ノロゲンゲAllolepis hollandiの体表面粘質物の研究I

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Studies on Body-surface Mucin of a Fish, "Norogenge" *Allolepis hollandi*—I Chemical Compositions of Glycoproteins and Glycopeptide*1

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Several glycoproteins were isolated under neutral conditions from the body-surface mucin of a fish, "Norogenge" *Allolepis hollandi*, purified and their chemical compositions examined. Pronase digestion of these glycoproteins gave two kinds of glycopeptides which were separated by DEAE-cellulose column chromatography. The first and major component contained glucosamine, neutral sugars (galactose and mannose), *N*-acetylneuraminic acid, and aspartic acid in molar ratios of 3 : 2 : 6–7 : 1. This glycopeptide contained some other amino acids as well. The linkage between carbohydrate and protein was deduced to be of the *N*-glycosidic type between asparagine and glucosamine.

Epithelial mucins are widely distributed in the cells of animals, especially in the lining cells of the cavities communicating with the environment. The mucins from digestive and respiratory tracts have been most intensively investigated and it was elucidated that the main components are glycoproteins containing sialic acid and sometimes sulfuric acid[1,2]. On the other hand, body-surface mucins of a fish have been much less studied and little is known about their properties and especially about their structures, although their chemical compositions were examined by several groups[3–10].

The structure of these body-surface mucins, especially that of carbohydrate moiety is very interesting from biological and also from chemotaxonomical standpoints.
The authors were interested in "norogenge" *Allolepis hollandi*, a fish living in the Japan Sea, whose body is covered with thick galatinous substance. In this paper, isolation and purification of glycoproteins from the body-surface mucin of "norogenge" are described together with the chemical compositions of one of the glycopeptides obtained after pronase digestion and the mode of linkage between carbohydrate and protein.

Materials and Methods

Materials Specimens of "norogenge" *Allolepis hollandi* were captured in the Bay of Toyama (Japan Sea) in March. Within a day or two the body-surface mucous layer was collected together with the thin skin. This mucin was stored in methanol in the cold (0°C), and used as starting material for glycoprotein preparation. Pronase-P was pur-

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Analytical methods Amino acid analysis was carried out with a Hitachi automatic amino acid analyzer Model KLA-2 or a Hitachi liquid chromatograph Model 034. Samples were hydrolyzed in 6 N HCl in sealed tubes at 110°C for 16–24 h. Total neutral sugar was determined by the anthrone method[11] or the orcinol method[12]. The contents of galactose and mannose were determined by gas liquid chromatography after hydrolysis in 2 N trifluoroacetic acid or in 2 N HCl at 120 or 100°C for 3.5 h, followed by reduction with sodium borohydride, and by acetylation with acetic anhydride and sodium acetate. Gas liquid chromatography was performed with a Hitachi gas chromatograph Model K-53 using the conditions of ALBERSHEIM *et al.*[13]. *N*-Acetylneuraminic acid was determined by the thiobarbituric acid method[14]. Glucosamine and galactosamine were determined by the automatic amino acid analyzer after hydrolysis in 4 N or 6 N HCl at 110°C for 6–16 h.

High-voltage paper electrophoresis was carried out on Toyo No. 51A paper in borate buffer (pH 8.0) and pyridine-acetic acid-water (1: 10: 89) at 20 V cm^{-1} for 2–3 h at 5°C. The glycopeptides were detected with 0.1% ninhydrin in *n*-butanol, and with the chlorination method[15].

Viscosity was determined using on Ostwald viscometer at 23°C.

