Journal of Chromatography, 510 (1990) 123–132
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 406

Specific adsorption of serine proteases on coated silica beads substituted with amidine derivatives

S. KHAMLICHI and D. MULLER*
LRM, CNRS URA 502, Université Paris-Nord, Avenue J.B. Clément, 93430 Villetaneuse (France)
R. FUKS
Chimie Générale I, Université Libre de Bruxelles, CP 206-4, Boulevard du Triomphe, B1050 Brussels (Belgium)
and
J. JOZEFONVICZ
LRM, CNRS URA 502, Université Paris-Nord, Avenue J.B. Clément, 93430 Villetaneuse (France)

ABSTRACT

Amidine derivatives interact with serine proteases, the inhibition being due to interactions between amidine functions and the active sites of the enzymes. Five differents types of amidine (substituted or unsubstituted) were coupled to coated silica beads, which had previously been coated with DEAE-dextran to minimize the non-specific interactions due to silanol groups. Coated silica functionalized with substituted amidines shows a strong affinity towards human plasmin. This affinity is probably due to hydrophobic interactions between the substituted amidine and the human plasmin structure. Coated silica grafted by *p*-aminobenzamide gives a specific interaction with human plasmin. The importance of ionic strength and the steric conformation of the ligand is discussed. This support was used to purify thrombin from crude preparations by high-performance affinity chromatography.

INTRODUCTION

Activation of coagulation factors is the central feature of blood coagulation, fibrinolysis, complement activation system and proteolytic digestion. The resulting serine proteases are able to hydrolyse specifically protein substrates. This hydrolysis generally begins by the interaction of the enzyme with the substrate implying an aspartic acid residue of the enzyme and the arginine residue of the substrate.

Amidine functions mimic the reactive binding site (arginine) of the substrate. Consequently, amidine derivatives are excellent inhibitors of serine proteases. Several studies have reported that benzamidine derivatives inhibit serine porteases in solution[1–3]. The primary step governing the binding of benzamidine to these enzymes is an ionic interaction of the cationic amidine moiety with an anionic amino acid side-

0021-9673/90/$03.50

chain in the enzyme structure, *e.g.*, carboxylic groups of an aspartic acid, leading to a reversible interaction[4]. Benzamidine derivatives have been immobilized on solid supports in order to prepare stationary phases for affinity chromatography[3,5]. On such supports, serine porteases such as thrombin, trypsin kallikrein and urokinase can be purified[5–7] by affinity chromatography.

To assess the relationship between amidine structure and affinity of enzymes, we prepared passivated dextran-coated silica supports bearing differents types of amidines (N,N′-substituted or unsubstituted) (Fig. 1). The affinity constants of plasmin and thrombin for these solide supports were determined using adsorption isotherms.

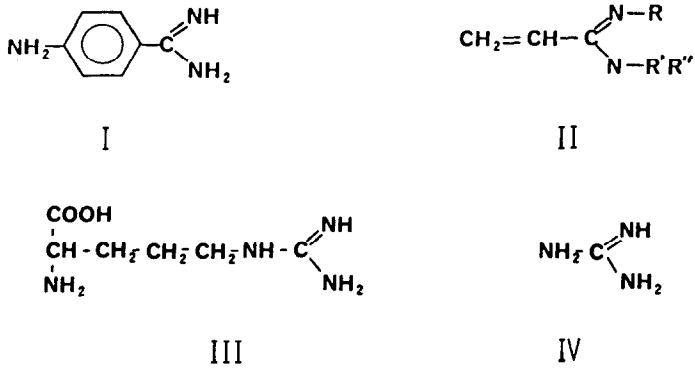

Fig. 1. Structure of amidine derivatives coupled on passivated silica beads. I = *p*-Aminobenzamidine. II = A_x; A_1 (N-*tert.*-butyl-N′-isopropylpropenamidine): R = $C(CH_3)_3$, R′ = H, R″ = $CH(CH_3)_2$; A_3 (N^1,N^2-triisopropylpropenamidine): R = R′ = R″ = $CH(CH_3)_2$. III = L-Arginine. IV = Guanidine.

