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# PREPARATION AND PROPERTIES OF INSOLUBLE FORMS OF BACTERIOPHAGE T4 LYSOZYME AND CHICKEN EGG WHITE LYSOZYME

**HARALD B. JENSEN**

*Department of Biochemistry*
*University of Bergen, Bergen, Norway*

**and**

**TALIA MIRON**

*Department of Biophysics*
*The Weizmann Institute of Science, Rehovot, Israel*

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Bacteriophage T4 lysozyme and chicken egg white lysozyme were covalently bound to cyanogen bromide activated Sepharose and to glutaraldehyde activated polyacrylhydrazido-Sepharose. The latter method seemed less favorable for T4 lysozyme, since the polyacrylhydrazido-agarose conjugate exhibited low activity compared to the agarose conjugate. Whole bacteria (*M. luteus* and chloroform-treated *E. coli B* cells) and the soluble uncross-linked peptidoglycan polymer from *M. luteus* were used as substrates. Both types of conjugates exhibited low specific activity (lytic activity) toward insoluble substrates (cells), but surprisingly high specific activity toward the soluble substrate (hydrolytic activity). Product analysis showed that the enzyme conjugates retained their specificity of action, i.e., the same products were formed, and their rates of production were the same as those observed with the soluble (native) enzyme. The cell wall disaccharide-tetrapeptide GlcNAc-MurNAc-L-ala-D-glu-($A_2$pm-D-Ala) ($C_6$) inhibits the hydrolytic activity of both the native and the agarose bound T4 lysozyme. Only a slightly increased thermal stability was observed upon immobilization of T4 lysozyme, whereas the stability of the enzyme during storage and handling was greatly improved. The pH optimum of the lytic activity of Sepharose-T4 lysozyme was shifted about 1 pH unit to the alkaline side, compared to that found for the soluble enzyme, whereas no pH shift was observed for the polyacrylhydrazido-Sepharose conjugate. The optimum of the hydrolytic activity of Sepharose-T4 lysozyme was shifted to the acidic side. The pH optima of the lytic activity of the various lysozymes toward the bacterial cells were all very similar (>7), and differed greatly from the pH optima (<6) observed for their hydrolytic activities toward the negatively charged soluble peptidoglycan polymer. It is proposed that the observed differences in pH optima primarily reflect the basically different properties measured, i.e., the $\beta(1\text{-}4)$ cleaving activity (hydrolytic activity), and dissolution process of the damaged cells (lytic activity).

## INTRODUCTION

Enzymes bound to water-insoluble supports are of great practical and theoretical interest. They can be readily manipulated and used repeatedly in columns and in batch reactors, from which they can be easily removed. The

*Abbreviations used*: GlcNAc, *N*-acetyl-D-glucosamine; MurNAc, *N*-acetylmuramic acid; $A_2$pm, meso-diaminopimelic acid. Enzyme: Lysozyme: EC 3.2.1.17.



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study of alterations in properties that result when enzyme molecules are bound to various solid supports has elucidated our knowledge concerning some of the principles underlying the kinetic behavior of enzymes, whose natural environment is of a highly complex and charged nature (1).

Of the many methods available for causing immobilization, the most widely used is the cyanogen bromide method for activation of polysaccharides (2). One of the drawbacks of this method is that the coupling of amines to the activated agarose introduces charged bonds. Moreover, these bonds are not quite stable, and leakage of functional groups has been observed (3). Recently, the preparation of polyacrylhydrazido-agarose has been described (4). This resin lacks charged groups and has a large number of modifiable groups. In addition to increasing the stability of the conjugate and the removal of charge, one assumes that the hydrophilic macromolecular spacer provides great separation of ligand from matrix without introducing hydrophobic interactions (4).

In this paper the preparation and properties of two water-insoluble forms of the lysozymes from chicken egg white and bacteriophage T4 infected *E. coli* cells are described. Coupling was performed to cyanogen bromide activated Sepharose and to glutaraldehyde activated polyacrylhydrazido-Sepharose (4,5). Whole bacteria, as well as a soluble peptidoglycan polymer, were used as substrates. Because of the particulate nature of support-bond enzyme preparations, soluble enzyme assay procedures, in particular spectrophotometric ones, usually have to be modified. Additional problems are met with when insoluble enzymes act on insoluble substrates. Ollis and Datta (6) measured the activity of lysozyme-polyacrylamide by a batch reaction, using *Micrococcus luteus* cells as substrates, removing samples after various times for determination of optical density at 440 nm. In the present work, we show that the recording of the turbidimetric changes caused by the lytic enzymes upon *M. luteus* cells and chloroform-sensitized *Escherichia coli* cells (7) can be obtained nearly as easily for the particulate as for the soluble enzymes.