Extraction and fractionation of glycoprotein mixture The mucin (about 4–5 kg) from 2000 fishes was homogenized in a mixer and suspended in 0.05 M phosphate buffer (pH 7.0, 20 *l*). The insoluble materials (mostly skin) were removed by centrifugation at 6000 rpm, and the supernatant containing approx. 0.5% of glycoproteins was used for isolation of glycoproteins and preparation of glycopeptides. Crude glycoproteins in phosphate buffer (approx. 1.5 g in 330 m*l* solution) was placed on a DEAE-cellulose column (5.1 cm × 73.5 cm) equilibrated with 0.05 M phosphate buffer (pH 7.0) and then eluted stepwisely with 0.05 M, 0.02 M and 0.5 M phosphate buffer (pH 7.0) and 0.1%NaOH as shown in Fig. 1. Fraction size was 20 m*l*. The fractions in each peak were collected and lyophilized after dialysis against water. P-1 (Fraction no. 65–95), 92 mg: P-2 (no. 108–127), 118 mg: P-3 (no. 180–195), 137 mg: P-4 (no. 199–215), 122 mg: P-5 (no. 600–660), 366 mg.

Preparation of glycopeptides by pronase digestion The following conditions were used for digestion of glycoproteins. To a 0.5–1% solution of substrate in 0.05 M phosphate buffer (pH 7.0) was added pronase-P (1/20–1/100 of substrate), and this solution was incubated at 37°C for 2 days maintaining the starting pH-value by occasional additions of dil. NaOH. After concentration of the digested solution followed by addition of approx. 5-fold volume of ethanol was obtained the glycopeptide mixture, which was digested once more under the same conditions as mentioned above.

The glycopeptide mixture precipitated by addition of 5-fold volume of ethanol was

desalted by gel filtration on a Sephadex G-25 column and lyophilized to give a mixture of glycopeptides (GP, 306 mg).

Fractionation of Crude Glycopeptides (GP) on DEAE-Cellulose A DEAE-cellulose column (4.2 cm×26.5 cm) was equilibrated with 0.05 M phosphate buffer (pH 7.0). The crude glycopeptide mixture (GP, 306 mg) was dissolved in the 0.05 M phosphate buffer and placed on the column. Elution was carried out stepwisely with the 0.05 M and 0.2 M buffer, and 10 m*l* fractions were collected. Elution diagram is shown in Fig. 2. The fractions in each peak were collected, concentrated to a small volume and desalted by gel filtration on a Sephadix G-25 column. The following glycopeptides were obtained by lyophilization.

GP-1 (Fraction no. 20–28), 91.1 mg: GP-2 (no. 105–112), 6.0 mg.

GP-1 was proved to be homogeneous electrophoretically. Its mobilities in the borate and pyridine-acetate buffers for 2 h under the conditions described in the experimental were 5.6 and 4.6 cm, respectively. GP-1 was eluted as a single peak behind that of blue dextran when gel-filtered through a Sephadex G-25 column. This indicates that the molecular weight of GP-1 is less than 5000 and deduced to be about 3000–4000 from the partition coefficient.

Results

Purification and Chemical Composition of Glycoproteins The body-surface mucin of "norogenge" was homogenized and suspended in 0.05 M phosphate buffer (pH 7.0) with its thin skin. The glycoproteins extracted with the phosphate buffer was fractionated

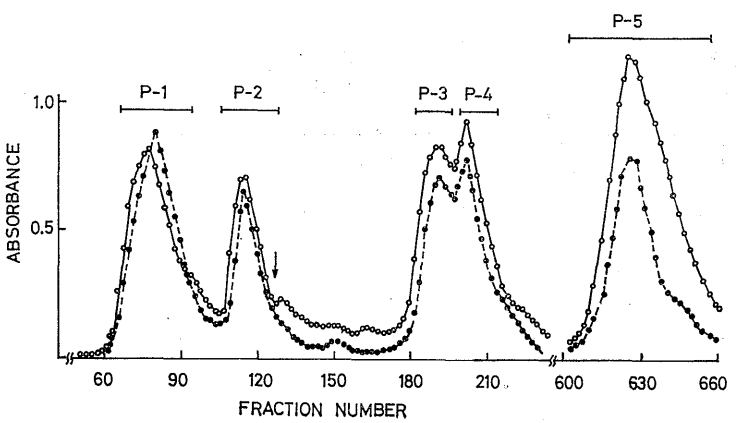

Fig. 1. Fractionation of glycoprotein mixture on DEAE-cellulose. Elution was carried out stepwisely with 0.05 M, 0.2 M and 0.5 M phosphate buffer (pH 7.0) and 0.1% NaOH, and monitored for protein by the method of LOWRY *et al.*[16] (○—○) and for sugar by the phenol-sulfuric acid method[17,18] (●—●). The arrow indicates the change of eluant from 0.05 M to 0.2 M phosphate buffer. Elution with 0.5 M phosphate buffer and 0.1% NaOH were started from fraction no. 300 and no. 415, respectively. The temperature was 4°C.

