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STAINING OF SERUM CONCANAVALIN A-BINDING PROTEINS AFTER MICRO TWO-DIMENSIONAL ELECTROPHORESIS AND BLOTTING

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SUMMARY

Human serum proteins separated by micro two-dimensional electrophoresis in the absence of denaturants were electrophoretically transferred to nitrocellulose sheets (blots), and glycoproteins on the blots were stained by sequential incubation with the lectin concanavalin A, the glycoprotein horseradish peroxidase and the substrates of peroxidase diaminobenzidine–hydrogen peroxide. The stained serum concanavalin A-binding glycoproteins were identified by referring to the "identification map" of human plasma proteins, which we have prepared by immunochemical staining of the proteins on the blots. The concanavalin A–peroxidase method applied for the blots after two-dimensional electrophoresis provided information on the location of serum concanavalin A-binding glycoproteins, and showed greater sensitivity than Coomassie Brilliant blue R-250 staining for several serum proteins.

INTRODUCTION

We have previously described the separation of human plasma or serum proteins by two-dimensional electrophoresis in the absence of denaturing agents [1, 2]. By developing a micro two-dimensional electrophoretic technique [3] and by adapting [4] the technique of electrophoretic transfer of proteins from slab gels to nitrocellulose sheets followed by immunochemical staining [5], we could identify 31 plasma proteins on the blots and an "identification map" was prepared [6].

However, immunochemical staining offered information only on the protein moiety of plasma proteins and not on the polysaccharide moiety, which might play important roles in the secretion of the proteins and in the binding of the proteins to cell receptors. For detection and isolation of glycoproteins, lectins that preferentially bind to specific sugar residues are employed. The most commonly

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used lectin is concanavalin A (Con A), which interacts with glycoproteins that have at least two non-substituted or 2-O-substituted α-mannosyl residues [7]. Hawkes [8] detected Con A-binding proteins after sodium dodecyl sulphate (SDS) gel electrophoresis and protein blotting. Staining of glycoproteins on nitrocellulose blots is preferable to staining on acrylamide gels, because of the absence of diffusion interference by the gel matrix. Furthermore, the blots can also be treated by staining methods with different specificity: by immunochemical staining, which is suitable for detection of one specific protein [4], or by colloidal gold staining, which is suitable for visualizing the general distribution of proteins [9].

In this paper, we describe the identification of human serum Con A-binding proteins. Serum proteins were two-dimensionally separated on micro slab gels and then electrophoretically transferred to nitrocellulose sheets. The blots were treated sequentially with Con A and peroxidase rather than with peroxidase-conjugated Con A, for better sensitivity. The stained spots on the blots were identified by comparing their locations with those of the identified proteins, which have been summarized in the "identification map" of human serum proteins [6].

EXPERIMENTAL

Materials

Human serum samples were obtained from healthy individuals; sucrose was added to give a concentration of 40% (w/v), and the samples were stored at −20°C. Purified human transferrin was purchased from Sigma. Con A and peroxidase-conjugated Con A were obtained from Seikagaku Kogyo (Tokyo, Japan). Horseradish peroxidases (type VI and crude) were from Sigma. Nitrocellulose sheets (0.45-μm pore size) were obtained from Schleicher and Schüll (Dassel, F.R.G.).

Micro two-dimensional electrophoresis

Microscale-multisample two-dimensional electrophoresis in the absence of denaturing agents was performed as described previously [3]. First-dimension isoelectric focusing was performed on capillary gel columns (35 mm×1.3 mm I.D.). A 4% acrylamide (0.2% bis) solution, containing 2% Ampholine pH 3.5–10, 0.5% Ampholine pH 3.5–5 and 0.05% ammonium persulphate, was used to prepare the capillary gels. The electrode solutions were 0.04 *M* sodium hydroxide (cathode) and 0.01 *M* phosphoric acid (anode). A serum sample (2 μl) was applied at the gel top (cathode side), and electrophoresis was performed at a constant current of 0.1 mA per tube for 20 min, and then at 300 V constant voltage for 40 min at 3°C. Second-dimension gradient polyacrylamide gel electrophoresis was performed in 4–17% acrylamide linear gradient (0.2–0.85% bis gradient) slab gels (38 mm long, 38 mm wide and 1.0 mm thick). The gradient gel buffer was 0.375 *M* Tris–HCl (pH 8.9) and the electrode buffer was 0.05 *M* Tris–0.38 *M* glycine (pH 8.3) containing bromophenol blue (BPB) as a marker dye. Electrophoresis was carried out at a constant current of 10 mA per slab for 1 h. In some cases, second-dimension gradient gel electrophoresis was performed in the

