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BRIEF COMMUNICATION

MICROHETEROGENEITY OF PLASMA GLYCOPROTEINS HEPARIN COFACTOR II AND ANTITHROMBIN III AND THEIR CARBOHYDRATE ANALYSIS

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INTRODUCTION

Antithrombin III is the most important protease inhibitor in the blood coagulation cascade. Its activity results from the formation of covalent complexes with Factor Xa and thrombin (FIIa) (1). The rate of this inhibition is accelerated up to 2,000-fold in the presence of catalyst, heparin (1). ATIII is a single-chain glycoprotein reportedly containing 9-17% of carbohydrate (2,3). Multiple isoforms of ATIII have been detected by the isoelectric focusing of plasma and purified protein and this microheterogeneity has been attributed to variable sialation (4-7).

Recently, a second heparin cofactor has been identified and purified from plasma (8-10). Heparin cofactor II (HCII) is also a glycoprotein, which is antigenically distinct from ATIII and inhibits only FIIa in the presence of heparin (8). Its carbohydrate structure and isoelectric point have not been reported. We confirm the presence of multiple IEF bands for HCII (8). Unlike ATIII it remains heterogeneous even after desialation. Ion-exchange chromatography with pulsed-amperometric detection is used for the first time, on microgram quantities of glycoprotein, to determine the concentration of each neutral sugar, amino sugar and sialic acid residue. The carbohydrate composition of ATIII and HCII are reported.

MATERIALS AND METHODS

Materials. Human blood plasma was obtained from the University of Iowa Hospital. IsoGel agarose IEF in pH 3-7 range was from FMC Co. Galactose oxidase (EC 1.1.3.9), neuraminidase (EC 3.2.1.18, type X), arylsulfatase (EC 3.1.6.1, type H5), agarose

Key words: microheterogeneity; antithrombin III; heparin cofactor II; carbohydrate analysis; pulsed amperometric detection

immobilized protease from Streptomyces griseus, thiobarbituric acid, and pI and Mr marker proteins were from Sigma Chemical Co. All other chemicals were reagent grades. Absorbance was measured on a Shimadzu 160 UV spectrophotometer. Electrophoresis was performed on a Bio-Rad PROTEAN II Slab cell and a 1405 cell using a Bio-Rad 3000/300 power supply. An EC #910 scanning densitometer was used.

Purification of ATIII and HCII. ATIII and HCII were purified to homogeneity according to previous procedures (9). Purity was assessed by scanning densitometry following electrophoresis on a 10% slab SDS-PAGE gel visualized by Coomassie blue staining. Concentration of ATIII and HCII were based on the absorbance at 280 nm, using molar extinction coefficients of 5.7 (11) and 7.5 (10) for 1% solution, respectively. A molecular weight of 56,000 for ATIII (11) and 65,600 for HCII was used (9). Pure HCII, obtained as a gift from Dr. M. Griffith, was used to prepare polyclonal antibodies in rabbits (12).

IEF-PAGE. The anolyte was 0.5 M acetic acid (pH 2.6) and catholyte was 0.1 M histidine (pH 7.3). A constant wattage of 10 W gave 600 V and 16 mA at the start and 1500 V and 2 mA after 60 min. A calibration curve of marker proteins was used to determine pI. Bands were visualized by 0.25% Coomasie blue dye solution in methanol or enzyme-linked immunoassay (12) after blotting to nitrocellulose membrane.

Enzymatic Analysis of ATIII and HCII. Asialo-ATIII and asialo-HCII were prepared by treating each glycoprotein (40-100 μg in 200 μL of water) with neuraminidase (0.1 U to 0.4 U in 150 μL of sodium acetate buffer, pH 5.5) for 2 to 24 h at 37°C. SDS and IEF-PAGE were used to monitor the reaction. HCII (75 μg) was also treated with immobilized protease (5 U, pH 7 sodium phosphate buffer) for 24 h at 30°C. SDS-PAGE was used to monitor its breakdown to glycopeptides. After filtering, to remove the immobilized protease, the solution of HCII-derived glycopeptides (40 μg) was treated exhaustively with 0.4 U neuraminidase for 24 h. Finally, asialo-HCII (50 μg) was treated with arylsulfatase (1 mU, pH 5 sodium acetate buffer) at 37°C for 24 h and the reaction was followed by IEF-PAGE.