on a DEAE-cellulose column (phosphate form) to give five glycoproteins (Fig. 1), whose chemical compositions are shown in Table 1. There is no characteristic difference among these glycoproteins in the composition of amino acids. These glycoproteins contained 4–8% neutral sugars, which were identified as mannose and galactose by gas chromatography after acid hydrolysis. Aminosugars were identified as glucosamine and galactosamine and determined after acid hydrolysis by amino acid analyzer. The content of sulfate could not be determined by the benzidine method[19] due to the presence of a large amount of protein.

Table 1. Chemical compositions of Glycoproteins isolated from body-surface mucin of "norogenge"

Amino acids*1	P–1	P–2	P–3	P–4	P–5
Asp	57	35	48	47	43
Thr	38	25	33	30	22
Ser	32	22	29	27	24
Glu	47	34	50	48	42
Pro	24	16	13	11	9
Gly	43	29	34	33	24
Ala	55	37	47	44	39
Val	34	23	37	30	30
Met	9	5	9	8	7
Ile	21	16	28	25	22
Leu	38	25	44	42	36
Tyr	16	9	15	13	12
Phe	17	11	19	16	12
Lys	34	23	30	28	21
His	28	19	10	9	9
Arg	17	11	18	17	14
Neutral sugars (%)	6.1	6.3	7.8	8.3	4.4
Glucosamine (Galactsamine) (%)	0.3(0.3)	0.2	0.2	0.1	0.1
N-Acetylneuraminic acid (%)	0.8	0.5	1.3	1.3	0.5
Uronic acid*2 (%)	0.6	0.5	1.4	1.6	2.3
Nitrogen (%)	11.88	8.25	11.40	10.65	9.91
Viscosity*3	0.20	0.12	0.20	0.25	0.21

*1 The contents of amino acids were expressed in terms of mol/100 mg sample.
*2 Determined as glucuronic acid.
*3 Viscosity number at c=0.4 g/100 m*l*.

Preparation and Purification of Glycopeptides The glycoprotein mixture extracted with the phosphate buffer was digested twice with pronase to give a glycopeptide mixture in a few % yield. Thus obtained glycopeptide mixture (GP) was fractionated on DEAE-cellulose (phosphate form) using phosphate buffer (pH 7.0) as an eluant as shown in Fig. 2. Only two glycopeptides (GP-1 and GP-2) were obtained. The yield of the glycopeptide mixture was about 1–2% from the glycoprotein mixtures.

As shown in Table 2, the carbohydrate moiety of glycoproteins from the body-surface mucin is composed of *N*-acetylneuraminic acid, neutral sugars and glucosamine. The neutral sugars were proved to be mannose and galactose by paper chromatography and

gas liquid chromatography. Pentose, uronic acid and sulfate could not be detected. Molecular weight of this glycopeptide was roughly estimated by gel filtration to be 3000–4000.

The results in Table 2 suggest that the carbohydrate moiety is attached to aspartic acid or threonine. In fact, the presence of *N*-glycosidic bond between asparagine and glucosamine was deduced for GP-1. After partial acid hydrolysis of this glycopeptide, the hydrolysate was examined by paper electrophoresis and an amino acid analyzer[22], and the spot and peak corresponding to synthesized 2-acetamido-1-*N*-(4′-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine[23] were detected, respectively. However, its isolation could not be done.

