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ORIGINAL PAPER

# Antioxidant activity of sulphated polysaccharide conjugates from abalone (*Haliotis discus hannai* Ino)

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**Abstract** The water-soluble sulphated polysaccharide conjugates were obtained from abalone viscera (*Haliotis discus hannai* Ino) by alkaline protease extraction followed by ethanol precipitation. Their antioxidant activities were evaluated in vitro by hydroxyl radicals scavenging activity, reducing power and metal chelating activity. Those various antioxidant activities were compared to standard antioxidants ascorbic acid and EDTA. The experimental results indicated that the crude extract having notable hydroxyl free radicals scavenging activity and moderate reducing power and chelating potency. The crude sulphated polysaccharide conjugates was enzymatically hydrolyzed by five commercially available proteases (trypsin, vernase, neutrase, pepsin and papain), and the resultant digests were tested for their antioxidant activities. Those proteolytic hydrolysates, although improving the hydroxyl radical scavenging activity in all cases except one, had lower reducing power and 3–15 times lower chelating ability than the native extract. Product derived from pepsin hydrolysate was fractionated by gel-filtration chromatography with sephadex G-100, giving two fractions containing sulphated polysaccharide conjugates termed ACP I and ACP II. The neutral monosaccharide composition of ACP I is rhamnose, fucose, xylose, mannose, galactose and glucose in a molar ratio of 1.00:45.14:4.00:5.36:33.18:2.15, with an average molecular weight of about 271 kDa. The neutral monosaccharide composition of ACP II is rhamnose, fucose, xylose, mannose, galactose and glucose in a molar ratio of 1.00:12.51:1.33:4.98:16.08:1.46, with an average molecular weight of about 6 kDa.

**Keywords** Abalone viscera (*Haliotis discus hannai* Ino) · Sulphated polysaccharide conjugates · Purification · Antioxidant activity · Protease

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## Introduction

Reactive oxygen species (ROS) have been implicated in the etiology of many diseases, including inflammatory disease, cancer, diabetes and ageing [1, 2]. In order to reduce the oxidative damage of ROS, many synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, *tert*-butyl-hydroquinone and propyl gallate are used widely at present. However, the applications of them are restricted due to potential hazards related to health [3, 4]. Therefore, enhancement of body's antioxidant defenses through natural and safe antioxidants would seem to provide a reasonable and practical approach to reduce the level of oxidative stress and retard the progress of many chronic diseases [5]. Published data indicate that natural polysaccharides and their conjugates in general have strong antioxidant activities and can be explored as novel potential antioxidants [6, 7].

The abalone (*Haliotis discus hannai* Ino) is large, single-shelled marine mollusks of genus *Haliotis* [8]. As an economically important food resource, abalones are widely cultured in China. Up to now, no investigation has been carried out on polysaccharides from abalone that may

possess the antioxidant activities. In this study, the antioxidant activities of crude sulphated polysaccharide conjugates (CPC) from the female abalone viscera were evaluated in vitro by hydroxyl radicals scavenging activity, reducing power and metal chelating activity. Literature reported that the bioactivity of polysaccharide conjugates depends on their chemical composition [9, 10]. To investigate whether there were some relationships between the chemical compositions and the bioactivities of abalone sulphated polysaccharide conjugates, five commercially available proteases (trypsin, vernase, neutrase, pepsin and papain) were used to hydrolyze the CPC and the digests with different chemical compositions were produced. The in vitro antioxidant activity of those hydrolysates was evaluated by the antioxidant assays described above and the effects of chemical compositions of abalone polysaccharide conjugates on their antioxidant activities were statistically analyzed by Bivariate Correlation. The typical polysaccharides from one proteolytic digest were purified by gel filtration and the neutral monosaccharide composition and molecular weight of which were determined.

