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Review

Separation methods for glycoprotein analysis and preparation

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Abstract

Several chromatographic methods have been developed for the isolation and characterization of glycoproteins. In these methods, affinity chromatography, a single-step method, or combined use with general chromatographic methods have now become essential for the purification of many biologically important glycoproteins, including α_1-acid glycoprotein, immunoglobulins, ceruloplasmin and erythropoietin. On the other hand, almost all glycoproteins exhibit polymorphism associated with their glycan moieties. This feature is wide-spread and has been observed in natural as well as in recombinant DNA glycoproteins. Recently, several sophisticated techniques – such as electromigration method (high-performance capillary electrophoresis) and chromatographic methods (two-dimensional polyacrylamide gel electrophoresis, high-pH anion-exchange chromatography with pulsed-amperometric detection) – have been introduced for qualitative or quantitative estimation of the microheterogeneity of glycoproteins. For gaining further insight into the structure–function relations for microheterogeneity, preparative chromatographic techniques that can yield sufficient quantities of glycoprotein variants must be developed. © 1997 Elsevier Science B.V.

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Contents

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1. Introduction

1.1. Chemistry and importance of glycoproteins in biological systems

Many proteins in cells and biological fluids are glycosylated and these glycoproteins are present in animals, plants, microorganisms and viruses. In glycoproteins, glycans are conjugated to peptide chains by two types of primary covalent linkages, N-glycosyl and O-glycosyl. The former is called an asparagine-linked sugar chain and contains an N-acetylglucosamine (GluNAc) residue that is linked to the amide group of asparagine residues of a polypeptide. The latter contains at its reducing end an N-acetylgalactosamine (GalNAc) residue that is linked to the hydroxyl group of either serine or threonine residues of a polypeptide. This linkage is called an O-linked sugar chain or mucin type. Some glycoproteins have both the N-linked and O-linked forms in their molecules (N, O-glycoproteins).

The addition of carbohydrate to a peptide chain will change the shape and size of the protein structure. Several important discoveries have revealed the following biological roles of glycans: (1) protection of polypeptide chains against proteolytic enzymes; (2) influence on heat stability, solubility, and many physico-chemical properties; (3) interaction with other proteins or non-protein components of the cell including control of the life-time of circulating glycoproteins and cells.

1.2. Microheterogeneity of glycans

Almost all glycoproteins exhibit polymorphism associated with their glycan moieties. This type of diversity is termed microheterogeneity and these different forms have recently been called glycoforms [1]. These variants were first characterized in the α_1-acid glycoprotein (AAG) from human serum by Schmid et al. [2] using electrophoresis. As shown in the structure of major oligosaccharides of AAG (Fig. 1), microheterogeneity was found to be due to the occurrence of di-, tri-, and tetra-antennary glycans of the N-acetyllactosamine type at the five glycosylation sites [3].

This feature is wide-spread and has been observed in natural as well as in recombinant DNA glycoproteins. The existence of microheterogeneity gives rise to many interesting questions regarding the origin of this phenomenon and its relevance for the biological functioning of the glycoproteins that can be distinguished.

In this chapter, we give an outline of the recently developed chromatographic and electromigration

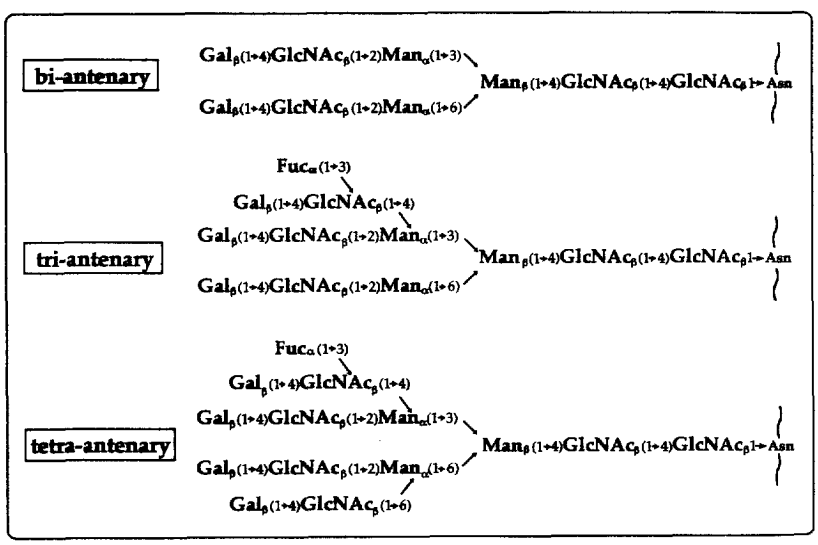

Fig. 1. Structure of the major oligosaccharides of AAG. Several NeuAc linked to galactose residues.

