PURIFICATION OF LYSOZYME BY AFFINITY CHROMATOGRAPHY

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1. Introduction

The ability of lysozyme to hydrolyze the $\beta(1-4)$ glycosidic linkages of chitin between the *N*-acetyl-D-glucosamine residues [1], made possible the selective adsorption and purification of the enzyme from different sources on insoluble chitin [2,3], chemically modified chitin [4,5] and chitin-coated cellulose [6]. Egg-white lysozyme was demonstrated to be inhibited by *N*-acetyl-D-glycosamine [7], with the β-anomer displaying a stronger inhibitory effect than the α-anomer [8]. In addition, the compound *p*-nitrophenyl-2-acetamido-2 deoxy-β-D-glycopyranoside was found to be unaffected by the enzyme, under conditions which hydrolyze other *N*-acetylglucosamine oligosaccharides [9]. Based on this evidence we have coupled *N*-acetyl-β-D-glucosamide to a modified Sepharose-4B matrix for the purification of lysozyme from several sources by affinity chromatography.

2. Materials and methods

The *N*-acetyl-β-D-glucosaminide-Sepharose-4B derivative was prepared as previously described [10]. The method consisted of activating Sepharose-4B with CNBr and coupling the extension arm 3,3'-diaminodipropylamine which was then succinylated. *p*-Nitrophenyl-*N*-acetyl-β-D-glucosaminide was hydrogenated under pressure and the *p*-aminophenyl derivative thus obtained was attached to the modified Sepharose matrix using 1-ethyl-3-(3-dimethylamino propyl) carbodiimide (EDC) as a condensing agent. Unreacted carboxyl groups on the matrix were blocked with 2-aminoethanol in the presence of EDC.

Analysis of the derivatives by microdetermination of Kjeldahl nitrogen [11] was done on samples extensively washed with pH 5.0 and 8.5 buffers, increased ethanol concentration from 70 to 100% and dried at 60°C for 12 hr under vacuum. The derivatives contained 9-13 μmoles of glycoside/ml of wet packed Sepharose. Chicken egg-white was obtained from a bakery supply company and was used without further purification. Human saliva and human milk was collected fresh and stored at −20°C. All crude lysozyme materials were centrifuged at 4°C for 30 min and at 20 000 *g* prior to storage. Human milk was previously defatted to prepare whey by adjusting the pH to 4.5 with 0.1 M acetic acid [12].

Chromatographic polypropylene columns of 4 × 0.7 cm (Bio-Rad Labs, Richmond, California) were prepared with the affinity adsorbent and were equilibrated in 0.05 M Na-acetate pH 5.2 (Buffer A) at 25°C. The crude extracts were loaded on the columns in the same buffer and one ml fractions were collected. Eluted protein was monitored by adsorption at 280 nm and the activity of lysozyme determined by the rate of lysis of Micrococcus lysodeikticus [13]. One unit of enzyme is equal to a decrease in absorbancy, at 450 nm of 1.0 per minute at pH 7.0 and 25°C. Protein concentration was determined by the method of Lowry et al. [14] using BSA as a standard.

3. Results and discussion

3.1. *Egg-white-lysozyme*

Egg-white solution (0.5 ml) in buffer A was loaded on an affinity column at a concentration of

Table 1
Purification of lysozyme from several crude extracts by affinity chromatography

Source	Enzyme Loaded total units	Recovery (%)	Enzyme Recovered Sp. Act., U/mg	Purification fold
Egg White	25.0	102	9.8	20.3
Human Saliva	1.5	101	5.4	90.0
Human Milk	5.2	87	44.0	49.0

2.1 mg/ml. The unadsorbed protein was washed out with 0.05 M Tris–HCl, pH 8.2, containing 0.1 M NaCl (buffer B) and further washed at pH 9.6 with 0.05 M Na-carbonate-bicarbonate (Buffer C). The enzyme was finally eluted with 0.1 M Na-carbonate bicarbonate pH 9.6 containing 0.3 M NaCl (Buffer D). The enzyme was completely recovered with a 20.3-fold purification and was at the level of chromatographic purity [15] when compared to the specific activity of 2X crystallized commercial lysozyme (Worthington, Freehold, N.J.). The results are shown in Table 1 and fig.1. Following these results, human lysozymes were selected for purification on this adsorbent since Jollès et al. [16] had shown that lysozyme from human milk and leucocytes are more strongly inhibited by *N*-acetylglucosamine than the hen enzyme.

3.2. *Human saliva lysozyme*

Five ml of saliva were adjusted to pH 5.2 with 1 ml of 0.3 M Na-acetate buffer to achieve the final concentration of buffer A, and were loaded on a column. The unadsorbed protein was eluted with buffer B and the enzyme recovered with buffer D. A 90-fold purification with 100% recovery resulted (see Table 1 and fig.2).

3.3. *Human milk lysozyme*

Five ml of milk were adjusted to pH 5.2 as indicated for the saliva extract. The material was loaded on a column and washed with buffers B and C and the lysozyme eluted with buffer D. There was an 87% recovery with a 49-fold purification.

Continuous washing of the columns with buffer containing 1 M NaCl did not elute additional protein.

