A Low Molecular Weight Carbohydrate Unit Isolated from Normal Human Plasma Ba-α_2-Glycoprotein*

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ABSTRACT: Low molecular weight glycopeptides were isolated from a proteolytic digest of human plasma Ba-α_2-glycoprotein by chromatography on a paper powder column, filtration through Sephadex G-25, adsorption on a talcum column, and by high-voltage electrophoresis. One of these glycopeptides was obtained in a highly purified state. It contained a disaccharide consisting of galactose and glucosamine.

In a recent study (Ishihara and Schmid, 1967), the isolation of the glycopeptides from a proteolytic digest of human plasma Ba-α_2-glycoprotein (Schmid and Bürgi, 1961) and their separation into two groups was described. The glycopeptides of one group revealed relatively high molecular weights, and their chemical composition varied within certain limits. Comparison of the chemical composition of the carbohydrate units of these peptides with that of the carbohydrate moiety of the parent protein revealed an unequal distribution of the galactose content. The present paper reports the characterization of the second group. One of these peptides distinguishes itself by a very low molecular weight and, especially, by its unusual chemical composition accounting for the balance in the mentioned galactose content.

Material and Methods

Ba-α_2-Glycoprotein was isolated in homogeneous state from Cohn fraction VI of pooled outdated normal human plasma (Schmid and Bürgi, 1961). After removal of its sialic acid by hydrolysis with 0.1 N H_2SO_4 at 80° for 1 hr (Satake *et al.*, 1965), the *sialyl-free glycoprotein* was digested with pepsin and pronase (Ishihara and Schmid, 1967). The digest was chromatographed on a paper powder column (Ishihara and Schmid, 1967; Kamiyama and Schmid, 1962a,b) and yielded, first, a fraction earlier referred to as peptide 1 (Ishihara and Schmid, 1967), the starting material for this study, that contained amino acids, peptides, and certain glycopeptides accounting for approximately 20% of the neutral hexoses and glucosamine of the original glycoprotein (Ishihara and Schmid, 1967) and, second, the glycopeptides with large molecular weights described earlier (Ishihara and Schmid, 1967).

Gel filtration through a Sephadex G-25 column (4.6 × 150 cm) was employed for the initial separation of the amino acids, peptides, and glycopeptides from each other using 4.4 g of starting material or fraction "peptide 1." As solvent 0.05 N acetic acid was utilized, and the flow rate was adjusted to 150 ml/hr.

Chromatography on a paper powder column (1.6 × 19 cm) served further to purify the glycopeptide fraction. A mixture of 1-butanol–acetic acid–water (12:3:5, v/v) was utilized as solvent. The flow rate was 6 ml/hr.

Fractionation on a talcum column (1.8 × 18 cm) in 1 N HCl served to separate inorganic salt and other low molecular weight substances from the DNP-glycopeptides which were eluted subsequently with a mixture of four parts of 95% ethanol plus one part of 1 N HCl. The above-mentioned three procedures were carried out at room temperature.

Analytical Techniques. Neutral hexoses were determined by the anthrone method (Scott and Melvin, 1953) and the hexosamines by the methods of Rimington (1940). For the measurement of the galactose: mannose ratio, the glycopeptides (2–3 mg) were hydrolyzed in sealed tubes with 3.0 ml of 1 N H_2SO_4 at 100° for 4 hr (Schmid, 1964). Following neutralization, passage through ion-exchange columns, and concentration, the resulting hydrolysate was applid to a Whatman No. 1 paper and chromatographed employing a mixture of benzene–1-butanol–pyridine–water (1:5:3:3). The method of Whistler and BeMiller (1962) was used for the titrimetric determination of the separated monosaccharides. Appropriate controls and references were included. Control paper strips were stained with aniline–phthalate (Ishihara and Schmid, 1967). The recovery of the neutral hexoxes using authentic monosaccharides was found to be about 80%. The amino-terminal amino acids were determined by Sanger's technique (Fraenkel-Conrat *et al.*, 1955) and the nonamino-terminal amino acids according to Satake *et al.* (1965). The molecular weight was measured by the method of Yphantis (1960).

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TABLE I: The Carbohydrate Content of the Glycopeptide Fractions Obtained by Gel Filtration on Sephadex G-25.

