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AFFINITY CHROMATOGRAPHY OF THROMBIN ON MODIFIED POLYSTYRENE RESINS

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SUMMARY

Insoluble polystyrenes substituted with sulphonate and L-arginyl methyl ester (PAOM) present substituents mimicking the reactive binding site of antithrombin III. These materials have a specific affinity for thrombin. The binding of the enzyme is reversible and the eluted thrombin remains active. Consequently, these resins can be used as stationary phases in affinity liquid chromatography in order to purify thrombin with a high biological activity. The influence of different characteristics of such polymers (substitution ratio, average particle size, affinity constant, synthesis conditions) on the purification performance is studied. Human prothrombin complex concentrate is activated and applied onto the gel. A purified human thrombin of high specific activity is separated with a high recovery of biological activity of the enzyme.

INTRODUCTION

It is well known that antithrombin III (ATIII) interacts strongly and specifically with thrombin (Th) in the coagulation system. The mechanism of this

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interaction is probably the irreversible formation of a stoichiometric complex ATIII—Th, requiring an arginyl site in the structure of ATIII for binding with a seryl group of the enzyme [1, 2]. The affinity of thrombin for other different ligands has been used previously for thrombin purification by affinity chromatography [3—5].

Moreover, it was previously demonstrated [6—7] that polystyrenes modified with L-arginyl methyl ester (PAOM) are insoluble materials mimicking the binding site of ATIII. The resins present antithrombic properties correlated to their specific interactions with thrombin in solution. The specific binding site of these materials can be used in affinity chromatography to purify or remove the enzyme. The binding of thrombin is reversible and the protein is eluted with a high recovery of its biological activity [8].

The present paper describes the synthesis of these stationary phases. The influence of the different characteristics of the resins on chromatographic performance is studied. Finally, these grafted polymers are used to purify thrombin from activated human prothrombin complex concentrate. Both the yield of the purification process and the specific activity of the purified protein are presented.

EXPERIMENTAL

Preparation of the stationary phases

The starting material is spherical porous styrene—divinylbenzene copolymer with 2% cross-linkage Biobeads S-X2 (200—400 mesh) supplied by Bio-Rad Labs. (Richmond, CA, U.S.A.). In a first step, polystyrene is chlorosulphonated using a procedure described previously [9]. The substitution of the polymer by L-arginyl methyl ester is realized using the following procedure: 24.37 g of arginyl methyl ester are mixed with 26.03 ml of triethylamine in 125 ml of different organic solvents at 40°C (Table I); 15 g of chlorosulphonated polystyrene are then introduced and 8.7 ml of triethylamine are progressively added to neutralize the release of HCl during the reaction. The heterogeneous mixture is stirred for several hours to obtain, according to the kinetics of the reaction, different degrees of substitution. In order to eliminate any im-

TABLE I

INFLUENCE OF SOME SYNTHESIS CONDITIONS ON THE L-ARGINYL METHYL ESTER CONTENT OF THE RESINS

Reaction temperature (°C)	Reaction time (h)	Solvent	Arginine content (mequiv./g)
25	24	Dichloromethane	0.15
40	24	Dichloromethane	0.36
40	96	Dichloromethane	1.02
40	96	Chloroform	0.11
40	96	Dimethylformaline	0.816
40	96	Acetonitrile	1.12
40	96	Dioxan	0

purities, the resins are filtered and washed at room temperature for 2 h, successively with 500 ml of the following solvents (the solvent used in the synthesis, dioxan, water, 1.5 *M* sodium chloride solution, citrate solution, Michaelis buffer and water). Then, the resins are dried under vacuum at 50°C. The substituted polymers are characterized by acidimetric titrations and elementary analysis.

Affinity chromatography of human purified thrombin

A chromatographic column (56 × 9 mm I.D.) is filled with 3 ml of a suspension of PAOM resin (dry weight = 1 g) as described previously [8]. Highly purified human α-thrombin (3000 U/mg) prepared in CNTS (Paris, France) by Dr. M.C. Boffa is applied to the top of the column. The flow is then stopped to let the protein interact with the stationary phase. Elution of the enzyme is performed using a linear salt gradient (0.1—2.5 *M* sodium chloride) in 0.05 *M* Tris—HCl buffer (pH = 7.4). The elution is followed by recording the optical density at 280 nm and by measuring the clotting activity of the collected fractions.

Purification of thrombin from activated human prothrombin complex concentrate

A column (200 × 10 mm I.D.) is packed with 5 g of dried resin. Aliquots of 5 ml of human prothrombin complex concentrate (CNTS) containing 60 U of factor II are used. The prothrombin is activated by addition of human brain tissue factor, calcium chloride and factor V, according to Benamon-Djiane et al. [10]. The incubated mixture is centrifuged (20 000 *g*) at 4°C for 3 h and dialysed against 0.05 *M* Tris—HCl—0.1 *M* sodium chloride buffer (pH 7.4). Then, the mixture containing 13 000 U of thrombin is applied to the top of the column and eluted using a linear salt gradient from 0.1 to 2.5 *M* sodium chloride in the same buffer. The eluted fractions are characterized as mentioned above. The specific activity of thrombin is the ratio of the biological activity (expressed in N.I.H. units, by reference to the international human thrombin standard from the National Institute of Health, Bethesda, MD, U.S.A.) to the amount of protein (mg) measured using the method of Lowry et al. [11]. The purity of the eluted enzyme is checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and by immunoelectrophoresis.

