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SEPARATION OF PLASMA MEMBRANE PROTEINS OF CULTURED HUMAN FIBROBLASTS BY AFFINITY CHROMATOGRAPHY ON BONDED MICROPARTICULATE SILICAS

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SUMMARY

Adsorbents for high-performance affinity chromatography were prepared by bonding proteins and reactive Procion triazine dyes to 3-isothiocyanatopropyl- and 3-aminopropylsilicas. The materials prepared were used successfully in the separation of hydrophobic plasma membrane proteins of cultured human fibroblasts. The data obtained show that the reaction of 3-isothiocyanatopropyltriethoxysilane (ITCPS) with the surface hydroxyl groups of silica yields a new and convenient route to preparing an "activated carrier" that is capable of coupling with potential affinity ligands containing amino functional groups. The reaction and bonding procedures of 3-isothiocyanatopropyltriethoxysilane and 3-aminopropyltriethoxysilane with silica were optimized with regard to a controlled and reproducible ligand density by adjusting the type of solvent and organic base as reaction catalyst. The ligand content of reactive triazine dyes directly coupled to 3-aminopropylsilica was significantly higher than that of the 6-aminohexyl derivatives coupled to 3-isothiocyanatopropylsilica. The stability of Procion Blue MX-R bonded to 3-aminopropylsilica and 3-isothiocyanatopropylsilica in phosphate-buffered aqueous solution at pH 5.0 and 8.0 was examined.

INTRODUCTION

Affinity ligands chemically bonded to microparticulate silica packings have been successfully applied in the high-performance liquid affinity chromatography (HPLAC) of biologically active substances, which is illustrated by the rapid growth of separations and purifications performed with these phases[1–18].

In this work we developed a novel and efficient method for preparing a variety of affinity silica packings. The method consists of a reaction between the bonded 3-

* This paper is dedicated to the initiator of this work, our co-worker, the late Dr. G. Brunner, who died during an expedition on Gasherbrunn II, Pakistan, in July 1982.

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isothiocyanatopropylsilica and an appropriate amino ligand, yielding the corresponding thiourea derivative. In this way concanavalin A, fetuin and mannan are bonded to silica. The proteins are well known ligands in the isolation of glycoproteins[14,16,18–21]. In addition to the proteins and glucosamine, triazine dyes, which have been shown to be group-specific ligands in the separation of enzymes[11], were bonded to 3-aminopropylsilica, and their 6-aminohexyl derivatives were bonded to 3-isothiocyanatopropylsilica. The chromatographic properties of triazine dye-bonded silicas were compared with those obtained on triazine dye-modified agarose. The synthesized materials were employed for the separation of membrane proteins involved in the regulation of the growth behaviour of mammalian cells.

EXPERIMENTAL

Materials and chemicals

The packing material was a 25 nm pore size type 250 Å (HPLC), 10 μm, from Grace, Worms, F.R.G. (lot No. QM 02-30). 3-Aminopropyltriethoxysilane was obtained from EGA, Steinheim, F.R.G. 3-Isothiocyanatopropyltriethoxysilane was prepared by a method described by Vogt[22]. Concanavalin A (type III), mannan, fetuin (type III), Triton X-100 and 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulphonate were obtained from Sigma, Munich, F.R.G., the Procion dyes Green H-E4BD, Orange H-ER and Red He-3B from ICI, Frankfurt, F.R.G., and the Procion dyes Blue MX-R, Yellow MX-4R and Cibacron Blue F3GA from Serva, Heidelberg, F.R.G. The dyes were used without further purification. The 6-aminohexyl derivatives of the dyes were prepared by reaction of 1,6-diaminohexane according to a procedure described elsewhere[1]. All chemicals and biochemicals were purchased from Sigma, unless stated otherwise. Acrylamide was obtained from Roth (Karlsruhe, F.R.G.).

