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High-performance anion-exchange chromatographic study of desialylated human α_1-acid glycoprotein variants

Development of a fractionation method for the protein slow variants

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ABSTRACT

The three main desialylated variants (F1, S and A) of human α_1-acid glycoprotein (AAG), a serum acute-phase reactant, were analysed by high-performance anion-exchange chromatography in order to determine their optimum separation conditions. The analysis consisted of three steps, as follows: (1) A desialylated commercial AAG was separated into one "fast"- and one "slow"-migrating fraction by preparative isoelectrofocusing. The "fast" and "slow" fractions were shown to contain the F1 variant and a mixture of the S and A variants, respectively. (2) The pH titration curves of these two fractions were then measured by strong anion-exchange chromatography with several buffer systems of increasing pH. From the data obtained, it was not possible to select the optimum conditions to separate the "fast" variant F1 from the "slow" variants A and S. However, the S and A variants were shown to ionize very differently. (3) The specific fractionation of the S and A variants was therefore carried out by anion-exchange chromatography under operating conditions based on the data obtained from the study of their pH titration curves. This was performed both with the "slow"-migrating fraction obtained by preparative isoelectrofocusing of commercial AAG and with an AAG (containing only variants S and A) purified from an individual serum on immobilized Cibacron Blue F3G-A. Identification of the fractionated proteins was achieved by analytical isoelectrofocusing.

INTRODUCTION

α_1-Acid glycoprotein (AAG), or orosomucoid, is an acute-phase reactant whose serum concentration increases in association with inflammatory states (for a review, see ref. 1). Its physiological function is still unknown but it is thought to act as a transport protein for basic drugs such as antidepressant drugs, neuroleptics and β-blockers, and also plays a role in immunoregulation (for a review, see ref. 1).

It has been well documented that AAG, which has a high carbohydrate content and a large number of sialyl residues, is very heterogeneous, consisting of subpopulations of different size, charge [2] and lectin-binding behaviour [3].

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One extensively studied case of the heterogeneity of AAG is the existence, after desialylation of the protein, of three main variants, distinguished by their electrophoretic migration in analytical isoelectrofocusing (IEF) as one "fast" and two "slow" bands [4], corresponding to the F1 and to the S and A variants, respectively. These variants differ in that numerous amino acids are substituted in the peptide chain [5]. This microheterogeneity is genetically determined and, depending on the relative concentrations of the variants in desialylated serum, three main phenotypes are observed for AAG in a general population: F1 S/A, F1/A and S/A, with expected frequencies of 47.9, 35.1 and 16.4%, respectively [4].

Elucidation of this heterogeneity, especially in the light of the possibility of functional differences between AAG variants, has recently been attempted. Commercial human AAG containing the three variants was fractionated after its desialylation, into a "fast" (F-AAG) and a "slow" AAG (S-AAG) by a preparative IEF technique [6,7]. The F- and S-AAG were shown to contain the F1 and a mixture of the S and A variants, respectively. Comparison of their drug-binding properties showed that S-AAG exhibits a higher binding capacity ($n \cdot k$, where n is the number of binding sites and k is the intrinsic affinity constant) than F-AAG for most of the drugs tested [6,7]. It was also proposed that only one of the two S-AAG variants was responsible for the high-affinity binding site. However, it was impossible to determine which variant, S or A, is involved, as they cannot be fractionated electrophoretically without denaturation, and because commercial AAG is a mixture of the phenotypes and contains equal proportions of the A and S variants.

Using high-performance ion-exchange chromatography (HPIEC), we studied the pH titration curves of the AAG variants in order to optimize their separation by this method. The results enabled us to develop an HPIEC method for the fractionation of S-AAG into its two charge variants and this fractionation was compared with the electrophoretic heterogeneity of S-AAG as shown by analytical IEF. The HPIEC methodology was also applied to the fractionation of an AAG purified in the laboratory from an individual serum of phenotype S/A by chromatography on immobilized Cibacron Blue F3G-A [8].

EXPERIMENTAL

Materials

Human serum containing AAG with the S/A phenotype (S/A-AAG) was obtained from a healthy donor and was frozen until use.

Materials were obtained from the following sources: human AAG (from Cohn fraction VI), *Clostridium perfringens* neuraminidase type X, Norite A charcoal and 1,3-bis[tris(hydroxymethyl)methylamino]propane (Bis–Tris-propane) were from Sigma (St. Louis, MO, U.S.A.), bovine serum albumin (grade A) from Calbiochem (San Diego, CA, U.S.A.), Cibacron Blue F3G-A from Fluka (Buchs, Switzerland), Sephadex G-100, DEAE-Sephadex, Mono Q HR 5/5 prepacked column, gradient gels PAA 4/30, low-molecular-mass marker protein kit, immobiline (pK 4.6 and pK 9.3), Gelbond PAG films, acrylamide and N,N′-methylenebisacrylamide from Pharmacia–LKB (Uppsala, Sweden), Coomassie Brilliant Blue R 250, urea and 2-mercaptoethanol from Merck (Darmstadt, Germany) and YM 10 membrane filter and Centricon 10 microconcentrator from Amicon (Danvers, MA, U.S.A.). All other reagents were of grade A or analytical-reagent grade and were purchased from local suppliers.

