Acetylation of Lysozyme

Part II. Mechanism of Lysis by Lysozyme

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Acetylated lysozyme retained full activity toward glycol chitin. However, lytic activity toward cells of Micrococcus lysodeikticus in neutral media decreased in proportion to the number of amino groups acetylated and its optimum pH shifted to the acid side with increase in the number of acetyl groups introduced. In the acid region below the optimum pH, the lytic activity was the same as that of untreated lysozyme. Very similar results were obtained using cell wall suspension.

The first step in lysis of bacterial cells by lysozyme seems to be an interaction between the positive charges of lysozyme and the negative charges on the surface of the bacterial cells. Acetylation of the free amino groups of lysozyme results in a diminution in the numbers of positive charges, causing a decrease in the interaction at neutral pH values. The second step of lysis is hydrolysis of the \(\beta-1,4\)-glucosaminide linkage in the polysaccharide of the cell wall. The last step is dissolution of the damaged cell wall.

Since it was reported that acetylation of the amino groups of the lysozyme molecule caused a remarkable decrease in the lytic activity, some of the amino groups have been considered to be essential for the activity of lysozyme. Many studies were carried out on the chemical modification of the amino groups in order to elucidate their role. In the previous paper, it was reported that acetylated lysozyme (Ac-lysozyme) retained full activity for the hydrolysis of glycol chitin and that the amino groups do not play any role in the enzymatic hydrolysis of the \(\beta-1,4\)-N-acetyl glucosaminide linkage of polysaccharide.

The present paper deals mainly with the lytic activity of Ac-lysozyme and the mechanism for the lysis of bacterial cells by lysozyme.

EXPERIMENTAL

Materials. Egg white lysozyme and glycol chitin were prepared by the methods described in the previous paper. The dried cells of Micrococcus lysodeikticus (lot. ATCC 4698) was donated by Eizai Co., Ltd.

Preparation of Ac-lysozyme. Acetylation, fractionation and determination of the free amino groups of Ac-lysozyme were carried out according to the methods described in the previous paper. In the present experiments, fractionation was done by CM-cellulose column chromatography with gradient elution, and each fraction was named by attaching the prefix G, such as G-1 and G-2, as did in the previous paper.

Oxidation of Lysozyme by N-bromosuccinimide. Oxidation of lysozyme by N-bromosuccinimide (NBS) was carried out according to the method described in a previous paper. Four moles of tryptophan residue per mole of lysozyme were oxidized in the NBS-oxidized lysozyme used. This preparation showed no \(\beta-1,4\)-N-acetyl glucosaminidase active.

Lytic Activity. Cells of *M. lysodeikticus* were suspended in 1/15 M buffer solutions at various pH values, and the turbidity of the suspension was adjusted to OD=0.6 at 530 mμ. To 5 ml of the suspension were added 2 ml of the buffer solution and 1 ml of 0.001% lysozyme solution. After incubation at 37°C for 10 min, the turbidity of the reaction mixture was promptly measured by a Hitachi Spectrophotometer ETO-9 at 530 mμ. Lytic activity was represented by the extent of the decrease in optical density of the suspension or as a relative value by taking the activity of untreated lysozyme as 100.

Lysis by Changing the pH of the Cell Suspension after Incubation with Lysozyme at pH 4.5. A cell suspension in 1/15 M acetate buffer, pH 4.5, was adjusted to OD=1.0 at 530 mμ. Ten ml of the suspension was mixed with 2 ml of 0.001% lysozyme solution. The reaction was allowed to proceed in four different ways, as follows:

a) Immediately after mixing, a 2-ml aliquot of the reaction mixture was added to 2 ml of 0.2 M phosphate buffer, pH 7.5, and was incubated for varied times.

b) Immediately after mixing, a 2-ml aliquot of the reaction mixture was added to 2 ml of 0.2 M acetate buffer, pH 4.5, and was incubated for varied times.

c) After incubation at 37°C for 20 or 40 min, a 2-ml aliquot of the reaction mixture was added to 2 ml of 0.2 M phosphate buffer, pH 4.5, and incubated for varied times.

d) After incubation at 37°C for 20 min, a 2-ml aliquot of the reaction mixture was added to 2 ml of 0.2 M acetate buffer, pH 4.5, and incubated for varied times.

In all cases, the decrease in the turbidity after the varied times was determined by measuring the optical density at 530 mμ.