Preparation of 3-isothiocyanatopropylsilica and 3-aminopropylsilica

A 50-g amount of silica 250 Å, 10 μm particle size, was activated under a reduced pressure of $< 10^{-3}$ mbar at 473°K for 12 h. After it had cooled to room temperature, a solution of 0.12 mol of dry 2,6-dimethylpyridine, 200 ml of dichloromethane and 0.12 mol of 3-aminopropyltriethoxysilane (or, added to the first two, 0.12 mol of 3-isothiocyanatopropyltriethoxysilane) was added to the silica still under vacuum in order to allow the reaction mixture to penetrate completely into the pore system. After refluxing the suspension under dry argon for 24 h, the silica was suspended and washed with dichloromethane, methanol and diethyl ether, in that order. Ligand densities of 3.6 μmol/m^2 for 3-aminopropylsilica and 3.5 μmol/m^2 for 3-isothiocyanatopropylsilica were established from elemental analysis. In accordance with the designation by other workers, modified silicas that are subjected to subsequent coupling of affinity ligands are called "activated carrier" (reaction 1).

Preparation of biospecific adsorbents

Bonding of concanavalin A, fetuin and mannan to the silica support was accomplished by reacting the SCN group of the 3-isothiocyanatopropyl-activated carrier with the amino functional groups of the proteins as follows:

$$\text{Si–OH} + (H_5C_2O)_3Si(CH_2)_3NCS \rightarrow \underset{\text{"activated carrier"}}{\text{Si–O–Si–}(CH_2)_3NCS} + H_2N\text{–ligand} \rightarrow \underset{\text{"affinity adsorbent"}}{\text{Si–O–Si–}(CH_2)_3NHC(S)NH\text{–ligand}} \quad (1)$$

A 3-g amount of 3-isothiocyanatopropylsilica was suspended in 40 ml of 0.15 *M* phosphate buffer (pH 8.0), then 250 mg of either fetuin or mannan, or 100 mg of concanavalin A, were added to the suspension, which was degassed under vacuum and stirred for 24 h at room temperature. The suspension containing the protein–silica was then poured on to a sintered-glass funnel and washed with an appropriate solution (to be used as an eluent) and stored in a refrigerator at 0–5°C.

Synthesis of triazine dye-bonded silicas was carried out by coupling the 6-aminohexyl derivative of the Procion dye to the 3-isothiocyanatopropyl-activated carrier or by direct bonding of the reactive triazine dye to 3-aminopropyl-activated silica according to the procedure already described.

A 3-g amount of 3-isothiocyanatopropyl silica was suspended in a solution of 20 ml 0.15 *M* phosphate buffer (pH 8.0) containing 300 mg of the 6-aminohexyl derivative of Blue MX-R, Orange H-ER, Yellow MX-4R, Red HE-3B, Green H-E4BD or Cibacron Blue F3GA. The slurry was degassed under vacuum, subjected to ultrasonic treatment for 5 min and then stirred at room temperature for 12 h. The product was decanted and washed thoroughly with water and methanol.

The bonded dye concentration was determined by a method described by Lowe *et al.*[1].

It should be noted that the reagent ITCPS offers the possibility of producing "activated ligands". We define these ligands as substances that are derivatized in such a manner that they may be used as modifying reagents for native silica, resulting in stable affinity silica packings (reaction 2). By this method, bonding of low-molecular-weight ligands (amino sugars, amino acids, etc.) as thioureapropyltriethoxysilyl derivatives was performed. The preparation and the properties of the materials prepared will be described elsewhere[23].

$$(H_5C_2O)_3Si(CH_2)_3NCS + H_2N\text{–ligand} \rightarrow \underset{\text{"activated ligand"}}{(H_5C_2O)_3Si(CH_2)_3NHC(S)NH\text{–ligand}} + \text{Si–OH} \rightarrow \underset{\text{"affinity adsorbent"}}{\text{Si–O–Si–}(CH_2)_3NHC(S)NH\text{–ligand}} \quad (2)$$

Chromatographic tests

Affinity packings were slurry-packed into stainless-steel columns (125 × 4 mm I.D.) using a suspension of 2.5 g of modified silica and 20 ml of water at a constant flow-rate during packing. All chromatographic experiments were carried out with a DuPont Series 8800 gradient controller, a DuPont 8800 pump, a Rheodyne 7125 injection valve with a 500-μl sample volume and a Biotronic BT 3030 UV detector of variable wavelength.

