

Separation of α -Acid Glycoprotein Glycoforms Using Affinity-Based Reversed Micellar Extraction and Separation

Jaehoon Choe,¹ Fuming Zhang,² Michael W. Wolff,^{2,3}
David W. Murhammer,³ Robert J. Linhardt,^{2,3} Jonathan S. Dordick¹

¹Department of Chemical Engineering, Rensselaer Polytechnic Institute, Troy, New York 12180; telephone: 518-276-6379; fax: 518-276-4030; e-mail: dordick@rpi.edu

²Department of Chemistry, and Division of Medicinal and Natural Products Chemistry, University of Iowa, Iowa City, Iowa 52242

³Department of Chemical and Biochemical Engineering, University of Iowa, Iowa City, Iowa 52242

Received 15 December 1999; accepted 23 April 2000

Abstract: A new method for the preparation of the glycoforms of bovine α_1 -acid glycoprotein (AGP) is described relying on affinity-reversed micellar extraction and separation (ARMES). This method has proven effective in separating structurally similar glycoproteins and separating glycoproteins from nonglycosylated proteins from natural sources. In this method, individual glycoforms complex with the lectin, concanavalin A (ConA) are extracted into an organic-phase reversed micellar solution formed by Aerosol OT (AOT). The purity of three AGP glycoforms isolated was assessed by hydroxyapatite high-performance liquid chromatography (HPLC), gel-permeation chromatography and SDS-PAGE. The glycan structure of the pure glycoforms was analyzed. Oligosaccharide mapping using capillary electrophoresis (CE) and PAGE showed the glycans obtained from each glycoform to be distinctly different. ARMES can be used for the semi-preparative scale resolution of the glycoforms of bovine AGP or other therapeutic glycoproteins. © 2000 John Wiley & Sons, Inc. *Biotechnol Bioeng* 70: 484–490, 2000.

Keywords: glycoprotein; glycoform; affinity-reversed micellar extraction and separation (ARMES); glycan analysis

INTRODUCTION

Glycoprotein separation and resolution is critical to understanding the structure, function, and heterogeneity of glycosyl groups on proteins (Kishino et al., 1992). Several chromatographic methods have been developed to separate glycoproteins from nonglycosylated proteins (Dakour et al., 1987; Liener et al., 1986). Most glycoproteins exhibit an array of heterogeneity, both with respect to sites of glycosyl chain attachment onto the polypeptide backbone as well as the sugar chains themselves (Johannes et al., 1995). Char-

acterization of these glycoforms, therefore, requires a highly selective and efficient method for resolution.

Recently, we developed a technique that combines the exquisite selectivity of biological affinity interactions with the scalability and processing ease of liquid–liquid extractions. Among the various affinity interactions available, ARMES (affinity-based reversed micellar extraction and separation) has been used to separate specific glycoproteins from mixtures of glycosylated (Choe et al., 1998) and nonglycosylated proteins (Paradkar and Dordick, 1991). Specifically, ARMES couples the natural glycosyl affinity of lectins [such as Concanavalin A (Con A)] with two-phase reversed micellar extraction. The result is a separation technique that magnifies the inherent selectivity of lectin–glycoprotein interactions and can enhance the small physicochemical differences between one glycoprotein and another, thereby leading to efficient separation.

In the present work, we demonstrate that ARMES can be used to *resolve* structurally similar glycoforms of the medically important glycoprotein, α_1 -acid glycoprotein (AGP). AGP is a serum glycoprotein that is synthesized in the liver (Schmid, 1975) and shows biantennary and triantennary glycan microheterogeneity (Küster et al., 1997). In acute-phase response in a variety of disease states, the AGP concentration in serum increases by several-fold, and the proportion of AGP glycoform having biantennary glycans increases in the early stage of an acute response (DeGraaf et al., 1993). In rheumatoid arthritis and cancer, the number of branches on *N*-linked glycans and the number of sialic acid and fucose residues can be altered, thereby influencing anti-inflammatory reactions (Mackiewicz and Mackiewicz, 1995). Because of the central importance of AGP in this biological response, information on the heterogeneity of AGP glycoforms may provide a highly sensitive and precise diagnostic of the aforementioned disease (Mackiewicz and Mackiewicz, 1995). Alpha-1-acid glycoprotein is also im-

Correspondence to: J. S. Dordick
Contract grant sponsors: NIH; NSF
Contract grant numbers: GM49056; BEF9614577

portant in drug delivery, as it is responsible for the binding of neutral and basic drugs in the blood (Kremer et al., 1988; Routledge et al., 1986). This interaction with drugs affects drug bioavailability and clearance, and is likely to be at least partly dependent on the nature of protein glycosylation (Jackson, 1982; Tinguely, 1985). Thus, resolution of AGP into individual glycoforms must also be important in monitoring effective drug delivery.