Purification of the S/A-AAG on immobilized Cibacron Blue F3G-A

The covalent coupling of Cibacron Blue F3G-A to Sephadex G-100 was performed according to Böhme *et al.* [9], with 0.85 g of dye per gram of dry gel at 80°C, in order to obtain maximum dye substitution. The determination of the amount of dye covalently bound to the matrix was carried out according to Chambers [10]. The S/A-AAG was purified from the serum essentially by the one-step chromatographic procedure on immobilized Cibacron Blue F3G-A described by Birkenmeier and Kopperschläger [8] in 10 m*M* sodium phosphate buffer (pH 5.8) and at 4°C. The purified protein was concentrated on a YM 10 membrane filter and dialysed against deionized water.

Charcoal treatment and desialylation of AAG

Before use, the pure S/A-AAG preparation and the commercial AAG were delipidated by charcoal treatment at pH 3.0 according to Evenson and Deutsch [11] and then lyophilized. Both proteins were then desialylated with neuraminidase as described by Eap *et al.* [6], dialysed against deionized water and lyophilized.

Prior to analytical IEF, the human whole serum used in the chromatographic procedure on immobilized Cibacron Blue was desialylated with neuraminidase as described by Eap *et al.* [4].

Spectrophotometry and protein assay

Absorbance determinations or spectral scans were performed on a Beckman Model DU-3 single-path recording spectrophotometer. Total protein concentrations were determined by the method of Lowry *et al.* [12] with bovine serum albumin (grade A) as a standard. The specific assay of AAG was carried out by an immunonephelometric method using a Beckman assay kit and instrument.

Electrophoretic techniques

The purity of the S/A-AAG preparation was checked by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) [13] using a Pharmacia GE 2-4/LS vertical gel electrophoresis apparatus. Analytical and preparative IEF were performed using an LKB 2117 Multiphor II electrophoresis apparatus.

Separation of the S- and F-AAG from the desialylated commercial AAG was performed by a preparative IEF technique in an immobilized pH 4.4–5.4 polyacrylamide gel gradient [6,7]. The separated F- and S-AAG were detected in strips of the gel by trichloroacetic acid fixation and Coomassie Brilliant Blue staining. The protein in each band was recovered from the remainder of the gel by electrophoretic elution into DEAE-Sephadex. After recovery from Sephadex, the two fractions were dialysed extensively against deionized water and lyophilized.

Analytical IEF of the desialylated AAG variants and AAG phenotyping of the desialylated whole serum were performed essentially as described by Eap and Baumann [14] in an immobilized pH 4.4–5.4 polyacrylamide gel gradient with 8 *M* urea and 2% (v/v) 2-mercaptoethanol. Detection of the variants was made by immunoblotting with two antibody steps [14] or by staining with Coomassie Brilliant Blue. The analytical IEF of the desialylated proteins also permitted the determination of the extent of desialylation. The relative proportion of each protein band in the gels was determined by scanning with an LKB 2202 Ultroscan laser densitometer.

High-performance ion-exchange chromatography (HPIEC)

HPIEC was performed on a Waters Assoc. liquid chromatography system equipped with two Model 6000-A pumps and a Model 660 solvent programmer capable of generating a gradient or step elution profile. Sample injections were carried out with a Waters Assoc. Model U6K injector and chromatograms were recorded by monitoring the absorbance of the eluent at 280 nm using a single-path UV monitor (Cecil Instruments, Cambridge, U.K.) fitted with a 10-mm path-length flow cell. The absorbance units full-scale (a.u.f.s.) were set between 0.02 and 0.5 as appropriate. Fractions were collected with a Gilson Model 203 fraction collector. The HPIEC system was operated at room temperature.

HPIEC of the desialylated AAGs was carried out using a Pharmacia Mono Q strong anion-exchange column (50 × 5 mm I.D.; 10 μm particle size).

In initial experiments, the pH titration curves of the AAG variants were determined using buffer systems of pH 7.0–10.0 (adjusted with 1 *M* hydrochloric acid), with the starting and final buffers containing 6.25 m*M* Bis–Tris-propane and with 0.15 or 0.35 *M* sodium chloride in the final buffer. The F- and S-AAG, obtained by the preparative IEF technique, were chromatographed with each buffer system.

