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HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY OF HUMAN THROMBIN ON MODIFIED POLYSTYRENE RESINS

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SUMMARY

Cross-linked polystyrenes modified with L-arginyl methyl ester mimic the binding site of antithrombin III and thrombin substrates. They can be used as stationary phases in high-performance affinity chromatography of thrombin. Under isocratic conditions, thrombin is strongly adsorbed on the resins when the sodium chloride concentration is lower than 0.5 *M*. The bound enzyme can be selectively desorbed when the salt concentration is raised to about 1.2 *M*. With a linear salt gradient, the specific elution of thrombin can be effected with a high recovery of its enzymatic activity. The decomposition products of thrombin, when treated with sodium dodecyl sulphate, are not retained by the stationary phase. The effects of the flow-rate and salt gradient slope on the adsorption and desorption of α-thrombin demonstrate the importance of kinetic parameters.

INTRODUCTION

Insoluble polystyrenes, substituted with L-arginyl methyl ester (PAOM), mimic the reactive binding site of antithrombin III and thrombin substrates towards thrombin[1,2]. Such polymers have a highly specific affinity for thrombin. It was observed that the binding of the enzyme is reversible[3,4]. Consequently, these resins were used as stationary phases in low-pressure affinity chromatography for the separation of purification of the active enzyme[5,6]. The highly purified thrombin was reversibly bound to the stationary phase and then eluted with a salt gradient. When human prothrombin complex concentrate was activated, pure thrombin was isolated by the same procedure in high yield and with a high specific activity.

The present paper describes the extension of this method to high-performance

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liquid chromatographic (HPLC) conditions. The polystyrenes possess suitable mechanical properties and, therefore, they can be used as stationary phases in high-performance affinity chromatography (HPAC) of thrombin. The elution behaviour of α-thrombin and of its decomposition products was studied. The active enzyme was first adsorbed on the resins and then eluted by raising the ionic strength of the eluent. The mechanism of the interaction between thrombin and these resins is discussed. The influence of kinetic parameters on the adsorption and desorption process is also demonstrated. Our study has led to a definition of the experimental conditions for the selective elution of thrombin in HPAC.

EXPERIMENTAL

The modified resins were prepared in two successive steps, as previously described. First, polystyrene, cross-linked with 2% divinylbenzene (Bio-Beads SX-2, 200–400 mesh) was chlorosulphonated[7]. Then, the beads were substituted with L-arginyl methyl ester[6]. The structure and composition of the resin (PAOM 20) are shown in Fig. 1. The affinity constant, K_T, between PAOM 20 and thrombin was determined from a Langmuir isotherm to be about $4 \cdot 10^6\ M^{-1}$.

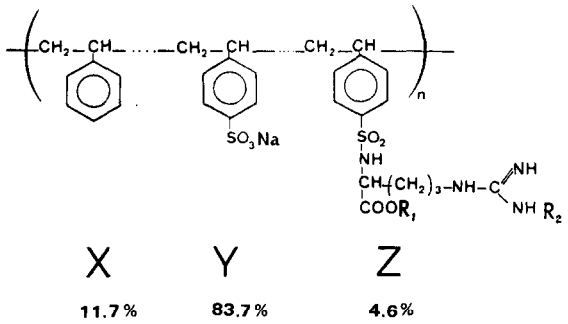

Fig. 1. Structure of polystyrene substituted by L-arginyl methyl ester.

The resins were successively washed with 2 *M* aqueous sodium chloride solution and Michaelis buffer (pH = 7.4) and then equilibrated in the chromatographic eluent, 0.05 *M* phosphate buffer + 2 *M* sodium chloride (pH = 7.4). The fine particles were eliminated by flotation. The diameter of the dry beads was *ca.* 50 μm. The chromatographic columns (25 cm × 0.7 cm I.D.) were packed by the slurry method with a suspension of 2 g of resin in the eluent. The column was equilibrated for several hours by elution at a flow-rate of 1 ml/min.

The HPLC apparatus consisted of a three-head (120°C) chromatographic pump (Merck LC21B), connected to a Rheodyne 7126 injection valve (sample loop 100 μl). A variable-wavelenght UV-VIS detector Merck-LC 313 and the gradient system were connected to an Epson QX-10 computer. The chromatographic signal was monitored, integrated and stored by the computer. All the equipment was provided by Merck-Clevenot (Nogent-sur-Marne, France).

