Journal of Chromatography, 210 (1981) 469–475
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,712

STUDY OF THE MOLECULAR INTERACTION BETWEEN LYSOZYME AND HEPARIN AND APPLICATION IN AFFINITY CHROMATOGRAPHY

E. BOSCHETTI*, P. GIROT and J. P. SECHERESSE
Réactifs IBF, 92390 Villeneuve-la-Garenne (France)
and
J. SAINT BLANCARD
CTSA, Laboratoire de Biochimie, 92141 Clamart (France)
(First received December 11th, 1980; revised manuscript received February 4th, 1981)

SUMMARY

Lysozyme interacts with heparin by giving a stable and insoluble complex that dissociates at increased ionic strength. The formation of the complex and its stability towards pH and sodium chloride concentration were studied in solution and in a heterogeneous phase by means of heparin immobilized on agarose beads. The two sets of results are in agreement and show that the interaction between heparin and lysozyme is essentially ionic. The practical consequence is the possibility of developing a one-step method of purification of egg-white lysozyme by affinity chromatography with a particularly high yield of biochemical activity.

INTRODUCTION

Heparin is a natural mucopolysaccharide that has the property of combining with a large number of natural and synthetic products[1]. Thus, it can combine with polycations, stains, alkaloids, certain antibiotics and certain proteins, *e.g.*, some coagulation factors or enzymes such as lysozyme. The complexes obtained must be distinguished into two groups: those which originate from non-specific interactions and those which are highly specific such as the heparin–fibronectin recently exploited for the isolation of the heparin-binding site of fibronectin[2]. This paper deals with non-specific interactions of heparin.

Lysozyme is an enzyme that lyses bacterial cell walls and also interacts with natural products, particularly polysaccharides[3]. Some of these affinities, such as that recently described for agarose[4], can be explained by the similarity of these polysaccharides to the natural substrates of lysozyme.

Heparin inhibition of lysozyme activity has been known since 1948[5]. Initial studies *in vitro* utilized a turbidimetric assay[6], but no attempt was made to elucidate the mechanism of action. Subsequently, Kerby and Eadie[7] attempted to demonstrate that this inhibition satisfied the admitted criteria of competitive inhibition. The inhibitory action of lysozyme *in vivo* on the anticoagulant properties of heparin was

0021-9673/81/0000–0000/$02.50

reported by Violle[8], who observed that coagulation power ceased when lytic action had disappeared and concluded that both properties arose from the same enzymatic entity. In 1953, Dumazert and Ghiglione[9] tried to demonstrate that this increased coagulation of blood *in vivo* was due to an antagonism that could be explained by the chemical affinity of the anionic groups on heparin for the basic groups on lysozyme. This hypothesis was based on the observation that when an excess of lysozyme was added to an aqueous heparin solution *in vitro* a flocculent precipitate formed, whereas the supernatant lost its coagulating activity. Nevertheless, as heat inactivation of lysozyme determined the disappearance of its coagulating effect *in vivo*[10], it was assumed that the anti-heparin action of lysozyme depended on its known mucolytic activity. In addition, when lysozyme was added to heparinized plasma[11], its behaviour was not consistent with the hypothesis of stoichiometric interaction between heparin and the enzyme.

In spite of the importance of a heparin–lysozyme interaction, this work was not continued since these early reports. The present results include a study of the formation of the heparin–lysozyme complex. The nature of the interactions was studied in aqueous solution and also in a heterogeneous reaction with heparin immobilized on a gel. This work has enabled us to propose a simple chromatographic technique for the purification of lysozyme from egg white.

EXPERIMENTAL

Heparin (170 USP units/mg), egg-white lysozyme (20,000 I.U./mg), heparin–Ultrogel A4R and N,N′-diacetylchitobiose were supplied by Réactifs I.B.F. (Pharm-industrie, Villeneuve-la-Garenne, France). Bacterial (*Micrococcus luteus*) cell walls were purchased from Sigma (St. Louis, MO, U.S.A.). Other reagents used were of analytical-reagent grade.

Succinylation of lysozyme

The method used was that reported by Gunther *et al.*[12]. Lysozyme (2 g) was dissolved in 200 ml of saturated sodium acetate solution at 20–25°C, succinic anhydride (600 mg) was added and mixing was continued for 2 h at 25°C. The solution was then dialysed against deionized water and the resulting solution was lyophilized.

