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Heterogeneity of α_1-acid glycoprotein in rheumatoid arthritis

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Abstract

α_1-Acid glycoprotein (AGP) or orosomucoid is a major serum glycoprotein, of unknown physiological function, which is classified as one of the positive acute phase reactants since its plasma concentration becomes elevated two- to five-fold in certain disease states. Additionally, the proportions and identities of the five asparaginyl-linked complex oligosaccharide chains are altered during several physiological and pathological conditions, which may be functionally significant. The key to studying the structural heterogeneity of AGP is to develop a procedure that will isolate AGP without structural degradation. We have developed 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-performance anion-exchange (HPAE) resin at pH 13. The analytical profile for normal AGP was consistent with that 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.

1. Introduction

Protein glycosylation is a major modification which occurs during and after translation and is characterised by extensive structural heterogeneity. In particular, glycosylated variants (glycoforms) of a single protein may be present which reflect the original source of the molecule and the particular physiological and biochemical conditions that existed in the body at the time of release. It is now recognised that the oligosaccharide chains of glycoproteins are important determinants of biological function (reviewed in Ref. [1]).

α_1-Acid glycoprotein (AGP) is a heavily glycosylated major component of blood plasma which has been extensively studied [2,3] but the physiological role of which is poorly understood. AGP isolated from the plasma of healthy people is heterogeneous. Isoforms may result from amino acid substitutions and due to glycoforms which contain varying ratios of the five types of oligosaccharide chain found in AGP [4,5]. These chains differ in size, degree of branching (bi-, tri- or tetra-antennary), monosaccharide composition and extent of sialylation and may be the likely sources of the macromolecule's structural

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and conformational heterogeneity [6]. In particular, individual glycoforms of AGP may have functional diversity in, for example, the processes of immunomodulation [7]. Injury to tissues causes the secretion of cytokines which regulate the expression of genes for AGP in hepatocytes [8] and thus, during several physiological and pathological conditions, the total concentration of AGP is increased by 2- to 5-fold [9]. In rheumatoid arthritis (RA), the major inducers of AGP synthesis appear to be tumour necrosis factor α [10] and interleukins -1 and -6 [11] which have both been found in the inflamed synovium. Additionally, the relative proportions of AGP glycoforms are altered in disease [12–15]. The populations of AGP glycoforms in RA are characterised by a decreased number of biantennary chains [15] and an increase both in the absolute amount of glycoforms containing fucose and in the number of fucose residues per molecule [16].

The key to studying the structural heterogeneity of AGP is to isolate the glycoprotein from plasma without structural degradation. Earlier studies of the immunomodulatory, drug-binding and conformational properties of AGP glycoforms have yielded inconclusive data. One explanation is that the methods of isolation which were employed caused damage to the oligosaccharide and polypeptide components [17]. 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-performance 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.

2. Experimental

2.1. Materials

Units of fresh frozen pooled normal human plasma were kindly supplied by Dr. Robin Fraser of the Scottish National Blood Transfusion Service (Law Hospital, Lanarkshire, UK); 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 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, UK) unless specified.

2.2. Precipitation of plasma by polyethyleneglycol (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 8×50 angle rotor of a MSE Hi-spin centrifuge at 20 000 g and 4°C for 30 min. The resulting supernatants were decanted and used as the starting material for the column chromatography purification stage.

2.3. Column chromatography

All chromatographic separations were performed using a 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, UK) at 280 nm.

The supernatants obtained by the PEG precipitation of the plasma were individually loaded onto a XK26 column (40×0.16 cm I.D.; Pharmacia), packed with Reactive Blue Sepharose,

which had been equilibrated with 0.05 *M* Tris–0.1 *M* 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 0.5 *M* potassium thiocyanate. Fig. 1A illustrates the typical profile for both normal and rheumatoid plasma. The non-bound peak of each sample was individually loaded onto a XK26 column, packed with Q Sepharose Fast Flow, which had been equilibrated with 20 m*M* Tris–0.075 *M* sodium chloride pH 6.5. A salt gradient (0.075 *M* to 0.5 *M* sodium chloride) was then passed down the column. The second of the three major peaks obtained (Fig. 1B) was found, by immunodiffusion, to contain AGP (see below). The latter peak from each sample was individually loaded onto a XK26 column, containing Red Sepharose (Pharmacia), which had been equilibrated with 0.03 *M* sodium acetate pH 5.7. A broad peak eluted immediately and was found, by immunodiffusion, to contain AGP (see below).