The influence of the substitution of the amidine function on the affinity of plasmin was investigated by comparison of the affinity constants of the different supports, in order to observe the steric hindrance of the substituent during the complex formation between the amidine and the enzyme. The affinity of these supports for plasminogen was used to study the specific interaction between the amidine function and the protein in solution. Finally, the supports were used in order to purify thrombin from commercial preparations by high-performance affinity chromatography (HPAC).

EXPERIMENTAL

Reagents

p-Aminobenzamidine (pABA) and L-arginine monohydrochloride were provided from Fluka (Buchs, Switzerland). 1,1′-Carbonyldiimidazole (CDI), used as a coupling agent, was obtained from Sigma (St. Louis, MO, U.S.A.). N-*tert.*-Butyl-N′-isopropylpropenamidine (A_1) and N-triisopropylpropenamidine (A_3) were prepared as described previously[8]. 1,4-Butanediol diglycidyl ether (BDGE) was purchased from Polysciences (Warrington, PA, U.S.A.). Silica beads, obtained from IBF Biotechnics (Villeneuve la Garenne, France), were in the size range 40–100 μm and the pore diameter was about 1250 Å. Dextran T40, batch No. 24512 (weight-average

molecular weight, M_w = 42 000; number-average molecular weight, M_n = 24 700; polydispersity index, $I = \bar{M}_w/\bar{M}_n$ = 1.70) was purchased from Pharmacia-France (Bois d'Arcy, France), human plasmin, human thrombin and the chromothrombin substrate from Diagnostica Stago (Asnières, France), the S 2251 substrate from Kabi-Vitrum (Stockholm, Sweden) and bovine thrombin [batch B 0941, 64 NIH.U/mg (*i.e.* activity of thrombin determined by comparison with standard from National Institute of Health, Bethesda, MD, U.S.A.)] from Hoffman-La Roche Diagnostica (Basle, Switzerland). Human plasminogen was purified from plasma by affinity chromatography on lysine-Sepharose according to the method of Deutsch and Mertz[9].

Preparation of affinity supports

Passivated silica beads (SID). In order to neutralize the anionic silanol groups on the silica surface, silica beads were coated with diethylaminoethyl-dextran as described previously[10]. The degree of substitution of the functionalized dextran polymer is 4% and the extent of polymeric coverage determined by elemental analysis corresponds to 3.6 g of carbon per 100 g of dry support. Passivated silica beads were coupled with several amidines as follows.

p-Aminobenzamidine (SID–pABA). A 2-g amount of SID was suspended in 20 ml of 1,4-dioxane and mixed with 1 g of CDI. The gel suspension was gently shaken at room temperature for 2 h, then the activated support was washed successively with 200 ml of 1,4-dioxane and 200 ml of 0.1 *M* carbonate buffer (pH 10.5) and resuspended in 20 ml of 0.1 *M* carbonate buffer containing 500 mg of pABA. The mixture was gently stirred at room temperature for 48 h. The solid support was filtered and washed successively with 200 ml of 0.1 *M* carbonate buffer (pH 10.5) and 200 ml of 0.05 *M* phosphate buffer (pH 7.5). The excess of activated groups was neutralized by suspending the supports in 0.1 *M* ethanolamine solution for 3 h. The final support was filtered and washed extensively with 200 ml of 0.05 *M* phosphate buffer (pH 7.5), filtred and dried under vacuum at 60°C.

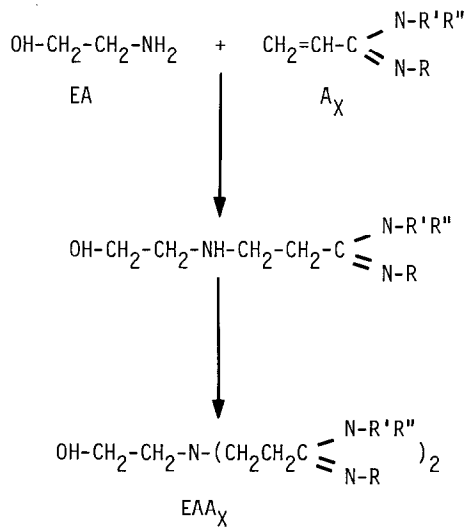

Fig. 2. Reaction of ethanolamine with substituted amidine.

