Chemical Studies on the Enzymatic Specificity of Goose Egg White Lysozyme*

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SUMMARY

In comparison with hen egg white lysozyme, goose lysozyme is known to be aberrant in both its structure and its enzymatic behavior in the presence of polymers of N-acetylglucosamine or cell suspensions of Micrococcus luteus. Our chemical studies show, however, that like the hen enzyme, goose lysozyme has muramidase activity. At several pH levels ranging from 3.5 to 7.1 the goose enzyme liberated the reducing ends of N-acetylmuramic acid residues in purified preparations of Escherichia coli and M. luteus peptidoglycans. In contrast to what is known about hen lysozyme, however, our results suggest that the goose enzyme has a distinct preference for N-acetylmuramic acid residues which are substituted with a peptide moiety. This difference in specificity towards the peptide portion of the peptidoglycan may be related to the biological function of lysozyme.

Extensive studies have been carried out on the structure and function of hen egg white lysozyme. Its complete amino acid sequence (1, 2) and three-dimensional conformation (3) have been determined. Chemical studies have established that it has muramidase activity (N-acetylmuramidase glycanohydrolase, EC 3.2.1.17). It carries out the hydrolysis of the β(1,4) glycosidic linkage between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan of bacterial cell walls (4). In addition the hen enzyme can bind polymers of N-acetylmuramic acid. It is inhibited by the monomer and trimer of N-acetylglucosamine (10) and to hydrolyze the tetramer and pentamer of this sugar (11).

Goose egg white lysozyme stands in marked contrast to the other avian lysozymes that have been examined. Partial amino acid sequence analysis (12), immunological cross-reactivity (13), and amino acid composition data (14, 15) indicate that this enzyme is radically different in structure. Even more striking is the fact that this enzyme appears to be insensitive to N-acetylglucosamine inhibitors (16, 17), incapable of hydrolyzing higher molecular weight polymers of this sugar (11) and unable to release the saccharides NAG-NAM² or NAG-NAM-NAG-NAM from the cell wall of Micrococcus luteus (18) although the hen enzyme accomplishes this readily. Because of its aberrant catalytic behavior we decided to examine the enzymatic specificity of goose lysozyme by chemical methods. Our results suggest that both hen and goose lysosymes have muramidase activity but that they differ in specificity towards the peptide portion of the peptidoglycan of the bacterial cell wall.

MATERIALS AND METHODS

Enzymes—Hen lysozyme (3 times crystallized) was obtained from Pentex Biochemicals. Goose lysozyme, isolated from Embden goose egg white, was a generous gift from Dr. R. Cannfield (Columbia University School of Medicine) and was purified by a procedure described previously (14).

Preparation of Peptidoglycan—From about 4 g of Escherichia coli M&74T2 grown in M9 medium (+ 4 μg per ml of thymidine), the envelope fraction was isolated as previously described (19). The envelope fraction was suspended in 80 ml of 0.01 N sodium phosphate buffer, pH 7.1, and 80 mg of trypsin were added in order to remove a specific protein which is covalently attached to the peptidoglycan (20). The mixture was incubated for 2 hours at 37°, and then centrifuged for 30 min at 100,000 × g. From the pellet, peptidoglycan was isolated using 4% SDS as described by Braun and Sieglin (20). The final peptidoglycan fraction was resuspended in 5 ml of H2O, and its chemical composition after acid hydrolysis in 6 N HCl at 106° is shown in Table I.

The abbreviations used are: SDS, sodium dodecyl sulfate; NAG, N-acetylglucosamine; NAM, N-acetylmuramic acid; NAG-NAM, disaccharide of NAG and NAM linked by means of a β(1,4) glycosidic bond. The free reducing end is on the residue furthest to the right; NAG-NAM-NAG-NAM, tetrasaccharide of alternating NAG and NAM linked together in the same manner as the disaccharide.

1 The abbreviations used are: SDS, sodium dodecyl sulfate; NAG, N-acetylglucosamine; NAM, N-acetylmuramic acid; NAG-NAM, disaccharide of NAG and NAM linked by means of a β(1,4) glycosidic bond. The free reducing end is on the residue furthest to the right; NAG-NAM-NAG-NAM, tetrasaccharide of alternating NAG and NAM linked together in the same manner as the disaccharide.