In the work presented herein, we show that ARMES (using Con A as the affinity ligand) can resolve the heterogeneous AGP into separate glycoforms. Bovine AGP was selected for this study as it has a complex glycan structure including the presence of a fourfold higher level of ConA-binding, bi-antennary structures than found in human AGP (Küster et al., 1998). Initial studies in our laboratory demonstrated that while a ConA-Sepharose affinity column could bind 100% of bovine AGP glycoforms (Choe et al., 1998), it was not possible to use this approach to separate bovine AGP into its glycoforms. The current study describes the successful separation of bovine AGP glycoforms using ARMES. The glycans contained in each glycoform were characterized by oligosaccharide mapping using polyacrylamide gel electrophoresis (PAGE) and capillary electrophoresis (CE) to assess the influence of glycan structure on the purification glycoforms with ARMES.

MATERIALS AND METHODS

Materials

Bovine serum α_1 -acid glycoprotein (AGP), Concanavalin A, Type IV (ConA), Sephacryl S-200HR, Sephadex G-25, Aerosol OT (AOT), and methyl- α -D-manno-pyranoside were obtained from Sigma Chemical Co. (St. Louis, MO). PNGase F was obtained from Boehringer Mannheim (Germany). SM-2 Bio-Beads, Bio-Scale CHT-I (7 \times 52 mm) and hydroxyapatite column were from Bio-Rad (Hercules, CA). Sephadex G10 was from Pharmacia (Uppsala, Sweden). Amido G Acid, monopotassium 7-amino-1, 3-naphthalenedisulfonic acid (AGA) was from Aldrich (Milwaukee, WI).

Affinity-Based Reversed Micellar Extraction of AGP

Alpha-1-acid glycoprotein (4.4 mg/ml) was combined in 25-mL vials with ConA (30.2 mg/mL) in 10 mL of 10 mM bistrispropane-HCl buffer (pH 7.0, 8.0, and 9.0) or 10 mM sodium acetate-acetic acid buffer (pH 5.0 and 6.0) in the presence of 100 mM NaCl. After 20 min, an equal volume of 40 mM AOT in isoctane was added and the vials were shaken for 15 min at 300 rpm. A clear-phase separation was achieved by centrifugation at 50g. The organic phase was saved and mixed with identical volume of back-extraction buffer (0.2M sodium pyrophosphate buffer, pH 8.5, with 0.2M NaCl and 0.5M methyl α -D-mannopyranoside (α MM)) for 20 min at 300 rpm. After centrifugation, the aqueous

phase was saved and the same volume of 30 mM HCl solution was added to reduce the pH to 5.5. All of the ConA was extracted into the organic phase after shaking with 40 mM AOT in isoctane. The aqueous phase was then dialyzed and lyophilized.

High-Performance Liquid Chromatographic Purification of AGP Glycoforms

After the affinity-based reversed micellar extraction of AGP, the purified AGP fractions were characterized by HPLC (Model SPD-10AV, Shimadzu, Japan) using a hydroxyapatite column. This column was pre-equilibrated with a 0.5 mM NaCl solution (unbuffered). Each AGP fraction (1 mg) was dissolved in 50 μ L of distilled water and injected into the HPLC system at a flow rate of 1 mg/mL. The resolution was achieved using a gradient elution shown in Figure 1a.

The purity and molecular weight of the AGP fractions were assessed by SDS-PAGE (12% gel) using a Mini-Protean II electrophoresis system from Bio-Rad Laboratories (Hercules, CA) and silver-staining.

Glycan Release by *N*-glycosidase F Digestion

Before performing glycan analysis, AGP fractions were further purified using a Sephacryl S-200 HR column (45 cm \times 1.5 cm i.d.) to remove low-molecular weight contaminants that might interfere with analysis. Alpha-1-acid glycoprotein samples were dissolved in column buffer (2 mM HEPES, 0.2% SDS, pH 7.5) and the AGP was eluted from the Sephacryl S-200 HR column in the void-volume fractions. These fractions were pooled, and the AGP was precipitated by adding 9 vol of 100% acetone and incubated overnight at -20°C and the precipitated protein was recovered by centrifugation.

The acetone-precipitated AGP was used without drying. It was resuspended in minimum volume of buffer 1 (20 mM HEPES, 1% SDS, pH 8.2) and β -mercaptoethanol (20 mM) was added. The sample was boiled for 5 min to dissolve the protein and then combined with 4 vol of buffer 2 (20 mM HEPES, 1.25% NP-40, pH 8.2) containing 50 mM EDTA. PNGase F (0.5 to 1.0 U/mL) was added to remove the glycan chains from AGP. The reaction was carried out overnight at 30°C and stopped by boiling for 3 min.

