Pentose-containing Polysaccharides from the Liver of the Squid, *Ommastrephes sloani pacificus*

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A few studies on the polysaccharides of invertebrate connective tissues have revealed several new types of mucopolysaccharide in these tissues[1)-4)]. However, very little is as yet known about the polysaccharides from other tissues of invertebrate animals.

In connection with the liver polysaccharide of invertebrates, Inoue[5)] obtained a sulfated heteropolysaccharide, which she named horatinsulfuric acid, from the liver of a marine gastropod, *Charonia lampas*, and Maki[6)] reported a sulfated polysaccharide-protein complex from the liver of the squid, *Ommastrephes sloani pacificus*. The sugar components of the squid liver polysaccharide were further investigated with paper chromatography by Rosenberg and Zamenhof[7)], but the properties of the polysaccharide have not yet been satisfactorily characterized.

In this study, polysaccharide fractions were isolated from the liver of *Ommastrephes sloani pacificus* by pronase digestion and subjected to a detailed chemical analysis.

Materials and Methods

Materials

Frozen squids, *Ommastrephes sloani pacificus*, were kindly supplied by Taiyo Gyogyo Co. Ltd., Tokyo. Pronase (EC class 3.4.4) was purchased from Kaken Kagaku Co. Ltd., Tokyo.

Analytical methods

Hexosamine and sulfate were analyzed as described previously[4)]. Glucosamine and galactosamine were separated and determined according to the method of Gardell[8)]. Hexose was determined by the anthrone reaction[9)], using galactose as the standard and subtracting the value representing the amount of methylpentose which was determined by the method of Gibbons[10)]. Pentose was determined by the orcinol reaction[11)], using xylose as the standard and subtracting the value representing the amount of uronic acid which was determined by the modified carbazole reaction[12)]. Sialic acid was analyzed

by the thiobarbiturate reaction[13], after hydrolysis with 0.1 N H_2SO_4 at 80° for 1 h. Protein was estimated by the method of Lowry *et al.*[14] with bovine serum albumin as the standard.

Chromatography on DEAE-cellulose

A DEAE-cellulose column (1.0×35cm) was equilibrated with 0.025 M sodium phosphate buffer (pH 6.0) and the sample was applied to the column in the same buffer. Stepwise elution was carried out with 0.025 M, 0.05 M, 0.1 M and 0.25 M sodium phosphate buffer (pH 6.0) and 0.1 N NaOH. The flow rate was 15 ml/h and 5 ml-fractions were collected. Aliquots of each fraction were analyzed by the phenol-sulfuric acid reaction[15].

Electrophoresis

Electrophoresis on cellulose acetate strips was performed in 1.0 M acetic acid-pyridine (pH 3.5) at 0.5 mA/cm for 15 min. Polysaccharides were detected with 0.5% toluidine blue in 3% acetic acid for 5 min[16].

Paper and gas-liquid chromatography

Uronic acid was identified by descending paper chromatography in *n*-butanol-acetic acid-water (4 : 1 : 2, by vol.) for 16 h[17], after hydrolysis with Dowex 50 (×8; 200–400 mesh, H^+ form) and 0.05 N HCl at 105° for 24 h according to Dziewiatkowski[18]. Uronic acid was detected by aniline hydrogen phthalate spray[19].

Gas-liquid chromatography of neutral sugars was carried out on a Shimazu chromatograph model GC-1C equipped with dual columns and a hydrogen flame ionization detector. A glass column (0.4×180 cm) packed with 2% XF-1105 on Gas-Chrom P (80–100 mesh) was used. The polysaccharide was hydrolysed with 1 N H_2SO_4 at 100° for 2 h and the hydrolysate was passed through a column of Amberlite MB-3 (1.0×10 cm). The effluent and washings were concentrated to dryness and the sugars were analyzed as alditol trifluoroacetyl derivatives by the method of Imanari *et al.*[20].