Analysis of carbohydrate components. For the analysis of hexose, two 100 μg samples of glycoprotein were each heated under nitrogen with 100 μl of 2 M trifluoroacetic acid for 6h at 100°C (13). Each of the hydrolyzed samples were cooled and passed through two columns in series: a (0.7 cm x 4.5 cm) of AG 1-X8,100-200 mesh (acetate form) and a AG 50W-X8, 100-200 mesh (H+ form). The columns were washed with a total of 10 ml water.

Abbreviations: AT III, antithrombin III; HCII, heparin cofactor II; FIIa, thrombin; IEF, isoelectric focusing; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; PAD, pulsed amperometric detection; TBA, thiobarbituric acid

The eluant from one set of columns was treated with galactose oxidase (5 U, 170 µL in 100 mM phosphate buffer, pH 7) for 40 min at 37°C. The samples were each lyophilized and dissolved in 500 µL of water.

Hexosamine was analyzed by heating glycoprotein (100 µg) under nitrogen in 4 M hydrochloric acid for 6h at 100°C (13). The hydrolyzate was lyophilized, and the residue was re-N-acetylated with 0.25 ml of a saturated solution of sodium bicarbonate and 0.05 ml of acetic anhydride. The sample was then passed through ion-exchange columns (as above), lyophilized and dissolved in 500 µL of water.

Sialic acid was analyzed by treating each glycoprotein sample (100 µg) with neuraminidase (0.3U) for 2h at 37°C. The reaction mixture was lyophilized, and the residue was dissolved in 500 µl of water. Free sialic acid was then analyzed chromatographically or by modified TBA assay (14) in which 2 mL of 5% hydrochloric acid in methyl cellosolve is added to 1.4 mL of the final assay mixture before measuring the absorbance.

Chromatography was performed essentially according to Bryan et al. (15) on an HPIC-AS6 (0.75 x 27.5 cm) anion-exchange column equipped with an HPIC-AG6 (0.75 x 7.5 cm) guard column. Sodium hydroxide (0.15 N) protected from carbon dioxide by an Ascarite trap was used at a flow rate of 0.4 ml/min to analyze hexose and hexosamine. Sialic acid was determined using 0.2 M sodium acetate in 0.2 M sodium hydroxide at a flow rate of 0.8 ml/min.

Table 1

Carbohydrate Analysis of Heparin Cofactor II

Sugar Component	Molar Ratio[a,b]	Amount[c]
Mannose	7.59 ± 0.87[d]	2.08 ± 0.24
Galactose	5.70 ± 0.46[d]	1.57 ± 0.13
N-acetylglucosamine	15.17 ± 1.83	5.10 ± 0.62
Sialic acid	5.69 ± 0.76[e]	2.68 ± 0.36

a. moles of monosaccharide/mole HCII
b. Mean standard deviation, average values of n = 3
c. µg sugar/100 µg HCII
d. Galactose was determined indirectly by subtraction of glucose measured after treatment with galactose oxidase from total amount of galactose and glucose
e. Sialic acid determined by TBA assay required more than 10 X greater glycoprotein sample and gave an identical molar ratio. Sialic acid measured was the same independent of amount of substrate, enzyme, length of treatment, or whether the intact glycoprotein or a glycopeptide mixture was examined.

The detector was a Dionex Ion-Chrom/Pulsed Amperometric Detector (PAD-I). A gold working electrode, a silver-silver chloride reference electrode, and a glassy carbon counter electrode were used. The potentials and times used were: E_1 = 0.2V; E_2 = 0.6V; E_3 = -0.8V; t_1 = 60 ms, t_2 = 60 ms, t_3 = 240 ms. Thin-layer chromatography of monosaccharides was run on zinc sulfate $7H_2O$ impregnated (10 wt%) silica plates (16).

