A three-step purification of human α_1-acid glycoprotein

Philippe Laurent, Laurent Miribel, Jacques Bienvenu, Charles Vallve and Philippe Arnaud*

Department of Immunology, Pasteur Institute of Lyon, 77 rue Pasteur, 69365 Lyon Cédex 02, France and **Department of Basic and Clinical Immunology and Microbiology, Medical University of South Carolina, Charleston, SC 29425, USA*

Received 9 December 1983; revised version received 26 January 1984

α_1-Acid glycoprotein (AGP) was purified to homogeneity by a 3-step procedure using pseudo-ligand affinity chromatography on immobilized Cibacron blue F3GA, Procion red HE3B, and preparative column isoelectric focusing. The overall yield of the combined techniques was 88%. Analysis of the purified AGP by lectin affinity chromatography on immobilized Con A and immunoaffinoelectrophoresis indicated that the most acidic form did not interact with the lectin, while the two more basic fractions possessed different affinities for Con A. In addition, 3 different populations of AGP were clearly separated by Con A affinity chromatography.

α_1-Acid glycoprotein *Orosomucoid* *Cibacron blue* *Procion red* *Preparative isoelectric focusing*

1. INTRODUCTION

α_1-Acid glycoprotein (AGP) is normally present in human plasma at concentrations of 0.50–1.00 g/l. These values may increase 2–4-fold during acute inflammatory states [1,2]. This response is typical of the class of plasma proteins known as acute phase reactants [3,4]. AGP is a 41-kDa plasma protein which is produced by the hepatocytes [5,6]. It is characterized among the other plasma proteins by its unusually high carbohydrate content (42%) which includes a large number of sialyl residues, its very acidic isoelectric point(s) (p*I* 2.7–3.5), and its microheterogeneity which has been attributed to different sialyl–galactosyl linkages [2,7–10]. In addition, a large number of amino acid substitutions, and a significant degree of homology with immunoglobulin have been reported [2,5]. Previous studies have provided evidence that AGP is capable of inhibiting platelet aggregation [11,12], phagocytosis of *Escherichia coli* and *Staphylococcus aureus* by neutrophils [13], and blastogenic transformation of human lymphocytes in the presence of phytohaemagglutinin [14]. In addition, AGP binds a number of therapeutic agents, and the increased plasma levels of AGP result in increased binding of a number of therapeutic drugs basically charged such as heparin, propranolol, chlorpromazine [15].

Most of the procedures for the isolation of human AGP use as a first step removal of most plasma protein by precipitation at pH 5, followed by submitting the supernatant to a combination of chromatographic procedures on DEAE– and/or CM–cellulose. Gel filtration chromatography on Sephadex G-100 is used as a final step [2]. Pseudo-ligand affinity chromatography on immobilised Cibacron blue F3-GA has been recently developed as a first step for protein fractionation, which prevents a possible alteration due to salt precipitation [16]. We here used pseudo-affinity chromatography on both immobilized Cibacron blue F3-GA and Procion red HA3B in combination with preparative isoelectric focusing (IEF) to isolate AGP from normal human plasma. Particular emphasis was placed on analysis of the interaction of the carbohydrates of AGP obtained from preparative IEF chromatography with free concanavalin A (Con A) using immunoaffino-

Published by Elsevier Science Publishers B.V
00145793/84/$3.00

electrophoresis and chromatography on immobilized Con A.