We have two main reasons for having an interest in insoluble lysozyme derivatives of various types. First, as easily removable agents they have proved very valuable when preparing low and high molecular weight fragments of bacterial cell wall sacculi. Second, affinity chromatography has recently been established as a technique for measuring quantitative parameters of macromolecule-ligand interactions (8–12). We have studied the specificity of action of T4 lysozyme by following its action on *E. coli B* cells (7,13) and a soluble uncross-linked peptidoglycan (14, 15). We expect that studies of the interactions between insolubilized lysozyme and various substrates and inhibitors will be another useful method for the elucidation of the mechanism and specificity of action of these enzymes.

## MATERIALS AND METHODS

Sepharose 4B was obtained from Pharmacia, Uppsala, Sweden. Glutaraldehyde, 25% aqueous solution, was obtained from TAAB Laboratories, Reading, England. Cyanogen bromide was obtained from Fluka, chicken egg white lysozyme (3× crystallized) from Worthington, and *M. luteus* cells, sprayed dried, from Miles Laboratories. *E. coli B* was from the laboratory stock of organisms.

The soluble uncross-linked radioactively labelled peptidoglycan was prepared from *M. luteus* cells (16). The label was in both aminosugar constituents [($^{14}$C)glycan peptidoglycan, specific radioactivity, 18 counts $min^{-1}pmol^{-1}$]. Purified bacteriophage T4 lysozyme was obtained from T4D-infected *E coli B* cells (7).

### *Preparation of Sepharose-Enzyme Conjugate*

Activation of Sepharose 4B was performed by using CNBr in organic solvent (17). Typically, Sepharose 4B was washed with water on a G3 disk sintered-glass funnel. Ten g (wet) was suspended in 25 ml 2 M $K_2CO_3$ at 0°C in a beaker equipped for magnetic stirring. To the slurry was added 1.0 ml of CNBr in *N,N*-dimethylformamide (1.0 g per ml). The reaction was carried out in an ice-water bath, with stirring for 90 sec, after which the gel was transferred to and washed on a G3 filter with 10 ml of 50% dimethylformamide in water (4°C) followed by cold water (500 ml). Immediately after activation, proteins were coupled by adding a freshly prepared solution of the enzyme (50 mg/20 ml 0.1 M $NaHCO_3$) to 10 g of activated Sepharose and slowly stirring for 16 h at 4°C. The conjugate was carefully washed with 0.1 M $NaHCO_3$ and water before being used. In general about 90% of the protein was bound.

### *Preparation of Polyacrylhydrazido-Sepharose-Enzyme Conjugate*

Polyacrylhydrazido-Sepharose was prepared as described (5), and the amount of hydrazide coupled was determined using an excess of trinitrobenzenesulfonic acid (5). T4 lysozyme and chicken egg white lysozyme were coupled to glutaraldehyde activated polyacrylhydrazido-agarose as described (5). Typically, polyhydrazido-Sepharose (2 g wet) was activated by suspending the resin in 10 ml of 7% glutaraldehyde in water for 2 h at 24°C. After washing with water on a glass filter (G3), the activated polymer was taken up in 7 ml of 0.03 M phosphate buffer, pH 6.8, containing 1.5 mg T4 lysozyme. The mixture was stirred slowly at 4°C for 16 h. In order to avoid unspecific binding to the carrier, reactive groups were blocked. The

insoluble enzyme was resuspended in 0.05 M hydrazine hydrate, pH 8.5, for 2 h at 24°C, and then washed on a filter.

Determination of bound proteins was performed on an amino acid analyzer after acid hydrolysis (6 N HCl, 110°C, for 22 h). Quantitation was also performed by measuring $A_{280\,nm}$ and enzyme activities in the original protein solution, the original solution after coupling, and the wash solution.

*Enzyme Activity Assays*

The action of the immobilized enzymes was followed turbidimetrically by incubation with (*a*) chloroform-treated *E. coli B* cells (7), using for all experiments a batch of substrate cells that was found to be fairly stable toward autolysis, and (*b*) *M. luteus* cells. The turbidimetric assay was carefully standardized, since the experimental conditions were found to have great influence on the apparent activities measured. A Spectronic 20 photometer equipped with a recorder was used. A small magnet was placed in the bottom of a standard 3-ml cuvette, and a magnet stirrer was placed immediately under the cuvette holder. Volumes down to 1 ml could now be tested, using a low stirring speed (100 rotations per min). The absorbance at 450 nm was then recorded continuously. One unit of activity was taken as the decrease in absorbance of 0.001 per min. Amounts of enzyme conjugate were determined by weighing, and experiments were performed in duplicate. All activities were measured in 0.05 M Tris buffer, pH 7.4, at room temperature unless otherwise stated. This method was found to be superior to others that were tested, e.g., the batch method described by Ollis and Datta (6), and attempts to separate the beads from the cell suspension before measuring the absorbance of the latter, both from a practical standpoint and when concerning the reproducibility of the assays.

