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Degradation of Bacterial Cell Walls by Immobilized Lysozyme

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ABSTRACT

Lysozyme was immobilized by two different methods in two different ways in order to obtain a preparation with an activity as high as possible toward a macromolecular substrate. The enzyme was bound as a Schiff base to a silicate carrier by using oxidized dextrans of different lengths as spacer and also was bound to controlled pore aminoglass via pyridino-4-aldehyde and BrCN. The latter preparations had activities up to 2.5% of the free lysozyme.

Index Entries: Lysozyme; immobilization; dextran; controlled pore glass.

INTRODUCTION

If the activity of the immobilized lysozyme is measured with a substrate of low molecular weight, it may be in the order of magnitude of the free enzyme (*1–6*).

However, studies with whole bacterial cells as substrate show a significantly lower activity owing to diffusion limitation and steric hindrance of the large particulate substrate (*1,2,4*). In another case (*7,8*), the substrate was partially adsorbed on the immobilizate and cosedimented together, thus simulating a higher activity.

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To optimize the activity of immobilized lysozyme toward macromolecular substrates, we investigated different spacers. Because the bacterial surface is negatively charged (*9,10*), we used spacers with a positive charge in a pyridinium ion. In another series of experiments, we used spacers of three different lengths to overcome steric hindrance and diffusion effects.

MATERIALS AND METHODS

Controlled pore glass (mesh size 100–200), lyophylized Micrococcus luteus cells, and linear dextran from Leuconostoc mesenteroides (clinical grade, molecular weight 35,600, 170,000, and 2,000,000) were obtained from Sigma Chemical Co., St. Louis, USA. Chicken eggwhite lysozyme (EC 3.2.1.17) 23,000 U/mg according to Shugar (*11*) was obtained from Boehringer Mannheim GmbH. The Gulsenit®, (a microporous carrier obtained from a magnesium silicate by acid treatment) was a gift from MAGINDAG Co., Vienna. All other reagents were obtained from E. Merck, Darmstadt. For turbidity measurements, a Hitachi 150–20 spectrophotometer was used.

Buffers Employed

Buffers for washing of the immobilizate: 0.1 *M* borate buffer, pH 8.0; 0.1*M* acetate buffer, pH 4.0; and 0.1*M* glycine buffer, pH 11.0. To every buffer NaCl (0.1 mol/L) was added.

Buffers of defined ionic strength for pH optimum measurement were prepared according to Miller and Golder (*12*).

Soluble enzyme was assayed according to the procedure of Weisner (*13*).

The activities of lysozyme are given in units according to Shugar (*11*). One unit will produce a decrease in optical density (OD) of 0.001/min under assay conditions.

The assay of the protein content of the immobilizate was carried out according to Jacobs (*14*).

Preparation of Amino Carriers

Controlled pore glass or Gulsenit® was coated with 3-aminopropyltriethoxysilane following the method of Weetall (*15*). Gulsenit® or controlled pore glass beads were treated for 1 h with 3% HNO_3 at 90°C, rinsed with water until the filtrate was neutral, and kept for several days in distilled water. The silicate carriers (20 g) were then mixed with 100 mL 10% aq. 3-aminopropyltriethoxysilane and the pH was quickly adjusted to 3.5 with 6*M* HCl. The suspension was gently agitated at 75°C for 2 h in a shaker. The coated carriers were filtered, rinsed with water, and dried overnight at 115°C.

INTRODUCING THE SPACER

Periodate Oxidation of Dextran

The procedure employed for the oxidation of dextran was a modification of the method of Miron and Wilchek (*16*).

Dextran (0.2 g) was stirred in 10 mL of freshly prepared 0.25*M* sodium periodate at 25°C for 3 h in the dark. The oxidized dextran was purified from an excess of $NaIO_4$ by dialysis against water.

