A Glycopeptide Isolated from Human Gastric Juice

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A glycopeptide containing 69% carbohydrate was isolated from human gastric juice. The complex was found to be homogeneous and to have mol.wt. 9600. The glycopeptide consisted of a protein core to which were linked, by *O*-glycosidic linkages to threonine and *N*-glycosidic linkages, carbohydrate side chains composed of *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose, mannose, fucose and sialic acid, in the proportions 2:10:7:4:12:1.

The secretions of the digestive tract contain a great number of glycoproteins (Buddecke, 1972). *N*-Acetyl-galactosamine, *N*-acetylglucosamine, galactose, glucose, mannose, fucose and sialic acid were identified as carbohydrate components of human gastric juice (Glass, 1967; Schrager, 1969). Several glycoproteins isolated from digestive-tract secretions were high-molecular-weight substances containing four major carbohydrate residues: *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose and fucose (Buddecke, 1972; Yoshizawa, 1972). So far, the existence of mannose in purified glycoproteins or glycopeptides from gastric juice has not been reported.

The present studies are concerned with the isolation and partial characterization of a glycopeptide containing mannose isolated from human gastric juice without proteolytic digestion.

Experimental

Glycopeptide preparation from gastric juice

Human gastric juice, collected under conditions of fasting and after gastrin stimulation, was pooled and dialysed extensively against distilled water, with toluene added to prevent bacterial growth. Non-diffusible gastric aspirate was centrifuged at 3000g for 20 min to remove the turbid material. Carbohydrate-containing complexes from the gastric juice were isolated by phenol/water extraction (Kuhn & Weicker, 1970), which requires the addition of an equal volume of 90% (v/v) phenol to the non-diffusible material containing 0.85% NaCl, and then shaking of the mixture at room temperature (20–25°C) for 5 h. Phenol extraction of the lower phase was repeated twice. Dialysis of the aqueous layer against water preceded freeze-drying. This extract, yielding about 350 mg/litre of juice, was made to 2% in water and centrifuged at 12000g for 3 h to remove insoluble material. The clear supernatant was fractionated with ethanol. Most of the glycoproteins

when precipitated at an ethanol concentration of 60% (v/v). The supernatant, which was about 10% of the phenol extract, was concentrated and further fractionated on a column (2.7 cm × 90 cm) of Bio-Gel P-60 and a column (1.7 cm × 70 cm) of Bio-Gel P-6, with water as the eluent. Fractions containing carbohydrate, which were retarded by the Bio-Gel P-60 column but not by the Bio-Gel P-6 column, were pooled and concentrated. The material, representing a 5% recovery of the phenol extract, is designated crude glycopeptide in this paper.

Analytical methods

Total hexose was measured by the phenol/H₂SO₄ method (Dubois *et al.*, 1956), with a galactose standard. Fucose was determined by the method of Gibbons (1955). Sialic acid was determined by Warren's (1959) thiobarbituric acid procedure, with *N*-acetylneuraminic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.) as standard. Analysis of hexosamines was based on the method of Elson & Morgan, as described by Boas (1953), after hydrolysis of the material in 4M-HCl at 100°C for 6 h and treatment described by Hotta *et al.* (1970). Analyses on the amino acid analyser were also performed for the hexosamines and hexosaminitols. Uronic acid was determined by the carbazole/H₂SO₄ method (Bitter & Muir, 1962). Lloyd's (1959) method was used to determine sulphate after its liberation by hydrolysis in 1M-HCl for 6 h. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. The *N*-terminal amino acid was determined by the dansyl chloride method, followed by t.l.c. (Hartley, 1970).

Amino acid analyses

Samples were hydrolysed in glass-distilled constant-boiling HCl at a concentration of 1 mg/0.5 ml in

sealed evacuated tubes at 107°C for 20h. Analyses were performed on a Nihon-Denshi JLC (Tokyo, Japan) amino acid analyser.

Gas-liquid chromatography

G.l.c. was carried out with Nihon-Denshi model JGC-1100 (Tokyo, Japan) equipped with dual flame-ionization detectors and N₂ carrier gas. Sugars were chromatographed as their trimethylsilyl derivatives after preparation as described by Sweeley *et al.* (1963).

Molecular-weight determinations

Sedimentation-velocity and equilibrium measurements were made on a 0.5% solution of glycopeptide material in 90mM-NaCl by using a synthetic-boundary cell and a multi-channel cell in a Beckman-Spinco model E ultracentrifuge at 56000 and 30000rev./min respectively. The molecular weights were calculated by taking 0.65 as the partial specific volume (Yphantis, 1960).

