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FRACTIONATION OF GLYCOPROTEINS ACCORDING TO LECTIN AFFINITY AND MOLECULAR SIZE USING A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SYSTEM WITH SEQUENTIALLY COUPLED COLUMNS

CARL A. K. BORREBAECK*, JOHN SOARES and BO MATTIASSON
Department of Pure and Applied Biochemistry, University of Lund, P.O. Box 740, S-220 07 Lund (Sweden)
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SUMMARY

The lectin phytohaemagglutinin was coupled to porous silica (10 μm) and used as adsorbent in a high-performance liquid affinity column sequentially coupled to a TSK-G 3000SW gel permeation column. This system was used for high-speed separation/analysis of human serum glycoproteins according to their lectin affinity and molecular size. Serum samples could be resolved in at least six different peaks representing glycoproteins exhibiting different molecular weights but with a carbohydrate content compatible with the specificity of phytohaemagglutinin.

INTRODUCTION

Lectins are carbohydrate binding proteins which are often used for the purification and analysis of glycoproteins by conventional affinity chromatography[1]. The advent of high-performance liquid affinity chromatography (HPLAC)[2,3] has made it possible to combine the specificity of affinity chromatography with the speed and resolution of high-performance liquid chromatography (HPLC).

In this paper we report an extension of the HPLAC technique and the development of a method that combines the specificity of HPLAC with the resolving power of high-performance gel permeation chromatography. Human serum glycoproteins were first allowed to bind to an affinity column containing phytohaemagglutinin (PHA), the lectin from red kidney beans, *Phaseolus vulgaris*, immobilized to porous silica. Bound glycoproteins were then eluted directly into a gel permeation column where the glycoproteins were subsequently separated according to their molecular size. The columns were both part of the HPLC system.

MATERIALS AND METHODS

Chemicals

LiChrospher Si 1000 (10 μm) was obtained from E. Merck (Darmstadt, F.R.G.). The gel permeation column was a TSK-G 3000SW (600 × 7.5 mm I.D.)

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from LKB (Bromma, Sweden). γ-Glycidoxypropyltrimethoxysilane (Silane Z 6040) was purchased from Dow Chemical (Midland, MI, U.S.A.) and 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride) from Fluka (Buchs, Switzerland). Human α_1-antitrypsin (high activity preparation) was obtained from Sigma (St. Louis, MO, U.S.A.). The gammaglobulin mixture from KABI AB (Stockholm, Sweden) was used as a source of IgG and contained less than 0.01% IgA.

PHA was isolated according to the method of Leavitt *et al.*[4] and was dialyzed against a 0.01 *M* phosphate buffer, pH 7.4 containing 0.15 *M* sodium chloride (PBS) before being immobilized to porous silica. Human blood serum was mixed with Kiesel Gel 60 (E. Merck) for 1 h to facilitate the removal of lipoproteins and other compounds that might irreversibly bind to silica. The Kiesel Gel 60 was removed by using a Millipore filtering apparatus equipped with a 0.45-μm filter.

Immobilization of PHA to tresyl-activated silica

The coupling was performed according to refs. 5 and 6. The tresyl-activation procedure was a modification of the procedure described in ref. 6. Briefly, 1.8 g of dry diol–silica was transferred to dry acetone on a glass filter funnel using the following washing steps in sequence: 3 × 50 ml of acetone–water (90:10), 3 × 50 ml of acetone, and 3 × 50 ml of dry acetone. The diol–silica was resuspended in 6 ml of dry acetone containing 200 μl of dry pyridine, and 36 μl of tresyl chloride was slowly added over a period of 1 min with vigorous magnetic stirring. The tresyl-activated silica was then washed with the following solutions in sequence: 3 × 50 ml of acetone, 3 × 50 ml of acetone-5 m*M* hydrochloric acid (70:30), 3 × 50 ml of acetone–5 m*M* hydrochloric acid (50:50), 3 × 50 ml of acetone–5 m*M* hydrochloric acid(30:70), 3 × 50 ml of acetone–5 m*M* hydrochloric acid (15:85), and 4 × 50 ml of 1 m*M* hydrochloric acid.

To immobilize the lectin, 1.6 g of dried tresyl-activated silica was mixed with 4 ml of PBS buffer containing 6.4 mg of PHA. The coupling took place overnight on a rocking table at 4°C. The silica was then suspended in 100 ml of 0.2 *M* Tris–HCl buffer, pH 7.5, to derivatize unreact tresyl groups.

Column packing and chromatographic conditions

The gel was packed into a 120 × 5 mm I.D. column by the slurry packing method using 50% sucrose as suspending medium.

An HPLC system comprising a Constametric 111 high pressure pump, a Spectromonitor 111 UV spectrophotometric detector (LDC, Riviera Beach, FL, U.S.A.) and a 7125 injector (Rheodyne, Cotati, CA, U.S.A.), equipped with either a 20-μl or a 200-μl loop, was used. To retain the maximum carbohydrate binding activity of PHA over a long period the lectin column was kept at 4°C.

