THE CARBOHYDRATE MOIETY OF JAPANESE MONKEY PEPSINOGENS. ITS COMPOSITION AND SITE OF ATTACHMENT TO PROTEIN

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Summary: Identification and determination of the carbohydrate component of Japanese monkey pepsinogens have been performed, and the amino acid sequence around the carbohydrate chain has been investigated. Glycopeptides were prepared by successive digestion of pepsinogens with thermolysin and aminopeptidases. Analyses of their carbohydrate composition by paper and gas-liquid chromatography showed the presence of 4 glucosamine, 6 galactose, 6-8 mannose, and 8-10 fucose residues per molecule of the carbohydrate chain, among which the high content of fucose is especially unique. The amino acid sequence of a major glycopeptide was deduced to be Ile-Gly-Ile-Gly-Thr-Pro-Gln-Ala-Asn, in which the asparagine residue is the site of attachment of the carbohydrate chain.

Japanese monkey pepsinogens have been purified from the gastric mucosa, and they were named as pepsinogens I, II, III-1, III-2, III-3, and C (1, 2). Pepsinogens I and II, which occupied about one-fifth of the total, contained carbohydrate, but the others did not. So far, carbohydrate-containing pepsinogen has not been found in other animals except for the following two cases. Chicken pepsinogen was reported to contain 6-7 moles of hexoses including 2 moles of glucosamine (3), and porcine pepsinogen to contain 3 moles of acid labile sugar, mainly glucose, (4) per mole of protein. However, no further studies on the composition and the site(s) of attachment to protein of these carbohydrates have been performed.

In this communication, we report the carbohydrate composition and the amino acid sequence of the isolated glycopeptide of Japanese monkey pepsinogen I. The results for pepsinogen I are solely shown, since those for pepsinogen II were essentially the same.

Materials and methods

Pepsinogens I and II were prepared from the gastric mucosa of Japanese monkey as described previously (1, 2). Thermolysin was purchased from Daiwa Kasei Co., Osaka. Aminopeptidase preparation purified from B. subtilis by the method of Matsumura et al. (5) was a generous gift from Dr. T. Yamamoto. It was a mixture of three aminopeptidases, e.g. aminopeptidases I, II, and III.

Isolation and purification of glycopeptide ..... Pepsinogen was digested successively with thermolysin and aminopeptidases according to the method of Ishii et al. (6). Pepsinogen (5 mg) was denatured by heating at 100° for 5 min, and then incubated with 75 μg of thermolysin in 6 ml of 0.01 M Tris-HCl buffer, pH 8.5, containing 0.02 M $CaCl_2$ at 65° for 4 hr. The reaction was stopped by heating at 100° for 5 min. Aminopeptidases (300 μg) and $MnCl_2$ (final 2 mM) were added to the reaction mixture and it was further incubated at 37° for 24 hr. The reaction was stopped by heating at 100° for 5 min. The digest was concentrated to 2 ml, then applied on a column (1.6 x 60 cm) of Sephadex G-25 previously equilibrated with 0.05 M ammonium acetate buffer, pH 5.5. The effluent fractions containing glycopeptide were pooled and concentrated under reduced pressure. The dried material was dissolved in 1 ml of 0.1 M pyridine acetate buffer, pH 4.7. Gel filtration on a Sephadex G-50 column (1.3 x 138 cm) was performed in the same buffer. The fractions containing glycopeptide were combined. Purity of the glycopeptide was examined by paper electrophoresis at pH 3.5. Buffer used was pyridine-acetic acid-water (1 : 10 : 90, v/v).

Identification and determination of carbohydrate ..... The glycopeptide was hydrolyzed in 1 ml of 1 N HCl at 100° for 7.5 hr in an evacuated sealed tube. Identification and quantitative analyses were performed by paper and gas-liquid chromatography. The solvent system used for paper chromatography was iso-butanol-pyridine-water-acetic acid (12 : 6 : 4 : 1, v/v). Each component separated on Tōyō Roshi No. 52 filter paper was extracted with water, and assayed by the phenol-sulfuric acid method (7). Gas-liquid chromatography of trimethylsilyl derivatives of sugars was carried out with a JEOL JGC-20KFP gas chromatograph on chromosorb W-AW (80-100 mesh) coated with a liquid phase containing 3 % of silicone OV-17. Hexosamine was identified and determined with a JEOL-6AH amino acid analyzer.

