ハタハタ(Arctoscopus japonicas)卵塊からの新規粘性タンパク質の分離

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Isolation of a Novel Viscous Protein from the Egg Mass of Japanese Sandfish (*Arctoscopus japonicas*)

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In the ovaries of mature Japanese sandfish (*Arctoscopus japonicas*), eggs are enveloped by a jelly-like mucilage to form an egg mass. In a part of Japan, the sandfish egg mass is regarded as a delicacy due to its unique texture. Here, we successfully isolated the mucilage from the egg mass by soaking it in hot water at 70℃. The freeze-dried mucilage was comprised of 72% protein, 1.4% carbohydrate, and 21% crude ash. Because removal of the ash by dialysis did not alter the viscosity of the mucilage, the stickiness was attributed to the protein content of the mucilage. The viscosity of the mucilage was not stable at high temperatures, but the heat stability was improved by the presence of 1～5% NaCl. Interestingly, electrophoretic analysis indicated that the mucilage contains a single predominant protein. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis indicated that the protein had an oligomeric structure, which might result in the viscosity of the mucilage. The N-terminal amino acid sequence of the protein was determined to be NH_2-GRRGDRERNQ, which matched neither to known viscous proteins nor to known proteins in fish species. Consequently, the characteristic viscosity of the sandfish egg mass might be attributed to a novel viscous protein.

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ハタハタ, 卵塊, 粘質物, 粘性, タンパク質

The distribution of Japanese sandfish (*Arctoscopus japonicas*) ranges from the Aleutian Islands to Okhotsk and the Japan seas. Japanese sandfish is consumed as broiled fish with salt and in a traditional soup dish in the Akita prefecture in Japan. Additionally, the sandfish is used as an ingredient of shottsuru, a fish sauce. In the spawning season, the mature sandfish lays an egg mass onto sargassum, a sea alga. The egg mass contains 500～1,500 eggs with a diameter of 3 mm, each of which is enveloped by a sticky membrane. The eggs are connected to each other by a filamentous portion of the membrane, and form a jelly - like egg mass. The cohesive egg mass entangles with algae. Once exposed to seawater, the egg mass hardens in 30 min, and settles on algae. However, when the egg mass in the matured sandfish ovary is broiled with salt or boiled, it does not harden, leaving the sticky texture intact. Due to its unique texture, the sandfish egg mass has been regarded traditionally as a delicacy. The viscosity of the egg mass is stable at high temperatures, whereas it is unstable in conditions of freezing and salt curing.

In this study, we isolated the uniquely viscous mucilage from the egg mass of the Japanese sandfish. Further, based on chemical characterization and electrophoretic analyses, we found a novel viscous protein, which is presumed to be the predominant factor contributing to the viscosity of the Japanese sandfish egg mass.

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Materials and methods

1. Fish

The Japanese sandfish (*A. japonicas* ; 3 ~ 4 years old) used in this study were caught by fixed fishing net at Kitaura Chisaki, Oga, Japan in December of 2003.

2. Isolation of mucilage from egg mass

The egg mass was collected from the ovaries of mature female fish, then soaked and stirred in 5-fold volume of water at 70℃. By stirring, the mucilage was isolated from the egg mass. The mucilage was freeze-dried, and used in the following experiments.

3. Composition analysis

(1) **Crude ash constituent** The sample was converted into ash by heating at 550℃ for 10 h using a muffle furnace (FM 37 ; Yamato Scientific, Tokyo, Japan). The ash was dissolved into 1 N HCl, and resultant solution was subjected to atomic absorption spectrochemical analysis using a Hitachi Z-6100 (Tokyo, Japan) to determine the sodium, potassium, magnesium, zinc, iron and copper contents.

(2) **Protein constituent** The protein content was determined by the Kjeldahl method using the Kjeltec Auto Sampler System 1035 Analyzer (Foss Tecator, Hoganas, Sweden). The protein amount was measured using a conversion factor of 6. 25.