presence of SDS. The gradient gel buffer contained 1% SDS, and the electrode buffer contained 0.1% SDS, in addition to the constituents mentioned above. Electrophoresis was run at a constant current of 10 mA per slab for ca. 60 min, until the band of BPB reached 5 mm from the bottom end of the slab gel [10].

Electrophoretic transfer

Construction details of the apparatus for electrophoretic transfer have been fully described elsewhere [4]. The apparatus is commercially available from Immunomedica (Tokyo). Four micro slab gels obtained after parallel two-dimensional electrophoresis were placed horizontally in the apparatus, and one nitrocellulose sheet (40×40 mm) was laid on each slab gel. Electrophoretic transfer was run 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 60 min. One blot was obtained from one slab gel.

Con A-peroxidase staining

The blots were treated as described previously [6], with some modifications for Con A staining. The optimum staining conditions were determined to be as follows. (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 at room temperature. (ii) Con A in saline (1 mg/ml, 250 μl) was added to the BSA solution and kept at room temperature for 60 min. (iii) Each blot was washed in saline (10 ml, five changes during 30 min). (iv) The blot was soaked in peroxidase solution (300 μg of Type VI peroxidase in 5 ml of saline) for 60 min at room temperature. (v) Washed in saline (10 ml, five changes during 30 min). (vi) Staining was carried out 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.

Dye staining

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

Quantitation

Quantitation of proteins on micro slab gels or on nitrocellulose blots was performed as described previously [11], with the following modifications in the apparatus and in the computer programs: a digitizer (Computer Eye Model TIF 64, Pax Electronica Japan), which has 64 gray levels, a 16-bit microcomputer (NEC PC-9801F) and a TV camera (NEMCO Model 130) were employed instead of those described in the previous paper. The microcomputer programs supplied from Pax Electronica Japan for the computer–peripheral machine interactions were used without modifications and only part of the operator–computer interactions were modified for our purpose in BASIC language. The details of the modified quantitation system have been described [12].

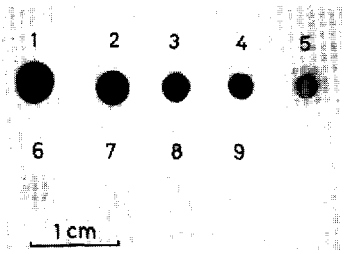

Fig. 1. Spot tests for Con A–peroxidase staining of purified human transferrin. A drop (2 μl) of transferrin solution was applied on a nitrocellulose sheet and then stained as described in Experimental. The amount of transferrin applied was: 1, 10 μg; 2, 2.5 μg; 3, 625 ng; 4, 156 ng; 5, 39 ng; 6, 9.8 ng; 7, 2.4 ng; 8, 0.6 ng; 9, 0.15 ng.

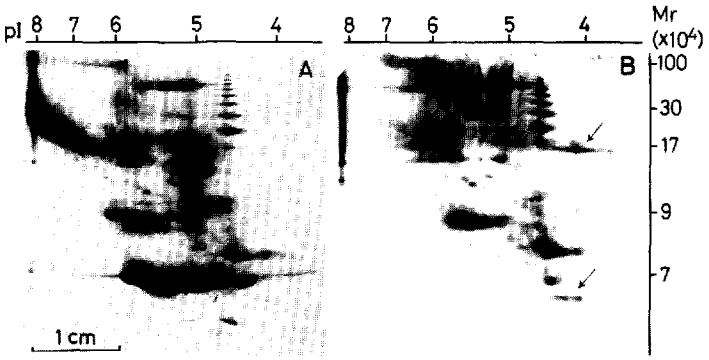

Fig. 2. Con A–peroxidase staining of human serum proteins. A sample of human serum containing 40% (w/v) sucrose (2 μl) was subjected to micro two-dimensional electrophoresis in the absence of denaturant. (A) One of the micro slab gels stained with Coomassie Brilliant blue R-250. (B) A nitrocellulose blot of the serum proteins from a micro slab gel. Glycoproteins on the blot were stained by the Con A–peroxidase method. Some of the glycoproteins that could not be clearly visualized by dye staining are shown by arrows.

