各種魚類の体表面粘質物中のグリコシダーゼ活性とそれから分離したβ-N-アセチルヘキソサミニダーゼの性質

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Occurrence of Glycosidases and Enzymatic Properties of β-*N*-Acetylhexosaminidases in the External Mucous Materials of Various Fishes

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Substantial glycosidase activities were found in all the external mucous materials of various fishes regardless of their habitats. β-*N*-Acetylhexosaminidase activity was the most dominant among glycosidase activities examined. Since β-*N*-acetylhexosaminidase occurred enough in the mucous materials to be isolated, individual β-*N*-acetylhexosaminidases were purified from the mucous materials of eel, loach, and stingray and their enzymatic properties were compared. During the course of the purification, these enzymes behaved quite similarly on Sephadex G-200 gel filtration and CM-cellulose chromatography. The molecular weights, pH optima, and pH stabilities of the enzymes were almost identical although the *Km* value of the stingray enzyme toward *p*-nitrophenyl β-*N*-acetylglucosaminide differed from that of the other enzymes. The above facts do not support a possibility that the glycosidases may be a product of bacteria which live in the mucous material but strongly support a possibility that the enzymes may be a secretion from the fish skin.

The mucous material covering a fish body has been postulated to play an important role for the protection of the living body against bacterial infection,[1–5] an inflammatory lesion,[6] and various stresses.[1,3,5–7] So far, glycoproteins,[8–12] lysozyme,[6,13,14] and agglutinins[2–4,15,16] have been found in the external mucous materials of fishes. In the course of studies on mucus glycoproteins, we unexpectedly found glycosidase activities in the mucous materials of fishes. This paper reports the glycosidase activities in the external mucous materials of various fishes and the enzymatic properties of β-*N*-acetylhexosaminidases isolated from the mucous materials of eel, loach, and stingray.

Materials and Methods

Substrates

p-Nitrophenyl β-D-*N*-acetylglucosaminide and *p*-nitrophenyl α-D-*N*-acetylgalactosaminide were purchased from Koch-Light Laboratories Ltd. *p*-Nitrophenyl α- and β-D-galactosides, *p*-nitrophenyl α-D-mannoside, and *p*-nitrophenyl β-D-glucuronide were purchased from Nakarai Chemicals Ltd.

Collection of External Mucous Materials of Fishes and Preparation of Acetone Powders

Living fishes were obtained directly from fishermen. The mucous material covering the fish body was immediately collected by shaving with an unsharpened knife, care being taken not to exfoliate the epithelial tissue. The mucous material was put into 10 volumes of acetone with stirring and the suspension was filtered. The residue on the filter was repeatedly washed with acetone and dried under reduced pressure. The dry residue was pulverized in a porcelain mortar. The acetone powder was stored in a freezer until use.

Extraction of Enzymes for Glycosidase Assays

The acetone powder (100 mg) was sufficiently swollen with a small volume of cold 0.05 M sodium citrate buffer, pH 5.0, and then homogenized with 3.0 m*l* of the same buffer for 2 min in a blender (Sakuma Seisakusho). The homogenate was centrifuged at 15,000×g for 30 min to obtain a clear extract which was kept in an ice chest and used within 1 h for the assays of glycosidase activities.

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Enzyme Assays

Activities of glycosidases were assayed at 37°C using the corresponding *p*-nitrophenyl glycoside as substrate. The enzyme solution (20–200 μl) was added to 0.5 m*l* of 2 mM *p*-nitrophenyl glycoside dissolved in 0.05 M sodium citrate buffer, pH 5.0. After incubation for a preset time, 3.0 m*l* of 0.2 M sodium borate buffer, pH 9.8, was added to stop the reaction and the absorbance of the resultant solution was measured at 400 nm. One unit of enzyme was defined as the amount of enzyme which hydrolyzes 1 μmol of *p*-nitrophenyl glycoside per min under the conditions described above.