procedures for the purification, separation and determination of glycoproteins and their glycoforms.

2. Chromatographic purification of glycoproteins

2.1. α_1-Acid glycoprotein (AAG)

AAG is a characteristic and dominant fraction of human serum sialoglycoproteins with a molecular mass of 40 000, an unusually high carbohydrate content (45%), and a large number of sialyl residues. Although its exact biological function is still unknown, AAG is an acute-phase reactant that increases following cancer, myocardial infarction, and congestive heart failure and has also been reported to play an important role in immunoregulation [4]. Many studies on methods for isolating AAG have been carried out. Previous purification methods used a combination of chromatographic procedures on diethylaminoethyl (DEAE)– and/or carboxymethyl–cellulose [5–7]. Gel permeation chromatography was used in final steps. These methods, however, are time-consuming and require a large volume of plasma or serum because of the low quantities recovered. Furthermore, there is a strong possibility that these methods will damage the oligosaccharide and polypeptide components. Moreover, separation of AAG and α_1-antitrypsin has been difficult, because the chromatographic behavior of these compounds during anion-exchange chromatography is similar [8].

Recently, successful procedures that avoid the desialylation and denaturation of AAG have utilized various affinity adsorbents [9,10] and dye–ligand chromatography [8,11,12]. Funae et al. [8] improved the technique for separating AAG and α_1-antitrypsin from human plasma by using high-performance liquid chromatography (HPLC) equipped with a hydroxyapatite column (Fig. 2) as the last-step after pseudo-ligand affinity chromatography using Blue–Sepharose CL-6B and Red–Sepharose CL-6B according to the method of Laurent et al. [11].

More recently, Hervé et al. [12,13] reported a one-step chromatographic purification method starting from a relatively large volume of human plasma using chromatography on immobilized Cibacron

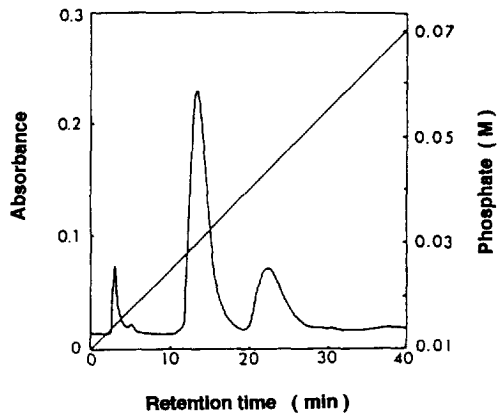

Fig. 2. Hydroxyapatite HPLC profile of the non-retained fraction with Red–Sepharose. Here, 400 μl of the non-retained fraction with Red–Sepharose containing 2 mg of protein was loaded on a hydroxyapatite column (10×0.6 cm I.D.). AAG was eluted by a linear gradient of phosphate at room temperature. Protein was monitored at 280 nm. AAG and α_1-antitrypsin were eluted as the second and third peaks, respectively (Ref. [8]).

Blue F3G-A to cross-linked Sephadex G-100. Although this procedure requires a dialysis step before the chromatography, it does not involve precipitation steps, which entail the risk of denaturing or loosing some of the AAG populations. The overall yield of the combined techniques was almost 80%. Furthermore, Hervé et al. [12] developed a rapid chromatographic method on an immobilized metal chelate affinity Cu(II) adsorbent for the purification and characterization of common (Fig. 3) and rare phenotypes of AAG.