Preparation of Cell Walls. Cell walls were prepared by the methods described by Dauson6) and Salton7) with minor modifications. Fifty ml of cell suspension (250 mg of dry cell) of *M. lysodeikticus* in deionized water was shaken with 50 ml of glass beads (0.11–0.12 mm in diameter) in a Braun Disintegrator Model MSK for 4 min at 4000 rpm. The glass beads were removed by filtration with a coarsely fused glass filter. After removing the intact cell by centrifugation at 3000 rpm, the cell walls were collected by centrifugation at 10,000 rpm. The cell wall preparation was digested with 2 mg of trypsin in 20 ml of 0.1 M phosphate buffer at pH 8.0 at 30°C for 20 hrs to remove protein moieties, then cell wall was collected by centrifugation at 10,000 rpm and washed thoroughly with deionized water prior to storage as a dense suspension.

Activity of Lysozyme toward Cell Walls. The turbidity of cell wall suspensions in 1/15 M buffer solution at various pH values was adjusted to OD=0.5 at 530 mμ. Five ml of each suspension was incubated with 1 ml of lysozyme solution (40 μg/ml) at 37°C. The dissolution of the cell walls was followed by measuring the turbidity at 530 mμ. Control tests were made using 1 ml of deionized water in place of lysozyme solution.

RESULTS

Lytic Activity of Ac-lysozyme. The lytic activity of Ac-lysozyme at neutral pH values decreased in proportion to the extent of the acetylation as shown in Table I. The optimum pH for the lytic activity of Ac-lysozyme toward cell suspensions shifted remarkably to the acid side with increase in the extent of acetylation as shown in Fig. 1. As described in the previous paper,4) the isoelectric point of Ac-lysozyme was lowered in proportion to the extent of acetylation. The magnitude of the shift of the optimum pH for the lytic activity of Ac-lysozyme was in good agreement with the difference in pH units between the isoelectric point of Ac-lysozyme and that of untreated lysozyme. In the region of pH 6–7, a marked decrease in the lytic activity of Ac-lysozyme was observed, while no difference in lytic activity between Ac-

<table>
<thead>
<tr>
<th>Table I. Lytic Activity of Ac-Lysosome</th>
<th>Relative activity* (%)</th>
<th>No. of free amino groups/mole of enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native lysozyme</td>
<td>100</td>
<td>7</td>
</tr>
<tr>
<td>G-1</td>
<td>3.6</td>
<td>0.3</td>
</tr>
<tr>
<td>G-2</td>
<td>9.3</td>
<td>1.1</td>
</tr>
<tr>
<td>G-3</td>
<td>12.5</td>
<td>1.8</td>
</tr>
<tr>
<td>G-4</td>
<td>24.0</td>
<td>2.4</td>
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* Activity was assayed at pH 6.0 and 37°C.

lysozyme and untreated lysozyme was found in the acid region below the optimum pH for the activity of each fraction (see Fig. 1).

**Fig. 1.** pH-Dependence of Lytic Activity of Ac-Lysozyme toward Bacterial Cells.


**Lysis by Changing the pH of the Cell Suspension after Incubation at pH 4.5.**

After the incubation of a bacterial cell suspension with lysozyme at pH 4.5 for 20~40 minutes, an abrupt reduction in turbidity was observed on adjusting the pH of the suspension to 7.0 by the addition of 0.2 M phosphate buffer, pH 7.5, as shown in Fig. 2. In the case of Ac-lysozyme, the turbidity of reaction mixture thereafter remained constant, independent of the incubation time. The abrupt dissolution of the suspension was observed with both Ac-lysozyme and untreated lysozyme. However, even if incubation with Ac-lysozyme at pH 4.5 was prolonged, no decrease at all was observed in the turbidity of the cell suspension.

NBS-oxidized lysozyme did not exhibit any lytic activity under these conditions.

**Fig. 2.** Lysis with Change in pH of Medium after Incubation at pH 4.5. Untreated Lysozyme:

- (1), incubated at pH 7.0; (2), changed pH to 7.0 after incubation for 20 min at pH 4.5; (3), changed pH to 7.0 after incubation for 40 min at pH 4.5; (6), incubated at pH 4.5. Ac-lysozyme (G-1); (4), changed pH to 7.0 after incubation for 40 min at pH 4.5; (5), changed pH to 7.0 after incubation for 20 min at pH 4.3; (7), incubated at pH 4.3; (8), incubated at pH 8.0.

**Dissolution of the Cell Walls by Lysozyme.**

The pH-dependences of the activity of Ac-lysozyme and untreated lysozyme toward cell walls were similar to those observed with intact cell walls.

**Fig. 3.** pH-Dependence of Cell Wall Lysis by Ac-Lysozyme.

One ml of enzyme solution (40 μg/ml) was added to 5 ml of cell wall suspension (OD_{530nm}=0.5).

cells as shown in Fig. 3. The rate of decrease in the turbidity of cell wall suspension at pH 4.5 was considerably higher than that observed with the lysis of intact cells as shown in Fig. 4. With prolonged incubation at pH 4.5, the cell wall suspension became almost completely dissolved.

