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FAST CHROMATOFOCUSING OF HUMAN SERUM PROTEINS WITH SPECIAL REFERENCE TO α_1-ANTITRYPSIN AND Gc-GLOBULIN

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SUMMARY

A new chromatofocusing medium, MonoP™, was used for fast (60 min or less) separations of human serum proteins. Separations in the broad pH interval 6.0–3.8 were analysed by fused rocket immunoelectrophoresis to identify a number of proteins, and by gradient gel electrophoresis to determine the molecular weight distribution of the eluted material. To illustrate further the high resolving power of chromatofocusing, narrow pH intervals of about 0.5 pH units were used to study the microheterogeneity of α_1-antitrypsin and Gc-globulin. Due to its high resolving power and preparative capacity, chromatofocusing is attractive as the first dimension in two-dimensional techniques for the resolution of complex protein mixtures.

INTRODUCTION

Chromatofocusing[1], a chromatographic analogue of isoelectric focusing, separates proteins on the basis of the net charge shift that takes place when the pH in the medium surrounding the protein molecules changes from a value above to a value below their respective isoelectric points, p*I*. The elution behaviour of a protein is thus closely related to its p*I*. Since pH intervals of less than 0.5 pH units can be generated, chromatofocusing is a powerful tool for the separation of closely related proteins, *e.g.*, genetic variants. Furthermore the preparative capacity of chromatofocusing allows large sample loads without appreciable loss of resolution. A single chromatogram thus provides enough material to proceed with a number of second-dimension analytical and/or preparative methods.

MonoP™ is a medium for chromatofocusing in the range pH 9–4. The small bead size (10 μm) and monodispersity of the matrix, MonoBeads™, provides the basis for fast (60 min or less) high-resolution separations. The macroporosity of the matrix allows separation of globular proteins with molecular weights up to 10 million[2].

Two-dimensional electrophoretic techniques based on isoelectric focusing in a polyacrylamide gel rod, followed by gradient polyacrylamide gel electrophoresis, have been developed for the analysis of complex protein mixtures[3,4]. Usually, electrophoresis in two dimensions is carried out under denaturing conditions. If, how-

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ever, non-denaturing conditions are to be employed, the sieving effect of the polyacrylamide gel used in the first-dimension isoelectric focusing imposes an upper limit on the molecular weight of the sample proteins that can be analyzed. Since it is technically difficult to measure the pH gradient in the first dimension, internal p*I* standards have to be included in the sample[5]. By using chromatofocusing for the p*I*-related separation many of these difficulties can be circumvented.

In this work we demonstrate the use of MonoP™ for the resolution of human serum proteins in a broad pH interval (pH 6.0–3.8), followed by analyses by fused rocket immunoelectrophoresis to identify some of the proteins, and by pore-gradient gel electrophoresis to determine the molecular weight distribution of the eluted material. Further to illustrate the high resolving power of chromatofocusing, narrow pH intervals of about 0.5 units were used to reveal the polymorphism of α_1-antitrypsin and Gc-globulin.

EXPERIMENTAL

Reagents

Pharmalyte® carrier ampholytes, Polybuffer™ chromatofocusing eluent, Agarose IEF, Agarose A, Blue Sepharose and rabbit antisera against human α_1-antitrypsin, albumin, transferrin and complement C3 were from Pharmacia (Uppsala, Sweden). Rabbit antisera against human Gc-globulin, α_2-macroglobulin and prealbumin were purchased from Dakopats (Copenhagen, Denmark). Human α_1-antitrypsin and rabbit antiserum against human haptoglobin were kindly provided by Dr. M. Inganäs, Biocell Laboratories, Uppsala. All other reagents were of analytical grade.

Equipment

Fast protein liquid chromatography (FPLC). A Pharmacia FPLC system, consisting of a MonoP HR 5/20 prepacked chromatofocusing column (20 × 0.5 cm), two P-500 pumps, a GP-250 gradient programmer, a V7 injector valve fitted with a Superloop™, a UV-1 UV-monitor fitted with a HR-10 flow cell and a FRAC-100 fraction collector, was used in all chromatographic experiments.

Electrophoresis. Isoelectric focusing and fused rocket immunoelectrophoresis were performed on a flat-bed apparatus (FBE 3000). Polyacrylamide gel electrophoresis (PAGE) was performed on a vertical apparatus (GE 2/4 LS). All from Pharmacia.

