Action of Lysozyme and Some Microbial Enzymes on Acid-Resistant Organisms

(Studies on Bacteriolytic Substances produced by Microorganisms, Part 5)

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Cells of an avian tubercle bacillus, when developed into weak acid-fastness were found to be easily susceptible to egg-white lysozyme but not sensitive to bacterial lysozyme. The cells of normal acid-fastness, on the contrary, were resistant to the egg-lysozyme and lysed only by successive application of egg-lysozyme and fungal enzymes. Heat treatment at about 60° changed the acid-fast cells to cells quite sensitive to lysozyme.

INTRODUCTION

The action of lysozyme has long been employed as a useful means in cytochemical studies of many species of bacteria^{1),2)}, but so far few reports have been published on the study of tubercle bacilli and lysozyme³⁾. Lysis of bacterial cell by lysozyme is attributed to the dissolution of a polysaccharide which plays a part in the maintenance of the cell-wall structure⁴⁾. Accordingly, it is doubtful whether complete lysis can be expected if this lysozyme sensitive polysaccharide in the cell wall were combined with other cell wall constituents into such a complex in which the susceptible polysaccharide could be protected from the direct attack of lysozyme. Concerning this, the effects of physical or chemical pretreatment of the cells and that of a combined-attack by lysozyme and other enzymes on the lysis were thought to be of special interest^{5),6),7)}. Recently, it has

been observed that a lysozyme sensitive polysaccharide really exists in several species of lysozyme-resistant bacteria^{8),9)}. Also, the application of enzymes other than lysozyme such as ribonuclease, lipase and phospholipase etc. has become available from studies concerning the composition of cell-wall⁹⁾. In addition to the enzymatic action, the lysozyme has been known from its basicity to cause an osmotical change to bacterial cells by a certain basic antibacterial polypeptide such as tyrocidin¹⁰⁾. Formerly, a killing action of lysozyme for tubercle bacilli had been proved^{3),11)}, but the existence of lysozyme-substrate polysaccharide has not yet been recognized hitherto. On the other hand, the physiological significance of fat and wax contained in acid-fast cells has attracted attention of some workers and, a lysis of the cells by lipolytic enzyme such as wax-decomposing enzyme "cerase" has been reported¹²⁾.

However, a further detailed investigation would be necessary for the structural features

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11) Q. N. Myrviik, R. S. Weiser and M. C. Kelly, *Am. Rev. Tuberc.*, **68**, 584 (1955).

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of carbohydrate components of the cell-wall of acid-fast bacilli similiary as in the case of many other bacterial cells. In this paper, some aspects of the cell-wall structure of an avian tubercle bacillus are demonstrated, using lysozyme and some microbial enzymes as analytical tools.

EXPERIMENTAL

Methods and Materials.

Cultures and preparation of suspension of washed bacteria; Some relations of acid-fastness with cultural conditions in the same strain of an avian tubercle bacillus are presented in Table I. Bacterial cells of both the acid-fast type and non acid-fast type were employed in the following experiments. Cellswere harvested and resuspended in a phosphate buffer solution after washing and grinding three times with distilled water. The turbidity of the cell-suspension was adjusted to 10% transmission in measuring optical density at 600 m μ with an electric spectrophotometer.

TABLE I. ACID-FASTNESS AND CULTURAL CONDITIONS

Medium	Glucose 2%	Tween 80 0.5%
	NH ₄ Cl 0.1%	
	Polypepton 0.5%	Salts*
Temperature	37°	37°
Growth-Time	12 hr	48 hr
Shape	rod	spherical
Acid-fastness	-, $\pm$	+

Shaking method was adopted

Salts*: Na₂HPO₄ 0.65, KH₂PO₄ 0.1, MgSO₄·7H₂O 0.02% (pH 7.0)

Enzyme preparations: Egg-white lysozyme was crystallized by the method of Alderton¹³⁾. Bacterial lysozyme was obtained from the culture of *Bacillus subtilis* and purified by the method using ion-exchange resin, previously reported¹⁴⁾. Enzymes of *Sclerotinia* fungus were extracted from the bran culture with a ten-fold volume of water and the extract was concentrated five times its volume and dialysed. Although the bran culture of *Sclerotinia* fungus possessed a lipase the aqueous extract had no lipase activity¹⁵⁾. The crystalline trypsin used here, was of an Armers product and commercial pancreatin was used as the extract with water.