The released glycans were recovered from the core protein by fractionation on the S-200 HR column (45 cm \times 1.5 cm i.d.) using the same running conditions as the AGP purification. Protein eluting from the column was monitored by measuring absorbance at 280 nm. The fractions containing released glycan were determined by reducing sugar assay (Mopper and Gindler, 1973). After the glycan fractions were pooled, 2% (v/v) of saturated KCl was added and the solution was incubated at 4°C overnight. The precipitated potassium-SDS salt was removed by centrifugation, and the glycans in the supernatant were passed over a 3-mL Biobead SM2 column to remove the residual nonionic de-

tergent. The column was washed with 5 column-volumes of water. The pooled fractions were freeze-dried and then individually desalted on a Sephadex G-10 column. Salt-free fractions containing glycan were pooled and freeze-dried.

Analysis of Glycans from AGP Glycoforms

Glycan-AGA conjugates were prepared by reductive amination in the presence of sodium cyanoborohydride (Lee et al., 1991; Wolff et al., 1999). The monopotassium salt of AGA (recrystallized from water) was dissolved in 15% acetic acid (5 mg/100 μ L), the purified glycan was added, and the mixture was incubated for 1 h at room temperature. Sodium cyanoborohydride (100 μ L of 1.0M) was added to the mixture and incubated for 12 h at 45°C. Excess AGA was removed using a Sephadex G-25 column (45 cm \times 1.5 cm i.d.) with detection at 247 nm. The fluorescently labeled glycan fractions were pooled and freeze-dried.

Electrophoresis was performed in a Mini-Protean II electrophoresis system from Bio-Rad Laboratories (Hercules, CA). The resolving gel was prepared with 5 mL of 22% acrylamide solution [11.2% (w/v) acrylamide, 0.5% (w/v) *N,N'*-bisacrylamide, and 1% (w/v) sucrose in resolving buffer (0.1M boric acid, 0.1M Tris, and 0.01M EDTA, pH 8.3)], 30 μ L of 10% ammonium persulfate (APS), and 5 μ L *N,N,N',N'*-tetramethylethylenediamine (TEMED). The stacking gel was prepared—2 mL of 4.75% (w/v) acrylamide, 0.25% (w/v) *N,N'*-bisacrylamide in resolving buffer, 30 μ L of 10% ammonium persulfate (APS), and 2 μ L *N,N,N',N'*-tetramethylethylenediamine (TEMED). The samples (5 μ L) were loaded with 5 μ L of 50% sucrose solution. Electrophoresis was conducted with the resolving buffer in the lower chamber and the upper buffer (1.25M glycine and 0.2M Tris) in the upper chamber of the gel electrophoresis apparatus at 200 V (constant voltage) for about 1 h. The gel was visualized in a UV-light chamber and photographed.

The labeled glycan was analyzed with a capillary electrophoresis system (Dionex, Sunnyvale, CA) at 25 kV by fluorescent detection (λ_{ex} of 250 nm and λ_{em} of 450 nm). Separation and analysis were carried out in a reversed polarity mode using a fused-silica capillary tube (55 cm in length and 50 μ m i.d.). The separation buffer contained 20 mM sodium phosphate pH 3.5. The sample was pressure injected (5 s, 5 psi) resulting in an injection volume of 0.5 μ L.

RESULTS AND DISCUSSION

Bovine AGP consists of multiple glycoforms as a result of having three *N*-glycosylation sites occupied by different glycans structures (Hunter and Games, 1995). The extensive heterogeneity of AGP glycans result in AGP glycoforms that bind ConA with different affinity (Bierhuizen et al., 1988). Therefore, AGP represents an excellent model to demonstrate the effectiveness of ARMES for resolving heterogeneous glycoforms. Affinity-reversed micellar extrac-

tion and separation consists of two distinct steps (Paradkar and Dordick, 1993). The first step involves contacting an aqueous solution of glycoprotein mixture and ConA with an organic phase, typically consisting of isooctane and a surfactant that results in a reversed micellar solution. Aerosol OT (AOT) has proven to be an effective surfactant giving protein extraction [into the reversed-micellar-organic phase (RMOP)] in high yields and maintaining protein activity and stability. The pH of the aqueous phase can be adjusted to ensure that glycoprotein extraction into the RMOP cannot occur without the formation of a ConA-glycoprotein complex. Once the ConA-glycoprotein complex is extracted into the RMOP, a second extraction in the presence of methyl α -D-mannopyranoside results in the transfer of the glycoprotein into a receiving aqueous phase, and purified from the initial mixture of glycoproteins. If the mixture represents a single glycoprotein consisting of several glycoforms, then such a separation would represent glycoform resolution.