## Materials and methods

### Materials

The viscera of female abalone were supplied by Dalian Zhangzidao Group Co. (Dalian, China), which was vacuum freeze-dried and crushed, and the powder was stored at 4 °C before used. Trypsin, vernase, neutrase and pepsin were purchased from Sanland Chemicals Co. (Xiamen, China). Papain and alkaline protease were purchased from Sangon biotechnology Co. (Shanghai, China). Ferrozine and bovine serum albumin (BSA) were purchased from Fluka Chemie Co. (Buchs, Switzerland). Trifluoroacetic acid (TFA) and the standard monosaccharides were purchased from Pharmacia Co. (Uppsala, Swenden). Dextrans of different molecular weights were purchased from Pharmacia Co. (Uppsala, Sweden). Sephadex G-100 was purchased from Pharmacia Co. (Uppsala, Swenden). DEAE Sepharose CL-6B was purchased from Amersham Co. (Uppsala, Swenden).

### Preparation and isolation of sulphated polysaccharide conjugates from abalone viscera

Two hundred grams of viscera powder was dissolved in 3,000 mL Borax-NaOH buffer (pH 10.0) containing 1.0 g alkaline protease. After incubating at 45 °C for 3 h, the mixture was boiled for 10 min to inactive enzyme, adjusted to neutral and centrifuged at 1,400 × *g* for 10 min. The supernatant was precipitated with 70% (v/v) ethanol at 4 °C for 12 h, and centrifuged at 1,400 × *g* for 10 min. After washing with deionized water for three times, the precipitate was vacuum freeze-dried, and 36.32 g CPC was obtained.

Crude sulphated polysaccharide conjugates was hydrolyzed by different proteases to remove protein content. Two grams of CPC was dissolved in 100 mL buffer containing 8,000 U different proteases (see Table 1). After 3 h of incubation at corresponding temperature, the reaction was terminated by boiling at 100 °C for 10 min. The hydrolysate was adjusted to neutral and centrifuged at 1,400 × *g* for 10 min. The supernatant was collected and precipitated with 70% (v/v) ethanol at 4 °C for 12 h. After washing with deionized water for three times, the precipitate was vacuum freeze-dried, and then the deproteinized sulphated polysaccharide conjugates (DPC) was obtained.

Two hundred and fifty milligrams of DPC derived from pepsin hydrolysate was dissolved in 20 mL 0.9% NaCl, and applied to sephadex G-100 column (2.5 cm × 80 cm) previously equilibrated with the 0.9% NaCl. Elution was performed with 0.9% NaCl at a flow rate of 30 mL/h, monitoring by sulfuric acid–phenol method. Two polysaccharide conjugate fractions (6 mL per fraction) coded ACP I and ACP II were pooled, dialyzed by 1000 Dalton membrane to remove salts, and vacuum freeze-dried for further experiments.

### Component analysis

Total sugars of the samples were determined by the phenol–sulfuric acid method, using glucose as the standard [11]. The protein contents of the samples were measured according to Lowry's method, using BSA as the standard [12]. Estimation of sulphate by modified barium chloride method and IR spectroscopy were carried out as described before, using kalium sulphate as standard [13]. The neutral monosaccharide composition of the sulphated polysaccharide conjugates was determined according the method described earlier [14]. Briefly, sample (5.0 mg) was hydrolyzed with 2 M TFA (1 mL) at 120 °C for 2 h in a sealed tube. After that, the removal of the excess amount of TFA was accomplished by co-evaporation at reduced pressure

**Table 1** Proteases hydrolysis conditions of CPC

| Enzymes | Buffer (0.2 M) | pH | Reaction temperature (°C) |
|---|---|---|---|
| Vernase | Citric acid–sodium citrate | 3.0 | 40 |
| Neturase | PBS | 7.0 | 50 |
| Papain | PBS | 7.0 | 65 |
| Trypsin | Tris–HCl | 8.0 | 37 |
| Pepsin | KCl–HCl | 2.0 | 37 |

with ethyl alcohol added after reaction. The subsequent treatment of the resultant dry hydrolysate with acetic anhydride and pyridine afforded the corresponding alditol acetate which was analyzed by a Agilent 6890 N gas chromatography (Agilent, USA) fitted with a flame ionization detector and a 112-88A7 HP-88 capillary column (100 m × 0.25 mm, 0.2 μm). The analytical conditions were 3 min at 180 °C, from 180 to 230 °C at 10 °C/min and held for 20 min at 230 °C, from 230 to 240 °C at 5 °C/min and held for 20 min at 240 °C and from 240 to 250 °C at 5 °C/min and held for 5 min at 250 °C.

