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Characterization of Nidamental Mucin from Japanese Common Squid *Todarodes pacificus*

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Abstract: The nidamental mucosubstance, 3 g by wet weight, of Japanese common squid *Todarodes pacificus* was solubilized with 0.4 M NaOH containing 0.1 M $NaBH_4$ at 4℃ for 4 h. A water-soluble mucin, 170 mg by dry weight, was isolated by precipitation with 50 % ethanol. The nidamental mucin comprised 16.4 % protein, 80.3 % sugar and 3.3 % ester sulfate by weight. About 72 % of the protein backbone was composed of threonine, proline and isoleucine in the molar ratio of about 2:1:1. The compositional feature of sugars was the presence of two methylated monosaccharides, 4-*O*-methylglucose and *N*-acetyl-4-*O*-methylglucosamine. The mucin had a long, rod-like structure with a molecular mass of 2,600 kDa and gave a very viscous solution in water. Before and after protease digestion of the mucin, no marked change of its molecular structure was observed. Sugar chains of the mucin were resistant to β-elimination, but were released by mild hydrazinolysis. A major component of the sugar chains was found to be a tetrasaccharide consisting of 1 mol each of galactose, fucose, 4-*O*-methylglucose and *N*-acetyl-4-*O*-methyl-glucosamine. These results suggest that the mucin plays a crucial role in the high viscosity and gel-forming properties of the nidamental mucosubstance.

Key words: *mucin, nidamental gland, squid, Todarodes pacificus, N-acetyl-4-O-methylglucosamine, 4-O-methylglucose*

Introduction

The nidamental gland mucosubstance of ommastrephid squid is assumed to form the jelly-like surface layer of egg masses up to 80 cm in diameter.[1,2] This surface layer is effective in preventing bacteria, protozoans, and crustaceans present in seawater from infesting the egg mass. We previously isolated a sulfated glycoprotein, mucin, from the nidamental mucosubstance of Argentine shortfin squid *Illex argentinus*.[3,4] Its protein backbone, which accounted for 18.7 % by weight, was characterized by extremely high levels of threonine, proline and isoleucine and was postulated to link *O*-glycosidically to sugar chains through threonine residues. An alkaline treatment of the mucin with 0.5 M NaOH at 25 ℃ for 24 h, however, failed to release *O*-glycosidically linked oligosaccharides, indicating that β-elimination did not occur. In addition, an unusual amino sugar was found in acid hydrolysates of the mucin.[3] and determined its chemical structure to be 4-*O*-methyl-D-glucosamine.[5] This unique mucin seems to be widely distributed in ommastrephid squid species, because they produce such large egg masses in seawater.[1,2]

One of the ommastrephid squid, Japanese common squid *Todarodes pacificus,* is a commercially important resource in Japan and two egg masses spawned by two captive *T. pacificus* females were obtained at the Usujiri Fisheries Laboratory of Hokkaido University.[2] As the first step of our biochemical study on egg mass mucin, we report here characterization of the nidamental mucin of *T. pacificus,* a possible precursor of the egg mass mucin.

Materials and Methods

Isolation of Alkali-solubilized Mucin from the Nidamental Mucosubstance

The nidamental glands of *T. pacificus* were obtained at the Usujiri Fisheries Laboratory, Hokkaido University and were stored at −30 ℃ until use. Nidamental mucin was isolated as a water-soluble form according to the method described previously.[3] Briefly, the gum-like mucosubstance of nidamental glands, 3 g by wet weight, was homogenized in 150 mL of distilled water and solubilized with 0.4 M NaOH containing 0.1 M $NaBH_4$ for 4 h at 4℃ while actively stirring. The resulting viscous solution was clarified by centrifugation and treated with 50 % ethanol in the presence of 0.2 M NaCl to precipitate an alkali-solubilized mucin. The fibrous precipitate was collected by

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a pincette, washed with 60 % ethanol in the presence of 0.2M NaCl and dissolved in water. This precipitation and solution was repeated twice. The mucin solution thus obtained was dialyzed extensively against water and lyophilized.

