A TWO-STEP PURIFICATION PROCEDURE FOR α_2-MACROGLOBULIN BASED ON PSEUDO-LIGAND AFFINITY CHROMATOGRAPHY

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1. Introduction

Alpha$_2$-macroglobulin (α_2M), a high-M_r (725 000) protein [1], is one of the major protease inhibitors in human plasma [2]. Classical procedures for its isolation involve ammonium sulfate and polyethylene glycol precipitation followed by gel filtration and/or ion-exchange chromatography [1,3,4]. Most of these techniques, which involve several sequential steps, result in yields of ~20%. In [5] α_2M was purified from ammonium sulfate precipitate using metal chelate chromatography, with an overall 22% recovery. In [6] α_2M was obtained by chromatography of human plasma on immobilized Cibacron blue followed by gel filtration, with 75% yield. Nevertheless, this procedure, as most of the others, is only applicable to plasma obtained from subjects homozygous for the phenotype 1 of haptoglobin, who represent only 20% of Caucasian individuals [7].

We report here a more convenient two-step procedure for the purification of α_2M. Plasma proteins are eluted from immobilized Cibacron blue F3GA at pH 5 by a salt concentration gradient which separates α_2M from haptoglobin (1, 1–2, and 2 phenotypes) and immunoglobulin M. The lower-M_r contaminants can then be removed easily by gel filtration.

2. Materials and methods

Human plasma from healthy volunteers with haptoglobin phenotypes 1, 1–2, and 2 was collected on citrate–soybean trypsin inhibitor according to [1] and centrifuged at 500 × *g* for 15 min at 4°C; 20 ml plasma was then dialyzed overnight at 4°C against 800 ml CH_3COOH/Na buffer (pH 5.0 at 25°C, ionic strength 0.05).

Affigel blue, a cross-linked agarose gel with covalently coupled reactive blue A dye (4.5 μmol dye/ml), was a generous gift of Bio-Rad Labs. Gel equilibrated with the acetate buffer was packed in a column (1.6 × 70 cm, Pharmacia Fine Chemicals, Piscataway NJ) equipped with plungers. The chromatographic experiment was run at room temperature. The flow rate was 20 ml/h and fractions of 1.25 ml were collected at 4°C. After loading of the sample (20 ml dialyzed plasma), the column was washed with 75 ml of the buffer, and a linear NaCl gradient (from 0.0–2.0 M in the acetate buffer, total vol. 225 ml) was applied using a Pharmacia GM10 gradient mixer. The column was then washed with an additional 60 ml 2.0 M NaCl in the acetate buffer. α_2M-containing fractions were pooled, concentrated to 5.0 ml under nitrogen pressure, then dialyzed against the buffer used for gel filtration.

Gel filtration experiments were performed at 4°C on a column (2.5 × 100 cm) containing 400 ml acrylamide–agarose gel (ACA-22, LKB Instruments, Rockville MD) equilibrated with 0.1 M H_3PO_4/K buffer (pH 6.5) at a flow rate of 10 ml/h. Fractions of 2 ml were collected.

Immunoelectrophoresis, fused rocket immunoelectrophoresis, and electroimmunoassay were performed according to [8–10]. Antisera were from DAKO (Accurate Chemical and Scientific, Westbury NY). Haptoglobin phenotypes were determined by polyacrylamide gel electrophoresis [11]. The trypsin-binding capacity of α_2M was measured as in [12]

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using soybean trypsin inhibitor and α-*N*-benzoyl-D,L-arginine–*p*-nitroanilide–HCl (BAPNA) all purchased from Sigma (St Louis MO). Trypsin from Sigma (type XI from bovine pancreas) was assayed by active site titration as in [13] using *p*-nitrophenyl-*p'*-guanidobenzoate–HCl (Sigma) and found to be 72% active.