Electrophoresis

SDS–polyacrylamide electrophoresis was performed as described by Laemmli[24] by using a gel gradient from 5 to 20% acrylamide. The samples were prepared for electrophoresis by precipitation with trichloroacetic acid (10%) followed by pre-

cipitation with cold acetone (4 volumes). The precipitates were resuspended in SDS–sample buffer [0.01 *M* Tris; 0.001 *M* EDTA; 4% (w/v) SDS; 5% (v/v) 2-mercaptoethanol; pH = 8.0] and boiled for 10 min.

Preparation of detergent extracts

Confluent cultures of human embryonal fibroblasts were washed twice with phosphate-buffered saline and the membrane proteins were solubilized in TMN buffer (20 m*M* Tris–HCl, pH 7.4; 0.14 *M* NaCl; 1 m*M* $MgCl_2$; 1 m*M* PMSF; 0.02% NaN_3; 1% Triton X-100) or 8 m*M* {3-[(3-cholamidopropyl)dimethylamino]-1-propanesulphonate} (CHAPS) for 1 h at 4°C. The extract was centrifuged at 100,000 *g* for 1 h and used immediately or stored at −30°C.

RESULTS AND DISCUSSION

Preparation of the "activated carriers" 3-isothiocyanatopropylsilica and 3-aminopropylsilica

Various experiments were carried out to determine the influence of solvents and organic bases on the modification of silica with 3-isothiocyanatopropyltriethoxysilane (I) and 3-aminopropyltriethoxysilane (II). Results were obtained that confirmed the conclusions drawn from studies of the optimum silanization of reversed-phase supports with *n*-alkyldimethylchlorosilanes under identical conditions[25]. After reaction with I in a non-polar, water-free medium (*e.g.*, toluene, in the absence of organic base, 24 h reflux), the ligand density was determined to be 1.95 μmol/m^2. The use of 2,6-dimethylpyridine as a basic catalyst in the more polar solvents dichloromethane (reaction temperature 313°K) or dimethylformamide (room temperature) for a reaction time of 24 h gave 3.47 and 3.38 μmol/m^2, respectively.

The modification in which II was used under identical conditions led to a value of 3.62 μmol/m^2. Comparison of this result with those of Gimpel[26] and Engelhardt and Mathes[27] (who achieved values of 4.7 and 4.97 μmol/m^2, respectively under non-dry conditions, thus allowing polymerization of the triethoxyorganosilane) demonstrates that this method offers the possibility of preparing monomolecular layers in high yields under mild conditions. The reproducibility of the procedure was established to be within the error limits of the elemental analysis (±0.3% for carbon, absolute value).

Preparation of dye-ligand affinity adsorbents by reaction of the triazine ring of Procion dyes with the activated carrier 3-aminopropylsilica (direct coupling) or by treatment of the activated carrier 3-isothiocyanatopropylsilica with 6-aminohexyltriazine derivatives (coupling via 6-aminohexyl spacer)

Preliminary attempts to bond triazine dyes directly on to diol-modified silica by the method of Lowe *et al.*[1] yielded only small ligand densities of attachment (0.5–1.0 μmol/g). A possible explanation of these low values, compared with the results achieved by Lowe on LiChrosorb Si 60 silica, is that the specific surface area of the Grace 250 Å silica is only 0.6 of that of LiChrosorb Si 60 ($S_{BET} \approx 500$ m^2/g). Second, the bonding procedure of 3-glycidoxypropyltrimethoxysilane to silica in aqueous solution at pH 3.5 results in multiple layers of organic groups and therefore in a higher concentration of diol groups per gram of silica. 3-Aminopropylsilica was

