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Note

Chromatographic separation of α_1-acid glycoprotein from α_1-antitrypsin by high-performance liquid chromatography using a hydroxyapatite column

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α_1-Acid glycoprotein (AGP) is normally present in human plasma; its level increases in acute inflammation, rheumatism and cancer [1, 2]. Human plasma AGP and derivatives of this protein produced by enzymatic cleavage of the glycosidic residues suppress a number of immune functions of mouse spleen cells in vitro at physiological concentrations [3]. The microheterogeneity of AGP depends on the heterogeneity of the carbohydrate moiety [4–6]. Differences in the microheterogeneity of AGP in healthy persons and patients with tumours are of interest. The usual way to purify AGP is with ion-exchange chromatography [5]. However, separation of AGP and α_1-antitrypsin (AT) is difficult using this technique, because the chromatographic behaviour of these compounds during anion-exchange chromatography is similar. Recently, high-performance liquid chromatography (HPLC) using a hydroxyapatite column performs good resolution of proteins that are not separable by an ion-exchange column [7, 8]. We used a hydroxyapatite column to separate AGP and AT. Here, we describe the separation of AGP and AT using pseudo-ligand affinity chromatography and hydroxyapatite HPLC from plasma of a patient with tumours.

EXPERIMENTAL

Material

Plasma was obtained from a 67-year-old man with bladder tumours of advanced metastases.

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Affinity chromatography

Pseudo-ligand affinity chromatography using Blue-Sepharose CL-6B (Pharmacia) and Red-Sepharose CL-6B (Pharmacia) was done using the method of Laurent et al. [9]. First, 2 ml of plasma were dialysed against 0.03 *M* sodium phosphate buffer (pH 7.0) overnight, followed by Blue-Sepharose chromatography (gel-bed volume, 20 ml) equilibrated with the same buffer at a flow-rate of 40 ml/h. The non-retained fraction (20—30 ml) from this chromatography was dialysed against 0.03 *M* sodium acetate buffer (pH 5.8) overnight, and then applied on a Red-Sepharose column (bed volume, 10 ml) equilibrated with the same buffer. The non-retained fraction (30—50 ml) from this chromatography was collected and concentrated to 2—3 ml with Amicon PM-10 and freeze-dried.

Hydroxyapatite HPLC

The freeze-dried sample was dissolved in 300 µl of 0.01 *M* sodium phosphate buffer (pH 7.0) containing 0.1 *M* sodium chloride, and injected into a HPLC apparatus (Toyo Soda, SP-8700, Tokyo, Japan) equipped with a hydroxyapatite column (KB-column, 10 × 0.6 cm I.D., Koken, Tokyo, Japan). HPLC was done at a flow-rate of 1.0 ml/min with a linear gradient of buffer A (0.01 *M* sodium phosphate, pH 7.0, containing 0.1 *M* sodium chloride) and buffer B (0.2 *M* sodium phosphate, pH 7.0, containing 0.1 *M* sodium chloride). Buffer B was incorporated into buffer A linearly to reach 50% in 60 min. Proteins were monitored at 280 nm.

Other methods

AGP and AT were measured on single radial immunodiffusion plates, such as NOR-Partigen-α_1-acid glycoprotein and NOR-Partigen-α_1-antitrypsin (Behring, Marburg, F.R.G.), respectively. Protein was assayed by the method of Lowry et al. [10]. Sodium dodecyl sulphate (SDS)—polyacrylamide gel electrophoresis was done using the method of Laemmli [11] on a slab separating gel of 1.5 mm thick containing 9% acrylamide. The apparent molecular weight was estimated using bovine serum albumin (68 000), glutamate dehydrogenase (53 000), ovalbumin (45 000) and α-chymotrypsinogen (25 000) as the standards.

RESULTS AND DISCUSSION

The yield of AGP from plasma was 65% by affinity chromatography using Blue- and Red-Sepharose. The partially purified AGP fraction obtained by Red-Sepharose chromatography contained AT. AGP was separated from cytochrome C, ovalbumin and β-lactoglobulin by HPLC using an anion-exchange column [12]; however, we could not separate AGP and AT in this way. Therefore, we tried to use a hydroxyapatite column. The HPLC profile of partially purified AGP with this column is shown in Fig. 1. Three fractions were obtained. The second peak (retention time, 13.5 min) and the third peak (retention time, 22.3 min) were identified as AGP and AT, respectively, with radial immunoplates. AGP and AT were separated within 30 min by HPLC using the hydroxyapatite column. The pressure of the KB-column was 3 bar at a flow-rate of 1 ml/min. It would be possible to elute AGP at a higher flow-rate,

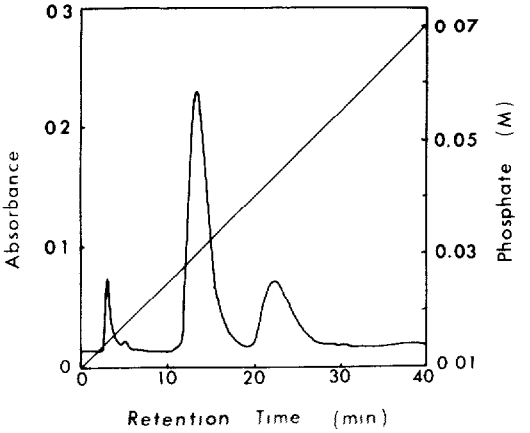

Fig. 1. Hydroxyapatite HPLC profile of the non-retained fraction with Red-Sepharose. Here, 400 μl of the non-retained fraction with Red-Sepharose containing 2 mg of protein was loaded on a hydroxyapatite column (10 × 0.6 cm I.D.) α_1-Acid glycoprotein was eluted by a linear gradient of phosphate at room temperature. Protein was monitored at 280 nm. α_1-Acid glycoprotein and α_1-antitrypsin were eluted as the second and third peaks, respectively.

Fig. 2. SDS—polyacrylamide gel electrophoresis of standard protein (track 1), α_1-antitrypsin (track 2), α_1-acid glycoprotein (track 3) and the non-retained fraction with Red-Sepharose (track 4).

because this column bears pressures up to 17 bar. When 10 mg of sample protein were loaded, resolution did not change. More than 90% of the AGP was recovered by HPLC using a hydroxyapatite column. If the guard column was sometimes changed with a new one, column efficiency was maintained for more than 100 samples. HPLC profiles were highly reproducible, even with different amounts of sample injected.

SDS—polyacrylamide slab gel electrophoresis of purified AGP and AT is shown in Fig. 2. AGP and AT gave single bands and their molecular weights were estimated to be 48 000 and 62 000, respectively, by comparing their electrophoretic mobility with the standard marker proteins. The diffuse proteins shown in track 4 at an approximate molecular weight of 50 000 were removed by hydroxyapatite HPLC. AGP consisted of microheterogeneous glycoproteins, so its band was broad.

This HPLC technique using a hydroxyapatite column is rapid, and makes it possible to separate and purify AGP and AT from a small amount of plasma.

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