. The autolysate of cells in culture broth had about 20% more lytic activity than that accumulated in the supernatant fluid of the culture broth. So, the excess of lytic activity found in the autolysate originated from cell wall-bound autolysin.

As mentioned above, the increment of lytic activity correlated with cell growth and NaCl-induced autolysis. So the lytic enzymes should play a role in cell growth and NaCl-induced autolysis.

FIG. 1. Cell Growth, NaCl-Induced Autolysis and Accumulation of Extracellular Lytic Enzyme Activity.

At desired periods, an aliquot of the culture broth was withdrawn and the turbidity and extracellular lytic enzyme activity were measured. NaCl-induced autolysis was induced by addition of 0.3 M NaCl, final concentration, to the withdrawn culture broth. Arrows indicate the addition of NaCl. ○, cell growth; ●, NaCl-induced autolysis; △, extracellular lytic enzyme activity.

Purification of extracellular lytic enzyme

Extracellular lytic enzyme was purified by the procedure described in MATERIALS AND METHODS. The gel filtrated fraction was purified about 500-fold from the first salted out fraction, and purified further by isoelectric focusing, as shown in Fig. 2. The activity was recovered in the fractions of pI 4.2.

Properties of extracellular lytic enzyme

1) *Optimum pH and temperature.* The optimum pH of extracellular lytic enzyme on cell walls was about pH 4.5, and it could not digest

cell walls at alkaline pHs above 9.0 or acidic pHs below 3.0, as shown in Fig. 3-A. The optimum temperature was about 35°C, as shown in Fig. 4-A.

2) *Stability of extracellular lytic enzyme.* As shown in Fig. 3-B, the extracellular lytic enzyme incubated at 4°C for 24 hr was stable between pH 4 and 8.

The extracellular lytic enzyme was completely denatured by heat treatment at 55°C for 5 min, and a 23% loss of activity was found at 45°C for 5 min, as shown in Fig. 4-B. These results indicated that the extracellular lytic enzyme was relatively sensitive to heat treatment, and had a narrow stable pH range.

FIG. 2. Electrophoresis of Extracellular Lytic Enzyme by Isoelectric Focusing.

The enzyme solution filtrated by Sephadex G-75 was applied on a 110 ml-LKB column, and electrophoresis was carried out with a potential gradient of 400 to 900 V for 48 hr, maintaining the column temperature at 4°C by circulating chilled water. Lytic enzyme activity was assayed as described in MATERIALS AND METHODS. ●, lytic enzyme activity; —, pH.

FIG. 3. Optimum pH and pH Stability of Extracellular Lytic Enzyme.

A, optimum pH; B, pH stability.

FIG. 4. Optimum Temperature and Heat Stability of Extracellular Lytic Enzyme.

A, optimum temperature; B, heat stability.

TABLE I. EFFECTS OF DIVALENT CATIONS AND EDTA-Na ON THE EXTRACELLULAR LYtic ENZYME ACTIVITY

Reagent	Concentration (M)	Relative activity (%)
None	—	100
MgSO ₄	1×10^{-3}	100
CaCl ₂	1×10^{-3}	100
BaCl ₂	1×10^{-3}	98
SrCl ₂	1×10^{-3}	98
CdCl ₂	1×10^{-3}	100
MnCl ₂	1×10^{-3}	100
FeSO ₄	1×10^{-3}	97
CoCl ₂	1×10^{-3}	100
NiSO ₄	1×10^{-3}	100
CuSO ₄	1×10^{-3}	26
EDTA-Na	1×10^{-3}	100

3) *Effects of divalent cations and EDTA-Na on extracellular lytic enzyme.* The activity of the extracellular lytic enzyme was not inhibited or accelerated by the divalent cations tested except Cu^{2+} that was known to be an enzyme inhibitor, as shown in Table I. And EDTA-Na had no effect on the activity. Therefore, the extracellular lytic enzyme did not require divalent cations for its activity. It was also

found that the enzyme was unstable in water and more than 0.10 M sodium ions was essential for the expression of its activity. But, its activity was not accelerated by addition of the high concentration of NaCl that was used to induce the autolysis.

4) *Substrate specificity of extracellular lytic enzyme.* Various materials were prepared or purchased for determining the substrate specificity of the extracellular lytic enzyme. The extracellular lytic enzyme could digest cell walls prepared from various kinds of clostridia. Whole cells of *C. saccharoperbutylacetonicum* were hardly lysed by the extracellular lytic enzyme, as shown in Table II. But in hypertonic conditions, the specific autolysis (NaCl-induced autolysis) of whole cells was accelerated by the addition of the extracellular lytic enzyme, as shown in Fig. 5. On the other hand, the glycan moiety prepared from peptidoglycan by using phage HM7 lysin (*N*-acetylmuramyl-L-alanine amidase)^{12,13} and ethylene glycol chitin (Seikagaku Kogyo) were not hydrolysed.