TABLE I

DYE CONTENTS OF THE AFFINITY SILICA PACKINGS

Dye	*Ligand content (μmol g^{-1})*	
	6-Aminohexyl derivatives coupled to 3-isothiocyanatopropylsilica	*Dye directly coupled to 3-aminopropylsilica*
Cibacron Blue F3GA	6.3	17.6
Procion Blue MX-R	8.2	22.3
Procion Red HE-3B	7.7	10.4
Procion Orange H-ER	6.1	17.6
Procion Yellow MX-4R	5.7	19.2
Procion Green H-E4BD	4.2	12.6

then chosen as a better starting material in an attempt to achieve higher densities of attachment of triazine dyes. The ligand contents of the products obtained by direct coupling are listed in Table I, and lie between 10.4 and 22.3 μmol/g, depending on the dye used. Treatment of 3-isothiocyanatopropylsilica with derivatives of 6-aminohexyltriazine led to significantly lower values (3–7 μmol/g), which are similar to those obtained by reaction of epoxide-modified silica and 6-aminohexyltriazine[10].

The stabilities of Procion Blue MX-R-modified 3-aminopropylsilica (III) and 6-aminohexyl-Procion Blue MX-R (IV)-modified 3-isothiocyanatopropylsilica were tested with regard to their future chromatographic use under the following conditions: (a) pH 5 (0.15 *M* phosphate buffer) and (b) pH 8 (0.15 *M* phosphate buffer). Table II shows the decrease in the dye content as a function of time for both substrates under both sets of conditions.

At pH 5, the dye content of III decreased slowly over the total period of observation, whereas at pH 8 only an initial decrease was observed. Material IV

TABLE II

DYE CONTENT OF AFFINITY SILICA PACKINGS AS A FUNCTION OF TIME

Material III			*Material IV*		
Time (h)	*μmol dye/g*		*Time (h)*	*μmol dye/g*	
	pH 5	*pH 8*		*pH 8*	*pH 5*
0	22.3	22.3	0	8.2	8.2
12	19.6	21.0	12	7.6	7.8
24	18.8	20.8	24	7.4	7.7
36	18.5	20.6	36	7.4	7.7
48	18.3	20.2	48	7.3	7.7
60	18.1	20.1	60	7.4	7.5

showed the latter behaviour for both pH values (*i.e.*, an initial decrease, followed by no further detectable loss). Triazine-modified agarose was also reported to lose about 38% of dye at pH 3.0 in 4 weeks at room temperature. The stability of this material at neutral or slightly basic pH is much better (loss of 1.3%)[28].

Proteins bonded to 3-isothiocyanatopropylsilica

The principal advantage of the 3-isothiocyanatopropyl unit lies in the ability of the NCS group to react selectively with the amino groups of proteins in the presence of water. This was tested by the reaction of IV with various amino acids (*e.g.*, L-proline, L-hydroxyproline, L-glutamine and L-histidine) in aqueous alkali medium (pH 8–8.5) at room temperature. Analysis then resulted in values of 1–2 μmol/m^2 for the amino acids. Similar values were found for cytidine (1.32 μmol/m^2) and glucosamine (1.57 μmol/m^2). For these low-molecular-weight compounds a higher ligand density is achieved by using the "activated ligand" for modification of native silica (reaction 2). Thus, surface reactions of the thioureapropyltriethoxysilyl derivatives of glucosamine, L-leucine, L-phenylethylamine, L-tryptophane and L-lysine were carried out, and the ligand densities were between 2.6 and 3.2 μmol/m^2 (ref. 23). However, reaction 2 cannot be used for proteins because of their multiple reactive groups, as these would be denatured by a derivatization step using ITCPS. Nevertheless, proteins are especially tightly bound on III with a high coupling efficiency. This is possibly due to a multi-site attachment (see Table III). A coupling yield of more than 90% at loads of 30 mg/g silica could be reached. Our assumption that this prepared concanavalin-silica was only partly modified was shown to be accurate by Renauer and Wieser[29], who used LiChrospher Si 500, 10 μm, as starting material and reaction 1 for immobilization of concanavalin A. A concanavalin A load of 75–80 mg/g silica was obtained.