Preparation of a Protease-resistant Fragment from Mucin

Nidamental mucin, 100 mg by dry weight, was dissolved in 100 mL of 0.5 M sodium acetate buffer (pH 8.0) containing 10 mM $CaCl_2$ and 3 % ethanol and was digested with 2 mg of actinase E from *Streptomyces griseus* (EC 3.4.24.4, Kaken Seiyaku Co.) for 17 h at 50 ℃. The protease digestion was stopped by addition of NaOH at 4℃ to a final concentration of 0.1 M, followed by incubation for 10 min. A protease-resistant fragment of the mucin was recovered by precipitation with 60 % ethanol in the presence of 0.2 M NaCl, dissolved in water and lyophilized.

Analytical Methods

Amino acid compositions, after hydrolysis of samples with 6 M HCl at 110 ℃ for 24 h, were determined by high-performance liquid chromatography (HPLC) with a TSK gel Aminopak column (Tosoh). Neutral and amino sugars, after hydrolysis of samples with 2.5 M trifluoroacetic acid (TFA) at 100 ℃ for 7 h,[6] were analyzed by the method of Yasuno et al.[7] Derivatization of monosaccharides was performed with a *p*-aminobenzoic ethyl ester (ABEE)-derivatized kit (Honen Corp.). ABEE-derivatized monosaccharides were subjected to reversed-phase HPLC on a 4.5 mm x 75 mm column of Honenpak C18 and were eluted with 0.02 % TFA containing 10 % acetonitrile or 0.2 M potassium borate buffer (pH 8.9) containing 6 % acetonitrile at a flow rate of 1.0 mL/min at 45 ℃. The effluent was monitored by a Tosoh FS-8010 fluorescence spectrophotometer with an excitation wavelength of 305 nm and an emission wavelength of 360 nm. L-Arabinose was used as an internal standard. The authentic compound of 4-*O*-methylglucosamine was obtained in our previous study[4] and that of 4-*O*-methylglucose was a gift from Dr Nishimura T. (Forestry and Forest Products Research Institute, Tsukuba).[8]

Determination of *N*-acetylhexosamine-terminating *O*-glycosidically linked sugar chains in the mucin was carried out with a modified Morgan-Elson reaction as reported by Bhavanandan et al.[9]

Moreover, the *N*-acetyl group of amino sugars was assayed by HPLC on a TSK gel OApak column (Tosoh) as acetic acid liberated by hydrolysis with 2 M HCl at 100 ℃ for 2 h[3] and ester sulfate was by the sodium rhodizonated method.[10]

Sedimentation velocity analysis was performed with an analytical ultracentrifuge (Hitachi model 282) at 50,000 rpm and at 28 ℃. The values of sedimentation coefficients were calculated by using a partial specific volume of 0.64 mg/mL for mucin.[11] Viscosity measurement was carried out at 28 ℃ with a Cannon-Fenske viscometer having an average shear gradient of about 1,000 sec^{-1}. Samples were dissolved in 0.02 M sodium phosphate buffer (pH 7.2) containing 0.1 M NaCl.

Fast atom bombardment (FAB) mass spectrometry was performed in the positive ion mode on a JEOL JMS SX-102 mass spectrometer.

Preparation of O-linked Sugar Chains from Mucin

O-linked sugar chains of the nidamental mucin were released by mild hydrazinolysis. In order to avoid excessive degradation of the sugar chains, the mucin (50 mg) was treated with 1.5 mL of anhydrous hydrazine at 60℃ for 50 h as reported by Kuraya and Hase[12] using a hydrazinolysis instrument "Hydraclub C-206" (Honen Corp.). After removal of hydrazine by repeated evaporation *in vacuo,* free amino groups of the released sugar chains were *N*-acetylated with acetic anhydride and saturated sodium bicarbonate solution. The reaction mixture was left in an ice bath for 15 min with occasional stirring and poured into a small column of Dowex 50W-X8 (H^+) resin. The column was washed with 5 bed volumes of water, and the passed-through fraction and the washings were combined and lyophilized.

