ELSEVIER

Journal of Chromatography B, 678 (1996) 1–14

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Two-step chromatographic purification of human plasma α_1-acid glycoprotein
Its application to the purification of rare phenotype samples of the protein and their study by chromatography on immobilized metal chelate affinity adsorbent

Françoise Hervé[a,*], Marie-Claude Millot[b], Chin B. Eap[c], Jean-Claude Duché[a], Jean-Paul Tillement[a]

[a]*Service Hospitalo-Universitaire de Pharmacologie de Paris XII, Centre Hospitalier Intercommunal de Créteil, 40 Avenue de Verdun, 94010 Créteil Cédex, France*

[b]*Laboratoire de Physico-Chimie des Biopolymères, Université Paris XII, Avenue du Général de Gaulle, 94010 Créteil Cédex, France*

[c]*Laboratoire de Biochimie, Clinique Psychiatrique Universitaire de Lausanne, Hôpital de Cery, CH-1008 Prilly-Lausanne, Switzerland*

Abstract

α_1-Acid glycoprotein (AAG) or orosomucoid was purified to homogeneity from human plasma by a separate two-step method using chromatography on immobilized Cibacron Blue F3G-A to cross-linked agarose and chromatography on hydroxyapatite. The conditions for the pre-purification of AAG by chromatography on immobilized Cibacron Blue F3G-A were first optimized using different buffer systems with different pH values. The overall yield of the combined techniques was 80% and ca. 12 mg of AAG were purified from an initial total amount of ca. 15 mg in a ca. 40 ml sample of human plasma. This method was applied to the purification of AAG samples corresponding to the three main phenotypes of the protein (F1*S/A, F1/A and S/A), from individual human plasma previously phenotyped for AAG. A study by isoelectric focusing with carrier ampholytes showed that the microheterogeneity of the purified F1*S/A, F1/A and S/A AAG samples was similar to that of AAG in the corresponding plasma, thus suggesting that no apparent desialylation of the glycoprotein occurred during the purification steps. This method was also applied to the purification of AAG samples corresponding to rare phenotypes of the protein (F1/A*AD, S/A*X_0 and F1/A*C1) and the interactions of these variants with immobilized copper(II) ions were then studied at pH 7, by chromatography on an iminodiacetate Sepharose-Cu(II) gel. It was found that the different variants encoded by the first of the two genes coding for AAG in humans (i.e. the F1 and S variants) interacted non-specifically with the immobilized ligand, whereas those encoded by the second gene of AAG (i.e. the A, AD, X_0 and C1 variants) strongly bound to immobilized Cu(II) ions. These results suggested that chromatography on an immobilized affinity Cu(II) adsorbent could be helpful to distinguish between the respective products of the two highly polymorphic genes which code for human AAG.

Keywords: α_1-Acid glycoprotein; Proteins

*Corresponding author.

0378-4347/96/$15.00
SSDI 0378-4347(95)00366-5

1. Introduction

Human α_1-acid glycoprotein (AAG; orosomucoid) is a heavily glycosylated component of blood plasma, normally present at concentrations of 0.36–1.46 mg/ml [1]. A high degree of heterogeneity is found both in the carbohydrate moiety and in the amino acid composition of AAG [2]. The former heterogeneity is mainly responsible for the microheterogeneity of native (sialylated) AAG demonstrated after isoelectric focusing (IEF) in an acidic pH range [3,4], and different electrophoretic patterns of either 5, 6, 7, 8 or 9 protein bands can be observed. The other heterogeneity yields the genetic variants of AAG [2]. These are revealed when the desialylated form of the protein is analyzed in IEF [5,6]. Three main variants, designated F1, S and A, are observed for AAG and identified on the basis of their different electrophoretic mobilities. This polymorphism is genetically determined [2] and three main phenotypes are observed for AAG in the human population, F1*S/A, F1/A and S/A, depending on the presence of two or three of the F1, S and A variants in plasma. Moreover, many other rare variants have been described for AAG [6].

Although its exact biological function is still unknown, AAG has been identified as the main plasma transport protein for basic drugs and has also been reported to play a role in immuno-regulation (for a review, see [7]). Evidence has been found that the structural heterogeneity of AAG is related to the existence of different functional forms of the protein. Functional diversity in the processes of immunomodulation has been reported for individual glycoforms of AAG [8]. Besides, we have recently demonstrated drug-binding heterogeneity between the three main genetic variants of AAG [9,10]. To assist us in this study, we have developed separate methodologies to purify AAG samples with different phenotypes from individual human plasma [11], and to fractionate the AAG variants [12–14]. The purification of AAG was performed by a one-step-chromatographic method on immobilized Cibacron Blue F3G-A to Sephadex G-100, and its fractionation into variants by chromatography on metal chelate affinity adsorbent (IMAC). However, the method used to purify AAG from plasma was not satisfactory in terms of easiness: the Cibacron-Blue-Sephadex gel must be synthesized under definite coupling conditions [15]; also, our experience was that the gel column could not be perfectly regenerated at the end of each cycle, rendering necessary the synthesis of a new gel for each AAG individual purification. We therefore developed another method to purify AAG, using Affi-Gel Blue (cross-linked agarose gel with covalently coupled Cibacron Blue F3G-A), a commercially available gel which can be perfectly regenerated [16]. We first studied the chromatography of human plasma on Affi-Gel Blue with different buffer systems, in order to optimize the pre-purification of AAG by this method. The results helped in the development of a separate two-step purification method of AAG, using Affi-Gel Blue chromatography of plasma as the first step for AAG fractionation, and chromatography on hydroxyapatite as the second step. The method thus developed was applied to the isolation of AAG samples corresponding to the three main phenotypes and also to rare phenotypes of the protein. In addition, the retention behaviour of the rare AAG phenotype samples on an immobilized copper(II) affinity adsorbent was investigated after their isolation from plasma.

2. Experimental

2.1. Materials

Blood samples were obtained from healthy subjects and collected into glass tubes containing sodium citrate. Blood was centrifuged immediately at 500 *g* for 20 min and the plasma fractions were frozen at −20°C until use. Selection of the individual plasma containing AAG with the three main phenotypes (F1*S/A, F1/A and S/A) was performed after AAG phenotyping, as described by Eap and Baumann [5]. The rare AAG phenotypes investigated (S/A^*X_0, F1/A*C1 and F1/A*AD) had previously been identified in genetic surveys [6,17].

Human AAG (from Cohn fraction VI), human α_1-antitrypsin, copper(II) chloride (ACS reagent) and urea ultrapure were from Sigma (St. Louis, MO, USA). *Clostridium perfringens* neuraminidase was from Boehringer (Mannheim, Germany). Immobiline

(pK_a 4.6 and 9.3), Ampholine carrier ampholytes (in the pH range 2.5–4.5), acrylamide, Gel Bond PAG films, gradient gels PAA 4/30, low molecular mass marker protein kit, columns PD 10 Sephadex G-25 M and chelating Sepharose Fast Flow (45–165 μm mean particle size, 22–30 μmol Cu^{2+} per ml gel) were from Pharmacia LKB (Uppsala, Sweden). Affi-Gel Blue gel (150–300 μm mean particle size; degree of substitution 5 μmol Cibacron Blue F3G-A per ml gel; albumin capacity 11 mg per ml gel), Biogel HT hydroxylapatite for chromatography (Control 42560 B) and Econocolumns were from Bio-Rad Laboratories (Richmond, CA, USA). Agarose A37 was from Industrie Biologique Française (Villeneuve-la-Garenne, France). N,N′-methylenebisacrylamide, ammonium persulphate, N,N,N′,N′-tetramethylenediamine, Coomassie Brilliant Blue R-250, 2-mercaptoethanol and imidazole were obtained from Merck (Darmstadt, Germany) and YM 10 membrane filters from Amicon (Danvers, MA, USA). All other reagents were of grade A or analytical-reagent grade and were purchased from local suppliers.