13) G. Alderton and H. L. Fevold, *J. Biol. Chem.*, **164**, 1 (1946).

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Measurement of lysis: A suspension of washed bacterial substrate was mixed with the various enzyme solutions and, after adjusting the pH with M/10 phosphate buffer, incubated at 37°. Lysis was followed by the turbidimetric method with the use of a spectrophotometer, or by weighing the dry weight of cells remaining after centrifuging.

Analytical methods: The increase in viscosity was measured by Ostwald's viscosimeter and the degree of liberation of nucleic acid (PNA) was determined by the 'Orcin-HCl' reaction. The release and dissolution of cell-wall mucopolysaccharide was estimated by measuring the turbidity which appeared by the addition of acidified horse serum¹⁶⁾.

Action of Lysozyme

From the experiments shown in Fig. 1. it is distinct that the non acid-fast cells are easily lysed by the action of lysozyme alone whereas, the acid-fast cells are not lysed even by the combined action of trypsin and

FIG. 1. Susceptibility of Acid-Fast and Non Acid-Fast Cells to Lysozyme (Incubation at 37°)

—○— L, Lysozyme 25 µg/ml
 - - × - - L+T, Lysozyme 25 µg/ml Trypsin 250 µg/ml
 - - △ - - T, Trypsin 250 µg/ml
 —●— Cont. No addition

TABLE II. ADSORPTION OF LYSOZYME TO CELLS

Cell Types	Lysozyme Activity		Rate of Adsorption %
	Initial no addition of cells	Remaining after addition of cells	
Non Acid-Fast	400	4	99
Acid-Fast	400	300	25
(Lysozyme Non-Sensitive Bacteria <i>E. coli</i>)	(400)	(356)	(11)

Treatment
 Cell suspension (10% transmission of optical density at 600 m μ) 10 ml.
 Buffer solution 9 ml.
 Lysozyme 1 ml.
 } at 37° for 5 min.

16) K. Meyer, *Physiol. Revs.*, **27**, 335 (1947).

lysozyme. In Table II, the difference in the degree of lysozyme adsorption of both types of cells is given. Thus, it can be supposed that the polysaccharide-substrate of lysozyme surely exists in cells of the non acid-fast type, as observed in the other lysozyme-sensitive bacterial cells which readily adsorb lysozyme.

Joint Action of Lysozyme with other Enzymes

As seen in Table III, egg-white lysozyme dissolves only 22~23% of the weight of the acid-fast cells even in a prolonged period of 18~20 hr. at pH 6.0, while the enzyme solution of *Sclerotinia* digests cells up to about 50% in the same conditions. Bacterial lysozyme possesses only scanty activity. However, a certain combined-use of these agents gave a particularly accelerated lysis action. When the *Sclerotinia* extract was combined with egg-white lysozyme in the above condition, it digested the cells up to ca. 70%, which is equal to the sum of lysis power of both agents. When however, the cells were pretreated with lysozyme for about 2 hrs. at pH 6.0 and centrifuged, resuspended in a buffer of pH 4.0 and then successively treated with *Sclerotinia* extract, lysis extended up to more than 80% within a period of 4 hours. From the results obtained, it was supposed that an inhibitory effect on the action of lysozyme had been caused by an acidic substance contained in the fungus extract, which can combine

with lysozyme. In the same procedure, pancreatic pretreatment plus *Sclerotinia* extract attained a ca. 60% lysis, and bacterial lysozyme plus *Sclerotinia* extract ca. 55% lysis. In these experiments *Sclerotinia* extract lost its activity upon heating at 100° for 10 minutes.

