Journal of Chromatography, 510 (1990) 71–81
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 355

Double-coated silica supports for high-performance affinity chromatography of proteins

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ABSTRACT

In order to prepare easily derivatizable supports for high-performance affinity chromatography (HPAC), the advantages of traditional polysaccharide-based supports were combined with the excellent mechanical properties of silica by coating the porous silica beads with a double layer of polysaccharide. The starting material was preliminarily impregnated with dextran or agarose, substituted with a calculated amount of positively charged diethylaminoethyl (DEAE) functions, in order to neutralize the cation-exchange capacity. These silica beads were then recoated by a second coupling with a native dextran or agarose so the DEAE functions introduced by the first coating could be overlayed and the coating state of the silica beads was further improved. The passivation of silica was confirmed by eluting standard proteins on the double-coated silica supports in high-performance size-exclusion chromatography. The elution of an acidic biopolymer, heparin, on different coated silica supports under gradient conditions demonstrated the major improvement of the native polymeric overlayer on the ionic properties of the support. These double-coated silica supports can also easily be activated by classical activation methods and coupled with active ligands (protein A and heparin). The active supports grafted by protein A were used in HPAC of human immunoglobulin G. The double-coated silica supports grafted by heparin were used in HPAC of human α-thrombin and human antithrombin III and for the purification of bovine thrombin from a commercial crude thrombin preparation.

INTRODUCTION

High-performance affinity chromatography (HPAC) combines the great specificity of affinity techniques with the efficiency, sensitivity and speed of operations of high-performance liquid chromatographic (HPLC) techniques. Although significant progress has been made in this area, applications of the technique have been limited owing to the lack of a good general and derivatizable support[1–3]. Such a support for HPAC of proteins requires minimum non-specific adsorption, a hydrophilic character, simple derivatization procedures with a broad range of ligand chemistries and an

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ability to tolerate different solvents and a rapid change in solvent composition necessary for efficient elution of proteins. In particular, the high elution rates used in HPAC require excellent mass-transfer properties and good mechanical properties of the stationary phase.

Polysaccharide-based supports have been successfully used for traditional affinity chromatography becaus of their good chromatographic properties and the simplicity of the activation procedures, but their mechanical instability limits their use in HPAC. In contrast, silica, commonly used for HPLC of proteins[4–7], is mechanically stable but contains acidic silanol groups, among other surface groups, that cause strong and often irreversible non-specific adsorption of proteins in aqueous media[4]. By coating polysaccharide on silica beads, we have combined the advantages of the traditional soft gel affinity supports with the excellent mechanical properties of silica supports.

We have previously reported the preparation of coated silica supports for the HPLC of biopolymers by impregnating silica beads with a polysaccharide (dextran or agarose), substituted by a calculated amount of diethylaminoethyl (DEAE) functions[8,9]. The DEAE-polymer monocoated silica supports present only minimal cation-exchange capacity and have been used in high-performance size-exclusion chromatography (HPSEC)[8] and HPAC of proteins[9]. However, it is difficult to obtain an exact balance between ion-exchange capacities of the DEAE-polymer and the native silica. The neutralization of the silanol functions requires an excess of positive charges and leads to a residual anion-exchange capacity.

In view of the above, we have prepared a new class of double-coated silica supports. After a preliminary coating with a DEAE-polymer, the silica beads are recoated with native dextran or agarose in order to overlay the positive DEAE groups on the surface. The minimization of non-specific adsorptions was investigated by HPSEC. Gradient elution of an acidic polymer, heparin, on the mono- and double-coated silica supports demonstrates the importance of the double coating. Because of their polysaccharidic surface, the techniques used for the immobilization of ligands on polysaccharide-based supports can be easily transferred to these supports. Active ligands (protein A and heparin) have been immobilized with a good yield by classical activation methods[10]. Comparison of the elutions of human IgG on mono- and double-coated silica supports grafted by protein A demonstrates the better performance of the double-coated silica support in HPAC. The elutions of human α-thrombin and human antithrombin III (AT-III) were carried out on the double-coated silica supports, grafted by heparin. Finally, the purification of bovine thrombin from a commercial crude thrombin preparation was achieved on this affinity sorbent by HPAC. The results of the purification are compared with those obtained on the commercial heparin grafted supports.