Results

Isolation of polysaccharides

The frozen squid was thawed and the large liver was removed. From 20 animals, 600 g of liver was obtained. The internal material of the liver was homogenized in a Waring blendor with 2 volumes of water. The homogenate was heated in a boiling water bath for 5 min and then centrifuged at 1,000 g for 20 min. The oily upper layer and residues were removed and 3 volumes of ethanol were added to the clear extracts. The precipitate collected by centrifugation was dissolved in 0.01 M calcium chloride and the pH of this solution was adjusted to 7.8. Pronase digestion was then carried out with 120 mg of pronase at 40° for 48 h. At the end of the digestion, insoluble materials were removed and trichloroacetic acid was added to a con-

centration of 10%. After centrifugation, the supernatant was dialyzed against water and the clear dialyzate was treated with a mixture of Lloyd's reagent and kaolin (3 : 1). The polysaccharides (PS) were precipitated from the concentrated solution by adding 4 volumes of ethanol in the presence of 1% sodium acetate. The yield was 710 mg. The analytical data showed that this material contained hexosamine, hexose, pentose, methylpentose and uronic acid (Table 1). The sialic acid content was negligible. Ester sulfate and protein (peptide) were detected.

Fractionation of the polysaccharides on a DEAE-cellulose column

The polysaccharides (100 mg) were fractionated on a DEAE-cellulose column. As shown in Fig. 1, the polysaccharides were separated to four peaks. The eluates, corresponding to each peak, were dialyzed and freeze-dried. The yields of Fractions I, II, III and IV were 17 mg, 14 mg, 26 mg and 27 mg, respectively.

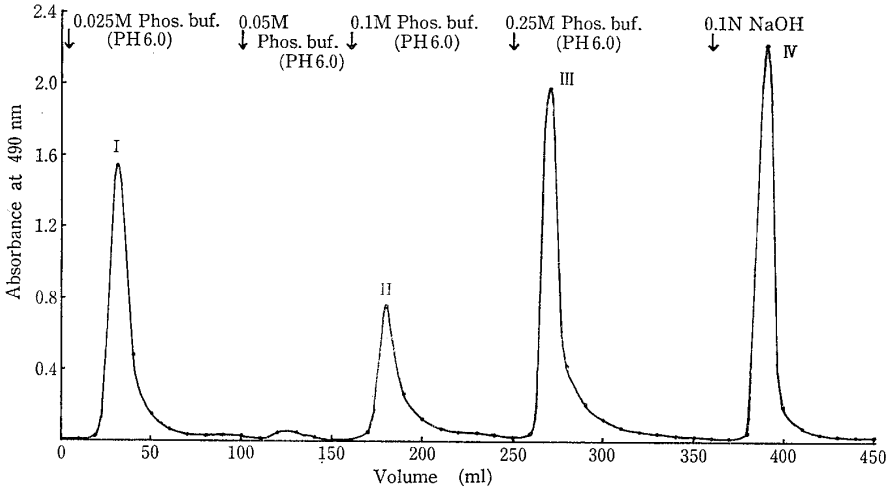

Fig. 1. DEAE-cellulose chromatography of the polysaccharides. Column size 1.0×35 cm. The sample (100 mg) was applied in 0.025 M sodium phosphate buffer (pH 6.0) and stepwise elution was performed as described in the text. The polysaccharide fractions were designated as Fractions I through IV.

Electrophoresis and chemical analysis of the polysaccharide fractions

Electrophoretic pattern on a cellulose acetate strip is shown in Fig. 2. Each polysaccharide fraction showed different mobilities migrating toward the anode except Fraction I which remained near the origin.

Chemical analysis of the polysaccharide fractions is summarized in Table 1. All the fractions contained hexosamine, hexose, pentose, methylpentose and uronic acid. Only a small amount of uronic acid was detected in Fraction I and the higher contents of this component were found in other fractions. The contents of ester sulfate of Fractions I and II were negligible, while the content of Fraction IV was