2 NAG, N-acetylglucosamine; NAM, N-acetylmuramic acid; NAG-NAM, disaccharide of NAG and NAM linked by means of a β(1,4) glycosidic bond.
Peptidoglycan from commercially available *M. luteus* cells (Worthington) was prepared in a similar manner. Lyophilized cells (200 mg) were suspended in 100 ml of 4% SDS and heated in a boiling water bath for 10 min. After centrifugation the pellet was subjected to the same treatment two more times. The pellet from the last SDS treatment was washed with H_2O and then incubated with protease (*Streptomyces griseus*, Sigma, 5130) for 2 hours in 0.2 m sodium phosphate buffer, pH 7.1, at a cell to enzyme weight ratio of 5:1. After centrifugation the pellet was washed with water, treated with trypsin under the same conditions as it was treated with protease, and subjected to the SDS treatment once again. The protease and trypsin digestions were repeated and followed by two more SDS heat treatments. After centrifugation the pellet was washed and resuspended in H_2O. The chemical composition of the recovered material after acid hydrolysis is shown in Table I.

### Digestion of Peptidoglycan by Lysozimes—The reaction mixtures in the experiments with *E. coli* consisted of 0.1 ml of buffer solution and either 0.25 or 0.20 ml of purified peptidoglycan suspension (1 mg per ml). To the mixture, either goose lysozyme (20 μg), hen lysozyme (2 μg), or no enzyme (control) was added. The experiments with purified *M. luteus* peptidoglycan (4.4 mg per ml) were carried out in a similar manner using 0.2 ml of peptidoglycan suspension and 20 μg of goose or hen lysozyme.

The experiments were carried out at three different pH levels with the use of 0.2 m sodium acetate buffer (pH 3.5 and pH 5.3) and 0.2 m sodium phosphate buffer (pH 7.1). After incubation for 20 hours at 38°, 0.2 ml of 1 m NaHCO_3 and 5 mg of sodium borohydride were added and the mixtures were kept overnight at room temperature (21). The next day concentrated HCl (0.5 ml) was added and the mixtures were sealed, without prior evacuation, and incubated for 24 hours at 106° to hydrolyze the peptidoglycan. In the experiments with *E. coli* peptidoglycan the whole sample was subjected to amino acid analysis, whereas only one-fifth of the sample was analyzed in the experiments with *M. luteus*.

The amounts of muramic acid, muramic acid, glucosamine, glucosaminol, and amino acids present in the acid hydrolysate were determined with a Beckman amino acid analyzer (120B) at 45° as described previously (21). The color values for glucosamine (10.6) and muramic acid (8.8) were calculated by subjecting known quantities of the sugars to hydrolysis in 6 x HCl in sealed tubes at 106° for 24 hours. The color value for muramic degradation was calculated in the identical manner using samples of muramic acid which had been subjected first to reduction by the sodium borohydride method described above.

In these experiments a small correction was needed in order to accurately calculate the amount of muramic acid present in the enzymatically treated preparations since hydrolysis of the substrate or protein alone gave rise to a small amount of material with chromatographic properties identical with muramic acid. This material was especially noticeable in the preparations of *M. luteus* peptidoglycan.

### RESULTS

**Enzymatic Activity of Goose Lysozyme on *E. coli* Peptidoglycan**—If goose lysozyme has muramidase activity, the chemical composition of the *E. coli* peptidoglycan will be altered after incubation with the enzyme followed by sodium borohydride reduction and acid hydrolysis. The enzymatic hydrolysis of the β(1,4) linkage between N-acetylmuramic acid and N-acetylglycosamine will liberate a reducing group on N-acetylmuramic acid which is susceptible to sodium borohydride reduction. Analysis of the reduced substrate after acid hydrolysis will reveal the presence of muramic acid in addition to the normally occurring muramic acid and glucosamine. The total amount of muramic acid plus muramic acid should equal the amount of muramic acid present before the sample was treated with enzyme. If goose lysozyme has N-acetylglycosaminidase activity on the peptidoglycan, glucosaminol, not muramic acid, should appear in our chemical analysis. Table II contains the data on the digestion of *E. coli* peptidoglycan by hen and goose lysozymes at pH 3.5, 5.3, and 7.1. At all three pH levels incubation of the substrate with goose lysozyme resulted in the appearance of muramic acid after reduction and acid hydrolysis. The extent of conversion of muramic acid to muramic acid was

<table>
<thead>
<tr>
<th>Table I</th>
<th>Chemical composition of principal components of purified peptidoglycan from <em>E. coli</em> and <em>M. luteus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Molar ratio</td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td>Observed</td>
<td>Expected</td>
</tr>
<tr>
<td>Muramic acid</td>
<td>0.9</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.4</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.9</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>1.0</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* The molar ratio is calculated assuming diaminopimelic acid = 1.0.
* The molar ratio is calculated assuming glutamic acid = 1.0.
* Based upon the data presented in Reference 15.
* Based upon the data presented in Reference 4. Lysine was not determined.