Analytical Methods

Protein was determined by the method of Lowry *et al.*[17] with crystalline bovine serum albumin as the standard. Sialic acid was determined by the thiobarbituric acid of Aminoff.[18] The molecular weights of β-*N*-acetylhexosaminidases were estimated by Sephadex G-200 gel filtration according to the procedure of Andrews.[19] The initial velocity of the hydrolysis of *p*-nitrophenyl β-*N*-acetylglucosaminide by β-*N*-acetylhexosaminidase was determined by the method of Algranati.[20]

Results

Glycosidase Activities in External Mucous Materials of Fishes

The major glycosidase activities found in the acetone powders of the external mucous materials of various fishes are summarized in Table 1. Various glycosidase activities were found in all of the mucous materials examined, regardless of the habitats of fishes such as freshwater, seawater, inland seas, and the Pacific Ocean. β-*N*-Acetylhexosaminidase activity was dominant throughout all the specimens. Comparatively low glycosidase activities were found in the mucous materials of the fishes which were caught in the southern Pacific Ocean. This low activities are considered to be due to the inactivation caused by freezing and thawing and during storage in a freezer.

Isolation of β-N-Acetylhexosaminidases from External Mucous Materials of Eel, Loach, and Stingray

1. Step 1. Extraction and ammonium sulfate precipitation—All operations were carried out at about 5°C. Each (4 g) of the acetone powders of the mucous materials was sufficiently swollen with a small volume of 0.05 M sodium citrate buffer, pH 5.0, and then homogenized with 200 m*l*

Table 1. Glycosidase activities in external mucous materials of various fishes

Fish name	Units/g of acetone powder					
	β-*N*-Achex	α-*N*-Acgal	β-Gal	α-Gal	α-Man	β-GA
Kingyo (gold fish, *Carassius auratus*)	7.5	1.6	1.1	0.5	0.5	0.3
Koi (carp, *Cyprinus carpio*)	7.3	1.4	0.6	0.3	0.5	0.2
Unagi (eel, *Anguilla japonica*)	6.5	1.0	0.6	0.2	0.3	0.2
Dojō (loach, *Misgurnus anguillicaudatus*)	5.7	0.8	0.5	0.2	0.3	0.2
Shiroguchi (White croaker, *Argyrosomus argentatus*)	8.1	2.0	1.4	0.8	0.8	0.3
Hiiragi (slimy, *Leiognathus nuchalis*)	7.1	0.8	0.6	0.4	0.5	0.3
Kochi (bartailed flathead, *Platycephalus indicus*)	6.8	1.1	0.7	0.3	0.4	0.3
Mahaze (goby, *Acanthogobius flavimanus*)	6.7	0.7	0.4	0.2	0.3	0.2
Kuchizoko (tonguefish, *Paraplagusia japonica*)	6.4	1.1	0.5	0.4	0.3	0.3
Akaei (stingray, *Dasyatis akajei*)	6.1	0.9	0.6	0.3	0.4	0.2
Hirame (flounder, *Paralichthys olivaceus*)	5.9	0.6	0.5	0.2	0.2	0.2
Maanago (conger eel, *Astroconger myriaster*)	5.3	0.9	0.7	0.3	0.3	0.2
Torafugu (puffer, *Takifugu rubripes*)	3.6	0.5	0.3	0.2	0.2	0.1
*Itoyori (golden thread, *Nemipterus virgatus*)	1.3	0.2	0.1	0.1	0.1	t
*Kihada (yellowfin tuna, *Thunnus albacares*)	1.0	0.1	t	t	t	t
*Shiira (dorado, *Coryphaena hippurus*)	1.0	0.1	t	t	t	t

* These fishes were caught in the southern Pacific Ocean. The external mucous materials collected there were kept in a frozen state for 2 months before their acetone powders were prepared. β-*N*-Achex, β-*N*-acetylhexosaminidase [EC 3.2.1.30]; α-*N*-Acgal, α-*N*-acetylgalactosaminidase [EC 3.2.1.49]; β-Gal, β-galactosidase [EC 3.2.1.23]; α-Gal, α-galactosidase [EC 3.2.1.22]; α-Man, α-mannosidase [EC 3.2.1.24]; β-GA, β-glucuronidase [EC 3.2.1.31]. t (trace): below 0.1 unit.