(3) **Carbohydrate constituent** The total carbohydrate content was determined by the orcinol -sulfuric acid method[1]. Mannose was used as the standard sugar.

4. Electrophoretic analysis

The sample was diluted in sodium dodecyl sulfate (SDS) sample buffer to a final concentration of 5 ㎎ /㎖, and then heated to 100℃ for 3 min. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method of LAEMMLI[2]on a 12.5% gel (NPU-12.5 ℓ, Pagel, ATTO, Tokyo, Japan). The molecular weight under denaturing conditions was determined using an LMW Molecular Weight Calibration Kit (GE Healthcare Bio-Sciences, Piscateway, NJ). Isoelectric focusing(IEF)was carried out in a Phast System (GE Healthcare Bio-Sciences) using a PhastGel IEF pH 3-9 (GE Healthcare Bio-Sciences). The isoelectric point (pI) was determined using a Broad Range Calibration Kit 3-10 (GE Healthcare Bio-Sciences). The proteins were detected by Coomassie brilliant blue (CBB) staining.

5. Amino acid composition analysis

The protein in the sample was hydrolyzed in 20% HCl (amino acid composition analysis grade) or 4 M methanesulfonic acid at 110℃ for 24 h. An amino acid automatic analyzer (JLC 500 ; JEOL Ltd., Tokyo, Japan) was used to measure the amino acids generated.

6. N-terminal amino acid sequence analysis

The N-terminal amino acid sequence was determined using a protein sequencer (PPSQ-10, Shimadzu, Kyoto, Japan). The BLAST program was used to perform a homology search of the amino acid sequence against a reference proteins database (National Center for Biotechnology Information) for similarity to previously reported proteins.

7. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis

The molecular mass of the protein obtained from the mucilage was determined by MALDI-TOF-MS using the Voyager System 4347 (Applied Biosystems, Foster City, CA). Sinapinic acid was dissolved in 30% acetonitrile containing 0.1% trifluoroacetic acid (TFA), and used as the matrix.

8. Detection of glycosylation of the protein by Periodic Acid Schiff (PAS) staining

Glycosylation of the protein in the mucilage was confirmed by PAS staining. As a positive control for glycosylation, horseradish peroxidase (Sigma-Aldrich, St. Louis, MO) and chicken egg albumin (Wako, Osaka, Japan) were used. The mucilage and positive control proteins were applied to a gel and subjected to SDS-PAGE. The gel was then stained with PAS using a Glycoprotein Detection Kit (Sigma-Aldrich) and finally, the banding profiles were compared to the gel stained with CBB.

9. Viscosity measurement of the mucilage

The mucilage was diluted to 0.1 and 0.05% with distilled water for viscosity measurements. Gelatin (Wako), guar gum (Sigma-Aldrich), and xanthan gum (Sigma-Aldrich) were used to compare the viscosities. The mucilage and reference solutions were left standing at 25℃ for 60 min, and then the viscosities of the solutions were determined using an Ostwald viscometer (inside diameter of capillary : 0.5 mm). The viscosity measurement of 25℃ water (0.8902 mPa · s) was employed as a viscosity reference.

Results

1. Chemical characterization of mucilage from the egg mass Japanese sandfish

When the egg mass was soaked in water at 70℃, the mass was easily separated into eggs and mucilage probably due to the swelling of the mucilage upon exposure to water. The mucilage sheared 2.5% of the weight of the solid content of the egg mass. The content of mucilage was determined to be 72% protein, 1.4% carbohydrate, and 21% crude ash (Table 1). The ash content of the mucilage contains sodium, potassium, calcium, and magnesium, whereas zinc, iron, and copper could not be detected (Table 2). The viscosity of the mucilage was not altered when the crude ash content was removed by dialysis (data not shown). Therefore, it was posited that the viscosity was predominantly due to the protein content of the mucilage. Interestingly, the mucilage exhibited a single protein band both on SDS-PAGE and IEF gels, which strongly suggests that the viscosity of the mucilage could be a feature of this protein (Fig. 1 a and b). The molecular mass and pI of the protein were determined to be 43 kDa and 5.2,

