Nidamental Gland Mucosubstance from the Squid *Illex argentinus*: Salt-soluble Component as a Mucin Complex

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Spawned eggs of the squid *Illex argentinus* are assumed to be wrapped up in fragile gels which are largely derived from the mucosubstance of nidamental glands. This mucosubstance is known to contain mucin-type glycoprotein as a main constituent. In this study, the soluble components of mucosubstance were prepared by sequential extraction with deionized water and 0.6 M NaCl, pH 7.2. The water-soluble component was poor in amino sugars and exhibited a low viscosity in solution, indicating the virtual absence of mucin. On the other hand, the salt-soluble component was relatively rich in amino sugars and had a high intrinsic viscosity of 10.0 dl/g. Moreover, sedimentation velocity analyses revealed that it comprised only a simple, sharp peak having a molecular weight of about 6.6×10^6 . When treated with 0.4 N NaOH, about 30% of the salt-soluble component was precipitated with 50% ethanol and identified as mucin. From these results, the salt-soluble component of mucosubstance was found to be a mucin complex which presumably exists in the gel structure of egg mass.

Key words: mucin, nidamental gland, squid, salt-soluble component

Nidamental gland mucosubstance of squid is responsible for the formation of jelly-like egg masses after spawning,^{1,2)} but little information is available on its chemical properties. Recently, we first reported the occurrence of mucin-type glycoprotein in the mucosubstance from the squid *Illex argentinus*,³⁾ which is considered to be closely related to the gel structure of egg mass. The mucin was successfully isolated by a mild alkaline treatment (0.4 N NaOH, 4°C, 4 h), followed by precipitation with 50% ethanol and it accounted for about 35% of the total mucosubstance. This squid mucin comprised 22.6% protein, 73.0% carbohydrate, and 4.4% ester sulfate and was characterized by its extremely high content of Thr-GalNAc linkage.

Although the mucosubstance contains mucin as a major protein, it is a complex mixture of many proteins. In order to further elucidate its property, the present paper deals with the fractionation of mucosubstance on the basis of solubility. It could be separated into water-soluble, salt-soluble, and insoluble fractions. Particularly, the salt-soluble fraction was featured by a high viscosity and thus analyzed with respect to chemical composition and macromolecular properties. It turned out that the salt-soluble component of mucosubstance is a mucin complex having a huge molecular weight.

Materials and Methods

Preparation of Nidamental Gland Mucosubstance

Frozen samples of the nidamental glands of mature squid *Illex argentinus* were collected at a fish market in Shioyama, Miyagi Prefecture, and stored at -30°C until use. Each sample weighed about 30 g on average. After thawing in air, the thin membrane of nidamental glands

was removed with scissors to obtain the mucosubstance.

Fractionation of the Mucosubstance

The mucosubstance, 3 g, was homogenized with 150 ml of deionized water. A water-soluble component was extracted from the homogenates for 18 h at 4°C while gently stirring and was obtained as the supernatant after centrifugation at $95,000 \times g$ for 40 min. Then, the precipitates were homogenized with 150 ml of 0.6 M NaCl containing 0.02 M sodium phosphate, pH 7.2, followed by incubation for 18 h at 4°C under good stirring. The resulting viscous solution was clarified by centrifugation as described above and treated with 50% ethanol in order to precipitate the salt-soluble component. The fibrous precipitate thus obtained was washed completely with 60% ethanol containing 0.1 M NaCl and dissolved in 0.6 M NaCl, pH 7.2.

Preparation of Mucin from the Salt-soluble Component

Mucin was prepared as described previously.³⁾ The salt-soluble component, 200 mg, was treated with 100 ml of 0.4 N NaOH containing 0.1 M NaBH₄ for 4 h at 4°C , followed by precipitation with 50% ethanol containing 0.2 M NaCl. The resulting precipitate, mucin, was collected by low-speed centrifugation and washed with 60% ethanol containing 0.1 M NaCl. This precipitate was dissolved in water, dialyzed against large volumes of water, and then lyophilized.