Coupling of Dextran to the Silicate Carrier

Aminosilane coated carrier (1.5 g) was mixed with the dialyzed aq. dextran solution and kept under vigorous shaking for 2 h at 25°C. Unbound dextran was removed by carefully rinsing with water until the filtrate gave no reaction with 2.4-dinitrophenylhydrazine. The dextran coupled carriers have to be prevented from becoming dry before coupling to the enzyme.

RESULTS

Coupling of Lysozyme to the Gulsenit® Carrier

Gulsenit®-dextran carrier (Fig. 1) was added to a solution of 55 mg lysozyme in 10 mL 0.1*M* borate buffer, pH 8.0. This type of buffer is an absolute requirement. Preliminary experiments showed that, compared to other buffers of the same ionic strength and pH, the enzyme activity can be increased sevenfold when the coupling procedure is carried out in borate buffer. The spacer is probably stabilized by borate. To protect the active site of the enzyme, 20 mg *N*-acetylglucosamine were added. The coupling reaction was carried out for 20 h at 37°C. To remove unbound enzyme, the product was filtered and washed with borate buffer pH 8.0 and with 0.1*M* aq. NaCl solution until the filtrate gave no reaction with ninhydrin and no enzyme activity could be detected.

Preparation of Lysozyme Pyridino Glass Beads

Preparation of pyridino glass beads according to Pittner et al. (*17*): Controlled pore glass (Fig. 2) was coated with 3-aminopropyltriethoxysilane as already described above. The coated glass beads were treated with 250 mL 2.5% pyridine-4-aldehyde in 0.25*M* phosphate buffer pH 7.0, stirred at room temperature for 1 h, washed carefully with water, and dried in a desiccator over NaOH. The completion of the reaction was indicated by a negative test with trinitrobenzenesulfonic acid. The beads were moistened with dry dioxane and treated with BrCN (1 mg/mL absolute dioxane). After 5 min of activation 20 mL of 0.2*M* borate buffer, pH 9, were added and the mixture stirred at room temperature for an additional 20 min as the pH was kept constant by manual titration with 2*M* NaOH.

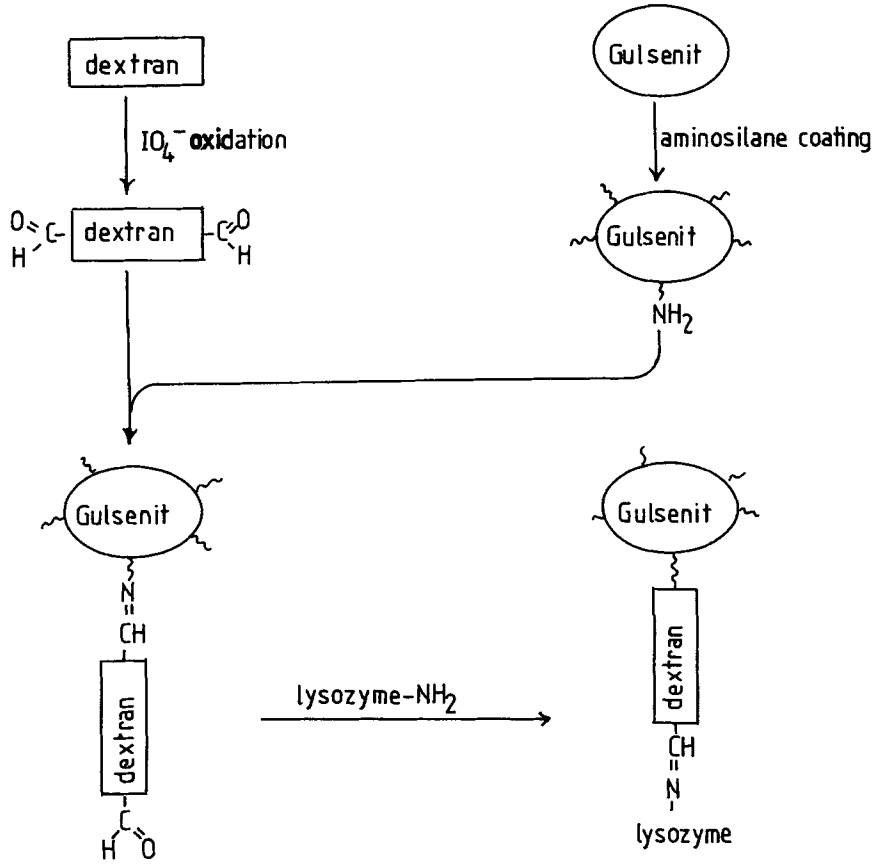

Fig. 1. Coupling of lysozyme to Gulsenit® carrier.