2.2. Column chromatography on Affi-Gel Blue and on hydroxyapatite

Chromatography of human plasma on Affi-Gel Blue was studied at 4°C using different sodium phosphate and acetate buffer systems in the pH range 5.8–5.0. An Affi-Gel Blue column (2.5 cm I.D., bed volume 80 ml) was equilibrated with the appropriate buffer for each pH value. In order to make possible a direct comparison of the results, a similar volume (12 ml) of the same human plasma was first dialyzed for 24 h at 4°C against each buffer system with several buffer changes, and then, a 10 ml portion of each dialyzed plasma was chromatographed with the appropriate buffer (the other 2 ml of plasma were used for protein assays and electrophoretic analysis). The dialyzed plasma was applied to the column at a flow-rate of 25 ml/h and fractions of 2 ml were collected. Elution of the non-bound plasma fraction was performed by application of several volumes of the equilibration buffer and was followed by spectrophotometry at 280 nm. At the end of each cycle, the Affi-Gel Blue was "stripped" of bound proteins by washing first with 2 *M* NaCl and then with 0.5 *M* potassium thiocyanate in the chromatographic buffer [16], before re-equilibration in the next buffer system.

After identification of the non-bound plasma proteins from Affi-Gel Blue chromatography, commercial preparations of these proteins were used to optimize their separation by chromatography on hydroxyapatite. The commercial proteins (ca. 4 mg each) were dissolved in 10 m*M* sodium phosphate, pH 7.0, containing 0.1 *M* NaCl, and separately applied to a Bio-Gel HT hydroxylapatite column (1.5 cm I.D., bed volume 20 ml) equilibrated with the same buffer. The column was run at room temperature (20°C) and at a flow-rate of 40 ml/h. Fractions of 2.7 ml were collected. After two bed volumes of the equilibration buffer, the elution of each commercial protein was carried out stepwise by washing the gel successively with 20, 30 and 40 m*M* sodium phosphate buffers, pH 7.0, containing 0.1 *M* NaCl. At the end of the cycle, a buffer (pH 7.0) consisting of 400 m*M* sodium phosphate, containing 0.5 *M* NaCl, was applied to regenerate the column.

2.3. Affinity chromatography of native (sialylated) AAG on immobilized copper(II) affinity adsorbent

This method has been described in detail previously [12,13], and in this report it is outlined only briefly. Chromatography was performed at room temperature (20°C), using small columns of iminodiacetate-Sepharose gel loaded with copper(II) ions [IDA-Cu(II) gel] (0.7 cm I.D., bed volume 8 ml) and equilibrated in a buffer (pH 7.0) of 20 m*M* sodium phosphate, containing 0.5 *M* NaCl (equilibration buffer E). The AAG samples used in chromatography were in the native (sialylated) form and consisted of rare AAG phenotype samples (S/A^*X_0, F1/A*C1 and F1/A*AD AAG). These samples had previously been isolated from individual human plasma by the two-step purification method described here. The AAG samples with rare phenotypes were all dissolved in buffer E (3–4 mg in 1 ml) and separately applied to the columns at a flow-rate of 8 ml/h. Fractions of 0.5 ml were collected. After several bed volumes of buffer E were applied to remove the non-bound sialylated AAG variant(s), a

second elution buffer, also pH 7.0, consisting of 20 m*M* imidazole in buffer E, was applied to remove the bound variant(s).

2.4. Protein determination

Total protein concentration was determined by the method of Lowry et al. [18] with bovine serum albumin (grade A) (Calbiochem, San Diego, CA, USA) as a standard. The specific determinations of AAG and of α_1-antitrypsin were carried out by an immunonephelemetric method using Behring assay kits and a Model 100 instrument.

2.5. Sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoelectrophoresis

These were carried out according to standard procedures [19,20], using gradient gel PAA 4/30 and agarose gel, respectively. Prior to analysis by SDS-PAGE, the proteins were reduced with 5% (v/v) 2-mercaptoethanol. Poly-specific antiserum against human whole plasma proteins and mono-specific antisera against human α_1-antitrypsin were from Behring (Marburg, Germany). Mono-specific antiserum against human AAG was from Dakopatts (Glostrup, Denmark).

2.6. Isoelectric focusing (IEF)

IEF was run with a LKB 2117 Multiphor II electrophoresis apparatus equipped with a 2297 Macrodrive 5 constant-power supply.

The microheterogeneity of native (sialylated) isolated AAG and of AAG in plasma was studied by analytical IEF with carrier ampholytes in the pH range 2.5–4.5. Thin-layer polyacrylamide gel slabs (0.5 mm thick) with a pH gradient of 2.5–4.5 were prepared according to Ref. [21]. The IEF was carried out by delivering a 5 W constant power for 3 h at 10°C and the steady-state conditions (350 V, 14 mA) were reached after 1.5 h of focusing. The catholyte and anolyte solutions were 0.4 *M* HEPES and 1 *M* H_3PO_4, respectively. The isolated AAG samples in the native (sialylated) form (20 μg in deionized water) and the plasma samples (20 or 40 μl) were applied at the cathodic end of the gel using small pieces of filter paper, which were removed after 1 h of focusing. The gels were fixed, stained with Coomassie Brilliant Blue R-250 and preserved according to [21], with a staining period lengthened from 10 to 45 min.

Analytical IEF of isolated AAG, after its desialylation with neuraminidase, was performed in an immobilized pH 4.4–5.4 polyacrylamide gel gradient (0.5 mm thick) supplemented with 8 *M* urea and 2% (v/v) 2-mercaptoethanol [5]. Desialylation of the isolated AAG samples was as follows: a 12-μl volume of a neuraminidase solution (1 U/ml) in a 5 m*M* sodium acetate buffer (pH 5.5), containing 0.9 m*M* $CaCl_2$ and 15.4 m*M* NaCl, was added to 50 μl of each AAG sample (120 μg of protein per 100 μl of acetate buffer). The mixture was incubated for 24 h at 37°C and then directly used in analytical IEF. The AAG samples (ca. 20 μg) were applied at the cathodic end of the gel using small pieces of filter paper, which were removed after 1 h of focusing. IEF was carried out at 10°C, using electrode strips soaked in deionized water. The conditions were set to 5 W, 5 mA and 500 V for 1 h, and then 5 W, 5 mA and 2000 V overnight. The desialylated AAG variants in the gel were detected by staining with Coomassie Brilliant Blue R-250. The relative proportion of each protein band in the gel was determined by scanning with an LKB 2202 Ultroscan laser densitometer.