Fraction	Weight		Neutral Hexose			Hexosamine			Ratio of Neutral Hexose: Hexosamine
	mg	%	Wt (mg)	Content (%)	Recov (%)	Wt (mg)	Content (%)	Recov (%)	
Starting material	4400	(100)	48	1.1	(100)	39	0.9	(100)	1.2
1	4.3	0.1	0.5	12	1	0.4	9.3	1.0	1.2
2	106	2.4	6.4	6	13	6	5.7	15	1.1
3	350	8.0	24	7	50	22	6.3	51	1.1
4–8	2350	54	Nil	—	—	Nil	—	—	—
Total	2810	64	31	—	64	28	—	67	—

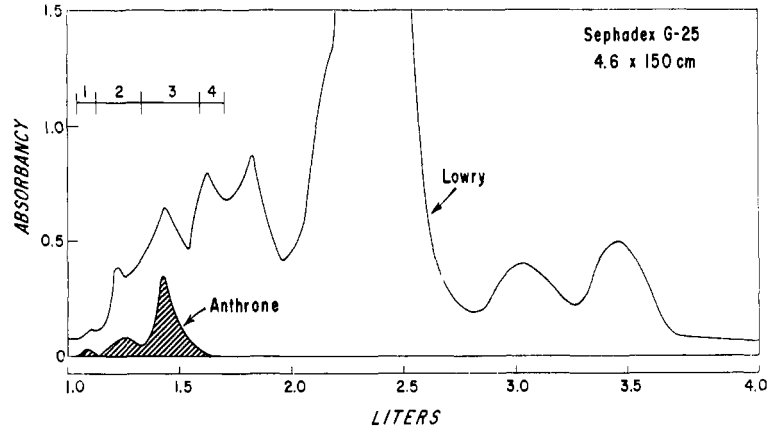

FIGURE 1: Isolation of the glycopeptides from 4.4 g of starting material by gel filtration through column of Sephadex G-25. The peptide content of each fraction was measured by the Lowry technique and that of neutral hexoses by the anthrone method. The number in the upper left corner refers to the resulting carbohydrate-containing fractions.

Results

The Carbohydrate Content and Galactose:Mannose Ratio of the Starting Material

The starting material was devoid of free neutral hexoses or hexosamine. However, after mild acid hydrolysis only galactose could be demonstrated. No mannose could be detected even when an aliquot of the hydrolysate containing a total of 50 μg of hexose was analyzed. Since the sensitivity of paper chromatography is about 1 μg, it was concluded that the percentage of mannose is less than 2% of the neutral sugars of this fraction. The content of hexose and hexosamine of the starting material was 1.1 and 0.9%, respectively (Table I).

Isolation of the Glycopeptides

Gel Filtration on Sephadex. The starting material (4.4 g) yielded on filtration through Sephadex G-25 eight fractions which accounted for 64% of its weight (Table I). Insoluble material remained on top of the column. The three fractions eluted first contained neutral hexoses (Figure 1). The weight of the two main glycopeptide fractions 2 and 3 was 106 and 350 mg, respectively. Their content of neutral hexose was 6 and 7%, respectively. The hexosamine content of these two fractions was 6%. The total amount of hexose and hexosamine recovered accounted for 65 and 73%, respectively, of that of the starting material. The galactose:mannose ratio of the mentioned three fractions indicated the lack of mannose.

Chromatographic Separation on Paper Powder Column. Fraction 3 (100 mg), obtained by gel filtration containing the major part of the recovered hexose, was further investigated by chromatography on a cellulose powder column. Four fractions were obtained which represented 90% of the weight (Figure 2). Most of the hexose was associated with the major peak which accounted for 30% of the weight of the applied fraction. The hexose content of the latter fraction was 10% and that of hexosamine 9%. Again no free hexose could be discerned. On high-voltage electrophoresis at pH 3.5, this major fraction yielded three main and three minor subfractions.

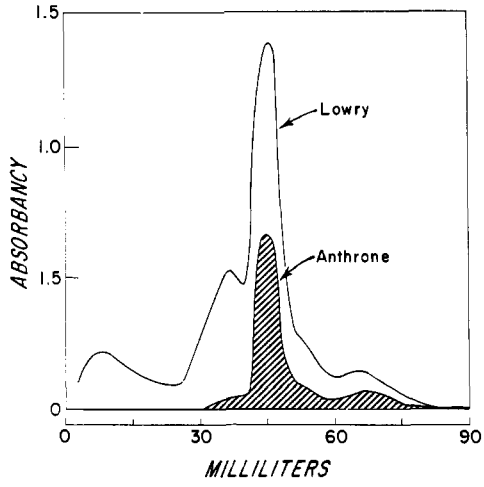

FIGURE 2: Purification of fraction 3, prepared as indicated in Figure 1, by chromatography on a paper powder column (1.6 × 19 cm) using a mixture of 1-butanol–acetic acid–water (12:3:5) as solvent.

Further Purification by Adsorption on Talcum. An aliquot of the above-mentioned major fraction (26.8 mg) was dinitrophenylated. Some of the formed DNP compounds which proved to be free of neutral hexose and probably were peptides could be extracted with ethyl acetate. The remaining aqueous solution with the carbohydrate-containing DNP compounds was passed through a talcum column. The colorless effluent and washings were free of sugar, confirming the earlier finding that free sugar was not present in the starting material or liberated by hydrolysis during this purification procedure.

After their elution from the talcum column and concentration, the DNP compounds were subjected to high-voltage electrophoresis at pH 3.5 in pyridine acetate buffer and resolved into six yellow bands. All DNP compounds moved toward the anode in contrast to galactose which, owing to electroendosmosis, migrated apparently cathodically. After elution from the paper and concentration, the weight of the two slowest moving fractions was 5.9 and 13.3 mg, amounting to 71% of the weight of the applied main fraction.

