イカ甲骨多糖体のゲル濾過像及び構成単糖について

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Gel-Filtration and Sugar Constituent of the Polysaccharide Extracted from the Internal Shell of Squid*2

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The internal shell of squid was extracted with hot 10 mM EDTA after being washed with acetone. The crude extract was dialyzed against water and freeze-dried.

The distribution of molecular weight was determined by column chromatographies on Sephadex G–75 and Sepharose 6B, 4B, and 2B. The data suggested that the crude extract was composed of polysaccharides of widely varying molecular weights and that the molecular weight of a major component was approximately 10^6.

After the crude extracts were acid-hydrolyzed and methanolyzed, the constituent monosaccharides were analyzed with gas chromatography. The results were as follows: L-arabinose, L-rhamnose, D-xylose, D-mannose, D-galactose, D-glucose, D-glucosamine, and D-galactosamine.

Uronic acid was also detected by chemical analysis but sialic acid was undetectable.

By the present author,[1] it was found that the crude extract obtained from the internal shell of squid contained polysaccharides and showed an antitumor activity against mouse Sarcoma-180.

It is likely that this antitumor activity is due to the polysaccharide moiety in this crude extract.[2,3]

The gel-filtration and sugar analysis of the crude extract were carried out in the present investigation.

Materials and Methods

Preparation of the Crude Extract

Fundamentally, the crude extract was obtained by the method similar to the procedure mentioned in the previous paper.[1]

The air dried internal shell of squid was pulverized and washed with acetone. The powder was extracted with hot 10 mM EDTA solution and filtered (Toyo No. 5B) with hyflosuper-cel. Then saturated $Ba(OH)_2$ solution was added to the filtrate. The precipitate obtained after standing over night was collected on a filter paper (Toyo No. 5B) with hyflosuper-cel and washed with water. The precipitate was dissolved in 10 mM EDTA solution and was dialyzed against deionized water. After the dialyzate solution was filtered (Whatman GF/C), the filtrate was freeze-dried and the pale brown powder was obtained. This lyophilized preparation was used in the present investigation as the crude extract.

Gel-Filtration

Columns of Sephadex G–75, Sepharose 6B, Sepharose 4B and Sepharose 2B were used.

Eluted fractions from the column were analyzed for polysaccharide by the phenol-sulfuric acid method and for the protein by the optical density at 280 nm.

Void volume (V_0) and total volume (V_t) of the column were estimated with blue dextran 2000 and urocanic acid respectively.[4]

Monosaccharide Analysis

After the crude extracts were hydrolyzed with 2N-HCl and methanolyzed with 5% methanol-HCl, the constituent monosaccharides were analyzed with gas chromatography.

Acid hydrolysis: The dried sample (20 mg) in the ampule was dissolved in 2 N-HCl (4 m*l*). After replacement with argon gas, the ampule was sealed and heated at 100°C for 2 h. The hydrolyzate was neutralyzed with $AgCO_3$ and filtered (Toyo No. 5B). The filtrate was evaporated to dryness with rotary evaporator, then the sample was dried on P_2O_5 in vacuo.

This dried sample was mixed with D-glucurono-lactone as the internal standard and mixed with 1 m*l* of TMS-reagent (The supernatant of the mixtures of; pyridine 10 m*l*, hexamethyldisilazane

*1 An Antitumor Substance Obtained from the Internal Shell of Squid-II. (イカ甲骨の抗腫瘍性物質について—II).

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4 m*l* and trimethylchlorosilane 2 m*l*). The mixture was heated at 70–80°C for 3 min. The supernatant of the reaction mixture was subjected to the gas chromatography.

Methanolysis: The dried sample (20 mg) in the ampule was mixed with 5% methanol-HCl (4 m*l*). After replacement with argon gas, the ampule was sealed and autoclaved at 120°C for 1 h. The methanolyzate was neutralyzed and treated according to the same procedures as in the case of acid hydrolysis.

Gas Chromatography

Nihon Denshi model JGC–750 was used. The operating conditions were as follows: Column; stainless steel column (0.3×300 cm) packed with 3% OV–17 on Shimalite W (80–100 mesh). Column temp; 100→225°C (6°C/min). Injection and detector temp; 230°C. Carrier gas; N_2. Detector; FID.

Estimation of Uronic Acid and Sialic Acid

The carbazol reaction method was used for the estimation of uronic acid.

The sialic acid was estimated by the following procedures. The sample was hydrolyzed with 0.1 N–H_2SO_4 at 80°C for 1 h. The hydrolyzate was passed through the column of Amberlite CG–400 (Formate). After the column was washed with water, the eluate by the 0.3 N-HCOOH was collected and sialic acid was estimated by the resorcin-HCl method.

Results and Discussion

Yield of the Crude Extract

In the previous experiment,[1] the acetone powder of the internal shell of squid was washed with hot water prior to EDTA extraction. However, the hot water extract contained a considerable amount of polysaccharides.