Prior to developing the ARMES process for AGP glycoform resolution, we needed to devise an analytical technique to confirm glycoform resolution. This was done using hydroxyapatite (HA) chromatography. Kishino et al. (1997) used HA chromatography to separate human AGP, which consists of five glycosylation sites with *N*-linked bi-, tri-, and tetra-antennary glycans. However, using the method of Kishino et al. (1997), we were unable to resolve bovine AGP glycoforms. ConA binds to bi-antennary glycans as well as high-mannose and hybrid-type glycans (Cummings, 1994). While ConA affinity chromatography was useful in resolving human AGP into glycoform fractions [nonbinding, weakly binding and strongly binding (Bierhuizen et al., 1988)], it was incapable of resolving the bovine AGP glycoforms (Choe et al., 1998). The *N*-linked glycans of AGP, prepared from various species, have recently been profiled by matrix-assisted laser desorption ionization-mass spectrometry (Küster et al., 1998). These results demonstrated that bovine AGP contained a fourfold higher percentage of bi-antennary glycans (weakly binding to ConA) than did human AGP. Following a number of trials, we identified appropriate elution conditions on the HA column to effect bovine AGP glycoform resolution. As shown in Figure 1a, elution of AGP yields three distinct fractions (A, B, and C). Using a 7-mm diameter HA column, 1 mg of AGP could be separated into the aforementioned three fractions in 160 min.

Resolution of AGP Glycoforms Using ARMES

AGP was extracted using ARMES at five different pH values (Table I) and the aqueous-forward-extraction phases and the aqueous-receiving phases were examined by HA chromatography. Prior to ARMES, ca. 83% (w/w) of bovine AGP consisted of glycoform A with the remainder distributed between glycoforms B and C (different commercial preparations of AGP show different glycoform compositions). Glycoform A could not be extracted by ARMES even though this glycoform could bind to ConA-Sepharose

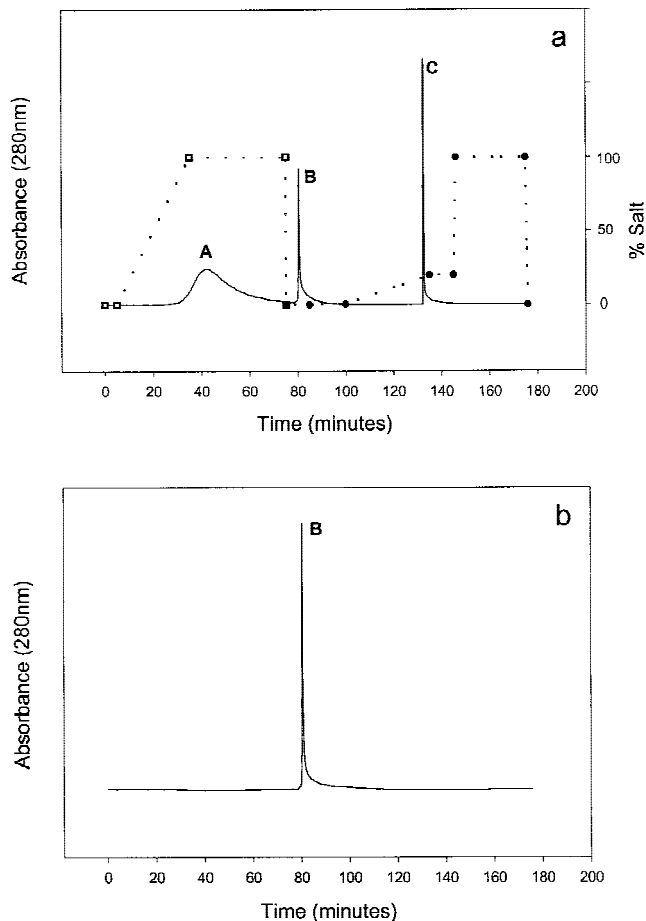


Figure 1. Separation of AGP by HPLC on HA column. (a) Separation of commercial AGP (a mixture of glycoforms) on HA-HPLC. The salt gradient used (·····) begins with NaCl (□) and then with pH 6.8 sodium phosphate (●) containing 0.5 mM MgCl₂. (b) Analysis of AGP glycoform B purified by ARMES using the same gradient described above.

(data not shown). At pH 9.0, glycoform B is extracted leaving glycoforms A and C in the aqueous phase. By lowering the pH to 5, glycoform C now can be extracted leaving only glycoform A in the aqueous phase. Figure 2 depicts a separation flowchart for AGP glycoforms. When bound to ConA, each glycoform affords a soluble complex having differences in pI and hydrophobicity, permitting their differential extraction into an organic phase. Affinity-reversed micellar extraction and separation, therefore, is capable of

Table I. Resolution of glycoform B by changing solution pH.