Sugar chains released by hydrazinolysis of the mucin were subjected to gel filtration on a 1.8 cm x 160 cm column of Bio-Gel P4 (Pharmacia) and eluted with water at a flow rate of 12.4 mL/h at 55 ℃. The effluent was monitored by the orcinol-sulfuric acid method[13] and appropriate fractions were collected and lyophilized. A major sugar chain was rechromatographed on the same Bio-Gel P4 column, derivatized with a *p*-aminobenzoic octyl ester (ABOE)-derivatized kit (Honen Corp.) and analyzed by reversed-phase HPLC on a 4.6 mm x 75 mm column of Honenpak C18. Chromatography was carried out at 45 ℃ with two solvents, A and B, as the eluent at a flow rate of 1.0 mL/min ; solvent A was a 75:25 mixture of water and acetonitrile and solvent B was a 55:45 mixture. The column was equilibrated with solvent A. After injection of a sample, the ratio of solvent B was 0 % for 10 min, then it was increased by a linear gradient of 10 -100

% in 60 min. The effluent was monitored with the fluorescence spectrophotometer as mentioned above.

Results and Discussion

Properties of Nidamental Mucin

From the nidamental mucosubstance of *T. pacificus,* mucin was easily isolated by solubilization with 0.4 M NaOH, followed by precipitation with 50 % ethanol in the presence of 0.2 M NaCl. About 170 mg of the nidamental mucin was obtained from 3 g (wet weight) of the mucosubstance. First, the mucin was analyzed for chemical constituents. The results are given in Table 1 in terms of residues per 1,000 total amino acid and sugar residues, confirming that the mucin has the compositional feature as a sulfated glycoprotein. It comprised 16.4 % protein, 80.3 % sugar and 3.3 % ester sulfate by weight and was characterized by the high contents of threonine, *N*-acetylglucosamine, *N*-acetylgalactosamine, fucose and galactose. Approximately 72 % of the protein backbone was composed of three amino acids, threonine, proline and isoleucine, which were present in the molar ratio of approximately 2:1:1. In contrast to serine residues, threonine residues accounted for more than one-third of the total amino acids and thus were assumed to be the main candidate for protein-sugar linkages in the mucin. The chromatographic separation of ABEE-derivatized monosaccharides from the mucin is given in Fig. 1. Of particular interest is the existence of two methylated monosaccharides and complete separation of them from other monosaccharides is achieved by using 0.02 % TFA containing 10 % acetonitrile as the eluent (Fig. 1B) ; peaks 11 and 13 are identified as 4-*O*-methylglucosamine and 4-*O*-methylglucose, respectively. 4-*O*-methylglucosamine as well as glucosamine and galactosamine was naturally present as *N*-acetylated form, because the equimolar amounts of acetic acid and amino sugars were obtained

Table 1. Chemical compositions of nidamental mucin and its protease-resistant fragment from Japanese common squid.

	Nidamental mucin	Protease-resistant fragment
Amino acid		
Asp	9	4
Thr	93	96
Ser	7	8
Glu	7	3
Pro	51	54
Gly	8	6
Ala	5	3
Cys	2	0
Val	9	7
Met	1	0
Ile	45	45
Leu	5	2
Tyr	2	1
Phe	4	0
His	5	6
Lys	6	2
Arg	3	0
Total	(262)	(237)
Neutral sugar		
Gal	200	197
Man	7	8
Glc	20	25
Xyl	5	9
Fuc	141	176
Rha	7	11
4-*O*-MeGlc	43	45
Total	(423)	(471)
Amino sugar		
GlcNAc	120	116
GalNAc	130	136
4-*O*-MeGlcNAc	65	40
Total	(315)	(292)
Total	1000	1000
Ester sulfate	56	68

Values are given in residues/1,000 total amino acid and sugar residues.