TABLE II. SUBSTRATE SPECIFICITY OF EXTRACELLULAR LYTIC ENZYME

Substrate	
<i>C. saccharoperbutylacetonicum</i>	
Whole cell	—
Heated cell	—
Cell wall	+
Peptidoglycan	+
Glycan moiety ^a	—
<i>C. sporogenes</i>	
Whole cell	—
Cell wall	+
<i>C. kairantoi</i>	
Whole cell	—
Cell wall	+
Ethylene glycol chitin	—

+, digested; —, not digested.

^a The glycan moiety was prepared from phage HM7 lysin-digested peptidoglycan.

FIG. 5. Acceleration of NaCl-Induced Autolysis of Washed Whole Cells by Addition of Extracellular Lytic Enzyme.

Harvested cells were washed with chilled 10 mM phosphate buffer (pH 6.0), and incubated at 30°C in the presence or absence of 0.3 M NaCl and purified extracellular lytic enzyme (2 units). ○, washed whole cells alone; ●, +extracellular lytic enzyme; △, +0.3 M NaCl; ▲, +extracellular lytic enzyme and 0.3 M NaCl.

Bond specificity of extracellular lytic enzyme

The extracellular lytic enzyme digested cell wall and peptidoglycan completely. As shown in Fig. 6, when peptidoglycan was used as substrate for the extracellular lytic enzyme, free reducing groups were released into the reaction mixture, proportional to the decrease of turbidity. However, free amino groups did not increase. This indicated that the extracellular lytic enzyme was either *N*-acetylmuramidase or *N*-acetylglucosaminidase, which hydrolysed the glycan chain and released free reducing groups.

The digested peptidoglycan was reduced with NaBH_4 and hydrolysed with 6 N HCl at 100°C for 12 hr to determine the bond specificity of the extracellular lytic enzyme.¹⁴ The hydrolysate was analyzed with an automatic amino acid analyzer (Type LC-R-2, Japan Electro Optics Lab.). As shown in Table III, the NaBH_4 -treated sample showed a decrease of 88% of *N*-acetylmuramic acid that became *N*-acetylmuramitol, compared with the non-

treated control. On the other hand, amino acids and *N*-acetylglucosamine were not reduced. This indicated that the extracellular lytic enzyme was an *N*-acetylmuramidase.

Comparison of cellular autolysin and extracellular lytic enzyme

Cellular autolysin prepared from crude cell wall as described in MATERIALS AND METHODS also had a single enzymatic activity. The cellular autolysin had a similar optimum pH, optimum temperature and stabilities to the extracellular lytic enzyme. This autolysin digested peptidoglycan and released only reducing groups into the reaction mixture, but free amino groups did not increase. The reducing sugar was determined to be *N*-acetylmuramic acid by the method describe above. Therefore this autolysin was also an *N*-acetylmuramidase, the same as the extracellular lytic enzyme, as shown in Table IV. Molecular weights of both enzymes determined by gel filtration on Sephadex G-100 (1.5×100 cm) were 44,000, as shown in Fig. 7.

FIG. 6. Release of Reducing Groups during Digestion of Peptidoglycan with Extracellular Lytic Enzyme.

Two mg of the peptidoglycan was suspended in 0.5 ml of 100 mM acetate buffer (pH 4.5) containing 10 units of lytic enzyme, and incubation was performed at 30°C. The reaction was stopped by heating for measurement of turbidity, amino groups and reducing groups. ○, turbidity; ■, reducing groups; ▲, amino groups.

TABLE III. ANALYSIS OF PEPTIDOGLYCAN DIGESTED WITH EXTRACELLULAR LYTIC ENZYME

Compound	No reduction (μmol/mg of peptidoglycan)	Reduction with NaBH ₄ (μmol/mg of peptidoglycan)
Muramic acid	0.363	0.038
Glucosamine	0.490	0.413
Glutamic acid	0.534	0.515
Alanine	1.110	1.071
Diaminopimelic acid	0.518	0.502

FIG. 7. Estimation of Molecular Weight of Extracellular Lytic Enzyme and Cellular Autolysin by Sephadex G-100 Gel Filtration.

One mg of each standard protein was applied on a column (1.5×100 cm) of Sephadex G-100 and the effluent volume was determined. A, chymotrypsinogen A; B, extracellular lytic enzyme; C, cellular autolysin; D, ovalbumine; E, γ -globulin.