3. Results

3.1. Chromatography of human plasma on Affi-Gel Blue

Fig. 1 shows the traces of the non-bound proteins from chromatography of 10-ml samples of the same human plasma on Affi-Gel Blue, using 10 m*M* sodium phosphate buffers with pH values of 5.8, 5.5 and 5.0 and 20 m*M* sodium acetate buffers with pH values of 5.5 and 5.0, respectively. We only studied the fraction of plasma proteins eluted from the Affi-Gel Blue column, because, under the conditions used, it was expected that most of the AAG would not be retained by the affinity gel, due to the very acidic isoelectric point of this glycoprotein (p*I* ca. 3) and its non-significant binding affinity for Cibacron

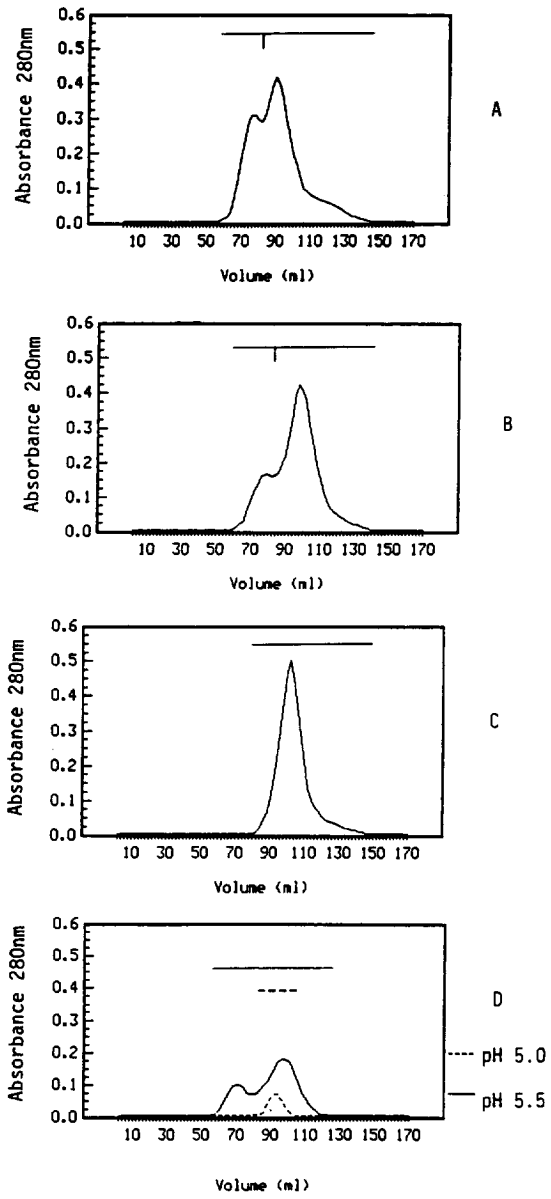

Fig. 1. Elution profiles of the non-bound proteins from chromatography of human plasma on Affi-Gel Blue with different buffer systems. The buffer systems used were 10 m*M* sodium phosphate with pH 5.8 (A), 5.5 (B) and 5.0 (C), and 20 m*M* sodium acetate with pH 5.5 and 5.0 (D). A similar volume (10 ml) of the same human plasma, previously dialyzed against the appropriate buffer, was used in each chromatographic experiment. The flow-rate was 25 ml/h and fractions of 2 ml were collected. All other details are described in Section 2. The horizontal full or dotted line above each trace indicates where fractions were collected. The minor and major protein peaks in (A) and (B) are delimited by a vertical line.

Blue F3G-A [16,22]. We did not investigate buffer pH values lower than 5.0, because the non-bound plasma protein from Affi-Gel Blue chromatography has been found to be negligible at these pH values [16]. Moreover, the use of strongly acidic buffers may result in some variations in the sialic acid content of AAG [23].

The elution profiles shown in Fig. 1 were different, depending upon the buffer system used. The non-bound proteins from Affi-Gel Blue chromatography using 10 m*M* sodium phosphate buffers with pH values of 5.8 and 5.5 (Figs. 1A and 1B) were both found to elute as two overlapping peaks, a minor peak and a major "retarded" one, while pH 5.0 (Fig. 1C) had only a single symmetrical peak. Also, by decreasing the phosphate buffer pH from 5.8 to 5.0, the elution volumes at the maximum of the peaks were found to increase slightly. The elution profiles of the non-bound plasma proteins eluted with 20 m*M* sodium acetate buffers with pH values of 5.5 and 5.0 (Fig. 1D) had a similar shape to those found at the same pH values using phosphate buffers. However, the chromatographic peaks indicated that a higher amount of protein was retained by the affinity gel by using acetate in the chromatographic buffer.

The results for (total) protein and AAG assays in each buffer system protein eluate, collected as indicated in Fig. 1 and concentrated on a YM 10 membrane filter, are summarized in Table 1. The yield of total protein eluted with 10 m*M* phosphate buffer, pH 5.8, was 2.6% and decreased accordingly with a more acidic buffer (pH values 5.5 and 5.0). The yield in the acetate buffer eluates showed a similar decrease when the pH was lowered, but was significantly less than that found with the corresponding phosphate buffers. These results agreed with the elution profiles shown in Fig. 1. The specific assay of AAG in the different eluates showed that ca. 85% of AAG was recovered from plasma using the phosphate buffers, but the yield was only 57% or less when chromatography was performed with the acetate buffers. Because of this, the acetate buffer plasma fractions were not studied any further.

SDS-PAGE analysis of the eluates with phosphate buffers showed the presence of only two major protein bands of respectively ca. 43 and 55 kDa (Fig. 2, lanes 1–4 and 6), the migration of the 43 kDa

Table 1
Total protein, AAG and α_1-AT concentrations and yields after chromatography with different buffer systems of a 10-ml sample of the same human plasma on Affi-Gel Blue

Buffer system	10 m*M* Sodium phosphate buffer with pH of			20 m*M* Sodium acetate buffer with pH of	
	5.8	5.5	5.0	5.5	5.0
Dialyzed human plasma					
Volume (ml)	10	10	10	10	10
Total protein (mg/ml)[a]	47.7	45.4	38.4	54.1	52.5
AAG (mg/ml)[b]	0.47	0.46	0.44	0.47	0.46
α_1-AT[b] (mg/ml)	1.82	1.71	1.53	ND	ND
Eluate from Affi-Gel Blue					
Volume (ml)	90	82	66	66	20
Volume after concentration by ultrafiltration (ml)	7.6	6.1	5.4	5.6	3.8
Total protein (mg/ml)[a,c]	1.61	1.34	1.06	0.74	0.21
AAG (mg/ml)[b,c]	0.53	0.64	0.70	0.48	0.20
α_1-AT (mg/ml)[b,c]	0.95	0.61	0.30	ND	ND
Yield of total protein (%)[c]	2.6	1.8	1.5	0.8	0.15
Yield of AAG (%)[c]	86	85	86	57	16.5
Yield of α_1-AT (%)[c]	40	22	11	ND	ND

[a] Total protein concentration determined by the method of Lowry et al. [18].
[b] AAG and α_1-AT concentrations determined by immunonephelemetric specific assays.
[c] Concentrations and yields determined after concentration of the eluate on a YM 10 membrane filter. ND, not determined.

protein band being identical with that of a standard commercial AAG. Using immunoelectrophoresis, the two protein species were identified as AAG and α_1-antitrypsin (α_1-AT) (data not shown, but those presented in Fig. 5A are typical). Separate analysis by SDS-PAGE of the minor and major chromatographic peaks from the phosphate buffers of pH 5.8 and 5.5 showed that the minor peak was enriched in AAG (Fig. 2, lanes 1 and 2) and the major "retarded" one in α_1-AT (lanes 3 and 4), indicating that most of the AAG was eluted ahead of α_1-AT. SDS-PAGE of the major peaks eluted at pH 5.8 and 5.5 also showed the presence of a faint protein band with a molecular mass of about 15 kDa, probably corresponding to traces of prealbumin [16,22].

