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Isolation and Characterization of Epidermal Mucus from *Hirudo nipponia*

Ju-Yun Lee, Cheol-O Joe and Ke-Won Kang*

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Taejon 305-701, Korea
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Abstract: The epidermal organ of the leech contains a complex glycoprotein molecule of mucus. The mucus excreted from annelids plays significant role in protection against desiccation and parasites. Mucus from the Korean native leech, *H. nipponia*, was investigated for biochemical characteristics for possible development of biomaterials of cosmetic and pharmaceutical agents. The leech skin mucus was heavily glycosylated mucin-like protein with a high molecular weight comprised 80% carbohydrate and 20% protein. Threonine, serine, and glycine were the major components of the isolated protein and these accounted for 50% of total amino acids. The carbohydrate portion contained glucosamine, galactosamine, galactose, glucose, mannose and sialic acid in oligosaccharide form linked with threonine and serine residues of the glycoprotein.
Key words: glycoprotein, *Hirudo nipponia*, mucin, skin.

Leech skin serves diverse vital functions. It is coated with a thick, protective mucus gel that is sticky, viscous, and elastic. It acts as a physical barrier against harmful agents in the external environments such as ultraviolet light and parasites (Saywer, 1986). This mucus gel material is secreted by unicellular mucus gland cells distributed irregularly on the leech skin and is very important in the life of this soft bodied animal for surviving both in the aquatic and nonaquatic environment. It was noted that the mucus probably acts as a buffer system outside the body since its secretion is largely stimulated by immersing in a noxious stimulant such as acid and salts. Pinching, severing or applying electric shock on the annelid results in a copious secretion of mucus (Ressler *et al.*, 1968).

The leech epidermal mucus contained heavily glycosylated, high molecular weight mucin-like protein. Mucins are secreted abundantly by various mucosal cells of other animals (Sheehan *et al.*, 1991; Corfield, 1992; Rose, 1992). The carbohydrate moieties of mucin exist as clustered oligosaccharide chains linked to the core proteins *via* *O*-glycosidic bonds (Amano *et al.*, 1991; Kitagawa *et al.*, 1991). Mucins are believed to play primarily protective roles, providing lubrication and particle clearance, as well as resistance to proteolytic actions and bacterial attacks (Lamblin *et al.*, 1991; Strous and Dekker, 1992). The present study has found that leech epidermal mucus contains characteristics similar to mucin isolated from various soft tissues of other animals.

*To whom correspondence should be addressed.
Tel: 82-42-869-2612, Fax: 82-42-869-2610
e-mail: kwkang@sorak.kaist.ac.kr

Materials and Methods

Collecting and cultivating leeches

Native Korean blood sucking leeches, *Hirudo nipponia* were collected from the lake at Chon-ju and cultivated in fish tanks in our laboratory. Fish tanks were maintained under aerobic conditions by continuous aeration. Leeches were periodically fed porcine blood (Hong *et al.*, 1993).

Collecting mucus

Mucus material was collected from liquid medium after porcine blood feeding. Feeding enhanced the secretion of mucus from the leech. The liquid medium was concentrated by ultrafiltration through 10 kD cut-off sized membrane and lyophilized. In order to stimulate the secretion of mucus, electrical shock, a low concentration of alcohol or table salts were applied.

Reduction of disulfide bonds

Solubilization of mucus was achieved by reduction of the disulfide bonds with dithiothreitol. Reduction buffer (6 M guanidine hydrochloride, 0.1 M Tris-HCl, 5 mM Na_2-EDTA, pH 8.0, containing 10 mM dithiothreitol) (Carlstedt *et al.*, 1993) was used for 5 h at 37℃. The alkylation was achieved by the addition of iodoacetamide and the sample was stored overnight in the

dark.

Isolation of mucin like glycoprotein

Solubilized sample was centrifuged at 10,000×g for 20 min and applied to a column (4×80 cm) of Bio-gel a-15m equilibrated with 0.1 M ammonium acetate buffer. Fractions of 5 ml were collected at room temperature at a flow of 20 to 30 ml/h. Tubes were pooled and analyzed for protein and neutral sugar. The high molecular weight fraction (5 to 10 mg) from Bio-gel a-15m was further fractionated on a column (1.5× 70 cm) of Sepharose-CL-2B equilibrated with 0.1 M ammonium acetate containing 0.1% SDS and 0.02% sodium azide.

