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HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY OF HUMAN SERUM CONCANAVALIN A BINDING PROTEINS

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SUMMARY

A high-performance concanavalin A (Con A) affinity column Gelpack GL-L55C (Hitachi Kasei Industries) was successfully used for the fractionation of human serum Con A-binding proteins. Serum proteins that have strong affinity to Con A (ca. 11% of the recovered proteins) could be fractionated within 80 min. By analysing the eluates from the column by micro two-dimensional electrophoresis, followed by blotting and Con A staining, the specificity of the column was effectively visualized. Although the protein-binding capacity of the column gradually decreased during repeated loading of serum or tissue extracts, the specificity of the column to Con A-binding proteins did not change. Serum lipoproteins have been eluted from the column with 6 *M* urea, suggesting that the capacity decrease is caused by the binding of lipids or lipoproteins to the column.

INTRODUCTION

Lectin affinity columns packed with agarose gel beads as supporting media have been employed for the chromatographic separation of glycoproteins [1]. Differential elution of glycoproteins with low and high affinity to the columns has also been reported [2]. Because of the wide applicability of lectin affinity chromatography, high-performance lectin columns packed with silica beads come into use for the rapid fractionation of glycoproteins. The properties of these columns have been evaluated mainly from the elution profiles of proteins [3–6].

In a preceding paper, we reported on the staining of concanavalin A (Con A)-binding proteins on nitrocellulose sheets after micro two-dimensional (2-D) electrophoresis and blotting [7]. Human serum Con A-binding proteins were iden-

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tified on the blots by reference to a protein map obtained by immunochemical staining [8].

This paper reports the application of the techniques of electrophoresis, blotting and lectin staining to the analysis of the eluate from a high-performance Con A column, packed with hydrophilic polymer beads Gelpack. The properties of the column are described in terms of the types of bound proteins.

EXPERIMENTAL

Materials

Human sera were obtained from healthy individuals and stored at −20°C. Con A was obtained from Seikagaku Kogyo (Tokyo, Japan). Human serum transferrin and horseradish peroxidase (Type VI) were from Sigma (St. Louis, MO, U.S.A.). Nitrocellulose sheets (0.45-μm pore size) were obtained from Schleicher and Schuell (Dassel, F.R.G.). Minicon CS15 concentrators were from Amicon (Danvers, MA, U.S.A.). A high-performance Con A affinity column GL-L55C (100 mm × 8 mm I.D.), packed with Con A-immobilized hydrophilic polymer support Gelpack, was obtained from Hitachi Kasei Industries (Tokyo, Japan). α-D-Methylmannoside and α-D-methylglucoside were from Wako (Tokyo, Japan). Rabbit antisera against human plasma proteins were obtained from Behring (Marburg, F.R.G.).

Chromatographic conditions

A high-performance liquid chromatographic (HPLC) system comprised of a Hitachi 655 solvent-delivery system, an injector (Rheodyne 7125) fitted with a 200-μl loop, a Hitachi 655-65 gradient controller, a Hitachi 638-41 UV monitor and a Hitachi 655-60 processor was used. The column was equilibrated with 50 m*M* Tris–HCl buffer (pH 7.2) containing 0.15 *M* sodium chloride and 0.02% sodium azide. Human serum samples ranging from 10 to 100 μl were loaded on the column, and non-adsorbed and weakly adsorbed proteins were eluted with the same buffer. Adsorbed glycoproteins were eluted with 0.2 *M* α-D-methylmannoside or 0.2 *M* α-D-methylglucoside in the same buffer. The flow-rate was 1.0 ml/min.