The results for α_1-AT assays in the phosphate buffers eluates showed that the yield of α_1-AT decreased from 40 to 10% by changing the buffer pH from 5.8 to 5.0 (Table 1). Comparing the results from the different chromatographic experiments on Affi-Gel Blue, it was clear that chromatography of plasma in 10 m*M* phosphate buffer, pH 5.0, was the most efficient as a first step for AAG purification: the yield of AAG from plasma at pH 5.0 was as high as at the other pH values (ca. 85%) and AAG was the least contaminated by α_1-AT.

3.2. Separation of AAG and α_1-AT by chromatography on hydroxyapatite

The separation of AAG from α_1-AT in the Affi-Gel Blue eluate was performed by chromatography on hydroxyapatite. The conditions for the separation were at first optimized using standard commercial preparations of the two proteins. The elution of each protein was carried out by washing the hydroxyapatite column successively with 10, 20, 30 and 40 m*M* sodium phosphate buffers, pH 7.0, containing 0.1 *M* NaCl. Hydroxyapatite chromatographic profiles of standard AAG and α_1-AT, used separately, are presented in Fig. 3. AAG was found to elute from the hydroxyapatite column with the 20 m*M* phosphate buffer and α_1-AT to partially elute with the 40 m*M* phosphate buffer. Finally, a (pH 7.0) 400 m*M* phosphate buffer step was used to wash off the tightly bound α_1-AT. Identical results were obtained when AAG and AT were mixed in equal proportions prior to chromatography. The recovery of AAG and

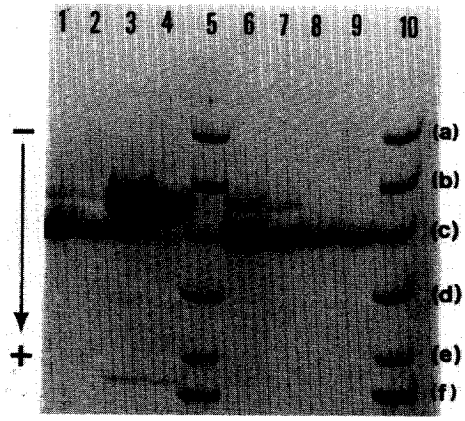

Fig. 2. SDS-PAGE analysis of selected fractions from Affi-Gel Blue and hydroxyapatite chromatography, after reduction of the proteins with 5% (v/v) 2-mercaptoethanol. Lanes 1–4 and 6, peaks of non-bound proteins from Affi-Gel Blue chromatography of human plasma (10 ml) with 10 m*M* phosphate buffers of different pH values: minor and major protein peaks eluted at pH 5.8 (lanes 1 and 3; each 7.5 μg) and at pH 5.5 (lanes 2 and 4; each 6 μg); protein peak eluted at pH 5.0 (lane 6; 6 μg). Lane 7, peak of non-bound proteins from Affi-Gel Blue chromatography of human plasma (36 ml) in 10 m*M* sodium phosphate buffer, pH 5.0 (6 μg), and lane 8, peak of purified AAG (5 μg) from hydroxyapatite chromatography of the Affi-Gel Blue fraction, in 20 m*M* phosphate buffer (pH 7.0), containing 0.1 *M* NaCl. Lane 9 contained standard commercial AAG (5 μg) and lanes 5 and 10 molecular mass markers: (a) phosphorylase b (94 kDa), (b) albumin (67 kDa), (c) ovalbumin (43 kDa), (d) carbonic anhydrase (30 kDa), (e) trypsin inhibitor (20 kDa) and (f) α-lactalbumin (14 kDa). Staining of the proteins was with Coomassie Brilliant Blue R-250.

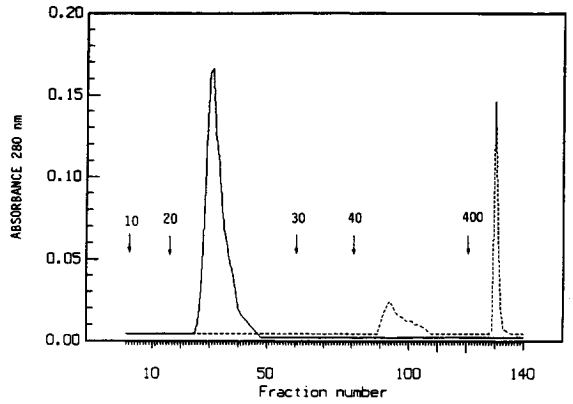

Fig. 3. Elution of standard commercial AAG and α_1-antitrypsin by chromatography on hydroxyapatite. The elution of commercial AAG (full line, 4.2 mg) and α_1-antitrypsin (dotted line, 4.5 mg) from hydroxyapatite was carried out by using successively 10, 20, 30 and 40 m*M* sodium phosphate buffers (pH 7.0), containing 0.1 *M* NaCl, and 400 m*M* phosphate buffer (pH 7.0), containing 0.5 *M* NaCl. The positions of changes in elution buffer are indicated by the arrows. All other details are described in Section 2.

α_1-AT was found to be 95% for both proteins. These results agreed with those of Funae et al. [24] who reported the separation of small amounts of AAG and α_1-AT by chromatography on hydroxyapatite, using an HPLC technique.

3.3. Two-step purification of AAG

The chromatographic results described above enabled us to develop a two-step purification procedure of AAG using a relatively large sample of human plasma (ca. 40 ml). In the first step, AAG was partially purified from plasma by chromatography on an Affi-Gel Blue column (2.5 cm I.D., bed volume 200 ml), using a 10 m*M* sodium phosphate buffer, pH 5.0. The protein eluate from Affi-Gel Blue was concentrated on a YM 10 membrane filter and then rapidly re-equilibrated in a pH 7.0 10 m*M* sodium phosphate buffer, containing 0.1 *M* NaCl, by filtration on columns PD 10 Sephadex G-25. The re-equilibrated eluate was then used directly in chromatography on a Bio-Gel HT hydroxylapatite column (1.5 cm I.D., bed volume 20 ml) also equilibrated with 10 m*M*, pH 7.0, sodium phosphate buffer, containing 0.1 *M* NaCl. The second chromatographic step on hydroxyapatite was then simplified. After application of approximately two volumes of the equilibration buffer, the elution of AAG was carried out with a 20 m*M* (pH 7.0) sodium phosphate buffer, containing 0.1 *M* NaCl, and next the gel column was regenerated by washing with a (pH 7.0) 400 m*M* sodium phosphate buffer, containing 0.5 *M* NaCl. Fig. 4 shows the traces from the two successive stages of AAG purification.

