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Separation and purification of low-molecular-weight chondroitin sulfates and their antioxidant properties

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Abstract

Low-molecular-weight chondroitin sulfate was obtained by degradation of chondroitin sulfate using hyaluronidase. Then, separated with sephadex G25, DEAE-52, and finally purified with AKATA superpeptide separation system and fluorescence-assisted carbohydrate electrophoresis. The main components detected by high performance gel-filtration chromatography were disaccharide, tetrasaccharide, hexasaccharide with molecular weight of 521, 1024 and 1527 Da, respectively. The anti-oxidant activity of these three oligosaccharides *in vitro* showed that the reducing power (maximum value at 10 mg/mL) and superoxide anion radical scavenging abilities were increased (maximum value at 4 mg/mL) with an increased in their concentration. There were no significant differences of the anti-oxidant properties between those three oligosaccharides.

Introduction

The applications of high-molecular-weight polysaccharides such as glycosaminoglycans are limited by their high molecular weight, high apparent viscosity, poor water solubility, as well as complex structures and conformations. Polysaccharides difficultly pass through organizational barriers to attach onto receptors in the cell interior (Liu et al., 2010). Regarding glycosaminoglycans, their major problem is poor bioavailability. Thus, low-molecular-weight glycosaminoglycans are attracted increasing attention particularly because of their biological activities. The activities of low-molecular-weight hyaluronic acid and heparin were discovered as early as in the 1990s, and extensive pertinent studies followed. However, reports on the activity of chondroitin sulfate are still limited.

Free-radical and active-oxygen generation contribute to tumor development, radiation-caused cancer, cardiovascular and cerebrovascular diseases, ischemia, organ reperfusion, drug poisoning, as well as the aging process (Valko et al., 2007). Glycosaminoglycans, particularly chondroitin-4-sulfate (C4S), possess anti-oxidant activity, ability to inhibit lipid peroxidation (Campo et al., 2008), and neuroprotective properties associated with its anti-oxidant and anti-inflammatory effects (Egea et al., 2010). High- and low-molecular-weight chondroitin sulfate have different or even opposite bioactivities.

Several studies have reported that chondroitin sulfate can relieve the pain and improve the functional status of osteoarthritis patients (McAlindon et al., 2000), and that chondroitin sulfate oligosaccharides generated with mammalian enzymes may possess pro-inflammatory potential (Jobe et al., 2003). Compared with high-molecular-weight chondroitin sulfate, the anti-oxidant properties of low-molecular-weight chondroitin sulfate is unclear, the main reason probably to be lack of separation method for low-molecular-weight chondroitin sulfate. A few literatures provided reference for low-molecular-weight chondroitin sulfate separation and purification.

The aim of the present study was to find an appropriate

way to separate and purify molecular-weight chondroitin sulfate and to determine its anti-oxidant activities *in vitro*.

Materials and Methods

Reagents and chemicals

C4S sodium salt from bovine trachea and bovine testicular hyaluronidase (437 U/mg; EC 3.2.1.35; Type I-S) were obtained from Sigma Chemical Co (China). All other chemicals and solvents used were of analytical grade.

Degradation of chondroitin sulfate

Chondroitin sulfate (200 mg) was digested with bovine testicular hyaluronidase (approximately 1.4×10^5 μL/L) in 10 mL of sodium phosphate buffer (5 mmol/L, pH 5.9) at 37°C for 22 hours. The reaction was stopped by boiling for 20 min. The sample was centrifuged at 4,000 rpm for 15 min, and the supernatant was recovered.

Separation of chondroitin sulfate degradation products by gel filtration

The supernatant was dialyzed using a dialysis bag (5000 Da) against deionized water for 48 hours, and the deionized water was changed every 4 hours. The sample outside the dialysis bag was concentrated, lyophilized, and stored at -20°C. About 1 mL of low-molecular-weight chondroitin sulfate (40 mg/mL) was fractionated on a sephadex G25 column (1.0 cm × 95 cm), using deionized water as an eluent at a flow rate of 4 mL/min. The eluate was automatically collected with a step collector (Redfrac 95) at 4 mL per tube. Analyses were performed by determining the uronic acid content in the eluate using the carbazole reaction method, and the same fraction was merged. After collection, each fraction was desalted on a sephadex G10 column (1.0 cm × 95 cm) against deionized water, and then lyophilized to gain samples.