Chromatographic results

Silicas modified with triazine dyes. Triazine dyes fixed to porous silicas are very effective in the separation and isolation of water-soluble proteins, especially enzymes.

TABLE III

PROTEIN LIGAND CONTENT OF AFFINITY SILICA PACKINGS

*Protein**	*Ligand content (mg protein/g silica)*
Concanavalin A	31
Fetuin	80
Mannan	33

* With concanavalin A the silica is probably not fully charged, as only 100 mg of protein was allowed to react with 3 g silica. The same amount of the same silica, when reacted with 250 mg of fetuin, resulted in 80 mg of protein per gram of silica. The content of bonded protein was given by the difference between the starting amount and the content of the washings (protein determination according to Lowry *et al.*[30]). With mannan, the determination was achieved by the increase in the carbon content of the reacted silica, as the protein content of the mannan molecule is small. The capacity of the concanavalin A silica for horseradish peroxidase was determined to be 4.8 mg/g column material, calculated by measurement of the breakthrough curve and determination of the eluted protein under non-binding conditions.

At present very little is known about the interactions between triazine dyes and hydrophobic proteins, such as the integral membrane proteins of the cell wall.

The experiments reported here were the first attempt in our laboratory to separate complex mixtures of detergent-solubilized membrane proteins by HPLAC. An important advantage of the method lies in the fact that the membrane proteins may be recovered in a functionally active form, owing to the mild conditions of the elution, and further that a selective enrichment of these proteins may be achieved by exploitation of their different selectivities with respect to different triazine dyes.

Three triazine- modified silicas were chosen for testing the selectivity of membrane proteins: 3-aminopropyl-bound Procion Blue MX-R, 3-aminopropyl-bound Procion Yellow MX-4R and 3-isothiocyanatopropyl-bound Procion Orange H-ER. The choice of these three and the composition of the eluent were based on experience gained in studies of the corresponding agarose supports[30]. *In vitro* studies showed that the compounds isolated from detergent cell extracts on agarose supports were still biologically active[31]. In the course of the chromatographic tests it was found that 3-aminopropyl-bound Procion Blue MX-R showed high affinity for serum components bound to the cell membrane (MW = 67,000). For elution 0.5 *M* NaCl was added to the starting 0.04 *M* phosphate buffer containing 0.5% Triton X-100. SDS polyacrylamide gel electrophoresis (PAGE) of the isolated material still showed small amounts of non-specifically adsorbed proteins at the still existing aminopropyl groups of the starting 3-aminopropyl silica. 3-Aminopropyl-bound Procion Yellow MX-4R failed to separate proteins from the detergent extracts. The elution conditions were the same as for 3-aminopropyl-bound Procion Blue MX-R. The use of a pH gradient from 8.0 to 5.0 also did not succeed in eluting the proteins (conditions as given in Fig. 1). However, further tests on the composition of the mobile phase must be carried out before conclusive statements as to the suitability of any particular dye ligand can be made. 3-Isothiocyanatopropyl-bound Procion Orange H-ER appeared to be especially specific for peripheral membrane proteins. Fig. 1 shows the chromatogram of 500 μl of protein extract (*ca.* 0.3 mg total protein content), eluted from 3-isothiocyanatopropyl-bound Procion Orange H-ER. Fig. 3b shows the SDS PAGE of fraction 5, collected from two chromatographic runs on 3-isothiocyanatopropyl-bound Procion Orange H-ER.

