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High-performance liquid chromatography of proteins on functional polysaccharide-coated silica supports*

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Dextran-coated silica supports are potentially excellent stationary phases for high-performance liquid chromatography of proteins. These supports combined the advantages of polysaccharide phases with the excellent mechanical characteristics of silica. The passivation of the inorganic phase was obtained by a coating with dextran or agarose substituted with a calculated amount of positively charged diethylaminoethyl (DEAE) functions. The passivation of the silica beads can be improved by a second coating with native polysaccharide. The supports presented minimal non-specific adsorption as tested by high-performance size-exclusion chromatography. Because of their polysaccharidic overlayer, these phases were easily grafted with active ligands like anticoagulant compounds (heparin or functional dextrans) or amidine derivatives and used in high-performance affinity chromatography. The affinity of these active supports for thrombin in solution was evaluated and their performances in affinity chromatography were determined. On these supports, the non-specific interactions were minimized and the importance of the specific binding between the active ligand and thrombin in the overall interaction mechanism was enhanced.

1. Introduction

High-performance liquid chromatography of biological compounds is mainly performed on silica-based supports for two main reasons: First, the mechanical properties of the inorganic material allow important mass transfers and the use of high elution flow rates. Secondly, these pellicular phases can be designed with controlled physicochemical characteristics (geometry and porosity) [1, 2]. However, these supports possess silanol functions at their surface which can develop several non-specific adsorptions with the biological compounds in solution [1]. These non-specific interactions can deeply disturb the mechanism involved in the separation process. High-performance affinity chromatography (HPAC) uses the great specificity of affinity techniques and the efficiency and speed of the HPLC techniques. Consequently, the supports for HPAC require minimum non-specific adsorption, an hydrophilic character, simple derivatization procedures with a broad range of ligand chemistries and should possess an ability to tolerate the different solvents and the changes in solvent composition necessary for the elution [3].

To combine the exceptional chromatographic properties of polysaccharide supports and the mechanical stability of silica, we have prepared new chromatographic supports by coating silica beads with polysaccharide polymers (dextran or agarose) substituted by a relatively small amount of positively charged diethylaminoethyl (DEAE) functions [4, 5]. The positively charged groups along the polymer chains neutralized the negatively charged silanol functions on the silica surface. Moreover, the ionic interactions facilitated the impregnation of the porous material by the polymer and improved the passivation. The coating polymers were crosslinked around the silica particles to assure a good stability. The passivation of such phases can be confirmed by the determination of the chromatographic characteristics of the supports in high-performance size-exclusion chromatography (HPSEC) [4]. The influence of the characteristics of the coating (molecular mass or substitution rate of the modified polymer and amount of the coating) was studied [4, 5].

However, it was difficult to obtain an exact balance between the ion-exchange capacity of the DEAE-polymers and the native silica. The neutralization of the silanol functions on the inorganic surface require an excess of positively charged groups and led to a residual anion-exchange capacity of the phase [6]. To obtain a better passivation, we prepared double-coated silica supports. After a preliminary coating, the silica beads were recoated with the native corresponding polysaccharide to overlay the excess of positively charged functions. The minimization of non-specific adsorption of the double-coated supports

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was demonstrated by the study of the elution conditions of several proteins with different isoelectric point or acidic polymer in HPSEC experiments [4–6].

Because of their polysaccharidic surface, these materials can easily be grafted with active ligands like anticoagulant products [5] or amidine derivatives [7] with a good yield by classical activation methods.

For example, dextran-coated silica grafted with heparin or functional dextrans exhibits a strong affinity for thrombin at low ionic strength. The active enzyme can be eluted specifically by using a salt gradient. Similarly, *p*-aminobenzamidine (*p*-ABA), arginine or guanidine immobilized on such phases interact with serine proteases like thrombin. The adsorbed enzyme can be desorbed by a competitive elution using arginine solution.