Kishino et al. [14] described a time-saving isolation method that is suitable for a large number of small-volume samples of human serum. AAG was purified from ten-fold diluted serum itself by a separate three-step chromatographic method using commercially available cartridge columns, a DEAE-M anion-exchange column (gel volume 1.0 ml), IC-SP cation-exchange column (gel volume 1.0 ml), and hydroxyapatite column (packed volume 0.4 g). They also developed a method for determining the level of monosaccharides in the carbohydrate moiety of purified AAG by using a high-pH anion-exchange chromatography with pulsed-amperometric detection (HPAEC-PAD) [15–17]. This purification method has several advantages compared to the previously reported techniques: pretreatment of serum (dialysis

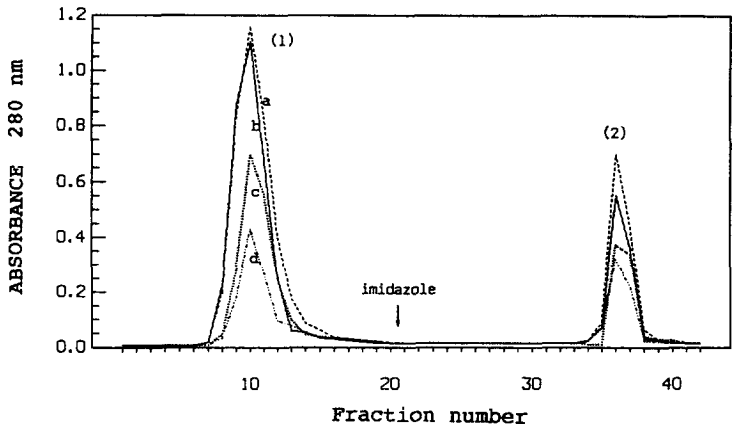

Fig. 3. Elution of the sialylated AAG variants by affinity chromatography of native samples on IDA-Cu(II) gel at pH 7. The affinity gel was packed into small glass columns (1.5 cm I.D.) up to a final bed volume of 10 ml. Native commercial AAG (c), or individually purified AAG samples with the F1S/A (b), F1/A (d), or S/A phenotype (a), were dissolved in buffer E (5–7 mg in 1.5 ml) and applied to the columns equilibrated with the same buffer at room temperature (20–23°C). The flow-rate was 10 ml/h. Fractions of 1.25 ml were collected and their respective absorbances were measured spectrophotometrically at 280 nm. After several volumes of buffer E had been applied to remove unbound protein(s), 20 m*M* imidazole was introduced (indicated by an arrow) to elute high-affinity protein(s). The total protein recovery was ca. 95% in all experiments. Peaks 1 and 2 from the different chromatographic experiments were separately pooled, concentrated and then dialysed against deionized water and finally lyophilized (Ref. [12]).

and precipitation steps are not needed); time required for purification (less than 1 h for two or three samples); volume of plasma needed for the purification of AAG and for the subsequent determination of monosaccharides (less than 1.0 ml); and the overall yield of the combined techniques (more than 80%).

Smith et al. [18] recently improved the previous technique by inserting an anion-exchange step (Q–Sepharose) between the two dye–ligand columns (Blue–Sepharose and Red–Sepharose) in order to completely separate AAG from α_1-antitrypsin in normal and rheumatoid plasma. The efficacy of this method was verified using HPAEC-PAD.

2.2. Immunoglobulins (Igs)

Igs are antibodies produced by B-lymphocytes when the immune system is challenged by an antigenic substance. Igs can be divided into five classes, IgG, IgA, IgD, IgM and IgE, based on differences in their molecular mass and chemical properties.

Traditionally, purification of Igs from sera or ascites has been accomplished by an initial precipitation followed by fractionation using gel permeation chromatography, ion-exchange chromatography, hydroxyapatite chromatography, or zone electrophoresis. These methods, however, are very time-consuming, extremely labor-intensive, and may damage the isolated product. Recently, an affinity chromatographic method using general or newly developed immobilized affinity ligands has been developed for the purification of Igs from the following various biological samples: human serum IgG (L-histidine in hollow fibre membranes [19]); human placenta IgG (fucoidan–Sepharose 4B [20]); bovine milk whey IgG (synthetic affinity ligand, thiophilic gel [21]); goat serum IgG (synthetic affinity ligand, Avid AL gel [22]); cat serum IgG (protein A–Sepharose [23]); human IgA (protein jacalin–Sepharose [24]); human IgD (protein jacalin–Sepharose [24]); human serum IgM (mannan binding protein–Sepharose 6B [25]); myeloma serum and ascites IgM (protein C1q–Sepharose [26]); ovine IgE (*Ascaris* extract–Sepharose 4B [27]); and polyclonal dog IgE (protein G–Sepharose [28]).

