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CHARACTERIZATION OF GLYCOPROTEIN OBTAINED FROM THE SKIN MUCUS OF AN ANTARCTIC FISH, *TREMATOMUS BERNACCHII*

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Abstract: The epithelial secretions were collected by scraping the skin of *Trematomus bernacchii* and seven species of temperate-water fishes and examined for the mucous glycoproteins by gel filtration on a Sepharose CL-4B column. In most cases, the glycoproteins were excluded at the void volume and separated from other mucous constitutents. The yields of the glycoproteins resolved were usually high (35–45%) in the following fishes secreting large quantities of thick mucus from their epidermis: *Callionymus lunatus*, *Misgurnus anguillicaudatus*, *Anguilla japonica* and *Leiognathus nuchalis*. A fish with hard scales, *Carassius auratus*, gave a mucous glycoprotein in a low yield (8.4%). Similarly, the glycoproteins of *T. bernacchii*, *Trachurus japonicus* and *Acanthogobius flavimanus* were obtained in their respective yields of 17.5%, 18.9%, and 14.2%.

The carbohydrate composition of the mucous glycoprotein in *T. bernacchii* was determined to consist of *N*-acetylneuraminic acid, *N*-acetylgalactosamine, galactose, mannose, fucose and glucose by analytical methods including thin-layer chromatography, high-performance liquid chromatography and gas-liquid chromatography. The molar ratio of *N*-acetylneuraminic acid, *N*-acetylgalactosamine and galactose (1.0 : 4.8 : 1.2) suggests that major carbohydrate chains are constituted by such mono- and disaccharide units as *N*-acetylgalactosamine, *N*-acetylneuraminyl-*N*-acetylgalactosamine, and galactosyl-*N*-acetylgalactosamine. These carbohydrate units may link through *O*-glycosidic linkage to threonine and serine residues in the core polypeptide. Assuming such a structure of the carbohydrate unit, it becomes probable that the mucous glycoprotein secreted from *T. bernacchii* epithelial tissue has a similar ability to depress freezing point for the antifreeze glycoproteins present in the serum.

1. Introduction

The suborder Notothenioidei is a group of teleosts predominant in the Antarctic region, occupying more than 90% of the fishes living in the area. The fishes of this group are so tolerant to freezing that they are not frozen until their body temperature decreased to approximately −2.2°C because they in general contain eight different antifreeze glycoproteins in their sera (DEVRIES, 1982). During the investigations on

the glycoproteins in the epithelial mucous secretions from temperate-water fishes, we had an opportunity to receive a typical Antarctic fish, *Trematomus bernacchii* belonging to Notothenioidei. This promoted us to examine whether or not the skin mucous glycoprotein is involved in the depression of freezing point in the body surface of the fish together with the serum glycoproteins which have been demonstrated to have a potent antifreezing ability. Therefore, we have purified the mucous glycoprotein from the skin mucus of *T. bernacchii* by Sepharose CL-4B gel filtration, and analyzed for the carbohydrate composition for a comparison with those of seven species of temperate-water fishes.

2. Experimental

2.1. *Materials*

T. bernacchii used in this study were collected near Syowa Station by the 26th Japanese Antarctic Research Expedition (1984/85). The samples were transported to our laboratory in the frozen state and kept at −20°C until analysis. *Trachurus japonicus*, *Callionymus lunatus*, *Acanthogobius flavimanus*, and *Leiognathus nuchalis* were caught at Amakusa, Kumamoto Prefecture. These fishes were separately kept in ice bags, immediately transported to our laboratory and kept at −20°C. Living *Misgurnus anguillicaudatus*, *Auguilla japonica* and *Carassius auratus* were purchased from fish-market and then frozen at −20°C until use.

2.2. *Collection of skin mucus*

A frozen fish was dipped into diluted acetic acid solution (pH 3). This treatment is effective in fixing the mucous secretion on the skin surface since coagulation of the mucus occurred during thawing of the fish. The coagulated mucus was scraped by using a spatula. In this operation, care must be taken not to exfoliate the epithelial tissue. The mucus collected was filtrated through a layer of cheese cloth, lyophilized, and used for the preparation of mucous glycoprotein.