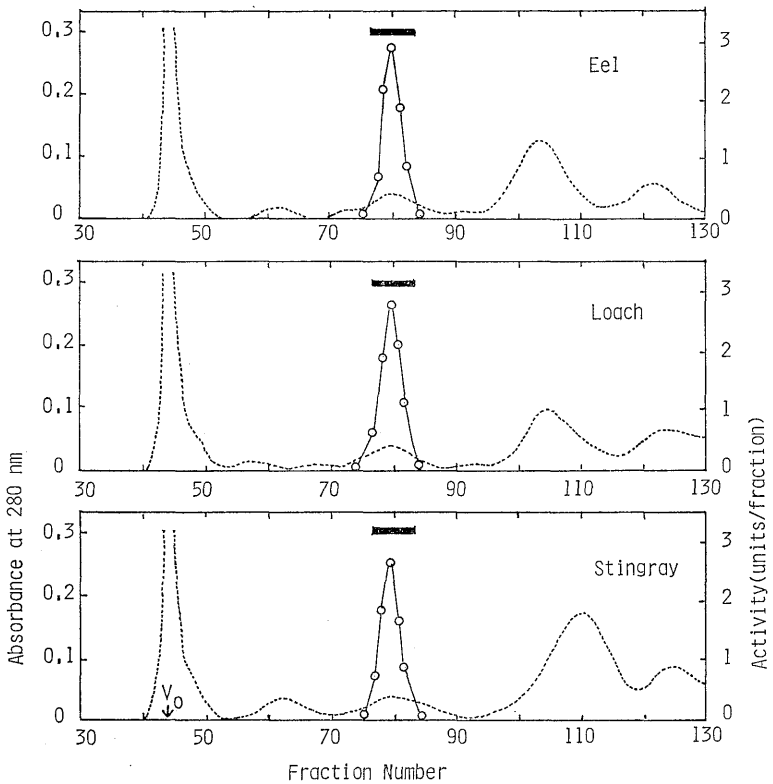

Fig. 1. Sephadex G-200 gel filtrations of the crude enzyme solutions obtained at Step 1. Each (10 m*l*) of the enzyme solutions was applied to a Sephadex G-200 column (3.4×70 cm) previously equilibrated with 0.05 M sodium citrate buffer, pH 5.0. The column was eluted with the same buffer at a flow rate of 14 m*l*/h and 5.0-m*l* fractions were collected. ------, absorption at 280 nm; ○, β-*N*-acetylhexosaminidase; Vo, void volume.

of the same buffer for 2 min in the blender. The homogenate was centrifuged at 15,000×g for 30 min. To the extract, solid ammonium sulfate was added to 35% saturation. After standing for 3 h, the precipitate was removed by centrifugation and more ammonium sulfate was added to the supernatant to 75% saturation. After standing overnight, the precipitate collected by centrifugation was dissolved in 5 m*l* of 0.05 M sodium citrate buffer, pH 5.0, and dialyzed against the same buffer. The dialyzed solution was centrifuged to obtain about 15 m*l* of a clear solution.

2. Step 2. Sephadex G-200 gel filtration—The above enzyme solutions were subjected to Sephadex G-200 gel filtration. The elution profiles are shown in Fig. 1. β-*N*-Acetylhexosaminidases from eel, loach, and stingray were eluted at the same elution volume which was 63% of the column volume. This indicated that these enzymes had about the same molecular weight. The first high peak was eluted at the void volume of the column and proved to be sialic acid-containing glycoprotein since all of the sialic acid present in the enzyme solution was found in the peak. The fractions containing β-*N*-acetylhexosaminidase activity as shown by horizontal bars were pooled.

3. Step 3. CM-52 chromatography—The enzyme solutions obtained at Step 2 were concentrated and equilibrated with 0.025 M sodium citrate buffer, pH 4.7, by ultrafiltration with a Diaflo PM-10 membrane. The concentrates were subjected to CM-52 chromatography. The elution profiles are shown in Fig. 2. The column was first eluted with the same buffer to wash off unadsorbed proteins, and then with a linear gradient made between 0.025 M sodium citrate buffer, pH 4.7 and 0.15 M sodium phosphate buffer, pH 6.7. The individual β-*N*-acetylhexosaminidases behaved quite similarly on the chromatography; these were eluted at pH about 5.2 and at a Na^+ concentration of about 0.075 M. The fractions containing β-*N*-acetylhexosaminidase activity as shown by horizontal bars were pooled and con-