Fractionation of the S-AAG and of the S/A-AAG preparation was carried out on the same anion-exchange column. The starting and final buffers were 6.25 m*M* Bis–Tris-propane chloride adjusted to pH 9.75 with 1 *M* hydrochloric acid, with the final buffer containing 0.15 *M* sodium chloride. A preprogrammed linear gradient was used for the chromatography and the appropriate peak fractions were collected, concentrated and re-equilibrated in deionized water or start buffer, using Centricon 10 microconcentrators, prior to their study by analytical IEF or their rechromatography.

RESULTS AND DISCUSSION

Purification of AAG from an individual serum on immobilized Cibacron Blue F3G-A

Measurement of the absorption spectrum of the Cibacron Blue F3G-A used showed a broad peak at 595 630 nm and the calculated molar absorptivity was 10.3 $l \cdot mmol^{-1}\ cm^{-1}$. This was compared with a value of 13.6 $l \cdot mmol^{-1}\ cm^{-1}$ obtained by Thompson and Stellwagen [15] using especially purified Cibacron Blue F3G-A, and a purity of 75.7% was calculated for the dye sample used in our experiments. This value was used to correct the values obtained for dye release in subsequent experiments.

Under the experimental coupling conditions used, a maximum of 150 μg of dye covalently bound per milligram of dry Cibacron Blue–Sephadex was found by hydrolysis of the substituted gel in 6 *M* hydrochloric acid [10]. This loading density is less than that used by Birkenmeier and Kopperschläger [8] in their chromatographic purification procedure for human AAG. Nevertheless, when the individual serum, dialysed against 10 m*M* sodium phosphate buffer (pH 5.8), was applied to the Cibacron Blue–Sephadex column, the only constituent detected in the breakthrough fraction was AAG. Fig. 1 shows the elution pattern obtained by chromatography of the individual serum and the presence of two peaks can be observed, a major peak and a minor "retarded" peak. Each peak was separately concentrated on an Amicon YM 10 membrane filter. The total protein concentration and the specific AAG concentration were determined in each peak and identical results were obtained with