With 0.1 M Na-acetate, pH 4.0 and 0.2 M NaCl the enzymes are fully recovered as was found by Jensen et al. [3]. As seen, pH and ionic strength can be manipulated to desorb the enzyme. Optimal loading capacity was found in the range of 5.0 and 8.0. When loading the chicken lysozyme in 0.1 M Tris–HCl, pH 8.5, 10% of the activity leached out with the unadsorbed protein. In the case of the human enzymes the purification was not complete, i.e., the milk lysozyme was purified by other investigators [17] to an extent of 2000 X. The partial purification was probably due

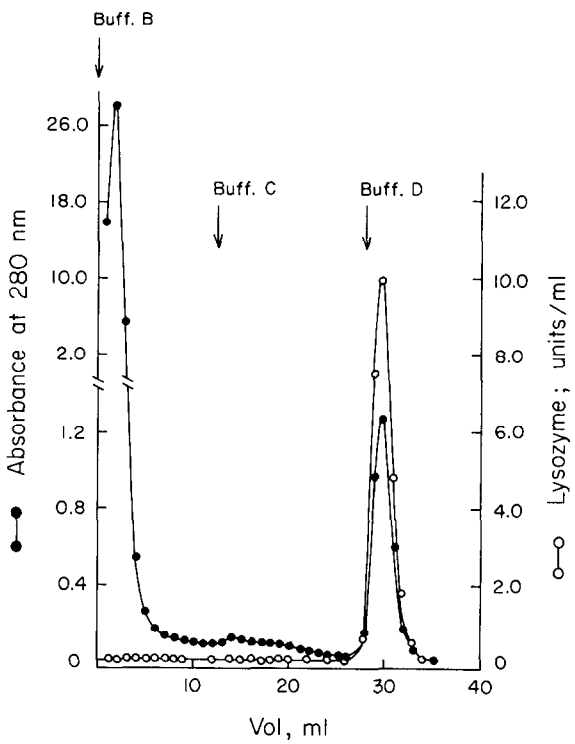

Fig.1. Affinity chromatography of chicken egg white lysozyme. The adsorption of lysozyme from a crude egg white preparation and its elution from the column is described in the text. The arrows indicate the change of buffer.

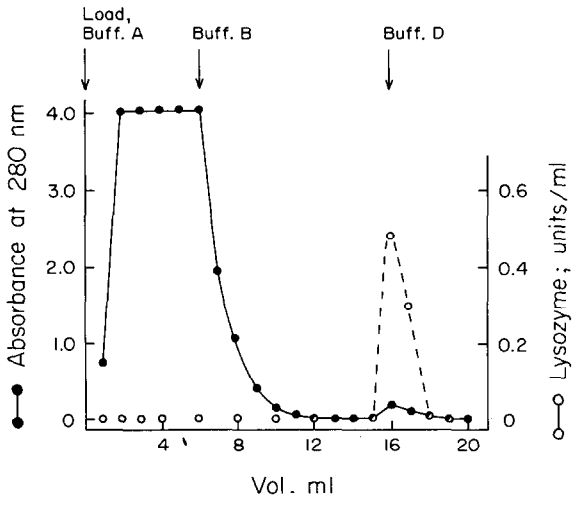

Fig.2. Affinity chromatography of human saliva lysozyme. The chromatographic procedures are described in the text.

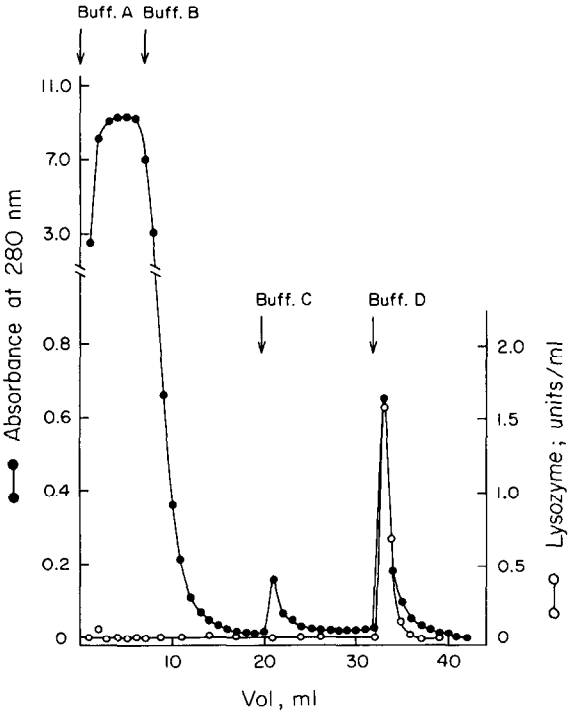

Fig.3. Affinity chromatography of human milk lysozyme. The chromatographic procedures are described in the text.

to the adsorption of other proteins present in milk or saliva which stereospecifically recognize the *N*-acetyl-β-glycosidic moiety [10]. The purification might be improved by addition to the eluant of *N*-acetylglucosamine or oligomers of hydrolyzed chitin (K_M: 10^{-5} to 10^{-8} M) which would preferentially interact with the multiple specific sites on the lysozyme molecule [18].

Acknowledgements

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