2.3. *Sepharose CL-4B chromatography*

Lyophilized mucus (100–150 mg) was dissolved in a small volume of 0.05 M NaOH in an ice bath for 10 min. To the viscous solution was added 20 m*l* of 0.05 M Tris-HCl buffer (pH 8.2) containing 0.2 M NaCl, and the mixture was subjected to gel filtration on a Sepharose CL-4B column. The fraction eluting at the void volume of the column was pooled, dialyzed against distilled water and lyophilized. The dried samples were stored at −20°C.

2.4. *Analytical methods*

Sialic acid was determined by the thiobarbituric acid method of UCHIDA *et al.* (1977) after hydrolysis with 0.1 N H_2SO_4 at 80°C for 30 min. Hexosamine was determined by the method of GATT and BERMAN (1966) after hydrolysis of the sample with 2 N HCl at 100°C for 16 h in an evacuated, sealed tube. Hexose was determined by the phenol-sulfuric acid method (MCKELVY and LEE, 1969). *N*-acetylneuraminic acid, D-galactosamine·HCl and D-galactose were used as the standard, respectively.

2.5. Identification of sugar components

Sialic acid in the skin mucous glycoprotein of *T. bernacchii* was hydrolyzed in diluted formic acid solution (pH 2) at 70°C for 1 h and identified by thin-layer chromatography (BUSCHER *et al.*, 1974) using a HPTLC silica gel 60 F_{254} plate and an Avicel SF plate. For hexosamine, the sample was hydrolyzed in 2 N HCl at 100°C for 16 h and the resultant free hexosamine was analyzed by thin-layer chromatography and high-performance liquid chromatography (BIDLINGMEYER *et al.*, 1984). Neutral sugars obtained by hydrolysis of the mucous glycoprotein with 1 N HCl at 100°C for 5 h was identified by gas-liquid chromatography (MCGINNIS, 1982).

3. Results and Discussion

It has been accepted that the mucus secreted from the epithelial tissue of fish is responsible for mechanical and biological functions in protecting the body from the infection of microorganisms, chemicals, stresses, and injuries. Thus far, the epithelial secretions of fishes have been reported to contain various biologically active substances including glycoproteins (ASAKAWA, 1972; FLETCHER and GRANT, 1968), lysozyme (ITAMI *et al.*, 1986; MURRAY and FLETCHER, 1976; OURTH, 1980; TAKAHASHI *et al.*, 1986), agglutinins (DI CONZA, 1970; SPITZER *et al.*, 1976; SUZUKI and KANEKO, 1986), lectins (ODA *et al.*, 1984), immunoglobulins (BRADSHAW *et al.*, 1971; FLETCHER and GRANT, 1969; LOBB and CLEM, 1981; OURTH, 1980), and glycosidases (NAKAGAWA *et al.*, 1987a, b). Among them, the epithelial glycoproteins, as a major constituent of the mucus have been assumed to play crucial roles due to their highly viscous properties and resistance to proteolysis in the defense against infection of microorganisms.

Working with an eel, *A. japonica*, we have purified a glycoprotein from the epithelial mucus by gel filtration using Sepharose 4B (ASAKAWA, 1972). Subsequent chemical and structural analyses demonstrated a glycoprotein in which approximately 400 disaccharide units, *N*-acetylneuraminyl-(α, 2→6)-*N*-acetylgalactosamine, are *O*-glycosidically linked to the hydroxy groups of threonine and serine residues in polypeptide core (ASAKAWA, 1983). The structure of the disaccharide unit was identical with that from ovine submaxillary glycoprotein, which is known to be one of typical mucins (GRAHAM and GOTTSCHALK, 1960).

In this study, we attempted to compare the chemical properties of a glycoprotein in the epithelial mucus of *T. bernacchii* with those of seven temperate-water fishes in order to explore the relation of the molecular characteristics of the glycoprotein to the adaptation of the fish to low temperature. The glycoprotein was purified from the skin mucus by gel filtration on a Sepharose CL-4B column, and the fractions were monitored for sialic acid, hexose, hexosamine, and protein (absorbance at 280 nm).