EXPERIMENTAL

Preparation of coated-silica supports and affinity sorbents

The starting silica beads (silica X015 M and silica X075 M; IBF Biotechnics, Villeneuve La Garenne, France) are mainly spherical (40–100 μm) and have average pore diameters of 1250 and 300 Å, respectively. The polysaccharides used in this study are agaroses (Indubiose A37 HAA and Indubiose A37 N) from IBF-Biotechnics and

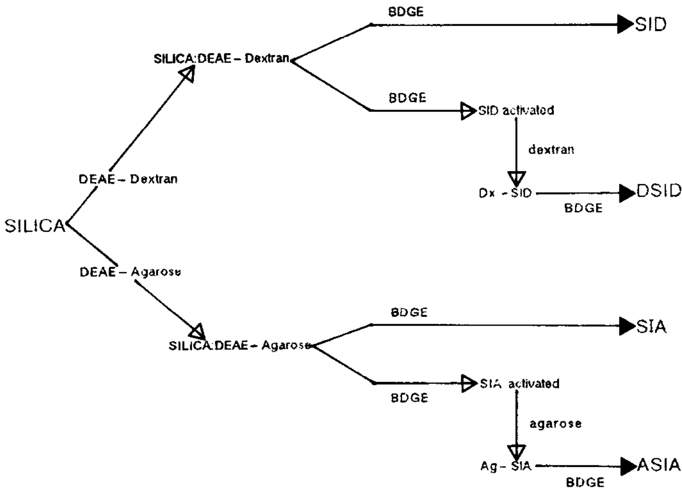


Fig. 1. Preparation of coated silica supports.

dextrans (T40, T70 and T500; Pharmacia France, Bois D'Arcy, France). The preparation of DEAE-polysaccharides and monocoated silica supports have been described previously[8,9].

The preparation of double-coated silica supports is illustrated in Fig. 1. A 10-g amount of the native silica was impregnated with 0.45 g of DEAE-agarose or 1.5 g of DEAE-dextran in 25 ml of doubly distilled water, the pH of which had been adjusted to 11.5, for 30 min at 80°C for DEAE-agarose and at room temperature for DEAE-dextran. The polymeric layer passivate the silica phases and increase the chemical stability of the supports towards the alkaline media under the experimental conditions. The packing was dried for 15 h at 80°C, then added to a solution of 284 mg (2.2 mmol) of 1,4-butanediol diglycidyl ether (BDGE) in 100 ml of diethyl ether. The mixture was stirred for 30 min at 40°C. After evaporation of the solvent, the silica powder was dried for 30 min at 80°C and then coupled with 0.45 g of agarose or 1.5 g of dextran in 30 ml of doubly distilled water (pH adjusted to 11.5) for 2 h at 80°C. After drying for 15 h at 80°C, the surface layer of coated silica was cross-linked with 71 mg (0.55 mmol) of BDGE in 100 ml of diethyl ether for 30 min at 40°C. The amount of polymer covering the material surface was determined by elemental analysis of carbon and expressed as the weight of coating polymer (mg of coating polymer per g of support).

The coated silica beads were actived using 1,1'-carbonyldiimidazole (CDI) or BDGE as activing agent[10]. Immobilization of protein A (IBF Biotechnics) and heparin (101 IU/mg; Institut Choay, Paris, France) was performed using the actived support suspended with the ligand in 0.1 *M* sodium carbonate buffer (pH 8.7) at room temperature. The amount of protein A immobilized was determined by Bradford's method[11] and the amount of heparin was determined by elemental analysis of sulphur. The adsorption capacity of the active supports was determined from the adsorption isotherms.