<table>
<thead>
<tr>
<th>Table II</th>
<th>Chemical composition of <em>E. coli</em> peptidoglycan after digestion with hen or goose lysozyme</th>
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</thead>
<tbody>
<tr>
<td>Muramic acid</td>
<td>Muramic acid</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>μmole</td>
</tr>
<tr>
<td>I. pH 3.5</td>
<td>0.05</td>
</tr>
<tr>
<td>a. Control</td>
<td>0.09</td>
</tr>
<tr>
<td>b. Goose</td>
<td>0.09</td>
</tr>
<tr>
<td>c. Hen</td>
<td>0.06</td>
</tr>
<tr>
<td>II. pH 5.3</td>
<td>0.12</td>
</tr>
<tr>
<td>a. Control</td>
<td>0.15</td>
</tr>
<tr>
<td>b. Goose</td>
<td>0.14</td>
</tr>
<tr>
<td>c. Hen</td>
<td>0.04</td>
</tr>
<tr>
<td>III. pH 7.1</td>
<td>0.05</td>
</tr>
<tr>
<td>a. Control</td>
<td>0.06</td>
</tr>
<tr>
<td>b. Goose</td>
<td>0.06</td>
</tr>
<tr>
<td>c. Hen</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* The amount of peptidoglycan solution subjected to lysozyme digestion was 0.2 ml.
* The amount of peptidoglycan solution subjected to lysozyme digestion was 0.25 ml.
The amount of muramic acid produced equaled the amount of muramic acid that was lost since the ratio of muramic acid plus muramicitol to glucosamine equals the ratio of muramic acid to glucosamine in the control sample. Goose lysozyme has no significant N-acetylglucosaminidase activity under these conditions since glucosaminidase was not observed.

At all three pH levels hen as well as goose lysozyme exhibited muramidase activity although the extent of conversion of muramic acid to muramicitol did not exceed 40%. More goose lysozyme than hen lysozyme, however, was used in the incubations and, in addition, no conclusions about the relative activities should be made from these exhaustive digest experiments.

Enzymatic Activity of Goose Lysozyme on M. luteus Peptidoglycan—Experiments similar to those carried out with the E. coli substrate showed that goose lysozyme has muramidase activity on M. luteus peptidoglycan. As seen in Table III incubations with goose lysozyme at pH 5.3 and 7.1 resulted in the appearance of muramicitol with a concomitant decrease in the amount of muramic acid. The amount of muramicitol produced at pH 3.5 is probably within the limits of our experimental error and we do not consider it significant. As in the case of the E. coli peptidoglycan studies no glucosaminidase was detected.

**DISCUSSION**

Our data demonstrate that goose lysozyme has muramidase activity on both E. coli and M. luteus peptidoglycans. This observation in light of other studies on the goose enzyme further suggests that the specificity of this lysozyme in contrast to what is known about hen lysozyme (22, 23) is restricted to N-acetylglucuronic acid residues whose lactyl group carries a peptide substituent. Dianoux and Jollès (18) observed that goose lysozyme would not release into solution NAG-NAM and NAG-NAM-NAG-NAM from M. luteus cell walls although the hen enzyme accomplished this readily. These observations are consistent with our hypothesis concerning the specificity of the goose enzyme. If goose lysozyme is specific for substituted NAG-NAM-NAG-NAM free of attached peptide would be expected.

Additional evidence that the specificity of goose lysozyme may be restricted to substituted N-acetylglucuronic acid residues is also indicated by the fact that purified NAG-NAM-NAG-NAM free of any attached peptide is readily hydrolyzed by hen lysozyme (24) but not digested by the goose enzyme (25). Finally, the ease with which goose lysozyme digests E. coli peptidoglycan may be related to the fact that almost all of its NAM residues are substituted with peptide (26) although differences in the degree of cross-linking or chemical composition between the E. coli and M. luteus peptidoglycans might also be important. Further studies on the digestion products themselves will help to settle these questions.

Very little is known about the role that the peptide portion of the peptidoglycan plays in determining the specificity of lysozyme. Howard and Glazer (22) speculated that papaya lysozyme had a distinct preference for unsubstituted N-acetylmuramic acid residues in the peptidoglycan of M. luteus, whereas hen lysozyme had a slight preference for the substituted sugars. The hen enzyme in fact is known to bind the tetrasaccharide NAG-NAM-NAG-NAM more strongly when it is substituted with the pentapeptide (23). Goose lysozyme may be an example of a muramidase with an even stronger preference for substituted NAM. This possible difference in specificity among these enzymes may be related to their antimicrobial function (27–30), since different species of bacteria vary in the extent to which NAM is substituted with a peptide.

Further investigations of the mode of interaction between bacterial cell wall peptidoglycans and lysozymes may lead to a better understanding of the adaptive significance of the amino acid sequence differences which exist among lysozymes from different species.

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