Table 1 Composition of mucilage from ovary of Japanese Sandfish

Component	Content (g/100 g)
Crude protein	71.9
Carbohydrates	1.4
Crude ash	20.7

Table 2 Metal ion composition of mucilage from the egg mass of the Japanese Sandfish

Metals	Content (g/100 g)
Na	7.4
K	0.5
Ca	0.3
Mg	0.1
Fe	ND
Zn	ND
Cu	ND

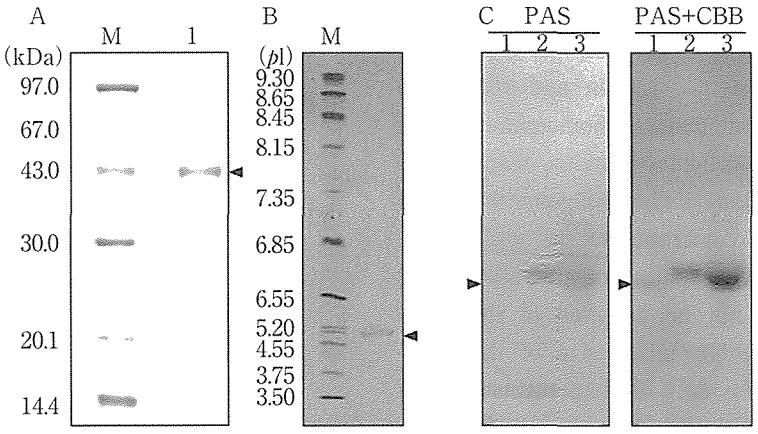

Fig. 1 Electrophoretic analyses of the mucilage from Japanese sandfish egg mass

(A) SDS-PAGE banding profile of the mucilage. Molecular mass of the mucilage protein was estimated to be 43 kDa based on the electrophoretic mobility of the mucilage protein (indicated with arrowhead, lane 1) and molecular mass standard proteins (lane M) concomitantly applied to the SDS-PAGE.

(B) IEF banding profile of the mucilage protein. The pI of the mucilage protein was estimated to be 5.2 based on the mobility of the mucilage protein (indicated with arrowhead, lane 1), and pI standard proteins (lane M).

(C) PAS staining of the mucilage protein separated on the SDS-PAGE. The mucilage protein (lane 1), horseradish peroxidase (lane 2), and chicken egg albumin (lane 3) were developed on the SDS-PAGE. The glycosylated proteins were stained with PAS stain (panel PAS). After the PAS stain, all proteins were stained with CBB (panel PAF+CBB). The mucilage protein could not be detected with the PAS stain, whereas the protein was stained with CBB.

respectively. The amino acid composition of the protein in the mucilage was determined with an amino acid automatic analyzer (Table 3). Further, the N-terminal amino acid sequence of the protein was determined to be NH_2-GRRGDRERNQ. This sequence exhibits similarity to some fungal and bacterial proteins, *i. e.*, YALI0F21362p from an alkane-assimilating yeast, *Yarrowia lipolytica* (accession number, XP_505706.1), whose function is unknown, and a putative ABC transport ATP-binding subunit protein from *Bordetella pertussis* (accession number, NP_880918.1). However, the sequence neither displayed similarity to known viscous proteins such as gelatin, nor to known fish proteins. Therefore, the protein found here is a novel viscous protein. PAS staining analysis indicated that the protein from the sandfish egg mass has no glycosylation (Fig. 1 c).

