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THROMBIN PURIFICATION BY ONE-STEP PREPARATIVE AFFINITY CHROMATOGRAPHY ON MODIFIED POLYSTYRENES

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SUMMARY

Insoluble polystyrenes substituted with sulphonate and L-arginyl methyl ester have been synthesized. Using their specific affinity for thrombin, we developed a simple one-step chromatographic procedure for thrombin purification. As a control, insoluble polystyrenes substituted only with sulphonate groups were tested. The results obtained confirmed the importance of the arginyl residues grafted onto these polymers to obtain an affinity matrix useful for purifying thrombin with a high specific activity and a good recovery.

INTRODUCTION

Thrombin interacts with its main physiological inhibitor, antithrombin III, by the formation of a complex using the seryl group of the enzyme active center and an arginyl site of the inhibitor[1]. Polystyrenes modified with L-arginyl methyl ester mimic the reactive site of the inhibitor and exhibit a specific affinity for thrombin[2], but the presence of sulphonate groups on the polymer lead also to a non-specific ionic interaction with the enzyme.

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In the present paper, we compare the behaviour of insoluble polystyrenes, modified either with L-arginyl methyl ester and sulphonate groups or only with sulphonate, groups, towards thrombin obtained by activation of prothrombin concentrate. A simple preparative purification method of thrombin by a one-step chromatographic procedure is described.

MATERIALS AND METHODS

Preparation of the resins

Polystyrenes grafted with sulphonate groups ($PSSO_3$) are obtained by total hydrolysis of chlorosulphonated polymers[3]. Part of the chlorosulphonated polymer is immediately substituted with L-arginyl methyl ester (PAOM) as previously described[4]. The chemical structures of PAOM and $PSSO_3$ resins, and their characteristics, are presented in Fig. 1. The resins are carefully crushed and the fine particles are eliminated by sedimentation. The mean size of the polymer beads are *ca.* 50 μm.

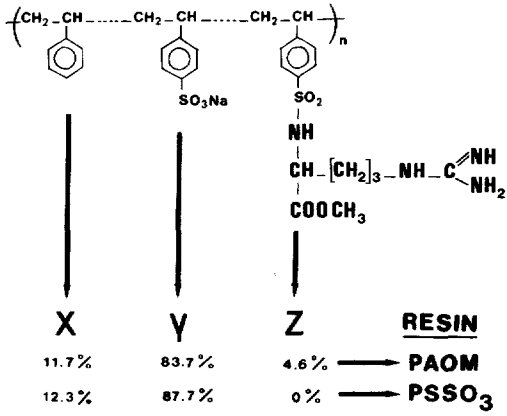

Fig. 1. Structure of PAOM and $PSSO_3$ resins.

Thrombin purification process

A column (200 × 10 mm I.D.) is packed with *ca.* 15 ml (corresponding to 4.5 g of dried resin) of a suspension of PAOM or $PSSO_3$ resin in 0.05 *M* Tris–HCl buffer and 0.1 *M* sodium chloride, pH 7.4. Commercial prothrombin complex concentrate is a partially purified human plasma fraction containing three coagulation factors: prothrombin (Factor II), Factor IX and Factor X. Prothrombin from prothrombin concentrate (batches 84 × 068 and 85 × 002, C.N.T.S., Paris, France) is activated in thrombin by the following procedure: at 37°C, human brain tissue factor, calcium chloride and bovine Factor V are added to the prothrombin solution according to Benamon *et al.*[5]. When the amount of generated thrombin reaches a steady level, the incubated mixture is centrifuged (20 000 *g*) at + 4°C for 3 h, and dialysed against 0.05 *M* Tris–HCl buffer and 0.1 *M* sodium chloride at pH 7.4.

Then, the mixture is applied onto the column, which had been pre-equilibrated with the dialysis buffer. Runs are performed at a flow-rate of 20 ml/h. After collection of the breakthrough, the column is extensively washed with the dialysis buffer until

the optical density at 280 nm comes down to 0.01; then, a linear gradient of sodium chloride from 0.1 to 2.5 *M* (40 ml in each chamber) buffered with 0.05 *M* Tris–HCl (pH 7.4) is applied to the column. The eluted fractions (2 ml) are collected and their optical density recorded at 280 nm using a LKB (Sweden) UV detector. The thrombin clotting activity of each fraction is measured on fibrinogen. The different fractions corresponding to the peaks of each chromatogram are pooled, concentrated and dialysed against 0.15 *M* sodium chloride and kept frozen at −80°C after characterization.

