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Isolation and Identification of Chondroitin Sulfates from the Mud Snail

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Chondroitin sulfates were isolated from the mud snail. For the quantitative analysis of enzymatic digestion products of isolated chondroitin sulfates, strong anion exchange-high performance liquid chromatography (SAX-HPLC) was performed. By the action of chondroitinase ABC, three unsaturated disaccharides 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-*O*S), 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi-6S) and 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔDi-4S) were produced from the mud snail chondroitin sulfates. The analysis showed that relative proportion of ΔDi-*O*S/ΔDi-6S/ΔDi-4S was 58.7/3.1/38.2. The immunomodulating activity of chondroitin sulfate was examined by cell proliferation assay and these results suggest that it might be a immunosuppressant.

Key words : Mud snail, *Cipangopaludina chinensis,* Chondroitin sulfates, Proteoglycan, Anion-exchange HPLC, Cell proliferation assay

INTRODUCTION

Chondroitin sulfates belong to the glycoaminoglycan (GAG) and occur in many vertebrates and invertebrate tissues as side chains of proteoglycans (Silbert and Sugumaran, 1995). Chondroitin sulfate is composed of repeating disaccharide units of glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc), and mainly bears sulfate groups at the 4- or 6-positions of the GalNAc residues. A chondroitin sulfate chain can often consist of various proportions of both 4-sulfated and 6-sulfated disaccharide repeats. Chondroitin sulfate is increasingly gaining attention as an important regulator of many biological process, such as cell migration, recognition, extracellular matrix deposition, and morphogenesis (Ruoslahti, 1989). In a recent study, chondroitin sulfate has been identified as a receptor for the *Plasmodium faciparum* infected erythrocytes (Rogerson *et al.*, 1995). Chondroitin sulfate is utilized as a medicine, food additive, cosmetic ingredient, health food and functional food. Recently Kim *et al.* (Kim *et al.*, 1996) isolated a unique glycoaminoglycan (GAG) from the giant african snail (*Achatina fulica*). Therefore, it could be important to determine the oligosaccharide composition of GAGs from the mud snail and to verify what kinds of GAGs are present in the mud snail. Other sulfated GAGs, heparan sulfates and dermatan sulfates are involved in the process of cell recognition and control of cell division (Nader *et al.*, 1984).

This paper reports the presence of chondroitin sulfate from the mud snail and the immunomodulating activity of the compound.

