ON THE MECHANISM OF LYSOZYME ACTION*

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Lysozyme, the purification and chemical properties of which have recently been described (1), was considered by Fleming (2) to be an enzyme. Its heat stability rather suggested a physico-chemical action. Furthermore, some substances, such as rattlesnake venom, saponin, digitonin, cholic, choleic, and desoxycholic acids, have been found to bring about appreciable, but incomplete, lysis of organisms sensitive to lysozyme.1 This may be explained by the finding that these organisms themselves contain lysozyme which is released by the above substances.2 Moreover, a very potent, highly purified lysozyme preparation produced no appreciable lowering of surface tension in water or saline, whereas a dilute solution of caprylic alcohol, which had a surface tension about 60 per cent that of water, had no lytic effect.

With suitable substrates no protease, kinase, amylase, lipase, or phosphatase activity could be demonstrated. On the other hand, the action of lysozyme upon Sarcina brings about progressive increase in non-protein nitrogen, inorganic phosphorus, and reducing substances.

We have been able to confirm the suggestion of Hallauer (4), that lysozyme acts on a mucoid fraction of the Sarcina. We believe that this action explains its bacteriolytic property and that

* A preliminary report was published in Science, 79, 61 (1934).

1 The organisms used were very resistant to solution either by autolysis or by the action of alkali.

2 According to Avery and Cullen (3), autolysis is prevented and the lytic action of bile salts on pneumococcus is abolished, if an autolysin, present in the bacteria, is inactivated.
the increase in non-protein nitrogen and inorganic phosphorus is
due to the release of material enclosed by the bacterial membrane.

From our results it appears that the action is on one of the sugar
linkages in certain mucoids. The nature of this linkage, which is
certainly not α-glucosidic, is unknown. The action is quite
specific, for a number of glucoproteins and polysaccharides are not
susceptible. The maximum liberation of reducing groups is,
with the Sarcinae fractions, about one-third of the total reducing
power after acid hydrolysis. It is conceivable that the first action
of the lysozyme on the Sarcinae consists in the breakdown of the
aggregates of the mucoid micelle in a manner comparable with the
first step of the action of diastase on starch (5).

In view of the relative abundance of lysozyme in egg white,
experiments were carried out with egg mucoids and polysaccharides
as substrates. It was found that these products were attacked in
the same way as the bacterial fractions only when the use of alka-
line hydrolysis in their preparation had been avoided. Similarly,
bacterial fractions lost their susceptibility after more drastic
alkaline hydrolysis. The idea that this may be due to the removal
of acetyl groups is supported by the observation that the treat-
ment of one of the poorer bacterial substrates with acetic anhydride
in pyridine doubled the amount of reducing substances liberated
by the same lysozyme preparation. This is significant in the light
of the recent work of Avery and Goebel (6) on the rôle of the acetyl
groups in the immunological behavior of pneumococcus poly-
saccharides and of the observation of Helferich, Iloff, and Streeck
(7) that, while emulsin is without action upon phenol-β, d-gluco-
saminide, the N-acetyl derivative is readily split.

In general, mucoids are resistant to the action of proteolytic
enzymes (8). The inhibitory effect of egg white preparations on
trypic activity (9, 10) is probably due to a mucoid. A sample of
highly active antitrypsin, kindly sent us by Dr. Swenson, showed
the properties and composition of an egg mucoid (without lyso-
zyme activity), and some of our egg mucoid preparations, tested by
Dr. Swenson, had antitryptic activity.

Avery and Cullen (3) have obtained an enzyme from various
types of pneumococcus, which renders the organism Gram-nega-
tive. In higher concentrations, the ferment causes lysis of the
bacterial membrane. In recent experiments, as yet unpublished,
Dubos (11) has shown that our lysozyme preparations have the same action. Although these enzymes are much alike in their chemical properties, they are probably not identical.

It is probable that enzyme-substrate pairs similar to this from egg white exist elsewhere. One such ferment was found in a commercial pepsin preparation which liberated reducing sugar from gastric mucin. This ferment was not identical with pepsin, by which it was destroyed. It may be identical with Castle's ferment (12).

**EXPERIMENTAL**

*Preparation of Lysozyme from Sarcina—Sarcinae, grown on large agar plates, were carefully washed with saline and distilled water, and dried with acetone and ether. The organisms were ground for*

12 hours with saline in a ball mill, during which treatment the larger part of the organisms remained intact. The mixture was centrifuged, acidified, again centrifuged, and the supernatant solution was precipitated with flavianic acid. The precipitate was dissolved in saline to make a 0.45 per cent solution which gave upon assay complete lysis up to a dilution of 1:64. This preparation was also active towards another lysozyme-sensitive organism (Sarcina lutea). Like lysozyme from egg white, it was inactive towards *Escherichia coli communis*. By use of the same procedure no lysozyme was obtained from the non-susceptible *Escherichia coli*.