*Digestion of Soluble, Uncross-Linked M. Luteus Peptidoglycan*

Digestion of the labelled *M. luteus* polymer was performed essentially as described in ref. (15), using 0.03 M phosphate buffer (pH 5.1–8.0) and 0.03 M sodium acetate buffer, pH 4.7, with a final volume of 45 $\mu$l. The enzyme conjugates were properly washed with the appropriate buffers before use. The reaction was started by addition of 1.9 nmol [$^{14}$C]glycan peptidoglycan. The solutions were agitated regularly and aliquot (13 $\mu$l) were removed using pipettes with an enlarged inlet opening. Time intervals were chosen according to the amount of active enzyme present in order to obtain a linear increase of products during the first part of the sampling period. The aliquots were spotted on Whatman 3 MM paper and immediately dried with hot air. Separation, detection, and quantitation of the reaction products were performed as described previously (14,15).

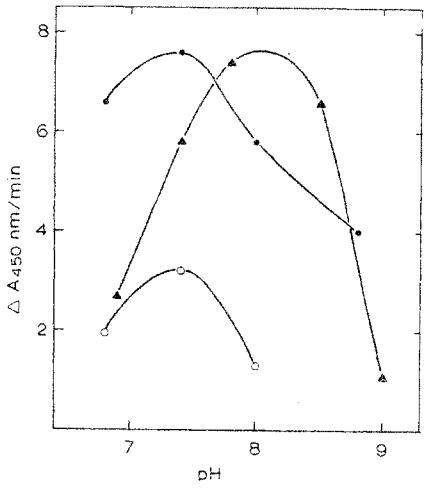


FIG. 1. Lytic activity of T4 lysozyme and T4 lysozyme conjugates as a function of pH. The reaction mixtures contained chloroform-treated *E. coli B* cells (initial absorbance at 450 nm about 0.7), either T4 lysozyme (1.2 ng), Sepharose-T4 lysozyme (12 mg wet weight, equivalent to 3.24 μg enzyme), or polyacrylhydrazido-agarose-T4 lysozyme (10 mg wet weight, equivalent to 4.6 μg enzyme) in 2 ml 0.03.M phosphate buffer at the pH indicated. The reactions were run at room temperature at a constant stirring speed (100 rpm). $A_{450\,nm}$ was recorded continuously and the turbidity change in the linear region, measured as $\Delta A_{450\,nm}$/min, was used to express the activity of the enzyme. Activities are given in μg/ml of soluble enzyme (●), and in mg enzyme/ml of Sepharose-T4 lysozyme (▲) and of polyacrylhydrazido-agarose T4 lysozyme (○).

## RESULTS

### *Lytic Activity of T4 Lysozyme Conjugates*

Enzymes immobilized on charged matrices often show pronounced pH shifts compared to the native enzymes (1). When chloroform treated *E. coli* cells are used as substrate, the pH optimum for Sepharose T4 lysozyme conjugate is shifted about one pH unit to the alkaline side, compared to that found for soluble T4 lysozyme (Fig. 1). No pH shift was observed for the polyacrylhydrazido-agarose conjugate (Fig. 1).

By setting the specific activity of the soluble enzyme at 1.0, it follows from the results (Fig. 1) that the specific activity of the agarose T4 lysozyme is about 0.001, and that of the polyacrylhydrazido-agarose T4 lysozyme is only about 0.0003. Rather low specific activities are to be expected, since in these cases both the enzymes and the substrate are insoluble. The polyacrylhydrazido-agarose derivatives of T4 lysozyme always exhibited a much lower specific activity than the agarose conjugate, however, indicating that the coupling conditions used were unfavorable for this enzyme.

*M. luteus* cells have been the most commonly used substrate for assaying the lytic activity of lysozymes. This organism is, however, a poor substrate for T4 lysozyme (18) (data not shown), and as expected, was virtually unaffected by the T4 lysozyme conjugates.

### *Hydrolytic Activity of Agarose T4 Lysozyme Conjugate*

The hydrolytic activity of T4 lysozyme upon a soluble, uncross-linked peptidoglycan from *M. luteus* cells can be followed by measuring the rate of

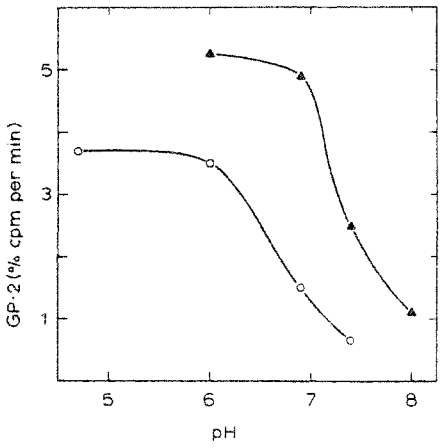


FIG. 2. Effect of pH on the time course of appearance of the cell wall glycopeptide GP-2. The reaction mixture contained [$^{14}$C]glycan labelled peptidoglycan (1.9 nmol) and either soluble T4 lysozyme (▲), 60 μg/ml, or Sepharose-T4 lysozyme (○), 56 μg/ml, in 0.03 M buffers at the pH indicated. Products were separated by paper chromatography, detected by using a chromatogram scanner, and subsequently counted in a liquid scintillation counter. The numbers refer to the radioactivity released as GP-2 during the initial stage of reaction expressed as percent of total radioactivity per min.

appearance of the main product of the reaction, the disaccharide-hexapeptide GP-2 (15). Compared with soluble T4 lysozyme, a pH shift toward the acidic side was observed with the agarose T4 lysozyme conjugate (Fig. 2). In both cases the pH optimum of the reaction was below 6. At pH 6.0 the rate of release of GP-2 was about 0.54 mol per mol soluble enzyme per sec.