Bond et al. [29] reported that human IgG preparations isolated by ion-exchange or protein G affinity chromatography differ in their glycosylation profiles. When examining the heavy chain of IgG, more sialic acid, galactose and GluNAc were detected in DEAE-purified IgG compared with that prepared by protein

G. Detection of sialic acid and GluNAc was also increased on light chains from IgG prepared by ion-exchange chromatography. They recommended that more than one purification method be used before undertaking an analysis of glycosylation changes.

2.3. Ceruloplasmin (CP)

CP is a serum α_2-glycoprotein that carries more than 95% of the copper present in plasma and is believed to have an active role in regulation of copper and iron homeostasis. It has been pointed out that fragmentation of CP during purification and storage hampered studies of its structure. The rapid degradation of purified CP reported by many laboratories may be largely due to the presence of one or more copurifying or contaminating proteases, at least one of which is a metalloproteinase.

Recently, Ehrenwald and Fox [30] have obtained a highly purified and non-labile CP from human plasma by combining the previously reported chromatographic steps with additional gel permeation and fast protein liquid chromatography (FPLC) steps. In the latter steps, further purification of CP by Sephadex G-50 chromatography and Mono Q FPLC were essential for the removal of plasma metalloproteinase and yielded a protein that was completely stable even after incubation at 37°C for 4 weeks.

A fast chromatographic method based on specific CP retention on aminoethyl (AE)–agarose has been developed by Wang et al. [31] and Calabrese et al. [32]. This single-step purification procedure for bovine serum CP is very fast, requiring only plasma dilution (about 20 times), followed by AE–agarose chromatography. However, in the case of handling a large volume (dozens of liters) of diluted plasma, clotting may occur during the chromatographic step, producing a decrease in the flow-rate and difficulties in regeneration. However, these limitations can be completely overcome by the following two common steps: precipitation with ammonium sulfate (at 35% and 55% saturation) followed by column chromatography on AE–agarose.

In contrast to the plasma/serum samples, whole bile is rich in bile pigments that interfere with CP sorption during affinity chromatography. Furthermore, purification of CP from normal human bile and a comparison with its serum counterpart from Wilson's disease may contribute to a better understanding of the physiological role of CP in copper metabolism. Verbina et al. [33] removed the bile pigments by 25% poly(ethylene glycol) (PEG) 6000 precipitation followed by chromatography of precipitated materials on a Sephadex G-100. The CP-containing fractions were pooled, reprecipitated by 25% PEG, pelleted in a centrifuge, and dissolved and fractionated by ion-exchange chromatography on a DEAE–Sepharose column. Subsequent affinity chromatography was carried out using a CP-specific sorbent. Biliary CP is represented by two molecular species: one that is identical to oxidase CP from normal human serum and another that is analogous to oxidase-lacking CP specific for the serum of carriers of Wilson's mutation.

2.4. Erythropoietin (EPO)

EPO, an acidic glycoprotein hormone, is synthesized in the kidney and circulates in the blood to stimulate red cell proliferation and differentiation in the bone marrow. Native human EPO was first purified from the urine of patients suffering from severe aplastic anemia by Miyake et al. [34]. Since then, several methods for the purification of urinary human EPO (uHuEPO) have been developed [35–37]. The method of Miyake et al. [34] uses phenol treatment and ethanol precipitation to avoid the effects of glycosidases and proteases, since urine is rich in these enzymes. These pretreatments, however, have resulted in a reduction in the apparent molecular weight and GluNAc content of uHuEPO [38]. Moreover, these purification methods [34–37] cannot be applied to urine containing less than 5 I.U./ml uHuEPO [38].