The efficiency of the purification method was monitored using SDS-PAGE and immunoelectrophoresis. SDS-PAGE of the plasma fraction from Affi-Gel Blue chromatography showed two major protein bands (Fig. 2, lane 7), which were identified as AAG and α_1-AT by immunoelectrophoresis (Fig. 5A). The fraction eluted with the 20 m*M* phosphate buffer from hydroxyapatite

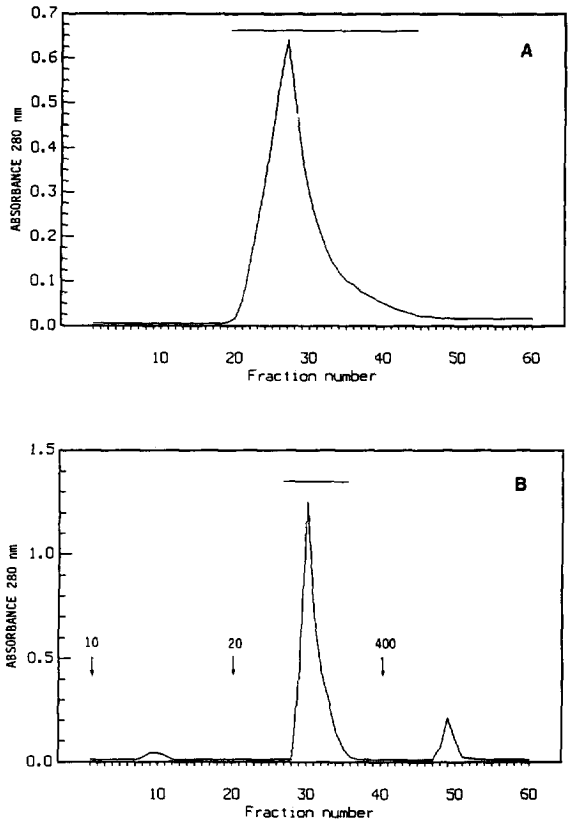

Fig. 4. Two-step purification of AAG from human plasma by chromatography using (A) Affi-Gel Blue and (B) hydroxyapatite. In (A), the elution of the non-bound plasma protein fraction from Affi-Gel Blue was carried out with a 10 m*M* sodium phosphate buffer, pH 5.0. Fractions of 6.7 ml were collected. The volume sample of dialyzed plasma used in chromatography was 36 ml. In (B), the Affi-Gel Blue fraction re-equilibrated in a 10 m*M* sodium phosphate buffer (pH 7.0), containing 0.1 *M* NaCl, was loaded on a hydroxyapatite column equilibrated with the same buffer. Elution of AAG was carried out with a 20 m*M* sodium phosphate buffer (pH 7.0), containing 0.1 *M* NaCl, and regeneration of the gel with a 400 m*M* sodium phosphate buffer (pH 7.0), containing 0.5 *M* NaCl. The positions of changes in elution buffer are indicated by the arrows. The horizontal line above each trace indicates where fractions were collected. All other details are described in the text.

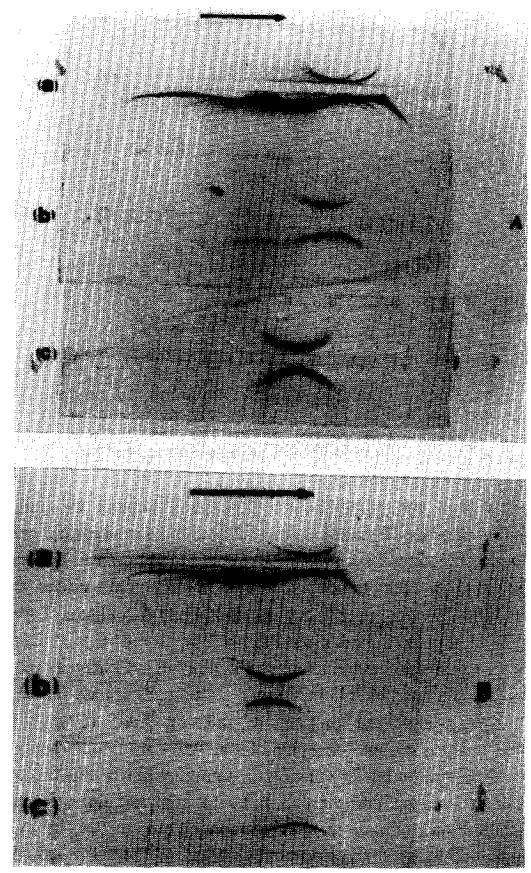

Fig. 5. Determination of the efficiency of purification by immunoelectrophoresis. The immunoelectrophoretic patterns of the non-bound plasma proteins from Affi-Gel Blue are shown in (A) and those of the 20 m*M* phosphate buffer peak from hydroxyapatite in (B). The antibodies were (a) a specific antiserum to human plasma proteins and monospecific antisera to (b) human AAG and (c) α_1-antitrypsin. Upper wells in (A) and (B): the Affi-Gel Blue fraction and the 20 m*M* phosphate buffer peak from hydroxyapatite, respectively. Lower wells in (A) and (B): (a) original human plasma, and (b) and (c) commercial AAG and α_1-antitrypsin, respectively.

chromatography contained AAG in a pure form: a single protein band with a molecular mass identical to that of standard commercial AAG was shown on SDS-PAGE (Fig. 2, lane 8) and a single arc-like immunoprecipitate was obtained on immunoelectrophoresis, using a specific antiserum to human plasma proteins and a monospecific antiserum to human AAG as antibodies, respectively (Fig. 5B). The hydroxyapatite fraction was negative for α_1-AT by immunoelectrophoresis (Fig. 5B). The AAG concentrations and yields at the two purification steps were determined by the specific assay of the protein and the results are shown in Table 2. About 85% of the AAG was recovered from plasma after chromatography on Affi-Gel Blue and the yield of the protein was 95% after hydroxyapatite chromatog-

Table 2
AAG concentration and overall yield after the two purification steps by chromatography on Affi-Gel Blue and hydroxyapatite

Material	Volume (ml)	Volume after concentration (ml)	AAG concentration[a] (mg/ml)	Overall yield (%)
Dialyzed human plasma	36	–	0.41	100
Eluate from Affi-Gel Blue	174	7.5	1.67[b]	85
Eluate from hydroxyapatite	24.3	–	0.49	81

[a] AAG concentration determined by an immunonephelemetric specific assay.
[b] AAG concentration in the Affi-Gel Blue eluate concentrated by ultrafiltration on a YM 10 membrane filter.

raphy. The overall yield of the combined techniques was 81% and ca. 12 mg of AAG were purified from an initial total amount of ca. 15 mg in the 36 ml sample of dialyzed plasma used.

3.4. Analytical IEF of individually purified AAG samples with different phenotypes

The two-step chromatographic method developed here was applied to the purification of AAG samples corresponding to the three main phenotypes of the protein (F1*S/A, F1/A and S/A, respectively) from individual human plasma samples previously phenotyped for AAG.