The agarose conjugate exhibited a rather high specific activity compared to that of the soluble enzyme. In this particular case (Fig. 2), about 70% of the apparent activity was retained at pH 6.0, and about 35% at pH 6.9 and 30% at pH 7.4. In general, other preparations retained about 50% activity at 6.0 and about 25% at pH 6.9.

### *Cell Wall Glycopeptides as Inhibitors for Immobilized T4 Lysozyme*

Several well-characterized low molecular weight cell wall glycopeptides have been shown to be good competitive inhibitors of T4 lysozyme (15). It was of vital importance for further use of immobilized T4 lysozyme in enzyme specificity studies that these glycopeptides also inhibit the enzyme conjugates. Digestion of *M. luteus* peptidoglycan polymer by agarose T4 lysozyme was therefore performed in the presence of the disaccharide-tetrapeptide GlcNAc-MurNAc-L-Ala-D-Glu($A_2$pm-D-Ala) ($C_6$). The rate of appearance of products was affected to nearly the same extent as that observed using the soluble enzyme (Table 1).

### *Stability of the T4 Lysozyme Conjugates*

One of the most troublesome properties of T4 lysozyme is its sensitivity towards dilution. The ionic strength of the dilution buffer must always be

TABLE 1. **Effect of the Cell Wall Glycopeptide C6 on the Time Course of Appearance of the Disaccharide Hexapeptide GP-2** [a]

| | | Product formed after | |
|---|---|---|---|
| | | 5 min | 10 min |
| T4 lysozyme | None | 18.5 | 26.0 |
| T4 lysozyme | C6, 2.0 mM | 1.9 | 4.1 |
| Sepharose-T4 lysozyme | None | 4.8 | 8.4 |
| Sepharose-T4 lysozyme | C6, 1.3 mM | 1.0 | 1.5 |

[a] The reaction mixture contained [$^{14}$C]glycan-labelled peptidoglycan (1.9 nmol) and either soluble T4 lysozyme (60 $\mu$g/ml) or Sepharose-T4 lysozyme (56 $\mu$g enzyme/ml) in 0.05 M ammonium acetate, pH 6.8. The values represent the radioactivity in GP-2 expressed as a percentage of total counts.

high, preferably above 0.1 M. This has also been reported by others (19). At lower ionic strength, both T4 lysozyme and chicken egg white lysozyme tend to absorb strongly to glass, which at low protein concentrations can be rather problematical. At high protein concentrations, T4 lysozyme tends to aggregate. The insoluble T4 lysozyme derivatives apparently do not possess these same properties. Thus there is no tendency of the conjugates to lose activity upon treatment with buffers of either high or low ionic strength. Moreover, they are very stable when stored in aqueous suspensions. It was observed, for example, that a Sepharose T4 lysozyme preparation apparently showed the same activity after storage for 16 months at 4°C in 0.05 phosphate buffer pH 6.8.

The thermal stability of immobilized T4 lysozyme was evaluated by heating for 2 min in 0.05 M phosphate buffer, pH 7.0, containing 0.6 M KCl, and measuring residual activity under standard conditions (25°C). The immobilized enzymes responded in a very similar manner to the soluble enzyme; only a slightly increased thermostability was indicated. No leakage of protein from the enzyme conjugates was observed under the same incubation conditions when washings were tested for UV-absorbing or ninhydrin positive material following acid hydrolysis and lytic activity. Similarly, no leakage of protein was observed when Sepharose T4 lysozyme was incubated in 0.05 M ammonium acetate or 0.05 M phosphate buffer, pH 6.8, for 24 h at room temperature (Fig. 3).

### *Digestion of M. Luteus Peptidoglycan by Columns of Agarose T4 Lysozyme*

One of the most useful properties of immobilized enzymes is that they can be packed in columns. The enzymic reaction is effectively stopped by