Separation of chondroitin sulfate degradation products by ion exchange

The freeze dried sample was solved into ultrapure water as 10 mg/mL. 1 mL of the sample was injected after 2 hours balance of DEAE-52 using ultrapure water. Then grade elution the column using 0-1 mol/L of NaCl in 75 min, peaks were collected under UV detector at 210 nm.

Purification of low-molecular-weight chondroitin sulfate by preparative electrophoresis

The collected sample from DEAE-52 was then separated by preparative electrophoresis according to the method described by elsewhere (Platzer et al., 1999).

A solution: Boric acid 0.1 mol/L, Tris 0.1 mol/L, EDTA 0.01 mol/L, pH 8.3.

Spacer gel (5%): Acrylamide 4.75 g/100 mL, bisacrylamide 0.25 g/100 mL, adjust pH to 6.3 using HCl. Each 3 mL of the spacer gel needs 100 μL of 10% ammonium presulfate and 10 μL of N, N, N', N'-tetramethyl-ethylenediamine (TEMED).

Separation gel (35%): Acrylamide 31.82 g/100 mL, bisacrylamide 3.18 g/100 mL and sucrose 15 g/100 mL. Each 10 mL of the separation gel 200 μL of 10% ammonium presulfate, 20 μL of N, N, N', N'-tetramethyl-ethylenediamine.

The spacer gel and separation gel were all made up by A solution. Buffer solution: Glycine 1.25 mol/L，Tris 0.2 mol/L，pH 8.3. Electrophoresis condition: Room temperature, 400 V, 4-5 hours.

The gel was dyed using 0.5% of toluidine blue dissolved in 2% of acetic acid solution, and was decolored by 2% of acetic acid solution. The gel with sample was then cut off and mashed, and put into 1 mL of 0.1 mol/L PBS, shaking for 12 hours, triple repeats were carried out and the liquid supernatant was then desalting using sephadex G10 (1.6 × 150 cm).

Purification of low-molecular-weight chondroitin sulfate by superdex peptide separation system

The collect sample was then purified by AKTA purification system with superdex peptide 10/300 GL as separation column. The elution buffer was ultrapure water, flow speed was 0.5 mL/min. Peaks were collected using UV detector at 210 nm.

Purity identification of low-molecular-weight chondroitin sulfate by fluorescent auxiliary sugar electrophoresis

Separated sample was then fluorescence labeled by ANTS (8- naphthylamine-1, 3, 6- trisulfonic acid), the procedure was as following: 60 μL of sample was injected into solution containing 3.75 μL of ANTS (0.2 mol/L, solvent was HAc-H2O = 3:17) and 5 μL of DMSO with 1.0 mol/L of $NaBH_3CN$ were dissolved in the solution. The mixtures were blended and set in water bath (40°C) for 16 hours. Then samples were detected by gel electrophoresis as showed above.

Molecular weight determination of final samples by high-performance gel-filtration chromatography

The molecular weight of the final samples was determined by high-performance gel-filtration chromatography using an Ultrahydrogel™ linear column (300 mm × 7.8 mm i.d. × 2). The other conditions were as follows: Mobile phase, 0.1 mol/L $NaNO_3$; flow rate, 0.9 mL/min; column temperature, 45°C; and detector, 2410 refractive index detector. The standard curve (log molecular weight vs. retention time) was then drawn.