Analytical Methods

Amino acid and amino sugar compositions were determined with an amino acid analyzer (Tosoh HPLC system) after hydrolyses of samples in 6 N HCl at 110°C for 24 h and in 4 N HCl at 100°C for 8 h, respectively. Tryptophan was analyzed after alkaline hydrolysis in the presence of

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4% thioglycol.⁴⁾ Neutral sugar composition was determined with a gas chromatograph (Shimazu GC-4C) as trifluoroacetyl derivatives of alditols after hydrolysis in 2.5 N trifluoroacetic acid at 100°C for 7 h⁵⁾; arabinose was used as an internal standard. Ester sulfate was analyzed by the sodium rhodizonate method.⁶⁾

Sedimentation velocity patterns were obtained with an analytical centrifuge (Hitachi model 282) at 55,000 rpm and at 25°C. Samples were dissolved in 0.6 M NaCl containing 0.02 M sodium phosphate, pH 7.2, at several concentrations between 0.036% and 0.076%. The value of sedimentation coefficient was calculated using a partial specific volume of 0.70 ml/g. Viscosity was measured on 5 ml sample solution with a Cannon-Fenske viscometer having an average shear gradient of about 1,000 sec⁻¹. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out on 3.5% gels containing 0.1% SDS and 3.5 M urea. Protein bands were visualized with Coomassie Brilliant Blue R-250 and molecular weight markers (Sigma) for SDS-PAGE were used as standard proteins.

Results and Discussion

Preparation of Soluble Components from the Mucosubstance

The mucosubstance of nidamental glands was composed of water-soluble, salt-soluble, and insoluble fractions in a weight ratio of 20:24:56. This ratio, however, was variable and depended on the conditions of extraction as well as the maturity of nidamental glands. Both soluble fractions were partially characterized with respect to their chemical compositions and electrophoretic properties. The salt-soluble component in 0.6 M NaCl was found to give a highly viscous solution and to precipitate as fibrous mass by the addition of 50% ethanol, while the water-soluble one gave no viscous solution and was not precipitated with 50% ethanol. Chemical analyses showed that the salt-soluble component was similar in composition to the whole mucosubstance, but not to the water-soluble one. The amino acid, neutral sugar, and amino sugar compositions are listed in Table 1, in terms of residues per 1,000 total residues. The salt-soluble component was relatively rich in threonine, fucose, galactose, and *N*-acetylgalactosamine as well as compound X which was reported in our previous paper³⁾ as an unidentified amino sugar. On the other hand, the water-soluble component was rich in 2-aminoethanesulfonic acid, taurine, and was very poor in amino sugars, indicating the abundance of nonprotein nitrogen and the virtual absence of mucin, respectively.

When examined by SDS-PAGE, both soluble components were observed to have several protein bands, but were apparently different in their electrophoretic patterns from each other (Fig. 1). The salt-soluble component was unique in having a protein band which remained at the gel top. This band largely disappeared by the reduction of disulfide bonds with dithiothreitol to give some new bands. The band pattern of the water-soluble component, however, did not change significantly before and after reduction with dithiothreitol.

Macromolecular Properties of the Salt-soluble Component

Table 1. Amino acid and carbohydrate compositions of squid nidamental gland mucosubstance and its soluble components (residues/1,000 total residues)

	Mucosubstance*1	Water-soluble component	Salt-soluble component	
			Whole	Mucin
Amino acid				
Tau	26	173	0	0
Asp	65	55	64	16
Thr	74	37	82	112
Ser	28	36	28	4
Glu	54	68	47	12
Pro	57	53	54	58
Gly	51	54	44	14
Ala	34	55	29	7
Gys/2	23	7	26	0
Val	36	31	34	13
Met	8	10	8	2
Ile	48	25	54	56
Leu	35	37	34	10
Tyr	21	16	21	5
Phe	21	16	20	7
Trp	3	1	2	0
Lys	51	42	50	15
His	14	12	9	3
Arg	22	22	20	7
Total	671	750	626	341
Neutral sugar				
Rha	24	7	21	41
Fuc	59	46	62	91
Rib	13	42	14	35
Xyl	24	12	28	46
Man	19	53	14	8
Glc	20	52	12	31
Gal	72	21	91	168
Total	231	233	242	420
Amino sugar				
GlcNAc	19	10	20	36
X*2	36	3	53	80
GalNAc	43	4	59	123
Total	98	17	132	239
Total	1000	1000	1000	1000

*1 Reference 3.