Micro two-dimensional electrophoresis

The HPLC eluate was fractionated, and aliquots of the fractions were added sucrose to give a concentration of 40% (w/v) and subjected to electrophoresis. In some cases, the fractions were concentrated with a Minicon CS15 concentrator, added sucrose and subjected to electrophoresis. Microscale multi-sample 2-D electrophoresis in the absence of the denaturing agents was performed as described previously [9]. First-dimension isoelectric focusing was carried out in capillary gel columns (35 mm × 1.3 mm I.D.) and second-dimension gradient polyacrylamide gel (4–17% linear gradient) electrophoresis was run on micro slab gels (38 mm long, 38 mm wide and 1.0 mm thick). In some cases, second-dimension electrophoresis was carried out in the presence of sodium dodecyl sulphate (SDS) [10].

Electrophoretic transfer

Construction details of the apparatus for electrophoretic transfer were fully described elsewhere [11]. Micro slab gels obtained after 2-D electrophoresis were placed in the apparatus, and one nitrocellulose sheet (cut to 40 mm×40 mm) was laid on each slab gel. Electrophoretic transfer was carried out at a constant current of 150 mA (initial voltage 12 V/cm) in 0.025 *M* Tris–0.19 *M* glycine (pH 8.3). For Con A-peroxidase staining, one blot was obtained from one slab gel after 60 min transfer time. For immunochemical staining, the transfer time was 10 min and five blots were obtained from one slab gel [11].

Con A-peroxidase staining

The blots obtained after 60 min transfer were treated as follows for the staining of Con A-binding proteins at room temperature [7]. (i) Each blot was placed in a plastic container with a lid (64×58×21 mm) and soaked in 5 ml of 3% bovine serum albumin (BSA) in saline (0.9% sodium chloride, 0.1% sodium azide, 10 m*M* Tris–HCl, pH 7.4) for 30 min. (ii) Con A in saline (1 mg/ml, 250 μl) was added to the BSA solution and the blot was kept for 60 min. (iii) The blot was washed in saline (10 ml, five changes during 30 min), then (iv) soaked in peroxidase solution (300 μg Type VI peroxidase in 5 ml saline) for 60 min, and (v) washed in saline (10 ml, five changes during 30 min). (vi) Staining was in 10 ml of 0.2 m*M* diaminobenzidine–saline supplemented with 0.1 ml of 3% hydrogen peroxide for 30 min. During the entire staining procedure, the container was gently shaken on a gyratory shaker.

Immunochemical staining

The blots obtained after 10 min transfer were treated as described previously [11] for immunochemical staining of human plasma proteins.

Dye staining

Polyacrylamide slab gels were stained in 0.1% Coomassie Brilliant Blue R-250 (CBB) in 50% (v/v) methanol–7% (v/v) acetic acid for 15 min before or after blotting and destained in 20% (v/v) methanol–7% (v/v) acetic acid for 120 min.

RESULTS AND DISCUSSION

Elution profiles of human serum proteins

Fig. 1 shows an elution profile of human serum proteins. Human serum (100 μl, ca. 7 mg of protein) was loaded on the high-performance Con A-affinity column equilibrated with 50 m*M* Tris–HCl buffer (pH 7.2) containing 0.15 *M* sodium chloride–0.02% sodium azide and eluted with the same buffer. The pass-through fraction appeared as a sharp UV-absorbing peak at the void volume of the column (3 ml), accompanied by small shoulders until the elution volume reached 10–20 ml. The adsorbed proteins were eluted with the buffer containing 0.2 *M* α-D-methylglucoside or 0.2 *M* α-D-methylmannoside as one peak. The time taken for the affinity chromatography was 80 min. The first peak (including