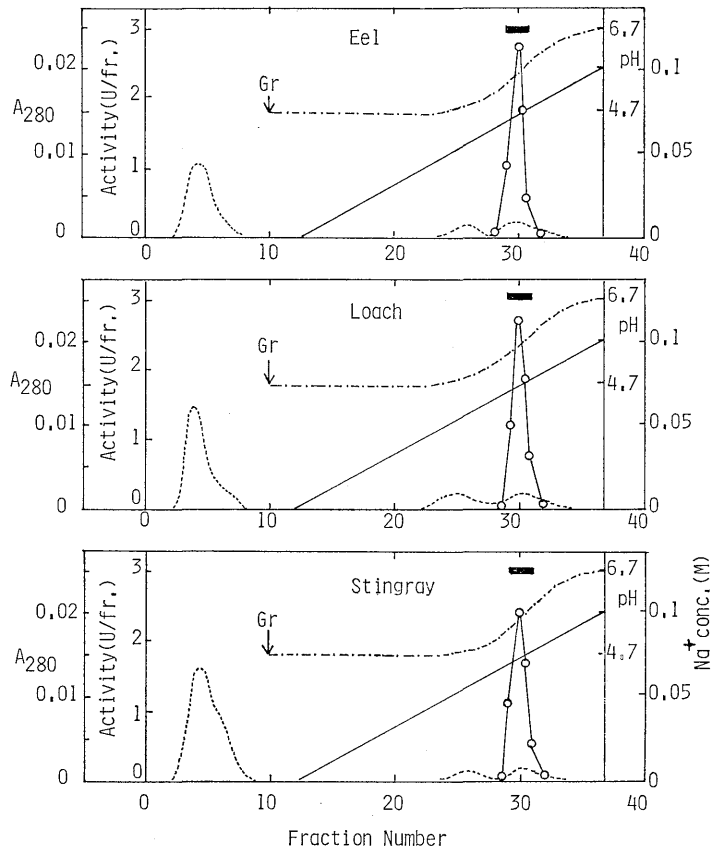

Fig. 2. CM-52 chromatographies of the β-*N*-acetylhexosaminidase preparations obtained at Step 2. Each (2 m*l*) of the concentrated and equilibrated enzyme solutions containing about 1.7 mg of protein was applied to a CM-cellulose (Whatman CM-52) column (1.5×12 cm) previously equilibrated with 0.025 M sodium citrate buffer, pH 4.7. The column was eluted at a flow rate of 10 ml/h with the same buffer and then with a gradient between 0.025 M sodium citrate buffer, pH 4.7, and 0.15 M sodium phosphate buffer, pH 6.7. ------, absorption at 280 nm; ○, β-*N*-acetylhexosaminidase; ——, concentration of Na^+; —·—, pH; Gr, at the start of gradient.

centrated by ultrafiltration. The purities of these enzymes were considered to be fairly homogeneous from disc gel electrophoresis at pH 9.5,[21] on which only one but faint band was observed since insufficient amounts of proteins were applied (limited amounts of the enzymes were available, data not shown). A summary of the specific activities and recoveries of the enzymes during the isolation procedure is given in Table 2.

Properties of β-N-Acetylhaxosaminidases from Eel, Loach, and Stingray

1. Molecular weight—The molecular weights of the β-*N*-acetylhexosaminidases were almost equal and estimated to be 120,000 by Sephadex G-200 gel filtration (Fig. 3).

2. pH optimum—The effect of pH on the activities of the enzymes was examined using *p*-nitrophenyl β-*N*-acetylglucosaminide as substrate in sodium citrate and sodium phosphate buffers (Fig. 4). Optimal activities of the enzymes occurred similarly at pH 4.0–4.5.

3. pH stability—The stabilities of the enzymes at various pH values were examined. The enzymes were incubated in buffers ranging from pH 4.0 to 9.5 for 3 h at 25°C and then the residual activities were assayed at pH 5.0. Untreated enzymes dissolved in 0.05 M sodium phosphate buffer, pH 7.0, were used as controls and were assumed to have 100% activity. As shown in Fig. 5, the pH-activity curves were analogous among these enzymes, showing that their activities were fairly stable at pH 6.5–7.5.

4. Effect of substrate concentration—The effect

Table 2. Purification of β-*N*-acetylhexosaminidases from acetone powders (4 g each) of external mucous materials of eel, loach, and stingray

Steps		Total activity units	Total protein mg	Specific activity units/mg	Reovery %
Crude extract:					
	Eel	26.0	219	0.12	100
	Loach	22.8	236	0.10	100
	Stingray	24.4	358	0.07	100
$(NH_4)_2SO_4$ ppt and dialysis:					
	Eel	19.0	38.4	0.49	73
	Loach	17.3	45.8	0.38	76
	Stingray	17.6	31.3	0.56	72
Sephadex G-200:					
	Eel	15.2	2.0	7.60	58
	Loach	14.4	2.2	6.55	63
	Stingray	14.1	1.9	7.42	58
CM-52:					
	Eel	10.1	0.25	40.4	39
	Loach	9.7	0.26	37.3	43
	Stingray	9.3	0.21	44.3	38