It was also demonstrated that the *Sclerotinia* enzyme extract attacked the polysaccharide fraction of the acid-fast cells to produce a small quantity of reducing sugar, decreasing the viscosity of the solution. As main sugars of the polysaccharide constituents glucosamine, glucose, mannose and rhamnose were detected by a paper-chromatographic test after acid hydrolysis.

Susceptibility of Heated Cells to Lysozyme

As described above, the sensitivity of acid-fast cells to lysozyme was very weak in an intact state, but sensitivity was found to be increased by heating the cells at about 60° for 10 min. in distilled water, thus bringing about the Nakamura effect¹⁷⁾. As shown in Fig. 2, the heated cell-suspension rather increases its turbidity in the earlier stage of incubation with lysozyme, as in the case with heat-killed cells of other lysozyme sensitive or non sensitive bacteria²³⁾, but prompt lysis occurs upon the addition of NaOH changing the pH from 6 to 12, concurrently increasing viscosity. However, the Nakamura effect did not appear with the cells heated at temperatures lower than 50° or higher than 70°. Also, the effect was not observed with the cells previously treated with a dilute HCl or NaOH solution. The degree of clearing, viscosity and the amount of nucleic acid released from the cells, increased in parallel

TABLE III. SINGLE OR JOINT ACTION OF LYSOZYME AND OTHER ENZYMES ON THE CELLS OF ACID-FAST TYPE

Cell Suspension +	18~20 hr (pH 6.0) 37°	Rate of Lysis
S	(pH 4.0)	46.0%
+ L	18~20 hr (pH 6.0) 37°	22.5
+ Panc.	"	19.5
+ Try.	"	2.0
+ Bact. L	"	9.5
+ (S+L)	"	70.6
+ L	2 hr (pH 6.0) S 4 hr (pH 4.0)	83.2
+ Panc.	" S "	59.7
+ Bact. L	" S "	54.8

(in Final concentration)

: *Sclerotinia* fungus-enzyme, 10 times water extracts of bran culture
 L: Egg-white lysozyme, 30 µg/ml
 Panc.: Pancreatin, 1% aqueous extract
 Try: Trypsin, 100 µg/ml
 Bact. L: Bacterial lysozyme ca. 50 µg/ml

Rate of Lysis = Cell-remains $\left(\frac{\text{Control} - \text{Test}}{\text{control}} \right) \times 100$

FIG. 2. Increase in Susceptibility of Cells to Lysozyme by Heat-Treatment, bringing about Nakamura Effect

17) O. Nakamura *Z. Immunitätsforsch.*, **38**, 425 (1923).

with the concentration of lysozyme added to the cell suspension, as presented in Fig. 3. As shown in Fig. 4, from the activity of clearing and releasing-PNA, the optimum pH of lysozyme action is about 6.0, while from the activity of increase in viscosity, the optimum pH seems to be in the vicinity of 7.0. In an incubation with lysozyme at 40° for 2 hr., the supernatant of the cell-suspension was found to give rise to turbidity

(incubation with cells for 2 hr)

FIG. 3. Relation of Lysozyme-Concentration with Nakamura Effect on Cells heated at 60° for 10 min.

FIG. 4. Effect of pH on Sensitivity of Heated Cells (60°, 10 min.) to Lysozyme, bringing Nakamura Effect

(during incubation at a conc. of 125 µg lysozyme/ml, for 2 hr)

FIG. 5. Release and Dissolution of Cell-Wall Mucopolysaccharide during Incubation with Lysozyme

upon the addition of an acidified horse serum. Hence, a certain kind of mucopolysaccharide was considered to be dissolved-out from the cell-walls during incubation. In Fig. 5 the turbidity appearing in such treatment with various concentrations of lysozyme is shown. It is noticeable, that in the curve there seems to exist optimal concentration to give maximal turbidity and an excess of lysozyme decrease, in proportion to remarkably the concentration, the turbidity. This phenomenon is most probably due to hydrolysis of mucopolysaccharide by an excess of lysozyme into the non-precipitable fraction with acid serum. With intact cells, the dissociation of mucopolysaccharide was found to be far weaker than that of heated cells. Also, the mucopolysaccharide revealed to contain a large amount of glucosamine as its main sugar component, by a paperchromatography following acid hydrolysis.