Extraction conditions	Area % of glycoform A	Area % of glycoform B	Area % of glycoform C
AGP before extraction	-(83)	82.4 ^a (14)	17.6 ^a (3)
AGP extracted at pH 5	0	91.4	8.6
AGP extracted at pH 6	0	95.5	4.5
AGP extracted at pH 7	0	96.0	4.0
AGP extracted at pH 8	0	97.6	2.4
AGP extracted at pH 9	0	100.0	0.0

^aCalculated from area % of glycoform B and C only.

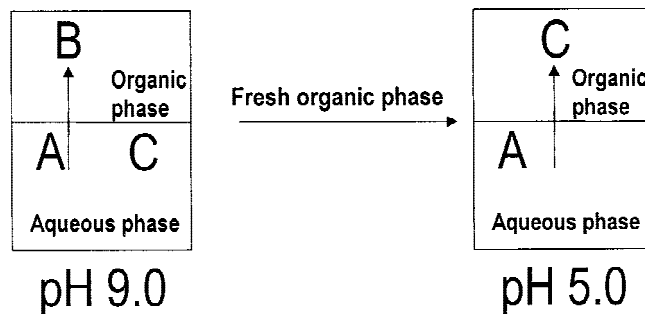


Figure 2. Scheme for the separation of AGP glycoforms. The first extraction of pH 9.0 removes B, after changing the organic phase the pH is decreased to 5 to extract C.

separating AGP glycoforms via a series of simple liquid-liquid extractions. In 1 h, 44 mg AGP was fractionated by ARMES. SDS-PAGE (data not shown) confirmed that all three AGP fractions were pure and identical to the commercial starting material affording a molecular weight of ca. 43 KDa. The ability of ARMES to resolve structurally similar glycoforms was next probed by examining the nature of the glycans on the resolved glycoforms.

Glycan Release and Separation

Alpha-1-acid glycoprotein glycoforms A, B and C were individually passed through a gel-permeation column to remove any low-molecular-weight contaminants that might interfere with glycan analysis. No contaminants were detected and a single symmetrical peak was observed at the void volume of the column (Fig. 3). The peak fractions (#18 to #35) were recovered and pooled corresponding to this for treatment with PNGase F. The same method was used to

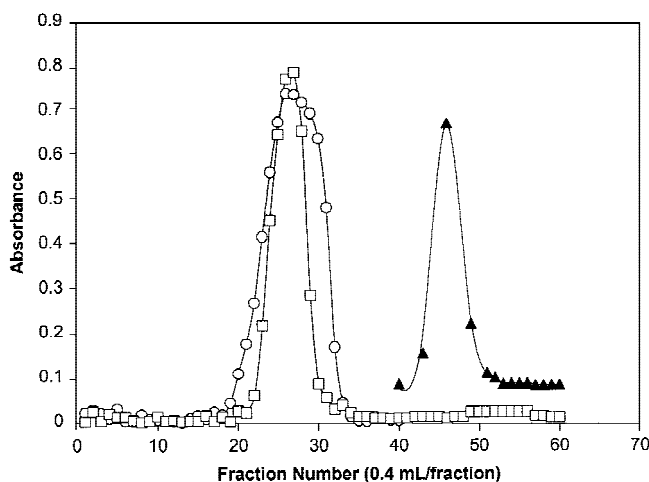


Figure 3. Gel-permeation chromatography of one respective AGP glycoform (A) before and after PNGase F digestion. The squares correspond to AGP glycoform A prior to PNGase F treatment as detected by protein absorbance at 280 nm. The circles correspond to AGP glycoform A following PNGase F treatment as detected by protein absorbance at 280 nm. The triangles correspond to glycans released from glycoform A following treatment with PNGase F using reducing sugar assay for reducing sugar by absorbance at 560 nm. Glycoforms B and C gave similar chromatograms.

prepare other AGP glycoforms. The AGP glycoforms recovered from the GPC column were individually treated with PNGase F to release their N-linked glycan chains and passed over the same column. While the protein again eluted in the void volume, a new peak was detected by reducing sugar assay in the column's total volume, corresponding to the released glycan (Fig. 3). The released glycan fractions located by the reducing sugar assay from each glycoform were recovered for glycan analysis.