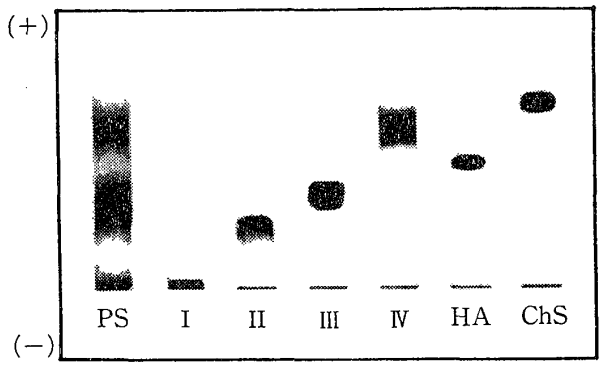

Fig. 2. Tracing of electrophoretogram on a cellulose acetate strip (Separax) of the polysaccharide fractions obtained from DEAE-cellulose chromatography. Electrophoresis was performed in 1.0 M acetic acid-pyridine (pH 3.5) at 0.5 mA/cm for 15 min. Polysaccharides were detected with 0.5% toluidine blue in 3% acetic acid. PS refers to the unfractionated polysaccharides and Fractions I through IV indicate the polysaccharide fractions designated in Fig. 1. HA and ChS are the standards, hyaluronic acid and chondroitin sulfate A and C, respectively.

Table 1. Chemical Analyses of Polysaccharide Fractions

Component	PS		I		II		III		IV	
	%	Molar ratio	%	Molar ratio	%	Molar ratio	%	Molar ratio	%	Molar ratio
Hexosamine	9.7		11.8		6.8		8.3		11.0	
Glucosamine		1.00		1.00		1.00		1.00		1.00
Galactosamine		0.46		0.31		0.49		1.08		0.88
Hexose	35.0		28.0		29.7		31.2		32.1	
Galactose		1.00		1.00		1.00		1.00		1.00
Mannose		0.42		0.68		0.38		0.38		0.30
Glucose		0.32		0.19		0.38		0.35		0.30
Pentose	11.0		6.9		12.6		16.6		9.0	
Xylose		1.00		1.00		1.00		1.00		1.00
Arabinose		0.69		0.31		0.79		0.82		0.57
Methylpentose	11.4		10.4		8.4		12.0		9.0	
Fucose		1.00		1.00		1.00		1.00		1.00
Rhamnose		0.33		0.16		0.50		0.43		0.51
Uronic acid	11.3		2.0		7.7		13.5		13.0	
Sialic acid	<0.2		—*		—		—		—	
Sulfate	3.1		Nil**		Nil		1.6		10.4	
Protein	7.4		7.5		5.0		3.3		5.7	

* Not determined. ** Not detected.

PS refers to the unfractionated polysaccharides, and I, II, III and IV indicate the polysaccharide fractions obtained from DEAE-cellulose chromatography as designated in Fig. 1.

considerably high.

Identification and composition of sugar components

The amino sugars in the polysaccharide fractions were identified as glucosamine and galactosamine by separation on a Dowex 50 column. These sugars were present in various ratios with fractions (Table 1).

Neutral sugars were identified by gas-liquid chromatography. As shown in Fig. 3, the polysaccharides (PS) gave seven peaks corresponding to rhamnose, fucose, arabinose, xylose, mannose, glucose and galactose. The seven peaks were also detected in other fractions.