2.4. Immunodiffusion

The location of AGP in the isolation procedure was identified by immunodiffusion on Ouchterlony plates (ICN Biochemicals, High Wycombe, UK). 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 e.g. 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.

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

SDS-PAGE was performed using 20 × 20 cm slabs and a 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 200 V for approximately 2 h in Tris-glycine buffer pH 8. The gel was stained with Coomassie Blue for 1 h then destained. The molecular mass 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 α_1-acid glycoprotein (41.0 kDa) and 200 μg human serum albumin (67.0 kDa).

2.6. Desalting

The purified AGP was desalted prior to glycosidase digestion. Each sample was dissolved in the minimum quantity (1–2 ml) of HPLC water and transferred in 400-μl aliquots to the sample chamber of 30 000 M_r cut-off sterile microspin filters (Anachem, Luton, UK). 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.

2.7. Glycosidase digestions

Each AGP sample was deglycosylated by treatment with the enzyme peptide: N-glycosidase F (PNGase F; Oxford Glycosystems, Oxford, UK) 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 denaturation of the polypeptide secondary structure by heat; the oligosaccharides released were separated from polypeptide material by ethanol precipitation [20].

2.8. High-performance anion-exchange chromatography (HPAE)

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 [21] using the DX-300 chromatography system supplied by