Chromatographic experiments

The HPLC apparatus was a Merck–Hitachi 655 A-12 gradient system from Lab Merck-Clevenot (Nogent sur Marne, France) with a Rheodyne 7126 injection valve, connected to an LMC UV–visible variable-wavelength monitor and a D2000 integrator. The solutions and the buffers were prepared with doubly distilled water, filtered through a 0.22-μm Millipore HA membrane. All chemicals were of analytical-reagent grade.

The passivation of native silica by the double coating was investigated by testing the performances of the coated silica supports in HPSEC. Molecular weight calibration graphs of standard proteins were obtained as described previously[8]. The size-exclusion retentions of the proteins on the supports were comfirmed by eluting several acidic or basic proteins [human albumin, human immunoglobulin G (IgG), cytochrome *c*, antithrombin III] under gradient conditions. The improvement in the ionic properties of the supports resulting from the double coating was also studied by injecting an acidic biopolymer, heparin, onto the double- and mono-coated silica supports. After washing with the initial buffer, the adsorbed substance was eluted by raising the salt concentration of the eluent.

The different biospecific affinity sorbents prepared from the double-coated silica support were used in chromatographic experiments. Huma IgG (12.5 mg/ml; Sigma), human antithrombin III (3.4 IU/mg; CRTS, Lille, France) and thrombin [1000 NIH.U/mg (*i.e.* activity of thrombin determined by comparison with standard from National Institute of Health, Bethesda, MD, U.S.A.); CNTS, Paris, France] were eluted on their respective affinity sorbents under gradient conditions. A 5-ml volume (23.5 mg of proteins) of crude bovine thrombin sample (Hoffmann-La Roche) was injected onto a column packed with 2.5 ml of heparin affinity sorbent and pre-equilibrated with the initial buffer [0.02 *M* disodium phosphate–0.15 *M* NaCl (pH 7.4)], at room temperature at flow-rate of 60 ml/h. The adsorbed thrombin was then eluted using a salt gradient.

In all chromatographic experiments, the eluted proteins were detected at 280 nm and the chromatographic fractions were collected in order to determine the yield of the separations. The concentrations of proteins were measured by Bradford's method[11]. The biological activity of AT-III was determined using Chromo-Thrombin from Diagnostica Stago (Asniéres-sur-Seine, France)[12]. The thrombin clotting activity of the eluted fractions was measured as described previously[13]. The fractions were also analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 20% gel using the PhastSystem (Pharmacia, Uppsala, Sweden).

RESULTS AND DISCUSSION

Preparation of coated silica support

The purpose of the modification of the silica surface is to minimize the non-specific adsorption of silica supports for biopolymers and to introduce a high concentration of easily activatable groups. This was achieved by coating the inorganic phases with polysaccharide. The cation-exchange capacity of the native silica is minimized by impregnation with dextran and agarose substituted by a calculated amount of positively charged functional DEAE groups[8,9]. The characteristics of the substituted polysaccharides used for the preparation of the different supports are

TABLE I

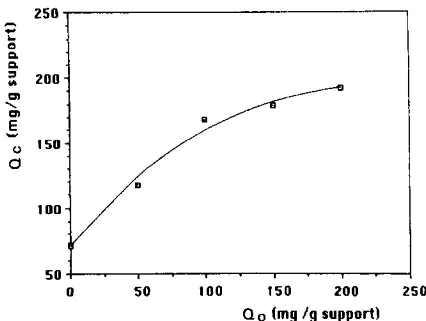
CHARACTERISTICS OF SUBSTITUTED POLYSACCHARIDES

Substituted polysaccharide	*Starting polymer*	*Mol.wt. (g/mol)*	*Percentage of DEAE units*
DDT404	Dextran T40	35 600	4.8
DDT705	Dextran T70	68 000	4.5
DDT50011	Dextran T500	488 000	7.2
DHAA1	Indubiose HAA	–	10.0
DNA1	Indubiose NA	–	10.0

presented in Table I. However, it is difficult to obtain an exact balance between the ion-exchange capacities of the native silica and the DEAE-polymer. Therefore, these silica beads impregnated with DEAE-polysaccharide were recoated by coupling a native dextran or agarose, in order to obtain supports with hydrophilic and non-ionic surfaces. The amount of polysaccharide coupled depends on the amount of polysaccharide used (Fig. 2). The use of a polymer with a high molecular weight is useful for raising the coating capacity (Fig. 3). The characteristics of the mono- and double-coated silica supports used in this study are presented in Table II.