The eluents were generally aqueous phosphate buffers of various pH values

and salt concentrations. The solutions were prepared from doubly distilled water, which was degassed and then filtered through a Type HA 0.45-μm membrane (Millipore, Velizy, France).

Highly pure α-thrombin (3000 U/mg) was kindly provided by M. C. Boffa (Centre National de Transfusion Sanguine, Paris, France); other proteins were supplied by Sigma (St. Louis, MO, U.S.A.). The decomposition products of α-thrombin were obtained by incubating the active enzyme at 50°C for *ca.* 2 h. A 2% sodium dodecyl sulphate (SDS) solution was necessary to dissociate the fragments obtained.

The α-thrombin sample (40–100 μl corresponding to 25–70 μg of pure enzyme) was injected into the HPLC system at 25°C. The proteins were allowed to interact with the stationary phase at low ionic strength. The absorbed α-thrombin was then eluted by raising the salt concentration of the eluent. The absorbance was detected at 230 nm. The chromatographic fractions were collected and then dialyzed against 0.15 *M* sodium chloride solution for several hours. The recovery of biological activity was determined by comparing the thrombin clotting time of human platelet poor plasma with these fractions or with that of the starting material[5].

RESULTS AND DISCUSSION

Elution of α-thrombin

Prior to the analysis of α-thrombin, some plasma proteins [albumin, immunoglobulin (IgG), antithrombin III] were injected into the column in order to test the chromatographic selectivity. Under isocratic donditions no retention was observed, even at 0.1 *M* sodium chloride, indicating that the resins exhibit only a poor affinity for these human proteins.

When the highly active enzyme was chromatographed under isocratic conditions, thrombin was strongly adsorbed from 0.1 *M* sodium chloride–phosphate buffer. Only a small loss of thrombin during this washing step was observed. However, a significant amount of enzyme was eluted with 0.5 *M* sodium chloride solution. When the ionic strength of the eluent was higher than 1.0 *M* sodium chloride the resin was unable to retain the protein.

A typical chromatogram of α-thrombin, under gradient-elution conditions, is shown in Fig. 2. This elution profile demonstrates that thrombin is strongly adsorbed at 0.1 *M* sodium chloride and can be desorbed by using a linear salt gradient from 0.1 to 2 *M* sodium chloride. Under these conditions, the salt concentration corresponding to the maximum of the peak is around 1.7 *M*. It should be noted that this salt concentration is related to the strength of the interaction, between thrombin and the particular PAOM resin. Therefore, it varies with the physicochemical characteristics of the resins. However, the kinetic aspects of adsorption and desorption processes are also important and can thus modify this elution profile.

The eluted fractions were collected and then dialyzed against 0.15 *M* sodium chloride aqueous solution for several hours at 4°C. The thrombin time of these dialyzed fractions was measured and compared with that of the starting sample. The recovery of the purification process, expressed as a percentage, could be calculated from a calibration curve[4]. It was generally between 60 and 70%. A loss of enzyme activity was unavoidable, since the separation was performed at room temperature. The recovery could be improved considerably if elution were effected at 4°C.

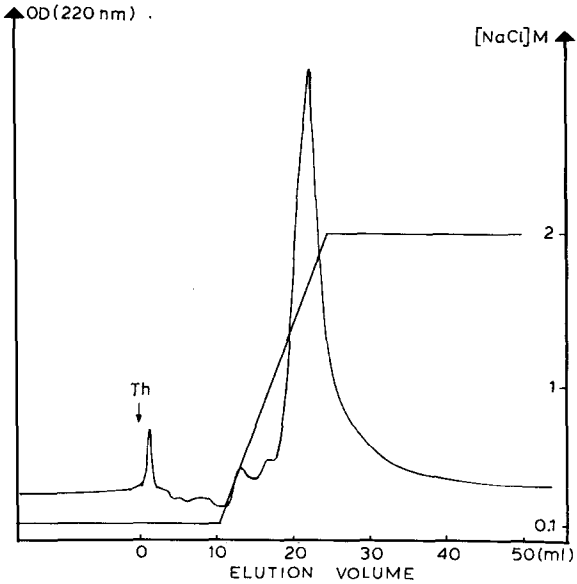

Fig. 2. Elution of human α-thrombin at 25°C on PAOM 20 resin with a linear gradient from 0.1 to 2 *M* sodium chloride. Eluent: 0.05 *M* phosphate buffer (pH = 7.4); flow-rate, 1 ml/min.