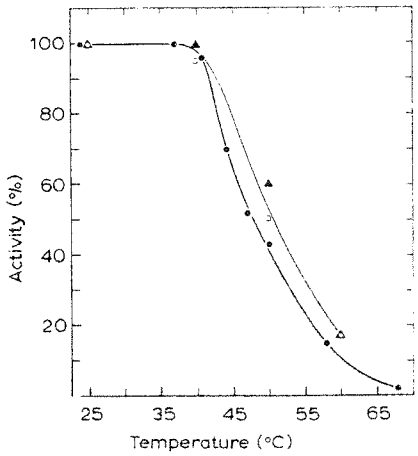


FIG. 3. Thermal stability of T4 lysozyme and T4 lysozyme conjugates. T4 lysozyme (●), Sepharose-T4 lysozyme, 10 mg wet weight, equivalent to 2.9 μg enzyme (○), and polyacrylhydrazido-agarose-T4 lysozyme, 30 mg wet weight, equivalent to 13.8 μg enzyme (▲), were incubated for 2 min in 0.05 M phosphate buffer, pH 7.0, containing 0.6 M KCl at the temperatures indicated. Residual lytic activities using chloroform-treated *E. coli B* cells as substrate were measured at pH 6.8 and 25°C, as described in the legend to Fig. 1. Activities are expressed as a percentage of that observed following incubation at 25°C.

elution and the enzymes can be used repeatedly and under identical conditions. Columns (0.5 × 6 cm) of agarose T4 lysozyme (0.29 mg per g) were packed and solutions of *M. luteus* peptidoglycan passed through. Digestion products were separated on paper or cellulose thin layer plates after direct application of the effluent from the column. It was thus found that the extent of digestion was determined by the period of time the substrate was in contact with the column material, and therefore, it could be controlled by varying the flow rate. The presence of the disaccharide tetrapeptide C6 on the column inhibited the hydrolytic activity of the agarose T4 lysozyme.

### *Lytic Activity of Chicken Egg White Lysozyme Conjugates*

*M. luteus* and sensitized *E. coli B* cells were both good substrates for chicken egg white lysozymes (Fig. 4 and Table 2). The enzyme conjugates exhibited relatively higher activities in the lower pH region than did the soluble enzyme, both compared to their activities at pH 7.4.

From Table 2 it can be seen that both enzyme conjugates have relatively low specific activities toward the cells, compared to the soluble enzyme. From the ratios of their activities on *E. coli* versus *M. luteus*, it may seem as if the polyacrylhydrazido-conjugate behaves more like the soluble enzyme. This may reflect a more flexible enzyme, which due to the spacer arms, may be more able to penetrate the complex Gram-negative cell wall.

### *Soluble M. luteus Peptidoglycan Polymer as Substrate for Chicken Egg White Lysozyme*

The hydrolytic activity of chicken egg white lysozyme upon the soluble uncross-linked peptidoglycan from *M. luteus* can be followed by analysis of

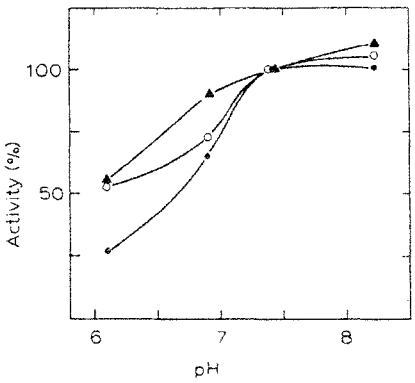


FIG. 4. Lytic activity of chicken egg white lysozyme conjugates as a function of pH. The reaction mixtures contained acetone-dried *M. luteus* cells (0.36 mg), either native chicken egg white lysozyme (●), 1 μg, Sepharose conjugate (○), about 10.0 mg, equivalent to 9.3 μg enzyme, or polyacrylhydrazido-agarose conjugate (▲), about 20.0 mg, equivalent to 56 μg enzyme, in 2 ml of 0.03 M phosphate buffer at the pH indicated. The assays were performed and recorded as described in the legend to Fig. 1. Activities are given as a percentage of those recorded at pH 7.4

the products of the reaction. Four major end products are formed (14,15), the disaccharide GlcNacβ(1-4)MurNAc, its corresponding β(1-4) linked dimer the tetrasaccharide, the disaccharide hexapeptide GP-2, and the tetrasaccharide hexapeptide GP-3. These compounds can be separated by partition chromatography (Fig. 5) and quantitated. Fractions I and II (Fig. 5) represent oligosaccharides and oligosaccharide peptides, which to a large extent are degraded further by the enzyme. As a measure of enzyme activity, we found it convenient to use the sum of the major end products released during the initial stage of the reactions, expressed as percent radioactivity released per min. Figure 6 shows the time course of appearance of the different products released when using Sepharose chicken egg white lysozyme. The results were nearly identical for both the soluble enzyme and the polyacrylhydrazido-agarose conjugate, i.e., the same products were formed (Fig. 5), and their relative rates of appearance (Fig. 6) were the same.

As with T4 lysozyme, chicken egg white lysozyme conjugates exhibited much higher specific activities when soluble peptidoglycan was used as

TABLE 2. **Enzyme Activity with *M. luteus* Cells and Chloroform-Treated *E. coli B* Cells as Substrate for Chicken Egg White Lysozyme Conjugates and Native Enzyme**[a]

| Enzyme | *E. coli B* cells | *M. luteus* cells | Ratio of activity *E. coli*/*M. luteus* |
|---|---|---|---|
| Native | 70 | 100 | 0.7 |
| Sepharose conjugate | 0.22 | 1.6 | 0.14 |
| Polyacrylhydrazido-agarose conjugate | 0.25 | 0.57 | 0.44 |

[a]The reaction conditions are given in Fig. 4, pH being 7.4. Initial absorbance of the *E. coli* substrate was about 0.7. Activities are expressed as $\Delta A_{450\,nm}$ per mg enzyme per ml.

