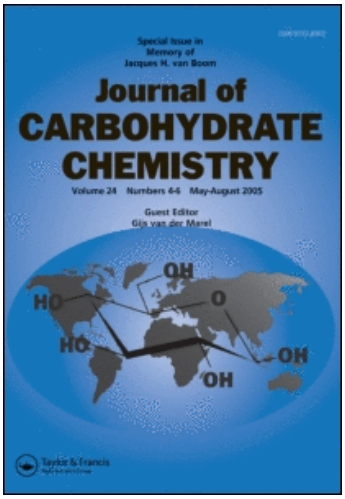

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THE NON-DEGRADATIVE ISOLATION OF α_1-ACID GLYCOPROTEIN FROM NORMAL AND RHEUMATOID PLASMA[1]

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ABSTRACT

We have developed a method for the purification of α_1-acid glycoprotein (AGP) using procedures unlikely to damage the glycoprotein structure. This was utilised to isolate AGP from samples of normal and rheumatoid plasma. The effectiveness of the purification procedure was examined by enzymatically deglycosylating each sample of AGP, separating the released oligosaccharides by chromatography on a pellicular high pH anion-exchange (HPAE) resin at pH 13 and detecting by a pulsed electrochemical (PED) method. The analytical profile for normal AGP was consistent with those previously reported thus indicating that the purification procedure did not denature the oligosaccharide chains of AGP; there was a noticeable difference between AGP in normal and rheumatoid plasma.

INTRODUCTION

α_1-Acid glycoprotein (AGP) is a major acute phase glycoprotein with five asparaginyl-linked complex oligosaccharide chains accounting for approximately 45% of the molecular weight.[2] In normal plasma, AGP exists as a heterogeneous population of glycoforms due to different ratios of N-linked complex oligosaccharide chains. These chains differ in size, degree of branching (di-, tri- or tetra- antennary), monosaccharide composition and extent of sialylation[3] and are the likely sources of the macromolecule's structural heterogeneity.

During several physiological and pathological conditions, not only is the total concentration of AGP altered[4] but the relative proportions of the normal AGP glycoforms have been found to change and abnormal glycoforms have been identified.[5-8] The populations of AGP glycoforms in RA are characterised by a decreased number of diantennary chains[8] and an increase both in the absolute amount of glycoforms containing fucose and in the number of fucose residues per molecule.[9]

The key to studying the structural heterogeneity of AGP is to develop a procedure that will isolate AGP without structural degradation. Earlier studies of the immunomodulatory, drug-binding and conformational properties of AGP have yielded inconclusive data because the methods of isolation which were employed caused damage to their oligosaccharide and polypeptide components.[10] AGP was isolated from normal and rheumatoid plasma by a method which avoids the desialylation and denaturation of AGP by using polyethylene glycol precipitation and purifying the mixed glycoform fraction by ion-exchange and dye-ligand chromatography. The efficiency of the method was verified using high pH anion-exchange (HPAE) chromatography and a pulsed electrochemical detection method (PED) which gave an oligosaccharide fingerprint for each AGP sample after enzymatic deglycosylation. The profile for AGP from normal sera was identical to published profiles which indicates that our purification procedure does not degrade the oligosaccharide chains of AGP. The rheumatoid trace differed indicating a disease specific oligosaccharide heterogeneity which may be functionally significant.[11,12]

RESULTS AND DISCUSSION

AGP was isolated from normal and rheumatoid plasma by a low pressure chromatographic isolation method which avoids desialylation and denaturation by using ion-exchange and dye-ligand chromatography. Figure 1 illustrates the traces from the successive stages of the AGP purification procedure. The average yields of AGP were 0.87 ± 0.125 mg/mL for normal human plasma and 1.39± 0.835 mg/mL for rheumatoid plasma which agree with published values.[10] The efficiency of the purification method was monitored using immunodiffusion (Figure 2). The initial plasma sample and the supernatant resulting from PEG precipitation (Figure 2; Well 1) both contained detectable quantities, by immunodiffusion, of serum albumin, AGP and α_1-antitrypsin. The albumin was selectively removed from the sample through its binding to Reactive Blue Sepharose; the non-bound fraction was negative for albumin by immunodiffusion (Figure 2; Well 2). Anion-exchange chromatography on Q-Sepharose fast flow partially