Finally, all these active supports were used as an example in HPLAC to separate or purify thrombin from commercial samples, and the chromatographic performances of the different functional supports were compared.

2. Materials and methods

2.1. Preparation of the supports

The silica-based supports were prepared in two stages as previously described [4]. Dextrans T40, T70 or T500, purchased from Pharmacia-France (Bois d'Arcy, France), or agaroses Indubiose HAA, kindly provided by IBF Biotechnics (Villeneuve la Garenne, France), were modified by a controlled substitution with 2-diethylaminoethyl chloride hydrochloride (Janssen Chemica, Pantin, France). In a first step, the silica beads X015M or X075M (particle size 40–100 μm, porosity 1250, or 30 nm, respectively) were impregnated with a concentrated solution of DEAE-polysaccharide and the polymer crosslinked to obtain a polymeric coverage of about 5 g of polymer for 100 g of dry support. The supports were eventually recoated with the corresponding native polysaccharide by using a similar procedure [6]. In a second step, the ligands were coupled by using 1,1′-carbonyldiimidazole (CDI) or 1,4-butanediol-diglycidyl-ether (BDGE) as activating agent [5]. Heparin H108 (Hep) with a specific anticoagulant activity of 108 IU mg^{-1} was kindly provided by Institut Choay (Paris, France). The functional dextrans were prepared in three successive substitution steps as previously described [8, 9]. These functional polymers (FD) exhibited an affinity for several plasma proteins [9, 10]. The different amidines derivatives, *p*-aminobenzamidine (*p*-ABA), arginine (Arg) or guanidine (Gua), were purchased from Sigma-France (La Verpillere, France). These compounds can be immobilized on the supports directly by using CDI activation. *p*-ABA was also coupled to the polysaccharidic layer via a spacer arm by using BDGE as a coupling agent (Fig. 1). Immobilization of the ligands was performed on 2.5 g of activated support suspended with the ligands in 12.5 ml of sodium carbonate buffer (0.1 M), pH 8.7 and treated at 20 °C with 300 mg of active ligand for 48 h. The supports were deactivated, filtered and washed with 300 ml of 0.05 M phosphate buffer (pH 7.5) and dried. The

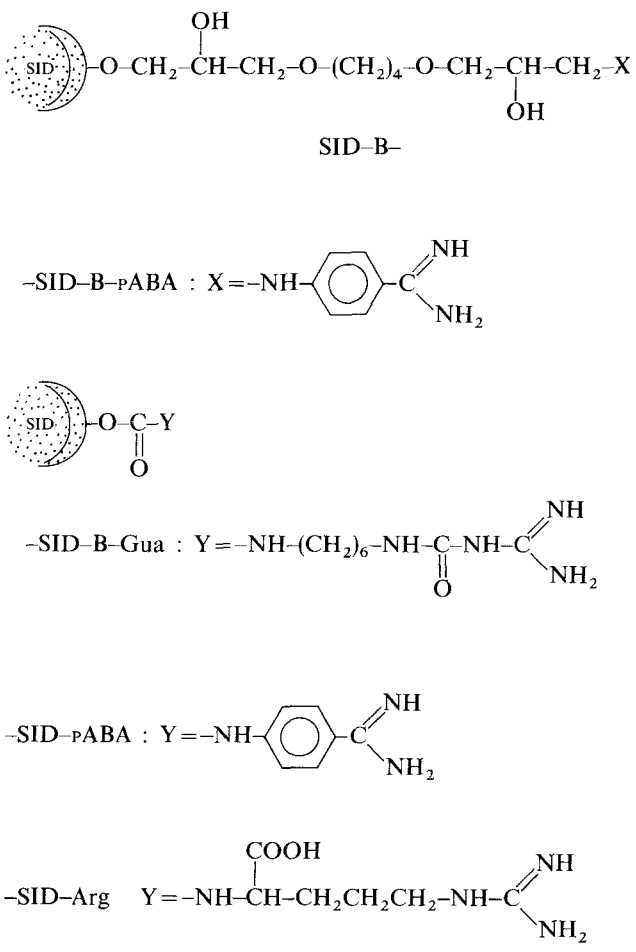

Figure 1 Structure of amidine coupled to dextran-coated silica.

substitution rates were evaluated by elemental analysis.