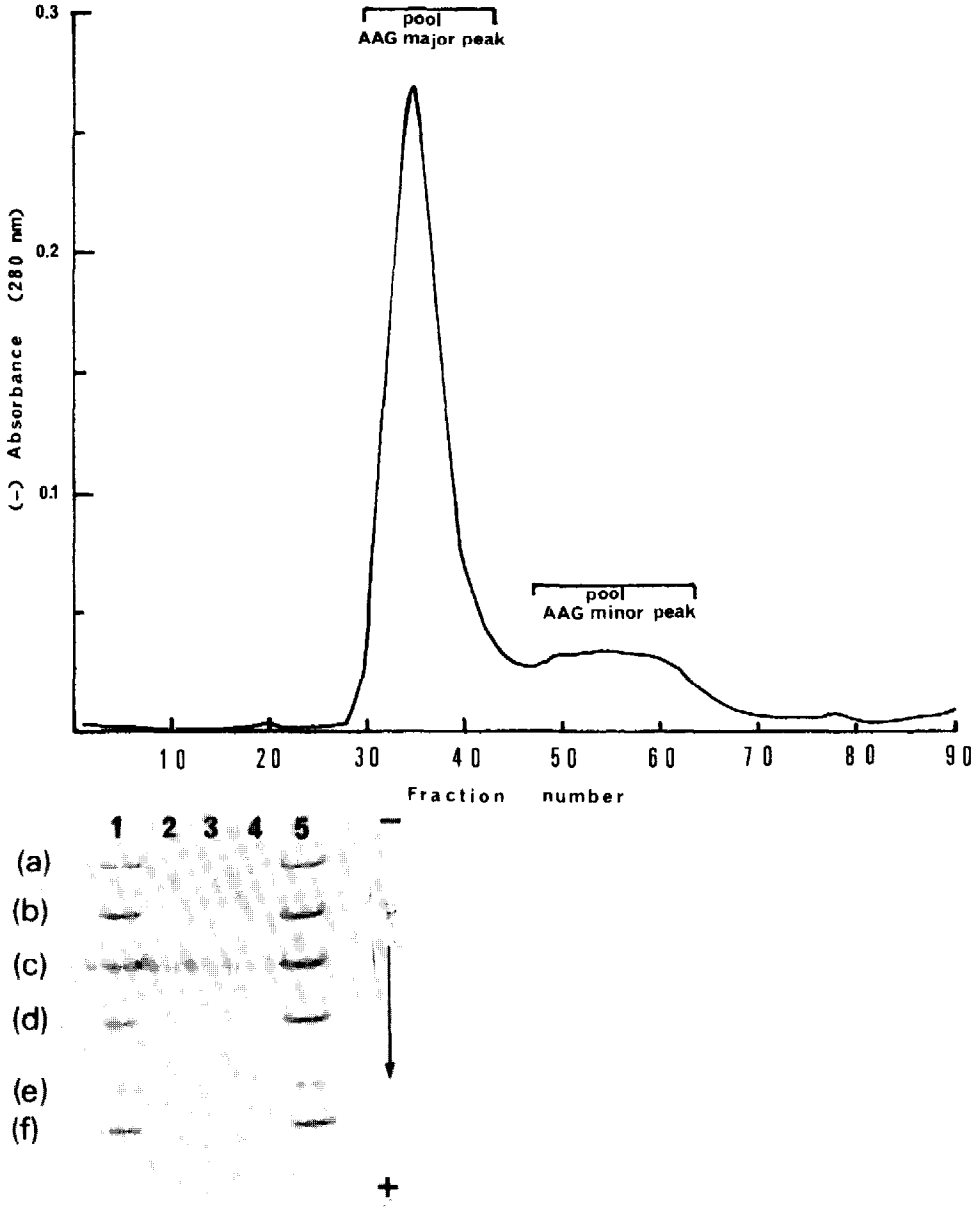

Fig. 1. Elution of AAG by chromatography of a single human serum sample on Cibacron Blue F3G-A-Sephadex G-100 gel at pH 5.8. The dye substitution in the gel was 150 μg of dye per mg of Sephadex. A 50-ml volume of the human serum was dialysed against 10 mM sodium phosphate buffer (pH 5.8) and applied to the gel column (50 × 5 cm I.D.) equilibrated with the same buffer at 4 C. The unbound protein was eluted in 10 mM sodium phosphate buffer (pH 5.8). The flow-rate was 50 ml/h and fractions of 6.8 ml were collected. The absorbance of each fraction was determined spectrophotometrically at 280 nm. The major and minor peaks of AAG were pooled separately as indicated and concentrated prior to their subjection to SDS-polyacrylamide gel electrophoresis. Inset: SDS-PAGE of the pooled fraction of AAG. Lanes: 1 and 5 = molecular mass (dalton) markers [(a) phosphorylase b (94 000), (b) albumin (67 000), (c) ovalbumin (43 000), (d) carbonic anhydrase (30 000), (e) trypsin inhibitor (20 000) and (f) α-lactalbumin (14 000)]; 2 = standard commercial AAG (5 μg); 3 = AAG major peak (5 μg); 4 = AAG minor peak (5 μg).

both assays. The major and minor peaks were shown to contain 88 and 12%, respectively, of the total amount of AAG purified on immobilized Cibacron Blue F3G-A. SDS-PAGE showed that the two peaks seemed to be composed of at least 90% pure AAG because, in both peaks, a single protein band was detected (Fig. 1, inset) and a molecular mass of 44 000 dalton was calculated, identical with that of a standard commercial AAG.

A similar fractionation of "native" AAG into more than one peak has already been described by Gianazza and Arnaud [16] during the chromatography of plasma proteins on commercially available immobilized Cibacron Blue (Affi-gel Blue). The origin of the existence of more than one peak for AAG in dye-affinity chromatography could be due to differences in the sugar chains of the protein sub-populations (for instance, the existence of partially desialylated "old" forms of AAG, physiologically present in the serum). This minor peak requires further study.

The major AAG peak corresponded to 70–75% of the total serum AAG as determined by immunospecific assays of the serum and the chromatographic peak. Hence the chromatography of the serum (50 ml) yielded 18 mg of AAG from a total amount of 25 mg of AAG.

This purified AAG (major peak) was dialysed against deionized water and delipidated. It was then desialylated with neuraminidase and subjected to analytical IEF on an immobilized pH 4.4–5.4 polyacrylamide gel gradient with 8 *M* urea and 2% (v/v) mercaptoethanol. The S/A-AAG was found to contain only the "slow" variants A and S (Fig. 2). This result agreed with the AAG phenotyping of the serum used (not shown). Several faint, more anodic bands were also observed after staining with Coomassie Brilliant Blue (Fig. 2), indicating the presence of a small proportion (less than 5%) of incompletely desialylated protein. The relative proportions of each variant in the S/A-AAG were 63 and 37% for the S and A variants, respectively, as found by scanning. These values agree with the relative proportions of the S and A variants in desialylated whole serum (60 and 40%, respectively).

Separation of S-AAG and F-AAG from desialylated commercial AAG by preparative IEF

The preparative IEF of commercial AAG, previously desialylated with neuraminidase, on an immobilized pH 4.4–5.4 polyacrylamide gel gradient allowed the separation of a "slow" S-AAG from a "fast" F-AAG [6,7]. The respective electrophoretic patterns of the F- and S-AAG, and that of the desialylated unfractionated commercial AAG, obtained by analytical IEF in the presence of 8 *M* urea and 2% (v/v) 2-mercaptoethanol are shown in Fig. 2. It can be seen that, whereas the F-AAG is homogeneous and contains only the F1 variant, the S-AAG is heterogeneous and is composed of the two A and S variants. The relative proportions of the F1, S and A variants in the commercial AAG were 32, 38 and 32%, respectively, as found by scanning. The relative proportions of the "slow" S and A variants were 50% each in the S-AAG and are in accordance with those found with the unfractionated protein.