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MATERIALS AND METHODS

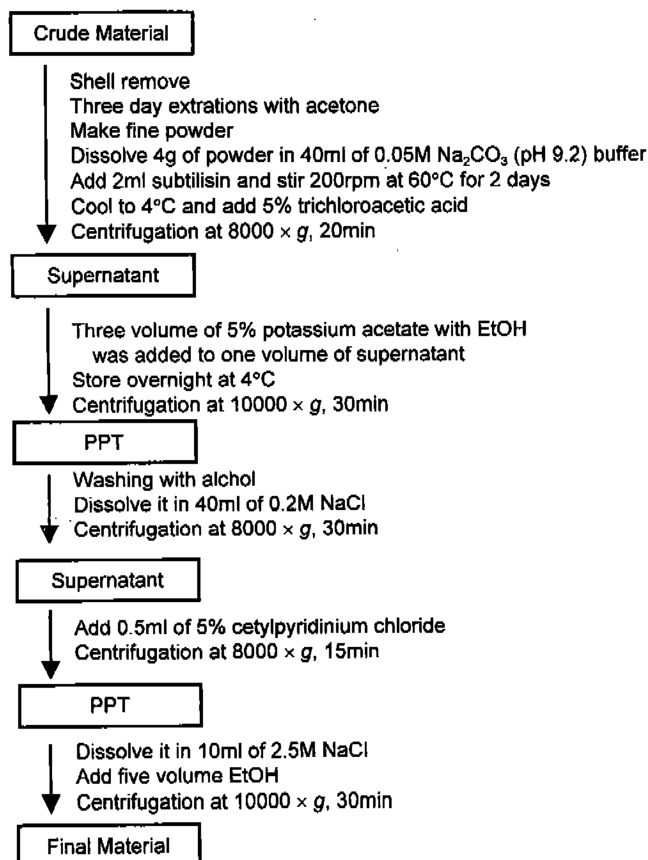
Materials

Mud snails (*Cipangopaludina chinensis*) were collected in Nonsan, Chungnam, Korea. Strong anion exchange HPLC (SAX-HPLC) was performed on a 5-μm Spherisorb 0.46×25 cm column from Phenomenex (Torells, CA, USA) using programmable pumps from Spectra Physics equipped with variable-wavelength UV detector and integrating recorder. Subtilisin and chondroitinase ABC were purchased from Sigma Co. (St. Louis, MO, USA). Standard unsaturated disaccharides 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-*O*S), 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔDi-4S) and 2-acetamide-2-deoxy-3-*O*-(β-D-

gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi-6S) were from Seikagaku (Tokyo, Japan). UV spectrometer (JASCO model V550) equipped with a thermostated cell was used. RPMI was purchased from GIBCO BRL (Grand Island, NY. USA).

Methods

Preparation of GAG: The GAG was prepared as shown in Scheme 1 (Kim *et al.*, 1996). The mud snail (*Cipangopaludina chinensis*) was defatted for 24 hr with acetone. This procedure was repeated three times. The powder of defatted snail (4 g) was suspended in 40 ml of 0.05 M sodium carbonate buffer (pH 9.2) containing 2 ml subtilisin and stirred for 48 hr at 60°C. The digestion mixture was cooled to 4°C, and trichloroacetic acid was added to a final concentration of 5%. The mixture was centrifuged for 20 min at 8,000× *g*. The recovered supernatant was mixed with three volumes of 5% potassium acetate in ethanol and allowed to stand at 4°C overnight. The precipitate was recovered by centrifugation for 30 min at 8,000× *g* and washed with absolute alcohol. The precipitate (1 g) was dissolved in 40 ml of 0.2 M NaCl. The insoluble material was removed by centrifugation for 30 min at 8,000× *g*. The supernatant was mixed with 0.5 ml of 5% cetylpyridinium chloride. After centrifugation the precipitate was dissolved in 10 ml of 2.5 M NaCl. To the above mixture 5 volumes of ethanol was added. The precipitate was collected by centrifugation for 30 min at 10,000× *g*. Uronic acid was analyzed by the modified carbazole assay (Kosakai and Yosizawa, 1979)



Scheme 1. Isolation of chondroitin sulfates from the mud snail.

Enzymatic digestion: GAG (0.1 mg) in 490 μl of 0.02 M sodium phosphate buffer (pH 8.0) was depolymerized by treating with 10 μl of 100 mU chondroitinase ABC at 37°C in the cuvette. The reaction was monitored by UV absorbance at 232 nm. The increase in UV absorbance is continually recorded as a function of time.

HPLC analysis of oligosaccharides: The composition of oligosaccharide produced from enzyme treatment was analyzed by SAX-HPLC (Linhardt *et al.*, 1988). The column (Spherisorb, 0.46×25 cm) was equilibrated with 5% of 2.0 M NaCl (Eluant 2, pH 3.5) in distilled water (Eluant 1, pH 3.5). After injection of the 10 μl volume of digestion mixture, the column was eluted isocratically at a flow rate of 1 ml/min with 5% of Eluant 2 in Eluant 1 for the first 5 min. For the next 50 min, the column was eluted with a linear gradient of Eluant 2 from 5% to 80%, monitoring $A_{232\ nm}$ at 0.01AUFS (absorbance unit at full scale). Each peak was identified by co-injection with disaccharide standard.