Characterization of the Glycopeptides

Hexose determination of the electrophoretically separated fractions demonstrated that only the two slowest moving compounds contained sugar. The hexose content of each of these two fractions was approximately 19% and that of the hexosamine about 17% accounting for 90% of that of the major fraction obtained by chromatography on paper powder column. The molecular weight of these fractions was found to be approximately 950 and 1000, respectively. One fraction possessed two amino-terminal amino acids and, therefore, was not analyzed further.

The other fraction (Table II) contained as amino-terminal amino acid only Asp and as nonamino-terminal amino acids Ala, Pro, Thr, and Val, each in about 1 mole/mole of glycopeptide. Moreover, this compound contained 1 mole each of galactose and glucosamine.

TABLE II: The Composition of the Small Glycopeptides of Ba-α_2-Glycoprotein.[a]

Mol Wt	950
Amino-terminal amino acid Asp	0.9
Nonamino-terminal amino acids	
Thr	0.8
Pro	1.2
Ala	1.0
Val	1.2
Hexose	1.0
Hexosamine	0.9

[a] Expressed in mole per mole of peptide.

Discussion

A glycopeptide with a low molecular weight carbohydrate unit was isolated in highly purified form from a proteolytic digest of human plasma Ba-α_2-glycoprotein. This carbohydrate unit is most likely present as a disaccharide because all studies on glycopeptides derived from plasma glycoproteins have demonstrated that the only linkage between carbohydrate and polypeptide moiety of this class of macromolecules is an *N*-glycosidic bond between the amide group of an asparaginyl and a glucosaminyl residue (Gottschalk and Graham, 1966; Neuberger *et al.*, 1966). From our earlier investigations on this glycoprotein involving partial hydrolysis of the protein itself and of the high molecular weight glycopeptides derived therefrom (Ishihara and Schmid, 1967) and partial elucidation of the monosaccharide sequence of the protein (Kamiyama and Schmid, 1961), it could be concluded that galactose probably occupies the terminal position of the low molecular weight glycopeptide decribed herein. As the sialyl residues were cleaved off specifically before proteolytic digestion of the globulin, it is possible that in the native Ba-α_2-glycoprotein sialic acid is attached to the mentioned monosaccharide.

The presence of other disaccharides as carbohydrate units (galactose-glucose and galactose-galactosamine) has been briefly reported for bovine glomerular basement membrane (Spiro, 1966) and porcine submaxillary mucoprotein (Katzman and Eylar, 1965). It should be noted that the glycopeptides with the latter disaccharides have been isolated by procedures that are completely different from the one employed in the present study.

The presence of galactose as the sole neutral hexose

in the low molecular weight glycopeptide of Ba-α_2-glycoprotein is also in agreement with the difference in the galactose content between this protein and the large molecular weight glycopeptides derived therefrom. In addition, the lack of mannose in the low molecular weight glycopeptide was also demonstrated. The low and high molecular weight carbohydrate units of Ba-α_2-glycoprotein (Ishihara and Schmid, 1967) and the carbohydrate units of many other glycoproteins serve to emphasize the wide heterogeneity of these units with regard to size and chemical composition.

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References

Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L. (1955), *Methods Biochem. Analy. 2*, 359.

Gottschalk, A., and Graham, E. R. B. (1966), *Proteins 4*, 95.

Ishihara, K., and Schmid, K. (1967), *Biochim. Biophys. Acta. (in press)*.

Kamiyama, S., and Schmid, K. (1961), *Biochim. Biophys. Acta 49*, 250.

Kamiyama, S., and Schmid, K. (1962a), *Biochim. Biophys. Acta 58*, 80.

Kamiyama, S., and Schmid, K. (1962b), *Biochim. Biophys. Acta 63*, 266.

Katzman, R. L., and Eylar, E. (1965), *Federation Proc. 24*, 231.

Neuberger, A., Gottschalk, A., and Marshall R. D. (1966), *in* Glycoproteins: Their Composition, Structure and Function, Vol. 5, Gottschalk, A., Ed., New York, N. Y., Elsevier, 273.

Rimington, C. (1940), *Biochem. J. 34*, 931.

Satake, M., Okuyama, T., Ishihara, K., and Schmid, K. (1965), *Biochem. J. 95*, 749.

Schmid, K. (1964), *Chimia 18*, 321.

Schmid, K., and Bürgi, W. (1961), *Biochim. Biophys. Acta 47*, 440.

Scott, T. A., and Melvin, E. H. (1953), *Anal. Chem. 25*, 1656.

Spiro, R. (1966), *Federation Proc. 25*, 409.

Whistler, R. L., and BeMiller, J. N. (1962), *Methods Carbohydrate Chem. 2*, 359.

Yphantis, D. A. (1960), *Ann. N. Y. Acad. Sci. 88*, 586.