### Determination of molecular weight

The polysaccharide conjugate (5.0 mg) was dissolved in 1.0 mL deionized water to 5 g/L solutions, applied to a gel-filtration chromatographic column of Sepharose CL-6B column (1.0 cm × 86 cm), eluted with NaCl (0.9%, w/w) solution at a flow rate of 5 mL/h, monitoring by sulfuric acid–phenol method. Preliminary calibration of the column was conducted using dextrans of different molecular weight.

### Determination of scavenging effect on hydroxyl radicals

Hydroxyl radical-scavenging activity was measured according to the method described before [15], with slightly modification. Briefly, the reaction mixture contained 1 mL phosphate buffer saline (pH 7.4, 0.15 mM), 0.2 mL safranine O (1.5 M), 1.0 mL EDTA-Fe(II) (6 mM), $H_2O_2$ (3%, v/v) and samples of varying concentrations. After incubating at 40 °C for 30 min, the absorbance of mixture was measured at 520 nm. The hydroxyl radical-scavenging activity was expressed as: scavenging activity (%) = $[(As - A_0)/(A - A_0)] \times 100\%$. Where As is the absorbance of the reaction mixture with sample, $A_0$ is the absorbance of the blank control and $A$ is the absorbance of the reaction mixture without sample. The $EC_{50}$ value (mg extract/mL) is the effective concentration at which the hydroxyl radicals were scavenged by 50%. Ascorbic acid (Vc) was used for a comparison.

### Determination of reducing power

The reducing power of the samples was evaluated according to the method described before [16], with slightly modification. Reaction mixtures contained 1.0 mL phosphate buffer saline (pH 6.6, 0.2 M), 1.0 mL potassium ferricyanide [$K_3Fe(CN)_6$] (1%, w/v) and samples of varying concentrations. After incubating at 50 °C for 20 min, 1.0 mL of trichloroacetic acid (10%, w/v) was added to the mixture, and then centrifuged at 1,400 × $g$ for 10 min. 2.5 mL upper layer of solution was collected and mixed with 2.5 mL deionized water and 0.5 mL $FeCl_3$ (0.1%, w/v). After incubating at room temperature for 10 min, the absorbance of mixture was measured at 700 nm. Higher absorbance of the reaction mixture indicated greater reductive potential. Vc was used for a comparison.

### Determination of $Fe^{2+}$-chelating ability

The $Fe^{2+}$-chelating ability of the samples was evaluated by the method of Dinis et al. [17]. The $Fe^{2+}$ was monitored by measuring the formation of ferrous iron–ferrozine complex. The reaction mixture contained 0.05 mL $FeCl_2$ (2 mM), 0.1 mL ferrozine (5 mM) and samples of varying concentrations. After shaking, the mixture was stored at room temperature for 10 min. The absorbance of the resulting solution was measured at 562 nm. The $Fe^{2+}$-chelating capability was expressed as: chelating ability (%) = $(1 - As/A) \times 100\%$. As is the absorbance of the reaction mixture with sample, $A$ is the absorbance of the reaction mixture without sample. A lower absorbance indicates a higher chelating power. The $EC_{50}$ value (mg extract/mL) is the effective concentration at which ferrous ions were chelated by 50%. EDTA was used for comparison.

### Statistical analysis

All the data were expressed as mean ± standard deviation of three replicates. Student's $t$-test and Bivariate Correlation were used for the statistical analysis. The values were considered to be significantly different when the $P$-value was less than 0.05.