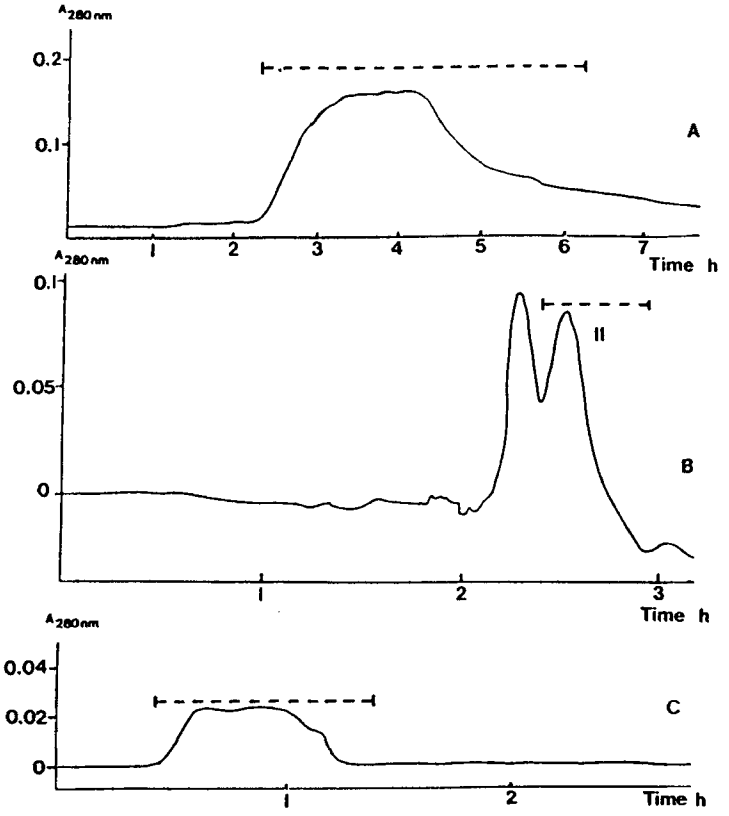

Figure 1. The three step isolation of AGP from plasma by low pressure chromatography using (A) Reactive Blue Sepharose (B) Q Sepharose Fast Flow and (C) Red Sepharose. The fraction collected is indicated by ---------.

separates AGP from the other components of the mixture, the most notable of which was α_1-antitrypsin (Figure 2; Well 3). Complete purification required the use of the Red Sepharose column (Figure 2; Well 4). Our three step purification therefore improves on previous work[13] by inserting an anion exchange step between the two dye ligand columns in order to separate completely AGP from α_1-antitrypsin.

The efficiency of the purification process was investigated further using gel electrophoresis to confirm that AGP was completely purified from other plasma components and that its molecular weight corresponded to that expected for the fully sialylated (and thus nondegraded) glycoprotein. Reference to Figure 3 shows that a darkly stained band corresponding to the molecular weight of AGP (41 kDa) is present at all the stages of sampling except in the precipitate from 40% PEG precipitation (Lane 3).

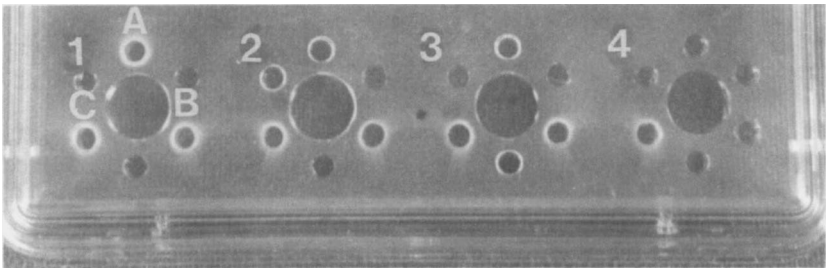

Figure 2. Determination of the efficiency of purification by immunodiffusion. The central wells contained (1) PEG supernatant (2) Unbound Reactive Blue Sepharose peak (3) Second peak eluted on Q Sepharose Fast Flow and (4) Unbound Red Sepharose peak. Each well was surrounded by antibody wells containing antisera to albumin (A), α_1-antitrypsin (B) and AGP (C) respectively.