The mobile phase for the columns consisted of 25 m*M* Tris–HCl buffer, pH 7.2, containing 0.5 m*M* each of manganese chloride and calcium chloride and 0.02% sodium azide. A 0.2 *M* glycine–HCl buffer, pH 2.8, containing 0.02% sodium azide, was used to elute glycoconjugates bound to the affinity column. The buffers were thoroughly degassed under vacuum and sonication immediately before use.

Column system operation

The affinity column and the gel permeation column were sequentially coupled

with a three-way connector piece. Using this three-way joint, it was possible to divert the unbound fraction of a sample injected into the affinity column to waste. After a sample had been injected into the lectin column, the flow-rate of 1 ml/min was continued for 6 min, allowing proteins not bound by the immobilized lectin to be diverted to waste. The affinity column was then eluted with a 5-ml pulse of 0.2 *M* glycine–HCl buffer, pH 2.8, at a flow-rate of 1 ml/min. Gel permeation chromatography proceeded at 200 μl/min using the eluted glycoproteins as samples. The time required to complete one analysis was 1–2 h.

Enzyme immunoassay

Fractions (100 μl) from the column system were collected in the 96 wells of a microtiter plate (Dynatech MicroELISA System M129B) and allowed to air-dry at 37°C. After the plate had been washed in PBS buffer the wells were challenged with 100 μl of a monospecific rabbit antiserum (diluted 1:500 in 10 m*M* phosphate buffer, pH 8.0, containing 0.5 *M* sodium chloride and 0.1% Tween 20) to a serum glycoprotein and incubated for 30 min. The plate was then washed and 100 μl of horseradish peroxidase labeled antiserum to rabbit antibodies (diluted 1:500 in 10 m*M* phosphate buffer, pH 8.0, containing 0.5 *M* sodium chloride and 0.1% Tween 20) (Dakopatts A/S, Copenhagen, Denmark) was added. After 30-min incubation the plate was finally washed and the enzyme substrate [0.4 m*M* 2,2-azinodi-(3-ethylbenzthiazoline sulphonic acid) diammonium salt (ABTS) and 0.3% hydrogen peroxide in 0.1 *M* sodium citrate buffer pH 4.0] was added. The plate was read spectrophotometrically at 405 nm.

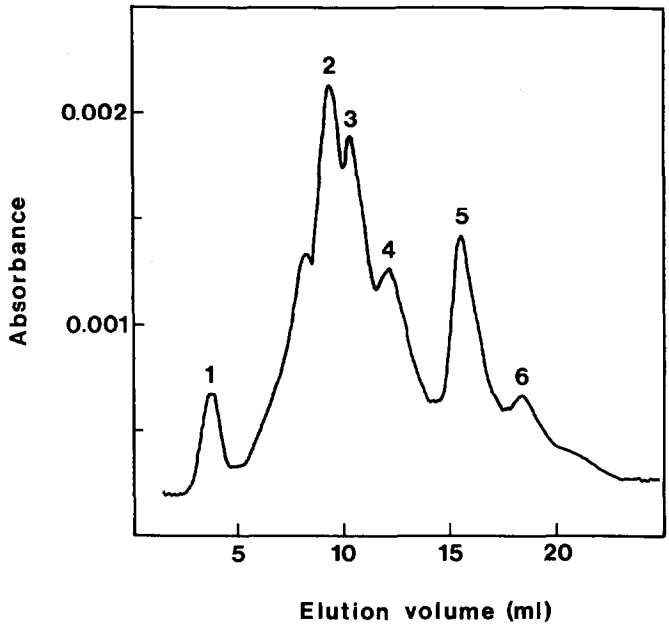

Fig. 1. Elution profile showing glycoproteins eluted from a system consisting of sequentially coupled affinity and gel permeation columns. The peaks represent human glycoproteins separated according to carbohydrate content and molecular weight.

RESULTS AND DISCUSSION

Fig. 1 shows the elution profile obtained when human blood serum diluted 1:3 in Tris–HCl buffer, pH 7.2, containing 0.5 m*M* each of calcium chloride and manganese chloride and 0.02% sodium azide, is run through the column system according to the procedure given in Materials and methods. Peaks 1 and 5 are also indicated to be present in blank runs performed by injecting 20 μl of running buffer instead of the sample. The large elution volume of peak 5 suggests that it could be partly composed of dissociated PHA subunits, which have a molecular weight of *ca.* 31,000. It is known that the PHA lectin contains no cystine disulfide bonds between the subunits[4], and it is thus possible that the elution step could remove PHA subunits not covalently bound to the silica. The height of peak 5 could, however, be reproduced after a few initial runs.