Characterization of the pooled fractions

Protein concentration is determined by the Lowry method[6]. Thrombin clotting activity is measured by addition of 0.1 ml of the eluent to 0.3 ml of a 0.2% human fibrinogen solution. Thrombin concentration is determined in NIH units by comparison to a standard curve. Electrophoresis in 7% polyacrylamide gels in sodium dodecyl sulphate (SDS) is carried out according to the method of Laemmli[7]. Highly purified human thrombin, 3000 NIH U/mg, provided by Dr. Boffa, C.N.T.S., Paris, France, is used as a control.

Presence of Factor IXa and Factor Xa is checked by the immunodiffusion method according to Ouchterlony[8], using rabbit anti-human Factor IX and Factor X antibodies (Stago, Asnières, France), which also react with activated Factor IX (factor IXa) and activated factor X (factor Xa). The rabbit anti-human prothrombin antibodies used (Behringwerke, Marburg, F.R.G.) react also with thrombin and prothrombin derivatives.

RESULTS AND DISCUSSION

Chromatography on PAOM resin

The elution diagram of activated prothrombin complex concentrate on PAOM resin is represented in Fig. 2. The breakthrough corresponds to peak 1 (P_1). Thrombin is eluted in one single peak (P_2) at 0.8 *M* sodium chloride ionic strength. This peak contains 69% of the amount of thrombin, tested by clotting activity, applied onto the column. The specific activity of thrombin recovered in the elution peak is 3000 U/mg (Table I). Electrophoresis on polyacrylamide gels reveals, in the eluted peak, one single band with the same mobility as the purified thrombin control (Fig. 3). No factor IXa or Xa, as possible contaminants, are detectable when checked by the immunodiffusion method (Fig. 4).

Chromatography on $PSSO_3$ resin

The elution diagram of activated prothrombin complex concentrate carried out on $PSSO_3$ resin is represented in Fig. 5. The breakthrough corresponds to peak 1 (P_1). Thrombin is eluted in one broad peak (P_3) at *ca.* 1.8 *M* sodium chloride ionic strength. The specific activity of thrombin is 915 NIH U/mg (Table I). No factor IXa or Xa are detectable when checked by the immunodiffusion method, but electrophoresis on polyacrylamide gels reveals the presence of thrombin and also of other contaminants (Fig. 6). The thrombin elution peak is preceded by a double peak (P_2) devoid of any thrombin activity, eluted within the gradient (Fig. 5).

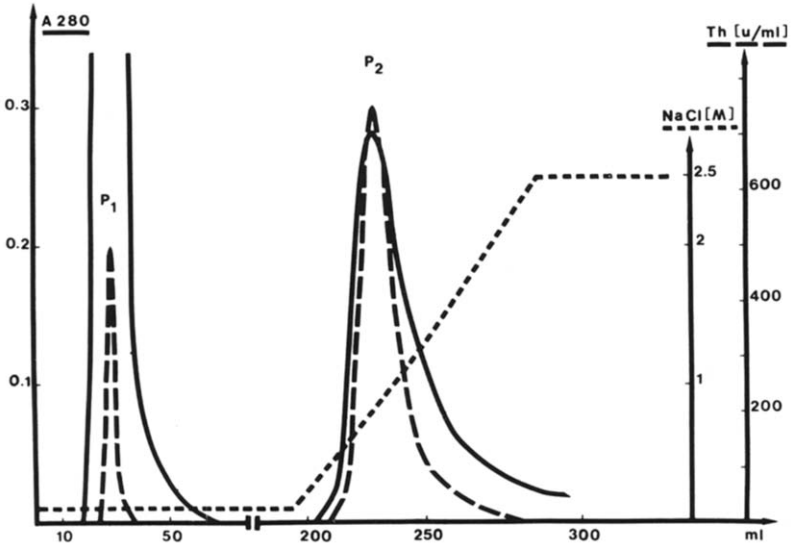

Fig. 2. Activated prothrombin complex chromatogram on PAOM resin. Peaks: P_1 = breakthrough; P_2 = eluted thrombin.

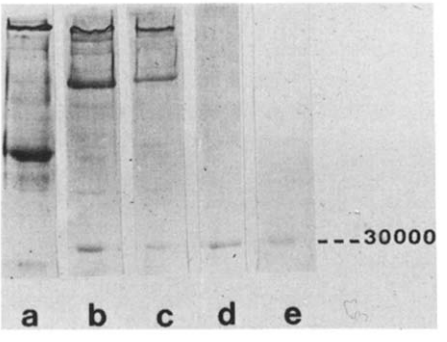

Fig. 3. SDS-polyacrylamide gel electrophoresis of the thrombin purification steps on PAOM resin. (a) Prothrombin complex concentrate; (b) activated prothrombin complex concentrate; (c) peak 1; (d) peak 2; (e) purified thrombin control.