Substituted amidine A1 (SID-B–A_1). The active support (SID-B–A_1) was prepared in two successive steps. The first step (Fig. 2) is the reaction of ethanolamine to A_1, as reported previously[11]. Secondly, the reaction product of addition of ethanolamine to A_1, EAA_1, was coupled to SID support using the following procedure: 2 g of SID were suspended in 20 ml of diethyl ether and 2 ml of BDGE were added. The suspension was gently stirred for 15 h at room temperature and the activated support was washed successively with 200 ml of diethyl ether and 200 ml of methylene chloride. The activated support was resuspended in 20 ml of methylene chloride containing 1 g of EAA_1 and the mixture was gently stirred at room temperature for 48 h. Then the support was washed successively with 200 ml of methylene chloride and 200 ml of 0.05 *M* phosphate buffer (pH 7.5). The excess of BDGE was inactivated in 0.1 *M* ethanolamine solution. Finally, the support was filtered and washed with 300 ml of 0.05 *M* phosphate buffer (pH 7.5), then isolated by filtering and dried under vacuum at 60°C.

Substituted amidine A_3 (SID-B–A_3). The coupling procedure was similar to that used for the SID-B–A_1 support.

p-Aminobenzamidine, using a spacer arm (SID-B–pABA). The step of support activation was similar to those described for SID-B–A_1 and SID-B–A_3 supports. The activated support was washed with 200 ml of 0.1 *M* carbonate buffer (pH 10.5). After filtration it was suspended in 20 ml of 0.1 *M* carbonate buffer (pH 10.5) containing 256 mg of pABA. The mixture was stirred at room temperature for 48 h. The final support was washed and filtered according to the method used for the activated support SID–pABA.

L-*Arginine (SID–Arg).* The protocol of the fixation procedure was similar to that for the SID–pABA support. The activated support was reacted with 500 mg of L-arginine in solution.

Guanidine (SID-B–Gua). This support was prepared in two successive steps. First, 2 g of SID were activated by 1 g of CDI as described above for the SID–pABA support, then the activated support was suspended in a solution of 20 ml of 0.1 *M* carbonate buffer (pH 10.5) containing 1.5 g of hexamethyldiamine (HMD). The mixture was gently stirred at room temperature for 48 h then the support was washed successively with carbonate buffer and 1,4-dioxane. The amine functions of HMD fixed on the support were reactivated with CDI (1 g of CDI per 2 g of support), then the activated support was suspended with 1 g of guanidinium carbonate dissolved in 20 ml of 0.1 *M* carbonate buffer (pH 10.5). The mixture was stirred at room temperature for 48 h. Finally, the support was washed with 0.05 *M* phosphate buffer and dried under vacuum at 60°C.

Characterization of the coated silica beads. The extent of substitution of the different supports by amidine derivatives was evaluated by elemental analysis (Service Central d'Analyse CNRS, Vernaison, France).

Adsorption isotherms and determination of affinity constants

Preliminary experiments were performed from a kinetic study to ascertain that human plasmin and thrombin were adsorbed at equilibrium after contact with silica beads for 30 and 20 min, respectively.

Adsorption of human plasmin. Isotherms were established from measurement of plasmin adsorption using the following procedure: 100 µl of support suspension (30–

100 mg/ml) were incubated with 900 μl of human plasmin solution at various concentrations (3–0.18 nKat/ml) in a polystyrene tube for 30 min at room temperature (1 nKat is the enzymatic activity, observed using CBS.3308, which releases 1 nmol of *p*-nitroaniline per second, under standard conditions). After sedimentation, the amount of residual enzyme was determined by taking 200 μl of supernatant and adding 620 μl of 0.05 *M* Tris–imidazole buffer (pH 7.5). Finally, 80 μl of chromogenic substrate S2251 were added and the mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 100 μl of pure acetic acid. The absorbance of the solution was read at 405 nm. The amount of adsorbed plasmin corresponds to the difference between the control and the remaining concentration of active enzyme in the supernatant.

Adsorption of human thrombin. A 100-μl volume of support suspension (5 mg/ml) was incubated with 500 μl of thrombin solution (concentration varying from 3 to 0.312 NIH.U/ml) for 20 min at room temperature. After sedimentation, the amount of residual thrombin was determined by adding 100 μl of supernatant to a mixture of 700 μl of 0.05 *M* phosphate buffer (0.1 *M* sodium chloride, pH 7.5) and 100 μl of the chromothrombin substrate. Time was measured after adding thrombin. The reaction was stopped after incubation for 3 min at 37°C, by adding 100 μl of pure acetic acid. The amount of adsorbed thrombin corresponds to the difference between the control and the ramaining concentration of active thrombin in the supernatant, determined by the absorbance at 405 nm.