Chromatographic pH titration curves of the desialylated AAG variants

In the ion-exchange chromatography of proteins, there is a correlation between the ionic strength needed for elution and the net charge of the protein. As the protein charge, and consequently the ionic strength at which it will be eluted, varies with pH,

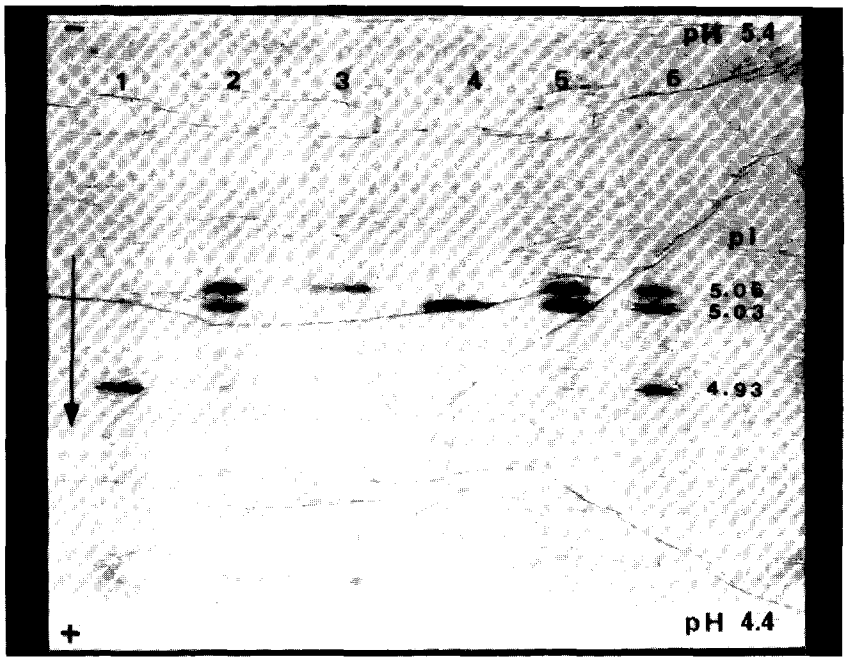

Fig. 2. Electrophoretic patterns of the different desialylated AAG samples on immobilized pH 4.4–5.4 polyacrylamide (4.85%, w/v) gel gradient with 8 *M* urea and 2% (v/v) 2-mercaptoethanol. Detection of the desialylated variants in the gel after fixation of the proteins in 11.5% (w/v) trichloroacetic acid and 3.5% (w/v) sulphosalicylic acid and staining with a 0.115% (w/v) Coomassie Brilliant Blue R 250 solution. Tracks: 1 = F-AAG isolated from desialylated commercial AAG by preparative IEF (20 μg); 2 = S-AAG isolated from desialylated commercial AAG by preparative IEF (20 μg); 3 = peak 2 (variant A) fractionated by HPIEC from S/A-AAG or S-AAG (20 μg); 4 = peak 1 (variant S) fractionated by HPIEC from S/A-AAG or S-AAG (20 μg); 5 = S/A-AAG purified on immobilized Cibacron Blue F3G-A (20 μg); 6 = desialylated commercial AAG (20 μg). The pH scale and the approximate isoionic point (p*I*) value for each variant are indicated.

titration curves are of great interest for predicting the elution pattern over a range of pH. The titration curves of F- and S-AAG were therefore determined separately by strong anion-exchange chromatography with buffer systems of different pH values, ranging from 7.0 to 10.0, to find the pH at which their titration curves are furthest apart and thus their separation will be optimum. The resolution of each buffer system was compared by plotting pH *versus* the amount of sodium chloride needed for elution (Fig. 3). The amounts of sodium chloride were deduced from the elution volume for the major peaks of interest. It can be seen that all the desialylated variants had a low negative charge because, even at pH 10, they eluted at a relatively low ionic strength. S-AAG began to be fractionated at pH 8.5 and, at higher pH, two well differentiated pH titration curves were observed, the lower curve corresponding to the titration of the A variant and the upper curve to that of the S variant.