RESULTS

Isoelectric focusing of proteins. The SDS-gel electrophoresis of ATIII and HCII each showed single major bands (Fig. 1). Molecular weights were estimated at 57,000 and 67,000, respectively, consistent with earlier reports (9,10,11). The purity was greater than 95% by scanning densitometer. The IEF gel of ATIII is shown in Fig. 1. Purified ATIII shows six to eight bands from pI 4.9 - 5.3 in IEF gel. The similar results were reported previously by performing IEF on plasma with immunofixation or immunoblotting techniques (6,7). After treatment with neuraminidase the IEF bands collapsed into a single major band (86% by scanning densitometry) of pI 5.9. IEF of HCII (Fig 1) shows five major bands pI 4.9 - 5.3 (8), while desialated HCII gives two major bands (35 and 57% by scanning densitometry) at pI 5.9 and 6.1. Immunoblotting of the IEF gel (Fig. 1B) showed all five major HCII bands (lane 1) and only the two major asialo-HCII bands (lane 2). Asialo-ATIII and asialo-

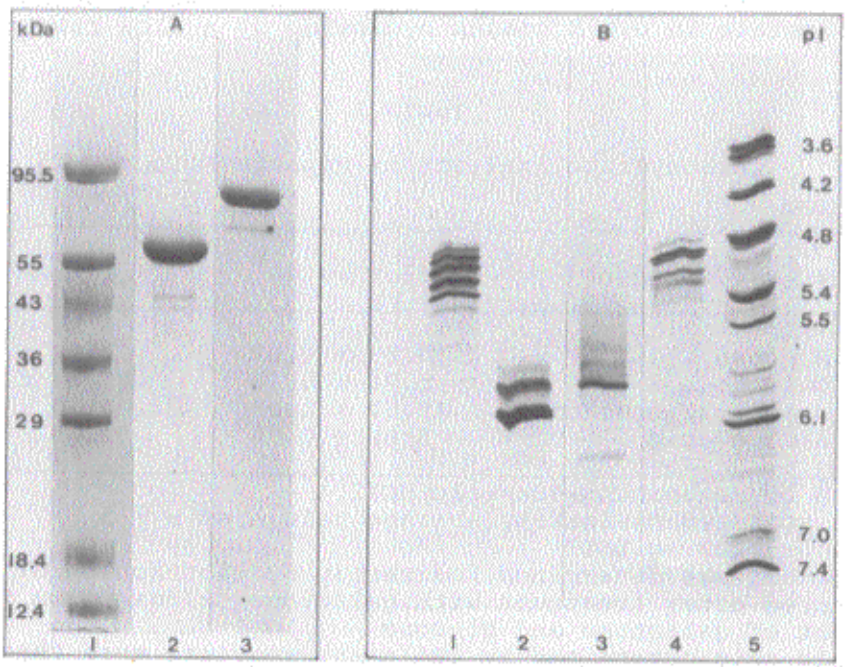

Fig. 1. SDS-PAGE (A) and agarose IEF gel (B) of ATIII and HCII. (A) Lanes: 1. Mr markers; 2. ATIII; 3. HCII. (B) Lanes: 1. HCII; 2. HCII (50 μg) after treatment with 0.4 U neuraminidase in acetate buffer (pH 5.5) for 24 hours; 3. ATIII (50 μg) after neuraminidase treatment; 4. IEF of ATIII; and 5. pI markers.

HCII both show single bands on SDS-PAGE having an apparent Mr reduced by 5,000.

Analysis of carbohydrates. ATIII and HCII were hydrolyzed, the sugars were identified by coelution with authentic sugars and were quantitated against a standard curve by ion-exchange chromatography (Fig. 2). To quantitate unresolved glucose and galactose, hydrolyzate was treated exhaustively with galactose oxidase. Each sugar was also collected from the ion-exchange column and its identity was confirmed by thin-layer chromatography. The molar ratio of mannose:galactose:N-acetylglucosamine:sialic acid in ATIII was determined as 3.0:2.2:4.6:1.7. The complete compositional analysis for HCII is given in Table 1.