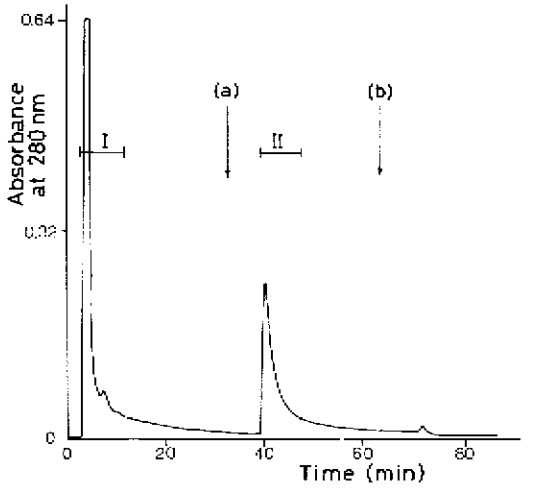

Fig. 1. Separation of human serum Con A-binding proteins on a Con A affinity column packed with hydrophilic polymer support Gelpack. The column was equilibrated and eluted with 0.05 *M* Tris–HCl buffer containing 0.15 *M* sodium chloride and 0.02% sodium azide. Human serum (100 μl, ca. 7 mg protein) was loaded on the column. At the time of arrow a, 0.2 *M* α-D-methylmannoside was further added to the buffer and at the arrow b, 0.05 *M* Tris–HCl buffer containing 1.0 *M* sodium chloride and 0.02% sodium azide (pH 7.2) was used. The flow-rate was 1 ml/min and the fractions indicated were collected: I, the pass-through fraction; II, the adsorbed fraction.

the tail) contained ca. 89% of the protein recovered and the second peak contained ca. 11%. This ratio of protein recovery was constant irrespective of the amount of serum protein applied (ranging from 0.7 to 7 mg).

Protein composition in the eluates analysed by micro 2-D electrophoresis

It has been reported that most of the serum glycoproteins have asparagine-linked oligosaccharides, which have in common three mannose residues in the core structure [12]. Assuming that all these proteins will bind to the Con A affinity column, ca. 40–50% of the applied serum proteins should be adsorbed. Since the actual quantity ratio of the adsorbed protein seemed to be too small, we examined the protein composition in the eluate of the Con A column. The eluate was collected in two fractions as shown in Fig. 1 and concentrated ca. twenty-fold with a Minicon CS15 concentrator, and aliquots (8 μl each) were subjected to micro 2-D electrophoresis in the absence of denaturing agents. Fig. 2A and B show the 2-D pattern of the pass-through fraction (fraction I) and the adsorbed fraction (fraction II), respectively. The ratio of the protein quantity loaded on the 2-D gels was set at 8:1, 160 μg for fraction I and 20 μg for fraction II, the same ratio as that recovered in the fractions. The stained proteins on the 2-D gels were identified according to the "identification map" of human plasma proteins [8], which has been prepared by immunochemical staining of the proteins after blotting. Fraction I (Fig. 2A) contained albumin, immunoglobulin G (IgG), transferrin, Gc-globulin, prealbumin and IgA as major protein species. Fraction II (Fig. 2B) contained IgM, IgG, α_2-macroglobulin, IgA, transferrin, ceruloplasmin,

