Acetylation of Lysozyme

Part I. Preparation, Fractionation and Properties of Acetylated Lysozyme

By Nobuyuki Yamasaki, Katsuya Hayashi and Masaru Funatsu

Laboratory of Biochemistry, Faculty of Agriculture, Kyushu University, Fukuoka
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In order to elucidate the significance of amino groups as active groups of lysozyme, egg white lysozyme was acetylated with acetic anhydride. The acetylated lysozyme was fractionated by CM-cellulose column chromatography. All acetylated lysozymes fractionated exhibited 120% relative activity toward glycol chitin at pH 5.6, while the optimum pH shifted to the alkaline side by 0.5 pH units. Trinitrophenylated lysozyme, which was derived from acetylated lysozyme with 1.1 free amino groups per mole and which contained no amino group itself, retained 75% relative activity toward glycol chitin. These results indicate that the amino groups in the lysozyme molecule are not involved in the active site.

Acetylated lysozyme behaved the same as the untreated enzyme except the susceptibility to proteolytic digestion.

Lysozyme contains seven amino groups, one of which is an N-terminal α-amino group and the other six ε-amino groups of lysine residues. The positions of the lysine residues in the primary structure of the lysozyme molecule have been elucidated. However, the significance of the amino groups in the action of lysozyme has not been established.

Fraenkel-Conrat reported that acetylation of the amino groups with acetic anhydride caused the inactivation of lysozyme. Leonis also reported that the lytic activity of lysozyme decreased in proportion to the number of amino groups acetylated or dinitrophenylated. In those experiments, conformational changes in the modified lysozyme were not investigated and the activity was assayed on the basis of the lysis of Micrococcus lysodeikticus.

It is interesting whether the decrease in the lytic activity is accompanied by a decrease in the activity for hydrolyzing β-1,4-N-acetyl glucosaminide linkage. As a part of the studies on the role of amino groups in lysozyme action, the present paper deals mainly with the acetylation of amino groups, fractionation of the acetylated derivatives by column chromatography and their properties.

EXPERIMENTAL

Materials. A lysozyme preparation isolated from hen’s egg white by the direct crystallization method was recrystallized five times and lyophilized. The preparation showed a single band on CM-cellulose column chromatography. Glycol chitin was synthesi-
Acetylation of lysozyme. To a mixture of 100 ml of 5% aqueous lysozyme solution and 100 ml of saturated sodium acetate solution was added dropwise 5 g of acetic anhydride over a period of 1 hr at 0°C with vigorous stirring. The reaction mixture was dialyzed successively against running water overnight and against deionized water for 2 days using Visking tube #18/32. The mixture was then centrifuged and the supernatant was lyophilized.

Fractionation of acetylated lysozyme. Four and a half grams of acetylated lysozyme were dissolved in 100 ml of 0.05 M phosphate buffer, pH 5.5, and the solution was applied to the top of a column (4.5 x 60 cm) of CM-cellulose equilibrated with the same buffer solution.

A) Stepwise elution: Ac-lysozyme adsorbed on the column was eluted stepwise with 0.05 M phosphate buffer with increase in pH from 6.0 to 8.0 and successively eluted with 0.1 M and 0.2 M phosphate buffers, pH 8.0.

B) Gradient elution: The column was developed by an eluent system with a linear gradient in pH, which originally consisted of 4.5 liters of 0.05 M phosphate buffer, pH 5.5, in the mixing chamber and 4.5 liters of 0.05 M phosphate buffer, pH 8.0, in the reservoir. After gradient elution, the column was eluted stepwise with increase in pH (above pH 8.0) and ionic strength. In both cases, (A) and (B), each fraction was further purified by rechromatography.

Estimation of number of free amino groups. The number of the free amino groups in Ac-lysozyme was determined spectrophotometrically using a specific reagent for amino groups, trinitrobenzenesulfonate. To 1 ml of 0.05% aqueous lysozyme solution were added 3 ml of 7 M urea in 0.1 M borate buffer, pH 8.0, and 1 ml of 0.3% trinitrobenzenesulfonate solution. After the reaction mixture was kept at 37°C for 3 hr in the dark, the optical density at 346 mμ was measured. In the blank, 1 ml of water was used in place of the enzyme solution. The concentration of Ac-lysozyme was determined by measuring the optical density at 280 mμ of a suitably diluted solution. The number of free amino groups was calculated from the ratio of the optical density at 346 mμ to that at 280 mμ using the molar extinction coefficients, 39,000 for lysozyme at 280 mμ and 12,500 for the trinitrophenylated lysine residue at 346 mμ.