*Chemical Observations during Lysis—Table I illustrates the increase in non-protein nitrogen of a mixture of lysozyme and bacteria killed by heating at 80° for 30 minutes.*

<table>
<thead>
<tr>
<th>Table I</th>
<th>Effect of Lysozyme on Non-Protein Nitrogen of Sarcina Suspension.</th>
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<tbody>
<tr>
<td></td>
<td>Total N</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>micrograms</td>
</tr>
<tr>
<td>Lysozyme + saline</td>
<td>614</td>
</tr>
<tr>
<td>Bacteria + “</td>
<td>956</td>
</tr>
<tr>
<td>“ + lysozyme</td>
<td>1570</td>
</tr>
</tbody>
</table>
incubation mixtures were precipitated with a quarter volume of 20 per cent trichloroacetic acid, and nitrogen was estimated in the filtrate by the micro-Kjeldahl method. Similar results were obtained with fresh or acetone-dried bacteria.

To test the possibility of lysozyme being a protease, it was incubated with casein, peptone, and a mixture of proteins prepared from the sensitive *Sarcinae* as substrates. No increase in carboxyl groups (13) could be observed. No phosphatase activity of lysozyme could be demonstrated, with use as substrates of magnesium hexosediphosphate, sodium β-glycerophosphate, lecithin, and a lipid mixture from the *Sarcinae*. Tests for lipase activity were negative, and fluoride, which inhibits lipase, did not inhibit lysozyme activity.

For the study of the increase in reducing material, substrates were prepared from the *Sarcinae* and from egg white. Folin-Wu (14) or Hagedorn-Jensen (15) sugar analyses were made on mixtures of equal volumes of (usually) 1 per cent substrate and 0.01 to 0.1 per cent lysozyme solutions incubated at 37°. A drop or two of toluene sufficed to avoid contamination.

*Experiments with Bacterial Substrates*—The bacterial substrates were alkaline hydrolysates of washed, acetone-dried organisms, fractionated by alcohol precipitation, containing 4 to 9 per cent nitrogen, depending upon the alkalinity and time of hydrolysis.

Acetone-dried *Sarcinae* were extracted successively with weak alkali, weak acid, and 90 per cent acetic acid. The residue was digested with 2.5 N NaOH at 100° for 10 to 30 minutes; the supernatant liquid was brought to 50 per cent alcohol; the precipitate was redissolved in water and brought to 75 per cent alcohol. A few drops of glacial acetic acid were added to promote precipitation. The precipitate was again dissolved in water and reprecipitated with 75 per cent alcohol and acetic acid, washed with acetone, and dried. The products formed slimy solutions which gave a very strong Molisch reaction, but gave no color with iodine and reduced Benedict’s solution only after acid hydrolysis.

One such solution contained 0.479 per cent dry material which had 0.72 per cent ash (0.95 per cent of the ash was phosphorus). The nitrogen content on an ash-free basis was 6.33 per cent (atomic ratio of N:P, 15.3). When incubated 40 hours with a
lysozyme preparation (acid 50 per cent acetone extract\(^3\) of egg white powder, containing both lysozyme and mucoid\(^4\)), the reducing sugar (Folin-Wu) of the mixture increased 126 micrograms per cc.

Table II represents a similar but more detailed experiment. Substrate A-404 contained 6.73 per cent nitrogen and 19.5 per cent reducing sugar as glucose after hydrolysis. Substrate A-

**Table II**

**Effect of Lysozyme on Reducing Power of Bacterial Sugar Complex**

Equal volumes of substrate (1 per cent) or saline, lysozyme (1 per cent) or saline, and \(\frac{1}{15}\) phosphate (pH 6 or 7). Hagedorn-Jensen sugar method after Zn(OH)\(_2\) precipitation.

<table>
<thead>
<tr>
<th></th>
<th>Total sugar per cc. mixture after acid hydrolysis</th>
<th>Per cent of total substrate sugar liberated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>With phosphate, pH 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 hr.</td>
</tr>
<tr>
<td>Lysozyme + saline</td>
<td>1250</td>
<td></td>
</tr>
<tr>
<td>Substrate A-404 + saline</td>
<td>975</td>
<td></td>
</tr>
<tr>
<td>&quot; A-405 + &quot;</td>
<td>1200</td>
<td></td>
</tr>
<tr>
<td>&quot; A-404 + lysozyme</td>
<td>2150</td>
<td>4.6</td>
</tr>
<tr>
<td>&quot; A-404 + &quot;</td>
<td>2150</td>
<td>17.7</td>
</tr>
<tr>
<td>&quot; A-405 + &quot;</td>
<td>2250</td>
<td>7.8</td>
</tr>
</tbody>
</table>

* Without toluene.