Inoue et al. [38] developed an improved method for the purification of uHuEPO with high in vivo activity. Their method involved two membrane filtration steps, Sephadex G-25, two DEAE–agarose steps, Sephadex G-75, wheat germ agglutine (WGA)–agarose, and reversed-phase HPLC. The two filtration steps were essential to prevent the coprecipitation of EPO with other urinary proteins during the concentration procedure. The Sephadex G-25 step and DEAE–agarose column were used for

the removal of low-molecular-mass materials such as pigments and lipids, and of EPO inactivated by desialylation. The Sephadex G-75 step was essential for further purification. As shown in Table 1, EPO rich in sialic acid specifically bound to the lectin column. Nucleic acids were removed by the final HPLC step. The uHuEPO obtained by this method had the same molecular mass (approximately 38 000) on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as recombinant HuEPO (rHuEPO) and revealed a specific in vivo activity of 160 000 I.U./mg.

On the other hand, Ghanem et al. [39] have developed an anti-EPO monoclonal antibody (Mab) and have purified a rHuEPO by using this Mab (immobilized on Sepharose 4B) and DEAE–Sepharose. A rHuEPO was obtained from the culture supernatants of human B-lymphoblastoid cells transfected by the human EPO gene. The combination of these two steps resulted in a highly purified rHuEPO with a global yield of about 50%.

2.5. Other glycoproteins

Affinity chromatography, a single-step method, or combination use with general chromatographic methods has now become essential for the purification of many glycoproteins. As shown in Table 2, several biologically important glycoproteins have recently been purified by using various affinity chromatography ligands.

3. Electromigration and chromatographic detection of microheterogeneity

Recently, several techniques such as high-performance capillary electrophoresis (HPCE), high resolution two-dimensional (2D)-PAGE, and HPAEC-PAD have been developed for qualitative or quantitative estimation of the microheterogeneity of glycoproteins. In these methods, the HPAEC-PAD system has been mainly used for the comparison of oligosaccharide chains and/or monosaccharide contents after enzyme digestion [e.g., peptide-N^4-(N-acetyl-β-D-glucosaminyl) asparagine amidase (PNGase) or endoglycosidase] or acid hydrolysis [e.g., trifluoroacetic acid (TFA)] of glycan chains.

3.1. High-performance capillary electrophoresis

HPCE is a relatively new tool for the analysis of biologically active materials. Since this technique can improve the resolution of complex mixtures under non-denaturing conditions, based on the differences in charge-to-mass ratios in a relatively short period of time, it has several advantages over conventional techniques such as HPLC, FPLC and other chromatographic techniques. However, it has been pointed out that electrophoretic separation of proteins in uncoated fused-silica capillaries has been more problematic due mainly to protein interactions either with the inner capillary wall or with other proteins. This problem has been circumvented through the use of chemical additives that either coat

Table 1
Purification of human erythropoietin from urine

Step	Protein (mg)	Activity (I.U.)	Specific activity (I.U./mg)	Yield (%)
Urine	Not measured	249 500	–	100
Sephadex G-25	2900	183 000	63	73
DEAE–agarose 1st	653	176 000	269	70
DEAE–agarose 2nd	560	168 840	301	68
Sephadex G-75	327	100 170	306	40
WGA–agarose	1.04	59 000	57 000	23
RPC C4	0.15	17 360	140 000	7

Urine (100 l) from aplastic anemia patients contained 2.5 I.U./ml erythropoietin as determined by RIA (Ref. [38]).

Table 2
Affinity chromatography ligands used for the purification of various glycoproteins