To help identify the components in the peaks, different human serum glycoproteins that are known to interact with PHA[7] were added separately to human blood serum and run through the column system. An increase in peak height would indicate the presence of the added protein in that peak. When 20 μl of a one-third dilution of human blood serum containing 3 mg/ml of added α_1-antitrypsin were run through the column system, peak 4 increased considerably in height. This indicated that peak 4 contained α_1-antitrypsin. When 200 μl of a one-third dilution of human blood serum containing 12 mg/ml of added human IgG preparation were run through the column system, peak 3 increased in height relative to the other peaks, indicating the presence of IgG in this peak. The enzyme immunoassay (see Materials and methods) was used independently to identify peak 3 as IgG and peak 4 as α_1-antitrypsin. Peak 2 was not unambiguously identified.

Fig. 2A shows the elution profile obtained when 20 μl of a one-third dilution of human blood serum were run through just the gel permeation column at a flow-rate of 200 μl/min. Fig. 2B shows the elution profile obtained when a 20 μl of a one-third dilution of human blood serum were run through the PHA column and then through the gel permeation column. Serum proteins that were not bound by PHA passed through the affinity column into the gel permeation column, giving an elution profile similar to that of Fig. 2A. After the appearance of the last peak in Fig. 2B, the serum glycoproteins that bound to the lectin column were eluted into the gel permeation column with glycine–HCl buffer, pH 2.8. An elution profile similar to Fig. 1 was obtained (data not shown).

It was observed that a ten-fold increase in sample size (200 μl of a one-third dilution of human blood serum) changed the elution profile shown in Fig. 1. In addition to the overall increase in height of the peaks, peaks 3 and 4 increased relative to peak 2. There was also a loss of resolution, exemplified by a merging of peaks 4 and 5. A possible explanation for the differences in relative peak heights between the two sample sizes is that the PHA lectin exhibited concentration-dependent differences in its affinity for the glycoproteins. This could cause changes in the relative peak heights compared with smaller sample sizes, where the available number of carbohydrate binding sites in the column was greater than the number of glycoproteins in the sample.

The use of lectins in the HPLAC step of this technique can help to resolve many types of biological substance, *e.g.* glycolipids, polysaccharides and membrane

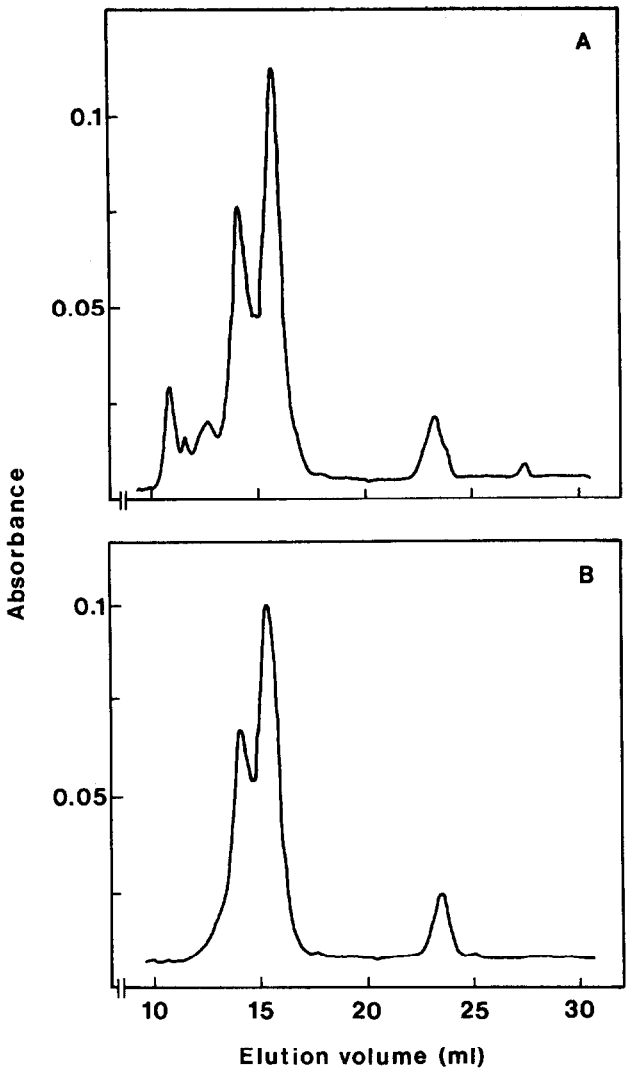

Fig. 2. (A) Elution profile showing the protein pattern obtained when a human serum sample was passed through a TSK-G 3000SW gel permeation column. (B) Elution profile showing the protein pattern obtained when a human serum sample was passed through the system consisting of an affinity column coupled to the TSK-G 3000SW gel permeation column. The pattern shows that certain glycoproteins have been removed by immobilized PHA in the affinity column.

glycoproteins. Sequentially coupled columns could also be used to reveal differences in, *e.g.*, the glycoprotein pattern of cell membranes, with respect to lectin binding and molecular weight.

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