Cell proliferation assay: Specific pathogen-free female Balb/C mice (6~8 weeks old) were used. The spleen cells isolated aseptically were resuspended in RPMI 1640 complete media containing 5% fetal bovine serum, 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 15 mM HEPES, and 50 μM 2-mercaptoethanol. The cells (2×10^5 cells/well) were transferred to each well of 96-well culture plate and cultured in a humidified 5% CO_2 incubator at 37°C for 72 hr in the presence of chondroitin sulfates and either 100 μg/ml of lipopolysaccahride or 1.0 μg/ml of concanavallin A. The proliferation of spleen cells was measured spectrometrically by using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega, WI, USA) under the manufacturer's instruction.

RESULTS and DISCUSSION

Isolation of chondroitin sulfates from the mud snail

The GAG component of the mud snail was isolated by protease digestion of defatted tissue and fractional precipitation (Scheme 1). The extraction of GAGs was followed by carbazole assay. This assay demonstrated that sample contained uronic acid. The uronic acid content of the defatted mud snail powder was approximately 24%.

Enzymatic degradation of chondroitin sulfates

The depolymerization of proteoglycans using various lyases has been a useful method for the structural analysis of GAGs. To identify the oligosaccharide composition of prepared GAG, the sample was treated with chondroitinase ABC to produce unsaturated disaccharides (Fig. 1). The depolymerization of sample by chondroitinase ABC was followed by measuring the change in absorbance at 232 nm. Chondroitinase ABC is an eliminase and as a result, introduces a double bond between carbons-4 and 5 of the glucuronic acid. The conjugated system of double bonds created by this elimination produces a chromophore at 232 nm in the resultant disaccharides (Fig. 2). The complete disappearance of this GAG from 1% agarose gel electrophoresis after digestion with chondroitinase ABC indicated that this GAG was completely degraded to disaccharides (data not shown). The results that mud snail chondroitin sulfates are totally degraded by chondroitinase ABC lead to the suggestion that they contain chondroitin 4-, 6- and O- sulfates.

Characterization of unsaturated disaccharides

Analysis of unsaturated disaccharides produced by chondroitinase ABC digestion is performed by using SAX-HPLC (Fig. 3). Three disaccharides were observed in the mud snail chondroitin sulfate. The structures of unsaturated oligosaccharides were first assigned by comparing elution times with those of authentic standards and the major peaks were confirmed by the co-injection of oligosaccharide standards. The separation of disaccharides is mostly dependent on the number of sulfate charges. The peak I in Fig. 3 was co-eluted with ΔDi-*OS* and the peak II was co-eluted ΔDi-6S. The peak III was co-eluted with ΔDi-4S. Interestingly, ΔDi-4S was eluted earlier than ΔDi-6S. Contrasting with the analysis of molluscs chondroitin sulfates that

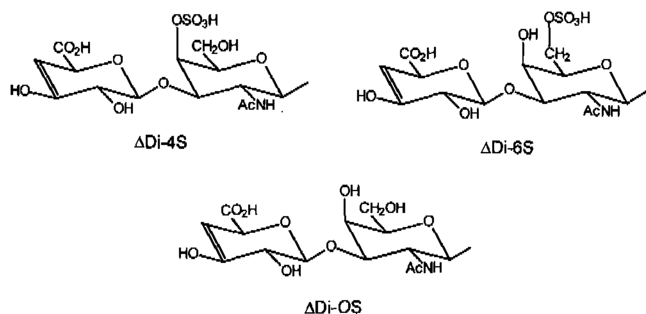


Fig. 1. Structures of unsaturated disaccharides formed from the mud snail GAG by the action of chondroitinase ABC.