The affinity of the active supports for thrombin was determined from the adsorption isotherms by using the Langmuir equations. These isotherms were established by using the following procedure: 100 µl of support suspension (100 mg ml^{-1}) was incubated with 500 ml of enzyme solution at various concentrations for 20 min at room temperature. This time of incubation was selected to reach a total adsorption of the enzyme with a minimal degradation. After sedimentation, the amount of residual enzyme was determined by using chromogenic methods or clotting methods [11, 12] and the purity was checked by SDS-PAGE performed as previously described [13].

2.2. Chromatographic experiments

The HPLC apparatus was a Merck-Hitachi 655 A-12 gradient system from Lab. Merck-Clevenot (Nogent sur Marne, France) connected to a variable wavelength monitor and a D2000 Hitachi integrator. The solutions and elution buffers were prepared with doubly distillated water, degased and filtered through a 0.22 µm Millipore HA membrane with reagents of analytical grade. The HPLC column (12.5 cm long, 0.4 cm ID) was packed with 1 g of active support by using a slurry procedure. A volume of 100 µl of enzyme solution (2 mg ml^{-1}) was injected. The eluted protein was detected at 280 nm and the chromatographic fractions were collected. The fractions were tested by SDS-PAGE [13] and no contaminants were detected. The amount of protein was measured by Bradsford's method [14] and the biological activity of the eluted fractions determined by chromogenic methods or clotting assays as previously described [11, 12].

3. Results and discussion

Native silica beads were coated with modified dextran or agarose (Fig. 2). The characteristics of the substituted polysaccharides used for the passivation and the mechanical behaviour of the starting silica are presented in Table I. The passivation of silica can be evaluated by HPSEC. Most of the standard proteins are normally eluted at low ionic strength (Tris buffer 0.02 M, NaCl 0.15 M, pH 7.4). These results show that the passivation obtained by a coating with a layer of

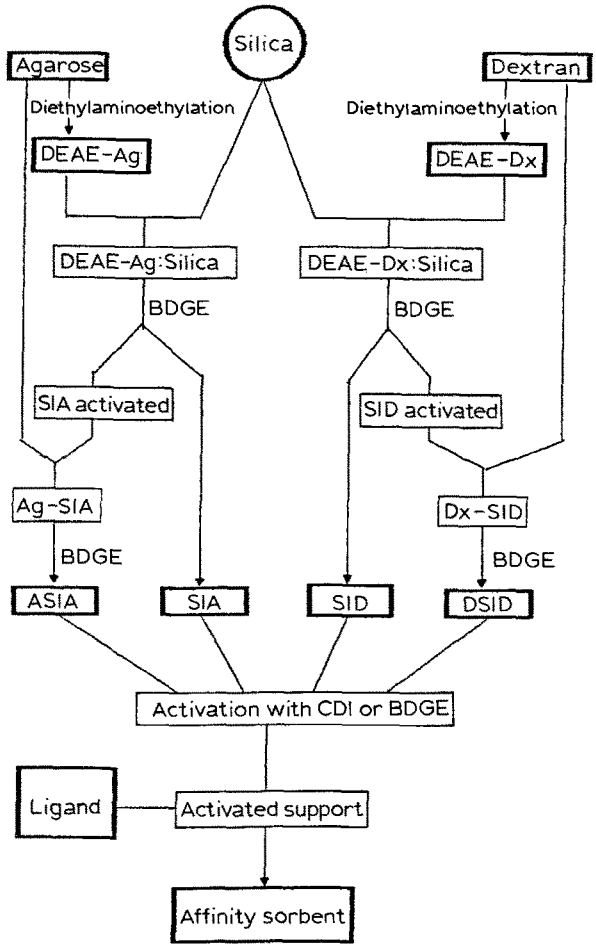

Figure 2 Diagram of coating of silica phases.