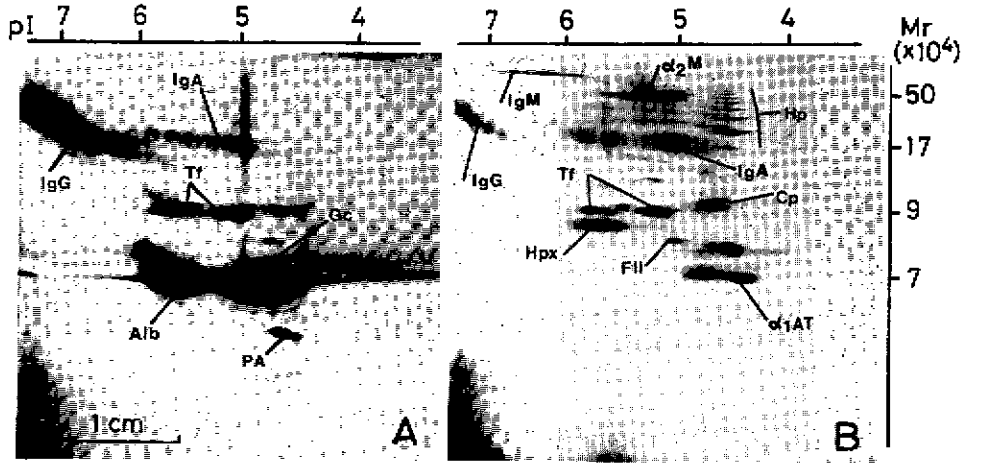

Fig. 2. Analysis of proteins in the eluate. The proteins in the fractions indicated in Fig. 1 were concentrated ca. twenty-fold with a Minicon CS15 concentrator, and aliquots (8 μl) were subjected to micro 2-D electrophoresis in the absence of denaturants. The gels were stained with CBB. (A) Proteins in the pass-through fraction (160 μg); (B) proteins in the adsorbed fraction (20 μg). Major serum proteins were identified according to the "identification map" of human plasma proteins [8]. Abbreviations: Tf = transferrin; Gc = Gc-globulin phenotype 1-1; Alb = albumin; PA = prealbumin; α_2M = α_2-macroglobulin; Hp = haptoglobin phenotype 2-2; Cp = ceruloplasmin; Hpx = hemopexin; FII = prothrombin; α_1AT = α_1-antitrypsin.

hemopexin, haptoglobin, prothrombin and α_1-antitrypsin as major components. Several glycoproteins, e.g. IgG, IgA and transferrin, were present in both fractions. In particular, most of IgG was in the pass-through fraction. Therefore, the reason for the low ratio of the adsorbed proteins could be that a part of serum glycoproteins had no or only weak interaction with Con A, although they have mannose residues in their oligosaccharide chains. We have already shown low accessibility of mannose residues of IgG and IgM, after separating them by 2-D electrophoresis under native conditions [7].

The 2-D patterns shown in Fig. 2A and B were compared with the pattern of the serum proteins loaded on the column. Most of the serum proteins were detected in one of the two patterns of the fractions, showing that they were recovered from the column, but low-density lipoprotein (LDL) and most of high-density lipoprotein (HDL) were not recovered.

Affinity of serum proteins to Con A visualized in nitrocellulose blots

The affinity of serum proteins to Con A was visualized by 2-D electrophoresis followed by blotting and lectin staining [7]. Aliquots (8 μl) of the twenty-fold concentrated fractions I and II were subjected to micro 2-D electrophoresis in the absence of denaturing agents, and the proteins on the slab gels were electrophoretically transferred to nitrocellulose sheets (blots). The blots were sequentially treated with Con A, peroxidase and diaminobenzidine–hydrogen peroxide, to stain Con A-binding proteins on the blots. Fig. 3 shows the results of blotting and lectin staining. The ratio of the density of a protein spot on a nitrocellulose sheet (Fig.