Glycoproteins	Samples	Affinity chromatography ligands and other chromatographic methods used for the purification	Reference No.
Interleukin 4 binding protein	Human urine	Protein-G	[40]
N-Acetylmuramyl-L-alanine amidase	Human serum	(1) The main substrate of the enzyme, (2) DEAE, (3) Superdex	[41]
Anti-bovine β2-glycoprotein I antibody	Sera from patients with antiphospholipid syndrome	(1) Heparin, (2) DEAE	[42]
Glycoprotein with Thomsen–Friedendreich activity	Human meconium	*Vicia unijuga* lectin, *Arachis hypogaea* lectin	[43]
New 85-KDa glycoprotein antigen	Human breast tumor	(1) DEAE, (2) Protein A–Sepharose-bound IgG	[44]
New subtype of membrane-bound guanyl cyclase	Bovine retinal rod	Wheat germ agglutinin	[45]
Urokinase plasminogen activator (uPA) receptor	Ascitic fluids from patients with ovarian cancer	Pro-uPA	[46]
Vitronectin	Chick egg yolk	(1) Hydroxyapatite, (2) DEAE, (3) Anti-vitronectin	[47]
Growth factor	Human breast cancer cell	(1) Heparin, (2) Reversed-phase HPLC	[48]
Collagenase	Bovine	(1) Heparin, (2) DEAE, (3) Green-agarose	[49]
INF-α subtypes, IFN-β	Human placental trophoblasts, fibroblasts, trophoblasts-derived malignant cell	(1) Anti-IFN globulins, (2) Cibracron Blue F3GA, (3) Con A	[50]

the wall or augment the buffer to reduce protein–wall interactions [51,52]. Moreover, it has been shown that the application of 1,4-diaminobutane (putrescine) as additive to the CE separation buffer is essential for the separation of the various glycoforms [53,54]. In the analysis of microheterogeneity of ovalbumin, Landers et al. [53] pointed out that the sodium borate buffer is proposed to play a key role in the separation by preferentially complexing with the diols of specific carbohydrate moieties on ovalbumin. Furthermore, they observed that addition of putrescine enhances resolution by slowing bulk flow through the capillary and allowing electrophoretic separation of what is deduced to be closely related glycoforms of ovalbumin. Several HPCE systems have since been applied to the analysis of carbohydrate-mediated heterogeneity, such as a recombinant tissue plasminogen activator [55], ovalbumin [53] and recombinant coagulation factor VIIa (recombinant coagulation factor VIIa, rFVIIa) [54].

3.2. Two-dimensional polyacrylamide gel electrophoresis

Since 2D-PAGE is highly responsive, this method proved useful to visualize, in a single experiment, changes in the charge and molecular weight associated with the glycosylation modification of proteins.

More recently, Gravel et al. [56] attempted to identify altered plasma/serum proteins in two groups of patients with different alcohol-related diseases: an actively drinking alcoholic patients group without liver disease and an alcohol cirrhotic patients group. An abnormal microheterogeneity of haptoglobin and α_1-antitrypsin was detected in the serum of all alcoholic patients. The carbohydrate deficient transferrin was also characterized using this method.

2D-PAGE has been used to investigate, the sialic acid content of AAG and its degree of microheterogeneity in AAG isolated from the pooled sera of patients with rheumatoid arthritis, or myocardial infarction or cancer, and of healthy volunteers [57]. All preparations showed considerable variation in the degree of heterogeneity by this method.

3.3. High-pH anion-exchange chromatography with pulsed-amperometric detection

HPAEC, a relatively newly developed method, has been used successfully to analyze covalently linked carbohydrates from natural and recombinant glycoproteins. Sensitivities in the low picomole range have been routinely achieved with either PAD or radiometric detection. A major advantage of HPAEC-PAD is its usefulness in analyzing both monosaccharides and all classes of oligosaccharides without derivatization. Smith and coworkers [18,58] confirmed the effectiveness of their purification procedure of AAG and observed that there was a noticeable difference between the profiles for AAG from normal and rheumatoid plasma by HPAEC-PAD. Klausen and Kornfelt [54] used this method for estimating the carbohydrate-mediated heterogeneity of the human rFVIIa.

Kishino et al. [14] determined the concentration of N-acetylneuraminic acid (NeuAc) and four monosaccharides (fucose, GluNAc, galactose and mannose) in purified serum AAG from healthy subjects and from patients with renal insufficiency or myocardial infarction (unpublished data). NeuAc separation was achieved on a column of Excelpak CHA-E II at a flow-rate of 0.75 ml/min and a column temperature of 50°C. The mobile phase was 1 m*M* H_2SO_4, and the reagent solution in the detection was 0.6 *M* NaOH. Monosaccharide separation was achieved on a column of Dionex Carbopak PA1 at a flow-rate of 1.0 ml/min and at room temperature. The detection was carried out at an isocratic NaOH concentration of 16 m*M*. As shown in Table 3, the concentrations of GluNAc, galactose and mannose in the AAG of patients with renal insufficiency were significantly higher than those in the AAG of healthy subjects. In the patients with myocardial infarction, more NeuAc, GluNAc and galactose were detected.