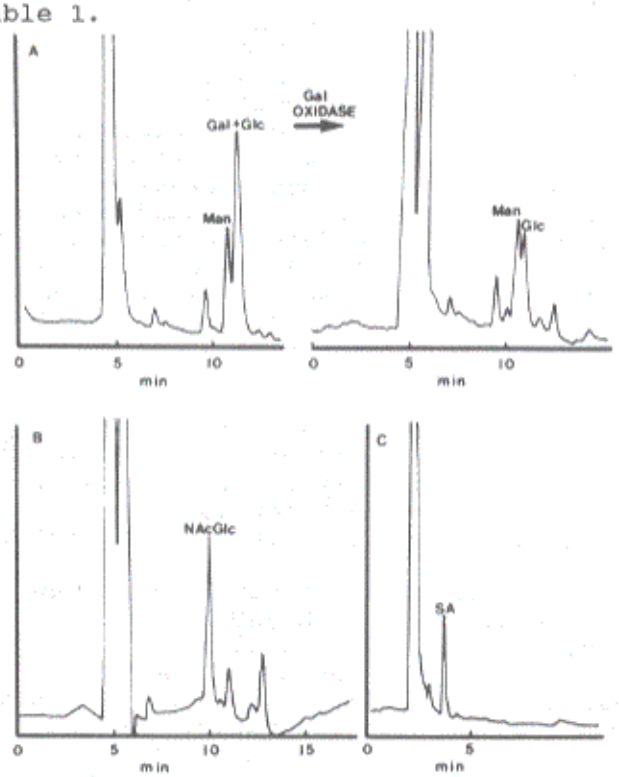

Fig. 2. Ion-exchange chromatography with PAD of HCII. (A) analysis of hexoses (mannose, Man; galactose, Gal; and glucose, Glc) in the presence and absence of galactose oxidase; (B) analysis of hexosamine (N-acetylglucosamine, NAcGlc); (C) analysis of sialic acid (SA).

DISCUSSION

Microheterogeneity among proteins is now recognized to be widespread and quite common, even though the reasons underlying it are not yet fully understood. IEF is a very powerful technique for resolving purified proteins into many additional bands. Although there is some disagreement on the pH shift of ATIII after desialation, this paper confirms the reported

(4-7) heterogeneity and indicates this heterogeneity is primarily the result of a variation of sialic acid content. Recently a physiological variant of ATIII with a reduced activity has been shown to lack one carbohydrate side chain emphasizing the importance of developing approaches to analyze the carbohydrate composition of plasma glycoproteins (17). In contrast to ATIII, two bands are observed following treatment of HCII with neuraminidase. Immunodetection using polyclonal antibodies prepared against pure HCII showed identical banding as observed by Coomassie staining, suggesting that all the bands visualized were indeed HCII. This polyclonal antibody preparation cross-reacted with only the two major bands formed on the desialation of HCII. This heterogeneity could be explained by incomplete desialation, variation in amino acid composition and(or) sequence, by the two recently identified sites of sulfation in tyrosine residues of HCII (18), or modification of oligosaccharide chains. Incomplete desialation is unlikely based on the following experiments: 1) Identical amounts of sialic acid were measured over a wide range of neuraminidase and glycoprotein concentrations and reaction times; 2) Identical amounts of sialic acid were measured following exhaustive neuraminidase treatment of intact HCII and its glycopeptide fragments; and 3) An accurate sialic acid analysis for ATIII using the same conditions, as well as similar reduction in the apparent Mr (measured by SDS-PAGE) of asialo-ATIII and asialo-HCII. To determine whether the sulfation of tyrosine was responsible for the heterogeneity of HCII, asialo-HCII was treated with arylsulfatase. Failure to detect any change in the two bands observed on IEF-PAGE suggests further studies will be required to establish this point. Finally, α_1-acid glycoprotein shows similar behavior when treated with neuraminidase (19) suggesting heterogeneity not explainable by differential sialation may be a common phenomenon.

The relative molar ratio of sugars measured for ATIII using ion-exchange chromatography with PAD is consistent with the reported structure of four identical N-linked oligosaccharide chains comprised of a molar ratio of mannose:galactose:N-acetylglucosamine:sialic acid of 3:2:4:2 (3). Kobata et al. (20) reported a similar oligosaccharide composition but one having reduced (mannose:sialic acid = 3.0:1.7) and variable sialation. These results on ATIII confirm the accuracy of monosaccharide analysis using ion-exchange chromatography with PAD. The analysis of the carbohydrate composition was also very reproducible giving standard deviations of under 10% for each sugar (n = 10). The glucose detected in both glycoprotein analyses (approximately 3.7 and 2.5% in ATIII and HCII respectively) might originate from glycolipid or lipoprotein contamination (21,22). There are several known glycans of the N-acetyllactosamine type (23). Our data, showing a molar ratio of mannose:galactose:N-acetylglucosamine:sialic acid of 4:3:8.3:3, indicate that HCII has a higher level of N-acetylglucosamine than ATIII. This suggests that HCII might not contain similar bi-antennary glycan type chains (23). Future study will be required to confirm the exact structure of HCII oligosaccharides and to fully understand the nature of its microheterogeneity.

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