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Coated silica and its behaviour in dye-affinity chromatography

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ABSTRACT

Cellulose-coated silica as a support for the high-performance affinity liquid chromatography of proteins was prepared. Cibacron Blue 3G-A as an affinity ligand was grafted onto silica beads coated with cellulose in one or two steps. The prepared supports were used for the purification of lactate dehydrogenase from a crude bovine extract using solutions of potassium chloride, NADH and Cibacron Blue–dextran T 10 as eluents of the bound enzyme. The sorption capacities of the dyed cellulose-coated silica for lactate dehydrogenase from bovine muscle were found to be much lower than those of Cibacron Blue–bead cellulose. The comparison of un-coated and coated silica supports showed that the non-specific interactions between the enzyme and the silica coated with cellulose were considerably reduced.

INTRODUCTION

Polysaccharide matrices such as cross-linked dextrans, agarose and cellulose as chromatographic supports for affinity chromatography of proteins [1] have been known for many years. During the last 15 years of the development of supports amenable to the demands of high-performance (pressure) affinity chromatography (HPAC), the time required for protein purification has decreased from hours to minutes [5] while the purity of the final product has increased. The traditional dextran and agarose supports used for affinity chromatography (AC) are not practical for HPAC owing to their mechanical instability with high-pressure flows.

The first practical HPAC systems for protein separation were based on porous silica beads [2] because they have excellent mechanical properties. However, the non-specific interaction between silanols and proteins on the silica surface requires a modification of these materials to decrease these interaction and to introduce functional groups for affinity ligand binding before their use as chromatographic supports [3,4]. For example, functional silane [5], poly(9-vinyladenine) [6], crown ethers–neutral macrocyclic polymers [7] and polyethyleneimines [8–10] have been used for coat-

ing the surface of silica and silica beads have been coated with polysaccharides (dextran or agarose) substituted by a calculated amount of positively charged diethylaminoethyl functions [3,11,12]. The last support (silica–DEAE-dextran) with the grafted reactive dye Procion Blue HE-GN was used in the dye-affinity chromatography of 6-phosphogluconate dehydrogenase [13]. Dextran-coated silica beads could be used in place of dextran- or agarose-based supports for the preparation of immobilized dye chromatographic supports with kinetically limited enzyme–dye interactions. Their capacity however, is, much lower than that found with agarose supports.

The characteristics of cellulose, such as reactivity, hydrophilicity, mechanical and microbial stabilities, absence of non-specific interaction and low price, are essential for supports for the affinity chromatography of proteins. The ability of cellulose to form solutions and a good sol–gel transition are very important for coating. The non-specific sorption of proteins on bead cellulose and the decrease in these non-specific interactions were studied in our previous work [14–16]. On the basis of this knowledge, we decided to examine cellulose as a coating material. We prepared cellulose triacetate-coated silica which was subsequently deacetylated. This support was used in the dye-affinity chromatography of lactate dehydrogenase (LDH).

EXPERIMENTAL

Materials

Silica gel (SG-100Na) was prepared in the laboratory with surface area 190 m^2 g^{-1} irregular, particle size 120–250 μm, mean pore diameter 25 nm and pore volume 1.15 cm^3 g^{-1}. Acetyl-cellulose (Rhodiafil Acetat-Faser, degree of substitution (DS) = 2.64, Deutsche Rhodiaceta, Freiburg, Germany) and Cibacron Blue 3G-A (CB) (C.I. Reactive Blue 2) were kindly provided by Ciba-Geigy (Basle, Switzerland) and Coomasie Blue G-250 by Serva (Heidelberg, Germany). Dextran T 10 was obtained from Pharmacia (Uppsala, Sweden) and was derivatized with Cibacron Blue 3G-A [14] (degree of substitution 106.3 μmol g^{-1}). Lyophilized LDH prepared from beef flank muscle [15] contained *ca.* 4.8 units of LDH (lactate:NAD oxidoreductase, E.C. 1.1.1.27) per milligram of solid material. The proteins used were bovine serum albumin (SEVAC, Prague, Czechoslovakia), ovalbumin (Fluka, Buchs, Switzerland), γ-globulin (IMUNA, Šarišské Michal'any, Czechoslovakia) and hog trypsin (Koch-Light, Colnbrook, U.K.).

Methods

The activity of LDH was established spectrophotometrically [14,17] and the protein content according to the method of Bradford [18].