Procedures

Phenotyping of serum by isoelectric focusing immunofixation. Serum samples were phenotyped by isoelectric focusing in Agarose IEF followed by immunofixation. The following gel formulations were used: A, Gc-globulin: Pharmalyte 2.5-5 and 4-6.5 with morpholinopropanesulphonic acid (MOPS) as a chemical spacer to flatten the pH gradient in the region 4.8–5.1[6]; B, α_1-antitrypsin: Pharmalyte 2.5–5 and 4–6.5 with acetamidoaminoethanesulphonic acid (ACES) to flatten the pH gradient in the region 4.4–4.8[7].

Preparation of samples for chromatofocusing. To remove albumin, 1 ml of serum was applied to a column (11.5 × 1.0 cm) packed with Blue Sepharose and

equilibrated in 50 m*M* Tris–HCl + 100 m*M* sodium chloride, pH 7.0. The albumin-depleted material was then applied to a PD-10 column (disposable Sephadex G-25 column) equilibrated in the chromatofocusing start buffer. After this buffer exchange the sample was applied to the MonoP column with the aid of a 10-ml Superloop™. Conditions for chromatofocusing are given in the figure legends.

Fused rocket immunoelectrophoresis. Gels, 1 mm thick, were cast with 300 μl of antiserum per 26 ml of 1% (w/v) Agarose A in Tris–5,5-diethylbarbiturate buffer (4.48:8.86, w/w) (I = 0.02), pH 8.6. Aliquots (6 μl) of the fractions were analysed by fused rocket immunoelectrophoresis according to standard techniques[8].

Polyacrylamide gradient gel electrophoresis (PAGE). Aliquots (25 μl) of the fractions were applied on ready-made 4–30% gradient polyacrylamide gels (PAA 4/30) equilibrated in 50 m*M* Tris–glycine buffer, pH 8.3. After electrophoresis for 2500 V h at 200 V the gels were fixed and stained with Coomassie Brilliant Blue R-250 according to standard procedures[9].

RESULTS AND DISCUSSION

Analysis of complex mixtures of proteins by two-dimensional techniques, which combine two different separation principles, are widely used. The most common method is a combination of a first-dimension isoelectric focusing in a polyacrylamide gel rod, followed by a second-dimension polyacrylamide gradient gel electrophoresis[3,4]. Electrophoresis in two dimensions is usually performed in the presence of denaturing agents, urea in the first dimension and sodium dodecyl sulphate in the second. By replacing isoelectric focusing in the first dimension with a high-performance, preparative chromatographic technique, such as chromatofocusing, a number of advantages are obtained.

The pH interval of a chromatogram is determined simply by equilibration of the MonoP column to the starting pH with the appropriate buffer (high pH) and subsequent elution with the eluent Polybuffer titrated to the final pH (low pH) of the gradient. The span of the self-generating pH gradient can be varied from about 3 pH units for broad pH intervals down to less than 0.5 pH unit for extreme resolution. The shape of the pH gradient and thus the characteristic elution pH for a certain sample component can be accurately determined by a simple pH measurement, eliminating the need for an internal isoelectric point standard.

It should be stressed however that the elution pH for a protein is not identical with the pH at which it is isoelectric. This may be explained by disproportionation of the buffer molecules giving a slightly higher pH inside the matrix pores than in the bulk solution. When the pH inside the pores falls to the pH for elution the protein molecules will thus be expelled into a mobile phase with a pH lower than the binding pH. Since the buffering capacity of the eluent is lower than that of the stationary phase, the linear velocity of the eluent down the column is much greater than the propagation of the pH profile. The protein molecule travelling down the column will thus soon reach a point where conditions for "binding" are met again. The difference in elution pH and isoelectric pH is a characteristic constant for each type of protein and is related to the charge *vs.* pH relationship around its isoelectric point. The more pronounced the change in net charge with a change in pH, the closer the pH of elution will be to the isoelectric pH.