Table 3
Analysis of N-acetylneuraminic acid (NeuAc) and monosaccharide level in purified α_1-acid glycoprotein (AAG) from plasma of healthy subjects and patients with renal insufficiency

	Healthy subjects (n=8)	Renal insufficiency (n=6)
Age (years)	65.8±12.5	60.2±7.2
AAG conc. (mg/ml)	0.79±0.14	1.01±0.19[a]
NeuAc (mg/g·AAG)	82.77±12.55	89.29±12.55
Fucose (mg/g·AAG)	9.84±3.08	12.79±3.37
GluNAc (mg/g·AAG)	113.01±10.07	135.44±10.51[b]
Galactose (mg/g·AAG)	76.97±4.23	88.98±5.61[b]
Mannose (mg/g·AAG)	49.14±2.57	57.26±3.80[b]

AAG concentration is determined by HPLC method with hydroxyapatite column. NeuAc and each monosaccharide concentration were determined by HPLC method with pulsed-amperometric deteciton.
The value on the table is mean±S.D..
[a] Significantly different ($p<0.05$) from healthy subjects.
[b] Significantly different ($p<0.01$) from healthy subjects (Ref. [14]).

The microheterogeneity of glycoproteins in healthy and/or disease states and its physiological significance require further investigation.

4. Chromatographic determination of AAG

Except for AAG, there are very few reports on chromatographic methods for determining glycoproteins in biological samples. Radial immunodiffusion (RID) utilizing the antibody against AAG has been widely used to determine AAG in serum because of its specificity. This method, however, is time-consuming and is not easily applicable to experimental animals. More recently, therefore, several simple and rapid HPLC methods have been developed.

Kremmer et al. [59] reported a method of determining human serum AAG based on solvent extraction by a chloroform–methanol mixture (2:1, v/v) and the anion-exchange HPLC method (Mono Q HR 5/5 column, FPLC system). After pretreatment of serum, 500 μl of the upper phase was applied into the FPLC system and AAG was eluted with a pH/NaCl gradient elution program. For measuring the serum AAG content the Mono Q HR 5/5 column was calibrated with commercial AAG in a range of 100–200 μg/500 μl of sample volume. Kremmer et al. [59] pointed out that the separation and purification of AAG from α_1-antitrypsin could be overcome by selecting a suitable sample preparation, an appropriate buffer system [N,N'-methylenebisacrylamide (Bis)–tris(hydroxymethyl)aminoethane (Tris)–propane] with a simultaneous pH/NaCl gradient elution program, and a macroporous anion-exchange with $-CH_2-N^+-(CH_3)_3$ functional group (Mono Q–Sepharose).

Kishino et al. [60] reported a rapid and sensitive determination method starting from the diluted serum itself. This procedure involved the anion-exchange step for clean-up serum (commercially available cartridge column, DEAE-M) and a hydroxyapatite HPLC system (Fig. 4). A linear relationship between the standard AAG concentration (x) and the peak height (y) was observed over the concentration range of 0.5–2.5 mg/ml serum. The coefficient of variation at 0.5 mg/ml AAG was 3.7% ($n=8$). A good correlation was observed between this HPLC method (y) and the conventional RID (x) ($y=1.009x+0.004$, $r=0.996$).

Reversed-phase HPLC for analysis of human urinary AAG, β_2-macroglobulin and albumin have been reported [61]. Urine samples (10–100 μl) were injected into the HPLC system (IGP PACK ODS column packed with polyporous glass) without any further precolumn procedure. A linear gradient of 1.0 ml TFA/l of water and 0.7 ml TFA/l of acetonitrile was used. The detection wavelength was 210 nm. The correlation between the HPLC assay (y) and laser nephelometric assay (x) of AAG was good ($y=1.06x-0.12$, $r=0.99$). The correlation between the radioimmunoassay (RIA) (y) and the HPLC assay (x) of β_2-macroglobulin was also good ($y=0.744x+0.083$, $r=0.99$).