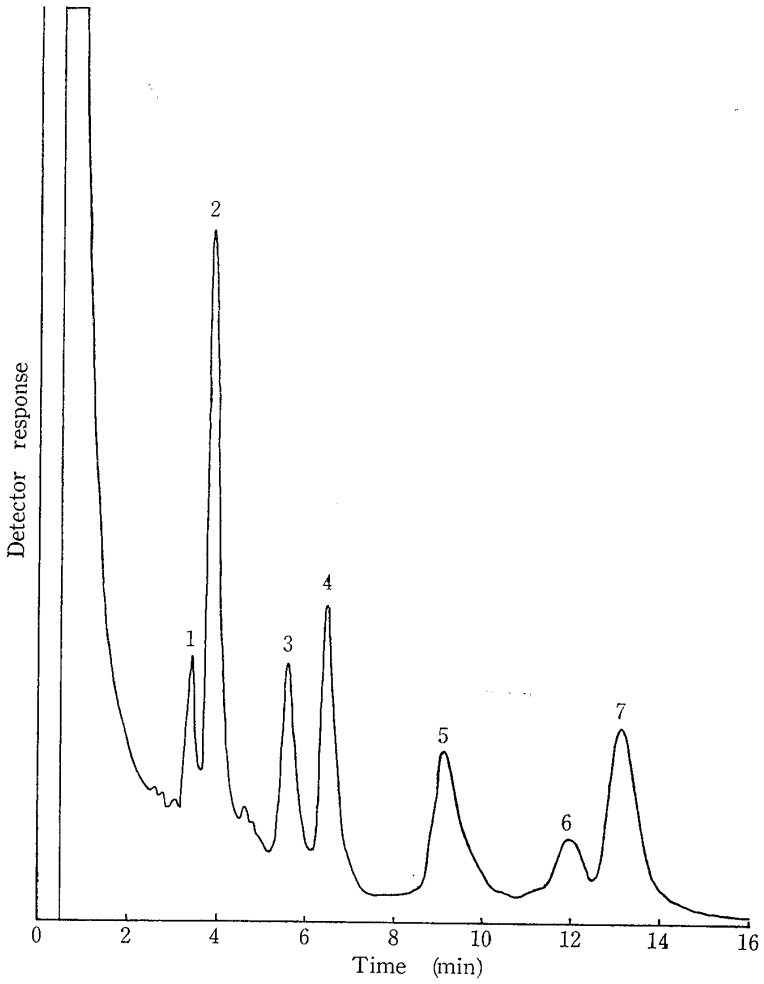

Fig. 3. Gas-liquid chromatogram for neutral sugars of the polysaccharide fraction. The polysaccharides (1 mg) were hydrolysed in 1 ml of 1 N H_2SO_4 at 100° for 2 h and the hydrolysate was deionized by passing it through a column of Amberlite MB-3 (1.0×10 cm). The effluent and washings (20 ml) were concentrated to dryness and neutral sugars were reduced to alditol with sodium borohydride followed by converting to trifluoroacetyl derivatives and then subjected to gas-liquid chromatography, according to Imanari *et al.*[20]. The followings are the assignments made by comparison with chromatograms of the standard sugars: 1, rhamnose; 2, fucose; 3, arabinose; 4, xylose; 5, mannose; 6, glucose; 7, galactose.

The molar ratios of these sugars calculated from the analysis of standard sugars are given in Table 1. Similar composition of neutral sugars was observed in Fractions II, III and IV, while that of Fraction I was considerably different from the others.

As for uronic acid, a single spot was obtained on a paper chromatogram and its Rf value was in accord with that of authentic glucuronic acid.

Discussion

In connection with the sugar components of the squid liver polysaccharide, the presence of seven sugars, glucosamine, galactosamine, galactose, xylose, arabinose, rhamnose and glucuronic acid, was demonstrated by previous analyses with paper chromatography[6)7)]. In the present study, further three sugars, mannose, glucose and fucose, were identified by gas-liquid chromatography. It has been reported that xylose or arabinose is involved in the linkage region between protein and carbohydrate in some mucopolysaccharides[21)-23)], and D-arabinose was isolated from an acidic polysaccharide obtained from the sponge, *Hippospongia gossypina*[24)]. However, a polysaccharide containing pentose as a constituent sugar has not yet been found in other animal tissues.

Fractions III and IV of the squid liver polysaccharide contained glucuronic acid and sulfate together with glucosamine and galactosamine, suggesting the presence of acidic mucopolysaccharides in the fractions. However, no clear band corresponding to hyaluronic acid or chondroitin sulfates could be detected on the electrophoretogram (Fig. 2), and the polysaccharide fractions were not degraded with testicular hyaluronidase. These results indicate that significant amounts of mucopolysaccharides may not be present in the squid liver polysaccharide fractions. There is a possibility that the sulfate groups may be derived from sulfated glycoproteins which have been reported to occur widely in other animal tissues[25)-29)].

We have previously isolated a non-sulfated chondroitin from squid skin[3)] and a novel chondroitin polysulfate (chondroitin sulfate E) from squid cartilage[4)30)], and suggested that the mucopolysaccharide composition in connective tissues of invertebrates may be different from that of vertebrates. It is interesting in relation to the polysaccharide distribution in invertebrates that a novel type of complex heteropolysaccharides was found in squid liver in this study.

Summary

1. The polysaccharides isolated from the liver of the squid, *Ommastrephes sloani pacificus*, by pronase digestion were fractionated

by DEAE-cellulose chromatography into four fractions which were practically homogeneous in electrophoresis on cellulose acetate strips.

2. All the fractions from DEAE-cellulose chromatography contained ten sugar components: glucosamine, galactosamine, galactose, xylose, arabinose, fucose, rhamnose and glucuronic acid. Such a complex polysaccharide containing pentose has not yet been found in other animal tissues.

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