RESULTS AND DISCUSSION

The coupling reaction of the chlorosulphonated polystyrene has taken place with the amino group of L-arginyl methyl ester; the structure of the final product is presented in Fig. 1. The influence of some synthesis parameters (temperature of reaction, reaction time, nature of organic solvent) on the arginine content of PAOM resins is shown in Table I.

Insoluble polystyrenes grafted with L-arginyl methyl ester present substituents mimicking the reactive binding site of ATIII. These materials have a specific affinity for thrombin, and the binding of the enzyme is reversible. The elution profile of purified thrombin using a PAOM resin as an affinity

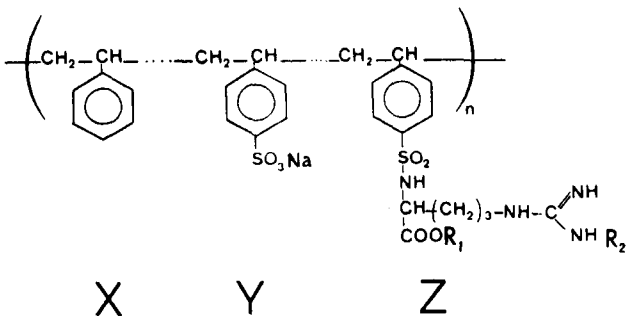

Fig. 1. Structure of polystyrene grafted with L-arginyl methyl ester.

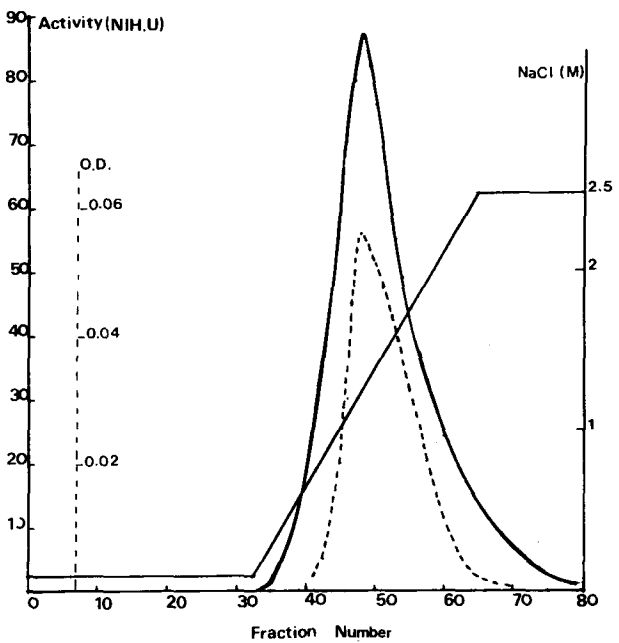

Fig. 2. Elution curve of human purified thrombin at 4°C on PAOM 21 resin. (——) Biological activity; (- - - -) optical density at 280 nm. Eluent: 0.05 *M* Tris—HCl buffer (pH 7.4); sodium chloride gradient from 0.1 to 2.5 *M*; flow-rate 20 ml/h.

chromatographic stationary phase is shown in Fig. 2. The protein is retained on the polymer at low ionic strength and is only desorbed when the salt concentration is increased to ca. 1.3 *M*. The enzyme is eluted in a single peak with the same specific activity as the starting material.

The physicochemical characteristics of the polymers influence their chromatographic performance. The degree of L-arginyl methyl ester substitution varies from 0.15 to 2.26 mequiv./g, depending on the synthesis conditions. When the degree of substitution is too high (PAOM 3), no significant amount of active thrombin is eluted (Table II). This could be explained by a strong and irreversible adsorption of the enzyme.

The affinity constant of the resins determined using isotherm adsorption curves [7] varies also with the synthesis conditions. When these constants

TABLE II

VARIATION IN BIOLOGICAL RECOVERY OF ELUTED THROMBIN WITH DEGREE OF SUBSTITUTION

Resin	Arginine content (mequiv./g)	K_{Th} ($\times 10^{-6}$) (l/M)	Recovery (%)
PAOM 1	0.15	3.97	80
PAOM 2	0.87	2.1	90
PAOM 3	2.26	3.5	0

TABLE III

VARIATION OF BIOLOGICAL RECOVERY OF ELUTED THROMBIN WITH AFFINITY CONSTANT OF RESINS

Resin	Arginine content (mequiv./g)	K_{Th} ($\times 10^{-6}$) (l/M)	Recovery (%)
PAOM 1	0.15	3.97	80
PAOM 2	0.87	2.1	90
PAOM 4	0.37	22	0

TABLE IV

EFFECT OF A SMALL AMOUNT OF WATER DURING SYNTHESIS ON THE BIOLOGICAL RECOVERY OF THROMBIN

Resin	Arginine content (mequiv./g)	K_{Th} ($\times 10^{-6}$) (l/M)	Water content (%, v/v)	Recovery (%)
PAOM 1	0.15	3.97	0	80
PAOM 2	0.87	2.1	0	90
PAOM 5	1.01	1.6	0.4	0

are too strong (PAOM 4), the active enzyme is totally retained by the solid phase (Table III).