Analytical procedures

Protein was determined by the Lowry method (Lowry *et al.*, 1951) with BSA as a standard. Total neutral sugars were estimated by the anthrone reaction (Gerhardt *et al.*, 1981) with glucose as standard. Sialic acid was measured by the resorcinol method (Jourdian *et al.*, 1971) with N-acetylneuraminic acid as a standard.

Estimation of molecular weight

The molecular weight of the mucin protein in solution was estimated by Sepharose CL-6B gel filtration chromatography using molecular weight markers as standard (carbonic anhydrase, 29,000; albumin, 66,000; alcohol dehydrogenase, 150,000; β-amylase, 200,000; apoferritin, 443,000; thyroglobulin, 669,000). The flow rate was 20 ml/h and 1.5×80 cm column was used. Void volume was determined with blue dextran. Molecular weight was estimated from plots of the log molecular weight versus relative elution volume of standard proteins.

Glycopeptide linkage analysis

The isolated mucin was treated with 0.5 M NaOH at 25℃ for 7 h. The increase in absorbance at 241 nm upon alkaline treatment was investigated (Sachdev *et al.*, 1978). Alkaline treatment was nearly completed after a 7 h treatment with 0.05 M NaOH at room temperature. The determination of the number of *O*-glycosidic linkages was based on the loss of serine and threonine residues against the increase of alanine residues following reductive β-elimination with 1 M $NaBH_4$ in 0.05 M NaOH at 45℃ for 22 h.

Amino acid and carbohydrate composition analysis

For amino acid analysis, samples were hydrolyzed with 3 M HCl at 110℃ for 24 h under nitrogen, dried in a Speed-Vac, and then analyzed with a Waters Pico-Tag HPLC system (Tarr, 1986). For carbohydrate analysis, an aquous solution of mucin was acidified by 5 M trifluoroacetic acid (1 : 1 volume) and heated at 100 ℃ for 4 h. The hydrolysates were evaporated to dryness in a Speed-Vac, and dissolved in water. After clarifying through the membrane (3 kD cut-off), the residues were analyzed for neutral sugars and hexosamines. Analyses were performed with high pressure liquid chromatography coupled to pulsed amperometric detection system (Hardy, 1989).

Results and Discussion

The surface of the leech epidermis is covered by a layer of mucus gel that is known to play a significant role for survival of primarily aqueous animals that are exposed occasionally to a terrestrial environment. Several methods were explored to stimulate the secretion of mucus from cultivated leeches. A low concentration of alcohol, salts, physical agitation and electrical shock proved to be good agents. Blood feeding brought about the replacement of the layer of mucus gel from the whole body. The mucus collected from the treatment was insoluble and highly viscous. After a single blood feeding, 2 mg of lyophilized dry weight yellowish-white colored mucus could be obtained from each adult leech. The dried mucus contained an equal portion of protein and carbohydrate by weight.

Fig. 1 shows the elution profile on Bio-gel a-15m indicating two distinct peaks with different ratios of protein and carbohydrate. Separate determinations of the protein and carbohydrate of the two fractions from Bio-gel a-15m revealed that peak I contained 35% protein and 65% neutral sugar, and peak II contained 65% protein and 35% neutral sugar, of total protein and carbohydrate by weight. The amino acid compositions of these fractions are shown in Table 1. Hydroxyamino acid (serine and threonine) comprised a large portion of peak I. This composition is definitely mucin-like in character. Cross-linked Sepharose 2B was utilized to further resolve the glycoprotein components in Bio-gel a-15m for peak I (Fig 2). Subsequent studies for amino acid composition were carried out from the high molecular weight glycoprotein of fraction Ia. The Ia fraction had mucin like glycoproteins which showed molecular mass of 480 kD from Sephaorose CL-6B gel filtration chromatography (Fig. 3).

