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ONE-STEP PURIFICATION OF RAT PLASMA α_1-ACID GLYCOPROTEIN BY ANTIBODY AFFINITY CHROMATOGRAPHY: APPLICATION TO NORMAL AND INFLAMED RAT SERA

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SUMMARY

Most purification procedures used previously to isolate α_1-acid glycoprotein (AGP) from plasma can lead to some alterations in its carbohydrate moiety. An immunoaffinity chromatographic method is proposed for purifying in one step rat plasma AGP without any detectable modification of its glycan moiety. Crossed immunoaffinoelectrophoresis with concanavalin A before and after purification showed identical patterns, suggesting no glycan selection during the purification. In the same way no desialylation occurred during the purification step. This immunoaffinity chromatographic procedure provided evidence of a decreased level of fucosyl residues in turpentine oil rat plasma AGP compared with normal rat plasma AGP.

INTRODUCTION

α_1-Acid glycoprotein (AGP) is a single-chain polypeptide containing about 45% carbohydrates. Human and rat AGP share some characteristic features concerning the glycan moiety, exhibiting two types of glycan microheterogeneities leading to different glycan variants: (i) variations in the degree of antennarity of the complex-type N-acetyllactosaminyl chains and (ii) the presence or not of either sialyl or fucosyl residues.

It has been shown that hormones, nutritional conditions and disease states amplify these glycan heterogeneities, but until now mainly qualitative methods have been used for such studies. It would be interesting to establish the precise nature of the alterations by structural studies. However, whereas the structures of the peptide and glycan moieties of human AGP have been extensively studied, as reviewed by Arnaud and Gianazza [1], few data concerning the structure of rat AGP are available. The complete amino acid sequence was deduced from the

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corresponding cDNA sequence [2] without any direct analysis of the peptidic sequence, and the structure of the glycan moiety was established by one group only [3].

To investigate the precise molecular basis of alterations in rat AGP, purification of this glycoprotein must be performed so that no alteration occurs, especially in the glycan moiety. Several methods have been reported for the isolation of AGP from specimens of human origin [4–7] and from rats [8,9]. However, most of them are time-consuming and could lead to partial desialylation of the glycoprotein by using an acidic precipitation in the first step of the purification procedure and/or to a selection of glycan subpopulations depending on the isoelectric pH value (pH_i) of the glycan variant and the pH of the buffer used.

We present in this paper a purification procedure using immunoaffinochromatography which allows a highly purified rat AGP to be obtained without any loss of sialyl residues or selection of glycan subpopulations. Application to sera withdrawn from normal and inflamed rats is presented.

EXPERIMENTAL

Animals

Male Sprague–Dawley rats (Charles River Lab., St. Aubin les Elbeufs, France) weighing 200–250 g and fed ad libitum were used. Inflamed rats were obtained by subcutaneous injection of 1 ml of turpentine oil 48 h before drawing blood. Blood was removed from the abdominal aorta under Pentothal anaesthesia. Pooled sera were prepared after centrifugation at 1000 *g* for 15 min.

Immunization

Antisera against rat AGP, rat albumin and rat total proteins were prepared in rabbits as described previously [10]. Rabbit antisera were stored at −20°C with 0.1% (w/v) sodium azide and 10% (v/v) glycerol. Rat albumin and rat AGP antibody titre (mg of antigen neutralized per ml of immunserum) were measured using reverse radial immunodiffusion (RID) according to Becker [11] and Durand et al. [12].

Immunosorbent

Specific immunoglobulins against rat AGP were purified by immunoaffinity chromatography using polyacrylamide agarose beads [ACA 22; Industrie Biologique Française (IBF), Villeneuve-la-Garenne, France], coupled to purified rat AGP according to the procedure of Ternynck and Avrameas [13]. Purified antibodies were concentrated at 5 mg/ml and dialysed overnight against 0.1 *M* borate coupling buffer (pH 8.3), containing 0.5 *M* sodium chloride at 4°C. The specific activity, expressed as the ratio between the antibody titre and the immunoglobulin concentration, was 0.11 mg of AGP neutralized per mg of immunoglobulin. Immunosorbent was prepared according to the Pharmacia procedure using 15 g of dried cyanogen bromide-activated Sepharose 4B (Pharmacia, Bois d'Arcy, France) and 260 mg of purified anti-rat AGP antibodies in order to obtain an approximate concentration of 5 mg/ml of gel. The immunoglobulin coupling yield,

calculated from the absorbance at 280 nm, was 99.5%. Immunosorbent was stored in borate coupling buffer with 0.1% sodium azide.