Furthermore, the precise molecular mass of the protein was determined by MALDI-TOF-MS (Fig. 2) which revealed 6 peaks with molecular masses of 20.2, 40.2, 60.5, 80.6, 120.9, and 160.6 kDa. The most predominant peak with a molecular mass of 40.2 kDa corresponds to the 43-kDa protein found by SDS-PAGE. Further, it can be hypothesized that the 80.6-, 120.9-, and 160.6-kDa proteins might be homodimers, homotrimers, and homotetramers of the 40.2-kDa proteins, respectively. Therefore, the formation of homooligomers of the 40.2-kDa proteins may contribute to the viscosity of mucilage. Proteins corresponding to the 20.2-, and 60.5-kDa peaks could not be detected in the SDS-PAGE. However, given the minor size of the 20.2-kDa protein peaks, the 60.5-kDa protein can be explained as a heterodimer of the 20.2-and 40.2-kDa proteins.

2. Physical characterization of the mucilage from the egg mass of the Japanese sandfish

The freeze-dried mucilage was soluble in water, NaOH solution, 1% SDS, and 8 M urea. An aqueous solution of the mucilage was clear and colorless, highly viscous, and stringy. The viscosity of the mucilage solution is summarized in Table 4. The viscosity of 0.1% mucilage solution was 2.01 mPa·s, which is higher than that of 0.1% gelatin solution, but lower than those of xanthan gum and guar gum solutions at the same concentrations. The viscosity of the sandfish mucilage solution was altered depending on the NaCl concentration and pH changes, whereas a change in viscosity could not be observed in gelatin and guar gum solutions. Therefore, it was presumed that the viscosity of the sandfish mucilage is affected by the presence of electrolytes. As shown in Table 5, the viscosity of the sandfish mucilage is also affected by heat. On the other hand, the heat tolerance of the viscosity was improved by the presence of 1-5% NaCl (Fig. 3).

Table 3 Amino acid composition of mucilage protein from the egg mass of Japanese Sandfish

Amino acid	Content (%)
aspartic acid	16.3
threonine	6.7
serine	7.2
glutamic acid	16.5
glycine	7.1
alanine	3.2
cysteine	2.8
valine	4.2
methionine	0.2
isoleucine	2.9
leucine	3.9
tyrosine	7.0
phenylalanine	5.5
histidine	1.0
lysine	5.9
arginine	9.8
proline	6.0

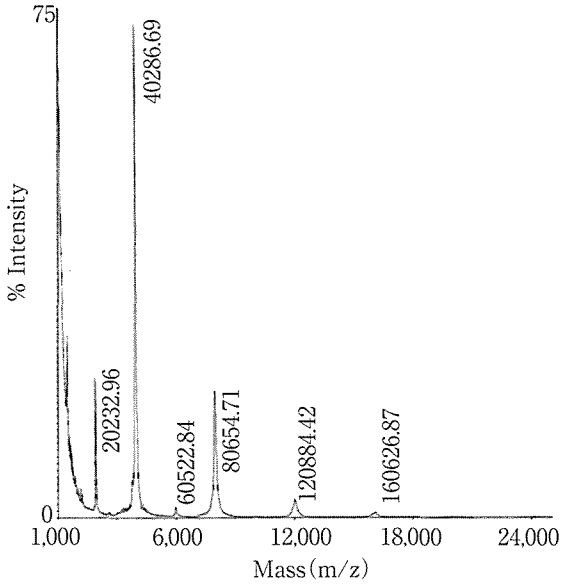

Fig. 2 MALDI-TOF-MS profile of the mucilage from Japanese sandfish egg mass

The mucilage was applied to the Voyager System 4347. The profile displayed predominant six peaks.

Table 4 Viscosity of the mucilage in various concentrations of NaCl and different pH conditions

(mPa·s [sP])

	Water	1 % NaCl	3 % NaCl	5 % NaCl	buffer pH 4.0	buffer pH 7.0	buffer pH 9.0
Mucilage protein (0.1%)	2.01	1.15	1.16	1.16	1.13	1.26	1.55
Gelatin (0.1%)	0.97	0.95	0.96	0.98	0.95	0.96	0.98
Guar gum (0.05%)	1.60	1.67	1.64	1.66	1.59	1.60	1.65
Xanthan gum (0.05%)	1.55	–	–	–	1.10	1.12	1.25

