Journal of Chromatography, 424 (1988) 385–391
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3979

Note

Affinity chromatography of human thrombin on modified silica

A.V. GAIDA, V.A. MONASTYRSKII and Yu.V. MAGEROVSKII

Research Institute of Haematology and Blood Transfusion, ul. Pushkina 45, 290044 Lvov (U.S.S.R.)

and

S.M. STAROVEROV and G.V. LISICHKIN*

Laboratory of Petrochemical Synthesis, Department of Chemistry, Lomonosov State University of Moscow, Leninskiye Gory, 119899 Moscow (U.S.S.R.)

(First received June 11th, 1987; revised manuscript received September 25th, 1987)

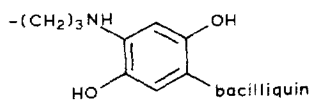
Affinity chromatography is one of the most efficient means of purifying human thrombin, a serine proteinase from blood serum, the main component of the coagulation system (EC 3.4.21.5). The procedure usually employs agarose gel derivatives modified with the residues of the thrombin inhibitors *p*-chlorobenzylamine [1] or *p*-aminobenzamidine [2], L-lysine and L-arginine amino acid residues relevant to the enzyme primary specificity and also with aliphatic α,ω-diamines [3]. Another reported adsorbent is polystyrene containing the methyl ester of L-arginine linked through its α-amino group and imitating the thrombin binding site in antithrombin III [4]. In addition, it has been demonstrated that serine proteinases of different origins may be purified by affinity chromatography on Silochrom coupled with proteinase inhibitors of broad specificity such as the polypeptides gramicidin S and bacitracin [5].

This study deals with the preparation and characteristics of affinity adsorbents for thrombin on Silochrom containing covalently bound residues of L-lysine, L-arginine, hexamethylenediamine, gramicidin S and bacitracin, and with the use of these adsorbents in thrombin purification.

0378-4347/88/$03.50

TABLE I

CHARACTERISTICS OF ADSORBENTS

Adsorbent	Ligand	Ligand concentration (μmol/g)
I	$-(CH_2)_3NH_2$	280
II	$-(CH_2)_3NH(CH_2)_6NH_2$	105
III	$-(CH_2)_3NHCH(COOH)(CH_2)_4NH_2$	135
IV	$-(CH_2)_3NHCH(COOH)(CH_2)_3C(=NH)NH_2$	120
V	$-(CH_2)_3OCH_2CH(OH)CH_2$-gramicidin S	4.3
VI	$-(CH_2)_3OCH_2CH(OH)CH_2$-bacitracin	5.0
VII		4.8

EXPERIMENTAL

Materials

Adsorbents II–IV were synthesized on the basis of γ-chloropropyl-Silochrom S-80, prepared as described elsewhere [6] by treating the matrix with ligand solution (0.6 mmol per gram of adsorbent) in dioxane–water–potassium carbonate (2:6:1) at 85°C for 1.5 h. Ligand incorporation was evaluated by adsorption of divalent copper ions [6].

Adsorbents V and VI were synthesized on the basis of γ-glycidohydroxypropyl-Silochrom S-80. Bacitracin (Sigma, St. Louis, MO, U.S.A.) was linked as in the synthesis of adsorbents II–IV using 50 μmol of ligand per gram of adsorbent. Gramicidin-Silochrom (adsorbent V) was prepared by treating Silochrom (10 g) with 60 ml of solution containing 59.5 ml of dimethylformamide, 0.5 ml of water, 0.075 g of potassium carbonate and 0.5 g of gramicidin S (425 mmol) at 80°C for 1.5 h. Incorporation of the antibiotics was evaluated after hydrolysis with 5.7 *M* hydrochloric acid at 105°C for 24 h using a Hitachi Model KLA-5 amino acid analyser. γ-Aminopropyl-Silochrom (adsorbent I) and bacilliquin-Silochrom (adsorbent VII) (bacilliquin is the commercial name for a crude extract of a mixture of bacitracins) were obtained from Biolar (Olaine, U.S.S.R.).

The characteristics of the adsorbents are given in Table I.

The original support Silochrom S-80 (Reakhim, U.S.S.R.) had a specific surface area of 80 m^2/g and an average pore diameter of 50 nm.

Thrombin was isolated from thromboplastin-activated extract of Cohn frac-

tion III obained in the presence of polyethylene glycol 4800 as described by Fenton et al. [7].