HPSEC performance of the supports

The different coated silicas were tested in HPSEC using several proteins with molecular weight from $6.5 \cdot 10^3$ to $700 \cdot 10^3$ g/mol. Blue dextran ($2 \cdot 10^6$ g/mol), glycyltyrosinamide (434 g/mol) and dinitrophenylalanine (255 g/mol) were used in order to determine the exclusion limits of the support. Most of the proteins were eluted between the exclusion limits of the supports, except cytochrome *c*, which was abnormally retained by the supports prepared from silica X075 M. This effect is probably related to the high p*I* value and the small size of this protein and indicates that some silanol functions inside the pores of the silica with smaller porosity are not



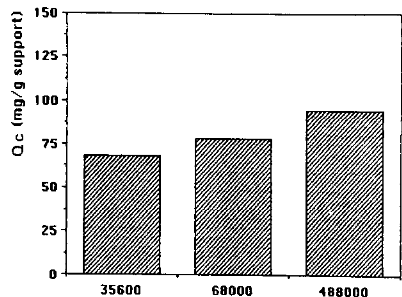


Fig. 2. Variation of the amount of dextran coupled with the initial amount of dextran used. Silica X015 M, dextran T500.

Fig. 3. Influence of the molecular weight (horizontal axis) of dextran used on the amount of coating. Silica X015 M.

TABLE II

CHARACTERISTICS OF COATED SILICA SUPPORTS

Support	*Silica porosity (Å)*	*Coating polymer*		*Amount of polymer fixed (mg/g support)*[a]
		Code	*Mol.wt. (g/mol)*	
SID15705	1250	DDT705	68 000	104
SID1550011	1250	DDT50011	488 000	101
SIA15HAA1	1250	DHAA1	–	45
DSID15M	1250	DDT50011	488 000	72 (1)
		Dextran T500	488 000	94 (2)
DSID75M	300	DDT705	68 000	68 (1)
		Dextran T70	68 000	90 (2)
ASIA15M	1250	DHAA1	–	45 (1)
		Indubiose HAA	–	35 (2)

[a] (1) First coating; (2) second coating.

completely masked. A comparison of the behaviour of two silicas with different porosities but passivated by the same dextran double coating is presented in Fig. 4. The slopes of the calibration graphs indicate that the support prepared with the 300-Å silica beads gives a better resolution for proteins in the range 10–100 kg/mol. However, the molecular weight limits of this support are slightly restricted compared with the exclusion limits of the 1250-Å pore-size unmodified silica packing. Because of their minimal non-specific adsorption and larger exclusion limits, the supports prepared from silica X015 M were chosen as starting materials for the preparation of affinity sorbents.

The normal elution behaviour of several acidic or basic proteins (human albumin, human IgG, human AT-III, cytochrome *c*) on the double-coated silica supports (DSID15M or ASIA15M), even at high ionic strength, demonstrates minimal non-specific adsorptions. Moreover, heparin is strongly adsorbed on the mono-coated silica support at 0.1 *M* sodium chloride (Fig. 5a), but is eluted by the same buffer on the

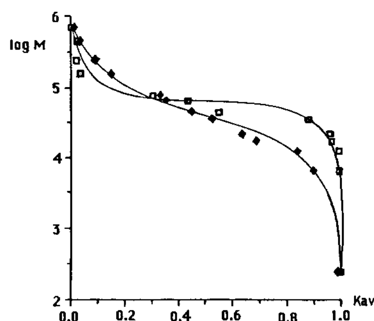


Fig. 4. Molecular weight (*M*) calibration graphs of standard proteins on (□) DSID75M and (◆) DSID15M supports. Column, 25 × 0.7 cm I.D.; flow-rate, 1 ml/min; eluent, 0.02 *M* Tris–HCl–0.15 *M* NaCl (pH 7.4). $K_{av} = (V_e - V_0)/(V_t - V_0)$.