Similarly, when the polymers are synthesized in the presence of water (0.4%), they are also unable to separate thrombin in affinity chromatography (Table IV). The properties of these polymers synthesized in the presence of water could be explained by a partial hydrolysis of the reagent during synthesis, leading to chemical modifications in the polymer structure. The chromatographic performance of PAOM resins are also strongly influenced by the average particle size of the polymer beads. The grafted phases have a natural spherical structure with a diameter between 20 and 150 μm. These materials give poor performances in chromatography. The same polymers are carefully crushed and the fine particles are eliminated by centrifugation. The solid structure of such polymer beads is changed, with a mean size of ca. 50 μm. The percentage recovery of active enzyme in the elution peak increases considerably when the polymers are crushed (Table V). These poly-

TABLE V

INFLUENCE OF SOLID STRUCTURE OF POLYMER BEADS ON CHROMATOGRAPHIC PERFORMANCE

RS = Recovery of active thrombin in the starting buffer; RE = recovery of active thrombin in the elution peak.

Resin	RS (%)	RE (%)
PAOM 1	80	0
PAOM 1 (crushed)	0	80
PAOM 6	25	25
PAOM 6 (crushed)	0	55

mers probably present an increase in their active surface, and interactions between the soluble enzyme and the solid phase are facilitated.

Consequently, the characteristics of an ideal resin for affinity chromatographic purification of thrombin can be summarized as follows. The polymers must be prepared with a degree of substitution between 0.15 and 0.87 mequiv./g arginyl methyl ester in the absence of water. The average particle size of the crushed resin is ca. 50 μm. The affinity constant for thrombin (K_{Th}) must have a medium value between $2 \cdot 10^6$ and $2 \cdot 10^7$ l/*M*.

A resin with these characteristics (PAOM 1) is then used to purify thrombin from activated human prothrombin complex concentrate. The elution pattern of the activated concentrate is shown in Fig. 3. Almost 75% of the enzyme activity applied onto the gel is eluted at a concentration of 0.8 *M* sodium chloride. The specific activity of the eluted enzyme is 2000 U/mg. The protein migrates as a single band in SDS-PAGE, performed as previously described [12] according to Laemmli [13]. No contaminants are revealed by immunoelectrophoresis using anti-human serum antibodies. About 15% of the enzyme does not bind to the solid phase and is eluted at a concentration of 0.1 *M* sodium chloride.

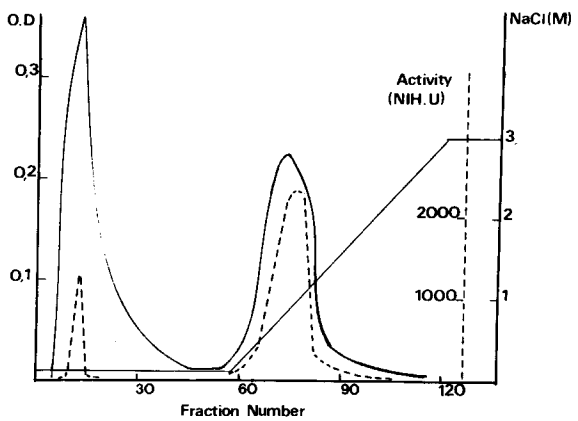

Fig. 3. Elution curve of human activated prothrombin complex, concentrated at 4°C on PAOM 21 resin. (- - - -) Biological activity; (——) optical density at 280 nm. Eluent: 0.05 *M* Tris—HCl buffer (pH 7.4); sodium chloride gradient from 0.1 to 2.5 *M*; flow-rate 20 ml/h.

The molarity necessary to desorb the bound thrombin is lower in this purification process than when purified enzyme is used. This could be due to non-specific interactions between the stationary phase and the other proteins present in the activated human prothrombin complex concentrate.

The method of purification described in this paper is remarkably quick and easy, with just one chromatographic run, and gives an enzyme with a high specific activity.

CONCLUSION

The synthetic biomaterials prepared by substitution of polystyrene with L-arginyl methyl ester and sulphonate present specific and reversible interactions with thrombin in solution. Using optimized synthesis conditions, it is possible to obtain a support for affinity chromatographic separation of human thrombin. The enzyme is eluted selectively without any significant loss of biological activity. This chromatographic process is used to separate thrombin from activated human prothrombin complex concentrate. The pure enzyme is collected in a single chromatographic run, with a high recovery of biological activity.

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