Assay of lysozyme. The assay of lysozyme activity was carried out by viscosimetry using glycol chitin as a substrate. The activity was represented in terms of relative values according to the method reported in the previous paper.

Measurement of optical rotatory dispersion. Twenty mg of Ac-lysozyme fractionated by gradient elution was dissolved in 1 ml of dilute acetic acid, pH 3.5, and 1 ml of 0.5 M acetic acid buffer, pH 4.5. Rudolph and Sons Model 200S Photoelectric Spectropolarimeter was used for the measurement of the optical rotatory dispersion. The values aₒ and bₒ were calculated by the Moffitt-Yang equation:

\[
[M]_k = \frac{3}{n^2 + 2} \cdot \frac{M}{100} \left( a \lambda_0^2 \frac{\lambda^2 - \lambda_0^2}{\lambda^2 - 2\lambda_0^2} + \frac{b \lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \right),
\]

where n is the refractive index of the solvent, M the average molecular weight per residue (assumed to be 100) and λₒ the absorption wavelength associated with the rotation (assumed to be 212 mμ).

Measurement of Difference Spectrum

1) Difference spectrum of the enzyme-substrate complex: The enzyme solution was prepared by the same method as that used in the measurement of optical rotatory dispersion. Two ml of 0.2% enzyme solution was added to 2 ml of 0.2% glycol chitin solution and the mixture was placed in the sample compartment in a 1-cm quartz cell. The reference cell was filled with 0.1% enzyme solution. The redshift difference spectrum was recorded over the wavelength range of 250~330 mμ. The intensity of the difference spectrum at 293 mμ represented a relative value, assuming the intensity of the enzyme-substrate complex of untreated lysozyme to be 100.

2) Difference spectrum between Ac-lysozyme and untreated lysozyme: 0.1% of Ac-lysozyme solution was placed in the reference compartment.
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and 0.1% of the untreated lysozyme solution in the sample compartment. The intensity of the spectrum at 293 m\(\mu\) gave the molar difference extinction coefficient, \(\Delta\epsilon\).

**Electrophoresis of Ac-lysozyme.** Fractions, G-1 and G-4 (G: Fraction from CM-cellulose column chromatography with gradient elution), were dialyzed against deionized water for 2 days and against buffer solutions at various pH values9) for 3 days at 4°C, changing the outer solution several times. The electrophoresis was carried out at 4°C using the Hitachi HDT-1 Tiselius type apparatus.

**Heat stability of Ac-lysozyme.** An aliquot of the Ac-lysozyme dissolved in 0.1 M phosphate buffer, pH 5.6, was placed in a test tube with a stopper. After keeping the solution in a boiling water bath for different times, it was cooled by tap water to room temperature. The concentrations of Ac-lysozyme solution incubated were 0.025% and 0.1%. When 0.1% Ac-lysozyme solution was used, the solution was diluted 4 times before assay of the activity.

The viscosimetric method was adopted for the assay. The activity was determined as a relative value, in per cent, taking the activity of unheated Ac-lysozyme to be 100. The same experiment was carried out using untreated lysozyme as a control.

**Effect of urea solution on the activity of Ac-lysozyme.** The activity of Ac-lysozyme was measured viscosimetrically in the presence of various amounts of urea. Ten ml of 0.08% glycol chitin dissolved in various concentrations of urea in 0.1 M phosphate buffer, pH 5.6, was allowed to react with 0.2 ml of 0.025% Ac-lysozyme dissolved in deionized water. The activity was determined as a relative value, in per cent, taking the activity in the absence of urea to be 100.

**Proteolytic Digestion of Ac-Lysozyme**

1) With trypsin, chymotrypsin and nagarse:
1.0 mg of protease was added to 100 mg of Ac-lysozyme dissolved in 10 ml of 0.1 M potassium chloride solution. Hydrolysis was followed at pH 7.9 and 37°C by titrating with 0.5 N sodium hydroxide using \(\text{pH-Statt (TTT-1c, Radiometer)}\). Fifty mg of Ac-lysozyme and 0.5 mg of protease were used in the case of Nagarse digestion.