When using chromatofocusing several second-dimension techniques can be applied to a single first-dimension separation. This is illustrated by the analysis of the molecular weight distribution of human serum proteins eluted in the range pH 6.0–3.8 (Fig. 1) and the analysis by fused rocket immunoelectrophoresis against eight

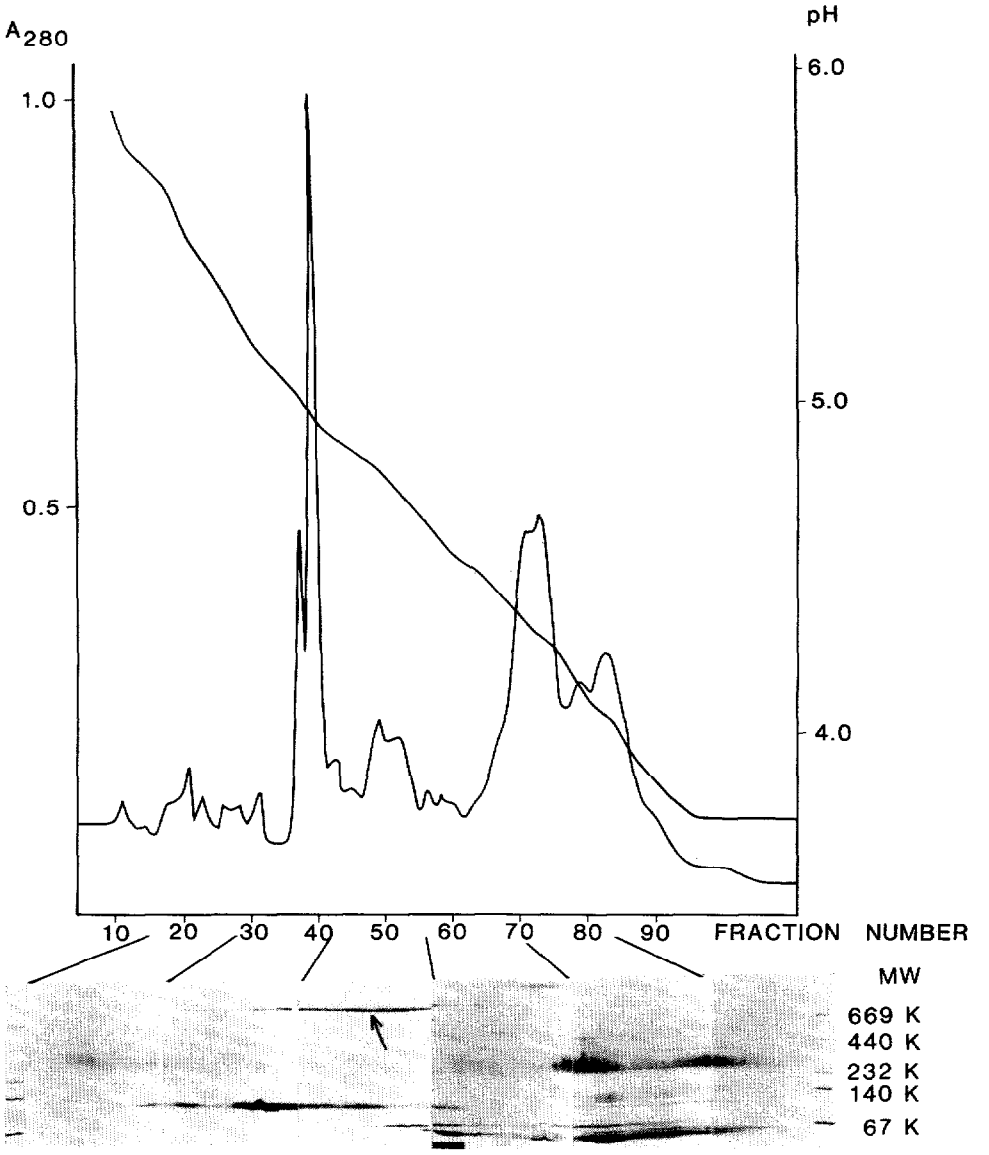
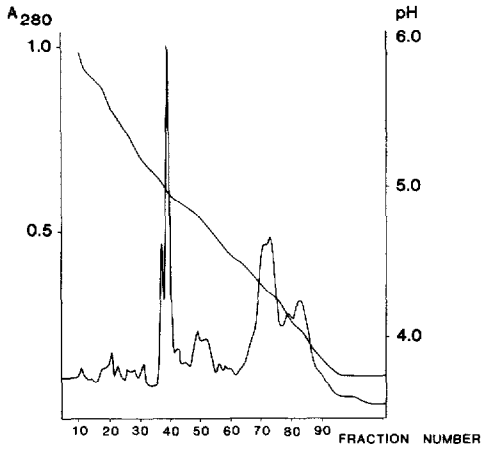