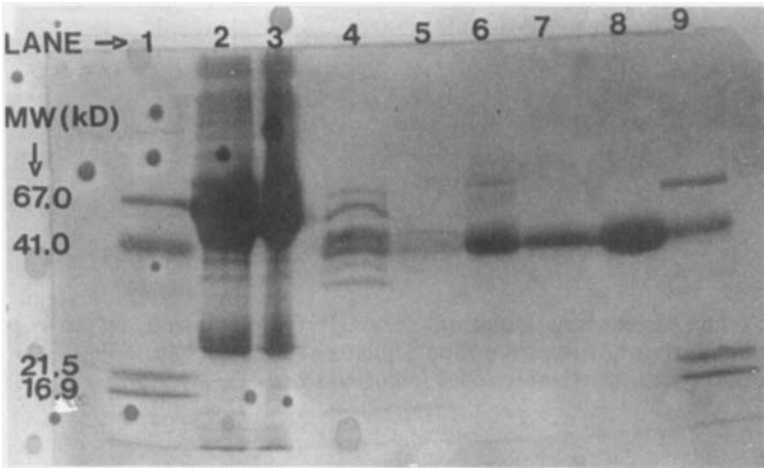

Figure 3. SDS-PAGE analysis of selected fractions from each stage of the purification process. The lanes contained (1) Molecular Weight Standards (2) Whole plasma (3) Precipitate from 40% PEG precipitation (4) Supernatant from 40% PEG precipitation (5) Unbound Reactive Blue Sepharose peak (6) Second peak eluted on Q Sepharose Fast Flow and (7) Unbound Red Sepharose peak (8) Unbound Red Sepharose peak (concentrated) (9) Molecular weight standards.

A 67.0 kDa band is not present after lane 4 thereby indicating that human serum albumin is completely removed by passage through Reactive Blue Sepharose. Only one protein band is present in the lanes (7 and 8) corresponding to the unbound peak eluted from the Red Sepharose confirming that it contained exclusively AGP.

The validity of our isolation procedure, in producing nondegraded oligosaccharide structures was determined using high pH anion-exchange (HPAE)

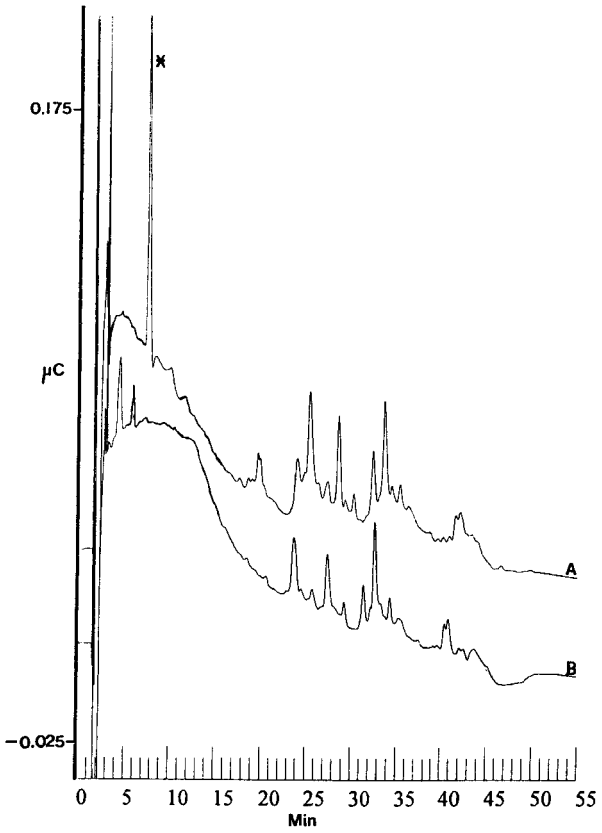

Figure 4. HPAE-PED profiles for the oligosaccharides released from (A) 100 μg AGP standard (B) 100 μg normal plasma by our purification method. Conditions are given in the Methods section.

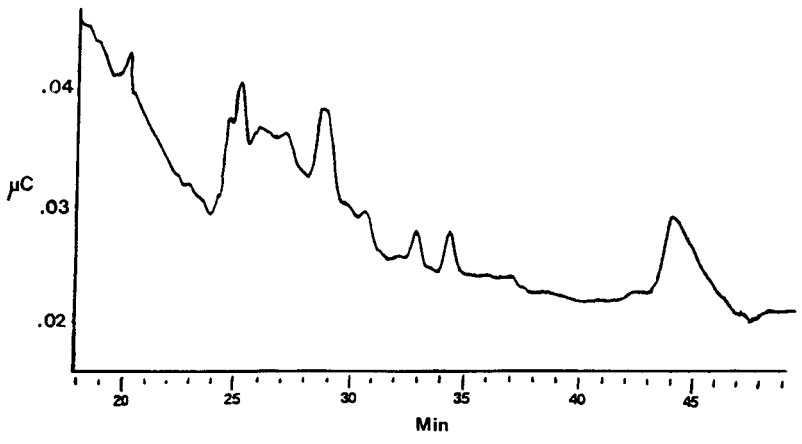

Figure 5. HPAE-PED profile for the oligosaccharides released from 10 μg of rheumatoid AGP. Conditions are given in the Methods section.