2) With pepsin: 0.5 mg of pepsin was added to 10 ml of 0.5% Ac-lysozyme dissolved in 0.1 M potassium chloride solution. Hydrolysis was followed at pH 2.5 and 40°C by titrating with 0.25 M hydrochloric acid. As a control, untreated lysozyme was digested under the same conditions.

**RESULTS**

**Fractionation of Acetylated Lysozyme**

A stepwise elution profile of Ac-lysozyme from a column of CM-cellulose is shown in Fig. 1. As can be seen in the figure, the acetylated lysozyme preparation was separated into eight fractions (S1 ~ S8). The three fractions which were eluted with buffer solution at pH 6.0 were subjected to rechromatography. As shown in Fig. 2-a, fraction S-1 (S: fraction from CM-cellulose column chromatography with stepwise elution) was separated into two fractions, S-1-1 and S-1-2, with 0.05 M phosphate buffer, pH 5.5. Each fraction obtained by rechromatography was further chromatographed under the same conditions in order to confirm the homogeneity. The results showed that the fraction obtained by rechromatography can be regarded to be homogeneous. One example is shown in Fig. 2-b. S-2 and S-3 were also each separated into two components by rechromatography at pH 6.0.

With gradient elution, the acetylated lysozyme can be fractionated into eight fractions.

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FIG. 2. (a): Rechromatograms of S-1, S-2 and S-3.

S-1 was eluted with 0.05 M phosphate buffer, pH 5.5; S-2 and S-3 were eluted with 0.05 M phosphate buffer, pH 6.0. (b): Rechromatogram of S-1-1. Eluent was 0.05 M phosphate buffer, pH 5.5.

FIG. 3. CM-cellulose Column Chromatogram of Ac-Lysozyme with Gradient Elution.

The gradient in pH was made by 4.5 liters of 0.05 M phosphate buffer, pH 5.5, and 4.5 liters of 0.05 M phosphate buffer pH 8.0. Four grams of Ac-lysozyme were loaded on the column (4.5 x 52 cm).
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Each fraction exhibited a single band on rechromatography. A chromatogram with gradient elution is shown in Fig. 3.

Activity of Ac-Lysozyme

All Ac-lysozyme fractions obtained chromatographically retained the original activity toward glycol chitin independently of the extent of acetylation. Fig. 4 shows the pH-dependence curve of Ac-lysozyme, which is very similar to that of untreated lysozyme. The pH-dependence curve for the activity of all Ac-lysozyme, however, shifted as a whole to alkaline side by 0.5 pH units. Trinitrophenylated preparation derived from G-2 exhibited 75% activity of the untreated enzyme toward glycol chitin.

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FIG. 4. pH-Dependence Curves for the Activity of Ac- and Untreated Lysozymes.

Assay was carried out by viscosimetry at 30°C. Buffer systems were 0.1 M acetate buffer over the range of pH 3.5 to 5.0 and 0.1 M phosphate buffer from pH 5.6 to 8.0.

- −: untreated
- ○−: Ac-lysozyme (G-1~G-6)

Determination of the Number of Free Amino Groups per Molecule

The number of free amino groups in each fraction obtained by gradient elution chromatography is shown in Table I. In fraction G-1 only 0.3~0.5 amino groups per mole could be trinitrophenylated, while in untreated lysozyme 6.7 groups could be trinitrophenylated. This indicates that in fraction G-1 more than 6 groups out of the 7 amino groups in each molecule were acetylated.

Electrophoresis

The fractions, G-1 and G-4, each showed a single boundary in free moving electrophoresis (Fig. 5). As shown in Fig. 6, the isoelectric points of G-1 and G-4 were calculated to be 7.8 and 9.5, respectively.

FIG. 5. Electrophoretic Pattern of Ac-Lysozyme, Measured in Phosphate Buffer at pH 6.1, 4°C.

Difference Spectrum of Enzyme-substrate Complex

The difference spectrum, in the ultraviolet region, of the Ac-lysozyme-substrate complex was of the same shape as that of the complex with untreated lysozyme. The intensity of

FIG. 6. Plot of Electrophoretic Mobilities of Ac-Lysozyme against pH.

The difference spectrum at 293 mp of the Ac-lysozyme-substrate complex was divided by that of the complex with untreated lysozyme. The quotient in percent represents the relative amount of the complex formed. The values are shown in Table II.