## Results and discussion

### Antioxidant activity of CPC

Natural polysaccharides do not always exist singly in plants, animals and microorganisms, but conjugate with other components, including protein, lipid and nucleic acid, etc. CPC was obtained from abalone viscera as a water-soluble extract by alkaline protease extraction. Through the extraction, the non-saccharide components were removed partly by chemical and enzymatic methods, and the sulphated polysaccharide conjugates were the main chemical components of CPC. There were 31.9% of carbohydrate, 18.6% of protein, 8.3% of sulphate and low abundance of fatty acids, lipoproteins and heavy metals in this extract. From the result we can see, the polysaccharides extracted contain sulphate groups. Indeed, the IR spectrum of this extract shows a band at 1,258/cm related to the > S = O stretching of the sulphate group [18]. Another band at 848/cm arising from secondary equatorial sulphate groups of

polysaccharides was also detected (see Fig. 1). In addition, the enzymatic hydrolysis techniques used could not remove the peptide chain bound to polysaccharides completely. The antioxidant activity of CPC was evaluated in vitro by hydroxyl radicals scavenging activity, reducing power and metal chelating activity

## Hydroxyl radicals scavenging ability

As shown in Fig. 2a, CPC was found to have the ability of scavenging hydroxyl radicals at concentrations between 0.4 and 2.0 mg/mL. The scavenging effects of CPC increased with increasing concentration. The $EC_{50}$ value of CPC for hydroxyl radicals scavenging activity was 1.44 mg/mL, which has no significant difference from scavenging effects of Vc (1.35 mg/mL). Wang and Luo reported that the scavenging abilities on hydroxyl radical of polysaccharide conjugate fractions from *Gynostemma pentaphyllum Makino* were about between 25 and 57% at 0.8 mg/mL [19]. In this study, the hydroxyl radical scavenging activity was 29.1% at 0.8 mg/mL, which was similar to that of report.

## Reducing power

Crude sulphated polysaccharide conjugates exhibited reducing power at all concentration points. The reducing capacity of the CPC increased with increasing concentration. However, as shown in Fig. 3a, the reducing capacity of CPC was lower than that of Vc. Tseng et al. reported that the hot water extracted and hot alkali extracted polysaccharides from mycelia showed reducing powers of 0.41–0.52 (absorbance at 700 nm) at 20 mg/mL [20]. In this study, the reducing power of CPC from abalone was 0.59 (absorbance at 700 nm) at 18 mg/mL, which were slightly higher than that of report.