405 contained 5.88 per cent nitrogen and 24.0 per cent reducing sugar.

\(^3\) An early procedure for preparation of lysozyme involved the extraction of 4 gm. lots of egg white powder overnight in the cold with 200 cc. of 50 per cent aqueous acetone containing 1 per cent HCl. The supernatant solution was then brought to 80 per cent acetone and a small amount of concentrated ammonia was added (to introduce salt) to facilitate flocculation. After standing in the refrigerator 24 hours, the precipitate was centrifuged off and washed with acetone and ether. The 350 to 400 mg. of dry white powder obtained, consisting chiefly of one of the egg mucoids, was easily soluble and had a lytic activity of 13 to 51 units per mg. This procedure was abandoned because of the cost of materials used.

\(^4\) In all preparations containing mixtures of mucoid and lysozyme high blank reductions were usually obtained, undoubtedly due to action of the enzyme upon the mucoid before the experimental period began.
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With highly purified lysozyme solutions in corresponding dilutions similar results were obtained.

*Experiments with Egg White Substrates*—A crude polysaccharide obtained by baryta hydrolysis\(^5\) gave no increase of reducing material after incubation with lysozyme. A similar but more pure material prepared according to Levene and Mori (16) gave the same negative results despite many variations in the experimental conditions. In the procedure of Levene and Mori the hydrolysis is carried out with 10 per cent \(\text{Ba(OH)}_2\) for 8 hours. We found that, if the egg white was heated for only 10 minutes with 2.5 N \(\text{NaOH}\), the resulting sugar complex was resistant to lysozyme.

**Table III**

*Effect of Lysozyme on Reducing Power of Egg Mucoid*

<table>
<thead>
<tr>
<th>Lysozyme + saline</th>
<th>Mucoid + saline</th>
<th>Lysozyme + lysozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar per cc. mixture</td>
<td>0 hr.</td>
<td>21 hrs.</td>
</tr>
<tr>
<td>micrograms</td>
<td>micrograms</td>
<td>micrograms</td>
</tr>
<tr>
<td>0</td>
<td>15</td>
<td>221</td>
</tr>
</tbody>
</table>

With 5 minute alkaline hydrolysis positive results were obtained, but even then the products gave only a small increase in reducing substances with the enzyme. However, when alkaline hydrolysis was avoided and a mucoid was prepared from acid aqueous or acid alcoholic extracts of the acetone-dried egg white powder, increases in reducing sugar comparable to those with bacterial materials were obtained.

Egg white, diluted four times and acidified with acetic acid, was poured into boiling water. The supernatant fluid was concentrated under reduced pressure and neutralized to maximal precipitation. The precipitate was extracted with 0.1 per cent HCl, and the extract treated with flavianic acid. The precipitated

\(^5\) We thank Dr. P. A. Levene for this material.
flavianates (containing most of the lysozyme) were removed and the clear supernatant solution poured into 6 volumes of alcohol. The results with this preparation (No. 80-B) are shown in Table III. It will be noticed that this material had a high reducing blank. It was found to contain lysozyme when tested with *Sarcina*. The non-protein nitrogen of the mixture increased 109 micrograms per cc. during the 21 hours.

The egg mucoid prepared by acetic acid-alcohol extraction (1) was likewise acted upon by lysozyme. A greater activity was observed in acid solution than in neutral. *Sarcina* swell markedly in acidic medium in the presence of lysozyme, but are not lyzed; upon neutralization they immediately dissolve, even after removal of the lysozyme (4,17).

We have found no action by lysozyme on starch, glycogen, cartilage, gastric mucin, the polysaccharide acid from the vitreous humor (18), osseomucoid, chitin, or a mucoid prepared from *Escherichia coli communis*.

**SUMMARY**

The lytic action of lysozyme on susceptible bacteria has been studied. Lysis cannot be explained on a physical basis; e.g., lowering of surface tension. Lysozyme has no protease, kinase, amylase, lipase, or phosphatase activity. It liberates reducing sugar from mucoids or polysaccharides of the susceptible *Sarcina* and from a mucoid fraction of egg white. The type of linkage attacked is not known.

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**BIBLIOGRAPHY**


* Controls with dilute filtered saliva showed a marked increase in reducing power.
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