The amino acid composition of fraction Ia and mucins from other sources are compared in Table 2. The leech epidermal mucus protein showed a very similar amino acid composition to cobra venom and canine tracheal excretion. The major amino acids were threonine, serine and glycine, which make up 46% of the total residues. The high contents of hydroxyamino acids

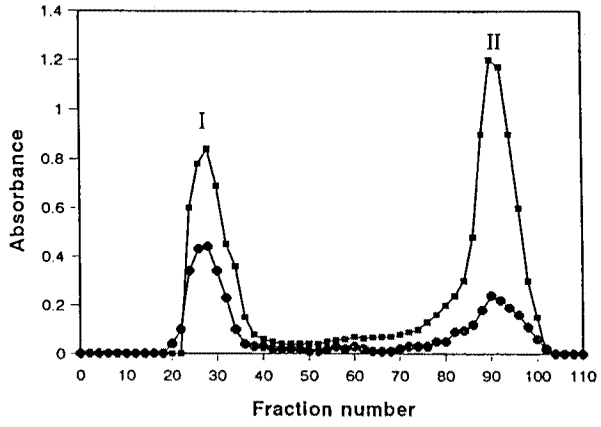

Fig. 1. Chromatography on Bio-gel a-15m of leech epidermal mucus. The 500 mg of sample was applied to a column (4×80 cm) in 0.1 M ammonium acetate buffer, pH 7.0. Fractions of 5 ml were collected at room temperature at a flow of 20 to 30 ml/h. The elution of protein was monitored by UV absorption peak at 280 nm. Hexose were assayed for each fraction by the anthrone reagent at 625 nm. Absorbace at 280 nm (■–■), Absorbance at 625 nm (●–●).

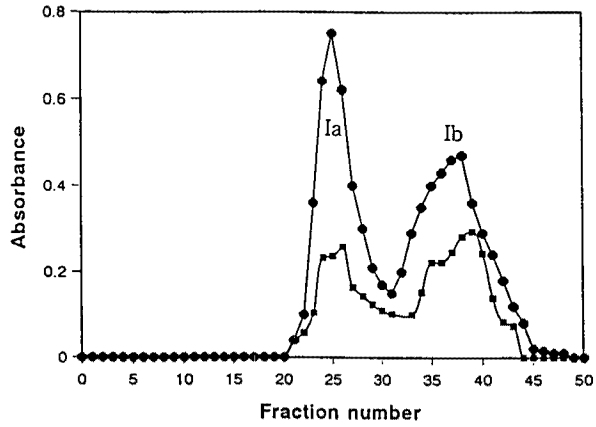

Fig. 2. Cross linked Sepharose-2B gel filtration chromatography. Peak I (Fig. 1) from Bio-gel a-15m was further fractionated on a column (1.5×70 cm) of Sepharose-CL-2B in 0.1 M ammonium acetate buffer, pH 7.0, containing 0.1% SDS and 0.02% sodium azide. 3 ml of each fractions that were generated a flow rate of 10 ml/h at room temperature were analyzed for protein and hexose. Absorbace at 280 nm (■–■), Absorbance at 625 nm (●–●).

Table 1. Percent amino acid composition of the mucus fractionated by Bio-gel a-15m gel filtration chromatography (fractions are shown in Fig. 1)

Amino acid	Fraction I	Fraction II
Asn	7.2	3.6
Gln	8.7	5.7
Ser	14.2	12.1
Gly	8.8	6.8
His	1.9	1.8
Arg	1.3	2.7
Thr	25.2	26.6
Ala	6.6	9.1
Pro	11.2	10.7
Tyr	1.0	0.3
Val	3.7	5.0
Met	1.2	2.0
Cys	ND	2.7
Ile	1.8	2.3
Leu	2.4	5.4
Phe	0.9	1.8
Lys	3.9	1.4
Trp	ND	ND

ND : not determined.

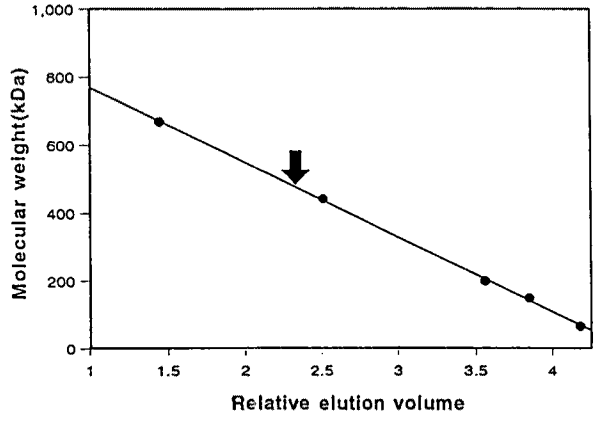

Fig. 3. Estimation of molecular weight of isolated glycoprotein using gel filtration chromatography with Sepharose CL-6B. Molecular weight standards were thyroglobulin (669,000), apoferritin (443,000), β-amylase (200,000), alcohol dehydrogenase (150,000) and bovine serum albumin (66,000). Relative elution volume of glycoprotein is shown by the arrow.

are potential *O*-glycosylation sites that is consistent with the high carbohydrate content. When we include the next two most abundant alanine and glutamate, two thirds of the protein consists of these amino acids. The isolated glycoprotein (fraction Ia) contained little amounts of methionine, tyrosine, lysine and histidine.