Amino acid analysis and amino acid sequence determination of glycopeptide ..... Amino acid analysis was performed according to the procedure of Spackman et al. with a JEOL amino acid analyzer (8). Amino acid sequence was determined by a modification (9) of the manual method of the sequential Edman degradation (10). Released PTH-amino acids were identified by thin layer chromatography (11).

Results and discussion

Isolation of glycopeptide ..... Pepsinogen was digested almost completely to free amino acids by thermolysin and aminopeptidases except for the part bearing the carbohydrate moiety. The glycopeptide was separated completely from free amino acids by Sephadex G-25 gel filtration, and was eluted as a symmetrical, single peak on Sephadex G-50 gel filtration. The glycopeptide was homogeneous as examined by paper electrophoresis at

Table I.

Amino acid and hexosamine composition of glycopeptide

Amino acid and hexosamine	Number of residues per molecule of glycopeptide[1]		Nearest integer[2]
	1 N HCl 100° 7.5 hr	6 N HCl 110° 24 hr	
Asp	1.00	1.00	1
Thr	0.14	0.97	1
Glu	0.90	1.11	1
Pro	nd	1.02	1
Gly	0.31	1.48	1-2
Ala	0.33	0.89	1
Ile	0.20	1.36	1-2
Glucosamine	4.10	1.14	4

1) The number of each amino acid residue was calculated assuming the number of aspartic acid to be 1.00 per molecule of glycopeptide.
2) The integers were obtained from 24-hr values, except that 7.5-hr value was used for glucosamine. nd : not determined.

pH 3.5 and chromatography on a Sephadex G-50 column. It contained all the carbohydrate originally present in the parent protein. It was thus assumed that the carbohydrate moiety was homogeneous and attached to the pepsinogen at a single site.

Amino acid and carbohydrate compositions of glycopeptide ..... Upon amino acid analyses of acid hydrolysates of the glycopeptide seven kinds of amino acids were found together with glucosamine (Table I). The number of each residue was calculated by assuming the number of aspartic acid to be one, and the peptide moiety appeared to be composed of 7 to 9 amino acid residues. When the glycopeptide was hydrolyzed in 1 N HCl at 100° for 7.5 hr, aspartic acid and glucosamine were shown to be quantitatively released. Therefore, based on this analysis, the number of glucosamine residue was estimated to be 4. Glucosamine, galactose, mannose, and fucose were identified and determined by paper chromatography

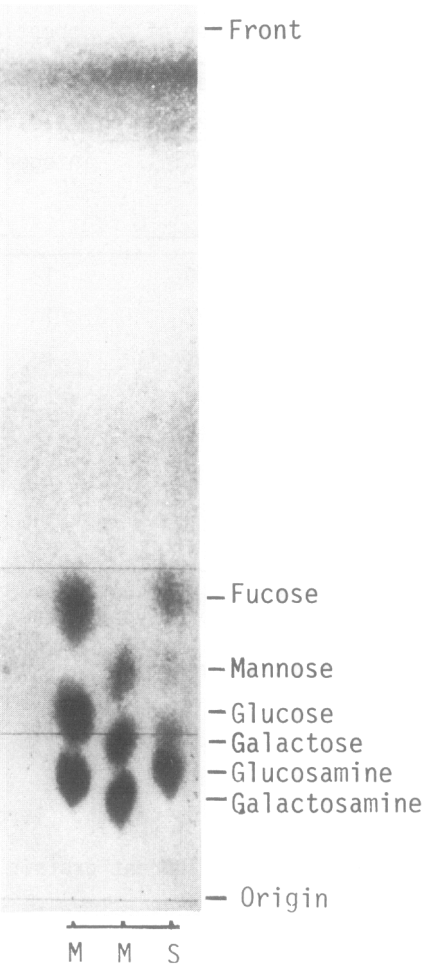

Fig. 1. Paper chromatogram of carbohydrate components in an acid hydrolyzate of the glycopeptide.