chromatography to provide an oligosaccharide map for each AGP preparation, by resolving the total oligosaccharide into the linkage and branch isomers of neutral and anionic oligosaccharides.[14] Separation by HPAE-PED is dependent upon the number of charges per oligosaccharide chain which normally correlates to the number of sialic acid (SA) residues, i.e. 20-30 min (2 SA normally diantennnary), 30-40 min (3 SA normally triantennary) and 40-50 min (4 SA normally tetraantennary). Thereafter, each sialylation band is further resolved on the basis of subtle isomeric differences such as the linkage between galactose and *N*-acetylglucosamine on the outer branches.[15] When used in conjunction with pulsed electrochemical detection (PED), HPAE can detect oligosaccharides down to picomole sensitivity without pre- or post column derivatisation.[14]

Samples of AGP were purified from normal and rheumatoid arthritis plasma and separately deglycosylated, along with a commercially available AGP (for comparison), using the enzyme PNGase F. The released oligosaccharides were separated using HPAE-PED (Figure 4 and 5). The oligosaccharide profiles in Figure 4 represent the oligosaccharides released from 100 μg of a commercially available AGP preparation (4A) and 100 μg of AGP purified from normal plasma by our purification method (4B). Figure 5 represents the oligosaccharides released from 10 μg of rheumatoid AGP. The analytical profile for normal AGP was consistent with those previously reported[16,17] thus indicating that the purification procedure did not degrade the oligosaccharide chains of AGP. Significantly, the profile from the commercial standard contains a peak at 7 min (asterisked in Figure 4A) which corresponds to sialic (*N*-acetylneuraminic) acid. In other words, the methods used to purify this AGP have resulted in partial desialylation. The glycosylation pattern for rheumatoid AGP (Figure 5) is significantly different from the normal trace both in the amount and identity of the oligosaccharide structures.

CONCLUSION

In this paper we report a method for the purification of AGP, using procedures unlikely to damage the glycoprotein structure, which was utilised to isolate AGP from samples of normal and rheumatoid plasma. The effectiveness of the purification procedure was examined by enzymatically deglycosylating each sample of AGP and separating the released oligosaccharides by chromatography on a pellicular high pH anion-exchange (HPAE) resin at pH 13. The analytical profile for normal AGP was consistent with those previously reported thus indicating that the purification procedure did not denature the oligosaccharide chains of AGP. Additionally, there was a noticeable difference between the profiles for AGP from normal and rheumatoid plasma.

EXPERIMENTAL

Materials . Units of fresh frozen plasma (three samples) were kindly supplied by Dr. Robin Fraser of the Scottish National Blood Transfusion Service (Law Hospital, Lanarkshire, U.K.); these donations had been found to be negative for the presence of hepatitis B surface antigen and antibodies to HIV and HCV. The plasma from rheumatoid arthritis patients (six samples) was a kind gift of Dr. Max Field of the Centre for Rheumatic Diseases at Glasgow Royal Infirmary. All chemicals, chromatographic media and immunobiologicals were purchased from Sigma, Poole, U.K. unless specified.

Precipitation of plasma by polyethylene glycol (PEG). The procedure was a modification of the method of Ingham.[18] PEG 3350 was very gradually added to the plasma, with constant magnetic stirring, to give a final concentration of 40% PEG (w/v). The solution was left stirring at room temperature for one hour then decanted into 50 mL capacity centrifuge tubes and left overnight at 4 °C. The centrifuge tubes were balanced, capped and centrifuged in the 8x50 angle rotor of a MSE Hi-spin centrifuge at 20000 g and 4 °C for 30 min. The resulting supernatants were decanted and used as the starting material for the column chromatography purification stage.

Column chromatography. All chromatographic separations were performed using an LKB 12000 Varioperspex peristaltic pump preset to give a flow rate of 1.0 mL/min and a single path UV monitor (Pharmacia, Milton Keynes, U.K.) at 280 nm. The supernatants obtained by the PEG precipitation of the plasma were individually loaded onto a XK26 column (40 x 0.16 cm I. D., Pharmacia), packed with Reactive Blue Sepharose, which had been equilibrated with 0.05M Tris/0.1M potassium chloride pH 7/0.02% sodium azide. Buffer was then washed through the column to elute all of the protein except for the human serum albumin which bound to the gel. The latter was eluted by washing the column with buffer containing 0.5M potassium thiocyanate. Figure 1A illustrates the typical profile for both normal and rheumatoid plasma. The non-bound peak of each sample was individually loaded onto an XK26 column, packed with Q Sepharose Fast Flow, which had been equilibrated with 20mM Tris/0.075M sodium chloride pH6.5. A salt gradient (0.075M to 0.5M sodium chloride) was then passed down the column. The second of the three major peaks obtained (Figure 1B) was found, by immunodiffusion, to contain AGP (see below). The latter peak from each sample was individually loaded onto an XK26 column, containing Red Sepharose (Pharmacia), which had been equilibrated with 0.03M sodium acetate pH 5.7. A broad peak eluted immediately and was found, by immunodiffusion, to contain AGP (see below).