Lectin- and glycoprotein-modified silicas. Based on the fact that membrane proteins that contain or bind mannose have a decisive influence on the regulation of cell growth[32,33], an attempt was made to develop an effective HPLC system for the isolation of such components. Concanavalin A is a well known ligand in the separation of glycoproteins and lectin because of its specificity for the mannose groups in these molecules. Moreover, the binding of concanavalin A to silica was performed by Borchert *et al.*[16] by three different methods. The chromatographic behaviour of the resulting materials was evaluated in the separation of sugars and glucoproteins. One of the tests, the separation of horseradish peroxidase, was also used by ourselves to allow a comparison of 3-isothiocyanatopropylsilica (as activated carrier) with epoxy-silica, tresylate-silica and aldehyde-silica. It was confirmed that the lectin–concanavalin A, after being fixed to silica via NCS groups (reaction 1) was also highly specific for bonding horseradish peroxidase (Fig. 2).

The binding capacity of concanavalin A-silica (30 mg of concanavalin A per gram of silica) for horseradish peroxidase was determined at 4.8 mg/per gram of support with a recovery of 98.5%.

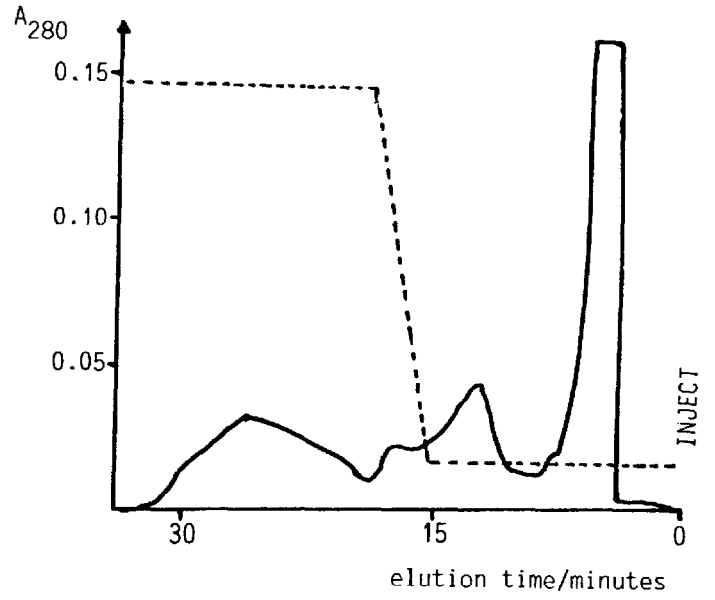

Fig. 1. Chromatogram of detergent extract of cultured human fibroblasts on Procion Orange H-ER 6-aminohexyl derivative bound to 3-isothiocyanatopropylsilica. Eluent (I) starting conditions of the gradient (A)/(B) = 20:80 (v/v). Gradient: change to eluent (II) = (A)/(C) = 20:80 (v/v) within 2 min. (A) 2.5% aq. Triton X-100, (B) 0.04 *M* phosphate buffer (pH 8.0), (C) 0.08 *M* phosphate buffer (pH 5.0). Flow-rate, 0.5 ml min^{-1}; detection, UV, 280 nm, 0.16 a.u.f.s.; sample amount, 0.3 mg.