Haemagglutination inhibition and immunological tests

The blood-group activity was tested by using the micro plate technique of haemagglutination inhibition described by Hamazaki & Hotta (1976). Immunological analyses were carried out by the micro Ouchterlony double-diffusion procedure described by Crowle (1958). Antisera against human serum, IgG,* IgM and IgA (Hyland Laboratories, Los Angeles, CA, U.S.A.) were used for the test.

Concanavalin A-Sepharose chromatography

Concanavalin A-Sepharose (Pharmacia, Uppsala, Sweden; 10mg of concanavalin A/ml of gel) was packed in a column and washed with buffer containing 0.2M- α -methyl glucoside, 1.0M-NaCl, 0.1mM-MnCl₂, 0.1mM-CaCl₂ and 18mM-ammonium acetate, pH7.0. The column was then washed completely with starting solution, containing 0.15M-NaCl, 0.1mM-MnCl₂, 0.1mM-CaCl₂ and 18mM-ammonium acetate buffer, pH7.0, until the eluate was hexose-free. After application of the sample to the column, the unadsorbed material was subsequently eluted with the starting buffer, before elution of the adsorbed material with the similar buffer containing 0.2M- α -methyl glucoside and 1.0M-NaCl described above.

Paper electrophoresis

Paper electrophoresis was performed on pre-washed Whatman 3MM paper in pyridine/acetic acid/water

* Abbreviations: IgG, IgM and IgA, immunoglobulins G, M and A.

(10:0.4:90, by vol.), pH6.3. A potential of 50V/cm width was applied. To determine the purity of the material, the paper was stained with ninhydrin and by the periodate/Schiff reaction.

Detection of O-glycosidic linkage

The sample was treated with 0.1M-NaOH containing 1M-NaBH₄ at 45°C for 15h, after which it was neutralized to pH5.0 with 4M-acetic acid. The sample was analysed for carbohydrate residues and amino acids. Part of the sample was chromatographed on Bio-Gel P-6.

Results and Discussion

The crude glycopeptide was chromatographed on an ECTEOLA-cellulose (Serva Feinbiochemica, Heidelberg, W. Germany) column (3cm×25cm) with a linear-gradient elution with NaCl and HCl (0–500mM; 1:1, v/v). Three main fractions (I, II and III) were obtained at 0mM-, 4mM- and 16mM-NaCl, and yielded 10–20, 55–60 and 5–19% respectively of the crude glycopeptide. The mannose-rich fraction II was chromatographed on a column (1.7cm×90cm) of Bio-Gel P-6 to remove the salt. Affinity chromatography on concanavalin A-Sepharose was used to fractionate further the glycopeptide as described in the Experimental section. The sample was divided into two fractions, one unadsorbed fraction (Fr-1) and one eluted with α -methyl glucoside (Fr-2), and the corresponding fractions were pooled. Both fractions were desalted by Bio-Gel P-6 chromatography. The yields of fractions Fr-1 and Fr-2 were 20–30% and 8–15% of the crude glycopeptide. A final purification of fractions Fr-1 and Fr-2 was achieved by paper electrophoresis: in this procedure, each material was applied to filter paper (58cm×20cm; 10mg of glycopeptide/electrophoretogram) and subjected to electrophoresis for 1h as described above. The areas corresponding to ninhydrin-positive spots of fractions Fr-1 and Fr-2 were eluted from the paper with water, pooled with corresponding material and concentrated. Re-electrophoresis was performed with the main fractions of Fr-1 and Fr-2. The major peak of fraction Fr-1 desalted through Bio-Gel P-6 showed only one spot; however, fraction Fr-2 still contained a slight impurity on electrophoresis. Further purification of this fraction was not achieved, since only small amounts of the material were available; quantitative analyses of the carbohydrate and amino acid components and alkali treatment were done. The yields of the purified fractions Fr-1 and Fr-2, designated glycopeptides 1 and 2 in this paper, were 10–15% and 5–7% respectively of the crude glycopeptide.

Glycopeptide 1 appeared to be homogeneous by the criteria of ultracentrifugation, electrophoresis and

Table 1. *Composition of glycopeptide*

Experimental details are given in the text. The results are expressed as g/100g of glycopeptide and mol/mol of glycopeptide (mol.wt. 9600). Hexose analyses were carried out by g.l.c.