Rat AGP purification

Normal or inflamed rat serum was filtered and applied on the immunosorbent packed into a column (40 cm × 15 mm I.D.) (Pharmacia) at room temperature. The flow-rate (20 ml/h) was chosen in order to obtain a minimum contact time of 30 min between antigen and immobilized antibodies. All the following steps were performed at 4 °C. After washing the immunosorbent with one column volume of 0.2 *M* Tris–HCl (pH 8.2)–0.5 *M* sodium chloride–0.5% Tween 80, then with ten column volumes of washing buffer [0.2 *M* Tris–HCl (pH 8.2)–0.5 *M* sodium chloride at a flow-rate of 40 ml/h, AGP was eluted using 0.2 *M* glycine–HCl buffer (pH 2.6), then immediately neutralized with 1 *M* K_2HPO_4 (one volume per four volumes of eluted solution), concentrated, extensively dialysed against distilled water and freeze-dried. Immunosorbent was regenerated using 0.2 *M* glycine–HCl buffer (pH 2.2)–0.5 *M* sodium chloride (two column volumes), then 0.1 *M* acetate buffer (pH 4.0) (two column volumes) and finally washing buffer (ten column volumes). The column was stored at 4 °C in washing buffer with 0.1% sodium azide.

Determination of α_1-acid glycoprotein

AGP was determined by RID according to Mancini et al. [14]. Rabbit monospecific anti-rat AGP (titre 0.40 mg/ml) was introduced at a 2% concentration in a 1% A 37 indubiose gel (IBF) and rat AGP purified as described previously [9,10] was used as a standard.

Evaluation of the degree of undersialylation

The degree of undersialylation of rat AGP was determined by using simultaneously two immunological methods of AGP quantification, viz., RID and Laurell's monodimensional electroimmunodiffusion (EIDm) [15] as described previously [10]. RID and EIDm were performed in a 1% A 37 indubiose gel containing 1% of anti-rat AGP (titre 0.40 mg/ml) and 4% (w/v) polyethylene glycol (PEG) 6000. The two calibration graphs were obtained with the same dilutions of pure native AGP (ranging from 25 to 100 mg/l).

Purity and identity of AGP preparation

Three methods were used in order to determine the purity and identity of rat AGP.

Crossed electroimmunodiffusion (EIDb). EIDb was performed according to Ganrot [16]. In the first dimension an amount of sample (5 μl) corresponding to about 1.5 μg of AGP was run in a 1% A 37 indubiose gel at 10 V/cm for 1.5 h. Migration in the second dimension was carried out in A 37 indubiose gel containing 4% (w/v) PEG 6000 and 2% of monospecific rabbit anti-rat AGP supplemented or not with 2% of rabbit antiserum to normal or inflamed rat serum at 2.5 V/cm for 16 h.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Electrophoresis was performed with 9% SDS-PAGE according to Laemmli [17]. Proteins were stained with Coomassie blue. Specific characterization of AGP was achieved by immunoblotting with electrotransfer on a Bio-Rad Labs. (Vitry/Seine, France) apparatus and double antibody staining with rabbit anti-rat AGP and gold-labelled goat anti-rabbit immunoglobulins using a commercial kit (Auroprobe TM BL plus; Janssen Pharmaceutica, Beerse, Belgium).

Immunoenzymatic assay of albumin. A 'sandwich'-type enzyme-linked immunosorbent assay (ELISA) was performed to determine possible contamination with rat serum albumin in the purified AGP solution. The polystyrene microtitration plates (Biokema, Nimes, France) were coated with 12.5 μg of purified monospecific rabbit anti-rat albumin in a 0.1 *M* hydrogencarbonate buffer (pH 9.0) and incubated for 4 h at 37°C. After washing with 0.15 *M* sodium chloride–0.5% Tween 20, the wells were filled with 250 μl of purified AGP solution at different dilutions in 0.15 *M* sodium chloride containing 5% (w/v) bovine serum albumin and incubated overnight at 37°C. After four washings, the wells were filled with 250 μl of phosphatase-labelled specific antibodies for a further incubation of 4 h at 37°C. Then after four washings bound conjugate was revealed using *p*-nitrophenyl phosphate at 1 mg/ml in 1 *M* diethanolamine buffer (pH (9.8)–1 mM magnesium chloride. The absorbance at 405 nm was then recorded with a semi-automated spectrophotometer (Bio-Tek Instruments, OSI, Paris, France). Purified rat albumin was used at different concentrations (5–60 μg/l) as a standard.