The succinylation reaction was repeated under identical conditions. Residual enzymatic activity was determined after lyophilization according to the method of Shugar[13].

Acetylation of lysozyme

Lysozyme (2 g) was dissolved in 200 ml of saturated sodium acetate solution at 20–25°C, acetic anhydride (2 ml) was added and mixing was continued for 2 h. The reaction mixture was then exhaustively dialysed against deionized water and subsequently lyophilized.

The reaction was repeated under identical conditions and residual enzymatic activity was determined after lyophilization according to the method of Shugar[13].

Heat denaturation of lysozyme

Lysozyme (100 mg) was dissolved in 10 ml of deionized water and the solution was heated at 80°C for 20 h. Assay using the method of Shugar[13] showed no activity.

Heparin-Ultrogel A4R affinity chromatography

These affinity chromatography experiments were performed with 16 mm I.D. columns. The buffered heparin–Ultrogel A4R suspension was poured into the column after removing dissolved gases under reduced pressure. The gel was then washed and equilibrated with phosphate buffer, acetate buffer or Tris–hydrochloric acid (see legends of Figs. 3 and 4). After loading the samples (lysozyme or egg white) on the column, elution was carried out with a linear sodium chloride concentration gradient, the slope of which was set at 20 m*M*/cm.

RESULTS AND DISCUSSION

When aqueous solutions of heparin and lysozyme are mixed, an insoluble complex forms. The purpose of these experiments was to determine the nature of the interactions leading to the formation of this complex. In practice, these insoluble lysozyme–heparin complexes are obtained by adding increasing amounts of lysozyme to a 0.2 mg/ml solution of heparin. After a 30-min incubation, the precipitate is removed by centrifugation and the supernatant is retained for absorbance measurements at 280 nm, used to determine the amount of residual lysozyme not involved in the insoluble complex.

The lysozyme–heparin interaction depends on the pH and ionic strength of the reaction medium. Fig. 1 shows the formation of the complex as a function of lysozyme concentration and buffer. It is noteworthy that the complex formation is almost quantitative at low ionic strength (0.05 *M* acetate and 0.05 *M* Tris–hydrochloric acid buffers). When using a high ionic strength buffer (0.05 *M* Tris–hydrochloric acid + 0.15 *M* sodium chloride), however, the complex does not form in the presence of low concentrations of lysozyme. This observation led us to study the effects of pH and ionic strength of the buffer on the formation of the insoluble complex.

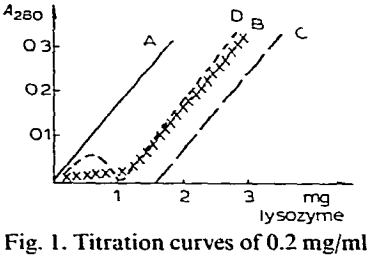

Fig. 1. Titration curves of 0.2 mg/ml of heparin with egg-white lysozyme. Heparin concentration 175 USP units/mg; lysozyme used at 20,000 I.U./mg. A, No heparin; B, 0.05 *M* acetate buffer (pH 4.5); C, 0.05 *M* Tris–HCl (pH 7.4); D, 0.05 *M* Tris–HCl (pH 7.4) containing 0.15 *M* NaCl.

The results are presented in Fig. 2, where it can be seen that there is a zone of pH from 2 to 10 in which the complex forms. Although this pH range for complex formation is wide, the stability of the complex towards ionic strength is low at high pH values and is high in an acidic medium. If the sodium chloride concentration is greater than 0.15 *M* at pH 10 the complex is resolubilized, whereas at pH 3 solubilization occurs only at sodium chloride concentrations greater than 0.7 *M*.

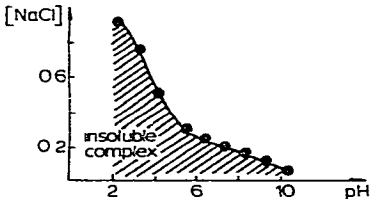

Fig. 2. Curve of lysozyme–heparin dissociation as a function of NaCl concentration; 0.2 mg/ml of heparin at 170 USP units/mg. Chicken egg-white lysozyme used at 20,000 I.U./mg. Temperature, 20°C.