Analysis of Fluorescent Glycan with Capillary and Gel Electrophoresis

The released glycans were purified by removing SDS and the nonionic detergent, and then desalted. The resulting mixture of pure glycans was fluorescently tagged with AGA, an anionic fluorophore, so that any neutral oligosaccharides would be given a charge for migration on CE and to improve their detection (Lee et al. 1991). Following tagging and the removal of excess AGA, oligosaccharide maps (e.g., fingerprints) of the released glycans of the individual glycoforms were prepared by CE (Fig. 4a-d). Under the reversed-polarity conditions used in these CE experiments, a small or a highly negatively charged glycan will show the fastest migration (Wolff et al., 1999). Coinjection experiments were also performed with the fluorescent glycans of the unfractionated (commercial) AGP and the fluorescent

glycans from each of the glycoforms. These experiments showed that each glycoform contained glycans found in the original AGP but in very different amounts. Peak 3 observed in each mixture of fluorescently labeled glycans was residual AGA tag and served as a useful internal standard. Three major peaks (11, 12, and 17) were observed in the electropherograms of fluorescent glycans of the original unfractionated AGP (Fig. 4a). The electropherogram of glycans from glycoform A (Fig. 4b) was very similar to electropherogram of the glycans obtained from the original unfractionated AGP, containing the same three major peaks 11, 12, and 17, suggesting glycoform A is the major component in the original unfractionated AGP. This is consistent with the high-isolated yield of glycoform A (83%) from the original AGP by hydroxyapatite chromatography compared to the lower recoveries of glycoforms B and C (14% and 3%, respectively). The fast migration of the glycans corresponding to 11 and 12, suggests that these glycans are either very small neutral or large highly charged glycans, which do not bind to ConA).

In electropherograms of glycoform B and C (Fig. 4c and d), only small amounts of major peaks (11, 12, and 17) observed in the original, unfractionated, AGP, were seen. Instead, additional peaks were observed that migrated faster on CE including 7 and 8 from glycoform B and 6, 8, and 9 from glycoform C. In addition, glycoforms B and C were

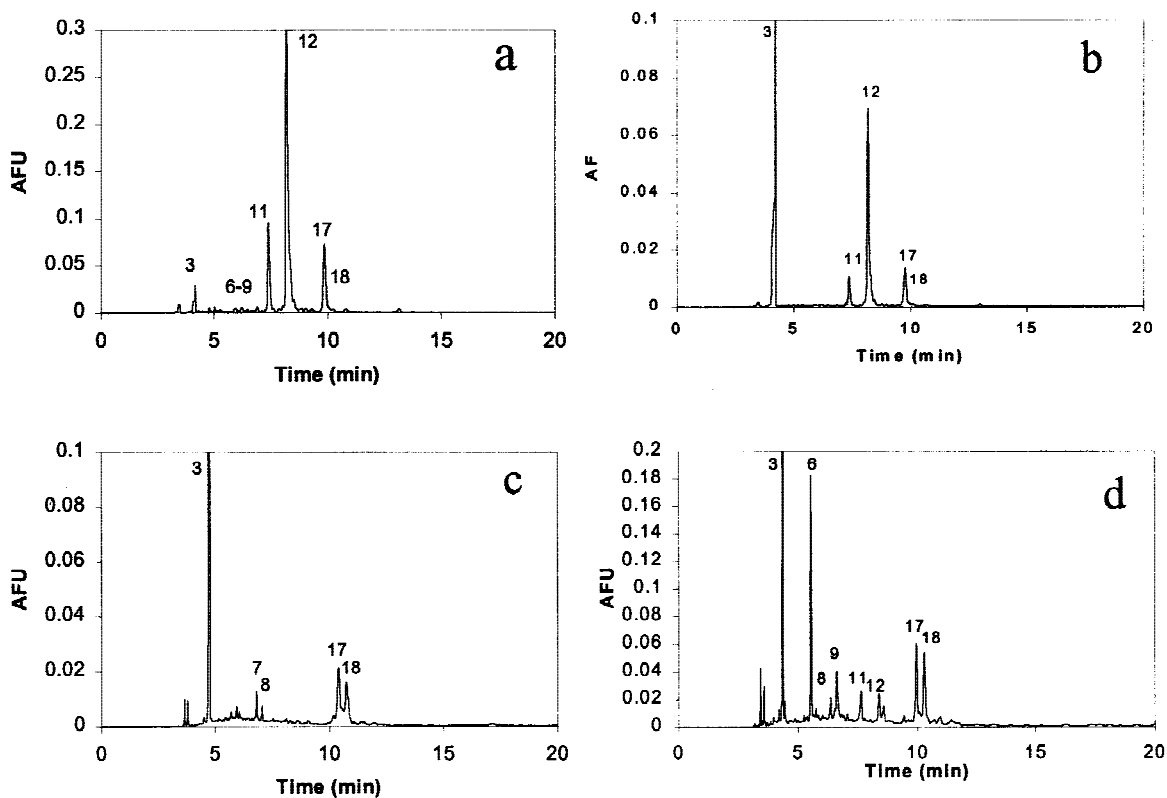


Figure 4. Electropherograms of fluorescent glycans from AGP and its glycoforms A, B, and C. (a) Electropherogram of fluorescent glycans from commercial AGP. (b) Electropherogram of fluorescent glycans from glycoform A. (c) Electropherogram of fluorescent glycans from glycoform B. (d) Electropherograms of fluorescent glycans from glycoform C.

both enriched with glycan, 18, eluting late in their electropherograms. The slow migration of the glycan corresponding to 18, suggests that this glycan corresponds to a large or an uncharged glycan (i.e., a desialated bi-antennary glycan capable of interacting with ConA).