DISCUSSION

The significance of effects of heat-treatment on susceptibility of acid-fast cells to lysozyme was recognized in the heat-treatment at about 60°. W. B. Redmond and his coworker¹⁸⁾ have reported on the autolysis of a tubercle bacillus grown in a nitrogen-deficient medium where autolytic enzymes were either unaffected or

18) W. B. Redmond and B. U. Bowman, *J. Bacteriol.*, **69**, 293 (1955).

little affected by heating at 56° for 30 min. In some gram-negative bacteria, it has been demonstrated that disorganization of the cell-wall brought about by physico-chemical^{5), 6), 7)} or a certain antibiotic treatment, results in the acceptance of lysozyme action¹⁹⁾. However, the polysaccharide substrate of lysozyme in the acid-fast cells present in the complex with other cellular constituents may not be considered to accept the action of lysozyme directly, after undergoing any change in the permeability-barriers by heat-treatment, as has been mentioned previously by M. E. Becker and S. E. Hartsell⁶⁾. It seems reasonable to assume that some autolytic enzymes which can split the complex structure of cell-surface substances would be activated by the heat-treatment, whereby a structural-site for the acceptance of lysozyme appears.

The difference between the action of egg-white and bacterial lysozyme on acid-fast cells indicates that the former enzyme²⁰⁾ has a comparatively broad specificity as a polyglucosaminidase for the bacterial substrates while the latter a somewhat limited specificity. From this fact, the use of the bacterial lysozyme may offer a new tool for a more fundamental study of the chemical constitution of bacterial cell-wall susceptible to lysozyme.

From the experiments described above, the main factors causing the synergistic effect within lysozyme and other enzymes are not considered to involve any of the following enzymes: protease, nuclease, lipase, phospholipase and esterases. But the factors are thought to be some sort of polysaccharidase commonly found in fungi just like those of *Sclerotinia* fungus,

such as chitinase, cellulase, lichenase, polyglucosanase²¹⁾ and protopectinase etc. Recently, A. Sohler and his coworkers²²⁾ reported that the different sensitivities of *Actinomycetales* to lysozyme, could be explained by the difference in carbohydrate composition of the cell-wall, and the lysozyme-resistant strain contained only a small amount of hexosamine contrary to its richness in arabinose and galactose. Lysozyme and other lytic factors should operate alternatively to dissolve the complex structure of the cell-wall, stepwisely removing the obstructive sites.

SUMMARY

(1) Non acid-fast cells of an avian tubercle bacillus were easily lysed by lysozyme, whereas acid-fast cells could be digested by a successive attack of lysozyme followed by *Sclerotinia*-enzyme. Bacterial lysozyme, trypsin and pancreatin, however, had no synergistic action with lysozyme. The synergistic factor contained in the *Sclerotinia*-enzyme extract was assumed to be a certain polysaccharidase.

2) The release of a mucopolysaccharide from the cell-wall during incubation with lysozyme increased by previously heating the cells at about 60° for 10 min. The effect of heat-treatment upon the sensitivity of cells to lysozyme, which was accompanied by a remarkable Nakamura effect, was concluded to be due to the activation of some autolytic enzymes.

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21) R. A. Aitken, B. P. Eddy, M. Ingram and C. Weurman, *Biochem. J.*, **64**, 63 (1957).

22) A. Sohler, A. H. Romano and W. J. Nickerson, *J. Bacteriol.*, **75**, 283 (1958).