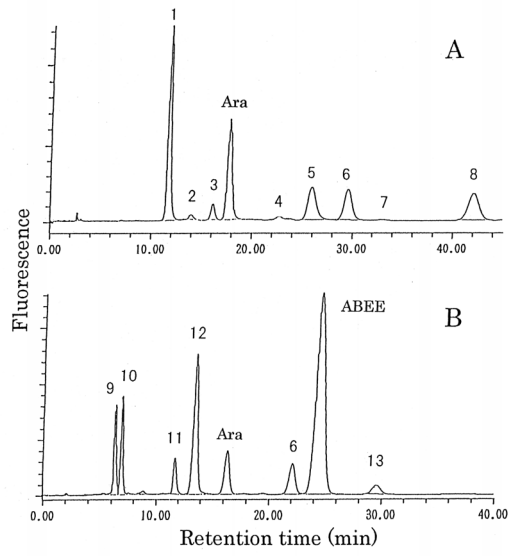

Figure 1. Chromatographic separation of ABEE-derivatized monosaccharides from nidamental mucin. The ABEE-derivatized monosaccharides were eluted from a Honenpak C18 column at 45 ℃ with (A) 0.2 M potassium borate buffer (pH 8.9) containing 6 % acetonitrile and (B) 0.02 % TFA containing 10 % acetonitrile at a flow rate of 1.0 mL/min. L- Arabinose (Ara) was used as an internal standard. The identities are as followes: 1, Gal ; 2, Man ; 3, Glc ; 4, Xyl ; 5, GlcNAc ; 6, Fuc ; 7, Rha ; 8, GalNAc ; 9, GlcN ; 10, GalN ; 11, 4-*O*-MeGlcN ; 12, Gal+Glc ; 13, 4-*O*-MeGlc.

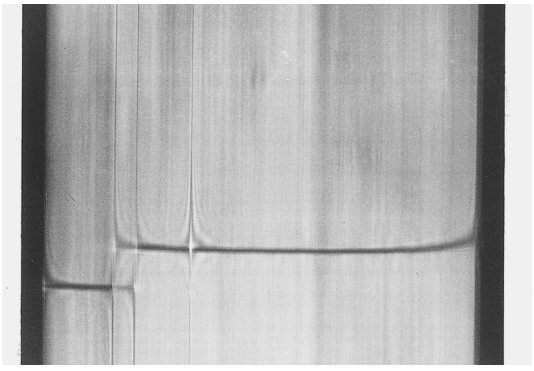

Figure 2. Sedimentation velocity pattern of nidamental mucin at 28 ℃. The mucin was dissolved in 0.02 M sodium phosphate buffer (pH 7.2) containing 0.1 M NaCl at a concentration of 0.030 %. The photograph was taken by the schlieren method at 13 min after the speed of 50,000 rpm was reached.

after acid hydrolysis of the mucin (data not shown). The nidamental mucin is found to be relatively rich in both methylated monosaccharides ; 65 residues/1,000 for. *N*-acetyl-4-*O*-methylglucosamine and 43 residues/1,000 for 4-*O*-methylglucose (Table1).

The molecular shape and molecular weight of nidamental mucin were estimated by both sedimentation velocity analysis and viscosity measurement. As given in Fig. 2, a single, sharp peak having a sedimentation constant of 16.9 S is observed by ultracentrifugal analysis of the mucin solution at 28 ℃. In the same temperature, it had a high intrinsic viscosity of 12.3 dL/g, which is presumably a minimum value because of the relatively high shear gradient (1,000 sec^{-1}) of the viscometer used. From the equation of Simha[14], the axial ratio of mucin molecules in solution as a prolate ellipsoid of evolution was calculated to be about 193. From these results, we calculated a molecular mass of the mucin to be about 2,600 kDa by using the Scheraga-Mandelkern equation[15] with a value of 3.42×10^6 for β.

The mucin was very resistant to proteolytic action and a large fragment was obtained by precipitaion with 60 % ethanol after actinase E digestion of the mucin at 50℃ for 17 h. The yield was about 86 %. As shown in Tables 1 and 2, there is no marked change in its chemical composition and macromolecular structure, respectively, before and after protease digestion of the mucin. Formation of the large, protease-resistant fragment having the extremely high contents of threonine, proline and isoleucine (Table 1) and a molecular mass of 2,200 kDa (Table 2) imply that molecular ends of the mucin are relatively poor in these amino acids and are removed by actinase E. Incidentally,

Table 2. Macromolecular properties of nidamental mucin and its protease-resistant fragment.

Mucin[*1]	[η](dL/g)	$S^0_{20,w}$(S)	Molecular mass (kDa)
Nidamental mucin	12.3	16.9	2,600
Protease-resistant fragment	14.4	14.3	2,200

[*1] Alkali-solubilized mucin.

the sum of threonine, proline and isoleucine is increased by the protease digestion from 72 % to 82 % of the total amino acids (Table 1).