Crossed immunoaffinoelectrophoresis with concanavalin A (CIAE-Con A)

CIAE-Con A was carried out as described previously [18,19] using concanavalin A (IBF) at a concentration of 200 μg/cm^2 in agarose type II (Sigma, La Verpilliere, France) in the first dimension and sample volumes of diluted sera or purified preparations containing 1.5 μg of AGP.

Carbohydrate composition

Quantitative carbohydrate analysis of purified AGP was performed by gas chromatography (GC) after methanolysis and trifluoroacetylation [20,21]. Sialic acid was also determined by colorimetric thiobarbituric assay according to Warren [22].

RESULTS

Immunosorbent binding capacity and yields of the AGP purification

The theorical binding capacity was determined from the antibody titre (mg of AGP neutralized per ml of antiserum) of purified immunserum determined by reverse RID [10] and the coupling yield. It was 0.5 mg per ml of immunosorbent whereas the experimental binding capacity was only 0.25 mg/ml. The difference may be explained by the loss of antibody-specific activity during the coupling procedure and/or by steric hindrance on the support. Using such a support, the final AGP purification yield was about 80%.

Purity of the AGP preparation

The identity of the isolated AGP was checked using SDS-PAGE (Fig. 1) and crossed electroimmunodiffusion (Fig. 2). AGP purified from normal rat showed an apparent molecular mass of 47 000 ± 3000, whereas the AGP pattern from inflamed rat gave two different bands, a major band of 43 000 ± 1000 and a minor band of 39 000. Applying immunoblotting after SDS-PAGE, the same electrophoresis patterns were found for serum and the corresponding purified AGP, indicating neither a change in the apparent molecular mass nor glycan alteration during the immunoaffinity procedure. Compared with the corresponding normal sera (Fig. 2A) the EIDb patterns obtained with purified AGP (Fig. 2B) did not show evidence of any contamination by other serum proteins. In order to evaluate in the purified solution the level of serum albumin, which is the major plasma protein and in fact could be the main contaminant, RID using rat albumin antisera was performed. No albumin was detected by this method. Nevertheless, using a more sensitive ELISA technique, we were able to detect less than 0.6% (w/w) albumin in the purified AGP.

As can be seen in Fig. 3, there was no change in the CIAE-Con A patterns before and after purification of normal or inflamed rat AGP, indicating preservation of the relative proportions of the different glycan variants during the purification steps. The ratio of the reactive form of Con A (Fig. 3, peak 4) to total AGP variants (peaks 1, 2, 3 and 4) was 0.33 ± 0.03 and 0.65 ± 0.08 for normal and inflamed rat, respectively, for both serum and purified AGP.