The microheterogeneity of the three main AAG phenotype samples in the native (sialylated) form was then studied and compared with that of the protein in the corresponding plasma, using analytical IEF with carrier ampholytes in the pH range 2.5–4.5. The electrophoretic patterns are shown in Fig. 6. The AAG bands were visualized by staining with Coomassie Brilliant Blue. Specific immunodetection of AAG was unnecessary in the case of plasma, because no interference between the electrophoretic migration of AAG and that of the other plasma proteins is observed in the pH range 2.5–4.5 [3,4]. Irrespective of the AAG or plasma sample used, AAG appeared to be very heterogeneous, consisting of several sub-populations that focused at a pH of ca. 3.4–3.7. A similar distribution of AAG bands was observed between the electrophoretic patterns obtained with the purified AAG samples (Fig. 6, lanes

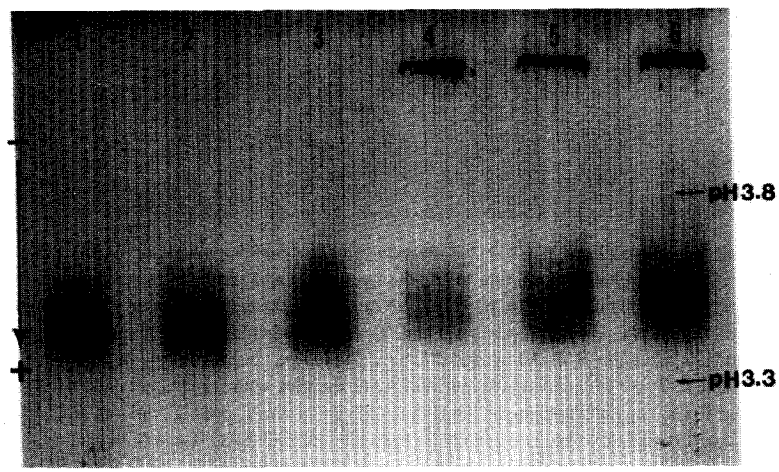

Fig. 6. Analytical IEF of the purified AAG samples with the F1*S/A, F1/A and S/A phenotypes in the native (sialylated) form and of their corresponding individual plasma on a polyacrylamide (6.06%, w/v) gel gradient with carrier ampholytes in the pH range 2.5–4.5. Lanes 1, 2 and 3: the purified AAG samples (20 μg) with the S/A, F1/A and F1*S/A phenotypes, respectively, in the native form. Lanes 4, 5 and 6: the corresponding individual human plasma containing AAG with the S/A (40 μl), F1/A (20 μl) and F1*S/A (20 μl) phenotype, respectively. Detection of the AAG bands in the gel was by staining with Coomassie Brilliant Blue R-250. The pH scale is indicated. The intense protein band observed after staining on the cathodic side of the gel in lanes 4–6 corresponds to other plasma proteins.

1–3) and their corresponding plasma (lanes 4–6), indicating that the purified AAG showed a micro-heterogeneity similar to that of the protein in plasma and that no apparent alterations of the sialic acid content of the protein occurred during its two-step purification.

Fig. 7A illustrates the genetic variants of the three main AAG phenotype samples, as obtained by analytical IEF in an immobilized, pH 4.4–5.4, polyacrylamide gel gradient and after desialylation of small amounts of the different samples. The desialylated AAG samples were found to contain either the three main genetic variants (F1*S/A AAG), or the F1 and the A variants (F1/A AAG) or the S and A variants (S/A AAG), respectively. These results agreed with the AAG phenotyping of the plasma used. The relative scanned proportions of each variant in the different AAG samples were: 43% F1, 25% S and 32% A for F1*S/A AAG; 61% F1 and 39% A for F1/A AAG; 63% S and 37% A for S/A AAG. These proportions were in agreement with those determined in desialylated whole plasma (data not shown), thus indicating that no apparent loss in a specific variant occurred during the two-step purification of AAG.

Besides, a correlation seems to exist between the phenotypes of desialylated AAG and the micro-heterogeneity of the protein in the native (sialylated) form. Comparison between the electrophoretic patterns of the three main AAG phenotype samples in the native (sialylated) form (Fig. 6, lanes 1–3) showed that the number of protein bands in the gel decreased from F1*S/A to F1/A AAG and from F1/A to S/A AAG. Also, the distribution of the protein bands for the S/A and F1/A AAG was slightly more anodic than that for the F1*S/A AAG. Montiel et al. [25] have previously observed a similar correlation between the microheterogeneity

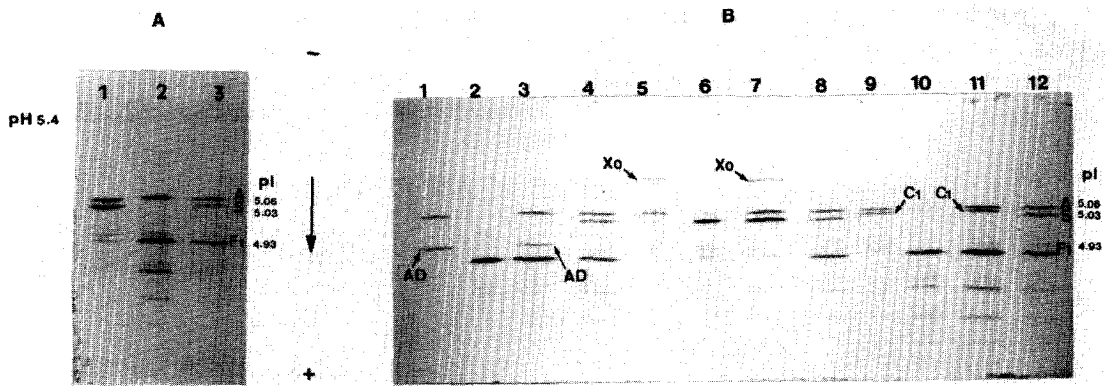

Fig. 7. Electrophoretic patterns on immobilized pH 4.4–5.4 polyacrylamide (4.85%, w/v) gel gradient with 8 *M* urea and 2% (v/v) 2-mercaptoethanol of isolated AAG samples corresponding to (A) the three main and (B) rare phenotypes of the protein, and of the isolated peaks 1 and 2, after affinity chromatography on IDA-Cu(II) gel of the rare AAG phenotypes samples. Prior to analytical IEF, small amounts of the different AAG samples and of the different peaks (1 and 2) were desialylated with neuraminidase. In (A), lanes 1, 2 and 3: individually purified AAG samples with the S/A, F1/A and F1*S/A phenotype, respectively (each 20 μg). In (B), lanes 4, 8 and 12: commercial AAG (20 μg). Lanes 3, 7 and 11: individually purified AAG samples with rare phenotypes, F1/A*AD, S/A*X_0 and F1/A*C1, respectively (each 20 μg), used in affinity chromatography on IDA-Cu(II) gel. Lane 1, peak 2 (variants A and AD, 20 μg) and lane 2, peak 1 (variant F1, 20 μg), fractionated by affinity chromatography of native F1/A*AD AAG. Lane 5, peak 2 (variants A and X_0, 20 μg) and lane 6, peak 1 (variant S, 20 μg), fractionated by affinity chromatography of native S/A*X_0 AAG. Lane 9, peak 2 (variants A and C1, 20 μg) and lane 10 (variant F1, 20 μg), fractionated by affinity chromatography of native F1/A*C1 AAG. The desialylated variants were detected in the gel by staining with Coomassie Brilliant Blue R-250. The pH scale and the approximate isoelectric point (p*I*) value for the three main, F1, S and A, variants of AAG are indicated. The protein bands corresponding to the rare AD, X_0 and C1 variants, respectively, are indicated. The faint, more anodic bands that were also observed after staining indicated the presence of a small proportion of incompletely desialylated protein: lane 2 in (A) and lanes 10, 11 and 12 in (B).

of AAG in non-treated human plasma and the F1*S/A, F1/A and S/A phenotypes of the protein in neuraminidase-treated plasma.