Preparations of cellulose-coated silica

For single coating, 7 g of silica gel were mixed and soaked with 12 ml of acetone solution containing 2 g of cellulose triacetate. After drying, the material was hydrolysed in 30 ml of 0.5 *M* sodium hydroxide solution at 20°C for 1 h, washed and dried. The cellulose content was 22% (w/w). For double coating, an additional 3 ml of acetone solution containing 0.3 g of cellulose triacetate were added to 4.2 g of the single-coated silica and then the subsequent steps as in the procedure described above were followed. The cellulose content was 31.3% (w/w).

Preparation of silica–CB

Wide-pore silica (pore diameter 200 nm, surface area 18 $m^2\ g^{-1}$, particle diameter 60–120 μm), modified with aminopropyltriethoxysilane, was treated with an excess of a 1% ethanolic solution of Cibacron Blue 3G-A at 60°C for 30 min, washed and dried.

Preparation of CB dyed silica–cellulose

The technique used for the attachment of dyes to bead cellulose [14] was applied to the preparation of the cellulose-coated silica: 2 g of silica–cellulose (SG–CEL) were suspended in 32 ml of water, then 0.12 g of CB in 2 ml water and 2.4 g of sodium chloride were added and the suspension was stirred at 60°C for 30 min. The temperature was subsequently elevated to 80°C, then 6 ml of 6% (w/w) sodium carbonate solution were added and the reaction was allowed to proceed for 2 h at the same temperature. The reaction mixture was neutralized and the unbound dye was washed out until the filtrate was colourless. The degree of substitution was determined spectrophotometrically at 630 nm [14] after the dissolution of CB-cellulose in cadmium tris(ethylenediamine)hydroxide solution.

Elution experiments

Dye-affinity chromatography of LDH on CB–SG–CEL (single-coated, DS = 5.8 μmol g^{-1} CB, and double-coated, DS = 8.4 μmol g^{-1} CB) was performed at ambient temperature using a column (2.5 × 1.1 cm I.D.) equilibrated with 20 m*M* phosphate buffer (pH 8.5). The solution of 0.2 ml of crude LDH (50 U, 8.2 mg protein) was applied. The unbound proteins were washed out with the equilibration buffer, followed by elution with 2 *M* potassium chloride in the equilibration buffer and finally with 1 m*M* NADH (or 50 μ*M* CB–dextran T 10) in the equilibration buffer. The flow-rate was 30 ml h^{-1} and both the total enzyme activity and the protein content were determined in 2-ml fractions.

The non-specific interaction SG–CEL–LDH was determined using a column (2.5 × 1.1 cm I.D.) with undyed support. After loading the same amount of crude LDH or other proteins the elution was performed only with the equilibration buffer.

Loading experiments and determination of binding capacity

The loading experiments were performed on the same columns as the elution experiments. After equilibration the columns were loaded with the solution of crude LDH (5 mg ml^{-1}) in the equilibration buffer at a flow-rate of 20 ml h^{-1}. The activity and protein content in effluent were determined as described above. When the column was saturated, the excess of the enzyme was washed out with the equilibration buffer and the bound LDH was eluted with 50 μ*M* CB-dextran T 10.

RESULTS AND DISCUSSION

Polymer-coated silica supports are potentially good stationary phases for HPAC separations of proteins. The hydrophilic polysaccharide layer neutralizes the negative properties of silica which might lead to, *e.g.*, non-specific interactions with proteins, sensitivity in alkaline media and low reactivity. In the present investigation we covered the surface of the macroporous silica with cellulose according to the

experimental procedure described above. Silica gel was mixed in an acetone solution of cellulose acetate and, after removing acetone, the coated silica gel was dried and the cellulose film was regenerated by saponification with a solution of sodium hydroxide. Silica coated with regenerated cellulose was then dried, whereby the secondary intermolecular bindings or hydrogen bridges were formed. The coating process is so mild that it does not influence the silica gel structure; this was confirmed by leaching out the deposited cellulose with 72% sulphuric acid, which fully restored the original silica gel surface. The silica was coated in one or two steps and thus supports with a lower non-specific interaction with proteins were prepared. The results of elution of crude LDH on three types of silica either coated or not coated with cellulose are given in Table I. The surface areas of cellulose-coated silica reveal that probably only part of the silica porous surface is covered with cellulose. Double coating has no influence on further decreasing the surface area. This finding is in contrast with the observation that the lowest non-specific sorption of the enzyme was found on the column with SG–CEL 2. Nevertheless, important differences between SG–CEL 2 and SG–CEL 1 were not found.