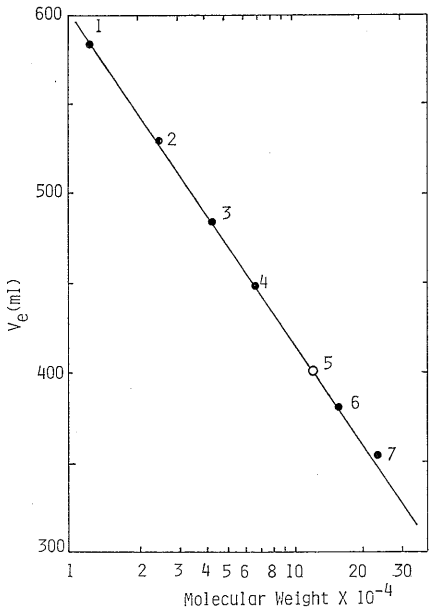

Fig. 3. Estimation of molecular weights of β-*N*-acetylhexosaminidases by Sephadex G-200 gel filtration. Gel filtration was carried out under the same conditions as described in the legend to Fig. 1 except that 2.5-m*l* fractions were collected. 1, cytochrome c; 2, chymotrypsinogen A; 3, ovalbumin; 4, bovine serum albumin; 5, β-*N*-acetylhexosaminidases from eel, loach, and stingray; 6, aldolase; 7, catalase.

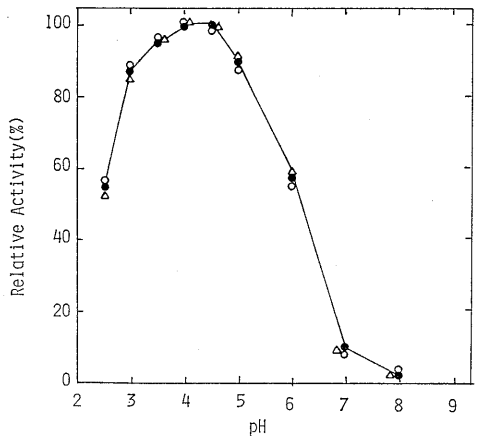

Fig. 4. Effect of pH on activities of β-*N*-acetylhexosaminidases. ●, eel; ○, loach; △, stingray. Buffers used: sodium citrate buffer for pH 2.5–6.0, sodium phosphate buffer for pH 7.0–8.0.

of varying substrate concentration on the velocity of hydrolysis of *p*-nitrophenyl β-*N*-acetylglucosaminide by the β-*N*-acetylhexosaminidases was measured at 37°C in 0.05 M sodium citrate buffer, pH 4.0. The Michaelis constants (*Km*) were determined from the Lineweaver-Burk plots shown in Fig. 6. The plots for β-*N*-acetylhexosaminidases from eel and loach almost coincided and the same *Km* value was determined to be 0.48 mM. On the other hand, the *Km* value for β-*N*-acetylhexosaminidase from stingray was determined to be 0.53 mM.

5. Effect of sodium chloride—The effect of sodium chloride on the activities of the enzymes

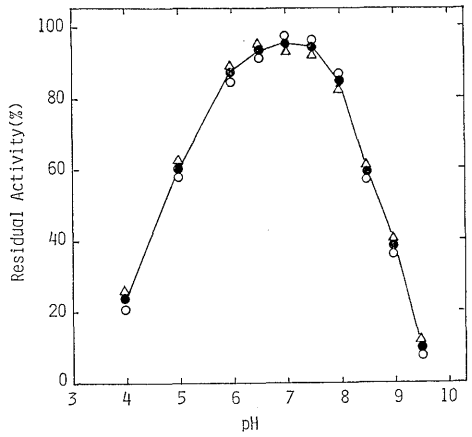

Fig. 5. Effect of pH on stabilities of *β-N*-acetylhexosaminidases. ●, eel; ○, loach; △, stingray. Buffers used: sodium citrate buffer for pH 4.0–6.0, sodium phosphate buffer for pH 6.5–8.5, ammonia-ammonium chloride buffer for pH 9.0–9.5.