Component	Glycopeptide	
	(g/100g)	(mol/mol)
Lysine	0.7	0.5
Histidine	0.5	0.3
Arginine	0.7	0.4
Aspartic acid	4.8	3.5
Threonine	3.7	3.0
Serine	2.6	2.4
Glutamic acid	3.9	2.5
Proline	4.7	3.9
Glycine	1.6	2.0
Alanine	1.4	1.5
Cysteine	0	
Valine	1.6	1.3
Methionine	0.2	0.1
Isoleucine	0.8	0.6
Leucine	1.9	1.4
Tyrosine	0.6	0.3
Phenylalanine	0.8	0.5
Galactose	12.6	6.7
Mannose	6.8	3.6
Fucose	19.7	11.5
<i>N</i> -Acetylgalactosamine	4.4	1.9
<i>N</i> -Acetylglucosamine	22.1	9.6
<i>N</i> -Acetylneuraminic acid	3.4	1.1

gel chromatography on Bio-Gel P-10. The molecular weight of the glycopeptide was calculated to be 9600 by the sedimentation-equilibrium method. Results of the quantitative analyses of the amino acid and carbohydrate components of the glycopeptide are given in Table 1. Total sugar and peptide accounted for 99.5% of the material dry weight. The peptide contained *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose, mannose, fucose and sialic acid in the proportions 2:10:7:4:12:1 for a mol.wt. of 9600. Glucose, xylose, uronic acid and sulphate could not be detected. The observation that the glycopeptide did not interact with concanavalin A suggests the possibility that the mannose residues can be substituted at the C-3, C-4 and C-6 positions or linked β -glycosidically (Goldstein *et al.*, 1973). On the other hand, in glycopeptide 2 the mannose residues were enriched relative to other sugars as compared with glycopeptide 1; the components were 10.8% galactose, 20.2% mannose, 5.3% fucose, 0.7% *N*-acetylgalactosamine, 21.3% *N*-acetylglucosamine and 5.7% *N*-acetylneuraminic acid. The amino acid composition of the glycopeptide was similar to that of glycopeptide 1, except that the aspartic acid (7.2%) and glutamic acid (5.0%) contents of the former were higher than that of glycopeptide 1. Cysteine was not

demonstrable in either peptide. After dansylation of glycopeptide 1, proline was the only *N*-terminal amino acid detected. The results indicate that glycopeptide 1 is composed of a single polypeptide chain. Glycopeptide 1 showed H human-blood-group activity, but not blood-group A and B activities, suggesting that neither *N*-acetylgalactosamine nor galactose residues occupied the terminal non-reducing end of the carbohydrate chains responsible for A or B blood-group activity. Alkali treatment of the peptide resulted in a partial loss of threonine (31%) and *N*-acetylgalactosamine (44%), but not of serine. The increase in α -aminobutyric acid and 2-acetamido-2-deoxygalactitol corresponded to 89 and 68% of the decrease in threonine and *N*-acetylgalactosamine respectively. The number of *O*-glycosidic linkages in the glycopeptide was calculated to be 0.9 per mol on the basis of the loss of threonine. Gel chromatography of the alkali-treated glycopeptide fraction indicated that more than half of the hexose in the glycopeptide was not split from the peptide chain by alkali treatment. This fact suggests the possibility that some of the carbohydrate chains in the glycopeptide are linked to the peptide backbone through an alkali-stable linkage, such as the *N*-acylglycosylamine type. The existence of the linkage is also suggested by the mannose residue. Both types of linkages, *N*-glycosidic and *O*-glycosidic, are proposed in glycopeptide 1. No decrease in the amount of serine and threonine was noted by alkali treatment of glycopeptide 2. The result indicates that the glycopeptide contains only the *N*-glycosidic type of linkage. Although it is obvious that glycopeptide 2 differs markedly from glycopeptide 1, further studies of glycopeptide 2 must be carried out to elucidate the details.

Because of the difficulty in obtaining substantial amounts of the glycopeptide from gastric juice, the size of oligosaccharide chain, the order and the structure of the carbohydrate and the number of the chains in glycopeptide 1 cannot as yet be stated. Gastric juice is a mixture of specific gastric compounds and contaminants, such as immunoglobulins and cell-membrane fragments (Schultz & Heremans, 1966; Buddecke, 1972). It is probable that the peptide is a digestion product of a glycoprotein by pepsin, since the *N*-terminal residue was proline. The occurrence of two different types of carbohydrate-peptide linkage in a protein has been observed in IgA and erythrocyte-membrane glycoproteins (Dawson & Clamp, 1968; Winzler, 1969; Hamazaki & Hotta, 1976). Glycopeptide 1 gave no precipitin reaction with anti-(human serum) and immunoglobulins and moreover showed H human-blood-group activity, suggesting that the peptide did not originate from immunoglobulins. The carbohydrate and amino acid compositions of the glycopeptide differ quantitatively from the erythrocyte glycoproteins (Winzler, 1969;

Hamazaki & Hotta, 1976). Although the origin of the glycopeptide is still unknown, it is most likely that the glycopeptide is part of a specific gastric glycoprotein degraded easily by pepsin.

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