Assays for antioxidant properties in vitro

Estimation of O2-• scavenging activity

About 1 mL of chondroitin sulfate,low-molecular-weight chondroitin sulfate 1, and low-molecular-weight chondroitin sulfate 2 with different concentrations was added to 2.4 mL of Tris-HCl buffer solution (pH 8.2), followed by 0.3 mL of 8 mol/L pyrogallol solution. After 5 min, one drop of 10 mol/L HCl solution was added to terminate the reaction and the absorbance was measured at 325 nm. The percentage scavenging effect was calculated as follows:

$$\% O_2^{-}\bullet \text{ scavenged} = (A_0 - A_{325}) / A_0 \times 100$$

where A_0 and A_{325} are the absorbance of deionized water and the sample or ascorbic acid, respectively

Estimation of reducing power

Each sample in water (1 mL) was mixed with 2.5 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The reaction mixture was incubated at 50°C for 20 min, and then 2.5 mL of 10% trichloroacetic acid was added. The mixture was centrifuged at 4,000 rpm for 10 min. The upper layer (2.5 mL) was mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% ferric chloride. The absorbance was measured at 700 nm against a blank. Ascorbic acid was used as a reference.

Results

After degradation by hyaluronidase, two chondroitin sulfate degradation products (low-molecular-weight chondroitin sulfate 1 and low-molecular-weight chondroitin sulfate 2) were obtained by separation through dialysis and gel filtration. The low-molecular-weight chondroitin sulfate 1 and low-molecular-weight chondroitin sulfate 2 yields were 22.7 and 44.4%, respectively. Their weight-average molecular weights as determined by high-performance gel-filtration chromatography were 4875 and 1326 Da, respectively (Table I). Low-molecular-weight chondroitin sulfate 1 showed a significantly higher polydispersity index, indicated a wider molecular weight range and a greater polydispersity degree.

The preparation of low-molecular-weight glycosaminoglycans are gaining increased attention. Some types of glycosaminoglycans with low-molecular-weight can effectively inhibit free radicals (Trabucchi et al., 2002), prevent lipid peroxidation (Trommer et al., 2003), and protect against DNA damage (Zhao et al., 2008). However, the anti-oxidant properties of low-molecular-weight chondroitin sulfate were unclear. Low-molecular-weight chondroitin sulfate is a kind of acidic oligosaccharide, it can be absorbed by the solid phase in NH_2-column, C18-column and silicon column very strongly, that made it very hard to elution sample. Thus, common separation methods, such as high performance liquid chromatography, for oligosaccharide separation are not suitable for low-molecular-weight chondroitin sulfate separation. It is necessary to construct a novel and efficiency way for low-molecular-weight chondroitin sulfate separation to support further research and to expending the application scope.

Considering the characteristics of low-molecular-weight chondroitin sulfate, the anion exchange chromatograph was selected for further separation. The low-molecular-weight chondroitin sulfate 2 was further separated by DEAE-52 (Figure 1).

The molecular weights of those three components were then detected by high-performance gel-filtration chromatography as 521 Da, 1024 Da and 1527 Da, separately (Figure 2). This showed that disaccharide, tetrasaccharide and hexasaccharide were the main components after a series of separation steps. The purity of these three components was then identified by fluorescence assisted carbohydrate electrophoresis. There was only one belt existed showed that the low-molecular-weight chondroitin sulfate has purified and each belt represent one pure component.

Both $O_2^{-}\bullet$ and its derived free radicals are cytotoxic; they can inflict damage to DNA and cell membrane. In this study, the $O_2^{-}\bullet$ scavenging activity of low-molecular-weight chondroitin sulfate 1 and low-molecular-weight chondroitin sulfate 2 were shown in Figure 3.