The carbohydrate compositon of the glycoprotein isolated is presented in Table 3. The ratio of carbohydrate to protein in various fractions was different from each isolation steps and the composition of the carbohydrate varies as well. For example, the crude extract contained 50% of carbohydrate by weight, over 40% of which was galactose, but the isolated mucin-like glycoprotein (fraction Ia) consisted 80% of carbohydrate with only 10% galactose. The isolated leech glycoprotein contains a high proportion of glucose and glucosamine suggesting large oligosaccharide chains.

Glycoprotein linkage analysis was performed by alkaline borohydride treatment of the isolated glycoprotein. The changes of reductive β-elimination with borohyd-

Table 2. Amino acid compositions of mucin-like glycoproteins from various sources

Amino acid	Leech[a]	Cobra venom[b]	Canine tracheal[c]	Ovine submaxillary[d]	Human cervical[e]
Asn	6.55	7.2	5.35	1.56	3.6
Gln	8.04	8.7	9.64	5.34	5.7
Ser	17.39	14.2	14.00	18.04	12.1
Gly	11.05	8.8	7.97	19.92	6.8
His	1.59	1.9	1.71	–	1.8
Arg	1.02	1.3	2.57	3.52	2.7
Thr	17.38	25.2	16.00	14.23	26.6
Ala	8.20	6.6	5.16	13.91	9.1
Pro	7.25	11.2	10.57	11.09	10.7
Tyr	1.04	1.0	1.06	–	0.3
Val	7.34	3.7	7.10	6.67	5.0
Met	1.16	1.2	2.86	–	2.0
Cys	ND	ND	ND	–	2.7
Ile	2.70	1.8	4.00	1.13	2.3
Leu	4.21	2.4	8.44	3.54	5.4
Phe	1.37	0.9	2.00	1.38	1.8
Lys	2.72	3.9	2.79	3	1.4
Trp	ND	ND	1.05	–	ND

[a] from the fraction Ia in the Fig. 2.
[b] Gawda *et al.*, 1994, [c] Sachdev *et al.*, 1978, [d] Hill *et al.*, 1977, [e] Yurewicz *et al.*, 1981.
ND : not determined.

Table 3. Percent carbohydrate composition of the leech epidermal glycoprotein

Carbohydrate	Crude mucus	Isolated glycoprotein[a]
Galactosamine	8.37	13.1
Glucosamine	14.8	17.8
Galactose	42.2	9.8
Glucose	24.3	47.1
Mannose	7.8	10.5
Sialic acid	2.6	1.6

[a] from the highest molecular weight fraction.

Table 4. Changes in the amino acid composition of the mucin after treatment with alkaline sodium borohydrate

Amino acids	Before treatment (nmol/100 nm)	After treatment (nmol/100 nm)	Difference (nmol/100 nm)
Serine	17.39	5.91	−11.48
Alanine	8.20	18.22	+10.02
Threonine	17.38	1.96	−15.42

ride on the amino acid composition is shown in Table 4. Approximately 66% of seryl and 88% of threonyl residues were β-eliminated and the total serine loss was closely matched with the gain of alanine. These results suggested that carbohydrate chains of the leech mucus glycoprotein were linked to the protein through *O*-glycosidic bonds involving galactosamine and seryl or threonyl residues (Sachdev *et al.*, 1978).

We have attempted to purify the leech epidermal mucus glycoprotein by liquid chromatographic methods. It was difficult to assess the purity of the mucin-like glycoprotein with physical methods because the glycoprotein had a very large molecular weight and was highly viscous and insoluble in aquous buffer solution. But the leech epidermal glycoprotein was very comparable with the snake venom (Gawda *et al.*, 1994) and ovine submaxillary gland mucins (Hill *et al.*, 1977) having a high proportion of serine and threonine. This mucus protein was a heavily *O*-glycosylated glycoprotein known as mucin from soft tissue in other sources, and a rather substantial quantity of mucin could be obtainable from the live leech in the laboratory.

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