3. Results and discussion

A combination of affinity chromatography on immobilized Cibacron blue F3GA and gel filtration has been proposed [6] for the purification of α_2M. They fractionated plasma proteins at pH 8.0 on a Sepharose matrix to which Cibacron blue was bound at the ratio of 2 μmol dye/ml gel. Under these conditions, α_2M is the first protein to be eluted. Using a more substituted matrix (4.5 μmol dye/ml gel) we have shown [14] that α_2M binds to the gel and is eluted only with increasing salt concentrations. Under these conditions, α_2M is eluted together with IgM and haptoglobin. Here, we found that when the column was equilibrated at pH 5 most of the plasma proteins were bound, but that during elution with a salt gradient haptoglobin and IgM were eluted over 1.25–1.5 M NaCl whereas α_2M was eluted over 1.6–2 M NaCl (fig.1). Haptoglobins of the 1, 1–2 and 2 phenotypes (the latter representing a major problem in α_2M purification [1,6,15,16]) were eluted at the same NaCl molarity (fig.2). Other high-M_r components such as C4, C1q, and fibrinogen were absent from the α_2M elution zone.

Analysis of the α_2M-containing fractions by immunoelectrophoresis (fig.3) and fused rocket immunoelectrophoresis (fig.2B) indicated that they were contaminated with pre-albumin, albumin, C3, ceruloplasmin, group-specific component, IgG, and monomeric IgA. All these contaminants were removed by an additional step of gel filtration on ACA-22 (fig.1B). α_2M was eluted ahead of the lower-M_r proteins and did not overlap with them as shown by fused rocket immunoelectrophoresis

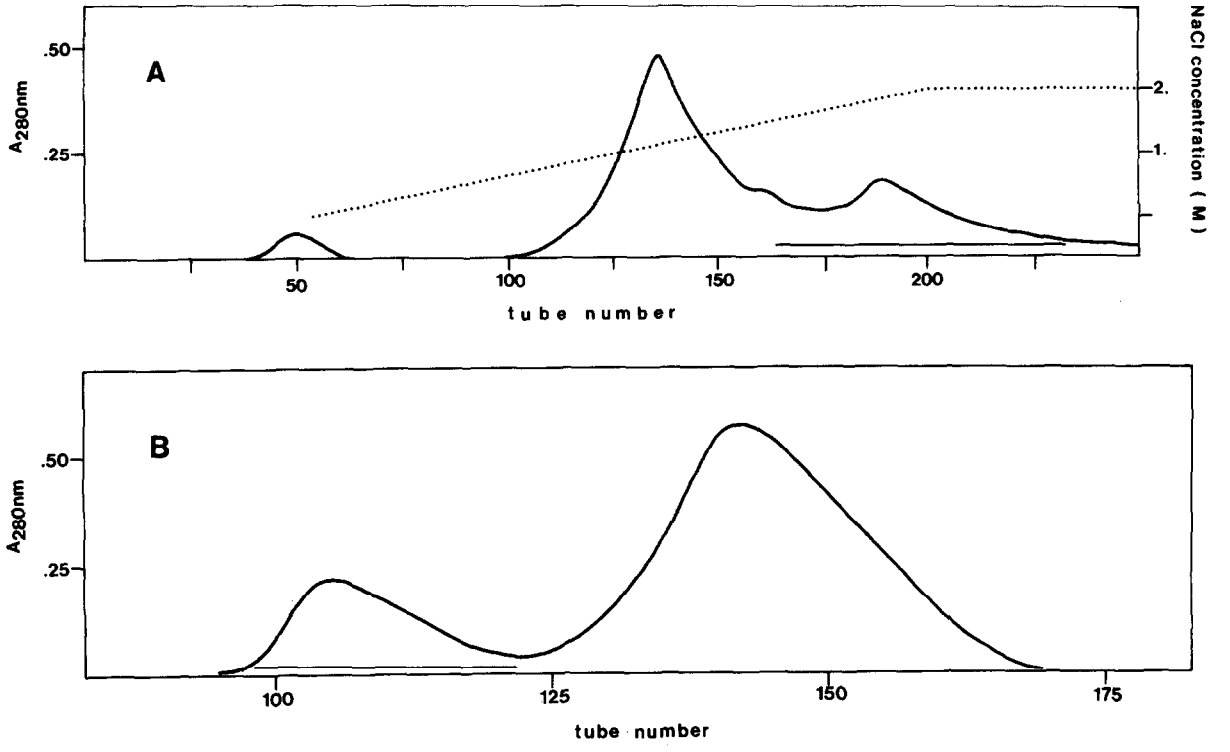

Fig.1. (A) Absorbance at 280 nm of plasma fractions obtained by chromatography on Affigel blue at pH 5. The bar indicates the position where α_2M was eluted. (B) Elution profile for the α_2M-containing fractions from A (tubes 176–225) after gel filtration on ACA-22. The bar indicates the position where α_2M was eluted. The difference in overall A_{280} between the two profiles is due to the different light path of the cells used.