Affinity constants were calculated from the initial slope and plateau of the isotherm on the basis of the Langmuir and Tempkin equations[12]. A computer program is able to establish the isotherm and to calculate the affinity constant of active supports for proteins.

Chromatographic procedure

The column (12.5 cm × 0.4 cm I.D.) was packed with 1 g of SID–pABA support using the slurry method. The liquid chromatographic apparatus consisted of a three-head (120°), chromatographic pump (Merck LC 21B) connected to a Rheodyne 7126 injection valve with a 100-μl sample loop. A variable-wavelength UV–visible detector (Merck-LC 313) and the gradient system are connected to an Epson QX-10 computer. The chromatographic signal is monitored, integrated and stored by the computer. All the equipment was provided by Merck-Clevenot (Nogent-sur-Marne, France). A volume of 50 μl (112.5 μg) of human plasminogen was injected onto the column of SID–pABA support. After adsorption of plasminogen, elution was achieved by competitive elution of arginine, aminocaproic acid or sodium chloride at varying molarity. The amount of plasminogen desorbed was determined from the surface area of the peak given by the computer. Elution of bovine thrombin was achieved as follows: 100 μl of crude thrombin (128 NIH.U) were injected onto the column in the initial buffer (0.05 *M* phosphate, pH 7.5). The column was washed with 20 ml of the initial eluent and the thrombin adsorbed was eluted by competitive elution using a 0.5 *M* arginine solution in the same buffer. The collected fractions were passed to a Sephadex G-25 column in order to separate arginine and thrombin. The specific activity of the eluted fraction was determined by clotting methods as reported previously[13].

All eluents were prepared from high-purity water (ELGA, Villeurbanne,

France), degassed and filtered through a Millipore (Velizy, France) HA 0.22-μm membrane.

RESULTS AND DISCUSSION

Adsorption of plasmin and plasminogen

Native silica beads were coated with dextran bearing a calculated amount of positively charged DEAE units in order to neutralize the negative charges of the silanol groups of native silica[10]. These coated silica supports exhibit two advantages: first, they supports have a minimum non-specific ion-exchange capacity, and second, ligands can easily be immobilized via the hydroxylic functions of the polysaccharide coating using conventional coupling methods. Unsubstituted amidines (*p*-aminobenzamidine, L-arginine and guanidine) and substituted amidines A_1 and A_3 (Fig. 1) were coupled on the coated silica supports which had previously been activated with CDI or BGDE (Fig. 3).

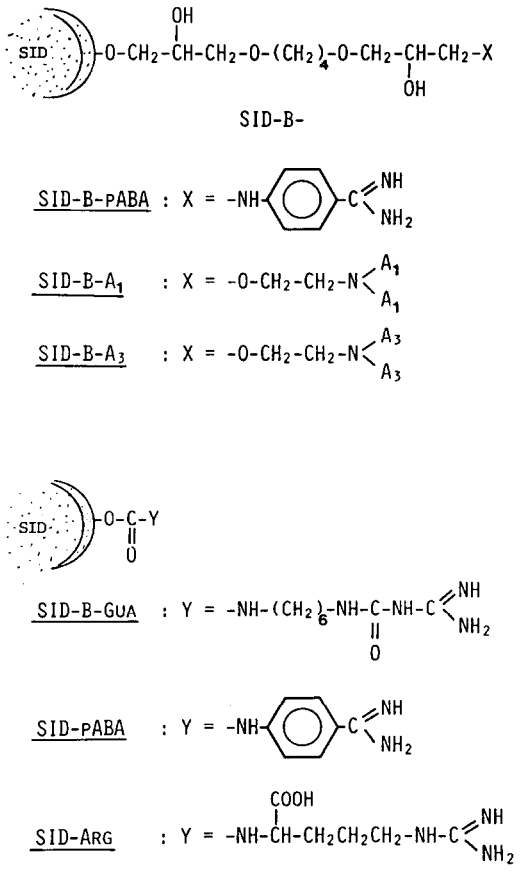

Fig. 3. Structure of the six active supports used in the adsorption and chromatographic studies.