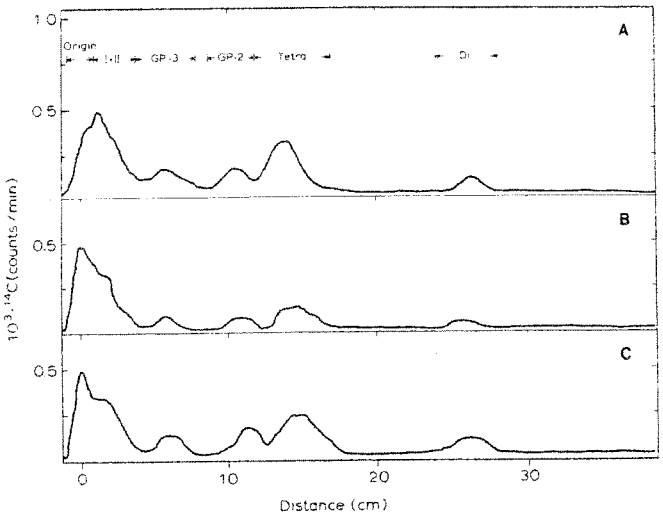


FIG. 5. Paper chromatographic separation of chicken egg white lysozyme digests of the peptidoglycan polymer. The reaction mixtures contained [$^{14}$C]glycan labelled peptidoglycan (1.9 nmol) at pH 6.1 and either native chicken egg white lysozyme, 16.6 $\mu$g/ml (a), Sepharose conjugate, 52 $\mu$g/ml, (b), or polyacrylhydrazido-agarose conjugate, 100 $\mu$g/ml (c). Aliquots (about 11,000 cpm) were spotted on Whatman 3 MM paper, and descending chromatograms were run with solvent butan-1-ol/glacial acetic acid/water, 4/1/5 (v/v/v), upper phase, for 45 h. Radioactive products were detected using a radiochromatogram scanner. The figure shows the scan after 5 min (a), 3 min (b), and 3 min (c) incubation.

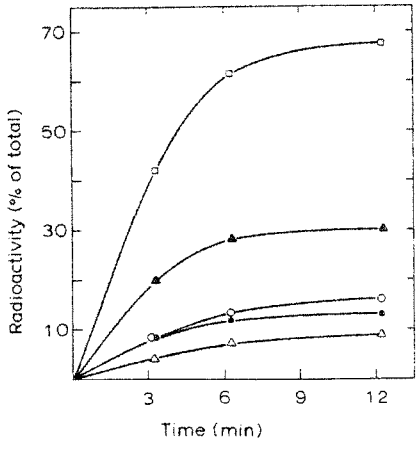


FIG. 6. The time course of appearance of products upon digestion of *M. luteus* peptidoglycan by Sepharose chicken egg white lysozyme. The reaction mixture contained [$^{14}$C]glycan labelled peptidoglycan (1.9 nmol) and Sepharose chicken lysozyme (52 $\mu$g enzyme/ml) in 0.03 M phosphate buffer, pH 6.1. Products were separated (see Fig. 5) and subsequently counted in a liquid scintillation counter. The values given are the counts found in the different products, expressed as a percentage of the total radioactivity applied to the paper. △, GlcNAc-MurNAc; ▲m GlcNAc-MurNAc-GlcNAc-MurNAc; ○, GP-2; ●, GP-3; □, sum of products.

TABLE 3. **Enzyme Activity with Soluble *M. luteus* Peptidoglycan as Substrate for Chicken Egg White Lysozyme**[a]

| | pH 4.7 | | pH 6.1 | | pH 6.9 | |
|---|---|---|---|---|---|---|
| Enzyme | Percent/min | Relative | Percent/min | Relative | Percent/min | Relative |
| Native | 14.0 | 1 | 26.0 | 1 | 16.0 | 1 |
| Sepharose conjugate | 4.2 | 0.30 | 6.4 | 0.25 | 3.8 | 0.24 |
| Polyacryl-hydrazido-agarose conjugate | 15.5 | 1.10 | 14.0 | 0.54 | 10.0 | 0.62 |

[a]The reaction mixtures contained [$^{14}$C]glycan labelled peptidoglycan (1.9 nmol) and either native enzyme (16.6 μg/ml), Sepharose conjugate (52 μg enzyme/ml) or polyacrylhydrazido-agarose conjugate (100 μg enzyme/ml). In each case the time course of appearance of end products, i.e., the tetrasaccharide, the disaccharide, GP-2, and GP-3, was determined as in Fig. 6. The numbers represent the percentage of radioactivity transformed to end products per min. For convenience the values are given per 16.6 μg enzyme/ml.

substrate in comparison to cells (Table 3). The pH optimum of the reaction was shifted more to the acidic side, compared to that observed using cells as substrate. Both at pH 6.9 and 6.1, the agarose and the polyacrylhydrazido-agarose conjugates exhibited about 25 and 55-60% activity, respectively, of that observed with the soluble enzyme. The polyacrylhydrazido-agarose conjugate exhibited an apparently slightly higher activity than the soluble enzyme at pH 4.7. This may reflect a shift in pH optimum.