Elution of decomposition products of thrombin

When the enzyme was incubated at 50°C for 2 h without any protein detergent, the elution profile of the active form was not significantly altered. However, when 2% sodium dodecyl sulphate (SDS) solution was added to the incubated mixture, after the same incubation, the bulk of the decomposition products was not retained by the stationary phase. A small amount of products was eluted in two small peaks by 0.5 and 1.7 *M* sodium chloride, respectively.

It is well known that thrombin is thermally unstable, and very easily degraded to several inactive macromolecular fragments[8]. These fragments remain associated by non-covalent interaction unless the mixture is treated by dissociating agents (SDS, urea, etc.). Therefore, in our experiments, the fragments of thrombin were associated and the mixture exhibited an affinity for PAOM, similar to that of the native form of thrombin. However, when these different forms were dissociated with SDS, the inactive fragments could be eluted, indicating a weak interaction with the resin.

It can be assumed that the PAOM resin has a specific affinity for either the active form of thrombin or its non-covalently associated decomposition products. In contrast, the dissociated fragments lose nearly all affinity for the resin. These preliminary results for degraded thrombin suggest that not only the specific sites of thrombin but also its non-specific polypeptide sequences are involved in the enzyme–resin interactions[9,10] and that a cooperative interaction of these different sites is probably predominant. These results need to be confirmed by experiments using pure inactive forms of thrombin.

TABLE I

INFLUENCE OF pH ON THE ADSORPTION AND THE DESORPTION OF ACTIVE THROMBIN

A_1, A_2 = peak areas at 0.1 and 2 *M* sodium chloride; C_{max} = sodium chloride concentration corresponding to the peak maximum in the linear gradient from 0.1 to 2 *M* sodium chloride.

pH	A_2/A_1	C_{max} *(M)*
5.5	3.8	0.87
6.5	4.7	1.1
7.4	2.5	1.2
8.5	2.4	1.17
9.5	2.1	1.05

Effect of the eluent pH

The amount of thrombin strongly bound to the stationary phase decreased with increasing pH. The ratio of the surface areas corresponding to the two peaks eluted by 0.1 and 2 *M* sodium chloride respectively showed that the enzyme was more strongly adsorbed when the pH is lower than the p*I* (7.3) of thrombin (Table I). This can be explained by the presence of positive charges on the protein at pH < p*I*, whereas the PAOM resins have a net negative charge in the same pH range, favouring non-specific interactions. The effect of pH on the desorption, under gradient conditions, is demonstrated in Table I and Fig. 3. The salt concentration corresponding to the elution peaks reaches a maximum at pH 7.4. At this (physiological) pH, the specific sites of thrombin are probably more accessible to the binding sites of the modified polymer. These effects of pH confirm the results previously obtained[5]. The specific enzyme–resin interactions are more important than the non-specific in-

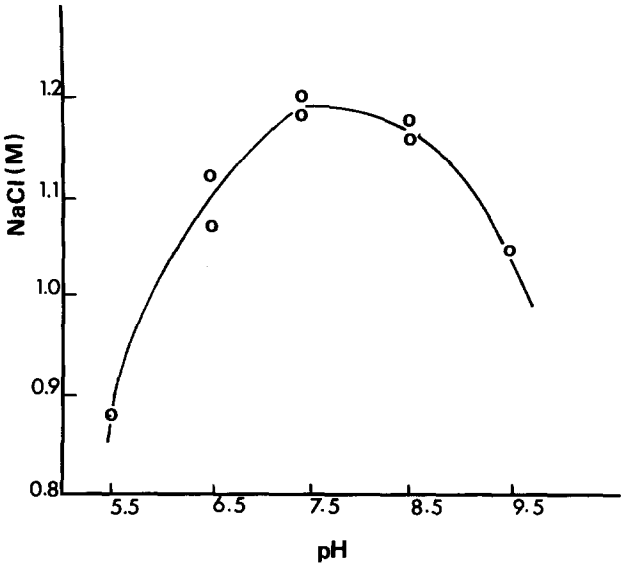

Fig. 3. Influence of pH on the desorption of active enzyme from PAOM 20 resin at 25°C. Conditions as in Fig. 2.