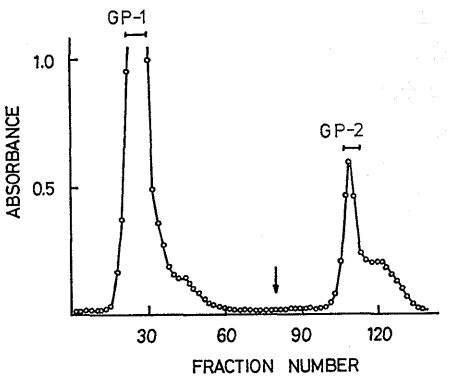

Fig. 2. Fractionation of GP on DEAE-cellulose. Elution was carried out with 0.05 M and 0.2 M phosphate buffer (pH 7.0), and monitored for sugar by the phenol-sulfuric acid method. Change of eluant is indicated by the arrow.

Table 2. Chemical composition of GP-1. Compositions are expressed in terms of molar ratio

Amino acids	
Aspartic acid	1.0
Threonine	0.64
Serine	0.48
Glutamic acid	0.29
Glycine	0.40
Alanine	0.17
Glucosamine	2.7
Galactosamine	0
N-Acetylneuraminic acid*	2.0
Neutral sugars	6.5

* Identified by comparing the Rf values of paper chromatography using *n*-butanol-pyridine-water (6 : 4 : 3, v/v) and ethyl acetate-acetic acid-water (3 : 1 : 3, v/v) as developing systems with the reported values including *N*-glycolyl- and *N,O*-diacetylneuraminic acids[20,21].

Discussion

Although main components of the body-surface mucin of fish seem to be glycoproteins, the general method of their isolation and purification has not been established due to diversity of the properties, that is, chemical compositions, and also due to lack of knowledge about them. At first, we tried to isolate the components of the body-surface mucin of "norogenge" under a neutral condition. Until now chemical compositions have been reported by some workers for the body-surface mucin of fishes themselves and for the

glycoproteins isolated from them. Carbohydrate contents vary from a few to 15% for the former[3,6-7] and from 25 to 30% for the latter[8,10]. Amino acid composition is also variable, although in general acidic one such as glutamic and aspartic acid, and hydroxy one such as threonine and serine are abundant[4,8,10].

The carbohydrate content of glycoproteins isolated from the body-surface mucin of "norogenge" was approx. 7–10%. The contents of neutral sugars, amino sugars, *N*-acetylneuraminic acid and uronic acid were 4.4–8.3, 0–0.6, 0.5–1.3 and 0.5–2.3%, respectively. However, comparing these values with their molar ratios in the glycopeptide (GP-1) the contents of amino sugars and *N*-acetylneuraminic acid must be lower than the actual ones. The low content of amino sugars may be due to its destruction during hydrolysis[24] and that of *N*-acetylneuraminic acid due to its incomplete release by hydrolysis. This deduction was also consistent with the fact that some glycoproteins obtained from "norogenge" had scarsely aminosugars, but any glycopeptide without aminosugars was not found. Neutral sugars were identified as galactose and mannose, and aminosugars as glucosamine and galactosamine. In the chemical compositions of these glycoproteins from "norogenge" low contents of carbohydrate are characteristic compared with those of plaice and eel[8,10].

As we were interested especially in the structure of carbohydrate moiety, direct pronase digestion of mixed glycoproteins were carried out to give almost only one glycopeptide unexpectedly, although the minor component was also present (6%). The result seems to indicate the homogeneity of sugar chain, but this is not the case as shown in a subsequent paper in detail. For example, the minor component, which contained galactosamine as aminosugar, was obtained in higher yield, when the mucin was extracted under alkaline conditions. Differing from the plaice and eel glycoproteins whose carbohydrate compositions have been elaborately described, absence of galactosemine and high content of mannose are characteristic to our glycoprotein.

The linkage between carbohydrate and protein was suggested to be *O*-glycosidic type in the case of the glycoprotein from eel because of high contents of hydroxy amino acids. In contrast to this fact, it was ascertained that in the case of GP-1 the carbohydrate chain attaches to the peptide chain through the *N*-glycosidic bond between asparagine and glucosamine. Amino acids adjacent to this aspartic acid are considered to be threonine, serine or glycine. On the other hand, in the case of minor glycopeptide the content of threonine was larger than that of aspartic acid, and *O*-glycosidic linkage must be also considered. Further studies along those lines are now in progress.

Acknowledgements

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