## DISCUSSION

T4 and chicken egg white lysozymes were bound to either cyanogen bromide activated Sepharose or to glutaraldehyde activated polyacrylhydrazido-agarose (2,4,5). The latter method seemed less favorable for T4 lysozyme, since the polyacrylhydrazido-agarose conjugate always exhibited much lower activity than the agarose conjugate. Glutaraldehyde is not specific for $NH_2$-groups but reacts also with SH-groups, for example (20). The loss of activity upon coupling is, therefore, in line with our finding that the two SH-groups in T4 lysozyme, together with one tryptophan group, are readily modified by dinitrophenylsulfenyl-chloride (T. Miron and H. Jensen, unpublished data), the modification being followed by loss of activity.

As expected, both types of conjugates exhibited rather low specific activity toward insoluble substrates (cells). The reduced enzymic activity that is usually observed when insoluble enzymes act on high-molecular-weight substrates is in most cases related to steric restrictions imposed by the

polymeric carrier (1). On the other hand, the enzyme conjugates retained surprisingly high specific activities toward the soluble, uncross-linked peptidoglycan polymer from *M. luteus*. This linear peptidoglycan polymer (16,14) has a molecular weight of about 40,000, corresponding to approximately 50 disaccharide units. On an average half the units are substituted by a hexapeptide on their *N*-acetylmuramic acid residues. It appears that the substitution by peptide side chains is not random; the peptidoglycan may consist of a mixture of chains, approximately half of which are substituted by peptide chains on most of their muramic acid residues, while the other half is made up of chains in which most of the muramic acid residues are unsubstituted (14). As a consequence these different types of chains are also differently charged. Considering the differences in structure and charge, various microenvironmental effects (e.g., partitioning effects) and mass transfer effects could therefore possibly be operative when insoluble enzyme derivatives are acting on the polymers. The relatively high specific activities of the enzyme conjugates, however, are clear indications of only minor diffusional and sterical limitations on the penetration of the substrate. The reasons why these effects are not manifested by this macromolecular substrate may be at least two-fold; the enzymes have very low turnover numbers, and the substrate is presumably unfolded due to the negative charges along the molecule.

In some instances it has been found that the specificity of action, i.e., the sites of attack of an enzyme, has been changed as a result of coupling to a solid support (1). Thus it was shown that while native trypsin hydrolyzed 15 lysyl peptide bonds in pepsinogen, the maximal number of bonds split by polyanionic trypsin did not exceed 10 (21). T4 and chicken egg white lysozyme have different substrate specificities and give rise to different products (14,15). T4 lysozyme cleaves glycosidic bonds only at muramic acid residues that are substituted with peptide side chains. Thus the main product of the polymer is the disaccharide hexapeptide (GP-2), together with the tetrasaccharide hexapeptide (GP-3) and other oligosaccharide peptides. Chicken egg white lysozyme produces four major products; in addition to GP-2 and GP-3, it gives the disaccharide GlcNAc$\beta$(1-4)MurNAc, and its corresponding (1-4) linked dimer, the tetrasaccharide. The product analysis following digestion of the polymer by the various lysozyme conjugates shows that in each case the same products were formed and the time course of their relative appearance was nearly identical to those observed using the corresponding native enzymes. The specificity of action is therefore seemingly fully retained after coupling. This is of particular value for the use of the enzyme conjugates in studies on their mechanism of action and specificity requirements.

The pronounced pH shifts often observed with immobilized enzymes have been related to the creation of a microenvironment around the enzyme

molecules that differs from the conditions in the bulk solution, the effect being caused by the electrostatic potential of the polyelectrolyte carrier (1,22). A similar type of reasoning has been adopted in attempts to explain the pH shifts observed when soluble enzymes act on insoluble substrates like cell walls (23). In this case the microenvironment found within the cell wall, which is usually negatively charged, presumably differs from that observed in the bulk solution. When an insoluble enzyme acts on an insoluble substrate, the resulting effects are not easily predictable. Using chloroform treated *E. coli B* cells as substrate, the pH optimum for the Sepharose-T4 lysozyme conjugate was shifted about one pH unit to the alkaline side, whereas no pH shift was observed for the polyacrylhydrazido-agarose conjugate (Fig. 1). The pH within a positively charged matrix is assumed to be higher than that measured in the bulk solution, which should favor a shift in pH optimum to the acidic side. This is observed using a soluble substrate (Fig. 2). When both enzymes and substrate are insoluble, however, reactions have to take place at or near their common surface. The microenvironment of both the interior matrix and the cell wall may thus become less important for the observed kinetics and other factors may therefore be more dominant.

