Purification and Characterization of the Polysaccharide Obtained from Squid Internal Shell^{*1}

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Polysaccharides A and B were isolated in pure form from the squid internal shell. The purification of the polysaccharide was carried out by using DEAE-cellulose column chromatography. These purified products were homogeneous when examined by gel-filtrations and ultracentrifugations.

The distribution constant values (K_d) of these purified polysaccharides, calculated by the Sepharose 4B column chromatography, were $K_d=0.90$ for polysaccharide A and $K_d=0.79$ for polysaccharide B. Approximate sedimentation coefficients were 1.96S for polysaccharide A and 3.20S for polysaccharide B. The infrared spectra of these purified polysaccharides A and B likewise indicated the presence of β -linkages (890 cm^{-1}). Both polysaccharides were composed of glucose as the sole constituent monosaccharide.

In the preceding paper,¹⁾ we reported the isolation procedure and the molecular weight distribution of the polysaccharide fraction from squid internal shell.

It has been shown that the polysaccharide contains residues of L-arabinose, L-rhamnose, D-xylose, D-mannose, D-galactose, D-glucose, D-glucosamine and D-galactosamine as constituent monosaccharides.

In the present experiments, a method is described for the isolation of homogeneous polysaccharides from squid internal shell using DEAE-cellulose column chromatography.

Purified polysaccharides prepared by this method were then characterized as to its physical and chemical composition.

Materials and Methods

Preparation of the Crude Extract

The crude polysaccharide extract was prepared from acetone-extracted squid internal shell by treatment with 0.01 M-EDTA solution as described in the previous paper.¹⁻²⁾

Ion-Exchange and Gel-Filtration Chromatographies

Ion-exchange chromatography was carried out on a column of DEAE-cellulose (Serva) and eluted with a stepwise gradient of NaCl.

Gel-filtration was done by using Sepharose 4B (Pharmacia). Elution was carried out with 0.5 M-NaCl solution. Void volume (V_0) and total volume ($V_t = V_0 + \text{internal volume}$) of the column were estimated with blue dextran 2000 and uronic acid respectively.

Fractions of eluate were analyzed, as necessary, for total carbohydrate and protein. Total carbohydrate was determined by phenol-sulphuric acid method (OD at 490 nm). Protein was determined by the UV absorption method (OD at 280 nm).

Elementary Analysis

The determinations of C, H and N were carried out by using CHN corder (Yanagimoto model MT-2).

Ultracentrifugal Analysis

Solution for ultracentrifugal characterization was prepared by dissolving purified polysaccharide in 0.1 M-NaCl solution. Sedimentation pattern was made on this solution with a Hitachi model 282 analytical ultracentrifuge.

Infrared Spectrum (IR)

The IR spectrum was obtained using the KBr pellet technique in a Hitachi model EPI-G2 spectrometer.

Monosaccharide Analysis

After the polysaccharide was hydrolyzed with

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2N-HCl and methanolized with 5% methanol-HCl, the constituent monosaccharides were analyzed with GLC as described in the previous paper.¹¹

Gas Chromatography (GLC)

Analysis was performed with a Nihon Denshi model JGC-750 equipped with dual flame ionization detectors, using stainless steel columns (0.3×300 cm), packed with 3% OV-17 on 80-100 mesh Shimalite (Shimadzu). The carrier gas was nitrogen. Sample was introduced at 100°C and the temperature was held for 2 min. The column temperature was programmed to rise to a maximum of 230°C at a rate of 6°C/min after the hold interval. The final temperature was maintained until all peaks were eluted.

Results and Discussion

The fractionation of the crude extract on DEAE-cellulose (Cl^-) was shown in Fig. 1. Three major peaks of the polysaccharide were separated. These peaks were pooled in three fractions (Fraction I, II and III) which were eluted with 0.01, 0.1 and 0.5 M-NaCl solutions respectively. These pools were complex mixtures of polysaccharides and were further fractionated by DEAE-cellulose (OH^-) column chromatography. Studies on Fraction-I were described in the present paper.

Fraction-I gave two peaks on DEAE-cellulose (OH^-) column chromatography (Fig. 2). The first peak (polysaccharide A) was eluted with water

Fig. 1. DEAE-cellulose column chromatography of the crude extract.

The crude extract (327 mg) was dissolved in 100 ml of 0.01 M-NaCl solution and applied to a column (3.8×80 cm) of DEAE-cellulose (Cl^-) equilibrated with 0.01 M-NaCl solution. Elution was performed with 0.01 M, 0.1 M, 0.5 M and 1 M-NaCl solution stepwisely at a flow rate of 2.5 ml/min. Fractions of 50 ml were collected and assayed for total carbohydrate (●—●) and for protein (○—○).

and the second peak (polysaccharide B) was eluted with 0.01 M-NaCl solution. These peaks were separately passed through Sepharose 4B columns and each was eluted as a single sharp peak (Figs. 3 and 4). The distribution constant (K_d) values⁸¹ of these purified polysaccharides were calculated by the Sepharose 4B gel-filtration analysis. The chromatograms suggested $K_d=0.90$ for polysaccharide A, and $K_d=0.79$ for polysaccharide B. It is assumed that the conformation of polysaccharide A is less expanded than that of polysaccharide B.