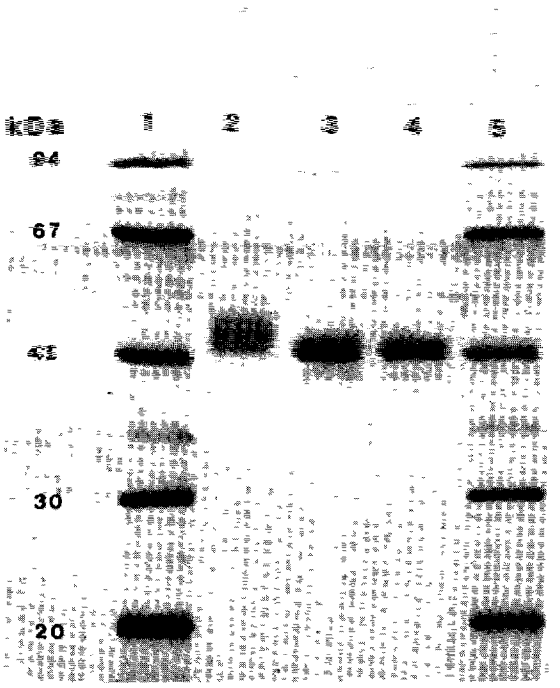
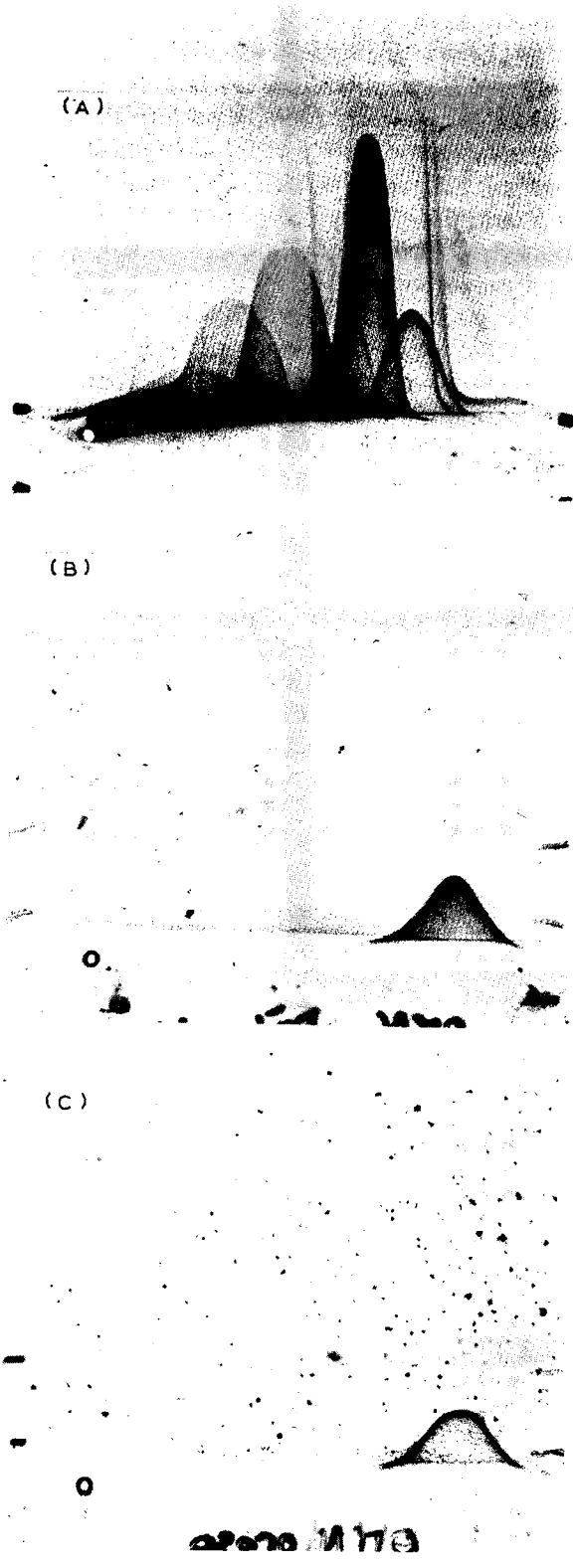


Fig. 1. SDS-PAGE of AGP purified from normal (AGPn) and inflamed (AGPi) rat serum. Lane 2, AGPn; lanes 3 and 4, AGPi; lanes 1 and 5, molecular mass markers (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase and soybean trypsin inhibitor).


(A)
(B)
O
(C)
O

No desialylation occurred during the purification, as demonstrated by an immunochemical procedure published elsewhere [10]. Both RID and EIDm were used for the determination of AGP concentration before and after purification. No underestimation by EIDm relative to RID was found, indicating a lack of desialylation of AGP during the immunoaffinity procedure.

Determination of specific absorbance ($A^{1\%}_{1\,cm}$)

Absorbance measurements were made at 278 nm on a Cary 118 instrument (Varian, Palo Alto, CA, U.S.A.) on three different preparations that had been extensively dialysed against water and freeze-dried before being dried to constant weight in vacuo over phosphorus pentoxide. The $A^{1\%}_{1\,cm}$ values at 278 nm for normal and inflamed rat AGP were 5.85 and 6.33, respectively. These results were higher than the value of 4.16 reported by Charlwood et al. [23].

Carbohydrate analysis

The results of carbohydrate analysis of purified rat AGP from normal and inflamed sera are given in Table I. Compared with normal AGP, the carbohydrate