Fig. 1. FPLC chromatofocusing of serum proteins in the pH interval 6.0–3.8 followed by PAGE. Sample: 1 ml of albumin-depleted serum. Starting buffer: 25 m*M* 2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol (Bis-Tris)–HCl, pH 6.3. Eluent: Polybuffer 74 (diluted 1:10)–HCl, pH 4.0. Fractions of 0.4 ml were collected. Flow-rate: 1 ml/min. Aliquots (25 μl) of the fractions were analysed by PAGE in 4–30% polyacrylamide gels under non-denaturing conditions. The arrow indicates α_2-macroglobulin (molecular weight, MW = 820,000).



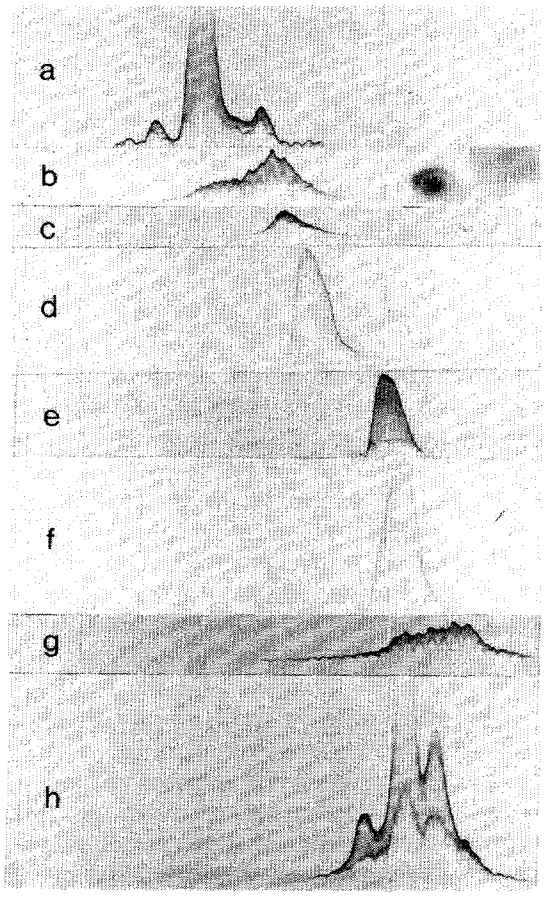


Fig. 2. FPLC chromatofocusing of serum proteins in the pH interval 6.0–3.8, followed by fused rocket immunoelectrophoresis. The chromatographic separation shown is the same as in Fig. 1. Aliquots (6 μl) of the fractions were analysed by fused rocket immunoelectrophoresis using monospecific antisera against: transferrin (a), α_2-macroglobulin (b), albumin (c), Gc-globulin (d), complement C3 (e), prealbumin (f), haptoglobin (g) and α_1-antitrypsin (h).

different antisera (Fig. 2) in which less than 20% of the fractionated sample is used. The same chromatogram (Fig. 1) also demonstrates the loading capacity of the MonoP column, the sample corresponding to 1 ml of albumin-depleted serum. Comparatively high sample loads can thus be used to increase the detectability of minor sample components in the second dimension.

The elution of α_2-macroglobulin (mol.wt. 820,000) and of an unidentified component with a molecular weight greater than one million (see Fig. 1) shows that the macroporous matrix allows separations in the molecular weight range up to several millions, where many plasma proteins are found under native conditions.

The ability to expand the pH gradient provides a powerful tool for the study of protein microheterogeneity. In this study we have selected Gc-globulin and α_1-antitrypsin which are eluted in the pH intervals 4.5–4.0 and 4.3–3.8, respectively. The human phenotypes of Gc-globulin (vitamin D-binding protein) are classified as Gc 2-2, 1-1 and 2-1, showing one, two and three bands, respectively, in a pH range of about