The reducing power is generally associated with the hydrogen-donating ability (Shimada et al., 1992). Many studies have confirmed that the reducing power is an important indicator of anti-oxidant property (Choi et al., 2010), and that because of, the anti-oxidant property and reducing power are concentration dependent. In

Table I

The molecular weight characteristics of chondroitin sulfate degradations by gel filtration column

Sample	Molecular weight	Number-average molecular weight	Peak-average molecular weight	Mw/Mn
LMWCS1	4875	2988	2712	1.632
LMWCS2	1326	960	1078	1.381

Low-molecular-weight chondroitin sulfate 1 (LMWCS1); Low-molecular-weight chondroitin sulfate 2 (LMWCS2)

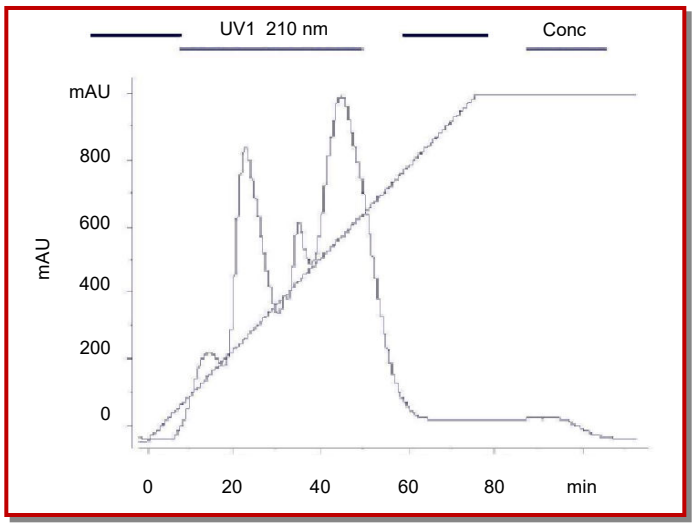

Figure 1: DEAE-52 separation results of low-molecular-weight chondroitin sulfate 2

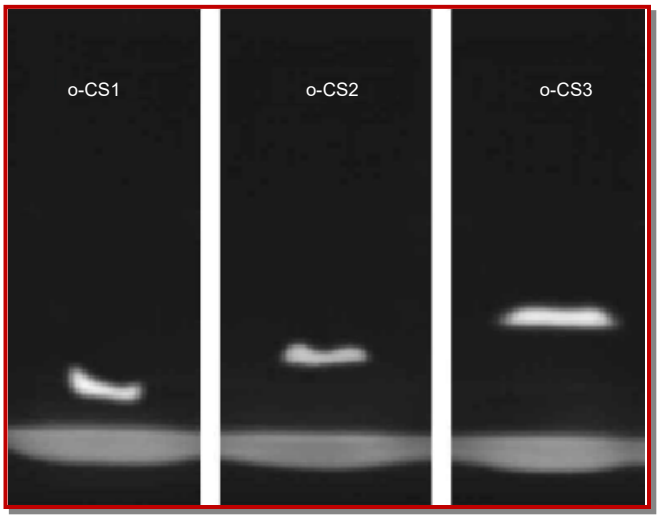

Figure 2: Preparative gel electrophoresis separation results O_2^{-}• is one of the major free radicals in biological systems, and it can produce components showed satisfactory O_2^{-}• scavenging activity, whereas the O_2^{-}• scavenging activity of chondroitin sulfate was almost zero (data not shown). These results further confirmed that the O_2^{-}• scavenging activity of low-molecular-weight chondroitin sulfate increased with an increased in its concentration and reached the maximum value at concentration of 4 mg/mL. There was no significant different of the O_2^{-}• scavenging activity between those three components

this study, Fe^{3+} in the form of ferricyanide was reduced to Fe^{2+}, which then reacted to produce Perl Prussian Queensland, which has a maximum absorbance at 700 nm. The absorbance strength was proportional to the Fe^{2+} concentration, the stronger absorbance value at 700 nm reflected higher anti-oxidant property. Analysis results of the absorbance strength of low-molecular-weight chondroitin sulfate in the ferricyanide system (Figure 4) revealed that the reducing power of low-molecular-weight chondroitin sulfate increased with an increased in its concentration and reached the maximum value at 10 mg/mL.