From these combined results, the alkali-solubilized mucin of *T. pacificus* was found to be similar in its molecular properties to that of *I. argentinus*.[3] The intact mucin in the mucosubstance appears to be associated with other proteins to form a huge, complex structure.[4] and was solubilized by the mild alkaline treatment. However, solubilization mechanism of the intact mucin remains to be solved.

Properties of a Major Oligosaccharide Released by Hydrazinolysis of Mucin

The nidamental mucin contains the methylated monosaccharides as described above and thus the structure of *O*-glycosidically linked sugar chains containing these unusual constituents is very interesting. To isolate such sugar chain, the mucin was treated with 0.5 M NaOH at 25℃ for 24 h. However, there was no sugar chain released by β-elimination reaction. This was confirmed by the finding that no significant change in the threonine content of mucin was observed before and after the alkaline

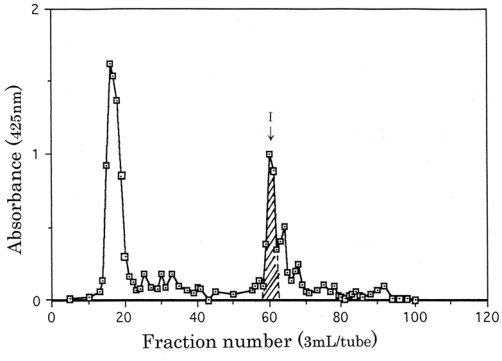

Figure 3. Gel filtration of oligosaccharides released by hydrazinolysis of nidamental mucin. The oligosaccharides were eluted from a Bio-Gel P4 (Pharmacia) column at 55 ℃ with distilled water at a flow rate of 12.4 mL/h. The effluent was monitored by the orcinol-sulfuric acid method. A major neutral oligosaccharide I was obtained.

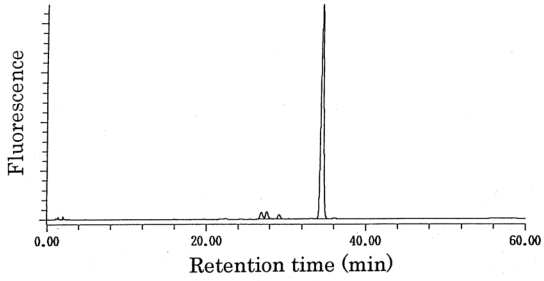

Figure 4. Chromatographic pattern of a major ABOE-derivatized oligosaccharide I released by hydrazinolysis of nidamental mucin. The oligosaccharide was eluted from a Honenpak C18 column at 45 ℃ with two solvents, A and B, at a flow rate of 1.0 mL/min as described in "Materials and Methods".

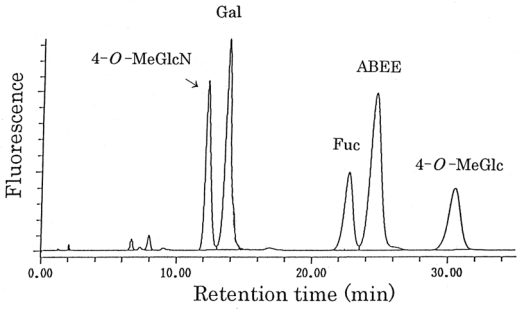

Figure 5. Chromatographic separation of ABEE-derivatized monosaccharides from oligosaccharide I. The HPLC conditions were the same as those described for (B) in the legend of Fig. 1.

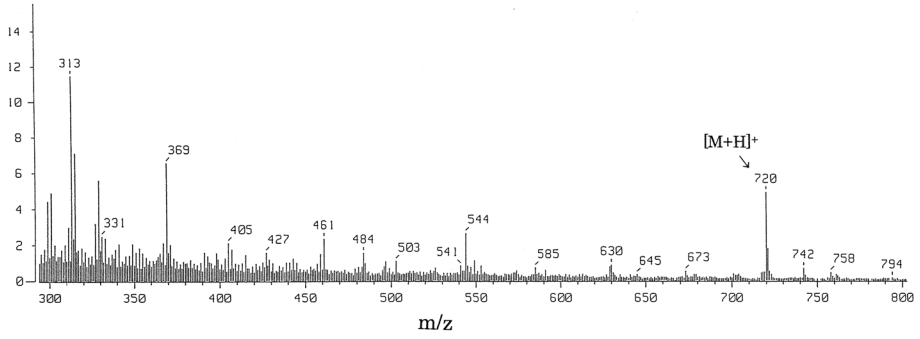

Figure 6. Positive mode FAB mass spectrum of oligosaccharide I.