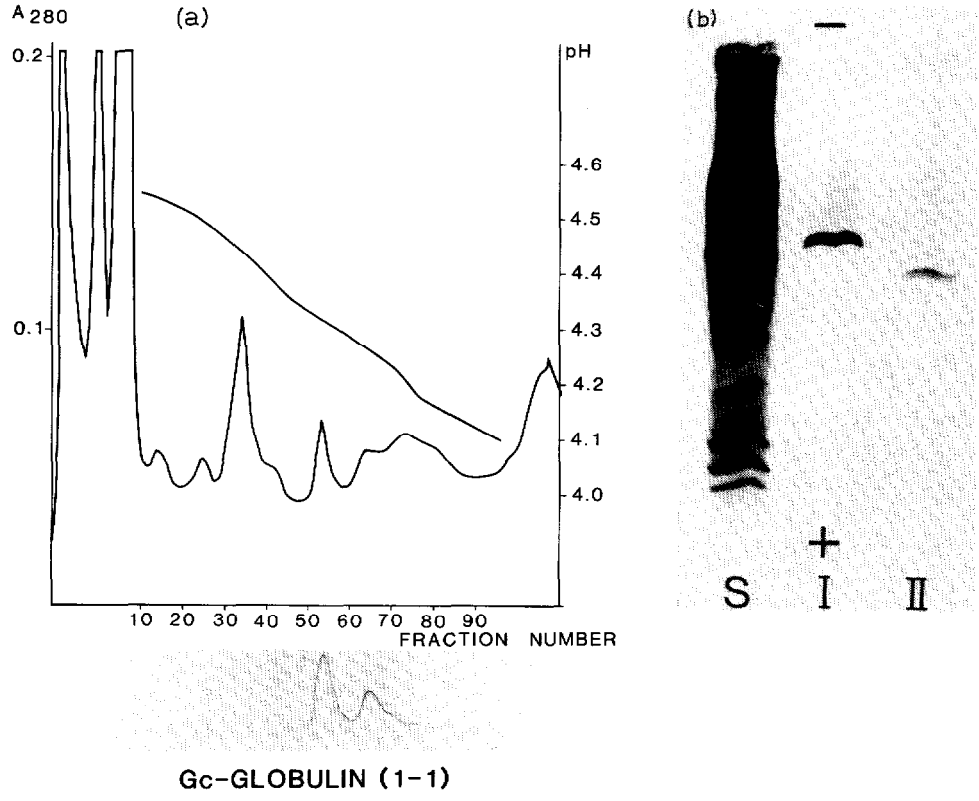


Fig. 3. FPLC chromatofocusing of serum (Gc-globulin type 1-1) in the pH interval 4.5–4.0, followed by fused rocket immunoelectrophoresis. Sample: 0.25 ml of albumin-depleted serum. Starting buffer: 25 m*M* methylpiperazine–HCl, pH 5.3. Eluent: Polybuffer 74 (diluted 1:20)–HCl, pH 4.2. Fractions of 0.3 ml were collected. Flow-rate: 1 ml/min. (a) Aliquots (6 μl) of the fractions were analysed by fused rocket immunoelectrophoresis using antiserum against Gc-globulin. (b) Fractions 51–53 (I) and 61–64 (II) were pooled and 20-μl aliquots of each pool were analysed by Agarose IEF as described in the Experimental section. S = Serum sample.