Figure 1 illustrates the elution profiles of glycoproteins in the skin mucus from *T. bernacchii* (Fig. 1, A) and five other fishes (Fig. 1, B–F) from a Sepharose CL-4B column. Distribution of sugars and protein in the fractions collected showed that the glycoproteins emerged at the void volume possessed the macromolecular nature and separated clearly from other constituents in the epithelial secretions. Further, the high contents of glycoprotein could be envisaged from the peaks for sugars and protein in the elution preofiles (Fig. 1, D–F) of the mucus from *M. anguillicaudatus*, *C. lunatus*,

and *L. nuchalis* that characteristically secrete large quantities of mucus giving rise to a thick layer on the skin surface. In contrast, peaks emerged at the void volume in the elution profiles of *A. flavimanus*, *C. auratus* (Fig. 1, B, C) and *T. japonicus* (data not shown) indicate that these fishes secrete small quantities of mucous glycoprotein forming a thin layer on the skin. The elution profile of *T. bernacchii* mucus shows that this fish falls under the category of the second group. These results were verified by the yields of mucous glycoproteins of fishes (Table 1). The data (Table 1) revealed that such fishes lacking scales or with degenerated scales in the integument as *C. lunatus*, *M. anguillicaudatus*, *A. japonica* and *L. nuchalis*, afforded high contents (35–45%) of the mucous glycoproteins. On the other hand, *C. auratus* with hard scales contained only 8.4% mucous glycoprotein. The content (17.5%) of the mucous glycoprotein from *T. bernacchii* with relatively soft scales was similar to those of *T. japonicus* (18.9%) and *A. flavimanus* (14.2%). From these results, the highly viscous glycoproteins in the mucous secretions are likely to compensate the scale-lacking fishes for protecting their skin from mechanical and for biological injuries by covering the surface.