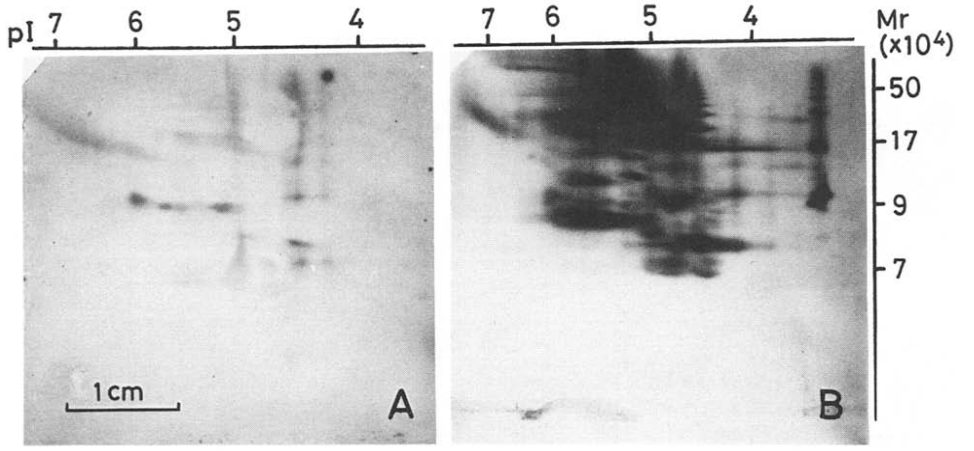

Fig. 3. Con A-peroxidase staining of proteins in the eluate. Proteins in the two fractions indicated in Fig. 1 were separated by 2-D electrophoresis, transferred to nitrocellulose sheets and stained by Con A staining. (A) Proteins in the pass-through fraction; (B) proteins in the adsorbed fraction.

3) to that on a slab gel (Fig. 2) represents the affinity level of the protein to Con A. Comparing Fig. 3A with Fig. 2A, it was ascertained that albumin, Gc-globulin and prealbumin have no affinity to Con A; they appeared as white spots negatively stained in a faint background colour. IgG, IgA and transferrin in fraction I were faintly stained by lectin staining, indicating that they have low affinity to Con A. Most of the proteins in fraction II were stained more densely by lectin staining (Fig. 3B) than by Coomassie staining (Fig. 2B), and minor serum glycoproteins were newly detected. These results demonstrated that serum glycoproteins that have high affinity to Con A have been fractionated by the high-performance Con A affinity column.

Differential elution of one protein species by the affinity column

Comparing the results shown in Fig. 2 and Fig. 3, it was obvious that protein molecules that have been immunochemically identified as one protein species could be separated into two fractions, the pass-through fraction and the adsorbed fraction. For example, IgG molecules in fraction I were only faintly stained by lectin staining, whereas those in fraction II were clearly stained. Similar subfractionation was observed for IgA and transferrin. In the case of IgA, the p*I* distribution was different between the two fractions: IgA molecules in fraction I were at p*I* 6.0–5.0, whereas those in fraction II were at p*I* 6.0–4.5. When purified human serum transferrin was subjected to affinity chromatography under the same conditions shown in Fig. 1, it was also separated into two fractions. Transferrin molecules in the two fractions were also different in p*I* distribution, and transferrin dimer was present only in the adsorbed fraction. These results showed that protein molecules that have been assumed to be one immunochemical protein species can further be separated into fractions according to the difference in affinity to Con A.

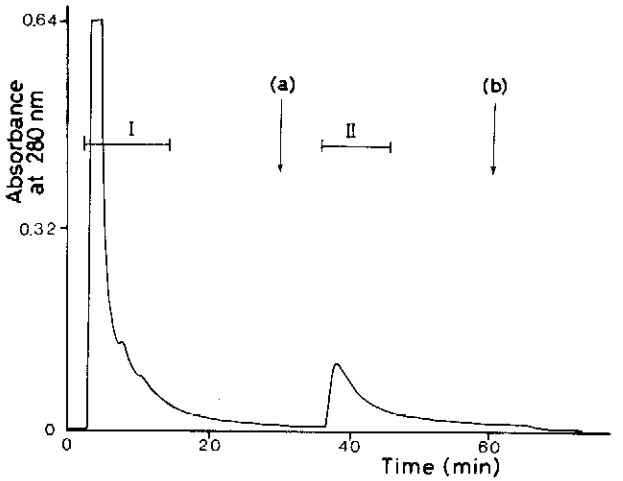

Fig. 4. Affinity chromatogram of human serum proteins. Human serum (100 μl) was loaded on the affinity column, which had been repeatedly used for affinity chromatography (ca. 200 mg of serum proteins had been loaded). The conditions for chromatography were the same as shown in Fig. 1. The fractions were collected as indicated: I, the pass-through fraction; II, the adsorbed fraction.