Identical results were obtained when the experiments were performed in the presence of 0.01 *M* N,N′-diacetylchitobiose. It can therefore be concluded that under these conditions the interactions leading to complex formation are principally ionic, especially as N,N′-diacetylchitobiose (a specific inhibitor of lysozyme activity) does not behave as a competitor for heparin.

The studies were extended to the heterogeneous phase, using heparin immobilized on an agarose gel (heparin–Ultrogel A4R) packed in a column. Consistent with the results obtained in the homogeneous phase, lysozyme which is adsorbed to immobilized heparin can be dissociated by eluting with a high ionic strength buffer.

The nature of column experiments was such that residual lysozyme could not be determined by absorbance measurements at 280 nm in the supernatant. We therefore determined the molarity of sodium chloride necessary to elute the adsorbed enzyme. In this series of experiments, we studied the behaviour of succinylated, acetylated and heat-denatured lysozyme. These modified enzymes could be studied only in the heterogeneous phase, as no insoluble complex is formed in homogeneous aqueous solution.

The results of these experiments are shown in Fig. 3. The curve obtained for the native enzyme is similar to that in homogeneous medium (Fig. 2). The molarity of sodium chloride required to elute the adsorbed enzyme is higher with decreasing pH. Succinylated and acetylated lysozyme behaved similarly, but at lower ionic strengths than those producing the equivalent result with the native enzyme.

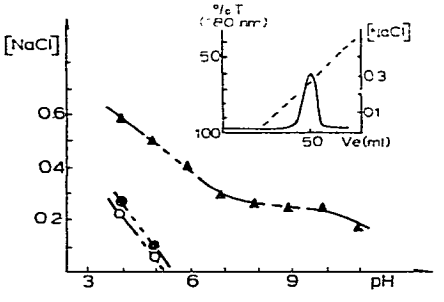

Fig. 3. Curves of lysozyme dissociation from heparin–Ultrogel A4R as a function of NaCl concentration and pH. ▲, Native lysozyme; ●, acetylated lysozyme; ○, succinylated lysozyme. All of the points on the curves were obtained by successive affinity chromatography on a heparin–Ultrogel column (see insert): column, 16 × 30 mm I.D.; sample, 10 mg of lysozyme in 1 ml of buffer; starting molarity of buffers (acetate, phosphate or Tris–HCl), 0.01 *M*; NaCl elution gradient, 0–1 *M*; flow-rate, 60 ml/h.

It may also be noted that at constant pH the sodium chloride concentration leading to complex dissociation decreases as the isoelectric point of the enzyme decreases.

More extensive results, including the behaviour of heat-denatured lysozyme, are given in Table I. These data may be interpreted on the basis of primarily ionic interactions, thus confirming our observations in the homogeneous phase. This relatively strong interaction is probably related to the cationic charge density of the enzyme and the complementary anionic charges of heparin. In this context it should be remembered that when the structure of the enzyme is modified in such a way as to leave its overall ionic charge intact (heat denaturation or treatment with dissociating agents such as urea or guanidine hydrochloride), it no longer complexes with heparin[14].

TABLE I

BEHAVIOUR OF NATIVE AND MODIFIED LYSOZYMES ON HEPARIN–ULTROGEL A4R

Lysozyme	*Specific activity (I.U./mg)*	*NaCl concentration required to elute adsorbed enzyme (M)*			
		pH 4	*pH 5*	*pH 6*	*pH 7*
Native ($pI \approx 10.5$)	20,000	0.6	0.5	0.4	0.3
Acetylated ($pI \approx 6$)	4000	0.25	0.1	N.A.*	N.A.
Succinylated ($pI \approx 4$)	3500	0.22	0.05	N.A.	N.A.
Denatured at 80°C**	0	0.13	N.A.	N.A.	N.A.

* N.A. = Not adsorbed.

** The isolectric point of heat-denatured lysozyme was not determined.

As a result of this strong interaction and the behaviour of lysozyme on heparin–Ultrogel A4R, we decided to exploit the present results for the preparation of egg-white lysozyme by affinity chromatography. The starting product was crude egg white treated in acidic medium and the experimental conditions chosen were those used for pure lysozyme.