Table 5 Thermal stability of the mucilage

(mPa·s [sP])

	min			
	0	10	20	40
Mucilage protein (0.1%)	2.01	1.92	1.86	1.77
Gelatin (0.1%)	0.97	0.95	0.95	0.93
Guar gum (0.05%)	1.60	1.58	1.57	1.53
Xanthan gum (0.05%)	1.55	1.55	1.52	1.51

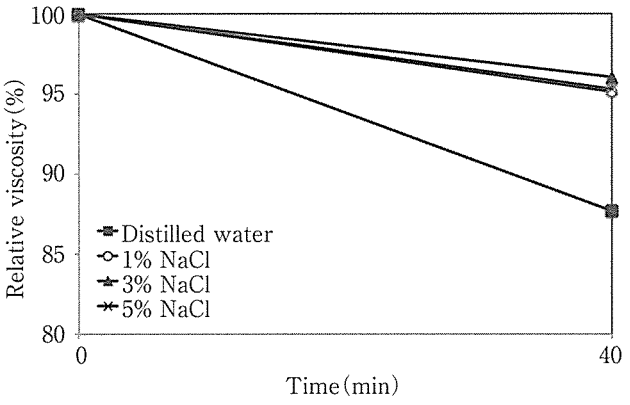

Fig. 3 Effect of the NaCl concentration on the heat stability of the mucilage viscosity

Discussion

In this study, we successfully isolated mucilage from the egg mass of Japanese sandfish. Interestingly, chemical characterization and electrophoretic analysis indicated that the viscosity of the mucilage might arise from a single protein. Gelatin and collagen are well known viscous proteins rich in glycine, alanine and proline[3]. The amino acid composition of the mucilage protein was not similar to that of gelatin or collagen. The N-terminal amino acid sequence of the sandfish mucilage protein displayed no similarity to known viscous proteins or to known fish proteins. Furthermore, the mucilage protein was identified as a simple protein without glycosylation, although some viscous proteins, such as oikosin from a larvacean tunicate, *Oikopleura dioica*[4] and dioscorin from the yam[5),6)], are highly glycosylated. Therefore, the sandfish mucilage protein found here is novel.

The mucilage connects the eggs with each other, resulting in the formation of an egg mass. The egg mass is soft, and has a tendency to be entangled with algae in seawater. Once the egg mass is exposed to seawater, it becomes hardened. Thus, the mucilage is essential to maintain the egg mass structure, and plays a role in hardening the egg mass in seawater. However, once the mucilage is soaked in hot water, the hardened mucilage softens. Amino acid composition analysis implied that the sandfish mucilage protein is glutamine-rich. These findings lead us to a hypothesis that a temperature-sensitive enzyme such as transglutaminase induces the formation of cross-linkage in the mucilage protein[7] that results in hardening of the egg mass. Cloning and complete nucleotide sequencing of the gene encoding the mucilage protein will be needed to clarify the precise role of mucilage protein on egg mass formation and hardening of the egg mass.

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ハタハタ(*Arctoscopus japonicas*)卵塊からの新規粘性タンパク質の分離

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ハタハタ (*Arctoscopus japonicas*) 卵巣内において卵は粘着性の高い膜に包まれ卵塊を形成している。本研究では，卵塊から粘質物を分離する方法を確立した。得られた粘質物は，72%のタンパク質，1.4%の糖質，そして21%の灰分を含んでいた。透析によって灰分を除いても粘質物の粘性に変化がなかったことから，その粘性にはタンパク質が関与しているものと推測された。粘質物の粘性は熱によって低下したが，塩の存在でその熱耐性が改善された。粘質物の電気泳動解析の結果，粘質に関与していると考えられるタンパク質はほぼ単一であった。質量分析の結果，2つから4つの同タンパク質が会合し，ホモ多量体を形成していることが見いだされ，これが粘性に関与しているものと考えられた。N末端アミノ酸配列分析の結果，本タンパク質は既知の粘性タンパク質あるいは既知の魚由来のタンパク質と異なる新規のタンパク質であると推測された。

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