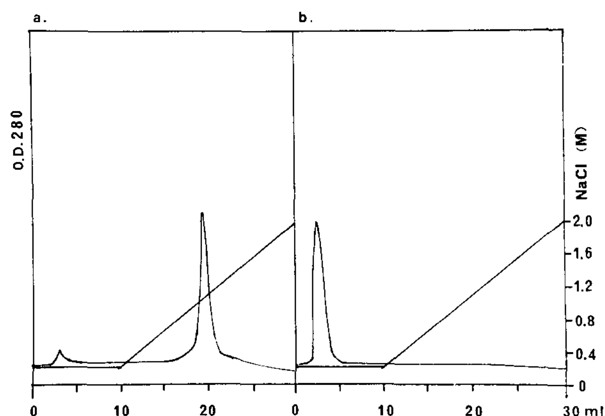


Fig. 5. Elution of 100 μl heparin (3 mg/ml) on (a) SID15M50011 and (b) DSID15M supports. Column, 5 × 0.7 cm I.D.; flow-rate, 1 ml/min; eluent, 0.02 *M* disodium phosphate (pH 7.4).

double-coated silica (Fig. 5b). This result demonstrates that the second coating masks the residual anion-exchange capacity of DEAE groups of the mono-coated phases.

Immobilization of active ligands

The immobilization of a broad range of ligands has been realized on

TABLE III

COUPLING CONDITIONS OF COATED SILICA SUPPORTS

Q_l = amount of ligand used (mg/g support); Q_a = amount of CDI used (mmol/g support).

Support	*Ligand*	Q_l	Q_a	*Time (h)*	*Yield (%)*
SID15M50011	Protein A	2.29	0.615	15	92
DSID15M	Protein A	2.29	0.615	15	93
DSID15M	Heparin	50	1.84	48	68
ASIA15M	Heparin	50	1.84	48	68

TABLE IV

CAPACITY OF THE AFFINITY SORBENTS

$[Q_l]$ = amount of ligand immobilized.

Stationary phase	$[Q_l]$ *(mg/g)*	*Protein adsorbed*	*Capacity (mg/g)*
Protein A–DSID15M	2	IgG	7
Heparin–DSID15M	28	Thrombin	13 000[a]
Heparin–DSID15M	28	AT-III	6.27

[a] NIH.U/g.

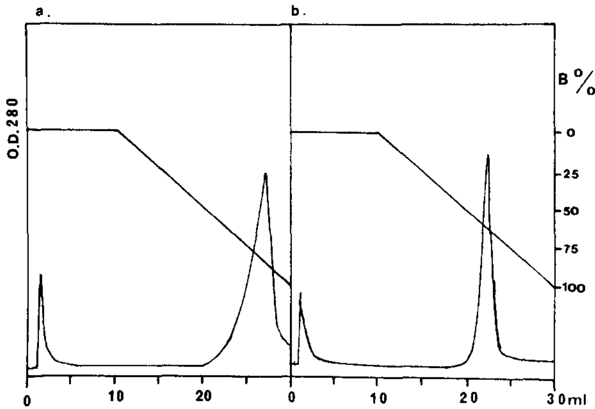


Fig. 6. Elution of 100 μl of human IgG (12.5 mg/ml) on (a) protein A–SID15M50011 and (b) protein A–DSID15M supports. Column, 5 × 0.7 cm I.D.; flow-rate, 1 ml/min; buffer A, 0.02 *M* Tris–HCl–0.15 *M* NaCl (pH 7.4); buffer B, 0.02 *M* glycine–HCl–0.15 *M* NaCl (pH 2.8).

polysaccharide-based supports[10]. These results can be easily adapted to the double-coated silica supports. The immobilization of protein A and heparin was performed with a high yield using CDI and BDGE as activating agents (Table III). The biopolymers immobilized on the double-coated silicas show a considerable adsorption capacity for the corresponding protein in solution (Table IV).