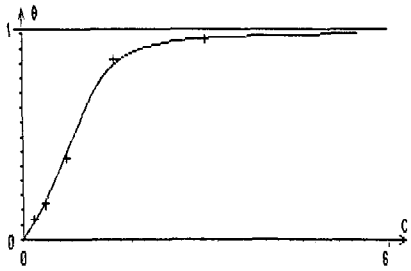

Fig. 4. Example of adsorption isotherm (Tempkin) from the computer program. C = Initial concentration of protein (U/ml); Γ = adsorbed protein[12]. $\theta = \Gamma/\Gamma_\infty$, where Γ_∞ is the concentration of adsorbed protein at saturation.

The affinity of these supports for human plasmin was studied by determining the affinity constants from Langmuir or Tempkin adsorption isotherms (Fig. 4). The affinity constants of the six supports tested are presented in Table I, and are between about 10^7 and 10^8 l/mol. These values demonstrate the strong affinity of all the supports for human plasmin. Comparison of the affinity constants of supports bearing an unsubstituted amidine (SID–pABA, SIB-B–pABA, SID–Arg and SID-B–Gua) shows that, in spite of the presence of the spacer arm, SID–pABA and SID-B–pABA supports have almost the same affinity constant (10^8 l/mol). The spacer arm has only a small influence on the adsorption process of human plasmin. SID-Arg and SID-B–Gua supports exhibit affinity constants in the same range. However the SID–pABA support is the most efficient adsorbent. Indeed, this support possesses the higher binding capacity and affinity constant. This difference in binding capacity is probably related to a better efficiency of the coupling reactions.

The affinity constants of SID-B–A_1 and SID-B–A_3 supports (Table I) show that the substitution of two or three hydrogens of the amidine function by R, R′ and

TABLE I

AFFINITY CONSTANTS AND BINDING CAPACITIES OF PASSIVATED SILICA BEADS FUNCTIONALIZED BY AMIDINE DERIVATIVES TO HUMAN PLASMIN

Material[a]	*Affinity constant* ($l\,mol^{-1}$)	*Binding capacity* (M/g)
SID–pABA (T)	$1.8 \cdot 10^8$	$2.9 \cdot 10^{-9}$
SID-B–pABA (T)	$9.5 \cdot 10^7$	$2.3 \cdot 10^{-9}$
SID–Arg (L)	$6.4 \cdot 10^7$	$1.2 \cdot 10^{-9}$
SID-B–Gua (L)	$1.1 \cdot 10^8$	$1.2 \cdot 10^{-9}$
SID-B–A_1 (L)	$3.2 \cdot 10^7$	$1.5 \cdot 10^{-9}$
SID-B–A_3 (T)	$4.2 \cdot 10^7$	$1.8 \cdot 10^{-9}$

[a] T = Tempkin isotherm; L = Langmuir isotherm.

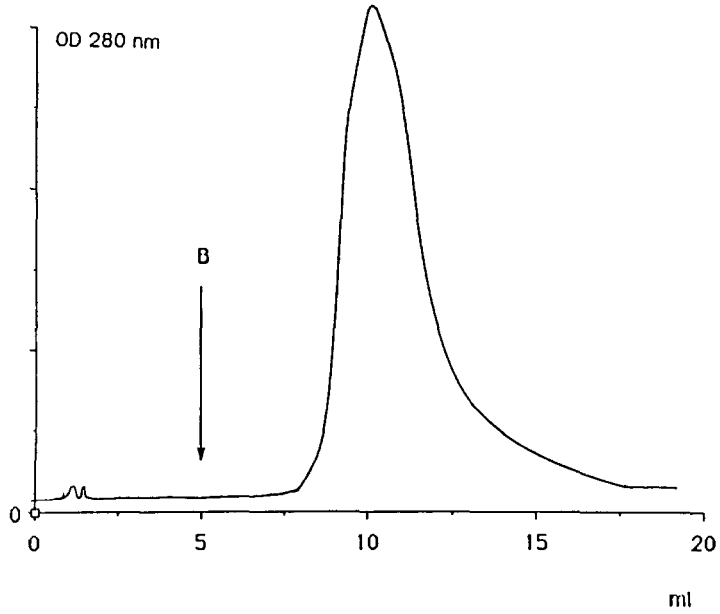

Fig. 5. HPAC profile of human plasminogen on SID–pABA support. Elution conditions: column, 12.5 × 0.4 cm I.D.; eluent 0.05 *M* phosphate buffer–0.1 *M* NaCl (pH 7.5); flow-rate, 1 ml/min; B = 0.5 *M* arginine in initial buffer.