Thrombin activity was evaluated from the coagulation time of 0.13% fibrinogen solution (70% coagulated protein) and expressed in NIH units.

Protein concentrations were determined with the use of Coomassie Brilliant Blue G-250 solution [8].

The following buffers were used in isolating thrombin: (1) 0.05 *M* sodium phosphate buffer–0.15 *M* sodium chloride (pH 6); (2) 0.05 *M* Tris–HCl buffer–0.15 *M* sodium chloride (pH 8); (3) 25% isopropanol in solution 2; (4), (5) 1 *M* sodium chloride in solutions 1 and 2, respectively; (6), (7) 0.25 *M* ϵ-aminocaproic acid in solutions 1 and 2, respectively; (8), (9) 1 *M* sodium chloride and 25% isopropanol in solutions 1 and 2, respectively.

Chromatography

Chromatography of thrombin was performed on non-siliconized 5.0×1.6 cm I.D. glass columns filled with an adsorbent equilibrated with solution 1 or 2. After application of thrombin solution the ballast proteins were removed and the enzyme was specifically desorbed by sequential elution with solutions 1, 4, 6 and 8 (at pH 6) or 2, 3, 5, 7 and 9 (at pH 8). Some experiments did not involve washing with solutions 3, 4 and 5–7. Fractions of 3 ml were collected and their protein content, absorbance at 280 nm and coagulating activity were determined. The thrombin-containing fractions were combined and dialysed against 0.15 *M* sodium chloride at 4°C overnight.

RESULTS AND DISCUSSION

Various techniques are available for modifying the surface of silica which permit the attachment of virtually any groups of atoms on spacers of the required length and hydrophobicity [9]. Nevertheless, silica adsorbents have rarely been used in purifying blood proteins. The main obstacles are non-specific adsorption on unmodified silanol groups [10] and the ability of unmodified silica to cause contact activation of the blood coagulation system, which leads eventually to the formation of fibrin gel and obstruction of the column. Both of these undesirable effects can be avoided by treating silica with silanes, of which the most useful is trimethylchlorosilane. A similar result can be achieved with certain other silanes, such as γ-chloropropyltrichlorosilane and γ-glycidohydroxypropyltriethoxysilane [6].

Investigation of the capacities of the prepared adsorbents (II–VI, Table I) and commercially available types (I and VII, Table I) showed that, despite their considerably different ligand contents, they have similar thrombin binding activities (Table II). The most likely explanation is that only a small part of the ligands might be involved in enzyme binding, the rest being inaccessible to the enzyme, apparently owing to steric hindrance. Thrombin adsorption on adsorbent I was unexpected as it had been shown earlier that thrombin binding to Sepharose containing immobilized aliphatic α,ω-diamines requires the number of methylene links to be not less than 4, i.e., the ω-amino group must be connected with

TABLE II

THROMBIN PURIFICATION BY CHROMATOGRAPHY ON MODIFIED SILICA AT pH 8 AND 6

pH	Adsorbent	Specific activity (NIH units/mg protein)		Purification factor	Yield of activity (%)	Adsorbent capacity (NIH units/g)
		Before purification	After purification			
8	I	225	945	4.2	75.0	3400
	II	190	1050	5.5	58.0	3500
	III	190	1250	6.6	100.0	5250
	IV	225	1330	5.9	83.0	5500
	V	154	1750	11.6	78.0	2000
	VI	154	941	6.2	96.0	3000
	VII	154	1200	7.9	58.0	3200
6	I	225	700	3.1	43.2	1750
	II	190	640	3.4	30.0	3000
	III	160	690	4.3	80.0	3300
	IV	160	660	4.1	60.0	850
	V	154	No adsorption			
	VI	154	No adsorption			
	VII	154	960	6.3	55.0	2760

the matrix by a spacer not shorter than five carbon atoms [3]. Thrombin binding to adsorbent I with a considerably shorter spacer arises form the nature of the silica matrix providing a tighter contact between the enzyme and the ligand than occurs with Sepharose.