Discussion

The above separation results showed that acidic oligosaccharide can be separated efficiently by combination of ion exchange and gel chromatograph. This property was similar as protein, which always be separated by combination of ion exchange and gel chromatograph. The probably reason may be the similar ionization electron ability for acid oligosaccharide as that of protein. Moreover, this study also provided a good reference for low-molecular-weight chondroitin sulfate separation in a large scale. From above results, *vitro* antioxidant tests revealed that the free-radical scavenging activities, reducing power of low-molecular -weight chondroitin sulfate were higher than those of chondroitin sulfate. Very few related references support this phenomenon. Some researchers have suggested that the carbonyl groups, charged groups, as well as, sulfation position and degree in the structure of chondroitin sulfate and low-molecular-weight chon-

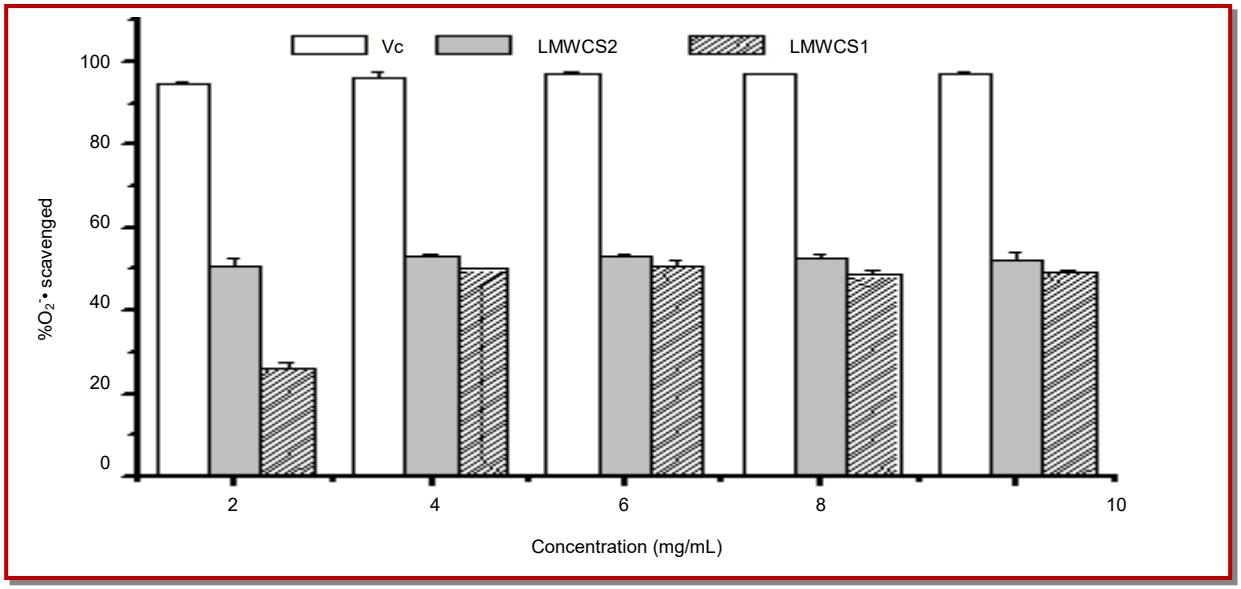

Figure 3: Scavenging activities of chondroitin sulfate (CS), low-molecular-weight chondroitin sulfate 1 (LMWCS1) and low-molecular-weight chondroitin sulfate2 (LMWCS2) on superoxide radical. The ascorbic acid (Vc) was used as a reference. Each value is the mean ± SD of triplicate measurements