treatment. Moreover, the modified Morgan-Elson reaction was negative for the mucin, suggesting the lack of terminal 3-*O*-substituted *N*-acetylgalactosamine which is present in all vertebrate mucins so far examined.[9] Then, mild hydrazinolysis with anhydrous hydrazine at 60 ℃ for 50 h was applied to this squid mucin. As shown in Fig. 3, the sugar chains released by hydrazinolysis, followed by *N*-acetylation of free amino groups, are separated into several oligosaccharides on a Bio-Gel P4 column, although the largest peak at the fractions 17 - 20 was found to contain mainly the mucin resistant to this mild hydrazionolysis. A major neutral oligosaccharide I at the fractions 59 - 61 was further analyzed by reversed-phase HPLC of its ABOE-derivative on a Honenpak C18 column ; Fig. 4 reveals the presence of a single peak together with negligibly small ones. The ABEE-derivatized monosaccharides of I are found by HPLC with the TFA solution as the eluent to consist of the equimolar amounts of galactose, fucose, 4-*O*-methylglucose and 4-*O*-methylglucosamine (Fig. 5). The occurrence of galactose in I was confirmed by HPLC with the borate buffer as the eluent, because ABEE-galactose and ABEE-glucose are not separated by HPLC with the TFA solution (Fig. 1B). On the other hand, the FAB mass spectrum of I reveals a peak at *m/z* 720 for $[M+H]^+$, indicating that its molecular weight is 719 (Fig. 6). These results imply that the oligosaccharide I is a tetrasaccharide containing 1 mol each of galactose, fucose, 4-*O*-methylglucose and *N*-acetyl-4-*O*-methylglucosamine. The lack of *N*-acetylgalactosamine in this tetrasaccharide is in contrast with the observation that mucin-type oligosaccharides are generally linked to threonine and/or serine residues in the protein backbone through *N*-acetylgalactosamine.[16] Thus, a possibility that the tetrasaccharide of nidamental mucin is linked to threonine residues through another monosaccharide than *N*-acetylgalactosamine is not excluded ; for instance, an oligosaccharide *O*-glycosidically linked to a serine residue through fucose was found in human factor IX.[17] Further structural studies on the isolated tetrasaccharide, which could not be released by β-elimination, are needed for

elucidation of a protein-sugar linkage in the nidamental mucin.

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スルメイカ包卵腺ムチンの性質

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スルメイカ（*Todarodes pacificus*）の包卵腺粘質物を希アルカリによって可溶化し，水溶性のムチンを単離した。粘質物 3g から，凍結乾燥ムチン 170mg が得られた。この包卵腺ムチンは硫酸化糖タンパク質の一種で，タンパク質 16.4％，糖質 80.3 % および 硫酸基 3.3 %（重量比）を含む。ムチンの水溶液は粘性が著しく高く，超遠心分析と粘度測定の結果，巨大な棒状分子（約 2,600 kDa）から成ることが判明した。さらに，このムチン分子はプロテアーゼ処理に対して強い抵抗性を示した。タンパク質部分はトレオニン，プロリンおよびイソロイシン（約 2:1:1）に著しく富み，この 3 種のアミノ酸が全アミノ酸の約 72 %（モル比）を占めていた。糖質部分は 2 種のメチル化糖，4-*O*-メチルグルコースと *N*-アセチル- 4-*O*-メチルグルコサミンをかなり多量に含み，糖鎖はβ - 位離脱反応に抵抗性を示したが，ヒドラジン分解によって遊離した。温和な分解条件下で遊離する中性糖鎖の主成分はガラクトース，フコースおよび上記 2 種のメチル化糖を各 1 モルずつ含むユニークな 4 糖であった。

キーワード：ムチン，包卵腺，スルメイカ，4-*O*-メチルグルコース，*N*-アセチル-4-*O*-メチルグルコサミン