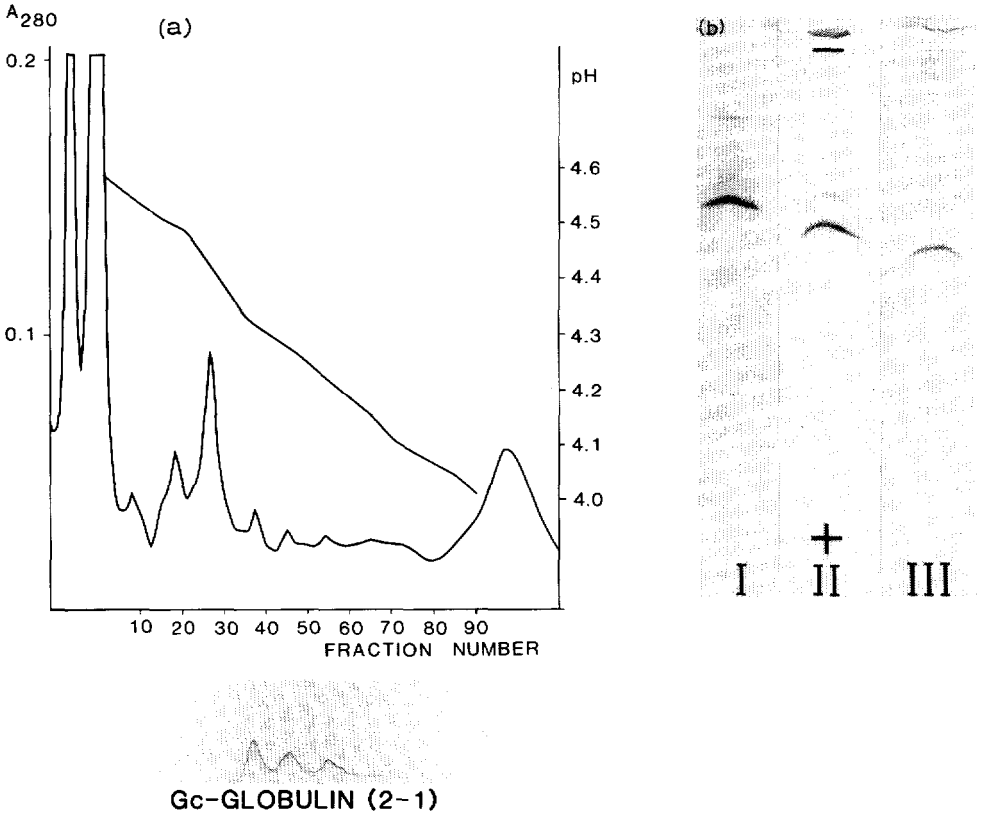


Fig. 4. FPLC chromatofocusing of serum (Gc-globulin type 2-1) in the pH interval 4.5–4.0, followed by fused rocket immunoelectrophoresis. For conditions see Fig. 3. (a) Aliquots (6 μl) of the fractions were analysed by fused rocket immunoelectrophoresis using antiserum against Gc-globulin. (b) Fractions 35–37 (I), 43–46 (II) and 52–55 (III) were pooled and 20-μl aliquots of each pool were analysed by Agarose IEF as described in the Experimental section.

4.95–5.1, when analysed by isoelectric focusing[10]. A number of patient sera were first screened by agarose isoelectric focusing-immunofixation[6] to identify the common Gc-globulin types. Figs. 3 and 4 show the results of chromatofocusing in the pH interval 4.5–4.0 of Gc-types 1-1 and 2-1 and subsequent analysis by fused rocket immunoelectrophoresis and agarose isoelectric focusing.

As pointed out earlier the elution pH of a component is not identical with the isoelectric pH. In the case of the Gc-globulin components, we found that the elution pH values were 4.32, 4.28 and 4.22 respectively (Figs. 3 and 4). These values are about 0.75 pH units lower than the isoelectric pH values of 5.10, 5.03 and 4.95 reported by others[10].

α_1-Antitrypsin is normally present in blood plasma as three charge-heterogeneous isoforms, with isoelectric points distributed over less than one pH unit. The pattern is further complicated by the heterogeneity of each isoform due to post-synthesis modification of the primary structure[11,12]. Figs. 5 and 6 show the α_1-antitrypsin profiles obtained for two different serum samples (M-type) separated by

