Lytic Enzymes for Gram-negative Bacteria Produced by *Bacillus subtilis* YT-25

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Cell walls of gram-negative bacteria are complex both in morphology and in chemical composition.^{1~3)} Many reports have been published on bacteriolytic enzymes but the vegetative cells of gram-negative bacteria are normally resistant to these enzymes.^{4,5)} Egg white lysozyme failed to lyse gram-negative bacteria without artificial detergents¹⁾ or chelating reagents such as ethylenediamine tetraacetic acid.^{6,7)}

Among soil bacteria which produce lytic enzymes toward *Pseudomonas aeruginosa* ATCC 10145, we obtained a strain YT-25 which strongly lysed the vegetative cells of gram-negative bacteria. This strain was identified as *Bacillus subtilis* YT-25. The lysis was caused by the combined action of lytic enzymes and a co-operating factor. This paper reports some properties of endo-N-acetylmuramidase produced by YT-25, and the effect of the co-operating factor.

Bacillus subtilis YT-25 was aerobically grown on nutrient broth containing 0.5% glucose at 30°C for 30 hr. The culture filtrate of YT-25 rapidly lysed the vegetative cells of *P. aeruginosa*, *Escherichia coli*, *Salmonella typhimurium* and *Klebsiella pneumoniae*.

Since the lytic activity of the culture filtrate decreased with dialysis, some dialyzable co-operating factor was supposed to play an important role in the lysis of cells. The co-operating factor was purified by Amberlite IRC-50, silicic acid and Sephadex G-25 column chromatographies to almost single band in electrophoresis with polyacrylamide gel. This substance, designated as NLF (Native Cell-Lytic Factor), is heat-stable,

dialyzable, soluble in methanol and has a peptide-like structure. Details of NLF will be reported in another paper.

Enzymes which lysed *P. aeruginosa* by the co-operation with NLF were then purified. The culture filtrate was applied to an Amberlite IRC-50 column and eluted by 0.5 M sodium phosphate dibasic. After re-chromatographed on the Amberlite IRC-50 column, *P. aeruginosa*-lytic fractions were applied to a column of SP-Sephadex C-25 equilibrated with 0.1 M acetate buffer pH 5.0. Linear gradient elution was performed with increasing concentration of sodium chloride from zero to 0.5 M in the same buffer. As shown in Fig. 1, *P. aeruginosa*-lytic frac-

FIG. 1. Purification of Lytic Enzymes on a SP-Sephadex C-25 Column.

P. aeruginosa-lytic activity was estimated by measuring the decrease in optical density at 660 nm after incubating 2 ml of cell suspension with 0.25 ml of each fraction (diluted two hundred times) and 0.25 ml of NLF solution (0.5 mg/ml) at 37°C for 10 min. The cell suspension was prepared as follows: *P. aeruginosa* was cultured in nutrient broth at 37°C for 12 hr on a shaker. The cells were harvested, washed with 0.01 M Veronal-HCl buffer, pH 7.0 and then suspended in the same buffer to give an optical density of 0.8 at 660 m μ .

Peptidoglycan-lytic activity was estimated by measuring the increase in reducing sugar after incubating 0.1 ml of each fraction with 0.3 ml of 0.1% peptidoglycan at 37°C for 5 hr.

○—○, O.D. at 280 nm; ●—●, *P. aeruginosa*-lytic activity; □—□, Peptidoglycan-lytic activity; ---, NaCl concentration.

tion was separated into Fractions A₁, A₂ and B. Among them, only the Fraction B hydrolyzed the purified peptidoglycan obtained

from *P. aeruginosa* according to Weidel *et al.*³⁾ with the release of reducing groups. The Fraction B was further purified by column chromatographies with SP-Sephadex C-25 and Sephadex G-75. The purified enzyme appeared to be homogeneous in electrophoresis with polyacrylamide gel. This enzyme was designated as B-enzyme. The purified B-enzyme failed to lyse the vegetative cells of *P. aeruginosa*, but the mixture of B-enzyme and NLF caused the lysis of *P. aeruginosa* cells as shown in Fig. 2.

FIG. 2. Effect of NLF for Lysis of *P. aeruginosa* by B-Enzyme.

Condition for cell lysis was same as shown in Fig. 1. ●—●, B-enzyme, final concentration was 10 μ g/ml; ▲—▲, NLF, final concentration was 50 μ g/ml; ○—○, B-enzyme, final concentration was 10 μ g/ml and NLF, final concentration was 50 μ g/ml; ■—■, Culture filtrate, 0.5 ml of the culture filtrate was added to 2 ml of the cell suspension; □—□, Control.

The B-enzyme cleaved the purified peptidoglycan obtained from *P. aeruginosa* with the release of reducing groups but no amino terminal groups. Maximal activity on the cleavage of peptidoglycan occurred at pH 4.5. The enzyme was stable at pH 3.5~6.0, and the lytic activity was inhibited by various heavy metal ions but not by monoiodoacetate, *p*-chloromercuribenzoate and ethylenediamine tetraacetate.

Lysozyme-like enzymes produced by *Bacillus* sp. were reported as 'bacterial lysozyme'.^{8~12)} Enzymatic properties of 'bacterial lysozyme' were investigated in detail by Satomura *et al.* and Okada *et al.*^{12,13)} The B-enzyme is similar to 'bacterial lysozyme' in bacteriolytic spectrum, *i.e.* the B-enzyme lysed *Micrococcus lysodeikticus* and *B. megaterium* but did not

Staphylococcus aureus. However, enzymatic properties of the B-enzyme were clearly different from that of the reported 'bacterial lysozyme' in pH stability¹²⁾ and susceptibilities to monoiodoacetate and *p*-chloromercuribenzoate.¹⁴⁾ These facts suggested that the B-enzyme is a new type of 'bacterial lysozyme'.

The mechanism of action of the B-enzyme on the peptidoglycan of *P. aeruginosa* was examined. Reduction of the liberated glycosidic groups with sodium borohydride according to Tipper *et al.*¹⁵⁾ resulted in a decrease of the amount of muramic acid and only muramitol was identified as the reduction product by an automatic amino acid analyzer. These results suggested that the B-enzyme hydrolyzed the N-acetylmuramyl-N-acetylglucosamine linkages in peptidoglycan, and caused to lyse the cells of *P. aeruginosa*.

The maximum turbidity reduction of *P. aeruginosa* suspension by the B-enzyme in the presence of NLF was inferior to that of the culture filtrate as shown in Fig. 2. This fact indicates that the other lytic substances which possess the significant action for gram-negative bacteria still remain in the culture filtrate. In fact, purified Fractions A₁ and A₂ were inactive on the peptidoglycan, but remarkably lysed the vegetative cells of *P. aeruginosa* in the presence of NLF. Properties of other lytic substances are now under investigation and will be reported elsewhere.

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