TABLE I

COMPARISON OF NON-SPECIFIC INTERACTIONS OF SILICA SUPPORTS

Silica support	Cellulose content (%, w/w)	Surface area ($m^2\ g^{-1}$)	Elution with equilibration buffer	
			LDH activity (%)	Proteins (%)
SG–100 Na	0	190	16.03	19.54
SG–CEL 1	22.0	131	89.0	79.0
SG–CEL 2	31.3	134	90.3	86.04

However, different non-specific sorptions of several proteins were observed, *e.g.*, about 44% of ovalbumin (p*I* 4.6), 1% of bovine serum albumin (p*I* 4.8), 58% of γ-globulin (p*I* 5.8) and 87% of trypsin (p*I* 10.8) were adsorbed on single-coated silica under the same conditions (20 m*M* phosphate buffer, pH 8.5). The results indicate that the non-specific interactions are not exclusively ionic in nature. Only the very strong sorption with trypsin might be explained by electrostatic effects (high p*I*). The lower non-specific sorption of proteins on SG–CEL 2 than on SG–CEL 1 (Table I) and the previous results suggest that the single-coated silica contains some accessible silanol groups.

Wide-pore silica (activated with aminopropyltriethoxysilane) and both coated supports (SG–CEL 1 and 2) were derivatized with the dye Cibacron Blue 3G-A. Derivatization of coated silica is based on etherification of the cellulose hydroxyl groups with the reactive dye under alkaline conditions. The bond between CB and cellulose is of high stability [19,20]. All dye-affinity chromatographic supports were then examined for the elution profile of crude LDH (Table II). The difficulties with the elution of enzyme and accompanying proteins from CB-silica were due to the non-specific interaction mentioned before. Neither wide pores nor modification of the surface with silane affected the elution of proteins. Protection of the silica surface

TABLE II

ELUTION OF LDH FROM CB–SILICA–CELLULOSE COLUMN

Sorbent	Content of CB (μmol g^{-1})	Elution with					
		Equilibration buffer		2 *M* KCl		1 m*M* NADH	
		LDH activity (%)	Proteins (%)	LDH activity (%)	Proteins (%)	LDH activity (%)	Proteins (%)
Silica–CB	–	45.0	22.3	0.9	0.1	12.0	3.0
CB–SG–CEL 1	5.9	5.5	54.5	3.9	1.0	96.5	13.4
CB–SG–CEL 2	8.4	17.7	65.0	33.9	2.9	48.0	7.6

with cellulose reduced these effects, as confirmed by the decreased elution of LDH (biospecific effects) and increased elution of proteins from CB–SG–CEL when using equilibration buffer as the elution agent. In contrast, elution of LDH with the biospecific eluent NADH (Table II) and with CB-dextran T 10 (not shown) was very effective. The elution characteristics of LDH from CB-coated silicas (Fig. 1a and b) indicated the differences between their sorption behaviour. Important differences were observed between the amounts of biospecifically bound enzyme on CB–SG–CEL 1 and 2. Whereas from CB–SG–CEL 2 the bound LDH was eluted with 2 *M* potassium chloride solution in good yield (almost 34%), from CB–SG–CEL 1 the yield was only 3.9%. On the other hand, the biospecific elution with 1 m*M* NADH (or alternatively with CB–dextran T 10) released the bound LDH quickly and completely from both supports.

Fig. 2 shows the frontal analysis profile for LDH on the dyed coated silicas. It can be seen that saturation of the coated columns with proteins depended on whether the surface was covered once or twice. The single-coated silica in CB-form was able to bind a greater amount of enzyme (9.06 nmol of LDH per gram of sorbent) than the double-coated silica (Table III). The concentrations of the accessible immobilized dye were determined on the basis of the saturation results under the conditions of monovalent interaction (LDH–dye). The values obtained were very low (0.15% or 0.1% of the total bound dye), much lower than those with bead cellulose supports (4.3% accessible dye from 6.03 μmol of total CB per gram of sorbent) [21]. This conclusion is in agreement with the results of Kroviarsky *et al.* [13], who compared dye–agarose with silica coated with dyed agarose or dextran supports in the binding of 6-phosphogluconate dehydrogenase. Clonis [22] compared the loading capacity of various dyed matrices, namely Procion Blue MX-R derivatives of wide-pore silica, TSK 65000 PW and Dynospheres XP-3507, for loading of LDH originating from crude muscle extract. The results showed that the capacities of silica gels were markedly lower (above 100 U ml^{-1} in the concentration range of the immobilized dye 5-10 μmol g^{-1}) than those of the other two matrices. This value is very close to the binding capacities of LDH to our coated silicas.