The pH titration curve of F-AAG shows that, at lower pH, the "fast" variant F1 seems to be slightly more negatively charged than the "slow" variants. This result

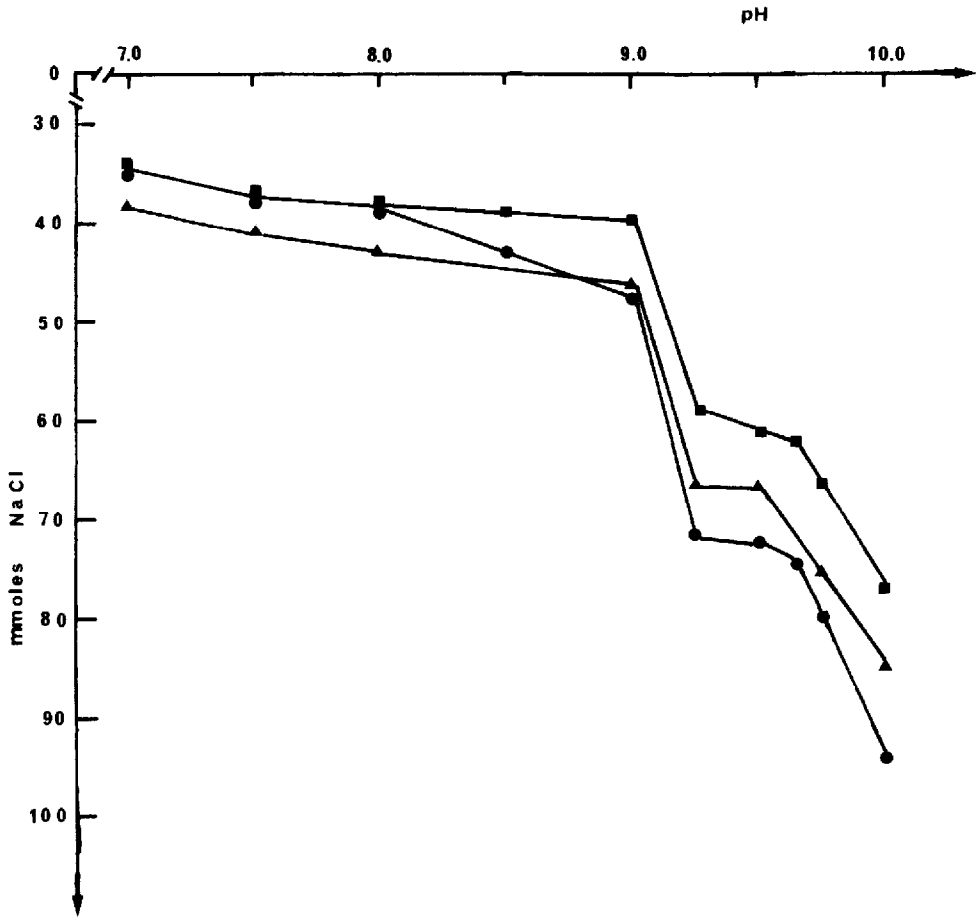

Fig. 3. pH titration curves for the desialylated AAG variants. The pH titration curves of the variants (▲) F1, (■) S and (●) A were determined in 6.25 m*M* Bis Tris-propane (starting and final) buffer systems, with different pH values ranging from 7.0 to 10.0, adjusted with 1 *M* hydrochloric acid. The final buffer contained 0.15 or 0.35 *M* sodium chloride. The F- and S-AAG (100–200 μg), purified by preparative IEF, were run on a prepacked Mono Q HR 5/5 column with a 20-min gradient time. The flow-rate was 1.0 ml/min and the a.u.f.s. was set to 0.05–0.2. The pH was plotted *versus* millimoles of sodium chloride, which were deduced from the elution volume of each major peak of interest.

agrees with the more anodic migration of the F1 variant in IEF electrophoresis. It can also be seen that, over the pH range considered, the titration curve of the F1 variant parallels that of the S variant. Consequently, at pH 9.0, the titration curve of the F1 variant falls between the upper (S) and the lower (A) titration curves.

Analysis of the pH titration curves of the AAG variants showed that these curves present different inflexion points. Three different inflexion points can be observed in Fig. 3, at pH 8–8.5, 9 and 9.5–9.75. The sharp drops at pH 9 and pH 9.5–9.75 are similar for the three titration curves, indicating that the AAG variants may not differ

greatly in their contents of amino acids ionizing at these pH values. However, the inflexion between pH 8 and 8.5 with variant A was not found with the F1 and S variants. The pH at which this change occurs could correspond to the ionization of a cysteine residue, present in variant A but not in variants F1 and S.