TABLE IV. PROPERTIES OF EXTRACELLULAR LYTIC ENZYME AND CELLULAR AUTOLYSIN

Enzymes	Optimum pH	Optimum temperature	pH stability	Heat stability	Molecular weight	Bond specificity
Extracellular lytic enzyme	4.5	35°C	4~8	40°C, 5 min	44,000	<i>N</i> -Acetylmuramidase
Cellular autolysin	4.5	35°C	4~8	50°C, 5 min	44,000	<i>N</i> -Acetylmuramidase

DISCUSSION

Bacterial autolysin activity is mainly classified into four types according to the bond specificities, i.e., *N*-acetylmuramidase, *N*-acetylglucosaminidase, *N*-acetylmuramyl-L-alanine amidase and peptidase. Many bacteria have *N*-acetylmuramyl-L-alanine amidase as the major autolysin. But some bacteria have only one autolysin, *N*-acetylmuramidase. Especially, *N*-acetylmuramidase of *Streptococcus faecalis* has been studied in detail, though the autolysin is firmly bound to the cell wall and hardly released into the supernatant fluid of the culture. In some bacteria, though *N*-acetylmuramidases are found in the supernatant fluid of the culture, the relationships with their autolysins have not been investigated. But some workers say that these bacterial lysozymes would be autolysins.¹⁵⁾ Extracellular lytic enzymes produced by *Staphylococcus aureus* are thought to be autolysins, because their enzymatic properties are very similar to cell-bound autolysins.⁴⁾

Extracellular lytic enzyme of *C. saccharoperbutylacetonicum*, *N*-acetylmuramidase, had very similar enzymatic properties to cell wall-bound autolysin. It would originate from cell wall-bound autolysin. And its accumulation in the supernatant fluid of the culture correlated with cell growth or cell division. Therefore, it is thought that the autolysin may be released into the supernatant fluid after acting on cell growth or cell division.

The autolysin of *C. saccharoperbutylacetonicum* could hardly lyse intact whole cells, but it accelerated the autolysis under hypertonic conditions. And autolysis of cells in the culture broth under hypertonic conditions was faster than that of washed cells in buffered hypertonic solution. These phenomena indicated that the autolysin could hardly attack the cell wall from the outside of intact cells, but could lyse the cell wall which is dissociated from the cell membrane by plasmolysis in hypertonic conditions.¹¹⁾ It may also be said that the intact and membrane-bound cell wall is not

attacked from the outside by the autolysin.

On the other hand, autolysin had another substrate specificity. Namely, it could not hydrolyse the glycan moiety prepared from phage HM7 lysin (*N*-acetylmuramyl-L-alanine amidase)-digested peptidoglycan. The autolysin may require the peptide moiety to hydrolyse the glycan chain, as reported for phage T4 lysin.¹⁶⁾ In our following work, we should make clarify the property that decides the high substrate specificity of the autolysin.

Acknowledgments. The authors thank to Professor Hongo (The Kumamoto Institute of Technology) for his helpful advice and Mr. Y. Kondo for his excellent assistance in this experiment.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

- 1) G. D. Shockman, J. S. Thompson and M. J. Conver, *Biochemistry*, **6**, 1054 (1967).
- 2) J. Coyette and J. M. Ghuyssen, *Biochemistry*, **9**, 2952 (1970).
- 3) K. J. Tipper, *J. Bacteriol.*, **97**, 837 (1969).
- 4) J. Coyette and G. D. Shockman, *J. Bacteriol.*, **114**, 34 (1973).
- 5) C. W. Forsberg and H. J. Rogers, *Nature* (London), **229**, 272 (1971).
- 6) I. Kusaka, *Protein, Nucleic Acid and Enzyme*, **20**, 518 (1972).
- 7) S. Ogata, *Nippon Nôgeikagaku Kaishi*, **50**, R69-R77 (1976).
- 8) M. Hongo and A. Murata, *Agric. Biol. Chem.*, **29**, 1135 (1965).
- 9) S. Ogata and M. Hongo, *J. Gen. Microbiol.*, **81**, 315 (1974).
- 10) S. Ogata, K. H. Choi and M. Hongo, *Agric. Biol. Chem.*, **39**, 1274 (1975).
- 11) S. Ogata, K. H. Choi and M. Hongo, *Microbiol. Immunol.*, **24**, 393 (1980).
- 12) S. Ogata, Y. Tahara and M. Hongo, *Agric. Biol. Chem.*, **39**, 1274 (1974).
- 13) S. Ogata, Y. Tahara and M. Hongo, *J. Gen. Appl. Microbiol.*, **21**, 65 (1975).
- 14) A. Tugita and M. Inoue, *J. Biol. Chem.*, **243**, 391 (1968).
- 15) H. J. Rogers, *Microbial Polysaccharides and Polysaccharases*, ed. by R. C. W. Berkeley, G. W. Gooday and D. C. Ellwood, Academic Press Inc., New York, N. Y., 1979, pp. 237~268.
- 16) D. Mirelman, C. Kleppe and H. B. Jensen, *Eur. J. Biochem.*, **55**, 369 (1975).