In the present experiment, the step of hot water washing was excluded, and the acetone powder was extracted with 10 mM EDTA solution directly. By such procedure, the yield of polysaccharide moieties was increased.

As shown in Fig. 1-A, when the acetone powder was washed with hot water, the crude extract obtained by hot EDTA extraction was calculated to 200 mg/500 g of acetone powder. On the contrary, hot water washings contained the crude substance more than 200 mg/500 g. From these results, it is clear that the procedure of hot water washing results in a loss of the polysaccharide.

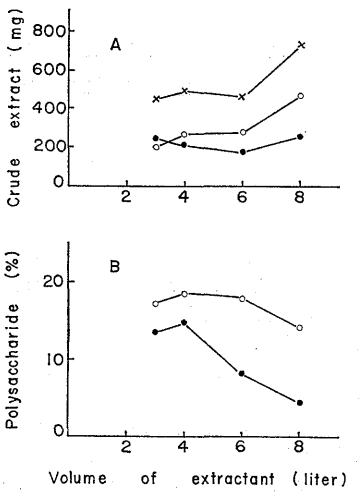

Fig. 1. Extraction of the polysaccharide from acetone powder with hot water and 10 mM EDTA.
Five hundred grams of acetone powder of the internal shell of squid were extracted with hot water (—○—) followed by hot 10 mM EDTA (—●—). A, Total extract (—×—). B, The yield of the polysaccharide moiety.
The polysaccharide content was indicated as per cent of the crude extract. The polysaccharide was determined by the phenol-sulfuric acid method using D-mannose as the standard.

Fig. 1-B shows that the per cent of the polysaccharide content of the crude extract was decreased by the increase of the extractant volume.

Gel-Filtration Analysis

As described in the previous paper,[1] the elution pattern of the column chromatography on Sephadex G–200 of the crude extract suggested that the one fraction of the polysaccharide was eluted at V_0 and the other fraction of the polysaccharide were eluted at various elution volumes between V_0 and V_t. It suggested that the crude extract was composed of the polysaccharides of wide varieties of the molecular weight.

In the present experiment, gel-filtration analysis on Sephadex G–75, Sepharose 6B, Sepharose 4B and Sepharose 2B were done for obtaining further knowledges about the molecular weight distribution of the polysaccharide moiety.

The elution pattern of the column chromatography on Sephadex G–75 was shown in Fig. 2. It shows that the polysaccharide was eluted at V_0 only, whereas the protein was eluted at V_0 and V_t. This result shows that the molecular weight of the polysaccharide was likely more than 50,000.

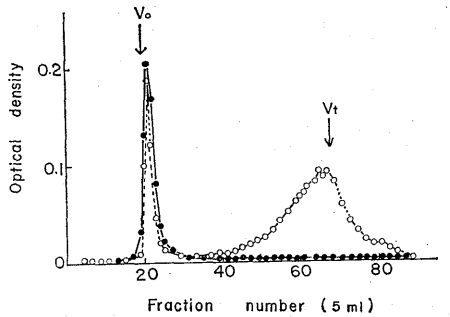

Fig. 2. Sephadex G–75 column chromatography of the crude extract.
The crude extract (20.3 mg) was dissolved in 5 m*l* of 0.5 M-NaCl. After filtration (Whatman GF/C), the solution was applied to the column (2.2×95.5 cm) and fractionated with 0.5 M-NaCl at a flow rate of 1 m*l*/min. Each 5 m*l* fractions was collected and analyzed.
Sugar was determined by the phenol-sulfuric acid method (—●—) at 490 nm. Protein was determined by the absorbance at 280 nm (---○---). Void volume (V_0) and total pore volume (V_t) of the column were indicated by arrows.

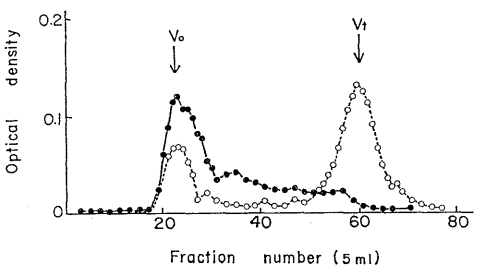

Fig. 3. Sepharose 6B column chromatography of the crude extract.
The crude extract (20.0 mg) was dissolved in 5 m*l* of 0.5 M-NaCl. After filtration (Whatman GF/C), the solution was applied to the column (2.2×85.2 cm) and fractionated with 0.5 M-NaCl at a flow rate of 25 m*l*/hr.
Refer to the foot-note in Fig. 2 for further details.

The elution pattern of the column chromatography on Sepharose 6B was shown in Fig. 3. A large part of the polysaccharide fraction was eluted at V_0 and the other fractions of the polysaccharide showed various elution volumes between V_0 and V_t. It suggests that a large part of the polysaccharide may have its molecular weight of 10^6.