The fluorescent glycans were next analyzed by polyacrylamide gel electrophoresis (PAGE) to estimate the molecular size of the released glycans (Fig. 5). Transillumination of PAGE electropherogram was used for detection. Excess fluorescent tag AGA, migrating farthest into the gel, was detected in each sample. An intense band high in the gel (indicated with the uppermost arrow and probably corresponding to the large glycans 11, 12, and 17) was observed for the original unfractionated AGP sample (lanes 2) and glycoform A (lane 3). The PAGE results, together with the CE analysis, strongly suggest that the glycans corresponding to 11 and 12 are large fully sialated tetra- and tri-antennary glycans, and 17 is missing a single sialic acid. Glycoform A, rich in such glycan structures would not be expected to interact with ConA. Glycoforms B and C were considerably more complex showing many additional bands corresponding to both higher molecular weight, uncharged (desialated), glycans (indicated by the second arrow probably corresponding to 18, a desialated, bi-antennary glycan) and lower molecular weight, glycans (indicated by the two lower arrows corresponding to 6–9, probably truncated gly-

cans). The glycan structures present in glycoforms B and C are expected to interact with ConA. The pH dependence in the extraction of glycoforms B and C with ARMES may result from either the number of ConA binding glycan chains on an AGP molecule effecting the stoichiometry of ConA-AGP interaction or differences in the position of interacting glycan on AGP resulting in charge shielding of the underlying protein altering the pI of the ConA-AGP complex and influencing its extraction at a particular pH. Additional work will be required to fully characterize these glycan structures and to understand the relationship of glycan valency and position on ARMES.

In conclusion, ARMES has been demonstrated to be an effective method for the semipreparative (10's of mg) scale resolution of the glycoforms of bovine AGP. The purity of these glycoforms was confirmed by HA-HPLC and the glycan maps of each were shown to be distinctive. This method might have future application for the preparation of pure glycoforms of therapeutic glycoproteins.

References

- Bierhuizen MFA, DeWit M, Govers ARL, Ferwerda W, Koeleman C, Pos C, Van Dijk W. 1988. Glycosylation of three molecular forms of human α_1 -acid glycoprotein having different interactions with concanavalin A. *Eur J Biochem* 175:387–394.
- Choe J, VanderNoot VA, Linhardt RJ, Dordick JS. 1998. Resolution of glycoproteins by affinity-based reversed micellar extraction and separation. *AIChE J* 44(11):2542–2548.
- Cummings RD. 1994. Use of lectins in analysis of glycoconjugates. In: Lennarz WJ and Hart GW, editors. *Methods in enzymology*, vol 230. New York: Academic Press. p 67–86.
- Dakour J, Lundblad A, Zopf D. 1987. Separation of blood group A-active oligosaccharides by high-pressure liquid affinity chromatography using a monoclonal antibody bound to concanavalin A silica. *Anal Biochem* 161(1):140–143.
- DeGaaf TW, Van der Stelt ME, Anbergen WG, Van Dijk W. 1993. Inflammation-induced expression of sialyl Lewis X-containing glycan structures on alpha 1-acid glycoprotein (orosomucoid) in human sera. *J Exp Med* 177:657–666.
- Hunter AP, Games DE. 1995. Evaluation of glycosylation site heterogeneity and selective identification of glycopeptides in proteolytic digests of bovine α_1 -acid glycoprotein by mass spectrometry. *Rapid Commun Mass Spectrom* 9:42–56.
- Jackson PR, Tucker GT, Woods HF. 1982. Altered plasma drug binding in cancer: Role of α_1 -acid glycoprotein. *Clin Pharmacol Ther* 32: 295–302.
- Johannes FG, Vliegthart JFG, Montreuil J. 1995. Primary structure of glycoprotein glycans. In: Montreuil J, Vliegthart JFG, Schachter H, editors. *Glycoproteins*. Amsterdam: Elsevier. p 23–24.
- Kishino S, Di Z, Sugawara M, Iseki K, Miyazaki K. 1992. Rapid and simple method for the determination of α_1 -acid glycoprotein in serum by column liquid chromatography. *J Chromatogr* 582:246–248.
- Kishino S, Nomura A, Saitoh M, Sugawara M, Iseki K, Kitabatake A, Miyazaki K. 1997. Single-step isolation method for six glycoforms of human α_1 -acid glycoprotein by hydroxylapatite chromatography and study of their binding capacities for disopyramide. *J Chromatogr B* 703:1–6.
- Kremer JM, Wilting J, Janssen LH. 1988. Drug binding to human alpha-1-acid glycoprotein in health and disease. *Pharmacol Rev* 40(1):1–47.