3.5. Affinity chromatography of rare AAG phenotype samples on immobilized copper(II) ions

We recently demonstrated the possibility of chromatography on immobilized copper(II) affinity adsorbent for the fractionation of the AAG variants, either in the native (sialylated) or the desialylated form [12,13], from individual AAG samples of the three main phenotypes. In this method, the A variant was found to strongly bind to immobilized copper(II) ions and was separated from the F1 and/or S variant(s), which showed no significant binding affinity for the transition metal ions.

Here, we investigated the interactions of AAG samples corresponding to three rare phenotypes of the protein, F1/A*AD, S/A*X_0, F1/A*C1, with copper(II) ions immobilized to an iminodiacetate-Sepharose [IDA-Cu(II) gel]. These samples had previously been isolated from individual human plasma by the two-step purification method described here and were used in affinity chromatography in the native (sialylated) form. Their variants are illustrated by the IEF patterns shown in Fig. 7B (lanes 3, 7 and 11) and obtained after desialylation of small amounts of these samples.

Fig. 8 illustrates the elution profiles obtained by affinity chromatography of the F1/A*AD, S/A*X_0 and F1/A*C1 AAG samples on IDA-Cu(II) gel equilibrated with a 20 m*M* sodium phosphate buffer (pH 7) containing 0.5 *M* NaCl (buffer E). Irrespective of the AAG sample used, the chromatography resolved only two protein peaks: a non-bound peak (peak 1) eluted with several volumes of the equilibration buffer and a bound peak (peak 2) eluted after the addition of 20 m*M* imidazole to buffer E enabling the elution of the high-affinity proteins. The elution volumes measured for each peak in the different experiments were very similar. The total recovery of native AAG in peaks 1 and 2 was ca. 95% in all the experiments. Integration showed that peaks 1 and 2 consisted respectively of ca. 69 and 31% of F1/A*AD AAG, 68 and 32% of S/A*X_0 AAG, and 70 and 30% of F1/A*C1 AAG.

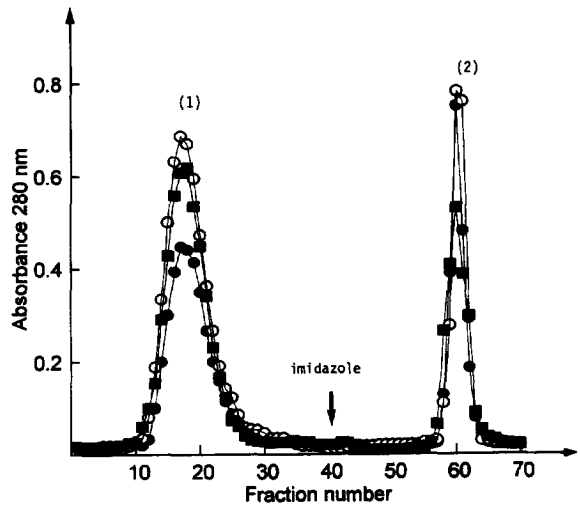

Fig. 8. Elution of the sialylated AAG variants by affinity chromatography of native AAG samples with rare phenotypes on IDA-Cu(II) gel at pH 7. The rare AAG phenotypes samples used in chromatography were S/A*X_0 AAG (●), F1/A*C1 AAG (○) and F1/A*AD AAG (■), and had been previously purified from individual human plasma by the two-step purification method described here. The different AAG samples (each ca. 4 mg) were used in the native (sialylated) form. The non-bound sialylated variant(s) (peak 1) were removed by application of several bed volumes of the equilibration buffer, consisting of 20 m*M* sodium phosphate buffer, pH 7.0, with 0.5 *M* NaCl. The high-affinity protein(s) (peak 2) were eluted after 20 m*M* imidazole was introduced (indicated by an arrow). All other details are described in Section 2.

After completion of chromatography, each peak was collected, concentrated on a YM 10 membrane filter, then dialyzed against deionized water and finally lyophilized.

The variants of the different chromatographic peaks were characterized by analytical IEF on an immobilized, pH 4.4–5.4, polyacrylamide gel gradient, after desialylation of a small amount of each peak with neuraminidase. The electrophoretic patterns are shown in Fig. 7B. Depending on the native AAG sample used in affinity chromatography, peak 1 was found to consist of only the F1 (F1/A*AD and F1/A*C1 AAG) or the S variant (S/A*X_0 AAG), and peak 2 of a mixture of the A and AD or the A and X_0 or the A and C1 variants.

The chromatographic recoveries of the variants, as

calculated earlier by integration of peaks 1 and 2, were in agreement with the proportion of each variant in the different AAG samples which was: F1 71%, A 18% and AD 11% for F1/A*AD AAG; S 74%, A 19% and X_0 7% for S/A*X_0 AAG; F1 69%, A 20% and C1 11% for F1/A*C1 AAG (as found by scanning the gel shown in Fig. 7B, lanes 3, 7 and 11).

It was concluded that the sialylated A and AD, A and X_0 and A and C1 variants corresponded respectively to the native AAG fraction strongly bound to IDA-Cu(II) gel. Conversely, the sialylated S or the sialylated F1 variant corresponded to the non-bound native AAG fraction, with no significant binding affinity for immobilized copper(II) ions.

As previously suggested [12,13], the observed microheterogeneity on binding to immobilized copper(II) ions between the AAG variants appears to be related to their separate genetic origin and thus to differences in their amino acid primary structure. The AAG system is controlled in humans by two different highly polymorphic genes, the AAG-A and the AAG-B/B′ genes as shown by Dente et al. [26], resulting in numerous genetic variants for AAG in the human population [6]. However, only two or three of the AAG variants (F1, S and A) are found with high frequencies in all the populations studied, all others being rare variants, geographically or ethnically restricted [6]. Genetic characterization of the main AAG variants has shown that the A variant is encoded by the AAG-B/B′ gene [27] and should differ from variants F1 and S, encoded by the other gene, by at least 22 amino acid substitutions, as deduced from the nucleotide sequences of the two genes [26].

Cysteinyl, histidyl and tryptophanyl residues are potent metal chelate-forming residues at the surface of proteins [28]. The A variant contains a single cysteinyl residue, which appears to be substituted by an arginyl residue in the F1 and S variants [26]. However, previous results obtained in the laboratory suggest that the A variant cysteinyl residue is neither accessible nor free, owing to covalent binding [12]. Furthermore, none of the amino acid substitutions described for the AAG variants involve histidyl and tryptophanyl residues [2,26] and thus, the heterogeneity between the A and the F1 or S variants on binding to immobilized copper(II) ions cannot be accounted for by differences in the total number of these residues. On the other hand, the differences in primary structure between the A and the F1 and S variants could involve differences in the surface accessibility and relative distribution of the metal chelate-forming groups (i.e. histidyl and tryptophanyl) on the surface of the variants, resulting in a more favourable surface topography for binding to transition metal ions for the A variant than for the F1 and S variants. This could therefore explain why the A variant and the F1 and S variants exhibit very different retention behaviours on immobilized copper(II) ions.

By contrast, the very high degree of primary sequence homology between AAG variants encoded by the same gene – these should differ by less than five amino acids [26] – could make us expect similar retention behaviours on IDA-Cu(II) gel. The chromatographic study of the rare AAG variants, AD, X_0 and C1, supports this proposal. These three rare variants have been assigned to the AAG-B/B′ gene, which also codes for the A variant [6]. Like the A variant, AD, X_0 and C1 were all found to strongly bind to immobilized copper(II) ions. Conversely, the F1 and the S variants, encoded by two alleles of the AAG-A gene, had no significant binding affinity for immobilized copper(II) ions.