The ultracentrifugal patterns of these polysaccharides indicated the presence of one com-

Fig. 2. DEAE-cellulose column chromatography of Fraction I.

Fraction I substance (15.4 mg) was dissolved in 5 ml of water and applied to a column (2.2×27.5 cm) of DEAE-cellulose (OH^-) equilibrated with water. Elution was performed with water, 0.01 M and 0.1 M-NaCl solutions stepwisely at a flow rate of 1.0 ml/min. Fractions of 5 ml were collected and assayed for total carbohydrate.

Fig. 3. Sepharose 4B column chromatography of polysaccharide A.

Fraction A (6.5 mg) in 2 ml of 0.5 M-NaCl solution was applied to a column (2.3×90 cm) of Sepharose 4B and eluted with 0.5 M-NaCl solution at a flow rate of 5 ml/h. Fractions of 5 ml were collected and assayed for total carbohydrate.

Fig. 4. Sepharose 4B column chromatography of polysaccharide B.

Fraction B (8.0 mg) in 3 ml of 0.5 M-NaCl solution was applied to a column of Sepharose 4B (2.2×87 cm) and eluted with 0.5 M-NaCl solution at a flow rate of 9.1 ml/h. Fractions of 5 ml were collected and assayed for total carbohydrate.

ponent each other, and schlieren patterns of these polysaccharides showed symmetric peaks (Fig. 5). Approximate sedimentation coefficients were calculated by the standard method.⁴¹ The values were 1.96S for polysaccharide A and 3.20S for polysaccharide B. This indicates that polysaccharide A is relatively low density as compared with polysaccharide B. The analytical data thus provide some support for proposal that polysaccharide A is relatively low molecular weight as compared with polysaccharide B.

IR spectra of these polysaccharides are shown in Fig. 6. IR absorption bands between 750–900 cm^{-1} are useful for the analysis of glycosidic linkage of polysaccharide. IR spectra of these purified polysaccharide A and B likewise indicate the presence of β -linkages (890 cm^{-1}) in these substances. IR absorption band at 1610 cm^{-1}

Fig. 5. Ultracentrifugal patterns for purified polysaccharides.

Sedimentation patterns were obtained at various times after the rotor had reached at maximum speed of 60,000 rpm at 25°C in 0.1 M-NaCl solution. Sedimentation is from right to left. The bar angles were indicated in the figure.

A Polysaccharide A; 37.0 mg/ml.

B Polysaccharide B; 13.4 mg/ml.

Fig. 6. Infrared spectra of purified polysaccharides. A Polysaccharide A, B Polysaccharide B.

Fig. 7. Gas chromatographic patterns of TMS-sugars from acid hydrolyzates of purified polysaccharides.

A Polysaccharide A, B Polysaccharide B.

Arrows indicate the peak of gluculono-lactone used as the reference.

Purified polysaccharide (10.0 mg) was hydrolyzed with 2 N-HCl (4 ml) at 100°C for 2h. The hydrolyzate was neutralized with AgCO₃.

suggests the presence of carboxylate in both polysaccharides. The C=O stretching band (1725 cm⁻¹) was observed in polysaccharide A. This indicates the possibility of the presence of acetyl or other acyl groups in polysaccharide A.

Chemical analyses showed that polysaccharide A contained 39.4% C, 5.6% H, 0.6% N and 5.5% ash whereas polysaccharide B contained 39.7% C, 5.9% H, 0.5% N and 4.6% ash. The analytical data indicate that these purified polysaccharides have no amino sugar as constituent sugars.

As shown in Fig. 7, chromatograms for polysaccharide A and B consisted two major peaks on acid hydrolysis.

The retention times (Rt) were relative to gluculonolactone used as the reference. The peaks with Rt 0.89 and Rt 0.97 are characteristics of α -glucose and β -glucose respectively.

As shown in Fig. 8, one major and one minor peaks were detected on methanolysis. The major peak with Rt 0.93 is characteristic of methylglucoside. A minor peak is assumed to be the

Fig. 8. Gas chromatographic patterns of TMS-sugars from methanolizates of purified polysaccharides.

A Polysaccharide A, B Polysaccharide B.

Arrows indicate the peak of gluculono-lactone used as the reference.

Purified polysaccharide (10.0 mg) was methanolized with 5% methanol-HCl (4 ml) at 120°C for 1 h. The methanolizate was neutralized with AgCO₃.

isomer of methyl-glucoside.

Although the adopted hydrolysis or methanolysis procedures compromise, which did not result in the quantitative release of various carbohydrate constituents of the polysaccharide, it seems to permit the release of monosaccharide such as aminosugar, neutral sugar and uronic acid from polysaccharide.

The analytical data for polysaccharide A and B suggest that these polysaccharides contain only glucose residue. However, the possibility that these polysaccharides might contain subfractions other than sugars corresponding to side-chain materials cannot be eliminated.

The structural determinations of these purified polysaccharides are presently in progress in our laboratory.

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