← Migration Direction

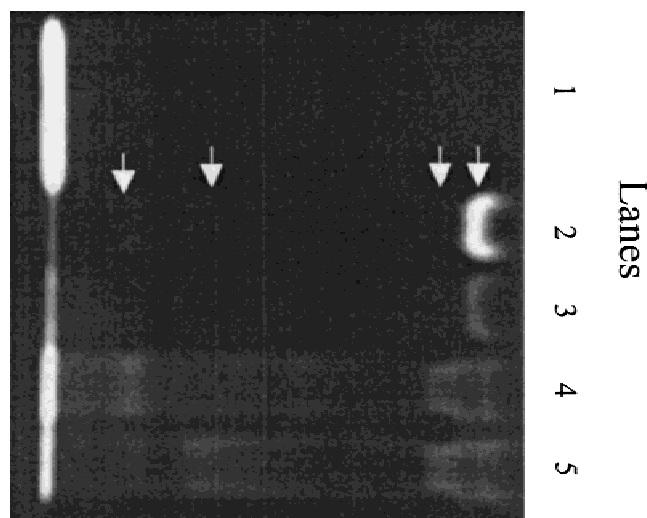


Figure 5. Analysis of fluorescent glycans by PAGE. An inverted image (bands appear bright and background dark in original gel under UV transillumination) of a gel is shown. Lane 1 corresponds to AGA standard showing a very intense band migrating to the bottom of the gel. Lane 2 contains AGA-tagged glycans from commercial AGP, the band highest in the gel (indicated by an arrow) corresponds to the major fluorescently labeled glycan. Lane 2 also contains very faint bands at positions indicated by the additional arrows. Lane 3 corresponding to glycoform A shows only the major glycan contained in lane 2 indicated by the uppermost arrow. Lanes 4 and 5 corresponding to fluorescently glycoform B and C, respectively, contain numerous additional glycans indicated by the arrows. All lanes contain excess AGA tag running at the bottom of the gel.

- Küster B, Wheeler SF, Hunter AP, Dwek RA, Harvey DJ. 1997. Sequencing of N-linked oligosaccharides directly from protein gels: In-gel deglycosylation followed by matrix-assisted laser desorption/ionization mass spectrometry and normal-phase high-performance liquid chromatography. *Anal Biochem* 250:82–101.
- Küster B, Hunter AP, Wheeler SF, Dwek RA, Harvey DJ. 1998. Structural determination of N-linked carbohydrates by matrix-assisted laser desorption/ionization-mass spectrometry following enzymatic release within sodium dodecyl sulphate-polyacrylamide electrophoresis gels: Application to species-specific glycosylation of α_1 -acid glycoprotein. *Electrophoresis* 19:1950–1959.
- Lee KB, Al-Hakim A, Loganathan D, Linhardt RJ. 1991. A new method for sequencing linear oligosaccharides on gels using charged, fluorescent conjugates. *Carbohydr Res* 214:155–168.
- Liener IE, Sharon N, Goldstein IJ. 1986. *Lectins: Properties, functions and applications in biology and medicine*. New York: Academic Press. p 294–357.
- Mackiewicz A, Mackiewicz K. 1995. Glycoform of serum α_1 -acid glycoprotein as markers of inflammation and cancer. *Glycoconjugate J* 12: 241–247.
- Mopper K, Gindler EM. 1973. A new noncorrosive dye reagent for automatic sugar chromatography. *Anal Biochem* 56:440–442.
- Paradkar VM, Dordick JS. 1991. Purification of glycoproteins by selective transport using concanavalin-mediated reverse micellar extraction. *Biotechnol Prog* 7(4):330–334.
- Paradkar VM, Dordick JS. 1993. Affinity-based reverse micellar extraction and separation (ARMES): A facile technique for the purification of peroxidase from soybean hulls. *Biotechnol Prog* 9:199–203.
- Routledge PA. 1986. The plasma binding of basic drugs. *Br J Clin Pharmacol* 22:499–506.
- Schmid K. 1975. Alpha-1-acid glycoprotein. In: Putnam FW, editor. *The plasma proteins vol 1*. New York: Academic Press. p 184–228.
- Tinguely D, Baumann P, Conti M, Jonzier-Perey M, Schopf J. 1985. Interindividual differences in the binding of antidepressives to plasma proteins: The role of variants of alpha-1-acid glycoprotein. *Eur J Clin Pharmacol* 27:661–666.
- Wolff MW, Murhammer DW, Linhardt RJ. 1999. Release and preparation of insect and unreduced N-linked oligosaccharides from Sf-9 insect cells. *Prep Biochem & Biotechnol* 29(1):1–21.