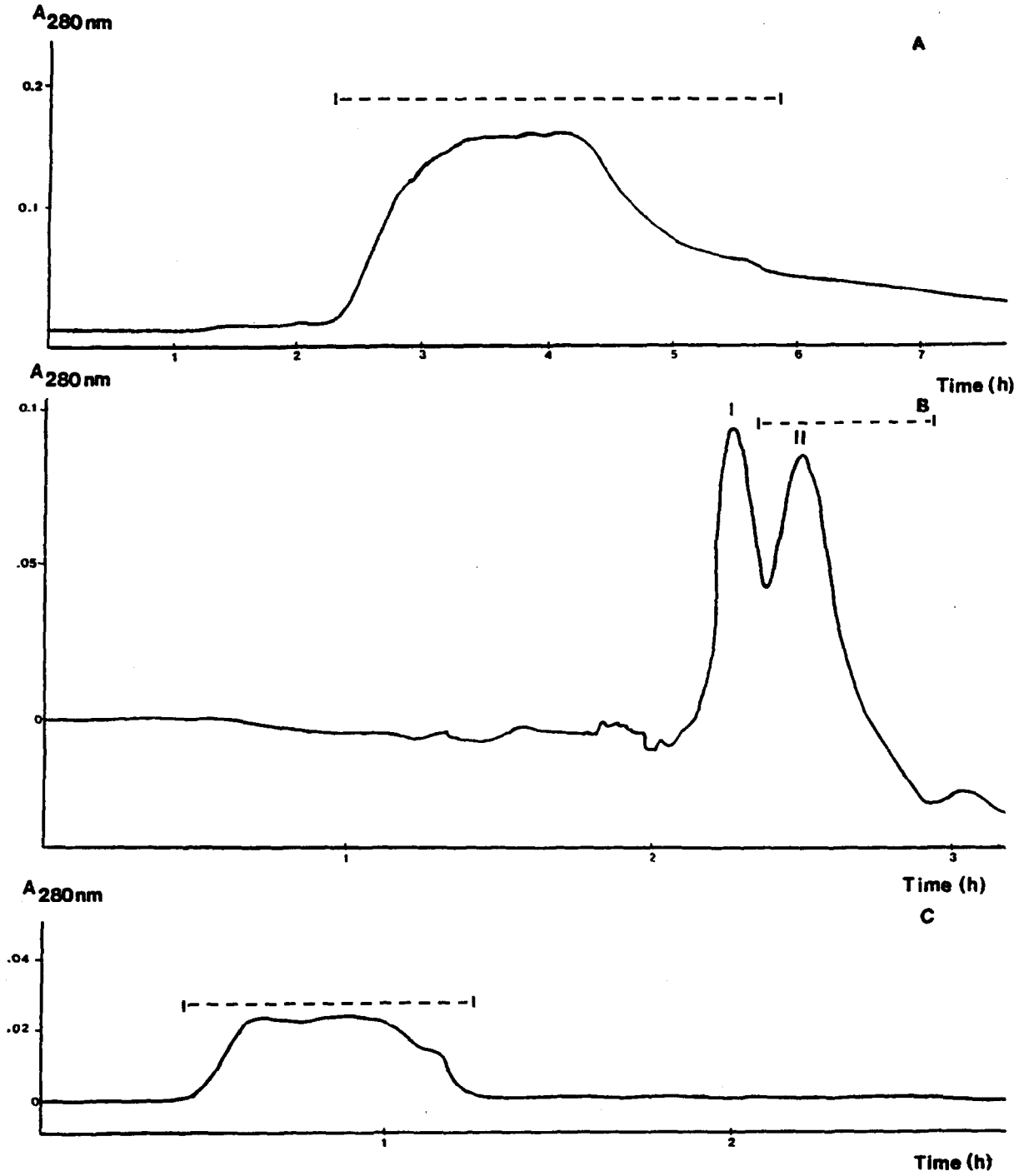

Fig. 1. The three-step isolation of AGP from plasma by low-pressure chromatography using (A) Reactive Blue Sepharose, (B) Q Sepharose Fast Flow, and (C) Red Sepharose. The horizontal dotted line above each trace indicates where fractions were collected.

Dionex (Camberley, UK). 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, UK) using AI-450 software. The oligosaccharide sample was applied to a CarboPac PA-100 column (25 × 0.4 cm I.D.; Dionex) equilibrated with a mixture of 10% solvent A (1 *M* sodium hydroxide)–5% solvent B (1 *M* sodium acetate)–85% solvent C (HPLC grade water; Rathburn, UK). 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 final conditions were continued for a further 5 min before the column was regenerated by changing 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 = 0 s, E = +0.05 V; 0.29 s, +0.05 V; 0.49 s, +0.05 V; 0.50 s, +0.05 V; 0.51 s, +0.6 V; 0.6 s, +0.6 V; 0.61 s, −0.6 V; 0.65 s, −0.6 V; 0.66 s, +0.05 V.

3. Results

3.1. Purification of AGP

Fig. 1 shows the traces from the successive stages of AGP purification. The efficiency of the purification method was monitored using immunodiffusion (Fig. 2). The initial plasma sample and the supernatant resulting from PEG precipitation both contained detectable quantities, by immunodiffusion, of serum albumin, AGP and α_1-antitrypsin (Fig. 2; wells 1 and 5). 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 (Fig. 2; well 6). Anion-exchange chromatography on Q-Sepharose fast flow did not completely separate AGP from the other components of the mixture, the most notable of which was α_1-antitrypsin (Fig. 2; well 7); complete purification required the use of the Red Sepharose column (Fig. 2; well 8).

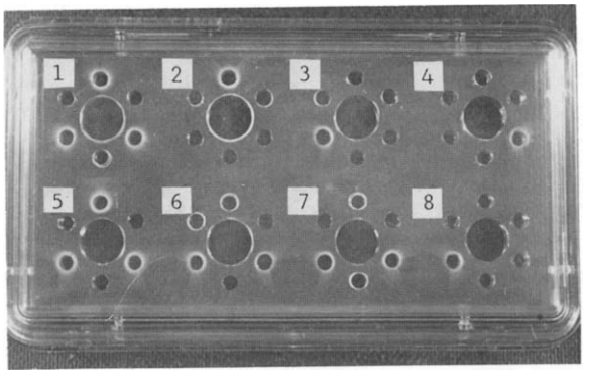

Fig. 2. Determination of the efficiency of purification by immunodiffusion. The central wells contained (1) original plasma, (2) standard human albumin, (3) standard AGP, (4) standard α_1-antitrypsin, (5) PEG supernatant, (6) unbound Reactive Blue Sepharose peak, (7) second peak eluted on Q Sepharose Fast Flow and (8) unbound Red Sepharose peak.