Elutions of proteins in HPAC

In order to study the performances of the supports in HPAC, several human proteins (IgG, AT-III and thrombin) were injected onto their respective affinity sorbents, prepared fom the double-coated silica supports. The conditions used for adsorption and desorption were similar to those used on classical supports, grafted by the same ligand.

A typical chromatogram of human IgG on protein A immobilized on dextran double-coated silica support (protein A–DSI15M) is presented in Fig. 6a. The protein was adsorbed at pH 7.4 (0.02 *M* Tris–HCl–0.15 *M* NaCl) and selectively desorbed by a decreasing pH gradient with 83% protein recovery. The comparison of the elution on dextran mono- and double-coated affinity supports (Fig. 6a and b) shows that IgG is

TABLE V

ELUTION CONDITIONS FOR HPAC OF PROTEINS

Stationary phase	*Protein eluted*	*Condition of desorption*	*Yield (%)*	*Fig.*
Protein A–SID15M	IgG	85% B	83	6a
Protein A–DSID15M	IgG	62% B	83	6b
Heparin–DSID15M	Thrombin	1 *M* NaCl	>80	7a
Heparin–DSID15M	AT-III	1.2 *M* NaCl	82	7b

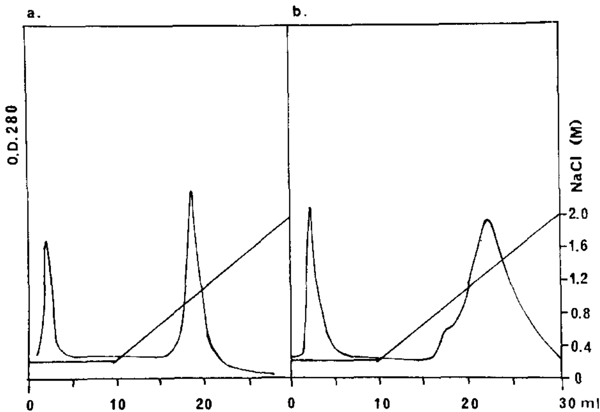


Fig. 7. Elution of (a) 100 μl of human thrombin (1000 NIH.U/ml) and (b) 100 μl of human antithrombin III (34 IU/ml) on heparin–SID15M support. Column, 5 × 0.7 cm I.D.; flow-rate, 1 ml/min; eluent, 0.02 *M* disodium phosphate (pH 7.4).

adsorbed and eluted with the same recovery on both supports (Table V). However, the narrower elution peak and the lower proportion of eluting buffer B at the peak maximum demonstrate the better performance of the double-coated stationary phase. The chromatograms are similar to those obtained on traditional phase, grafted by protein A[14].

HPAC elutions of human thrombin (Fig. 7a) and human AT-III (Fig. 7b) were carried out on heparin immobilized on the dextran double-coated support (heparin–DSID15M). The two proteins were strongly adsorbed at low ionic strength and selectively desorbed by increasing the salt concentration. The eluted fractions were collected and their biological activities were measured, demonstrating a good recovery (>80%) (Table V). The recovery of the enzymatic activity of α-thrombin was reduced because slight inactivation of the enzyme occurs during the separation at room temperature.

A 100-μl volume of AT-III solution was successively injected 20 times onto the

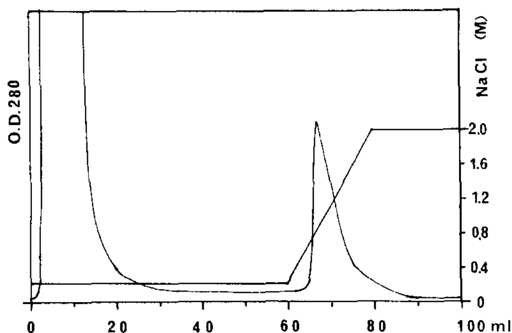


Fig. 8. Purification of bovine thrombin (5 ml) by HPAC on heparin–DSID15M support. Column, 5 × 0.7 cm I.D.; flow-rate, 1 ml/min; eluent, 0.02 *M* disodium phosphate (pH 7.4).