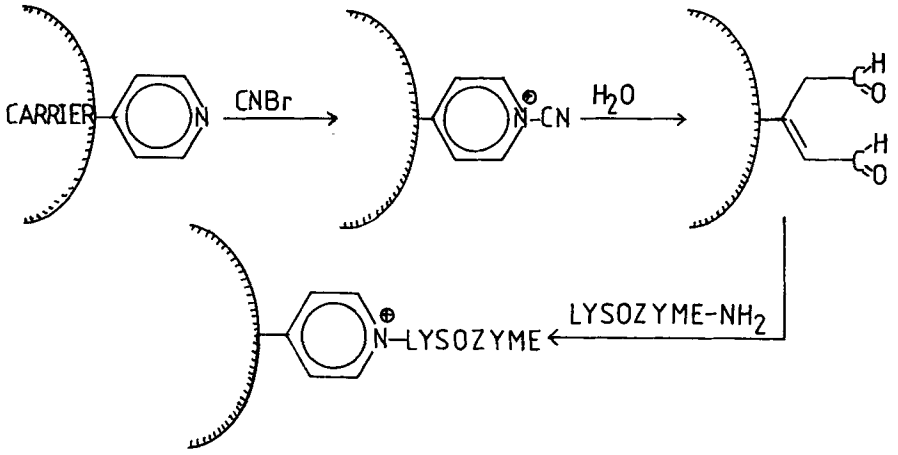

Fig. 2. Coupling of lysozyme to pyridino glass beads.

Coupling of the Enzyme

Lysozyme (350 mg) and 70g of *N*-acetylglucosamine were dissolved in 70 mL acetate buffer, pH 5. The freshly prepared activated glass beads (3.5 g) were added, and the suspension was kept under shaking for 20 h. After washing the product at pH 11, alternating washing steps at pH 5 and at pH 8 were repeated until no protein could be found in the washings.

Assay of the Immobilized Enzyme

To obtain the activities of the immobilized lysozyme, batches of 100 mg carrier were incubated under shaking at 37°C in 5 mL of a suspension, containing 0.2 mg *M. luteus*/mL phosphate buffer, pH 6.3 and an ionic strength of $I=0.1$. To correct for bacterial cell autolysis and adsorption phenomena that might influence the results, blanks contained carriers coupled to lysine instead of lysozyme. The decrease in turbidity of the bacterial suspension was measured spectrophotometrically in the supernatant at 546 nm. Substrate could be degraded within 20 min nearly quantitatively.

Immobilization of Lysozyme on Silicate Carrier Using Dextran Spacers of Different Length

The enzymatic activity and the amount of bound protein depends on the chain length of the spacer.

The amount of bound lysozyme increases with increasing spacer length, but the specific activity decreases, whereas the total activity remains approximately constant. This observation can be explained by the assumption that the macromolecular substrate reacts only with the lysozyme molecules on the surface and cannot diffuse into the dextran layer (Table 1).