The results obtained indicate that cheap cellulose-coated silica can be prepared from cellulose triacetate by a very simple method and used as a support for the dye-affinity chromatography of enzymes. The dyed matrix is highly stable, as appar-

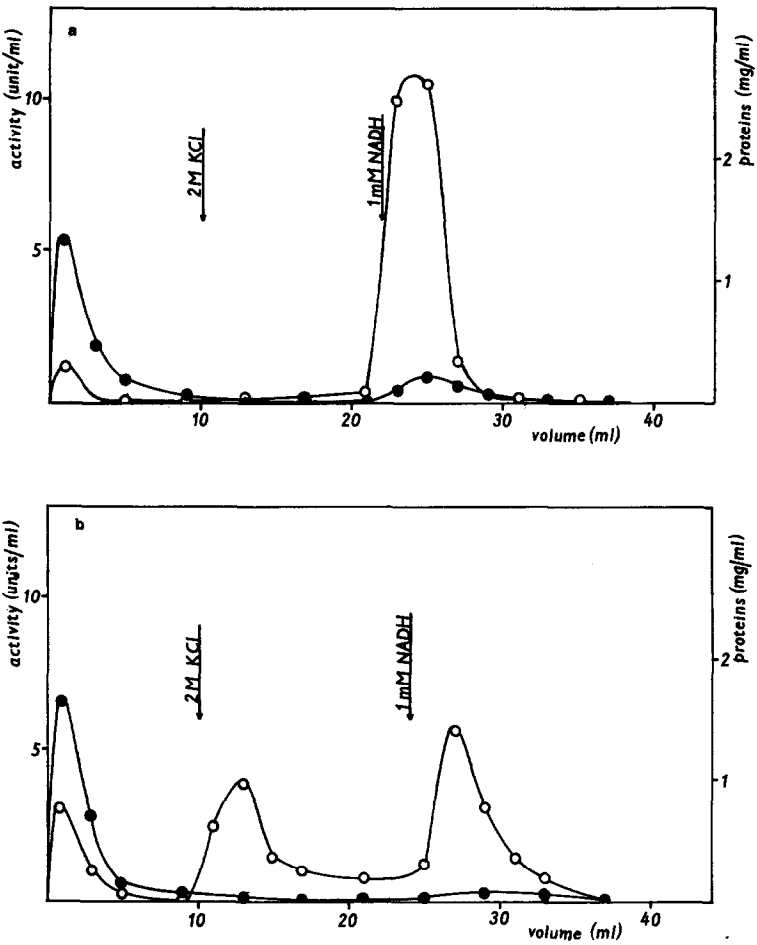

Fig. 1. Elution of LDH from CB–silica–cellulose columns: (a) CB–SG–CEL 1; (b) CB–SG–CEL 2. The procedure was performed on the columns of 2.5 × 1.1 cm I.D.; 0.2 ml of crude LDH (50 U, 8.2 mg protein) was loaded and after removing the unbound protein with the equilibration buffer, LDH was eluted with 2 *M* potassium chloride solution and 1 m*M* NADH. The LDH activity (○) and protein content (●) were determined.

TABLE III

SATURATION PROCESS OF LDH

Sorbent	Concentration of total CB (μmol g^{-1})	Concentration of sorbed LDH (nmol g^{-1})	Accessible dye (%)	Specific activity of LDH (U mg^{-1})
CB–SG–CEL 1	5.9	9.06	0.15	188
CB–SG–CEL 2	8.4	8.69	0.10	227

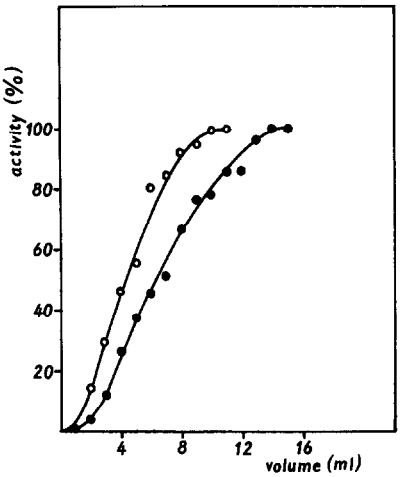

Fig. 2. Saturation process. The size of the column was as in the elution experiments. The supports were loaded with the solution of crude LDH (5 mg/ml, 20 U/ml) using a flow-rate of 20 ml/h. The activity of LDH was determined in 2-ml fractions. Support: (●) CB–SG–CEL 1; (○) CB–SG–CEL 2.

ent changes in its behaviour and release of the dye into the effluent were not observed even after many experiments performed on the same column. The protection of the silica surface with cellulose may prevent non-specific interactions with proteins and facilitate the binding of the reactive dye on the surface of the support. When the coating with cellulose was performed in two steps, the non-specific sorption of the proteins and the binding capacity of LDH to the dyed support were lower, but the separated product was purer (Table III). The present study was performed under low-pressure conditions and it would be of interest to perform experiments with cellulose-coated silica under high-performance liquid chromatographic conditions.

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