RESULTS AND DISCUSSION

Method of Con A–peroxidase staining

In the method of Hawkes [8], the nitrocellulose sheets after electrophoretic transfer (blots) were first incubated with Con A and then incubated with peroxidase. This method exploits the facts (i) that at the pH employed (pH 7.4) Con A is a tetramer with four binding sites and (ii) that horseradish peroxidase itself is a Con A-binding glycoprotein [13]. However, as a generally applicable method for the detection of lectin-binding proteins, the use of lectins covalently labelled with peroxidase seemed to be suitable. We compared the sensitivity of the two methods by spot tests using purified human serum transferrin as an example of a Con A-binding protein. Serial dilutions of transferrin were applied as 2-μl drops on a dry nitrocellulose sheet, and the sheet was treated as described in Experimental. In the case of the covalently bound Con A–peroxidase method, the complex was used instead of Con A in step ii so as to give the same Con A concentration, and steps iv and v were deleted. Fig. 1 shows one example of the results

of the spot tests, exmploying the method of sequential addition of Con A and peroxidase (Sigma, Type VI). Transferrin as low as 39 ng per spot (8 ng/mm^2) was detectable by the method. The grade of peroxidase affected the sensitivity of the sequential addition method; when a peroxidase preparation of lower purity (Sigma, crude) was employed to give the same total peroxidase activity unit, the sensitivity was 156 ng per spot (30 ng/mm^2). When the method of Con A–peroxidase conjugate (Seikagaku Kogyo) was employed, the sensitivity was also 156 ng/spot (30 ng/mm^2). The density and the area of the spots on nitrocellulose sheets obtained by these spot tests were quantitated using a TV camera–microcomputer system [12]. From these results, the sequential addition method was employed for the detection of Con A-binding proteins on the blots after micro two-dimensional electrophoresis.

Identification of serum glycoproteins

Fig. 2A shows an example of the micro two-dimensional gel pattern of human serum proteins separated in the absence of denaturants and stained with Coomassie Brilliant blue. Some of the slab gels run at the same time as the gel shown in Fig. 2A were used for protein blotting and subjected to Con A–peroxidase staining. An example of the pattern of Con A-binding proteins is shown in Fig. 2B. As shown in Fig. 2, most of the serum proteins detectable on the slab gels with Coomassie Brilliant blue were also detected by Con A–peroxidase staining. Furthermore, several serum proteins were stained more clearly than in the case of Coomassie Brilliant blue staining (arrows in Fig. 2B).

In order to identify Con A-binding proteins, the pattern of Fig. 2B was compared with the identification map of human plasma proteins [6] prepared by immunochemical staining of the proteins after blotting. The identification map copied on tracing paper was laid on a photograph of the pattern of Fig. 2B and, by superimposing the protein spots, Con A-binding proteins were identified. The results of the identification of Con A-binding serum proteins are summarized in Fig. 3. The location of fibronectin is also shown in Fig. 3, which was recently identified immunochemically. Twenty-two serum Con A-binding proteins were identified on the two-dimensional pattern and four major serum proteins, albumin, Gc-globulin, prealbumin and high-density lipoprotein (HDL), were not stained.

The reports on the carbohydrate content of plasma proteins have been collected and reviewed [14]. The structure of oligosaccharide moiety of plasma proteins has also been studied and reviewed [15]. The results shown in Fig. 3 are generally consistent with the previous reports and provide information on the serum glycoproteins that contain α-D-mannopyranosyl residues in their oligosaccharide moiety.