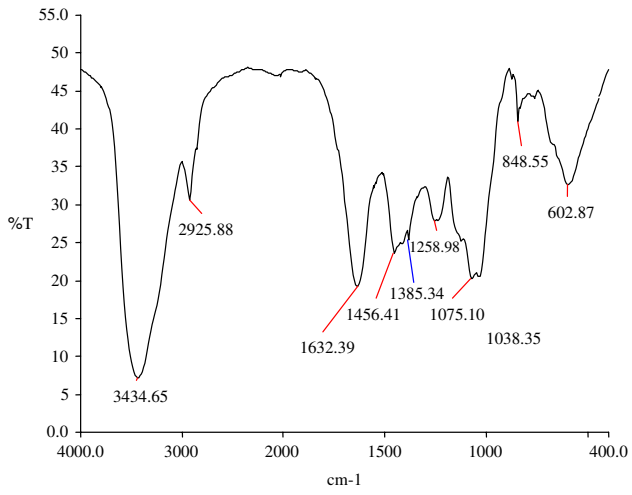


**Fig. 1** IR spectrum of CPC

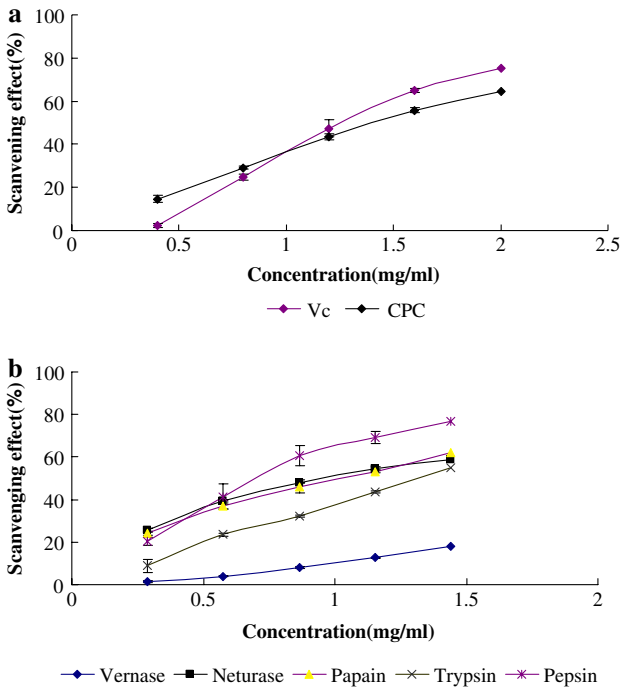


**Fig. 2** Scavenging effects of CPC (**a**) and DPCs (**b**) on hydroxyl radicals

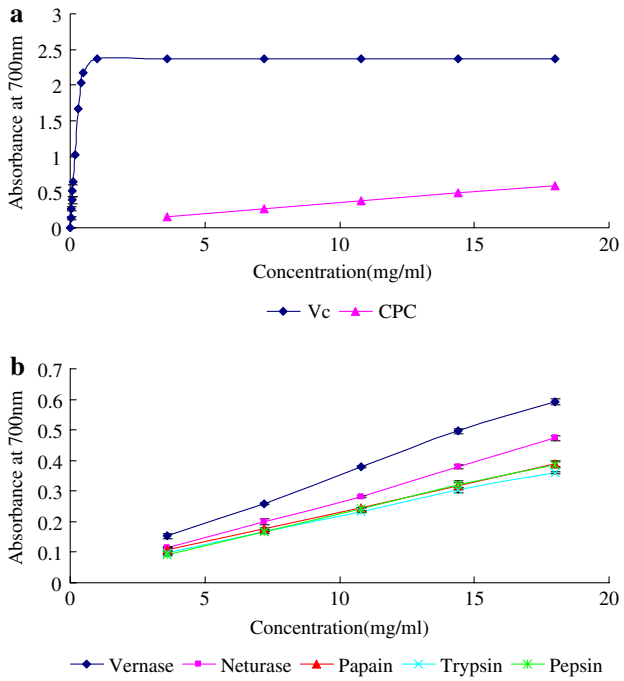


**Fig. 3** Reducing powers of CPC (**a**) and DPCs (**b**)

## Chelating ability

Crude sulphated polysaccharide conjugates showed chelating ability at concentrations between 25 and 125 μg/mL. The chelating ability of CPC increased with increasing

concentration. As shown in Fig. 4a, the chelating activity of CPC was ten times lower than that of EDTA. Tseng et al. reported that at 5 mg/mL, chelating ability of polysaccharides from mature and baby Ling chih were 73.0–75.6%, whereas chelating abilities of polysaccharides from mycelia and filtrate were 50.8–68.1% and 41.1–47.5%, respectively [20]. Meanwhile, Li et al. reported that chelating ability of polysaccharides from *Lycium barbarum* fruits was 89.7% at 250 μg/mL [21]. In this study, the chelating ability of CPC was 94.7% when the concentration was 100 μg/mL, which was obviously higher than those of reports.