4. Conclusion

The two-step purification of AAG described offers several advantages over previously reported techniques (for a review see [29]). It does not involve precipitation steps which entail the risk of denaturing or loosing some (of the) AAG (populations). It is relatively simple compared to other methods, because only two (instead of generally three or four [24,30,31]) different chromatographic steps are involved. These two steps can also be carried out immediately: the pre-purified AAG sample from the first Affi-Gel Blue step can be re-equilibrated in the buffer used in the second hydroxyapatite step, either by rapid filtration on PD 10 columns or with the addition of NaCl and diluted NaOH, since the two chromatographic buffers used in each step are very similar. One may also note that the isolated AAG from hydroxyapatite can be immediately separated

into variants by chromatography on IDA-Cu(II) gel after the addition of NaCl up to 0.5 *M* to the hydroxyapatite fraction. The overall AAG yield of this purification method is about 80%, which is higher than that obtained with the two-step chromatographic method of Succari et al. [23]. Finally, the chromatographic gels are commercially available and easily regenerated. Our purification method also provides an AAG without apparent alteration of its sialylated sub-populations, as demonstrated by analytical IEF.

The two-step chromatographic method thus developed was applied to the purification of individual AAG samples with common and rare phenotypes. The high degree of polymorphism of the two genes, AAG-A and AAG-B/B′, coding for human AAG may confuse their respective products. When no family study is possible, the differentiation of the AAG variants relies only on quantitative differences [6]: the variant bands with strong intensities detected in analytical IEF are assigned to the AAG-A gene and the other bands with weaker intensities to the AAG-B/B′gene, the two genes having slightly different coding potentials [32]. On the other hand, chromatography on immobilized copper(II) chelate affinity adsorbent (IMAC) of rare AAG phenotype samples, as performed here, and of the three main AAG phenotypes, as performed previously [12–14], has shown the high-affinity binding of the variants from the AAG-B/B′ gene (A, AD, C1 and X_0) to immobilized copper(II) ions, while those from the AAG-A gene (F1 and S) did not bind to the transition metal ions. Thus, the IMAC method could help to distinguish between the products of the AAG-A and AAG-B/B′ genes. This possibility needs, however, to be confirmed by testing a larger range of rare variants from the two genes of human AAG in affinity chromatography on IDA-Cu(II) gel.

Acknowledgments

This research was supported in part by research grants from Réseau de Pharmacologie Clinique (Ministère de la Santé) and Direction de la Recherche de l'Education Nationale (France). The laboratory is associated with the University of Paris XII (DRED EA 427).

References

[1] P.G. Blain, J.C. Mucklow, M.D. Rawlins, D.F. Roberts, P.A. Routledge and D.G. Shand, Br. J. Clin. Pharmacol., 20 (1985) 500.
[2] K. Schmid, in F.W. Putnam (Editor), The Plasma Proteins, Structure, Function and Genetic Control, Vol.I (4), Academic Press, New York, 2nd ed., 1975, p. 183.
[3] E.G. Berger, S.R. Wyss, R.B. Nimberg and K. Schmid, Hoppe-Seylers Z. Physiol. Chem., 361 (1980) 1567.
[4] D. Tinguely, P. Baumann and J. Schöpf, J. Chromatogr., 229 (1982) 319.
[5] C.B. Eap and P. Baumann, Electrophoresis, 8 (1988) 333.
[6] I. Yuasa, S. Weidinger, K. Umetsu, K. Suenaga, G. Ishimoto, C.B. Eap, J.-C. Duché and P. Baumann, Vox Sang., 64 (1993) 47.
[7] J.M.H. Kremer, J. Wilting and L.H.M Janssen, Pharmacol. Rev., 40 (1988) 1.
[8] O. Pos, R.A.J. Oostendorf, M.E. van der Stelt, R.J. Scheper and W. Van Dijk, Inflammation, 14 (1990) 133.
[9] F. Hervé, E. Gomas, J.-C. Duché and J.-P. Tillement, Br. J. Clin. Pharmacol., 36 (1993) 241.
[10] F. Hervé, C. Marché, J. Barré, E. Albengres and J.-P. Tillement, Can. J. Physiol. Pharmacol., 72 (Suppl. 1) (1994) 75.
[11] F. Hervé, J.-C. Duché, N. Sportes and J.-P. Tillement, J. Chromatogr., 539 (1991) 401.
[12] F. Hervé, J.-C. Duché, J. Barré, M.-C. Millot and J.-P. Tillement, J. Chromatogr., 577 (1992) 43.
[13] F. Hervé, E. Gomas, J.-C. Duché and J.-P. Tillement, J. Chromatogr., 615 (1993) 47.
[14] M.-C. Millot, F. Hervé and B. Sebille, J. Chromatogr. B, 664 (1995) 55.
[15] H.J. Böhme, G. Kopperschläger, J. Schulz and E. Hofmann, J. Chromatogr., 69 (1972) 209.
[16] E. Gianazza and P. Arnaud, Biochem. J., 203 (1982) 637.
[17] C.B. Eap, C. Cuendet and P. Baumann, Hum. Genet., 80 (1988) 183
[18] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
[19] U.K. Laemmli, Nature, 227 (1970) 680.
[20] P. Grabar and C.A. Williams, Biochim. Biophys. Acta, 10 (1953) 193.
[21] LKB Application Note 250, Pharmacia-LKB, Uppsala, 1977.
[22] G. Birkenmeier and G. Kopperschlaeger, in J.F. Stoltz and C. Rivat (Editors), Biotechnology of Plasma Proteins: Fractionation and Applications, Editions INSERM, Paris, 1989, p. 217.
[23] M. Succari, M.J. Foglietti and F. Percheron, J. Chromatogr., 341 (1985) 457.
[24] Y. Funae, S. Wada, S. Imaoka, S. Hirotsune, M. Tominaga, S. Tanaka, T. Kishimoto and M. Maekawa, J. Chromatogr., 381 (1986) 149.
[25] M.D. Montiel, A. Carracedo, I. Lopez-Rodriguez, M.S. Rodriguez-Calvo, J.-L. Blasquez-Caeiro and L. Concheiro, Hum. Hered., 38 (1988) 353.

[26] L. Dente, M.G. Pizza, A. Metspalu and R. Cortese, EMBO J., 6 (1987) 2289.
[27] L. Tomei, C.B. Eap, P. Baumann and L. Dente, Hum. Genet., 84 (1989) 89.
[28] J. Porath, Protein Expression Purif., 3 (1992) 263.
[29] T.W. De Graaf, M.E. van der Stelt, M.G. Anbergen and W. van Dijk, J. Exp. Med., 177 (1993) 657.
[30] P. Laurent, L. Miribel, J. Bienvenu, C. Vallve and P. Arnaud, FEBS Lett., 168 (1984) 79.
[31] K.D. Smith, M.A. Elliott, H.G. Elliott, C.M. McLaughlin, P. Wightman and G.C. Wood, J. Chromatogr. B, 661 (1994) 7.
[32] L. Dente, U. Ruther, M. Tripodi, E. Wagner and R. Cortese, Gene Dev., 2 (1988) 259.