modified polysaccharide was generally efficient. In fact, the amount of polymer covering the silica surface is the most important characteristic of a good coating, it has to be around 10 g of modified dextran or 5 g of modified agarose for 100 g of dry support. Because of its smaller solubility, the coating with modified agarose was experimentally more difficult to obtain, but the passivation, especially for the large porosity materials, was more efficient. The abnormal retentions observed for cytochrom-C on the dextran-coated silica SiD75 showed that the silica surface inside the smaller pores was only partly covered. The interactions developed by heparin were stronger and due

TABLE I Characteristics of the coated silica supports (Si15 and Si75 are silica with a 1250 and 30 nm porosity respectively. Polymer type: nature of the native polysaccharide (dextran T40, T70 to T500 or indubiose HAA). % of DEAE: % of sugar units substituted by DEAE functions. Coating: amount of polymeric coating in mg per g of dry support.)

	Silica		Polymer		
	Porosity	Granul.	Type	% DEAE	Coating
SiD15-1	1250	40–100	T40	7.0	90
SiD75-2	300	40–100	T40	4.8	48
SiD15-3	1250	40–100	T70	4.4	119
SiD15-4	1250	40–100	T500	4.5	121
DSiD-5[a]	1250	40–100	T500	7.2[b]	166[c]
SiA15-6	1250	40–100	HAA1	10.0	45
ASiA-7[a]	1250	40–100	HAA1	10.0[b]	80[c]

[a] Double-coated silica phases.
[b] % of DEAE of the first layer.
[c] Amount of the double coating.

to the presence of an excess of positively charged groups available on the surface. This effect was completely masked by the second coating.

The exclusion limits for proteins on the different kinds of supports prepared from the large porosity silica (1250) were all in the range 10 000–200 000 g mol^{-1}. The porosity of the material was weakly affected by the coating, especially for the 30 nm silica beads. This decrease in the exclusion limits of the phases was more important for the double-coated supports. Consequently, the larger porosity materials coated with dextran (T500) substituted at 5% by DEAE functions were selected for the HPAC experiments.

These supports, activated by CDI, were coupled with anticoagulant materials or amidine derivatives. The coupling yields of heparin, functional dextrans, or *p*-aminobenzamidine obtained on the different supports coated by modified dextran or agarose were similar to those obtained with the traditional polysaccharide phases.

The coated silica supports grafted with heparin or functional dextran derivatives (SiD-Hep or SiD-FD, respectively) exhibited a strong affinity for human thrombin in solution. The affinity constants calculated from the adsorption isotherms are presented in Table II. In the chromatographic experiments, thrombin was adsorbed in the initial buffer and desorbed during the salt gradient. The differences observed in the salt concentration at the maximum of the elution peak (Table III) are related to the strength of the interaction between the immobilized ligand and the enzyme in solution. About 60% of the enzymatic activity was recovered in the elution peak. The loss in the enzymatic activity was due to thermal denaturation of thrombin and can be decreased with the use of higher elution flow rates.

TABLE II Affinity constant and binding capacity of human thrombin on the different supports

Support	Affinity constant[a]	Binding cap.[b]
SiD-Hep	3.6×10^5	1.8
SiD-FD	8.5×10^5	3.0
SiD-pABA	1.7×10^8	0.4
SiD-B-pABA	8.4×10^7	0.23
SiD-Arg	6.7×10^7	0.21
SiD-Gua	5.2×10^7	0.20

[a] Affinity constant of human thrombin in 1 mol^{-1}.
[b] Binding capacity expressed in mg of thrombin per g of dry support.