TABLE II. THE AMOUNT ENZYME-SUBSTRATE COMPLEX OF AC-LYSOZYME AT pH 4.5

<table>
<thead>
<tr>
<th>Components</th>
<th>E-S Complex (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native lysozyme</td>
<td>100</td>
</tr>
<tr>
<td>S-1-1</td>
<td>100</td>
</tr>
<tr>
<td>S-1-2</td>
<td>98</td>
</tr>
<tr>
<td>S-2-1</td>
<td>90</td>
</tr>
<tr>
<td>S-2-2</td>
<td>90</td>
</tr>
<tr>
<td>S-3-1</td>
<td>90</td>
</tr>
<tr>
<td>S-3-2</td>
<td>97</td>
</tr>
</tbody>
</table>

FIG. 7. Difference Spectrum between Ac-Lysozyme (S-2-2) and Untreated Lysozyme at pH 4.5.

Untreated lysozyme solution was placed in the sample compartment, and Ac-lysozyme solution in the reference compartment.

TABLE III. MOLAR EXTINCTION COEFFICIENT OF DIFFERENCE SPECTRUM OF AC-LYSOZYME AT 293 mp AND 260 mp

<table>
<thead>
<tr>
<th>Components</th>
<th>$\epsilon_{293\text{mp}}$</th>
<th>$\epsilon_{260\text{mp}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-1-1</td>
<td>537</td>
<td>1890</td>
</tr>
<tr>
<td>S-1-2</td>
<td>552</td>
<td>1620</td>
</tr>
<tr>
<td>S-2-1</td>
<td>520</td>
<td>1180</td>
</tr>
<tr>
<td>S-2-2</td>
<td>520</td>
<td>1170</td>
</tr>
<tr>
<td>S-3-1</td>
<td>460</td>
<td>1100</td>
</tr>
<tr>
<td>S-3-2</td>
<td>400</td>
<td>730</td>
</tr>
</tbody>
</table>

Optical Rotatory Dispersion

The values of the Moffitt Yang's parameters, $a_0$ and $b_0$, remained unchanged at any degree of acetylation (Table IV). This fact indicates that no conformational change in the main chain of the lysozyme molecule occurred in the process of acetylation.
TABLE IV. MOFFITT YANG'S PARAMETERS OF AC-LYSOZYME AT pH 4.5

<table>
<thead>
<tr>
<th>Components</th>
<th>$a_0$</th>
<th>$b_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative lysozyme</td>
<td>-260</td>
<td>-145</td>
</tr>
<tr>
<td>G-1</td>
<td>-243</td>
<td>-120</td>
</tr>
<tr>
<td>G-2</td>
<td>-265</td>
<td>-140</td>
</tr>
<tr>
<td>G-3</td>
<td>-264</td>
<td>-145</td>
</tr>
<tr>
<td>G-4</td>
<td>-266</td>
<td>-135</td>
</tr>
</tbody>
</table>

FIG. 8. Heat Stability of Lysozymes

Assay was carried out at pH 5.6 and 30°C by viscometry, after incubation in boiling water bath. Concentrations of lysozymes were 0.025% (a) and 0.1% (b).
- ■: untreated lysozyme
- ○: Ac-lysozyme

FIG. 9. Effect of Urea on the Activity of Ac-Lysozyme.

Assay was carried out in the presence of urea at pH 5.6 and 30°C.
- ■: untreated lysozyme
- ○: Ac-lysozyme (G-1)

Heat Stability of Ac-Lysozyme

The heat stability of Ac-lysozyme at pH 5.6 in a boiling water bath was exactly the same as that of untreated lysozyme as can be seen in Fig. 8. The heat stability was not affected by the concentration of the enzyme incubated.

Effect of Urea on Activity of Ac-Lysozyme

The activity of Ac-lysozyme decreased in...
urea solution in proportion to the concentration of urea. This agreed exactly with the results obtained with untreated lysozyme, as shown in Fig. 9.

**Proteolytic Digestion of Ac-Lysozyme**

As shown in Figs. 10 and 11, Ac-lysozyme was hydrolyzed to a great extent by Nagarse, trypsin or chymotrypsin, while untreated lysozyme was resistant to the attack of these enzymes. Nearly all of the bonds to be cleaved were hydrolyzed by trypsin within 1 hr under experimental conditions. Ac-lysozyme was more easily hydrolyzed by pepsin at pH 2.5 than untreated lysozyme.