TABLE VI

PURIFICATIOH OF BOVINE THROMBIN

Parameter	*Starting material*	*Desorption peak I*	*Desorption peak II*
Volume (ml)	5	15	12
Protein (mg/ml)	4.7	1.4	0.05
Thrombin (NIH.U/ml)	600	4	104.4
Specific activity (NIH.U/mg)	64	2.9	2130
Yield (%)	–	4	83.5
Purification factor	–	–	33.3

heparin-immobilized column under the same elution conditions; similar chromatograms were obtained and no apparent change in the elution recovery was observable during these elutions. This result demonstrates the excellent chemical stability of the coated-silica supports and the absence of ligand bleeding under these elution conditions.

This support was also used in order to purify bovine thrombin from a commercial crude preparation (Fig. 8). Thrombin is eluted at an ionic strength of about 1 *M* NaCl as a single, fairly sharp peak. No additional material was eluted at higher

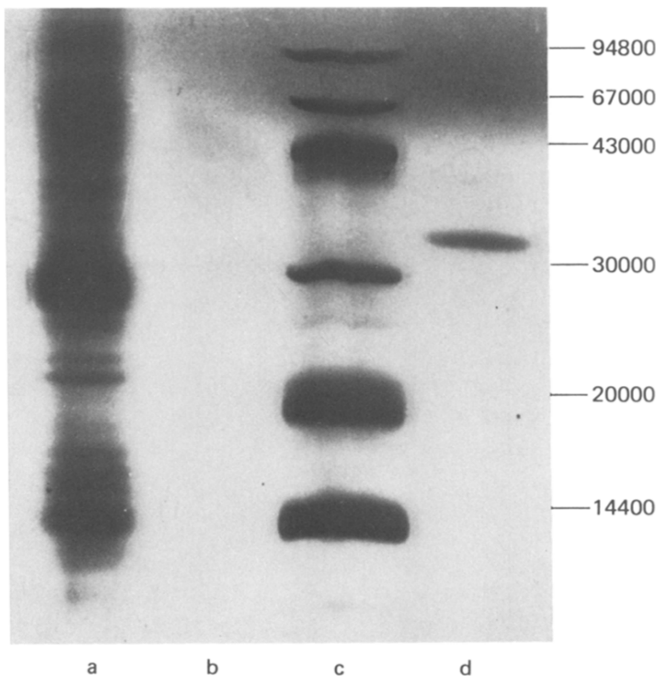


Fig. 9. SDS-PAGE. (a) Crude thrombin; (b) background; (c) standard proteins; (d) purified thrombin.

ionic strength. The enzyme activity, determined by clotting assay, coincides with the protein peak. The results of the different purifications are given in Table VI. The recovery of biological activity in these experiments was about 78% and the total yield of purified thrombin was 0.56 mg, with a specific activity of about 2100 NIH.U/mg. The purity of thrombin was confirmed by SDS-PAGE (Fig. 9). These results are similar to those obtained on commercial heparin-grafted phases[15]. The small difference in recovery is probably due to a slight inactivation of the enzyme during the separation at room temperature.

CONCLUSIONS

The cation-exchange capacity of silica beads can be minimized by a preliminary impregnation of the inorganic material using a hydrophilic polymer with a relatively low percentage of units bearing positively charged DEAE groups. The impregnated silica can be recoated by coupling a native polysaccharide in order to overlay the DEAE units and to improve the coating state. The introduction of a hydrophilic and non-ionic polymeric layer on the silica surface minimizes the non-specific adsorptions and introdues a high concentration of activatable hydroxylic functions. The modified supports can be easily grafted with active ligands by the classical coupling techniques. The affinity sorbents prepared from the double-coated silica supports can be used in HPAC of proteins. In comparison with mono-coated silica supports, these double-coated silicas exhibit better performances in HPAC and HPAC purifications of proteins from a crude material can be carried out on these affinity sorbents. The separations are similar to those obtained on the corresponding traditional matrices. Moreover, because of the mechanical properties of the starting material, these double-coated silica supports can easily be used for scaling up the purification of proteins by HPAC.

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