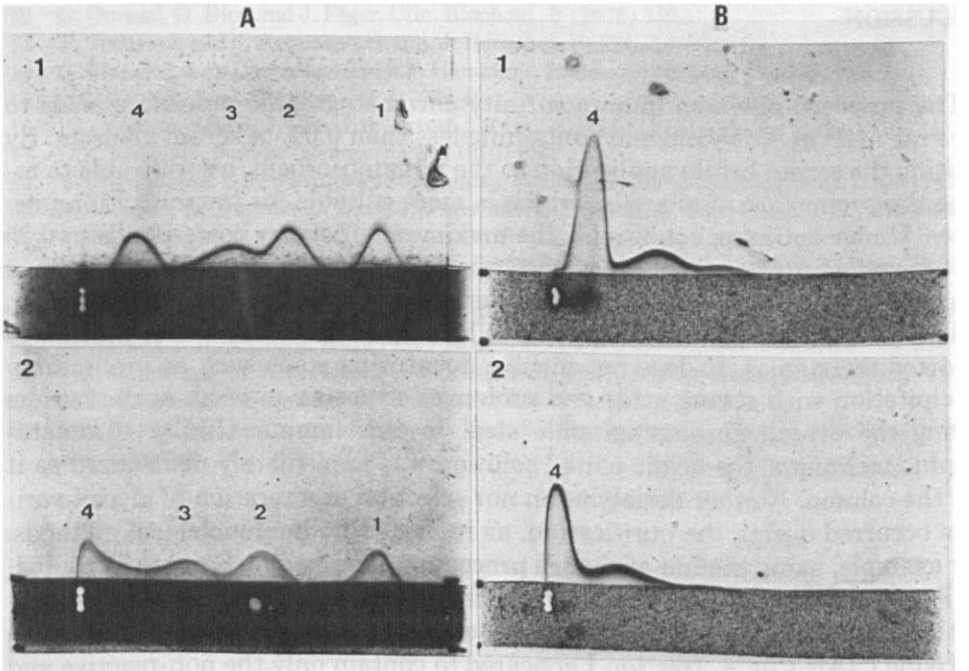


Fig. 3. Crossed immunoaffinoelectrophoresis with concanavalin A of (1) unpurified and (2) purified AGP from (A) normal and (B) inflamed rats. The percentages of isotype 4 were 0.33 ± 0.03 and 0.65 ± 0.08 for normal and inflamed rat AGP, respectively.

Fig. 2. Crossed electroimmunodiffusion (EIDb) of purified AGP from normal rats (B and C) and corresponding pooled sera (A). EIDb was performed as described in the text using total serum proteins antiserum (A and B) supplemented with α_1-acid glycoprotein antiserum (A, B and C).

TABLE I

CARBOHYDRATE COMPOSITION OF AGP ISOLATED FROM NORMAL RAT SERA (AGPn) AND FROM INFLAMED RAT SERA (AGPi)

Sample	Molar ratio[a]				
	Fuc	Gal	Man	GlcNAc	NeuAc
AGPn	0.23 ± 0.01	2.61 ± 0.03	3	3.00 ± 0.08	3.54 ± 0.15
AGPi	0.08 ± 0.01	2.24 ± 0.20	3	2.96 ± 0.21	3.18 ± 0.02

[a]Calculated on the basis of three mannose per oligosaccharide (mean ± S.D., $n=6$).

composition of inflamed AGP, which is described here for the first time, showed a significant decrease in fucosyl residues with a slight decrease in both galactosyl and sialyl residues. Considering the molar ratio of galactosyl and sialyl residues, we observed about 0.9 supplementary sialyl residue per oligosaccharide. This suggests the simultaneous presence of two sialyl residues for some outer chains, confirming the data of Yoshima et al. [3].

DISCUSSION

The proposed one-step immunoaffinity chromatographic procedure leads to pure rat AGP in 80% yield and containing less than 0.6% of serum albumin. By diluting the serum before application to the immunosorbent, we were able to reduce the proportion of albumin, which is more suitable for immunization purposes. Under optimum conditions, the maximum efficiency corresponds to 0.25 mg of purified AGP per ml of immunosorbent, so that 10 mg can be purified in a single pass, which is satisfactory for further structural studies of the glycoprotein.

This purification procedure appears to have some advantages over previously reported techniques. It does not involve denaturing steps such as preliminary precipitation with strong acids and prolonged exposure to weak acidic buffers during the overall chromatographic step. In this immunoaffinity chromatographic technique, the acidic eluted solution was immediately neutralized as it left the column. Neither desialylation nor selection or alteration of glycan variants occurred during the purification, as indicated by immunological methods. For example, using the ion-exchange procedure described by Shibata et al. [5], AGP was obtained from phenobarbital-treated rat pooled sera with carboxymethylcellulose in two fractions: a non-retained fraction I and a retained fraction II. Using CIAE-Con A, fraction I appeared to contain only the non-reactive and weakly reactive forms whereas the fraction II contained only the reactive form. By adding the two patterns, we were able to reconstitute the pattern of the native AGP (unpublished results).

The absence of modification or selection in the glycan structure is essential for structural and biological studies, as it has been shown that the immunomodulatory role of human AGP probably depends on its glycan structure [24,25].

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