These data agree with the differences between the primary structures of the variants. Human AAG is encoded by two different genes [17], thus explaining the multiple amino acid substitutions found in the primary sequence of AAG [5]. Genetic studies at the protein level in population [4] have shown that variants F1 and S are encoded by two different alleles of the same gene, and would differ only by few (five) amino acids, thus explaining their very similar pH titration curves. Variant A is encoded by the other gene and would differ from the other two variants by more amino acids (at least 22). Examination of the possible amino acid substitutions described for AAG [5] shows that only eight of them would be significant from an ionization point of view and, as most of these involve arginine residues which would not be negatively charged at pH values lower than 10, it becomes obvious why the pH titration curves of the three variants are similar. Given these data, it has not been possible to find optimum conditions for the separation of the "fast" F1 variant from the "slow" S and A variants by strong anion-exchange chromatography. Nevertheless, the specific change in the pH titration curve of the A variant between pH 8 and 8.5 could correspond to the titration of the lone cysteine residue at position 149, which could be substituted by arginine residues in the F1 and S variant molecules. Therefore, the major difference between the titration curves for the S and A variants could be used to optimize their fractionation.

Fractionation of S-AAG and of the pure S/A-AAG preparation

We applied the resolving power of HPIEC to the resolution of S-AAG and S/A-AAG variants. It was found that fractionation of the two proteins at pH 9.75 on the strong anion-exchange column resolves two major peaks, 1 and 2, corresponding to the "slow" variants S and A (Fig. 4). This pH was used, rather than pH 10, because the proteins lost structural integrity at pH 10.5.

Integration showed that these peaks comprised *ca.* 50% each for the total S-AAG and 62.5 and 37.5%, respectively, for the S/A-AAG. The relative proportion of peak 2 in S/A-AAG is less than that in S-AAG. This seems to be due to the fact that, whereas S/A-AAG was purified from an individual serum containing a genetically determined proportion of each variant, S-AAG was prepared from a standard commercial protein which corresponds to a mixture of AAGs of different phenotypes.

The elution diagram also showed the presence of a minor "retarded" peak, which seems to correspond to an incompletely desialylated protein (faint anodic band in Fig. 2, track 3), thus explaining its delayed elution from the anion-exchange column.

Peaks 1 and 2 were isolated and rechromatography showed that they are genuine peaks with different elution volumes (Fig. 5). Characterization of peaks 1 and 2 by analytical IEF (Fig. 2) shows they are essentially pure: peak 1 comigrates with the S variant and peak 2 with the A variant. The elution volumes needed to elute the variant S in peak 1 and the variant A in peak 2 are in accordance with their respective pH titration curves.