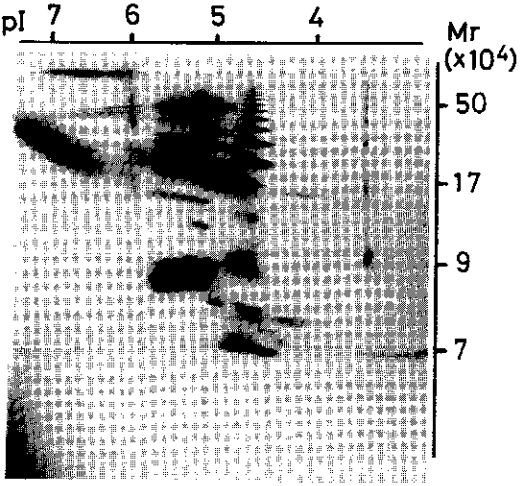

Fig. 5. Analysis of proteins in the eluate. The proteins in fraction II (the adsorbed fraction) of Fig. 4 were concentrated and subjected to micro 2-D electrophoresis. The protein composition was the same as that shown in Fig. 2B.

Gradient elution of Con A-binding proteins

Serum proteins adsorbed on the affinity column were eluted with a concentration gradient of α-D-methylmannoside in various concentration ranges. Broad protein peaks were observed in the elution profiles, indicating the separation of adsorbed proteins. About 50% of the adsorbed proteins was eluted at α-D-methylmannoside concentrations below 0.01 *M*. Although the protein species in the eluates have not been analysed by electrophoresis, gradient elution should be useful for the affinity-dependent separation of serum proteins, as suggested in Figs. 2 and 3.

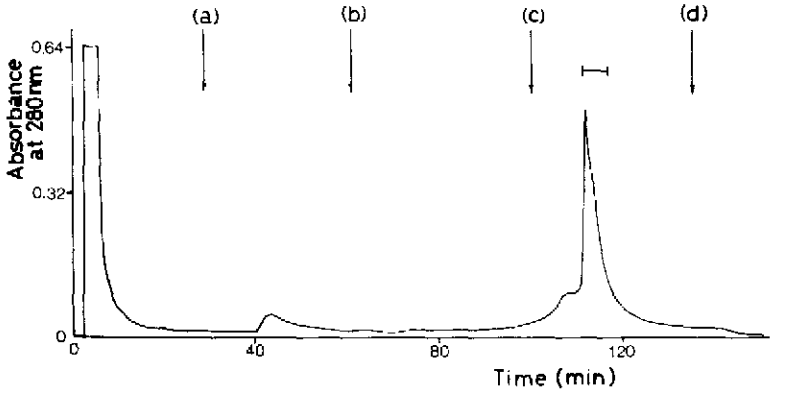

Fig. 6. Elutions of proteins non-specifically bound to the column. Human serum (100 μl) was loaded on the column and eluted as shown in Fig. 1 until arrow b. At arrow b, the buffer containing 0.1% SDS was used; at arrow c, the buffer containing 6 *M* urea; at arrow d, the initial buffer.