The mechanism of lysis by lysozyme has been discussed by many authors (23, 25–29). Nakamura (30) in 1923 noticed that the lytic activity of human lysozyme upon Gram-positive bacteria (*M. luteus*) is composed of at least two factors, one, the catalytic activity of the enzyme, and two, dissolution of damaged cells. Thus cells exposed to lysozyme at about pH 5 do not lyse before the pH is raised to about 10. The cells then lyse very rapidly. Also, in many other instances it has been shown that the enzyme has acted, e.g., reducing sugars become exposed, without lysis taking place (13, 31).

Yamasaki et al. (24) divide the process of lysis by lysozyme into three successive steps; the first step is the binding of the enzyme to the surface of the cells by electrostatic interaction due to surface charges, the second is the $\beta$(1-4) glycosidic bond cleaving activity of the enzyme, and the third is a physiochemical dissolution of the enzymatically damaged cell walls. Since the specific catalytic activities (step 2) of T4 and chicken egg white lysozyme are low, factors influencing steps 1 and 3 in the lytic process may become dominant. Thus Yamasaki et al. (24) found that acetylation of the amino groups of chicken egg white lysozyme caused a decrease of its lytic activity toward *M. luteus* cells and purified *M. luteus* peptidoglycan at neutral pH with a concomitant shift in pH optimum to the acid side. This behavior was interpreted in terms of lower initial electrostatic interaction between the enzyme and the negatively charged cell wall, since acetylated lysozyme retained full hydrolytic activity toward the neutral substrate glycol chitin (pH optimum 5.2).

It has also been shown that the susceptibility of cell walls to lysis by lysozyme increases with the negative charge on the cell walls (32). It is

possible, therefore, that a stronger initial interaction between the positively charged T4 lysozyme Sepharose conjugate and the negatively charged cell wall of *E. coli* may explain the observed pH shift to the alkaline side. Unequal distribution of charged molecules other than hydronium is to be expected, however, and it must be recalled that the lytic activity of both T4 and chicken egg white lysozyme is greatly and mutually influenced both by pH and ionic strength and also by certain membrane-stabilizing agents (13, 27).

Several authors have observed that the pH optimum of the lytic activity of various lysozymes toward bacterial cells and cell walls differed greatly from the pH optimum observed for hydrolytic activities toward oligosaccharides (13,23,25,27,33). Thus the reaction with oligosaccharides like glycolchitin has an apparent optimum at about pH 5, irrespective of ionic strength, whereas the lytic activities on bacterial cells can present a maximum at 7 or higher, the position being strongly dependent on the ionic strength. To explain these observations, Mauriel and Douzon (23) took into consideration the polyanionic structure of the bacterial cell wall and its possible influence on the physical properties of the environment of the catalytic reaction. They claim that the lytic activity of (human) lysozyme toward *M. luteus* cells can be satisfactorily explained when analyzed in terms of the catalytic implications of the electrostatic potential developed by the negatively charged cell wall.

In this connection it is very interesting to note that the present work shows that the pH optimum changes in the same manner when substituting cells with cell wall polymer as substrate, and that both soluble and insoluble enzymes behave in the same or in a very similar way. The *M. luteus* peptidoglycan polymer is strongly negatively charged, and the large shift in pH optimum therefore cannot be explained merely by differences in initial electrostatic interactions. Moreover, the same shift in pH optimum to below 6 was observed with soluble T4 and chicken egg white lysozyme, using purified peptidoglycans from *E. coli* and *Bacillus megaterium* cells as substrate, when measuring hydrolytic activities in terms of liberation of reducing sugars (H. Jensen, unpublished results). The explanation therefore probably lies in the basis for the different assays used: the turbidimetric assay for whole cells and cell walls, and the product analysis or reducing power measurements in the case of peptidoglycan or soluble peptidoglycan polymer. The latter methods are directly related to the number of bonds being hydrolyzed, whereas the turbidimetric assay measures the dissolution of enzymatically damaged cells, and this property is only indirectly connected to the enzymatically catalyzed reaction.

These aspects of the turbidimetric assay have been discussed in some detail by Gorin et al. (29), who clearly showed that this method provides a

large amplification factor of the catalytic reaction, i.e., a minute amount of chemical reaction causes an easily observed physical change. Thus the enzyme concentrations used in turbidimetric assays are usually in the order of 1% of those used with soluble substrates. Therefore it is very likely that factors influencing the dissolution process may have great influence on the apparent lytic activity of the enzyme. These aspects of the assay used were not considered by Maurel and Douzon (23). From the present results and the discussion above, it seems obvious that the pH profiles of the lytic activities reflect the combined effects on the catalytic activities and the dissolution process of the damaged cells, with the latter being by far the most important.

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