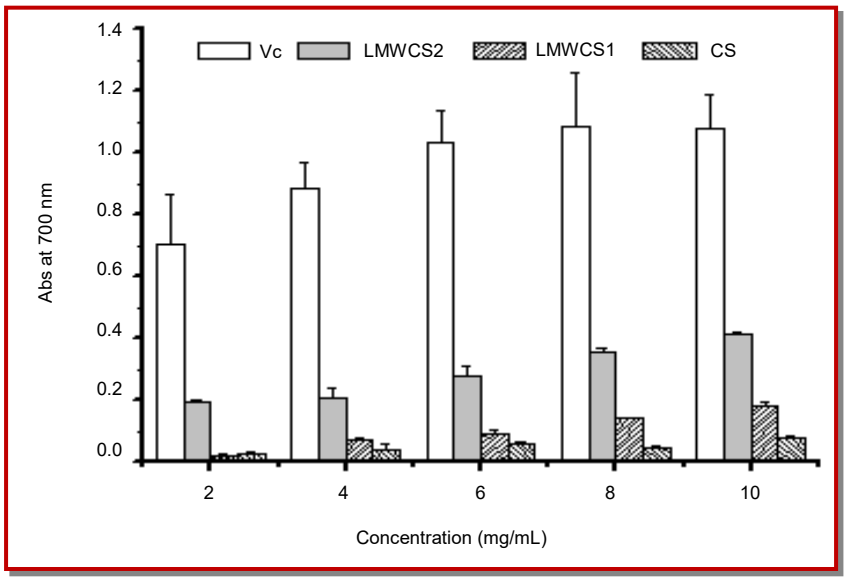

Figure 4: The reducing powers of chondroitin sulfate (CS), low-molecular-weight chondroitin sulfate 1 (LMWCS1) and low-molecular-weight chondroitin sulfate 2 (LMWCS2). The ascorbic acid (Vc) was used as a reference. Each value is the mean ± SD of triplicate measurements

droitin sulfate may play roles in their anti-oxidant properties (Duh, 1998; Campo, et al., 2004a; Campo, et al., 2004b; Campo, et al., 2003; Mark et al.,1990). Some others researchers reported that mole-cular weight of sulfated polysaccharides has some relationship with their activity. In mammalian GAGs, enzymatically digested hyaluronate showed higher radical scavenging activity than intact HA (Kim et al., 2008), and HA polysaccharides with different branching degrees of exhibit different biological activities (Stern, 2003). Low-molecular-weight (3–10 kDa) disaccharides have unique biological activities compared with high-molecular-weight (6–20 kDa) ones, which can inhibit the anchorage-dependent growth of tumor cells. Regarding sulfated polysaccharides, Nishino et al., reported that high-molecular-weight fucan (27–58 kDa) had higher anticoagulant activity than low-molecular-weight fucan (about 10 kDa) (Nishino et al., 1991). This finding could be attributed to the fact that linear sulfated fucan required significantly longer chains than mammalian GAGs to achieve anticoagulant activity(Pomin et al., 2005). The antiviral activity is also related to the molecular weight, which was increased with an increased in molecular weight (Witvrouw et al., 1997). However, few studies have reported on the relationship between anti-oxidant properties and molecular weight.

We speculated that the anti-oxidant properties of CS might be related to its molecular weight to some extent, i.e., short chains may be required to achieve anti-oxidant property.

Interestingly, our results showed that low-molecular-weight chondroitin sulfate had high solubility (data not shown) and can be quickly dissolved in water. However, the chondroitin sulfate polymer needed to be strongly stirred to be dissolved. We speculated that the extent of biological absorption of low-molecular-weight chondroitin sulfate was superior to that of high-molecular-weight chondroitin sulfate *in vivo* because of the good water solubility and small size of low-molecular-weight chondroitin sulfate.

The anti-oxidant properties of low-molecular-weight chondroitin sulfate can serve as a reference for research on the activities and clinical applications of low-molecular-weight glycosaminoglycans. Other bioactivities of low-molecular-weight chondroitin sulfate warrant further studies to maximize its potential benefits. Given the safety and nutritive content of low-molecular-weight chondroitin sulfate, its applications as a functional food ingredient and food additive are also promising. Low-molecular-weight chondroitin sulfate can also be used as a natural anti-oxidant.

Conclusion

Low-molecular-weight chondroitin sulfate was firstly separated, purified and identified as disaccharide, tetrasaccharide and hexasaccharide, which showed higher anti-oxidant activity than chondroitin sulfate. This finding supported the potential application of low-molecular-weight chondroitin sulfate as anti-oxidant and also displayed a beneficial way for the acidity oligosaccharide separation.

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Conflict of Interest

Authors declare no conflict of interest

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