The elution pattern of the column chromatography on Sepharose 4B was shown in Fig. 4. A large part of the polysaccharide was eluted near V_t and the other fractions were eluted between V_0

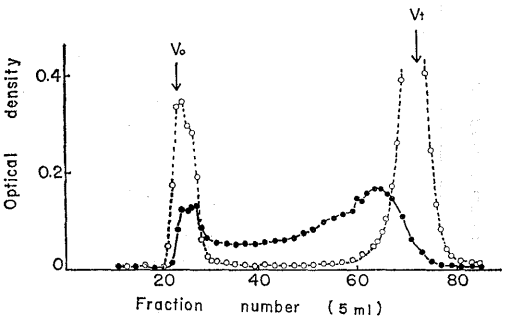

Fig. 4. Sepharose 4B column chromatography of the crude extract.
The crude extract (31.5 mg) was dissolved in 5 m*l* of 0.5 M-NaCl. After filtration (Whatman GF/C) the solution was applied to the column (2.2×93.5 cm) and fractionated with 0.5 M-NaCl at a flow rate of 4.3 m*l*/hr.
Refer to the foot-note in Fig. 2 for further details.

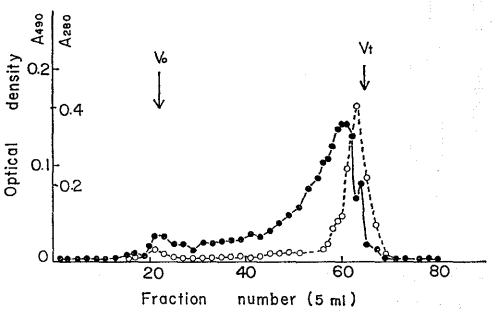

Fig. 5. Sepharose 2B column chromatography of the crude extract.
The crude extract (20.6 mg) was dissolved in 5 m*l* of 0.5 M-NaCl. After filtration (Whatman GF/C), the solution was applied to the column (2.2×82.0 cm) and fractionated with 0.5 M-NaCl at a flow rate of 4.0 m*l*/hr.
Refer to the foot-note in Fig. 2 for further details.

and V_t. This result shows that the molecular weight of the main fraction of the polysaccharide was approximately 2×10^6.

The elution pattern of the column chromatography on Sepharose 2B was shown in Fig. 5 which showed that almost all polysaccharides were eluted near V_t. This result also indicates that the polysaccharide has its molecular weight of 10^6.

The above mentioned results suggested that the crude extract was composed of the polysaccharides of wide varieties of the molecular weight, and a major component has its molecular weight of 10^6.

Analysis of the Constituent Monosaccharide

After the crude extracts were acid-hydrolyzed

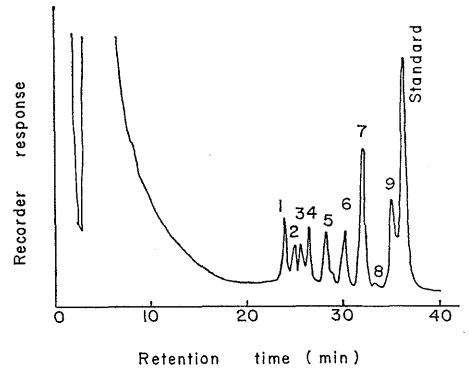

Fig. 6. Gas chromatography of acid hydrolyzate of the crude extract.
The details were described in the text.

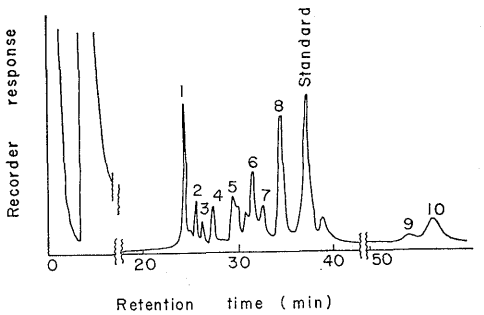

Fig. 7. Gas chromatography of methanolyzate of the crude extract.
The details were described in the text.

and methanolyzed, the constituent monosaccharides were analyzed with gas chromatography.

Fig. 6 shows the gas chromatogram of TMS-sugars obtained with acid hydrolyzate. Relative Rt was estimated by using glucurono-lactone as the standard. Each peaks was identified by comparing the relative Rt of the sample to that of authentic sugar. The result was as follows: 1. L-arabinose, 2. L-rhamnose, 3. unidentified, 4. D-xylose, 5. D-mannose, 6. D-galactose, 7. D-glucose, 8. D-glucosamine, 9. unidentified.

Fig. 7 shows the gas chromatogram of TMS-sugars obtained with methanolysis. Each peaks was identified by the same procedure as in acid-hydrolysis experiment. The result was as follows: 5. D-mannose (α), 8. D-glucose (α, β), 9. D-galactosamine (α), 10. D-glucosamine (α), 1, 2, 3, 4, 6, 7. unidentified.

Gas chromatographic peaks indicated in Figs. 6 and 7 were also identified by the method of authentic sugar addition. The identification procedure of unidentified peaks is now undertaken.

Uronic Acid and Sialic Acid

The presence of uronic acid in the extract was positive on the carbazol reaction but that of sialic acid was negative on the orcinol-HCl reaction.

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