(fig.2B) and by immunoelectrophoresis (fig.3). Determination of the yield by Laurell rocket immunoelectrophoresis and measurement of trypsin-inhibitory capacity is indicated in table 1. During the process of purification (despite exposure of α_2M to pH 5), no significant modification of the trypsin-binding capacity of α_2M occurred, and the purified protein retained its functional properties.

The purification procedure we have described appears to have several advantages over previously reported techniques:

(1) It does not involve time-consuming or denaturing steps, and the chromatographic gels used are commercially available.

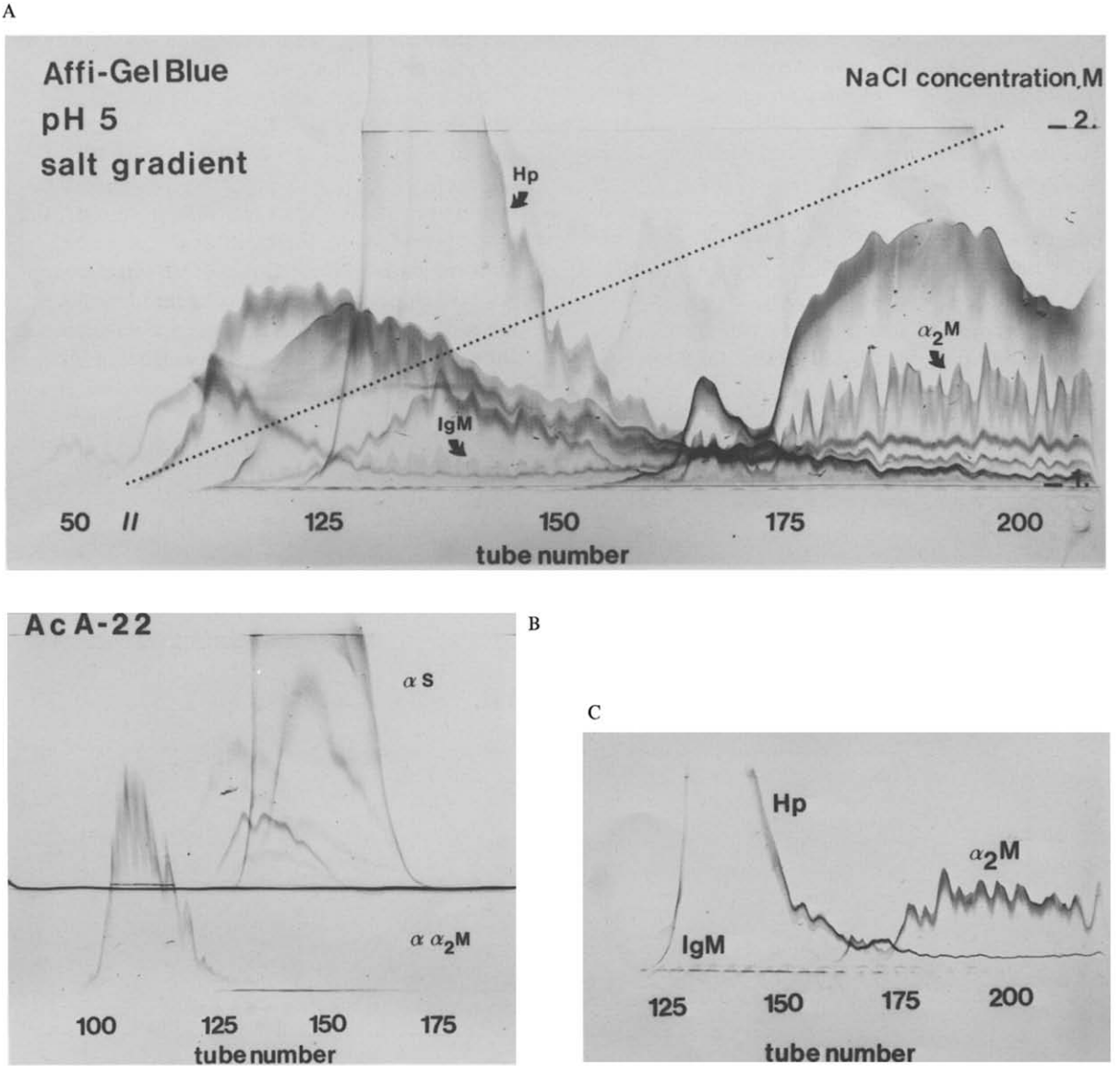

Fig.2. (A) Fused rocket immunoelectrophoresis of plasma fractions eluted from the Affigel blue column. Conditions: 1% agarose in barbital buffer (pH 8.6) containing total antihuman antiserum (DAKO) 50 µl/ml. Sample deposition: 3 µl. Run for 3 h at 350 V. (B) Fused rocket immunoelectrophoresis of the fractions eluted from the ACA-22 column. Bottom layer: antiserum to α_2M, 10 µl/ml ($\alpha\alpha_2$M). Top layer: total antihuman antiserum, 20 µl/ml (S). (C) Fused rocket immunoelectrophoresis showing the separation between haptoglobin (phenotype 2-2) and α_2M on Affigel blue.

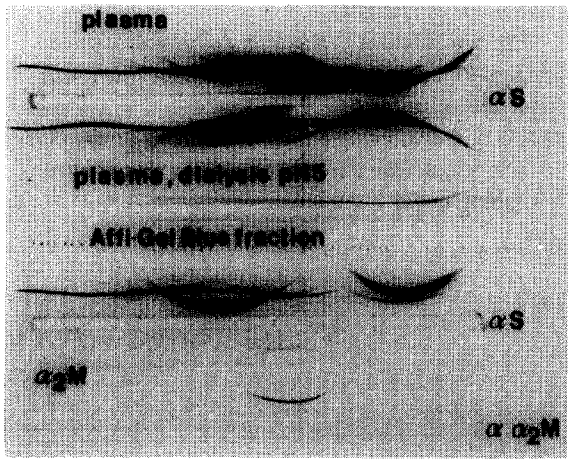

Fig.3. Immunoelectrophoresis of whole plasma, fractions containing α_2M from the Affigel blue column (tubes 176–225), and fractions containing α_2M from the ACA-22 column (tubes 100–120). αS, total antihuman antiserum; $\alpha\alpha_2$M, antiserum to α_2M.

(2) It is applicable to plasma obtained from donors either homozygous or heterozygous for the 2 variant of haptoglobin.

(3) It provides α_2M with an overall yield of 60% and with preservation of its biological activity. This is important in view of the continuing interest in the structure and biological role of α_2M [17], not only as a protease inhibitor but also as a modulator of the immune response [18,19].

(4) Our procedure illustrates the versatility of pseudoligand affinity chromatography as a tool for the fractionation of human plasma proteins [6,14,20,21].

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Table 1
α_2M concentration, biological activity and yields after the different purification steps

Material	Vol. (ml)	α_2M (ml/dl)[a]	Yield (%)	Trypsin binding capacity[b]	Yield (%)
Dialyzed human plasma	20	250	100	0.410	100
Pool of Affigel blue fractions (176–225)	62.5	64	80	0.104	79.2
Pool of ACA 22 fractions (100–120)	40	75	60	0.120	58.5

[a] α_2M concentration measured by Laurell rocket immunoelectrophoresis [10] using a protein standard obtained from Calbiochem-Behring (La Jolla CA; lot no. 75425)

[b] Trypsin binding capacity measured as in [12]. The reaction between bound trypsin and BAPNA was stopped after 15 min incubation

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