Immunodiffusion. The location of AGP in the isolation procedure was identified by immunodiffusion on Ouchterlony plates (ICN Biochemicals, High Wycombe,U.K.). Aliquots of samples obtained from various stages of the purification procedure were placed individually in separate antigen wells cut on an agarose plate. Each was surrounded by antibody wells containing antisera to AGP, α_1-antitrypsin and albumin respectively. The presence of AGP in a sample was indicated by the appearance of a white line of precipitation between the wells containing the sample and the antibodies to AGP.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed using 20cm x 20cm slabs and an 8-18% acrylamide gradient resolving gel/6% acrylamide stacking gel according to the method of Laemmli.[19] The samples were prepared by boiling for 2 min in Laemmli sample buffer. Each sample was added separately to the gel which was run at 200V for approximately 2 h in Tris glycine buffer pH 8. The gel was stained with Coomassie Blue for 1 h then destained. The molecular weight markers were injected as a 1 mL solution containing 200 μg myoglobin (16.9 kDa), 200 μg α_1-antitrypsin (21.5 kDa), 400 μg AGP (41.0 kDa) and 200 μg human serum albumin (67.0 kDa).

Desalting. The purified AGP was desalted prior to glycosidase digestion. Each sample was dissolved in the minimum quantity of HPLC water and transferred in 400 μL aliquots to the sample chamber of 30,000 MW cut off sterile microspin filters (Anachem, Luton, U.K.). After centrifugation at 2000 g for 10 min, 250 μL of HPLC water was added to the filter to dissolve the salt free AGP sample. The whole process was repeated for the remainder of the original solution and the aliquots were combined and dried down.

Glycosidase digestions. Each AGP sample was deglycosylated by treatment with the enzyme peptide: N glycosidase F (PNGase F, Oxford Glycosystems, Oxford. U.K.) which catalyses the hydrolysis of the N,N^1-diacetylchitobiose bond adjacent to the asparagine residues of all mammalian N-linked oligosaccharides. Complete deglycosylation required prior denaturation of the polypeptide secondary structure by heat then digestion with PNGase F in 20mM sodium phosphate pH 7.5 buffer at 37 °C for 48 h. Initially, 2U of the enzyme was added with a further unit added after 24 h. The released oligosaccharides were separated from polypeptide material by ethanol precipitation.[20] Hydrolysis of the deglycosylated protein with 2M trifluoroacetic acid (100 °C for 4 h) and subsequent analysis by HPAE-PED[14] demonstrated the complete absence of monosaccharides. Hence deglycosylation is complete.

High pH anion-exchange (HPAE) chromatography. The oxyanion forms of the oligosaccharides were separated by chromatography on a pellicular HPAE

resin at pH 13 and detected by a pulsed electrochemical method[14] using the DX-300 chromatography system supplied by Dionex (Camberley, U.K.). The latter system consisted of an advanced gradient pump, pulsed electrochemical detector (PED-2) and an anion micro-membrane suppressor (AMMS-MPIC) controlled via an advanced computer interface by a Vtech 486SX 25 (Viglen, U.K.) using AI-450 software. The oligosaccharide sample was applied to a CarboPac PA-100 column (25 x 0.4 cm I.D., Dionex) equilibrated with a mixture of 10% solvent A (1M sodium hydroxide)/5% solvent B (1M sodium acetate)/85% solvent C (HPLC Grade Water; Rathburn, Murrayburn, U.K.). This solvent elution was continued for 10 min after which a linear gradient of 10%A/5%B/85%C to 10%A/20%B/70%C was achieved over 40 min. The limit solvent was continued for a further 5 min before the column was regenerated by moving to 50%A/50%C in 1 min and holding for a further 10 min. Thereafter, the initial conditions were resumed. The flow-rate was 1.0 mL/min at room temperature. Detection was by a pulsed electrochemical detector using the following pulse potentials and durations : Time = 0s, E = +0.05V; 0.29s, +0.05V; 0.49s, +0.05V; 0.50s, +0.05V; 0.51s, +0.6V; 0.6s, +0.6V; 0.61s, -0.6V; 0.65s, -0.6V; 0.66s, +0.05V.

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