Thrombin binding to the adsorbents studied is greatly influenced by pH. Thus, at pH 8 the amount of bound enzyme, yield of activity and extent of purification are higher than those at pH 6 (Table II). A particularly pronounced difference in binding is observed with adsorbents V and VI, with which no adsorption occurs at pH 6. At pH higher than 7.5 the thrombin molecule is known to undergo a conformational change, leading to a greater number of hydrophobic residues being exposed to the protein globule surface [11]. As hydrophobic forces play a certain role in the interaction of thrombin with all the ligands used, one can conclude that it is different enzyme conformations that give rise to differences in thrombin binding at pH 6 and 8. Also, it should be noted that, although adsorbents VI and VII contain similar ligands, the latter does bind thrombin at pH 6 (Table II). The tighter binding to adsorbent VII prepared by sequential treatment of γ-aminopropyl-Silochrom with benzoquinone and alkaline bacilliquin solution [12] may be accounted for by non-specific adsorption on the trimethylene chains and non-substituted aminopropyl and benzoquinone groups increasing thrombin binding to the ligand.

Tentative experiments have demonstrated that elution with 1 *M* sodium chloride solution results in desorption of up to 40% of thrombin with a low degree of purity from adsorbents I and III–VI but fails to elute the enzyme bound to adsor-

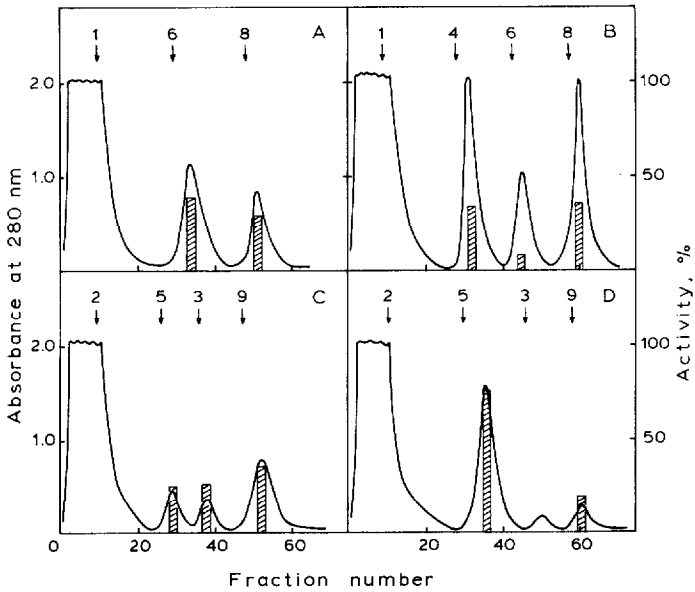


Fig. 1. Chromatography of thrombin on adsorbents II (A), III (B), V (C) and VI (D) at pH 6 (A and B) and 8 (C and D). Arrows indicate the change of eluents. Solid lines and bars designate absorbance at 280 nm and total activity of combined fractions, respectively.

bents II and VII. A 0.25 *M* solution of ϵ-aminocaproic acid employed as a biospecific eluent failed to produce any significant desorption and purification with any of the adsorbents except adsorbent III. On sequential elution with these solutions, each of them desorbed only part of the enzyme, the yield and purity being low in both eluates (Fig. 1).

Such strong adsorption of thrombin on adsorbents II–IV distinguishes them from the earlier described types containing similar ligands immobilized on cyanogen bromide-actived Sepharose [3] as the thrombin can be completely eluted with saline solution with an ionic strength of 0.15 or with 0.2 *M* L-lysine solution. This difference in binding efficiency is likely to result from the introduction of a trimethylene spacer in adsorbents II–IV (Table I), non-specific adsorption on which enhances thrombin binding to the affinity ligand. However, it cannot be denied that the silica matrix itself plays a certain role in binding. The strong thrombin binding to adsorbent III containing L-lysine residues and the lack of enzyme desorption by ϵ-aminocaproic acid solution (Figs. 1B and 2A) allow, in addition, the separation of plasminogen impurities which also bind to the sorbent and can be completely eluted with 0.25 *M* ϵ-aminocaproic acid solution.

Optimal conditions for thrombin desorption and removal of ballast proteins were selected for each of the adsorbents tested in order to achieve a higher purity and specific activity. The best purification was provided by sequential elution with solutions 7 and 9 for adsorbent III (Fig. 2A), with solutions 3 and 9 for adsorbents V–VII (Fig. 2C and D) and with solution 9 for adsorbents I, II and IV (Fig. 2B). Such an elution sequence allows thrombin to be eluted in narrow peaks containing 58–100% activity depending on the adsorbent. The specific ac-