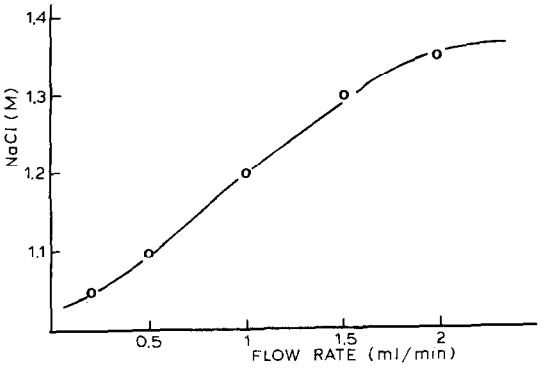

Fig. 4. Influence of flow-rate on the desorption of active thrombin from PAOM 20 resin at 25°C. Conditions as in Fig. 2.

teraction at pH = p*I*. At other pH values these non-specific interactions can play a predominant rôle in the adsorption process, particularly at a low ionic strength and pH.

Influence of kinetic effects

The influence of the flow-rate and gradient slope on protein elution clearly demonstrates that kinetic effects are essential in the interaction process. The amount of thrombin adsorbed on the stationary phase increased when the flow-rate was decreased from 2 to 0.2 ml/min. In the desorption process, a similar effect is observed, as shown in Fig. 4. The sodium chloride concentration, corresponding to the elution

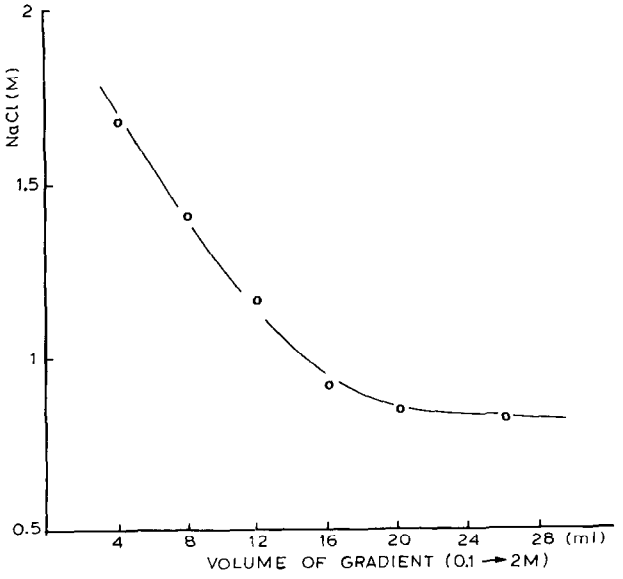

Fig. 5. Influence of the volume corresponding to the gradient on the desorption of active thrombin from PAOM 20 resin at 25°C. Conditions as in Fig. 2.

peak, increased with the flow-rate. This result indicates that the desorption of the enzyme becomes increasingly difficult as the flow-rate increases.

Finally, the volume of eluent corresponding to the gradient also influences the chromatographic behaviour (Fig. 5). When this volume was increased from 4 to 26 ml at the same flow-rate, the salt concentration corresponding to the peak maximum progressively decreased and reached an equilibrium value. The results indicate that the desorption process is relatively slow. When the gradient is too steep, the enzyme remains adsorbed in spite of the increased ionic strength. It can be concluded, therefore, that the kinetic parameters are of great importance in the HPAC of thrombin.

CONCLUSION

Our chromatographic procedure offers a number of advantages for investigating the adsorption and desorption of thrombin on modified polymers. Analysis of this enzyme is performed with very small quantities and in a short time, minimizing losses of biological activity. Both the specific and non-specific binding sites of the enzyme are involved in the enzyme-resin interactions. The high specificity of thrombin toward the PAOM resins requires a cooperative binding of these different sites.

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