2. MATERIALS AND METHODS

Human plasma was obtained from healthy blood donors with their informed consent. After centrifugation at 500 × *g* for 15 min, sodium azide (final concentration 0.2%) and soybean trypsin inhibitor (50 mg/dl) were added. The plasma was kept frozen at −20°C until used. Forty ml of plasma were dialyzed overnight against 0.03 M sodium phosphate (pH 7.0, $I = 0.05$). A column (100 × 2.5 cm) was filled with Affigel blue (a gift from Biorad, Richmond, CA) equilibrated in the same buffer. The bed volume of settled gel represented 400 ml. After application of the sample, the column was washed first with 2.5 bed vols of the equilibration buffer, then with a linear salt gradient from 0 to 1.0 M NaCl in the equilibration buffer. The column was run at room temperature, at a flow rate of 30 ml/h, and 4.8-ml fractions were collected at 4°C. The fractions containing AGP were pooled, concentrated under nitrogen pressure by ultrafiltration on an Amicon PM10 filter (Amicon, Denver, CO) and then dialyzed overnight against acetic acid–sodium acetate buffer (pH 5.8, $I = 0.05$). A column (20 × 1.6 cm) was filled with Red Sepharose CL-6B (a gift from Pharmacia, Piscataway, NJ) equilibrated with the same buffer. After application of the sample, the column was washed first with 2 bed vols of equilibration buffer, then with a linear salt gradient from 0 to 1.0 M NaCl in the equilibration buffer. The column was run at room temperature at a flow rate of 30 ml/h. The fractions containing AGP were desalted by overnight dialysis against 1% glycine (w/v), and applied on a preparative IEF column (LKB 8100 type 110 ml column, LKB, Orsay, France). The final concentration of the carrier ampholytes was 2% (w/v) (Pharmalyte, pH range 2.5–5, Pharmacia), and stabilized in a 5–50% linear sucrose gradient. The electrode solutions were prepared as in [15], and the electrophoresis performed at 4°C at constant power (7 W), and limiting voltage preset at 1700 V. Fractions were collected from the bottom of the column at a flow rate of 10 ml/h. The elution profile of each chromatography run was monitored at 280 nm, and specific determination of proteins was performed by fused rocket immunoelectrophoresis using antisera against human AGP and whole human serum. Immunoaffino-electrophoresis, immunoelectrophoresis, fused rocket immunoelectrophoresis, SDS-polyacrylamide gel electrophoresis (SDS–PAGE), total protein measurement and Con A affinity chromatography were carried out according to standard procedures [18–22].

3. RESULTS AND DISCUSSION

A combination of pseudo-affinity chromatography on immobilized Cibacron blue F3GA and Procion red HE3B and preparative IEF is proposed for the purification of AGP. Fig.1 shows the elution profiles of the 3 chromatographic steps. AGP is eluted in the non-bound fraction, from the Cibacron blue column together with α_1-antitrypsin (A1AT), prealbumin, α_2-HS glycoprotein, group-

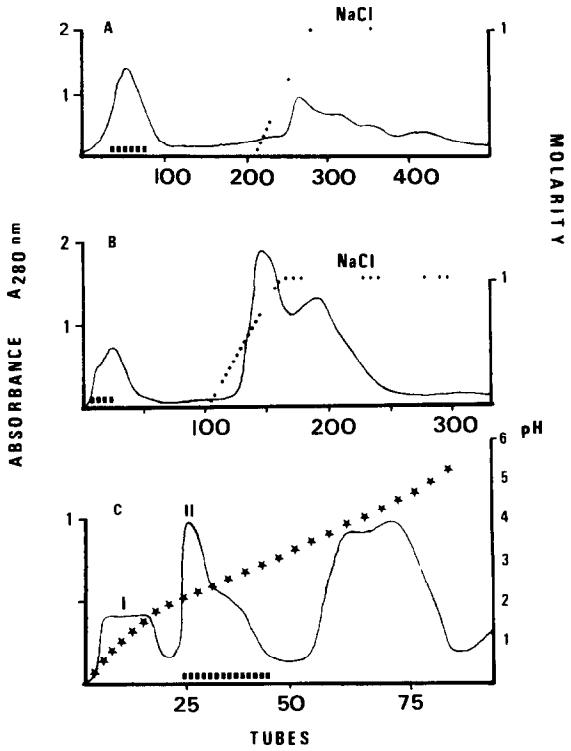

Fig.1. Absorbance at 280 nm of plasma fractions obtained by chromatography on Affigel blue (A), Procion red (B) and preparative column IEF (C). (▪ ▪ ▪) Position where AGP was eluted; (· · · ·) development of salt gradient; (★) development of pH gradient

specific component, transferrin, ceruloplasmin, IgA, and some IgG [16]. We found that when the Red Sepharose was equilibrated with sodium acetate buffer (pH 5.8), most of these proteins tightly bound on the dye ligand with the exception of AGP, A1AT and a trace amount of prealbumin. Analysis of the unbound fractions from the Red Sepharose column by fused rocket immunoelectrophoresis (fig.2) indicated that A1AT is weakly retarded and most of the AGP was eluted in part ahead of A1AT. Considering the very acidic p*I* of AGP, these contaminants were easily removed by an additional step of preparative IEF (IEF column). Fig.1,3 shows that AGP was eluted in the second peak of the elution profile of the IEF column. The first peak corresponded to the absorbance of the anodic solution.

No contaminants could be detected by using a series of monospecific antisera against human proteins, by SDS–PAGE, and immunoelectrophoresis. Determination of the yield by immunonephelometry is indicated in table 1.