The efficiency of the purification process was also checked using SDS-PAGE. Reference to Fig. 3 shows that a darkly stained band corresponding to the molecular mass of AGP (41 kDa) is present at all the stages of sampling

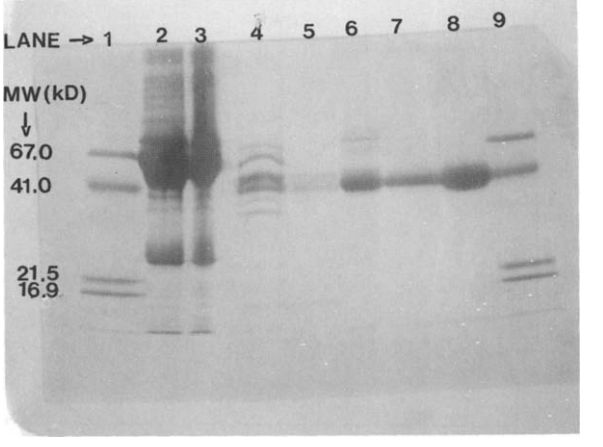

Fig. 3. SDS-PAGE analysis of selected fractions from each stage of the purification process. The lanes contained (1) molecular mass standards, (2) whole plasma, (3) precipitate from 40% PEG precipitation, (4) supernatant from 40% PEG precipitation, (5) unbound Reactive Blue Sepharose peak, (6) second peak eluted on Q Sepharose Fast Flow, (7) unbound Red Sepharose peak, (8) unbound Red Sepharose peak (concentrated), and (9) molecular mass standards.

except in the precipitate from the 40% PEG precipitation (lane 3). 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.

3.2. Yield of AGP

The average yield of AGP was 0.87 ± 0.125 mg/ml for four isolations from pooled normal human plasma and 1.39 ± 0.835 mg/ml for seven isolations from individual samples of rheumatoid plasma.

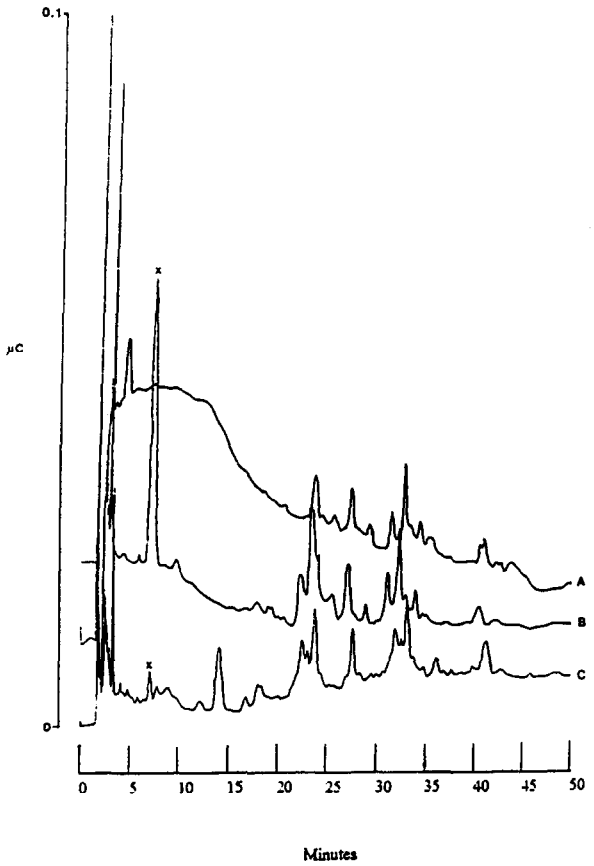

Fig. 4. HPAE-PED profiles for the oligosaccharides released from (A) 100 μg normal plasma by our purification method, (B) 100 μg AGP standard, (C) 50 μg standard AGP oligosaccharide library. Conditions are given in the Experimental section.

3.3. High-performance anion-exchange chromatography

Samples of AGP were purified from normal and rheumatoid arthritis plasma and separately deglycosylated, along with a commercially available AGP, using the enzyme PNGase F. The released oligosaccharides were separated using HPAE-PED (Fig. 4) and their traces contrasted with each other and a commercially available AGP oligosaccharide standard. Fig. 5 shows the profile for the oligosaccharides of rheumatoid AGP.

The oligosaccharide profiles in Fig. 4 represent the oligosaccharides released from 100 μg of AGP purified from normal plasma (trace A), 100 μg of a commercially available AGP preparation (trace B) and 50 μg of a standard AGP oligosaccharide library (Oxford Glycosystems, Oxford, UK) (trace C). Fig. 5 represents the oligosaccharides released from 10 μg of rheumatoid AGP.