![Fig. 11. Peptic Digestion of Ac-Lysozyme. Digestion was carried out at pH 2.5 and 40 °C.](image)

**DISCUSSION**

Since Fraenkel-Conrat\(^2\) reported that acetylation of the free amino groups of the lysozyme molecule caused the loss of the lysis activity toward *M. lysodeikticus*, the amino groups have been considered to be essential for the action of lysozyme. Wauters and Leonis\(^3\) attempted to elucidate the role of the amino groups on the enzymatic action of lysozyme by means of chemical modification. However, in their experiments, the activity of the lysozyme derivatives was assayed only by lysis of bacterial cells. When cell suspensions are used as a substrate, several factors seem to be involved in the assay of the activity, besides the actual enzymatic action of lysozyme.

Therefore, a much simpler substrate should be adopted for assay purposes when investigating the role of the amino groups of lysozyme. A water-soluble substrate, glycol chitin, which consists only of N-acetyl glucosamine units, seems to satisfy this requirement.

Acetylation of the amino groups did not affect the activity of lysozyme toward glycol chitin. For instance, fractions S-1 and G-1 exhibited activity as high as 120\% at pH 5.6 in contradiction to the findings of Fraenkel-Conrat, and Wauters and Leonis. The optimum pH for the activity of Ac-lysozyme shifted to the alkaline side by 0.5 pH units, but the shape of the pH-dependence curve of the activity of Ac-lysozyme was the same as that of the untreated enzyme. The shift in the optimum pH, however, did not depend upon the isoelectric points of the preparations; the magnitude of the shift of the optimum pH cannot be related to the extent of acetylation of the amino groups. The number of acetylated amino groups of each fraction was calculated by estimation of the number of unmodified amino groups by trinitrophenylation in the presence of 3 M urea.

Digestion was carried out at pH 2.5 and 40 °C.

\(-\bigcirc-\): Ac-lysozyme (G-1)

\(-\bullet-\): untreated lysozyme

The elution order of Ac-lysozyme from the column of CM-cellulose was in accord with the extent of acetylation as expected. When fraction G-2 which contained 1.1 free amino groups per molecule was completely trinitrophenylated, it still retained 75\% relative activity toward glycol chitin. This indicates that amino groups do not participate in the enzymatic activity of lysozyme.

From carboxymethylation of lysine residues under various conditions, Kravchenko\(^11\) has concluded that three lysine residues are essential for the maintenance of the unique conformation of lysozyme. In the case of acetylation, however, no evidence for any conformational change in the main peptide chain could be obtained by measurement of

the optical rotatory dispersion, heat stability or stability in a concentrated urea solution. Kravchenko’s conclusion, therefore, seems to be reached on inadequate evidence, from the introduction of charged group, the carboxymethyl group, onto the amino groups.

Although it is concluded that no conformational change in the main peptide chain occurred, there was some evidence for changes in the side chain arrangements of the lysozyme molecule due to the acetylation of the amino groups. A difference spectrum with $\Delta \epsilon_{292} = 500$ was observed when the ultraviolet spectrum of acetylated lysozyme was compared with that of the untreated. This difference spectrum may indicate some change in the side chain arrangements near the modified groups, since the loss of positive charge of the amino groups leads to the appearance of a red-shift in the spectrum, and the complete exposure of one buried tryptophan leads to a blue-shift difference spectrum with $\Delta \epsilon_{292} = 1,500$. Furthermore, Ac-lysozyme was more easily hydrolyzed by various proteases regardless of their substrate specificity. This suggests that the increase in digestibility of Ac-lysozyme might be caused by a change in the side chain arrangement, even though great digestibility could also be attributable to the loss of positive charge of the lysozyme molecule.

Recently, Johnson and Philips\textsuperscript{12) 13) } presented the main chain configuration of crystallized lysozyme and the apparent binding site of the inhibitor, N,N'-diacetylchitobiose. The lysine residue at position 97 is involved in the apparent binding site for the inhibitor. This suggests that the lysine residue at position 97 may play a part in the catalytic hydrolysis of glycol chitin. The complete analysis of the three dimensional structure of the crystallized lysozyme-substrate complex, however, demonstrated that none of the lysine residues were involved in either the binding site or the catalytic site of lysozyme.\textsuperscript{13) }

It is thus concluded that the amino groups are neither the catalytic nor binding group of lysozyme and that the side chain arrangement near the amino groups modified is not responsible for the activity of lysozyme.

The inconsistency of the results on the activity of Ac-lysozyme reported in several papers including the present paper will be discussed in the following paper.