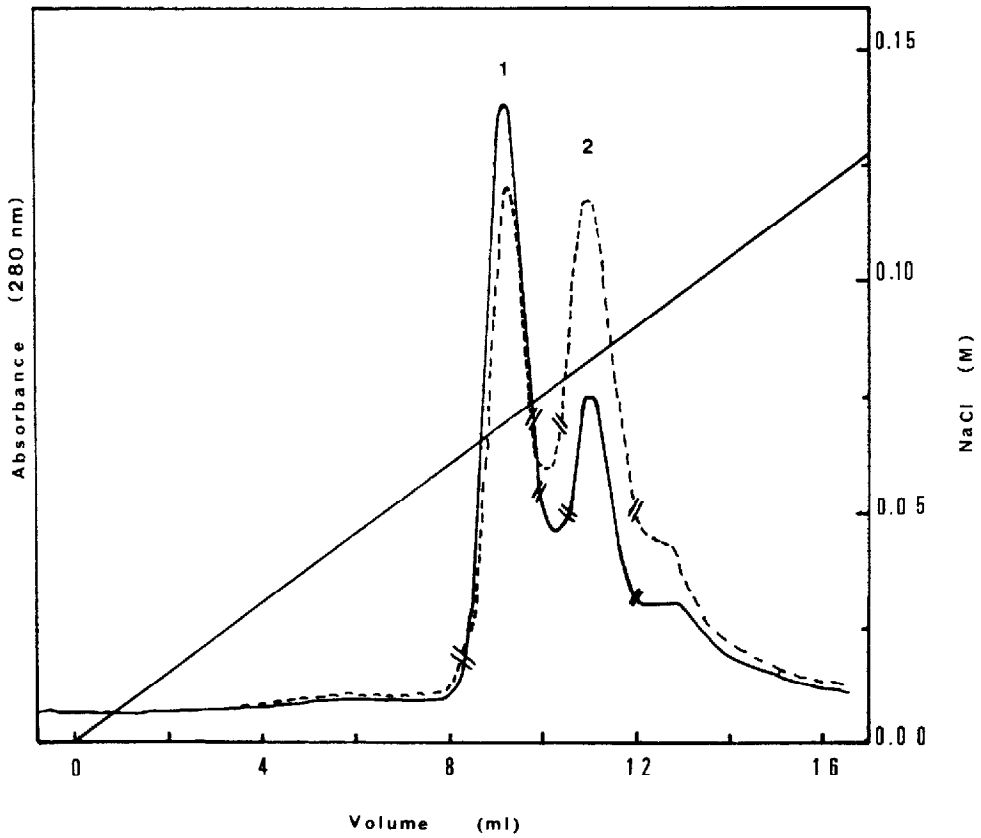

Fig. 4. HPIEC fractionation of S- or S,A-AAG on a prepacked Mono Q HR 5/5 column. S-AAG (dashed line) was isolated by preparative IEF of commercial AAG and S,A-AAG (solid line) was purified from the individual serum by chromatography on immobilized Cibacron Blue F3G-A. The starting buffer was 6.25 m*M* Bis–Tris-propane (pH 9.75) and final buffer was starting buffer containing 0.15 *M* sodium chloride; flow-rate, 1.0 ml/min; a.u.f.s., 0.5. Peaks 1 and 2 were collected as indicated by hatch marks in the elution profile.

CONCLUSION

This method allows the simple and rapid purification of large amounts of the "slow" variants of desialylated AAG. As these variants appear to be the more interesting with respect to drug binding [6,7], this purification method would greatly aid the study of their individual drug-binding properties.

Depending on the phenotype (F1,A or S,A or F1 S/A) of AAG preparations from individuals, the HPIEC method described here could be used to separate the S from the A variant and the preparative IEF technique could be used to separate the F1 from the A variant, but both techniques are required for the F1 S/A phenotype and commercial mixtures.

It was also found that the one-step chromatographic procedure on immobilized

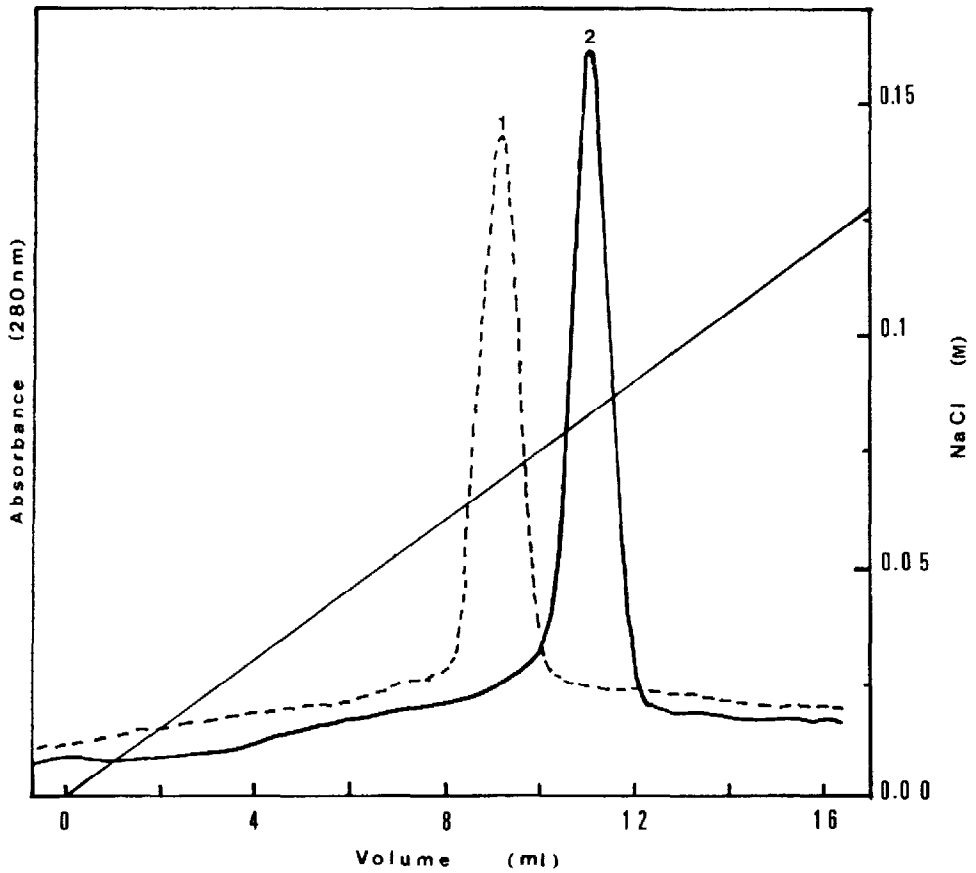

Fig. 5. HPIEC re-injection of peaks 1 (dashed line) and 2 (solid line) isolated from S/A-AAG in Fig. 4. Starting and final buffers and flow-rate as in Fig. 4; a.u.f.s., 0.1 and 0.05 for peaks 1 and 2, respectively. Prior to their injection, the proteins were re-equilibrated in starting buffer on a Centricon 10 microconcentrator.

Cibacron Blue F3G-A [8] is a valuable tool for purifying a "native" AAG with high yield from an individual serum. This procedure could be useful for comparing the drug-binding properties of individual "native" proteins corresponding to the different AAG phenotypes.

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