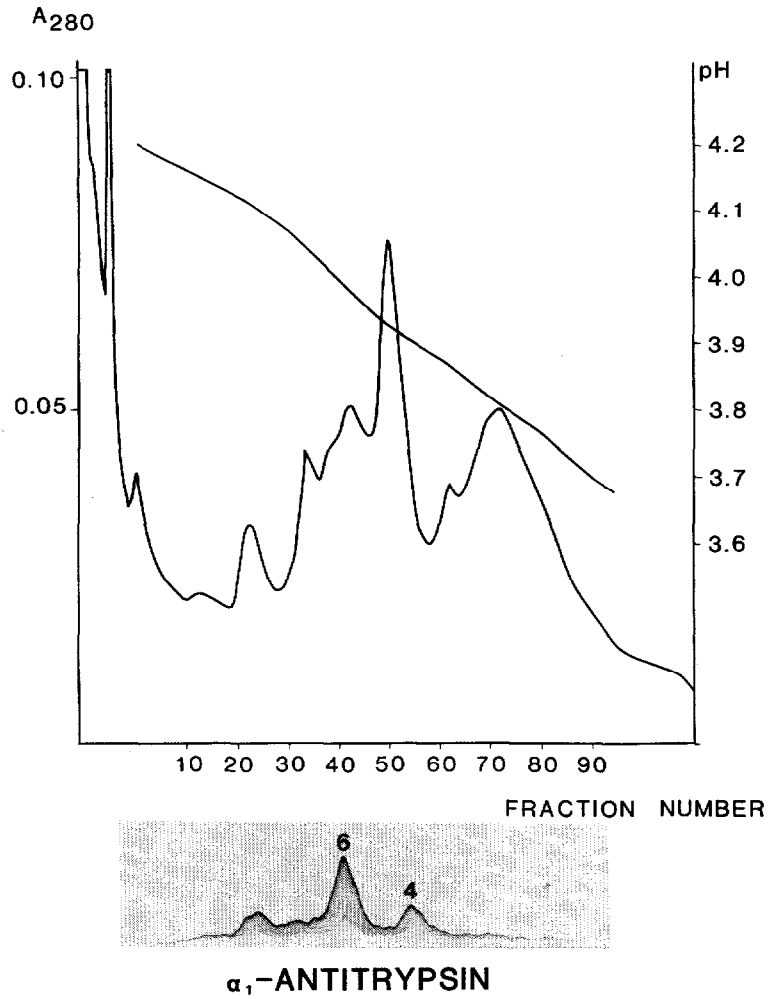


Fig. 5. FPLC chromatofocusing of serum in the pH interval 4.3–3.8, followed by fused rocket immunoelectrophoresis using antiserum against α_1-antitrypsin. Sample: 0.25 ml of albumin-depleted serum. Starting buffer: 25 m*M* methylpiperazine–HCl, pH 5.0. Eluent: Polybuffer 74 (diluted 1:16)–HCl, pH 4.0. Fractions of 0.3 ml were collected. Flow-rate: 1.0 ml/min. Aliquots (6 μl) of the fractions were analysed by fused rocket immunoelectrophoresis using antiserum against α_1-antitrypsin.

chromatofocusing in the pH interval 4.3–3.8. The main components have been tentatively assigned to the bands 6 and 4, in their order of elution, as described by Vaughan *et al.*[12].

Fig. 7 shows a separation of purified human α_1-antitrypsin to illustrate the extreme resolution that can be obtained by chromatofocusing in narrow pH intervals. To increase peak sharpness further and to counteract the buffer disproportionating effect discussed above, the zwitterionic substance taurin (2-aminoethanesulphonic acid) was added to the eluent[13]. Under these conditions the elution pH values of the α_1-antitrypsin components were about 0.3 pH units below their respective isoelectric