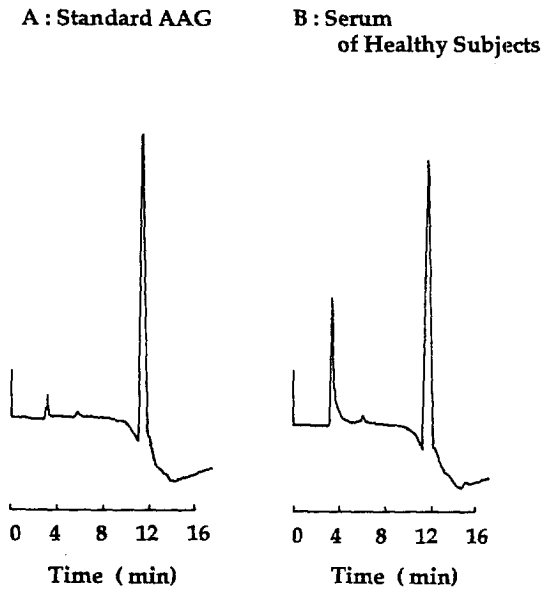

Fig. 4. Hydroxyapatite HPLC profiles of standard AAG and serum from healthy subject. A 250 μl aliquot of serum was diluted to 50 ml with 0.01 *M* citrate–phosphate buffer (pH 4.0) (buffer A), and then the whole mixture was loaded on to the DEAE-M column. After washing the column with 5 ml of buffer A, AAG was eluted with 1 ml of 0.15 *M* citrate–phosphate buffer (pH 4.0) (buffer B), and then 50 μl of the eluate was injected into the HPLC system equipped with multi-wavelength UV detector set at 280 nm. Separation was achieved on an A-7610 hydroxyapatite column (10×0.76 cm I.D.) with a linear gradient of potassium phosphate buffer (pH 5.2) at flow-rate of 1.0 ml/min; 0.35 *M* phosphate buffer was added to 0.01 *M* phosphate buffer linearly up to 50% in 10 min (from Ref. [60] with permission).

5. Conclusion

The availability of improved and sophisticated methods for the isolation and characterization of glycoproteins and its derived glycans has paved the way for study to clarify the structure–function relations for carbohydrate chains of glycoproteins. Recently, we developed a single-step HPLC system (hydroxyapatite column) for the isolation of six AAG variants, which is a sufficient quantitie for studying changes in the carbohydrate moiety and the drug binding activity. In the six variants obtained from normal human serum, variants rich in NeuAc were present in relative abundance in the female AAG.

Furthermore, variants containing a highly branched glycan chain (e.g., tetra-antennary glycan chain in Fig. 1) had a significantly lower affinity for the cationic drug disopyramide. For gaining further insight into the structure–function relations for microheterogeneity, preparative chromatographic techniques that can yield sufficient quantities of glycoprotein variants must be developed.

6. List of abbreviations

AAG	α_1-Acid glycoprotein
AE–agarose	Aminoethyl agarose
CP	Ceruloplasmin
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
DEAE–agarose	Diethylaminoethyl agarose
DEAE–cellulose	Diethylaminoethyl cellulose
DEAE–Sepharose	Diethylaminoethyl Sepharose
EPO	Erythropoietin
FPLC	Fast protein liquid chromatography
GluNAc	N-Acetylglucosamine
HPAEC	High-pH anion-exchange chromatography
HPAEC-PAD	High-pH anion-exchange chromatography with pulsed-amperometric detection
HPCE	High-performance capillary electrophoresis
HPLC	High-performance liquid chromatography
IDA-Cu(II) gel	Iminodiacetate-copper(II) gel
Igs	Immunoglobulin(s)
Mab	Monoclonal antibody
NeuAc	N-Acetylneuraminic acid
PEG	Polyethyleneglycol
PNGase	Peptide-N^4-(N-acetyl-β-D-glucosaminyl) asparagine amidase
rFVIIa	Recombinant coagulation factor VIIa
RIA	Radioimmunoassay
RID	Radial immunodiffusion
rHuEPO	Recombinant human erythropoietin
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TFA	Trifluoroacetic acid
uHuEPO	Urinary human erythropoietin
WGA–agarose	Wheat germ agglutine agarose

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