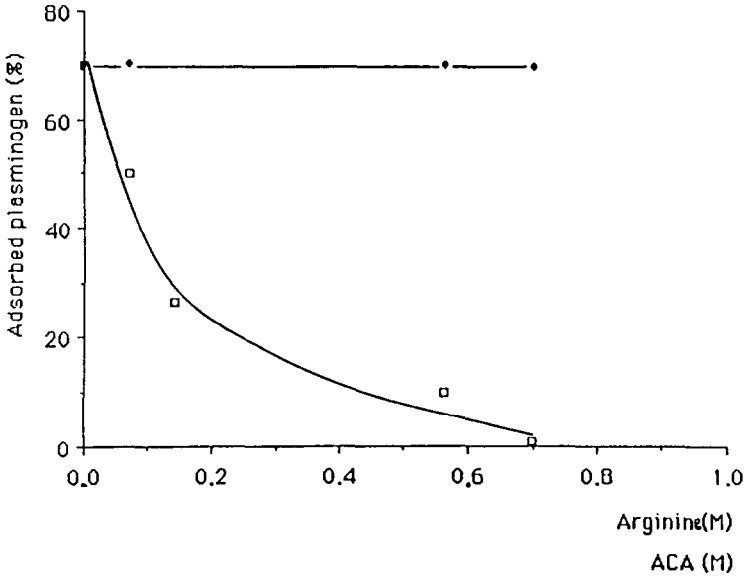

Fig. 6. Elution of human plasminogen on SID–pABA support, with (□) arginine and (●) aminocaproic acid (ACA) solutions.

R″ groups (see Fig. 1) decreases slightly the affinity of the supports towards plasmin. However, this affinity is still strong ($K_{aff} = 10^7$ lmol^{-1}). The hydrophobic character of the substituents (isopropyl, *tert.*-butyl) can explain this high affinity. The interactions between supports such SID-B–A_1 or SID-B–A_3 for plasmin are probably due to hydrophobic interactions taking place between the substituted amidines and the enzyme. Moreover, the presence of hydrophobic sites adjacent to the ionic binding site of serine proteases has been reported by Andrews *et al.*[4].

Results of the chromatographic elution of human plasminogen on SID–pABA support are presented in Figs. 5 and 6. Human plasminogen is adsorbed at low ionic strength and can be desorbed in a single peak with 0.5 *M* arginine solution (Fig. 5). The elution of plasminogen with arginine solutions at different molarity is presented in Fig. 6. Competition between arginine in solution and the coupled amidine allows the desorption of the plasminogen from the stationary phase. In addition, an increase in the ionic strength of the eluent is unable to desorb the plasminogen. These results demonstrate that the affinity between the protein and the support is strong and not due only to ionic interactions. This specific interaction probably requires the cooperation of hydrophobic (aromatic rings of p-ABA) and hydrogen interactions. Finally, the strong affinity of human plasminogen for SID–pABA support, cannot be broken by the competitive elution of aminocaproic acid, which has unsubstituted amine groups (Fig. 6), indicating that the steric conformation of the amidine function is very important in the interaction between the *p*-aminobenzamidine groups fixed on the support and the protein.