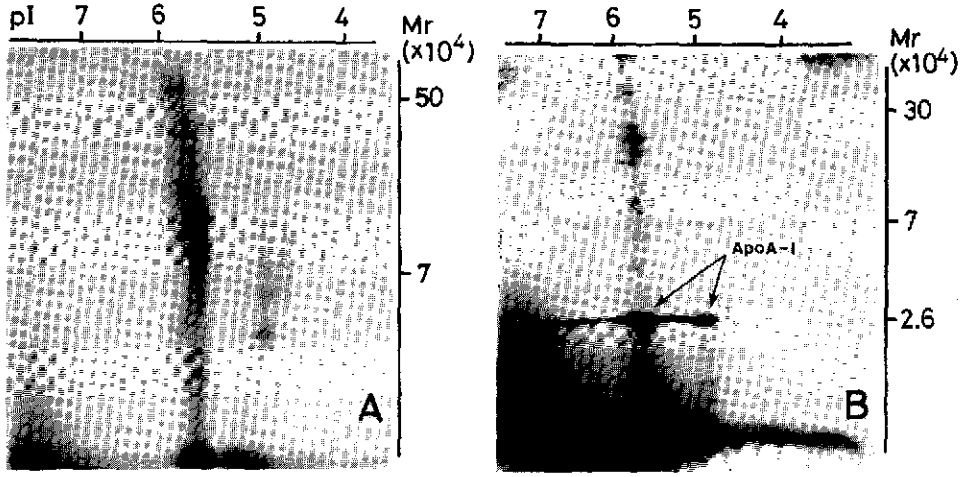

Fig. 7. Analysis of proteins in the eluate. The proteins eluted with 6 *M* urea were concentrated and subjected to micro 2-D electrophoresis. (A) Proteins in 6 *M* urea eluate analysed in the absence of denaturants; (B) proteins in 6 *M* urea eluate analysed in the absence of denaturant in the first dimension of 2-D electrophoresis and in the presence of SDS in the second dimension. Apolipoprotein A-I (ApoA-I) was immunochemically identified in separate experiments and appeared at the positions arrowed in the figure.

Repeated use of the affinity column

Repeated loading of the columns led to a gradual decrease in the amount of proteins adsorbed. Fig. 4 shows the elution profile of serum proteins after 30 cycles of affinity chromatography, when a total of ca. 200 mg of serum proteins had been loaded on the column. From the elution profile, the amount of proteins adsorbed was calculated to be ca. 5% of that of the pass-through proteins and a decrease of the capacity of the column was observed. However, the protein species adsorbed on the column did not change (Fig. 5), indicating no change in the specificity of the column.

Analysis of non-specifically bound proteins

After repeated loading of serum proteins and tissue extracts, the capacity of the Con A column decreased as shown in Fig. 6. Since we supposed non-specific binding of proteins on the column to be one reason for the decrease of capacity, we tried to elute these proteins with SDS or urea. SDS (0.1%) had almost no effect on the elution of the proteins from the column, but proteins up to 1.5 mg could be eluted with 6 *M* urea. The proteins were concentrated and subjected to micro 2-D electrophoresis. The proteins in this fraction showed the characteristics of lipoproteins, LDL and HDL, when analysed in the absence of denaturants (Fig. 7A) or in the presence of SDS in the second dimension (Fig. 7B) [13]. Further, the major spot arrowed in Fig. 7B was immunochemically identified as apo A-I (a major apolipoprotein of HDL). These results suggest that the column has bound serum lipoproteins. Since the decrease of column capacity was obvious after loading of turbid (abundant in lipids) samples and also because the recovery of lipoproteins in the eluate was very low, we assume that the column non-specifically binds lipids and lipoproteins that interfered with the binding of serum glycoproteins to Con A. The capacity of the column did not recover after the elution of lipoproteins. It is possible that Con A covalently bound on the resin is denatured by the treatment with SDS and 6 *M* urea. For the prolonged use of the column, removal of lipid from the samples before loading might be necessary.

Selection of lectin affinity columns for glycoprotein purification

High-performance lectin affinity columns packed with hydrophilic polymer gels, in which each lectin has an affinity to a specific structure of oligosaccharide chains, have become commercially available. These columns will be effective for the purification of glycoproteins if their specificity can be confirmed. The specificity of the high-performance Con A column to serum proteins agreed very well with that predicted by 2-D electrophoresis followed by Con A staining [7]. We have reported on lectin staining of 2-D separated serum glycoproteins employing several lectins labelled with fluorescein isothiocyanate [14]. The technique will be useful for the selection of lectin affinity columns in glycoprotein purification.

CONCLUSIONS

The high-performance Con A affinity column, packed with Con A-immobilized hydrophilic polymer support Gelpack, is useful for the rapid fractionation of human serum Con A-binding proteins. Serum protein species eluted with α-D-methylmannoside from the Con A affinity column coincided very well with those intensely stained on the blots after micro 2-D electrophoresis and Con A staining, and the results visualized the specificity of the affinity column.

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