Detection of spot : silver nitrate-NaOH.

M : a mixture of standard sugars.

S : sample.

(Fig. 1) and gas-liquid chromatography. The relative number of each residue was calculated assuming that of glucosamine as 4.0 (Table II). The composition is unique especially in high content of fucose, and appears

Table II.

Carbohydrate composition of glycopeptide

	Number of residues per molecule of glycopeptide[1)]	Nearest integer
Glucosamine	4.0	4
Galactose	6.4 (6.0[2)])	6 (6[2)])
Mannose	5.7 (7.9[2)])	6 (8[2)])
Fucose	7.8 (9.5[2)])	8 (10[2)])
Sialic acid[3)]	0.05	0

1) Each value was obtained by chemical analysis after paper chromatography.
2) Values obtained by gas-liquid chromatography. The number of residue was calculated assuming the number of galactose residue to be 6.0.
3) Glycopeptide was hydrolyzed in 0.1 N H_2SO_4 at 80° for 40 min. Sialic acid was determined by the thiobarbituric acid method (13).

to be related to blood group substances. Sialic acid content was so low that it does not seem to be the component of the carbohydrate moiety. Based on these results, the molecular weight of the carbohydrate chain should be around 4,000-5,000.

Amino acid sequence of glycopeptide ..... Manual Edman degradation was performed using 100 nmoles of the glycopeptide. The yields of the major PTH-amino acids identified during each cycle of degradation are given in Table III. From cycle 3 to 7, two kinds of PTH-amino acids were detected in each step. This indicates that the peptide moiety of the glycopeptide was a mixture of the following two peptide fragments derived from the same carbohydrate attachment site:

Ile-Gly-Ile-Gly-Thr-Pro-Ala-Gln-Asx

and Ile-Gly-Thr-Pro-Ala-Gln-Asx.

The C-terminal residue is thought to be asparagine, to which the carbohydrate chain was bound. The presence of the carbohydrate chain is thought to have inhibited further digestion of the peptide moiety by aminopeptidases. On the other hand, the Gly-Ile bond seems to have been partially hydrolyzed by thermolysin to give the heptapeptide. The amino

Table III.

Manual Edman degradation of glycopeptide

Cycle	Yield (%)[1)]	Residue identified
1	40	Ile
2	32	Gly
3	17	Ile, Thr
4	13	Gly, Pro
5	8	Thr, Ala
6	7	Pro, Gln
7	4	Ala, Asx[2)]
8	nd	Gln
9	nd	Asx[2)]

1) Yields of the major PTH-amino acids were determined spectrophotometrically at 269 nm. No correction was made for either overlap or background.
2) Identified only as dansyl-derivative (14). No PTH-amino acid was found, indicating the attachment of the carbohydrate chain to this residue.
nd : not determined.

acid sequence of the nonapeptide is identical with that of residues 18 to 26 of porcine pepsin determined by Tang et al. (12), except that in porcine pepsin residue 26 is aspartic acid. The presence of asparagine rather than aspartic acid at this position seems to enable Japanese monkey pepsinogen to have the carbohydrate moiety. It is interesting to note that in porcine pepsin aspartic acid-26 is followed by the sequence Phe-Thr and is located near one of the active site aspartic acid (Asp-32) (15). Presumably monkey pepsinogens I and II would also have a sequence Asn-X-Thr (or Ser), which is a characteristic sequence where asparagine residue is the site of carbohydrate attachment (16). Pepsins I and II have much lower specific activity toward hemoglobin than other monkey pepsins which have no carbohydrate (1). This may be due to the inhibitory effect of the carbohydrate moiety which is attached near the active site.

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