Experiments were run at various pH values and elution was carried out in each instance with linear sodium chloride gradients, *i.e.*, elution with increasing ionic strength. The results are summarized in Fig. 4 and Table II. Although the interaction is stronger at lower pH values (the enzyme is eluted at a sodium chloride concentration greater than 0.6 *M*), the peak eluted at pH 4 and containing all enzymatic activity also contains at least one other protein. This is not surprising, as the polyanion support can adsorb a number of proteins by ion exchange. At higher pH values, the enzyme is eluted at lower sodium chloride concentration and can be totally separated from contaminants, thus yielding a very high specific activity (see Table II).

The method of affinity chromatography for the preparation of lysozyme from egg white is important for several reasons in addition to that of basic research. Lysozyme is currently produced by non-specific fractionation methods, such as fractional precipitation, purification on an adsorbent[15] or crystallization[16]. These conventional procedures are time consuming, often lead to losses of activity and gener-

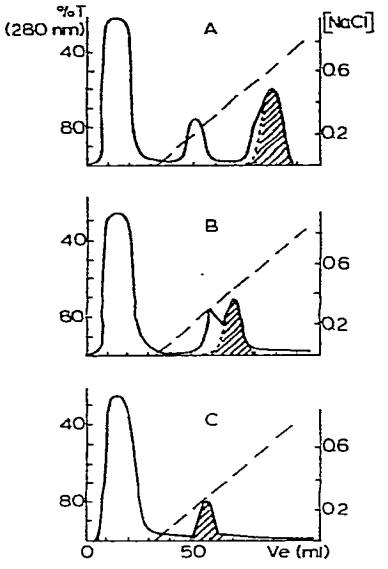

Fig. 4. Preparation of egg-white lysozyme by affinity chromatography on heparin–Ultrogel A4R at different pH values. Column, 16 × 37 mm I.D.; sample, 50 mg of egg-white protein; buffers, 0.05 *M* sodium acetate buffer pH 4 (A) and pH 6.2 (B) and 0.01 *M* phosphate buffer pH 7.4 (C); NaCl elution gradient, linear from 0 to 1 *M*; flow-rate, 75 ml/h; temperature, 22°C. The hatched portion indicated the presence of lysozyme activity.

TABLE II

AFFINITY CHROMATOGRAPHY PURIFICATION OF LYSOZYME FROM EGG WHITE

50 mg of proteins were used. Elution was carried out with a linear NaCl concentration gradient from 0 to 1 *M* in all instances.

pH	*NaCl concentration for elution (M)*	*Lysozyme obtained (mg)*	*Total activity (I.U.)*	*Specific activity (I.U./mg)*	*Yield (%)*	*Purification factor*
4	0.6	6.4	38,400	6000	76.8	6.0
6.2	0.4	6.1	39,650	6500	79.2	6.5
7.4	0.25	2.1	42,000	20,000	84.0	20.0

ally give low yields. It is therefore understandable why lysozyme producers have attempted to improve this type of preparation and reduce the number of steps involved. In 1974, Sternberg and Herschberger[17] proposed a method of selective precipitation with water-soluble polyacrylic acid; in the same year, Yoshimoto and Tsuru[18] proposed an affinity chromatographic process for the first time. This process involved the immobilization of a *Micrococcus luteus* cell wall hydrolysate on an agarose-based support.

In spite of the interest of these developments, it was difficult to industrialize the first process because of the complexity involved in the recovery of polyacrylic acid. In

the affinity chromatographic procedure, the problems were the preparation of large amounts of cell wall hydrolysate, the immobilization and the periodic renewal of the moderately stable chromatographic support.

The process described here has the following advantages: a scientific approach to the separation of lysozyme, the fact that it is a one-step operation, the yields are high and the support is commercially available, is extremely stable and is easily regenerated. Even though these advantages are partially counterbalanced by the very large amounts of egg-white lysozyme produced annually, they should nonetheless be considered for the preparation of the enzyme from other human biological fluids, such as urine, saliva, placenta, maternal milk and leukocytes.

ACKNOWLEDGEMENT

We thank Dr. M. Monsigny for his recommendations and useful comments during the preparation of this manuscript.

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