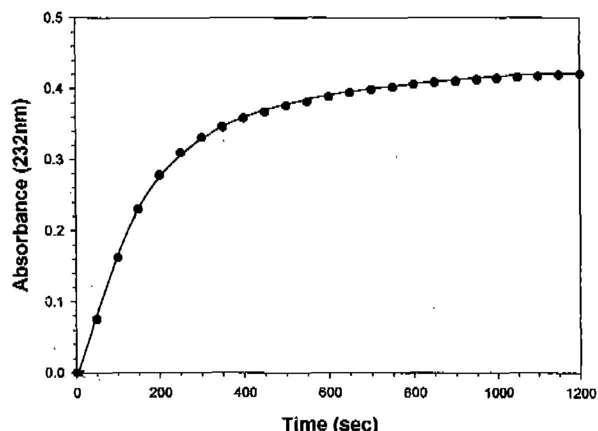


Fig. 2. Degradation of the mud snail GAG by chondroitinase ABC.

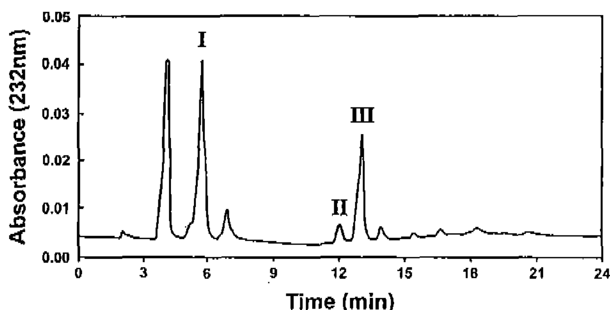


Fig. 3. The SAX-HPLC analysis of the mud snail GAG on treatment with chondroitinase ABC.

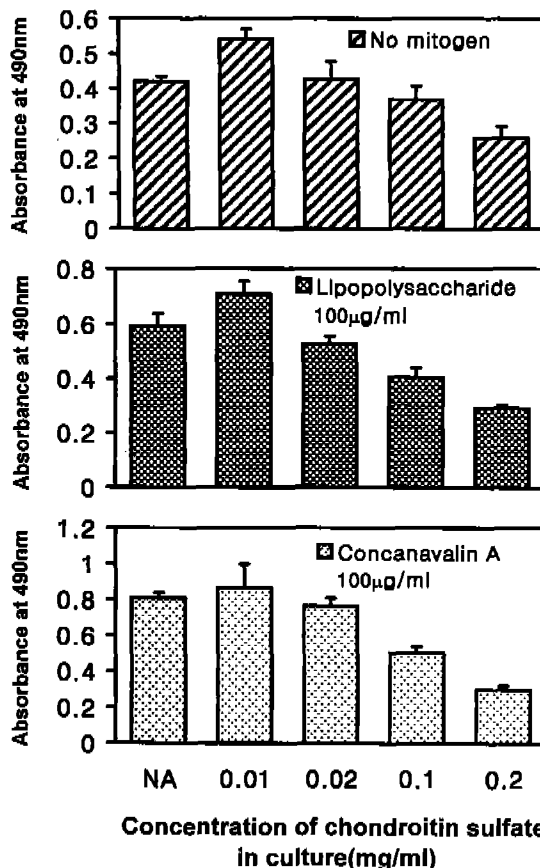


Fig. 4. Effect of chondroitin sulfate on cell proliferation. Values represent the mean±SD of quadruplicate.

showed ΔDi-OS present in approximately 2%, this analysis showed that significant amounts of ΔDi-OS were present in the mud snail chondroitin sulfate (Nader *et al.*, 1984). The relative proportion of ΔDi-OS:ΔDi-6S:ΔDi-4S was 58.7:3.1:38.2.

Cell proliferation assay

Fig. 4 shows the immunosuppressive effect of chondroitin sulfates from mud snail. When a B-cell mitogen, lipopolysaccharide, was added in the culture, chondroitin sulfate partially suppressed the lymphoproliferative response of spleen cells in a dose-dependent manner. Likewise chondroitin sulfate also suppressed in the lymphoproliferative response of spleen cells to a T-cell mitogen, concanavallin A, in a dose-dependent manner. Interestingly, a low concentration of chondroitin sulfate (0.01 mg/ml) slightly stimulated the proliferation. Taken together, chondroitin sulfate appeared to suppress both B-and T-cells in the present study.

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