TABLE III HPLC conditions of human thrombin

Chromatography of human thrombin		
Support	Molarity[a]	Yield[b](%)
SiD-FD	NaCl 1.2 M	68
SiD-Hep	NaCl 1.0 M	65
SiD-pABA	Arg 0.5 M	80

[a] Molarity : concentration of NaCl or Arginine in the eluant used for the desorption.
[b] Yield : % of enzymatic activity recovered.

These supports were also used for the purification of bovine thrombin from a commercial sample (Fig. 3). The enzyme was strongly adsorbed in the initial buffer whereas most of the contaminant compounds were washed out in the first peak. The enzyme was specifically desorbed during the salt gradient and collected. The purification yields obtained on the two types of active support were similar (Table IV) and the specific enzymatic activity of the purified enzyme was remarkly high. The comparison between the two active phases indicates that functional dextrans display stronger interactions. However, the lowest recovery in the purification process reveals the simultaneous adsorption of contaminant proteins in the initial buffer. These proteins can be desorbed by detergent (Tween 20) in the washing buffer (Fig. 4). In fact, beside their specific interactions, functional dextrans also displayed non-specific adsorption towards thrombin.

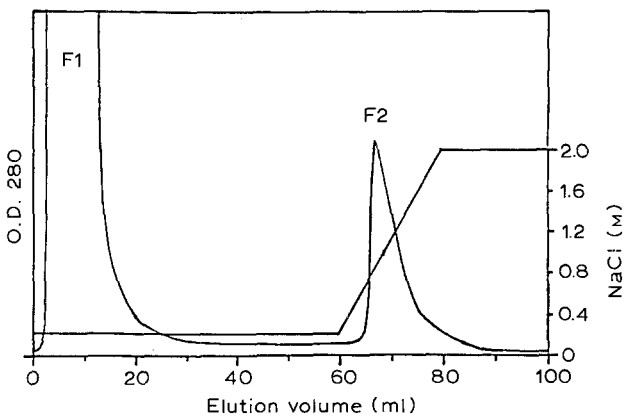

Figure 3 Purification of bovine thrombin by HPAC on SiD-Hep. Column 5 cm by 0.7 cm ID, flow rate 1 ml mn^{-1}. Initial eluent: phosph. buffer 0.02 M, NaCl 0.1 M, pH 7.4.

TABLE IV Purification of bovine thrombin by HPAC

Purification of bovine thrombin		
Support	Recovery[a](%)	Specific activity[b]
SiD-FD	28	1800
SiD-Hep	54	2100
SiD-pABA	75	1200

[a] Recovery: % of enzymatic activity of the purified fractions.
[b] Specific activity of thrombin expressed in NIH.U mg^{-1}.

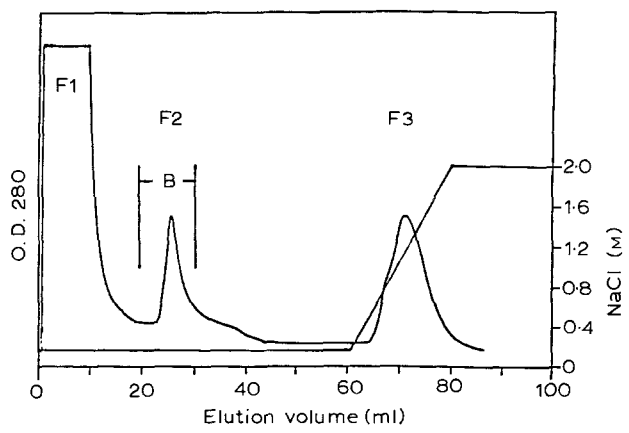

Figure 4 Purification of bovine thrombin by HPAC on SiD-FD. Elution conditions similar to Fig. 3. Buffer B: 0.015% Tween 20 in initial buffer.