Carbohydrate compositions of the mucous glycoproteins from *T. bernacchii* and

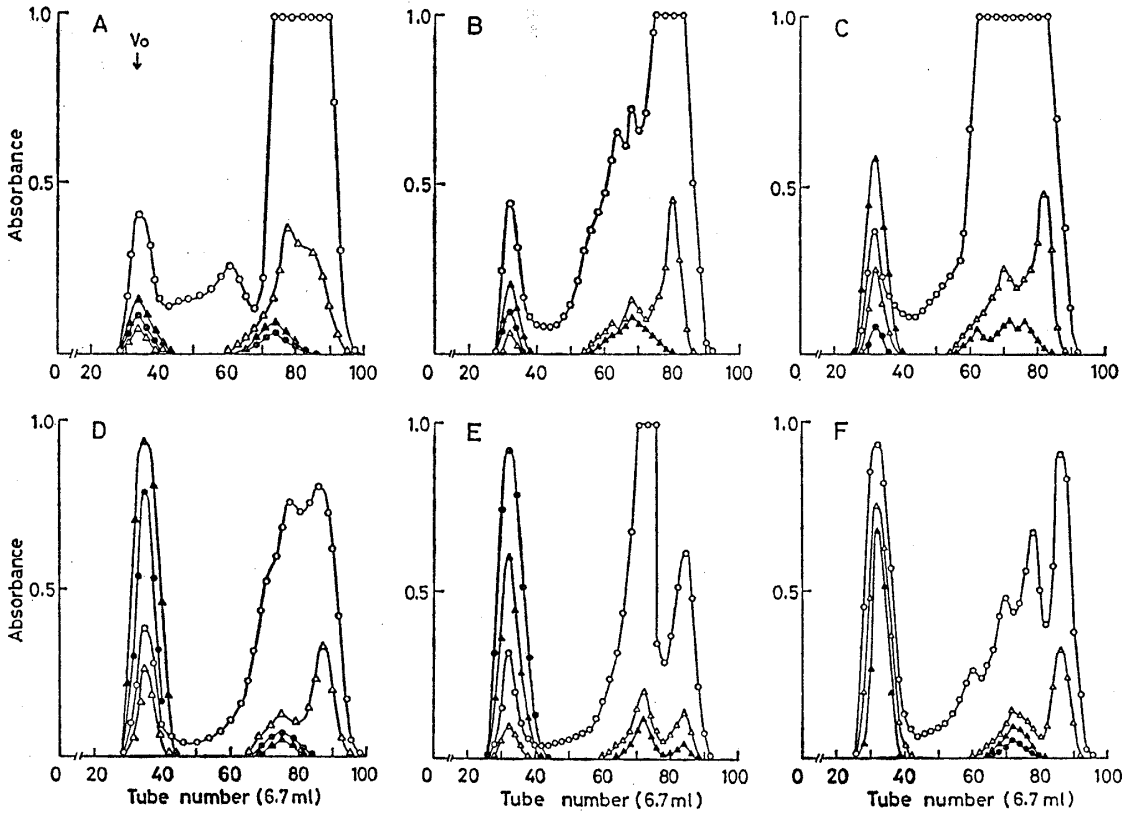

Fig. 1. Elution profiles of skin mucous glycoproteins from a Sepharose CL-4B column. The mucus sample was applied to a column (2.6×110 cm) of Sepharose CL-4B and eluted with 0.05 M Tris-HCl buffer (pH 8.2) containing 0.2 M NaCl. Fractions of 6.7 ml were collected. A, T. bernacchii; *B*, A. flavimanus; *C*, C. auratus; *D*, M. anguillicaudatus; *E*, C. lunatus; *F*, L. nuchalis. —○—, *absorbance at 280 nm;* —●—, *sialic acid;* —△—, *hexose;* —▲—, *hexosamine.*

seven other fishes are summarized in Table 2. The mucous glycoproteins of *A. japonica*, *M. anguillicaudatus* and *C. lunatus* were characterized by their high content of sialic acid while those of *C. auratus*, *T. japonicus* and *A. flavimanus* were found to contain only a small amount of sialic acid. The glycoprotein from *L. nuchalis* mucus was lacking sialic acid in its sugar constituents. The occurrence of similar mucous glycoproteins lacking sialic acid has been reported in the epithelial secretions of *Gymnura japonica*, *Raja kenojei* and *Pleuronichthys cornutus* (NAKAGAWA *et al.*, 1988).

The mucous glycoprotein of *T. bernacchii* contained only 1.7% sialic acid, comparable with the contents in those of *C. auratus*, *T. japonicus* and *A. flavimanus*. It also contained fucose, galactose, mannose, and a small amount of glucose (Fig. 2). Sialic acid and hexosamine in the mucous glycoprotein of *T. bernacchii* were identified to be *N*-acetylneuraminic acid and *N*-acetylgalactosamine, respectively, by thin-layer chromatography and high-performance liquid chromatography (Tables 3 and 4). The analytical values of *N*-acetyleneuraminic acid, *N*-acetylgalactosamine and galactose gave the molar ratio of 1.0 : 4.8 : 1.2.

Table 1. Yields of the skin mucous glycoproteins from 8 species of fishes by Sepharose CL-4B gel filtration.

Fish	Yield (%)[a]
Trematomus bernacchii	17.5
Callionymus lunatus	44.6
Misgurnus anguillicaudatus	41.0
Anguilla japonica	38.1
Leiognathus nuchalis	35.0
Trachurus japonicus	18.9
Acanthogobius flavimanus	14.2
Carassius auratus	8.4

[a] Yield (%)=(mucous glycoprotein obtained/crude mucus applied) ×100. The figures indicate the mean values from 3 runs.

Table 2. Carbohydrate compositions of the skin mucous glycoproteins from 8 species of fishes.

Fish	Percent in skin mucous glycoprotein			
	Sialic acid[a]	*N*-acetyl-hexosamine[b]	Hexose[c]	Total
Trematomus bernacchii	1.7	5.8	1.1	8.6
Misgurnus anguillicaudatus	15.1	30.9	1.7	47.8
Anguilla japonica	17.2	12.2	1.4	30.8
Leiognathus nuchalis	0	12.4	9.4	21.8
Callionymus lunatus	8.0	9.0	1.1	18.1
Carassius auratus	1.7	9.0	5.2	15.9
Trachurus japonicus	1.1	6.1	3.9	11.0
Acanthogobius flavimanus	1.2	2.5	0.6	4.2

[a] as *N*-acetylneuraminic acid.
[b] as *N*-acetylgalactosamine.
[c] as galactose.