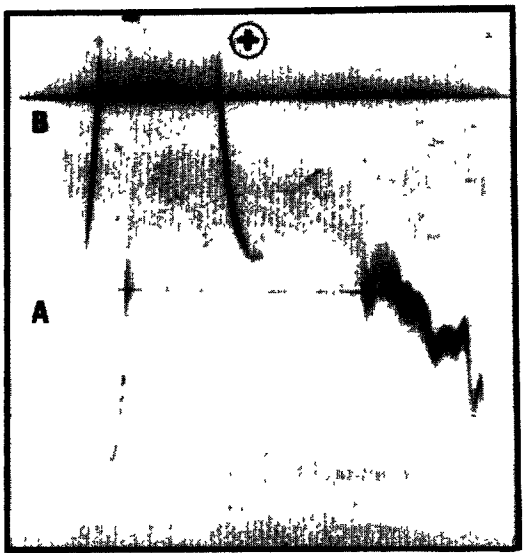

Fig.2. Fused rocket immunoelectrophoresis of tubes 10–50 eluted from Procion red, and developed against human α_1-antitrypsin (20 μl/ml, A), and human AGP (20 μl/ml, B). Gel was 1% agarose (1.5 mm thick) in 7.88 mM sodium barbital/1.40 mM barbital/93.67 mM glycine/46.6 mM Tris (pH 8.6). Three μl of samples were applied to the wells. The plates were run overnight at 2 V/cm, then pressed, washed twice, dried and stained. Anode at the top.

Fig.3. Fused rocket immunoelectrophoresis of tubes 20–81 collected from preparative column IEF. For technical details see legend to fig.2.

The immunoaffino-electrophoresis (fig.4) applied to the study of the different fractions containing AGP separated by the IEF column indicated that the most acidic subpopulations of AGP did not bind Con A. These data indicate that the interaction of AGP for Con A parallels the p*I* of the protein and suggests that the sugar configuration is responsible for the different p*I* values of the protein. Furthermore, the same fractions pooled and applied on an immobilized Con A–Sepharose column revealed 3 clearly separated AGP populations: Con A tightly bound, weakly retarded, and non-reactive. Similar results

Table 1

AGP concentration and overall yield after the different purification steps

Material	Volume (ml)	g/l	Overall yield (%)
Dialyzed human plasma	40	1.10	100
Pool of Affigel blue fractions (tubes 45–80)	168	0.25	95
Pool of Procion red (tubes 10–25)	80	0.51	93
Pool of column IEF (tubes 18–44)	26	1.50	88

AGP concentration was measured by immunonephelometry using a protein standard obtained from Hyland Travenol (Derfield, IL)

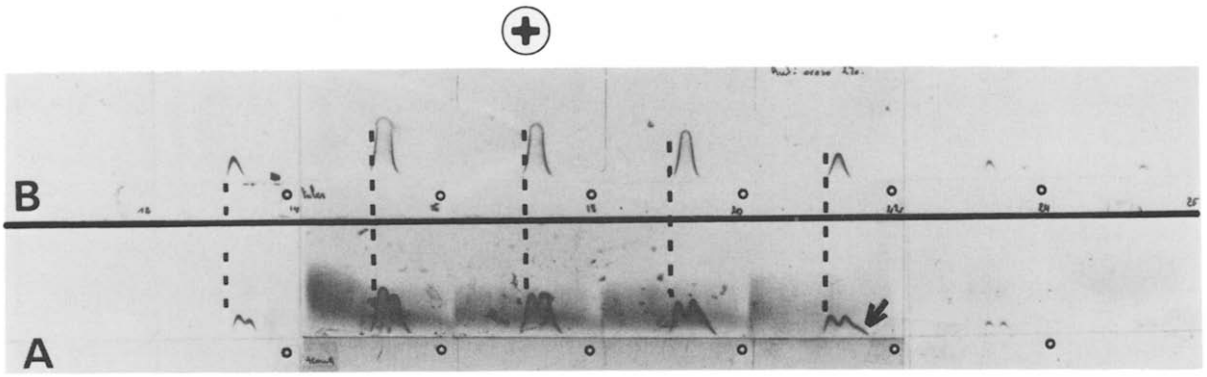

Fig.4. Crossed affinoimmuno-electrophoresis developed against human AGP. (A) Control experiment without lectin, (B) with free Con A in the first-dimension gel. The lectin concentration was 0.143 mg Con A per cm^2. For other technical details see legend to fig.2.

were observed in [8]. AGP affinity for Con A is related to its heteroglycanic structure. As demonstrated in [7] and [8] the sugars of AGP exhibit 5 different structures: biantennary chain, triantennary chain, triantennary chain plus fucose, tetra-antennary, tetra-antennary plus fucose. Increasing complexity in the glycanic structure of AGP results in decreasing affinity for Con A.