*2 An unidentified amino sugar was determined as a glucosamine equivalent (Reference 3).

In the solvent of 0.6 M NaCl, pH 7.2, the molecular shape and weight of the salt-soluble component were estimated by both sedimentation velocity analysis and viscometry. As given in Fig. 2, the homogeneity of the salt-soluble component is evident; a single, sharp peak having a sedimentation constant of 27.7 S is detected by ultracentrifugal analysis. Moreover, it has a high intrinsic viscosity of 10.0 dl/g at 25°C (Fig. 3), indicating that the axial ratio of salt-soluble component in solution as a prolate ellipsoid of evolution was about 180 from the equation of Simha.⁷⁾ This high viscosity remained almost constant after heat treatment (100°C, 10 min). From these results, the molecular weight was calculated to be about 6.6×10^6 using the Scheraga-Mandelkern equation⁸⁾ with a value of 3.40×10^6 for β .

Then, we tried to isolate a mucin from the salt-soluble component by treatment with 0.4 N NaOH, followed by precipitation with 50% ethanol. The fibrous precipitate thus obtained was found to be a water-soluble sulfated

Fig. 1. SDS-polyacrylamide gel electrophoresis of the soluble components from squid nidamental gland mucosubstance.

Each gel was visualized with Coomassie Brilliant Blue R-250. W, water-soluble component; S, salt-soluble component; DTT, dithiothreitol.

Fig. 2. Sedimentation velocity pattern of the salt-soluble component of squid nidamental gland mucosubstance at 25°C.

The sample was dissolved in 0.6 M NaCl-0.02 M sodium phosphate, pH 7.2, at a concentration of 0.05%. The photograph was taken at 17 min after the speed of 55,000 rpm was reached.

glycoprotein, mucin, comprising 22.6% protein, 73.1% carbohydrate, and 4.3% ester sulfate by weight. The yield was about 30% of the salt-soluble component. As shown in Table 1, the mucin is characterized by high levels of threonine, proline, isoleucine, fucose, galactose, *N*-acetyl-galactosamine, and compound X. Furthermore, it has a high intrinsic viscosity of 9.0 dl/g in the solvent of 0.6 M NaCl, pH 7.2, (Fig. 3) and could not penetrate a 3.5% gel when examined by SDS-PAGE (data not shown). These chemical properties are almost identical to those of mucin from the whole mucosubstance as reported previously.³⁾

These composite results have clearly shown that the salt-soluble component of mucosubstance from mature squid is a mucin complex; mucin is associated with other con-

Fig. 3. Intrinsic viscosity of the salt-soluble component of squid nidamental gland mucosubstance at 25°C in the solvent of 0.6 M NaCl containing 0.02 M sodium phosphate, pH 7.2.

○—○: salt-soluble component, ●—●: mucin from the salt-soluble component.

stituents, such as several proteins detected by SDS-PAGE (Fig. 1), to form the salt-soluble component with the high intrinsic viscosity of 10.0 dl/g and the huge molecular weight of 6.6×10^6 . The salt-soluble component from immature squid, however, was observed to have a larger molecular weight (data not shown), indicating that the degree of association between mucin and other proteins was variable according to the maturity of nidamental glands. This mucin complex presumably exists in the egg mass gels. We assume that further aggregation of mucin with other constituents results in the formation of insoluble components. In fact, a preliminary experiment confirmed our assumption that a considerable amount of mucin exists in the insoluble fraction of mucosubstance. The detailed structure of nidamental gland mucin and its complexes remains to be clarified.

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