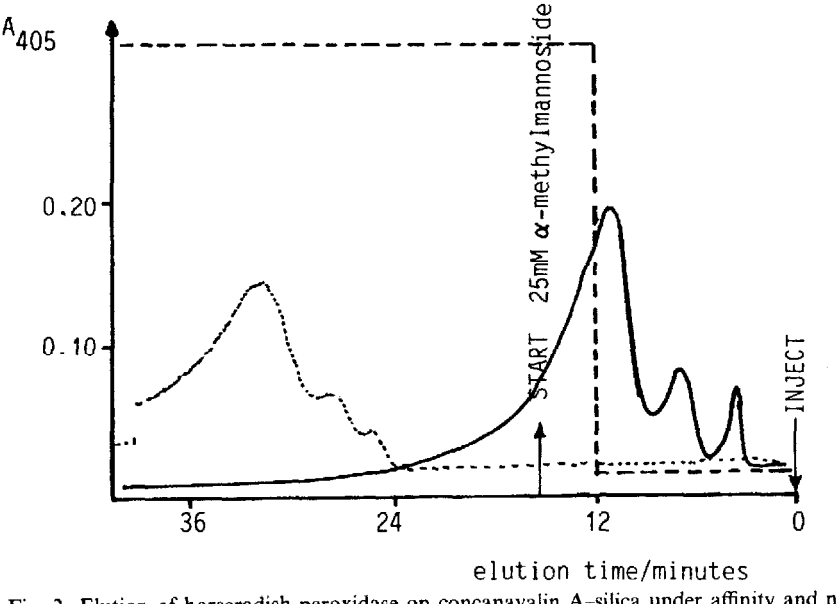

Fig. 2. Elution of horseradish peroxidase on concanavalin A–silica under affinity and non-affinity conditions. Eluent: ---------, buffer A [0.05 *M* NaOAc(pH 5.1), 0.50 *M* NaCl, 1 m*M* $CaCl_2$, 1 m*M* $MnCl_2$]; ———, 2.5 m*M* methylmannoside in buffer A; ---------, 25 m*M* methylmannoside in buffer A. Flow-rate, 0.5 ml min^{-1}; detection, UV, 405 nm, 0.32 a.u.f.s.; sample amount, 0.8 mg.

Borchert *et al.*[16] found a capacity of about 12 mg of peroxidase per gram of column material (Concanavalin A–silica, 1000 Å, 60 mg/g SiO_2). This indicates that by use of the 250 Å silica in our preparation the accessibility of the binding sites of the bonded concanavalin A is diminished.

SDS PAGE of the membrane proteins isolated from the 3-isothiocyanatopropyl-bound concanavalin A support showed that these were identical with the proteins isolated by using the mannose-specific *Lens cullinaris* Sepharose 4B (see Fig. 3a).

The mannan supports prepared in this work had a higher concentration of mannose-binding membrane proteins. SDS PAGE identified proteins with molecular weights of *ca.* 30,000, 28,500, 18,000 and 16,000 in the eluted mannoside peak (Fig. 4).

It is likely that the four substances are subunits of a large mannose-specific protein. Affinity chromatography on mannose–Sepharose 4B under native conditions[30,31] separates mannose-binding proteins obtained from rat liver and human liver; these proteins have a molecular weight of >900,000 (rat) and >1,000,000

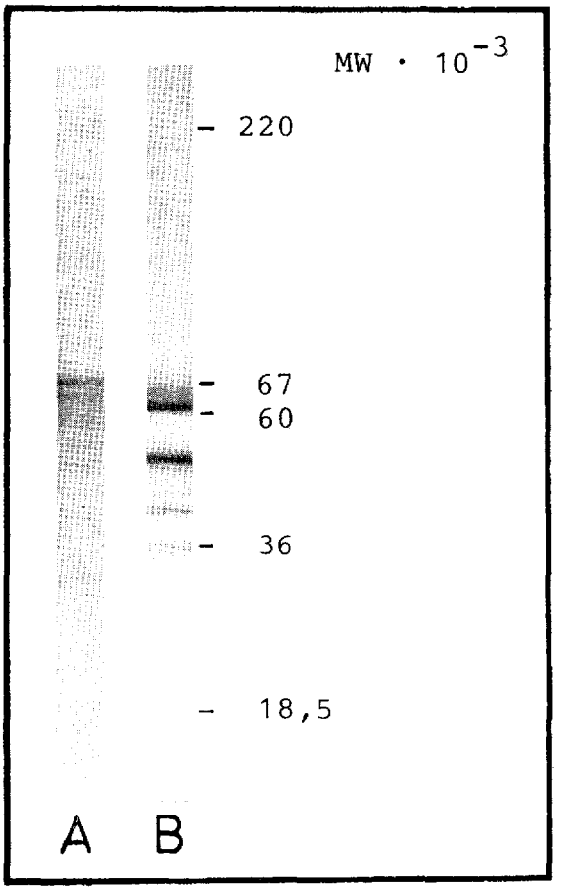

Fig. 3. SDS PAGE of human plasma membrane protein extracts separated on (A) concanavalin A silica with 0.5 *M* methylmannoside as eluent; (B) Procion Orange H-ER–silica with 0.08 *M* phosphate buffer (pH 5.0) as eluent.