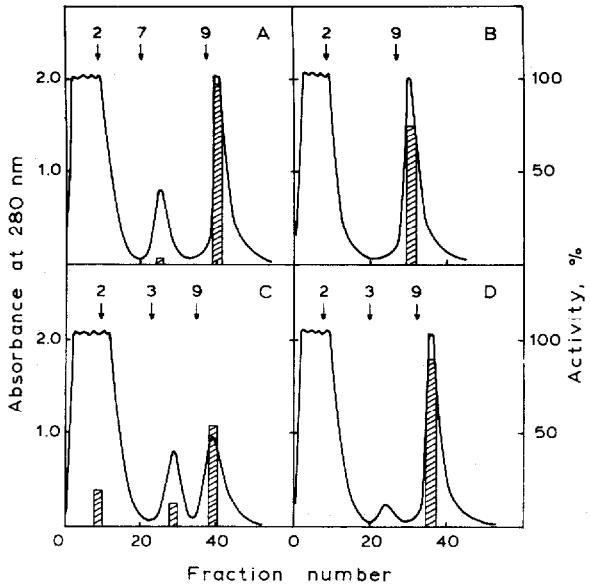


Fig. 2. Chromatography on adsorbents III (A), II (B), V (C) and VI (D) at pH 8. Other details as in Fig. 1.

tivity increases in this instance by 4.2–11.6 times up to 945–1750 NIH units per milligram of protein (Table II).

Crude thrombin extracts with low specific coagulating activity that had not been preliminarily concentrated and purified by ion-exchange chromatography were subjected to purification by chromatography on biospecific sorbents. As conventional adsorbents fail to extract thrombin from solutions in which its content or specific activity is low, affinity chromatography is normally performed in the final steps of thrombin purification, the enzyme solutions used having specific activities of 1250–3000 NIH units per milligram of protein [1, 4]. The use of the proposed adsorbents, which bind thrombin more tightly, allows one to isolate the enzyme with high activity directly from crude extracts. This is particularly true of adsorbents containing immobilized L-lysine and L-arginine residues, which allow the isolation of thrombin with a specific coagulating activity of 1250–1330 NIH units per milligram of protein. Although these values are lower than those achieved with gramicidin-Silochrom (adsorbent V, Table II), their use is warranted by their easier synthesis and greater availability.

CONCLUSION

Biospecific adsorbents prepared by covalent modification of Silochrom containing γ-chloropropyl or γ-glycidohydroxypropyl groups with hexamethylenediamine, L-lysine, L-arginine, gramicidin S or bacitracin can reversibly bind thrombin directly from crude extracts. The enzyme is selectively eluted without substantial losses of specific activity in a yield of 60–100%, its specific coagulating activity increasing 4.2–11.6 fold and reaching a value of 945–1750 NIH units per

milligram of protein. Thrombin adsorption is greatly affected by pH, the silica matrix and the spacer connecting the ligand and the support.

REFERENCES

1 N.A. Gracheva, L.D. Uzhinova, A.L. Kurts and N.A. Plate, Bioorg. Khim., 7 (1981) 1560.
2 G. Schmer, Z. Physiol. Chem., 353 (1972) 810.
3 M.W.C. Hatton and E. Regoeczi, Biochim. Biophys. Acta, 427 (1976) 575.
4 X.J. Yu, A.M. Fischer, D. Muller, A. Bros, J. Tapon-Bretaudiere and J. Jozefonvicz, J. Chromatogr., 376 (1986) 429.
5 V.M. Stepanov, G.N. Rudenskaya, A.V. Gaida and A.L. Osterman, J. Biochem. Biophys. Methods, 5 (1981) 177.
6 V.A. Malinovskii, S.M. Staroverov and G.V.Lisichkin, Zh. Obshch. Khim., 55 (1985) 2767.
7 J.V. Fenton, II, M.J. Fasco, A.B. Stackrow, D.L. Aronson, A.M. Young and J.S. Finlayson, J. Biol. Chem., 252 (1977) 3587.
8 M.M. Bradford, Anal. Biochem., 72 (1976) 248.
9 G.V. Lisichkin (Editor), Modifitsirovannye Kremnezemy v Sorbtsii, Kastalize i Khromatograffi (Modified Silica in Adsorption, Catalysis and Chromatography), 1986, 248 pp.
10 M. Kimura, J. Satoh, M. Ueno, I. Horikoshi and N. Sakuragawa, J. Pharm. Soc. Jpn., 103 (1983) 411.
11 G.B. Villanueva, Thromb. Haemost., 46 (1981) 46.
12 V.M. Stepanov, G.N. Rudenskaya, V.K. Akparov and A.V. Gaida, U.S. Pat., 4 264 738 (1981).