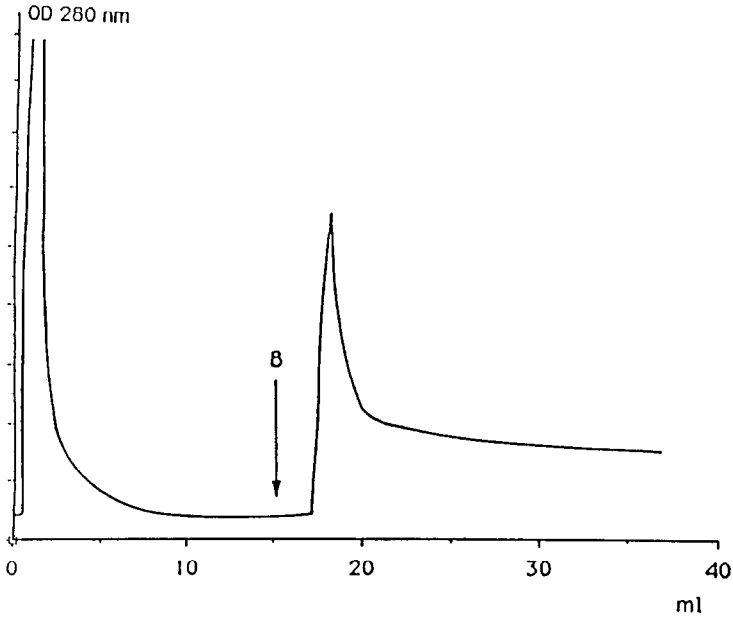

Fig. 7. Purification of bovine thrombin by HPAC on SID–pABA. Elution conditions: column, 12.5 × 0.4 cm I.D.; sample, 100 μg of crude thrombin (128 NIH.U); eluent 0.05 *M* phosphate buffer–0.1 *M* NaCl (pH 7.5); flow-rate, 1 ml/min; B = 0.5 *M* arginine in initial buffer.

Adsorption and separation of bovine thrombin

The affinity of thrombin for the SID–pABA support was evaluated from the adsorption isotherms. The calculated affinity constant is about $1.7 \cdot 10^8$ l mol^{-1}. This active support was used in HPAC in order to purify thrombin. Crude thrombin preparations were eluted on a column packed with SID–pABA support. As shown in Fig. 7, thrombin is adsorbed at low ionic strength and desorbed specifically by competitive elution using 0.5 *M* arginine solution. The specific activity of the purified thrombin is about 1200 NIH.U/mg. The yield of this separation is about 75%.

CONCLUSION

Amidine derivatives can be coupled on silica beads previously passivated with DEAE-dextran. These solid supports exhibit minimum non-specific ionic interactions with proteins in solution. As expected, such coated silica supports functionalized by amidine derivatives have a strong affinity for human plasmin. In addition, substituted amidines (A1, A3) immobilized on coated silica beads have a strong affinity for human plasmin. This affinity is probably due to hydrophobic interactions between the substituted amidine residues and the human plasmin in solution. The specific interaction between *p*-aminobenzamidine and the serine protease is probably the result of several types of interactions. SID–pABA is a good stationary phase for separating and purifying different serine proteases such as thrombin, trypsin and tissue plasminogen activator (tPPA) by HPAC. The good mechanical properties of these supports, particularly the excellent resistance to hydrostatic pressure, allow their use in HPAC and the separation process can easily be scaled up.

REFERENCES

1 F. Markwardt, H. Landmann and P. Walsmann, *Eur. J. Biochem.*, 6 (1968) 502–506.
2 J. Sturzebecher, P. Walsmann, B. Voigt and G. Wagner, *Thromb. Res.*, 36 (1984) 457–467.
3 H. F. Hixon and A. H. Nishikawa, *Arch. Biochem. Biophys.*, 154 (1973) 501–509.
4 J. Andrews, D. Koman and D. Bing, *J. Med. Chem.*, 21 (1978) 1202–1207.
5 A. Kanamori, S. Nobuko and I. Matsumoto, *Chem. Pharm. Bull.*, 35 (1987) 3777–3783.
6 L. Heimark and E. W. Davie, *Biochemistry*, 18 (1979) 5743–5750.
7 L. Holmberg, B. Bladh and B. Astedt, *Biochim. Biophys. Acta*, 445 (1976) 215–222.
8 R. Fuks, *Tetrahedron*, 29 (1973) 2147–2151.
9 D. G. Deutsch and E. T. Mertz, *Science (Washington, D.C.)*, 170 (1970) 1095–1096.
10 X. Santarelli, D. Muller and J. Jozefonvicz, *J. Chromatogr.*, 443 (1988) 55–62.
11 R. Fuks, B. Tremerie, S. Khamlichi, D. Muller and J. Jozefonvicz, *Synth. Commun.*, submitted for publication.
12 S. J. Thomson and G. Weeb, in *Heterogeneous Catalysis*, Wiley, New York, 1968, pp. 22–27.
13 C. Boisson, C., D. Gulino, J. Jozefonvicz and A. M. Fischer, *Thromb. Res.*, 34 (1984) 269–276.