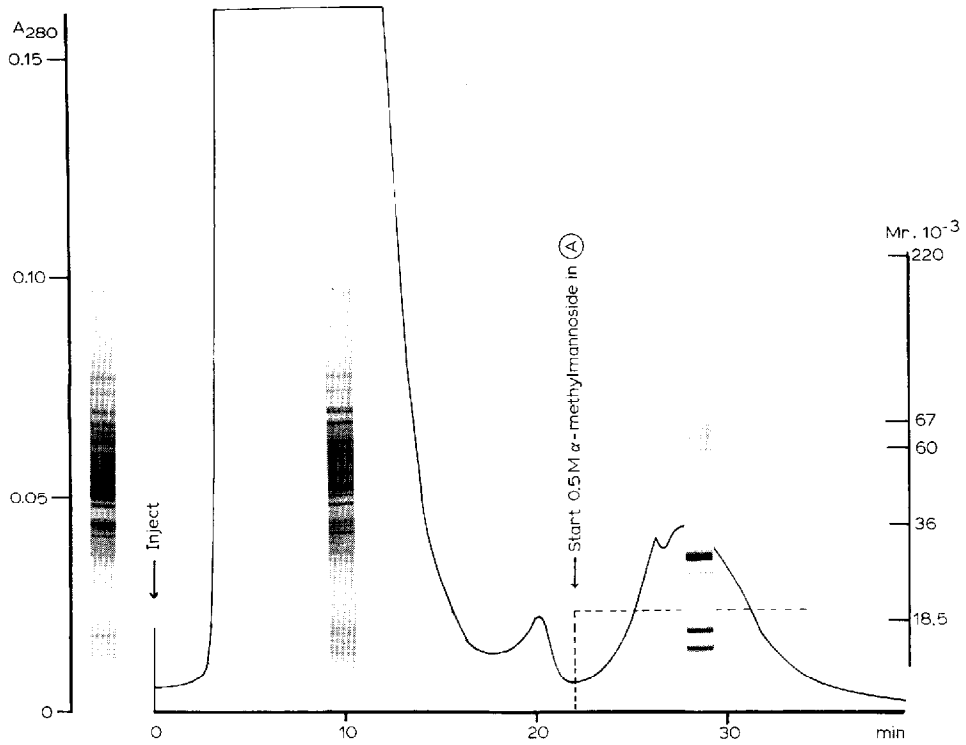

Fig. 4. Separation of human plasma membrane proteins on mannan–silica and SDS PAGE of the collected peak fractions. Eluent: ———, buffer A [30 m*M* phosphate buffer (pH 7.4) 0.025% Triton X-100]; ----------, 0.5 *M* methylmannoside in buffer A. Flow-rate, 0.5 ml min^{-1}; detection, UV, 280 nm, 0.16 a.u.f.s.; sample, 0.5 ml Triton X-100 extract from human embryonal lung fibroblasts (0.4 mg protein/ml). SDS PAGE: Laemmli system: gel, linear gradient 5–20% acrylamide; sample, reduced; detection: Coommassie Blue R 250.

(human) and dissociate into subunits of 34,000 and $(34,000)_n$ (rat) and 30,000 (human). After reduction and alkylation, polypeptide chains of 63,000 and 32,000 daltons were observed for the material isolated from rat liver and 28,000, 30,500, 31,500 and 70,000 daltons for the protein from human liver[30].

In summary, the use of 3-isothiocyanatopropylsilica as an activated carrier for the immobilization of bioaffinity ligands resulted in materials suitable for analytical and micropreparative use in HPLAC.

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