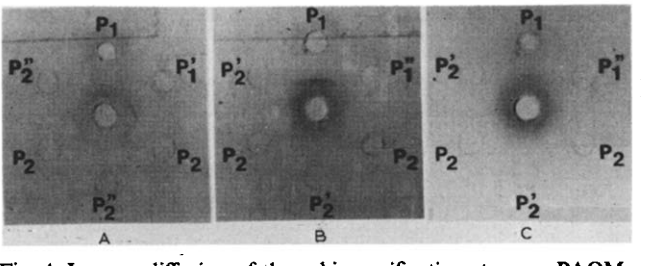

Fig. 4. Immunodiffusion of thrombin purification steps on PAOM resin. In the central well (A) specific anti-Factor II antiserum, (B) specific anti-Factor X antiserum, (C) specific anti-Factor IX antiserum. Peak 1 is tested at the following dilutions: (P_1) 1/1; (P_1') 1/5; (P_1'') 1/10. Peak 2 is tested at the following dilutions: (P_2) 1/1; (P_2') 1/2; (P_2'') 1/10.

TABLE I

THROMBIN PURIFICATION

	V (ml)	*Total thrombin (u)*	*Total proteins (Lowry) (mg)*	*Specific activity (thrombin u/mg)*	*Yield (%)*	*Purification*
PAOM						
Activated prothrombin concentrate	5.6	12000	102.5	117		
Peak 2	4.35*	8300	2.685	3091	69	26
$PSSO_3$						
Activated prothrombin concentrate	4	5000	50.2	99.6		
Peak 3	2.13*	1951	2.13	915	39	9

* After concentration.

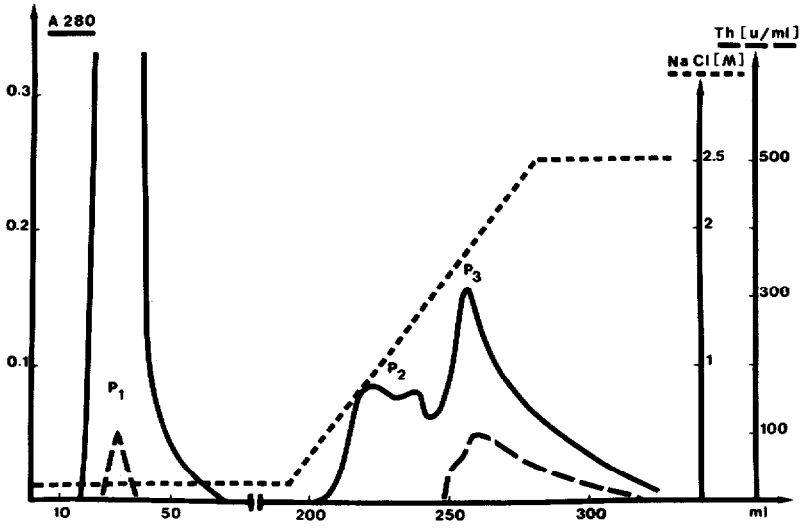

Fig. 5. Activated prothrombin complex chromatogram on $PSSO_3$ resin. Peaks: P_1 = breakthrough; P_2 = eluted peak without any clotting activity; P_3 = eluted thrombin.

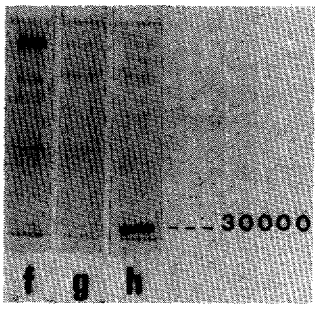

Fig. 6. SDS-polyacrylamide gel electrophoresis of the thrombin purification steps on $PSSO_3$ resin: (f) Peak 1; (g) peak 2; (h) peak 3.

Comparison of the two resins

Both $PSSO_3$ and PAOM resins exhibit a high affinity for thrombin, probably due to the presence of numerous negative charges. As a matter of fact, in most of the classical thrombin purification methods, ion-exchange chromatography is an important step, nevertheless preceded by other steps of fractionation[9].

When this type of chromatography is used as a one-step procedure, as in the case of $PSSO_3$ resin, thrombin is only roughly purified with a low specific activity (900 NIH U/mg), and numerous contaminants are detectable by polyacrylamide gel electrophoresis. By contrast, thrombin obtained after one chromatographic run on PAOM resin exhibits a high specific activity (3000 NIH U/mg), and the purity of the enzyme, as checked by polyacrylamide gel electrophoresis, is good. The yield of this purification procedure is remarkably high. The good performance of PAOM resin in thrombin purification must be due to the presence of arginyl groups, selectively interacting with thrombin. These results lead us to propose this insoluble resin as a useful simple tool for thrombin purification.

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