**DISCUSSION**

Free amino groups, one N-terminal α amino group and six lysine amino groups, have been considered to be essential for lysozyme activity. The acetylation of lysozyme, however, did not cause any decrease in the activity toward glycol chitin as described in the previous paper. However, the lytic activity of Ac-lysozyme toward intact cells and cell walls at neutral pH values decreased in proportion to the number of acetyl groups introduced onto the amino groups. This finding accords with the results reported by Wauters and Leonis. A more precise study on the pH-dependence of the lytic activity of Ac-lysozyme preparations which had been acetylated to various extents revealed that the lytic activity of Ac-lysozyme in the acid region below the optimum pH was identical with that of untreated lysozyme. It had once been believed that the lysis of bacterial cells by lysozyme consisted of two successive processes; first, the hydrolysis of the mucopolysaccharide of the cell walls, and second, the decomposition and dissolution of cell structure by autolysis with proteases contained in the cell protoplasm.

Gerschwind and Li reported that guanidylation of the ε-amino groups of the lysine residues of lysozyme with O-isothiourea did not cause any change in lytic activity. They concluded that the positive charge of the lysine residues was essential for the enzymatic activity of lysozyme, because the lysine residues retain their positive charge after this modification.

In the present experiments, the following factors, therefore, were taken into account to explain the loss in lytic activity of Ac-lysozyme toward bacterial cell wall:

1. loss of affinity of Ac-lysozyme for bacterial cell walls,
2. loss of enzymatic activity toward the mucopolysaccharide of cell walls and
3. inhibition of cell proteases by Ac-lysozyme.

Evidence was provided in the previous paper that all of the amino groups of lysozyme were not indispensable for β-1,4-N-acetyl glucosaminidase activity. This was confirmed by the fact that the cell wall fraction of *M. lysodeikticus* could be almost completely dissolved by Ac-lysozyme with prolonged incubation, even at pH 4.5 (Fig. 4). Hydrolysis of mucopolysaccharide occurs at the β-1,4-linkage between the C1 of N-acetyl muramic acid and the C4 of N-acetyl glucosamine. It is well known that lysozyme hydrolyzes the β-1,4-N-acetyl glucosaminidase linkage. Therefore, β-1,4-N-acetyl muramidase activity can be represented by the β-1,4-N-acetyl glucosaminidase activity which was measured using glycol chitin as a substrate. Similar conclusion was obtained by Sharon with comparing the binding of oligosaccharide derived from cell wall to that of oligosaccharide of N-acetyl glucosamine. These facts indicate

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9) N. Sharon, personal communication.
that retention of full activity for hydrolyzing the β-1,4-linkage is not sufficient for the appearance of rapid lysis at neutral pH values by lysozyme.

The contribution of proteases in the cell to lysis was ruled out because the cell wall fraction, from which protein components other than constituents of the cell walls were removed by trypic digestion, was easily dissolved by lysozyme. In addition, cells of _M. lysodeikticus_ used were previously irradiated by ultraviolet light and dried by acetone. This might cause the denaturation of protein in the cell. The lysis suddenly accelerated on the adjustment of the pH of the medium to 7 after the incubation of the cell suspension with either Ac-lysozyme or untreated lysozyme at pH 4.5 (see Fig. 2). This indicates that the dissolution process in lysis may be driven by hydroxyl ions in the alkaline medium.

The remaining factor explaining the great decrease in lytic activity of Ac-lysozyme at neutral pH values may be connected with the positive charge of the enzyme. In fact, the loss in lytic activity at neutral pH values was exactly proportional to the acetyl groups introduced and the lowering of the isoelectric point of Ac-lysozyme. The surface of the bacterial cells is considered to be negatively charged. This would facilitate the contact of the lysozyme molecule with cells by electrostatic interaction. It is, therefore, obvious that the interaction force is lost proportionally with the number of amino groups acetylated.

It is concluded that the lysis of bacterial cells by lysozyme consists of three different steps; the first step is the binding of lysozyme to the surface of the cells by electrostatic interaction due to their charges, the second is the true enzymatic action of lysozyme, splitting β-1,4-linkages, and the third is a physicochemical dissolution of the enzymatically damaged cell walls followed by release of the protoplasmic material from bacterial cells. The decrease in the lytic activity of Ac-lysozyme toward cell materials at neutral pH values was caused by the loss of positive charge on acetylation; that is, the first step was lacking in the action of Ac-lysozyme, though the second step was maintained unchanged.