Our purification procedure appears to have several advantages over previously reported techniques. First, it does not involve denaturing steps such as acidic precipitation and/or exposure to strongly denaturing agents. The overall yield is about 88%. The chromatographic gels used are commercially available. In addition, it provides an AGP without apparent alteration of the carbohydrate structure as demonstrated by affinity techniques on Con A. This is important in view of the biological role suggested for this protein as drug-carrier [15] and modulator of the immune response [1,2,12,23]. The understanding of the relationships between the structure and immunomodulative functions of AGP is of great interest. The carbohydrate moieties of AGP may play a key role in its biological functions. The AGP prepared according to our procedure may provide a reliable native material to investigate its immunoregulatory role as suggested by authors in [23]. These authors particularly mention the enhanced potency of the agalacto/asialo derivatives in suppressing a number of immune functions of mouse spleen cells in vitro. Finally, this purification procedure illustrates the versatility of pseudo-ligand affinity chromatography for protein purification under close to physiological conditions.

ACKNOWLEDGEMENTS

P.L. was supported by a fellowship from the College of Graduate Studies, Medical University of South Carolina, on leave of absence from the Pasteur Institute, Lyon, France, during the initiation of this work. We thank C. Court for her excellent technical assistance. Supported in part by Grant IPL 1982. L.M. was a post-doctoral fellow of the Pasteur Institute of Lyon, France.

REFERENCES

[1] Kushner, I. (1982) Ann. NY Acad. Sci. 389, 39–48
[2] Schmid, K. (1975) in: The Plasma Proteins (Putnam, F.W. ed) vol.I, pp.183–228, Academic Press, New York.
[3] Koj, A. (1974) in: Structure and Function of Plasma Proteins (Allison, A.C. ed) vol.1, pp.73–125, Plenum, London.
[4] Jamieson, J.C., Morrison, K.E., Molasky, D. and Turchen, B. (1975) Can. J. Biochem. 53, 401–414.
[5] Schmid, K., Kaufman, H., Isemura, S., Bauer, F., Emura, J., Motoyama, T., Ischigura, M. and Nanno, S. (1973) Biochemistry 12, 2711–2722.
[6] Nagashima, M., Urban, J. and Schreiber, G. (1980) J. Biol. Chem. 255, 4951–4956.
[7] Bayard, B. and Kerckaert, J.P. (1980) Biochem. Biophys. Res. Commun. 95, 777–784.

[8] Fournet, B., Montreuil, J., Strecker, G., Dorland, L., Haverkamp, J., Vliegenthart, J.F.G., Binette, J.P. and Schmid, K. (1978) Biochemistry 17, 5206–5214.
[9] Schmid, K., Binette, J.P., Kamiyama, S., Pfister, V. and Takahashi (1962) Biochemistry 1, 959.
[10] Nicollet, I., Lebreton, J.P., Fontaine, M. and Hiron, M. (1981) Biochim. Biophys. Acta 668, 235–245.
[11] Snyder, S. and Coodley, E.L. (1976) Arch. Int. Med. 136, 778–781.
[12] Costello, M., Fiedel, B.A. and Gewurz, H. (1979) Nature 281, 667–668.
[13] Van Oss, C.J., Gilman, C.F., Bronson, P.M. and Broder, J.R. (1974) Immunol. Commun. 3, 321–328.
[14] Chui, K.M., Mortensen, R.F., Osmand, A.P. and Gewurz, H. (1977) Immunology 32, 992–1005.
[15] Piafsky, K.M., Borga, I., Odar-Cederlof, I., Johansson, C. and Sjoqvist, F. (1978) N. Engl. J. Med. 99, 1435–1439.
[16] Gianazza, E. and Arnaud, P. (1982) Biochem. J. 203, 637–641.
[17] Vesterberg, O., Wadstrom, T., Vesterberg, K., Svensson, H. and Malmgren, B. (1967) Biochim. Biophys. Acta 133, 435–445.
[18] Bog Hansen, T.C. (1983) Scand. J. Immunol. 17, suppl.10, 243–253.
[19] Svendsen, P.J. (1973) Scand. J. Immunol. 2, suppl.1, 69–70.
[20] Laemmli, U.K. (1970) Nature 227, 680–685.
[21] Murthy, R.J. and Hercz, A. (1973) FEBS Lett. 32, 243–246.
[22] Bradford, M. (1976) Anal. Biochem. 15, 45–52.
[23] Bennett, M. and Schmid, K. (1980) Proc. Natl. Acad. Sci. USA 77, 6109–6113.