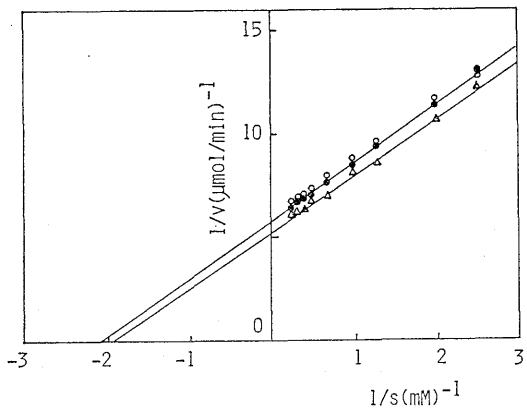

Fig. 6. Lineweaver-Burk plots for hydrolysis of *p*-nitrophenyl *β-N*-acetylglucosaminide by *β-N*-acetylhexosaminidases. ●, eel; ○, loach; △, stingray.

was examined since *β-N*-acetylhexosaminidase isolated from the liver of marine top shell was remarkably activated by sodium chloride.[22] As shown in Fig. 7, practically no activation by sodium chloride was observed for the enzymes.

Examination on Activities of Protease and Neuraminidase

The activities of protease and neuraminidase were examined using the enzyme solutions obtained from eel, loach, and stingray at the end of Step 1. Slightly positive results of protease activity were

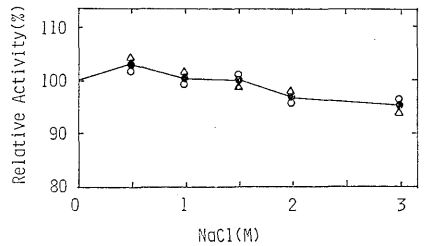

Fig. 7. Effect of sodium chloride on activities of *β-N*-acetylhexosaminidases. ●, eel; ○, loach; △, stingray.

observed for the individual enzyme solutions when 0.2 m*l* portions of the solutions were incubated with 1 mg each of Azocoll[23] for 48 h at 25°C in the presence of toluene.

For examination of neuraminidase activity, 0.2-m*l* portions of the individual enzyme solutions were incubated with 1 mg each of keratan sulfate, which was isolated from whale cartilage and contained 14.5% of *N*-acetylneuraminic acid,[24] for 48 h at 25°C in the presence of toluene and assayed for free *N*-acetylneuraminic acid by the thiobarbituric acid reaction.[18] No neuraminidase activity was observed for the enzyme solutions.

Discussion

The external mucous materials collected were treated with acetone to prepare their acetone powders for preservation of the glycosidase activities until use. A little inactivation of the activities might occur by the acetone treatment.[25]

Glycosidases were found in all the external mucous materials of fishes examined. *β-N*-Acetylhexosaminidase, which was the most dominant glycosidase, occurred enough in the mucous materials for the isolation of the enzyme. Therefore, *β-N*-acetylhexosaminidases were isolated from the mucous materials of eel, loach, and stingray and their enzymatic properties were compared. These enzymes closely resembled with each other in the properties except that the *Km* value for the stingray enzyme differed from that for the eel and loach enzymes.

There are two possibilities about the origin of glycosidases; one is a secretion from fish skin and the other is a product of bacteria which live in the external mucous material. The facts that glycosidases occur in all the external mucous materials of fishes regardless of their habitats and that the enzymatic properties of *β-N*-acetylhexosaminidases isolated from different fishes are quite similar

strongly support the possibility of a secretion from fish skin.

Glycoproteins, lysozyme, and the substances related to the protection of fish body have been found in the external mucous materials. These are secretions from fish skin and postulated to play an important role for the protection of fish body against bacterial infection, an inflammatory lesion, and various stresses.

The carbohydrate chains of sialic acid-containing glycoproteins, which were found in the external mucous materials of eel, loach, and stingray and separated on Sephadex G-200 gel filtration (first peaks in Fig. 1), can not be hydrolyzed by glycosidases in the absence of neuraminidase since the nonreducing termini of the carbohydrate chains are occupied by sialic acid.[9,11,12] Actually, no neuraminidase activity was found in the present study in the mucous materials of eel, loach, and stingray.

Glycosidases play an important role in catabolic degradation[26-28] of complex carbohydrates such as glycoproteins, proteoglycans, and glycolipids, which are quite ubiquitous in the animal organism. Hence, these natural substrates of glycosidases other than the sialic acid-containing glycoprotein must exist in fish skin.

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