Differences in staining intensity between dye stain and glycoprotein stain

As shown in Fig. 2A and B, most of the serum proteins detectable with Coomassie Brilliant blue on polyacrylamide slab gels were also stained by Con A and peroxidase. However, the density distribution pattern of Con A-binding proteins (Fig. 2B) was considerably different from that of Coomassie Brilliant blue-stained

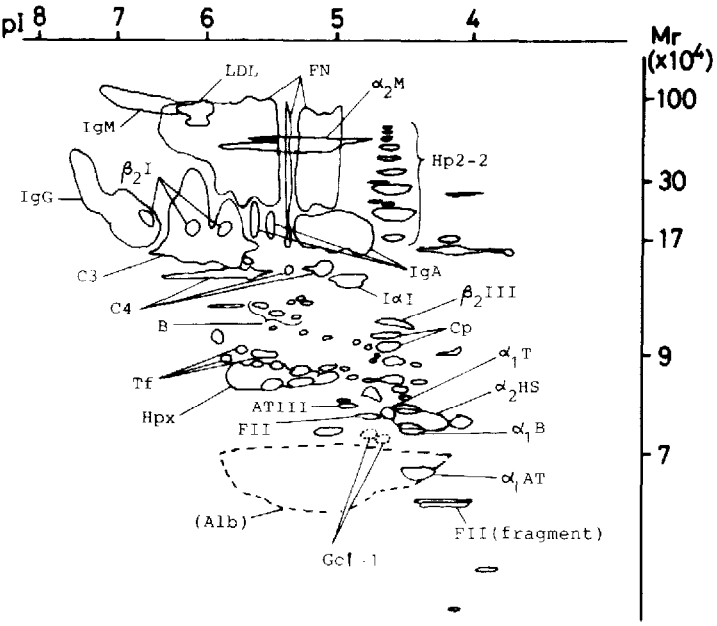

Fig. 3. Map of human serum Con A-binding proteins. The stained spots in Fig. 2B were identified by superimposing the figure on the identification map of human plasma proteins, which has been prepared by blotting and immunochemical staining [6]. Abbreviations: LDL = low-density lipoproteins; FN = fibronectin; α_2M = α_2-macroglobulin; β_2I = β_2-glycoprotein I; β_2III = β_2-glycoprotein III, Hp2-2 = haptoglobin phenotype 2-2; IαI = inter-α-trypsin inhibitor; C3 = complement C3; C4 = complement C4; B = complement factor B (C3 activator); Tf = transferrin; Cp = ceruloplasmin; Hpx = hemopexin; ATIII = antithrombin III; FII = prothrombin; α_1T = α_1T-glycoprotein; α_2HS = α_2HS-glycoprotein; α_1B = α_1B-glycoprotein; α_1AT = α_1-antitrypsin; Gc1-1 = Gc-globulin phenotype 1-1; Alb = albumin. Gc-globulin was not stained by Con A staining, but could be visualized as white spots in a faint brown background, as shown by dashed lines. The position of albumin was simulated from the pattern of dye-stained proteins, and is also shown by dashed lines.

proteins (Fig. 2A). For example, immunoglobulin G and transferrin, which were densely stained with Coomassie Brilliant blue on the slab gels, were only faintly stained by the glycoprotein staining method. In contrast, hemopexin was more densely stained by the glycoprotein stain than by dye stain. Several serum proteins arrowed in Fig. 2B became more clearly detectable by the glycoprotein stain. These results should reflect the differences in the affinity of serum glycoproteins to Con A, which might be affected by the following factors: (i) the content of mannose residues in the protein; (ii) the structural accessibility of mannose residues to Con A.

Glycoprotein stain after SDS electrophoresis

Fig. 4A shows the Coomassie Brilliant blue-stained gel pattern of human serum proteins separated by two-dimensional electrophoresis employing SDS in the second dimension [10]. Fig. 4B is the corresponding glycoprotein pattern stained on a nitrocellulose sheet after electrophoretic transfer. Some of the serum glycoproteins have been immunochemically identified, and their location is shown