## Antioxidant activity of DPCs

Literature reported that chemical composition of polysaccharide conjugates could play an important role in the bioactivity [9, 10]. To obtain sulphated polysaccharide conjugates with different chemical composition, five commercial proteases were used to hydrolyze CPC, respectively. Fig. 5 shows that DPCs derived from different hydrolysates have different carbohydrate, protein and sulphate contents. The effects of chemical compositions on their antioxidant activities in vitro were evaluated. As shown in Figs. 2b, 3b and 4b, DPCs exhibited different hydroxyl radicals scavenging activities, reducing powers and chelating abilities. However, Bivariate Correlation analysis between antioxidant activities and chemical composition (the contents of polysaccharide, protein and sulphate) showed negative results, which means there were no significant relationship between them.

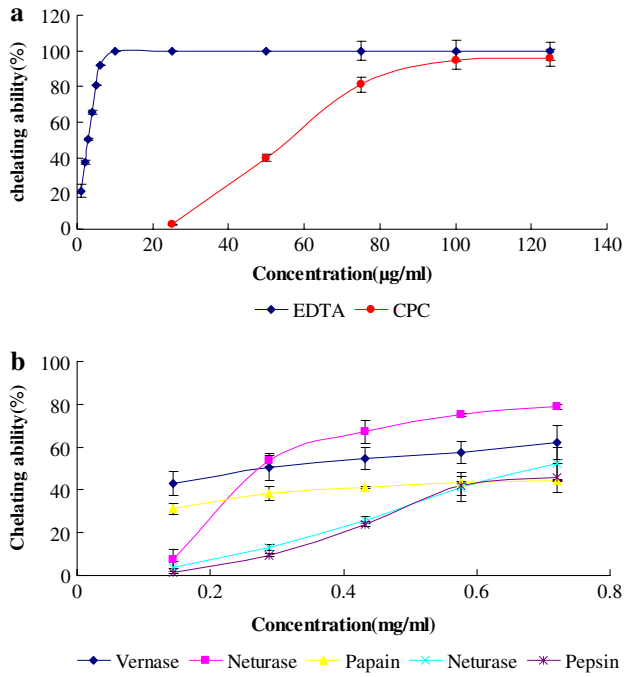


**Fig. 4** Chelating ability of CPC (**a**) and DPCs (**b**)

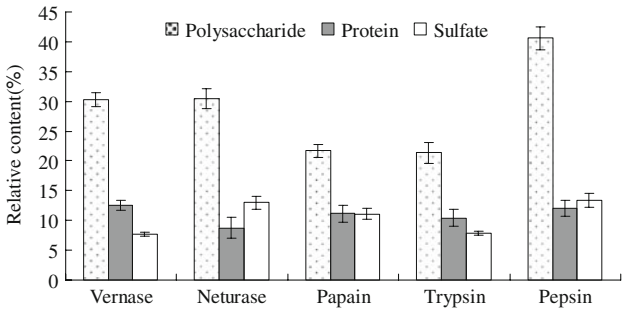


**Fig. 5** Carbohydrate and protein contents of DPCs derived from different protease hydrolysate

The data of antioxidant activities of DPC and CPCs were summarized in Table 2. As the table shows, except the hydroxyl radicals scavenging activities, nearly all DPCs demonstrated weaker antioxidant activities than CPC. The reason for such phenomenon could be that the crude extracts (CPC) were rich in some low molecular weight components having higher antioxidant activity, such as oligosaccharides, peptides, amino acids, fatty acids, lipoproteins and heavy metals, but which were removed partially during purification. Literature reported that the purified polysaccharide conjugates fractions exhibited less antioxidant activity than the crude extracts, which are similar with our results [19, 22].