4. Discussion

The natural heterogeneity of the oligosaccharides of glycoproteins requires that chromatography plays an essential part in the isolation and structural elucidation. The key to studying the structural heterogeneity of AGP, from different sources, is to isolate the glycoprotein from plasma without structural degradation; more specifically to avoid exposure to strongly acidic conditions which causes desialylation. Many methods for the isolation and purification of AGP have been reported (reviewed in Ref. [16]) which have resulted in AGP preparations with different physical-chemical properties due to structural degradation. The method of Succari et al. [22] avoided the latter and was also advantageous in that it kept the number of stages involved to a minimum. Other recent successful procedures have utilised various affinity adsorbents [23,24].

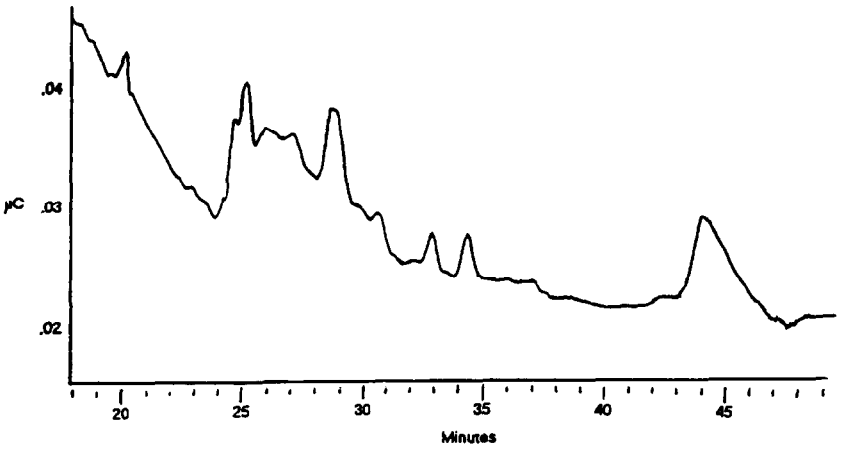

Fig. 5. HPAE-PED profile for the oligosaccharides released from 10 μg of rheumatoid AGP. Conditions are given in the Experimental section.

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. 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. These values agree with published values [17]. The efficiency of the purification process was determined using both immunodiffusion (Fig. 2) and SDS-PAGE (Fig. 3) to confirm that AGP was isolated without contamination from other serum proteins.

The validity of our isolation procedure, in producing non-degraded oligosaccharide structures was determined using high pH anion-exchange (HPAE) 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 [21]. When used in conjunction with pulsed electrochemical detection (PED), HPAE can detect oligosaccharides down to picomole sensitivity without pre- or post-column derivatisation [21]. HPAE-PED profiles were generated for the oligosaccharides released from AGP isolated from normal and rheumatoid plasma (by our purification method) and from a commercial (Sigma) standard.

The HPAE analytical profile for normal AGP (Fig. 4A) was consistent with those previously reported [25] and those for commercially available AGP (Fig. 4B) and an AGP oligosaccharide library (Fig. 4C). This indicates that our purification procedure isolated AGP intact and did not denature the oligosaccharide chains. Additionally, the latter two traces are significant in that they both contain a peak at circa 7 min (marked x in Fig. 4) which corresponds to sialic (N-acetylneuraminic) acid. In other words, the methods used to either purify or deglycosylate the AGP used in Figs. 4B and 4C have resulted in desialylation. The glycosylation pattern for rheumatoid AGP (Fig. 5) is significantly different from the normal trace both in the amount and identity of the oligosaccharide structures.

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 biantennnary), 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 [26]. Thus the rheumatoid AGP sample (Fig. 5) appears to contain more bi- and tetra-antennary chains than normal plasma although the increased biantennary population between 20

and 30 min may also be due to chains with fucosylated triantennary structures. Fucosylation results in a decrease in retention time probably as a result of charge masking. A population consisting of fucosylated tri- and tetra-antennary structures would agree with previous studies which have found AGP glycoforms in RA to be characterised by a decreased number of biantennary chains [15] and an increase both in the absolute amount of glycoforms containing fucose and in the number of fucose residues per molecule [16].

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