Coated silica supports grafted by the unsubstituted amidine derivatives gave a strong and specific interaction with human thrombin. The affinity constants and the binding capacity determined from the adsorption isotherms are shown in Table II. The high values of the affinity constants observed on the supports grafted by *p*-aminobenzamidine seem to demonstrate that the aromatic ring of the ligand also interacted with some hydrophobic residues of the enzyme. The support SiD-B-pABA, where the amidine is coupled to the coated silica via a spacer arm, presented a similar affinity but a smaller binding capacity in comparison with the corresponding support obtained by the direct grafting of *p*-aminobenzamidine. The spacer arm probably led to a great flexibility of the grafted ligand but also to a weaker reactivity during the coupling reaction. In the chromatographic experiments, the enzyme was strongly adsorbed by the active supports and could not eluted by the salt gradient. However, the adsorbed enzyme was desorbed by a competitive elution of arginine 0.5 M (Fig. 5). The pure enzyme was obtained by a size exclusion separation by using a Sephadex G25 column. The recovery of the enzymatic activity and the specific activity of the enzyme after these two separation steps were high (Table III).

This support was also used to purify bovine thrombin from a commercial crude extract. In our experimental conditions, most of the contaminant compounds were washed out by the initial buffer in a first elution peak. The adsorbed enzyme was desorbed by using arginine as a competitive agent. After the separation on the Sephadex column, the recovery and the specific activity of the active enzyme were important (Table IV), but the binding capacity of this active support was notably smaller than those exhibited by the heparin grafted silica.

The purifications obtained on these active coated silica supports grafted by anticoagulant compounds or amidine were similar to those observed on similar affinity soft gels [15–18], but they could be realized in a shorter time. However, this decrease was also limited by the kinetic parameters of the heterogeneous adsorption process.

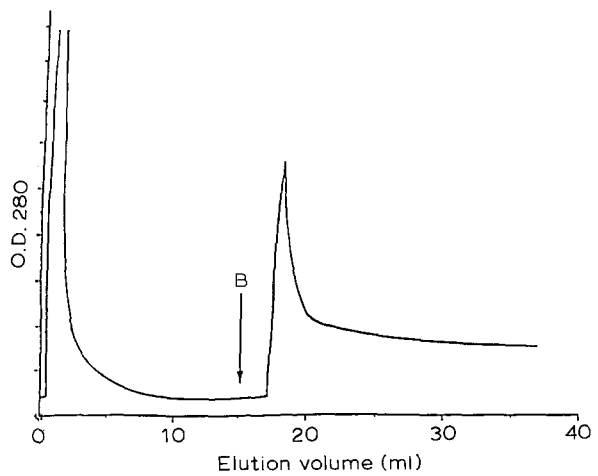

Figure 5 Purification of bovine thrombin by HPAC on SiD-pABA. Column 12.5 cm by 0.4 cm ID, flow rate 1 ml mn^{-1}. Eluent A: phosph. buffer 0.02 M, NaCl 0.1 M, pH 7.4. Eluent B: arginine 0.5 M in buffer A.

4. Conclusion

The passivation of silica was obtained by a coating of the inorganic beads with polysaccharide substituted by a calculated amount of positively charged DEAE functions. This passivation led to supports presenting minimal non-specific adsorptions with proteins in solution. Moreover, a second coating of the coated silica with native polysaccharide can improve the passivation. Because of their polysaccharidic layer, these supports can easily be grafted with active ligands such as anticoagulant compounds or amidine derivatives. The active stationary phases developed strong and specific interactions for human thrombin. On the coated silica beads grafted by heparine or functional dextrans, the adsorbed enzyme was desorbed by the increase of the ionic strength of the eluent. Thrombin was also strongly adsorbed on the supports grafted by amidine derivatives and could only be desorbed by the use of a competitive agent.

These affinity phases were used for the purification of bovine thrombin from a commercial crude extract. The chromatographic profiles and the characteristics of the separation were similar to those observed on the corresponding soft gel supports. Moreover, because of the mechanical behaviour of the silica, the separations could be realized at high flow rate and the purification processes could easily have been scaled up.

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