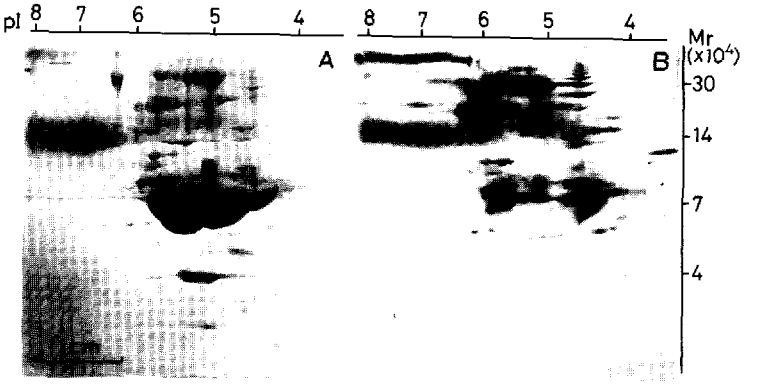

Fig. 4. Con A-peroxidase staining of human serum proteins separated by two-dimensional electrophoresis in the presence of SDS in the second dimension: the same human serum sample (2 μl) as shown in Fig. 2 was used. (A) One of the slab gels stained with Coomassie Brilliant blue R-250. (B) A nitrocellulose blot of the serum proteins. The proteins were stained by Con A-peroxidase method.

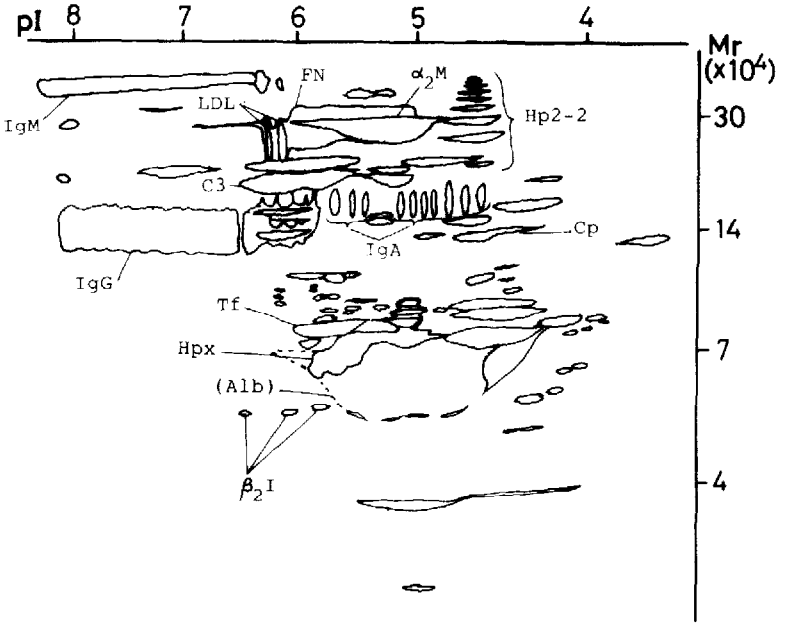

Fig. 5. Map of human serum Con A-binding proteins separated by two-dimensional electrophoresis in the presence of SDS in the second dimension. The positions of immunochemically identified proteins are shown. Abbreviations are the same as in Fig. 3.

in Fig. 5. Immunoglobulins G and M, which were not clearly stained in Fig. 2B, were densely stained in Fig. 4B. The staining intensity of serum proteins generally increased when SDS was used in the second dimension. These results suggest that the differences in staining efficiency between serum glycoproteins separated under native conditions may in part reflect the differences in the accessibility of mannose residues to Con A.

Relation of lectin staining and affinity chromatography

Since serum proteins were separated under native conditions, the staining intensity of the glycoproteins shown in Fig. 2B may be related to the affinity of native glycoproteins to Con A. In cases of purification of native serum glycoproteins, affinity chromatography using Con A as a ligand has been reported. Therefore, it will be possible to predict the affinity of glycoproteins to Con A affinity columns by comparing Fig. 2A and B. We have applied serum proteins to high-performance lectin affinity chromatography and analysed the eluates by micro two-dimensional electrophoresis followed by blotting and Con A–peroxidase staining. The affinity of serum proteins in the Con A-affinity column coincided very well with the staining intensity on the blots shown in Fig. 2B. These results will be described in detail elsewhere.

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