Immobilization of Lysozyme on Glass Beads under Various Coupling Conditions

For activation, the pyridino glass beads were treated with varying amounts of BrCN solution (1 g/mL absolute dioxane). For the coupling procedure of the lysozyme, various buffers between pH 4 and 8 were

Table 1
The Immobilization of Lysozyme

Molecular weight of the dextran	Activity/g wet carrier	Protein bound mg/g wet carrier	Specific activity of bound lysozyme compared to free enzyme
35,600	720 U	2.2	1.5%
170,000	440 U	4.4	0.45%
2,000,000	670 U	8.17	0.42%

used. Optimal results were obtained by activation with a BrCN solution (4 mL) and coupling of the enzyme at pH 5. The amount of bound protein was 1.5–2.5 mg/g carrier and had an activity of 840–1420 U/g immobilizate. This is 2.9% specific activity of immobilized lysozyme compared to free lysozyme. If coupling was performed at higher pH, lysozyme was also adsorbed unspecifically.

DISCUSSION

Compared to some other data known from the literature (*2,4–6*) *M. luteus* could be degraded within a rather short time (about 20 min) by our immobilizates. There is also a report of lysozyme immobilized on a copolymer of styrol/vinylbenzoic acid which has a much higher activity (*7,8*). Using *M. luteus* as substrate, we could not reproduce this activity. Under the given assay condition, the suspended substrate and immobilizate cosedimented in the cuvet and could not be separated (Moser, I. and Dworsky, P., unpublished). This complicates the assay, reduces the precision, and simulates a higher activity. In accordance to this, the authors recommended to use substrates of low mol wt for the assay of the activity of the immobilizate (*8*).

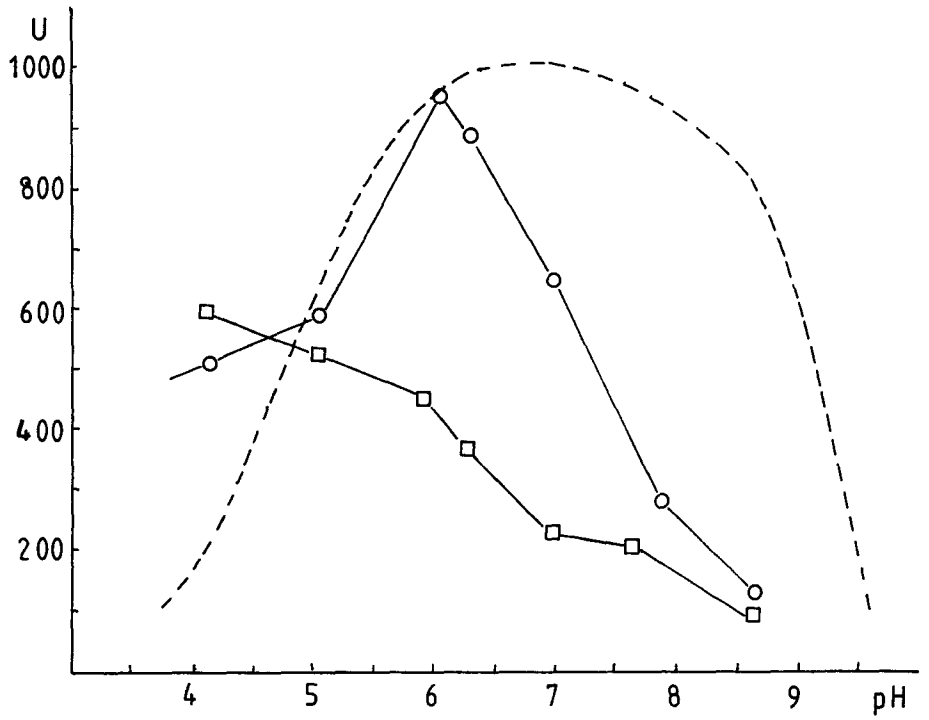

Fig. 3. pH optima of immobilized and soluble lysozyme ionic strength = 0.1; ○—○ lysozyme coupled to pyridino glass beads; □—□ lysozyme coupled in Gulsenit® carrier; and ——— soluble lysozyme.

If the lysozyme is immobilized as described in this paper, the pH optimum is shifted to lower values compared to the free enzyme (Fig. 3). This effect is probably caused by an electrical field that is generated by the positively-charged carrier. Such a field would increase the local pH in the microenvironment of the enzyme molecule and thus simulate a lower pH optimum (*18*).

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