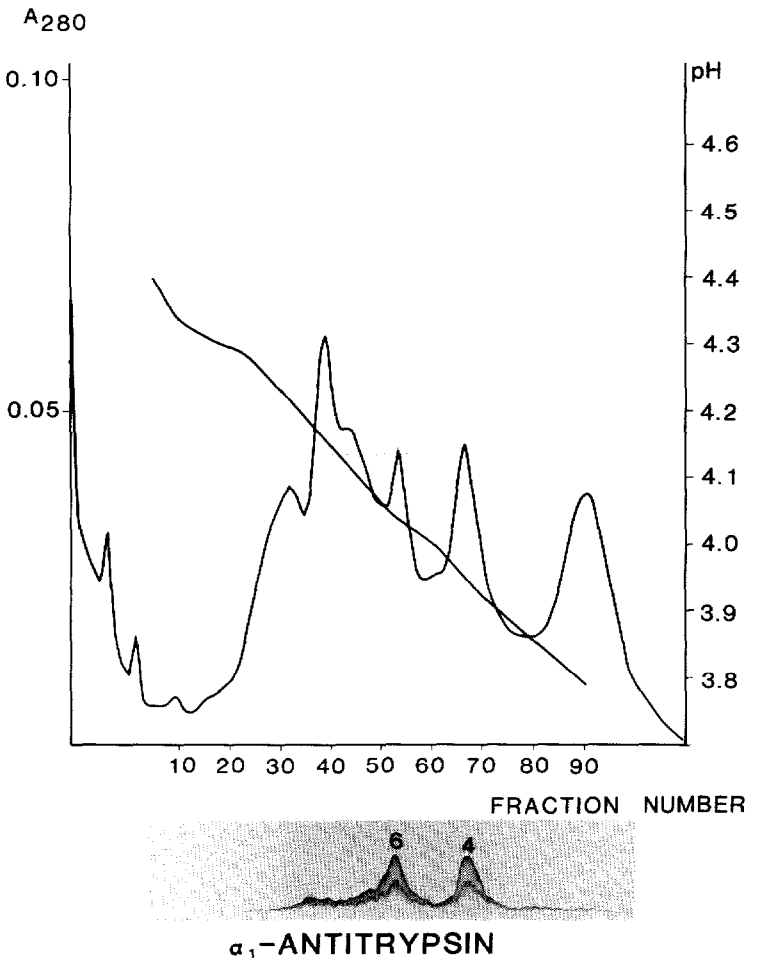


Fig. 6. FPLC chromatofocusing of serum in the pH interval 4.3–3.8, followed by fused rocket immunoelectrophoresis with antiserum against α_1-antitrypsin. All conditions as in Fig. 5.

points[14]. When taurin was not included (Figs. 5 and 6) the displacement was about 0.5 pH units. The peaks 6, 4 and 2 in Fig. 7 should then correspond to the main components of the isoforms I, II and III, the charge difference between successive peaks being one negative charge due to an increased sialic acid content[12]. In this chromatogram 95 fractions were collected over the range pH 4.3–3.8, giving an elution pH "resolution" of 0.005 pH units.

The high resolving power of chromatofocusing in combination with its preparative capacity makes chromatofocusing attractive as the first dimension in two-dimensional techniques for the resolution of complex protein mixtures. We have combined chromatofocusing with the analytical techniques of polyacrylamide gel electrophoresis and immunoelectrophoresis. However, preparative techniques such as gel filtration or affinity chromatography can also be used without elaborate sample treatment, since the separated material from the first dimension is in free solution.

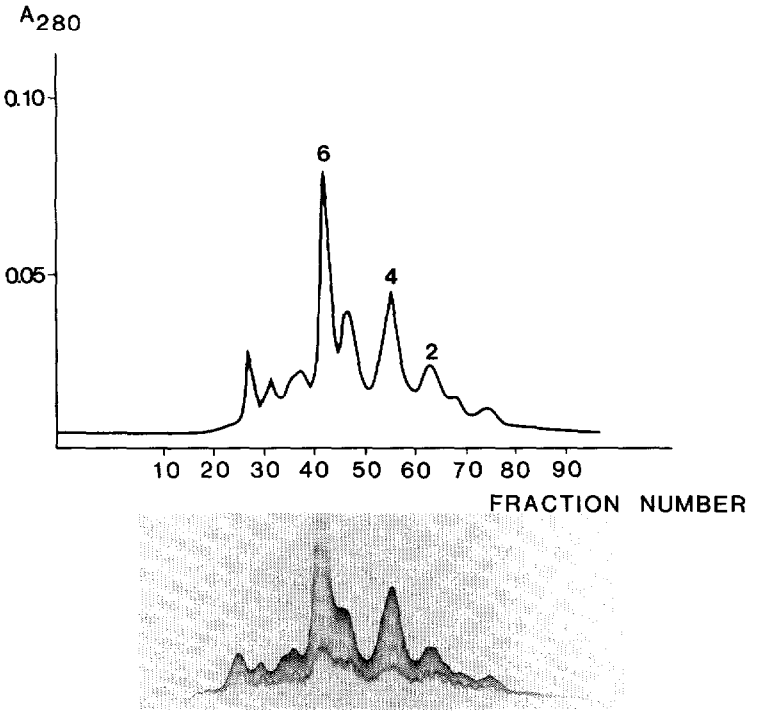


Fig. 7. FPLC chromatofocusing of purified human α_1-antitrypsin in the presence of taurin. Sample: 1.2 mg of human α_1-antitrypsin. Starting buffer: 25 m*M* methylpiperazine–HCl + 5% (w/v) taurin, pH 5.0. Eluent: Polybuffer 74 (diluted 1:16)–HCl, 5% (w/v) taurin, pH 4.0. Fractions of 0.3 ml were collected. Flow-rate: 1.0 ml/min. Aliquots (6 μl) of the fractions were analysed by fused rocket immunoelectrophoresis with antiserum against α_1-antitrypsin. The peaks were numbered as explained in the text.

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