## Chemical composition and molecular weight of the abalone viscera sulphated polysaccharide conjugates

Deproteinized sulphated polysaccharide conjugate derived from pepsin hydrolysate showed the highest carbohydrate content (see Fig. 5), which were fractionated by gel-filtration chromatography with sephadex G-100, ACP I and ACP II were obtained from NaCl elute, respectively (Fig. 6). The polysaccharide conjugate ACP I had a higher sulphated carbohydrate content (74.7% in ACP I and 60.0%

**Table 2** $EC_{50}$ values of sulphated polysaccharide conjugates from abalone in antioxidant properties

| | Scavenging activity ($EC_{50}$, mg/ml) | Reducing power[a] (A700 nm = 0.2, mg/ml) | Chelating ability ($EC_{50}$, mg/ml) |
|---|---|---|---|
| CPC | 1.43 ± 0.03 | 5.44 ± 0.07 | 0.05 ± 0.01 |
| DPC (vernase) | 3.38 ± 0.14 | 5.53 ± 0.09 | 0.34 ± 0.27 |
| DPC (neturase) | 0.87 ± 0.02 | 7.39 ± 0.15 | 0.28 ± 0.03 |
| DPC (papain) | 0.94 ± 0.04 | 8.71 ± 0.15 | 0.17 ± 0.02 |
| DPC (trypsin) | 1.32 ± 0.01 | 9.35 ± 0.15 | 0.75 ± 0.18 |
| DPC (pepsin) | 0.71 ± 0.03 | 8.95 ± 0.24 | 0.75 ± 0.04 |

[a] Reducing power value was used the sample concentration when the absorbance at 700 nm was 0.2. Results are presented as mean ± standard deviations ($n = 3$)

**Table 3** Monosaccharide composition and molar ratios of ACP I and ACP II

| | Rhamnose | Fucose | Xylose | Mannose | Galactose | Glucose |
|---|---|---|---|---|---|---|
| ACP I | 1.00 ± 0.03 | 45.14 ± 2.07 | 4.00 ± 0.16 | 5.36 ± 0.22 | 33.18 ± 1.29 | 2.15 ± 0.08 |
| ACP II | 1.00 ± 0.04 | 12.51 ± 0.58 | 1.33 ± 0.04 | 4.98 ± 0.22 | 16.08 ± 0.77 | 1.46 ± 0.06 |

Results are presented as mean ± standard deviations ($n = 3$)

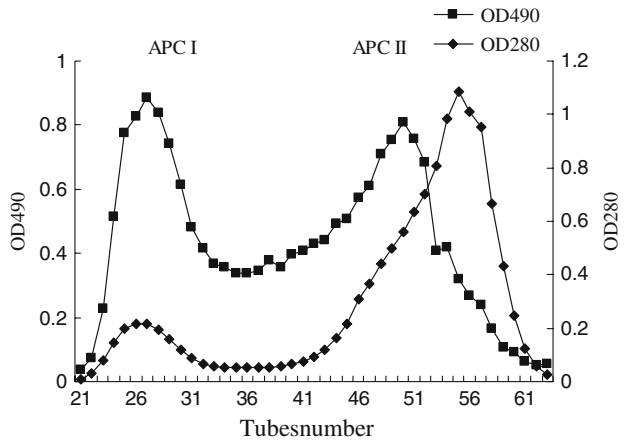


**Fig. 6** Sephadex G-100 column chromatogram of DPC derived from pepsin hydrolysate

in ACP II) but lower protein content (1.7% in ACP I and 15.6% in ACP II) than ACP II. According to GC analysis, ACP I and ACP II were composed of the same neutral monosaccharide with different molar ratios (see Table 3). The weight-average molecular weights were calculated to be 271 and 6 kDa for ACP I and ACP II, according to the calibration curve with standard dextrans.

## Conclusions

In this study, several in vitro assays were applied to evaluate the antioxidant potential of sulphated polysaccharide conjugates from abalone viscera. It was found that chemical composition of abalone sulphated polysaccharide conjugates could play an important role in the antioxidant activity. However, Bivariate Correlation analysis between antioxidant activity and carbohydrate, protein and sulphate content showed negative results. In addition, the experimental outcomes revealed that the purified sulphated polysaccharide conjugates exhibited less antioxidant activity than the crude extracts. Further structural analysis and evaluation of the bioactivities of the sulphated polysaccharide conjugates from the abalone will be important for their application in food fields.

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