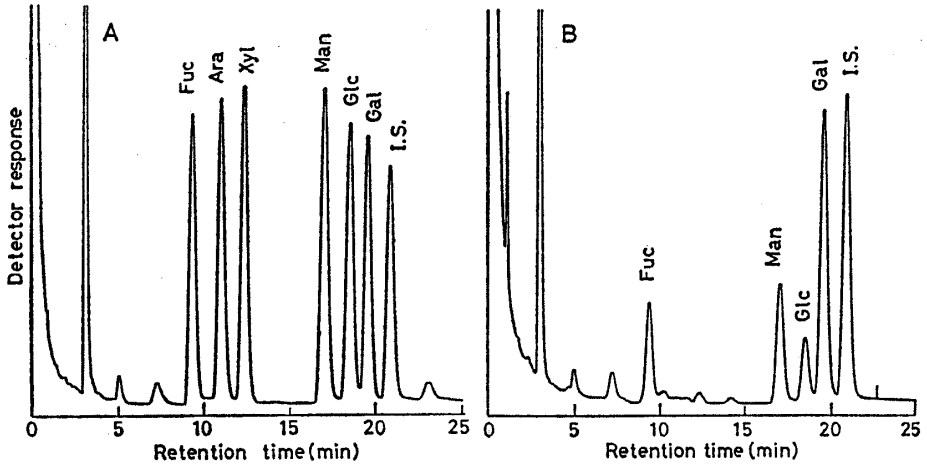

Fig. 2. Identification of neutral sugars from the skin mucous glycoprotein of T. bernacchii *by gas-liquid chromatography. A, a standard mixture of neutral sugars; B, neutral sugars from the mucous glycoprotein of* T. bernacchii. *Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Glc, glucose; Gal, galactose; I.S., internal standard myo-inositol. Condition: column, glass column (0.3×200 cm) pakced with 2% DEGA stabilized on Chromosorb HP (100/120 mesh); pressure of carrier gas, N_2 0.6 kg/cm²; column temperature, from 210°C to 240°C at a program rate of 3°C/min.*

Table 3. Identification of sialic acid from the skin mucous glycoprotein of T. bernacchii *by thin-layer chromatography.*

Sample	HPTLC F_{254}[a)] (Rf)	Avicel SF[b)] (Rf)
T. bernacchii glycoprotein	0.30	0.56
N-acetylneuraminic acid	0.30	0.55
N-glycolylneuraminic acid	0.21	0.45

a) HPTLC silica gel 60 F_{254} plate with *n*-propanol/water (7:3, v/v).
b) Avicel SF plate with *n*-butanol/*n*-propanol/0.1 N HCl (1:2:1, v/v/v).

Table 4. Identification of hexosamine from the skin mucous glycoprotein of T. bernacchii *by thin-layer chromatography and high-performance liquid chromatography.*

Sample	TLC[a)] (Rf)	HPLC[b)] (Rt, min)
T. bernacchii glycoprotein	0.30	4.22
Galactosamine	0.31	4.22
Glucosamine	0.37	4.51

a) Avicel SF plate with *n*-butanol/pyridine/0.1 N HCl (5:3:2, v/v/v).
b) Pico-Tag column (Waters).

All or a large portion of the *N*-acetylgalactosamine occurring in mucous glycoproteins is involved in the carbohydrate-protein linkage (ASAKAWA, 1977, 1983; NAKAGAWA *et al.*, 1988). From the above results, the carbohydrate chains of *T. bernacchii* are assumed to be *N*-acetylgalactosamine, *N*-acetylneuraminyl-*N*-acetylgalactosamine,

galactosyl-*N*-acetylgalactosamine, etc. These mono- and disaccharides units must be linked through *N*-acetylgalactosamine to the hydroxy groups of threonine and serine residues in the core protein. The minor carbohydrate components such as mannose, fucose and glucose may be attached to the units as the side chains.

As regards the antifreeze glycoproteins in serum of *T. bernacchii*, galactose-(β, 1→3)-*N*-acetylgalactosamine attached to threonine residue has been demonstrated as the major structural unit (FEENEY and YEH, 1978; DEVRIES, 1982). Hence, if such a structural feature of mucous glycoproteins from *T. bernacchii* and other fishes is valid, their close structural similarity to serum antifreeze glycoproteins would provide an important clue to investigate further the relation of piscine epithelial glycoproteins to their ability to depress freezing point.

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