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Chondroitin sulphate extracted from antler cartilage using high hydrostatic pressure and enzymatic hydrolysis

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ABSTRACT

Chondroitin sulphate (CS), a major glycosaminoglycan, is an essential component of the extracellular matrix in cartilaginous tissues. Wapiti velvet antlers are a rich source of these molecules. The purpose of the present study was to develop an effective isolation procedure of CS from fresh velvet antlers using a combination of high hydrostatic pressure (100 MPa) and enzymatic hydrolysis (papain). High CS extractability (95.1 ± 2.5%) of total uronic acid was obtained following incubation (4 h at 50 °C) with papain at pH 6.0 in 100 MPa compared to low extractability (19 ± 1.1%) in ambient pressure (0.1 MPa). Antler CS fractions were isolated by Sephacryl S-300 chromatography and identified by western blot using an anti-CS monoclonal antibody. The antler CS fraction did not aggregate with hyaluronic acid in CL-2B chromatography and possessed DPPH radical scavenging activity at 78.3 ± 1.5%. The results indicated that high hydrostatic pressure and enzymatic hydrolysis procedure may be a useful tool for the isolation of CS from antler cartilaginous tissues.

1. Introduction

Antlers from deer species have unique mammalian structures, where there is annual occurrence of cycle of growth, maturation, mineralisation, casting and regeneration [4]. Growing antlers are composed of different types of tissues including cartilaginous and osseous tissues surrounded by velvet connective tissues. Cartilage in antlers comprises of collagen and proteoglycans as its major extracellular matrix constituents, and aggrecan as the predominant proteoglycan [27,12]. The structure of aggrecan has a protein core of approximately 200 kDa molecular weight in which glycosaminoglycan (GAG) chains containing approximately, 100 chondroitin sulphate (CS) chains (MW 10–25 kDa), 30–60 keratin sulphate (KS) chains (MW 3–15 kDa), and N- and O-linked oligosaccharides are covalently attached [10]. CS is one of the GAGs composed of the alternating sugars D-glucuronic acid (GlcA) and *N*-acetyl-D-galactosamine (GalNAc). As a major GAG of aggrecan molecules in the antler, CS accounts for approximately 92% of total GAGs with relatively small amounts of KS [29,25]. Thus, CS is an important component of the extracellular matrix in antler cartilage.

Due to its negative charge, CS is responsible for water retention in the cartilage, which is important for pressure resistance. Physiologically, CS increases hyaluronan production by human synovial cells to maintain viscosity in the synovial fluid [6]. It also has many functional properties for the prevention of osteoarthritis, such as modifying the chondrocyte apoptosis process, improving the anabolic/catabolic balance of the extracellular cartilage matrix, reducing pro-inflammatory and catabolic factors, and stimulating the anabolic processes involved in new cartilage formation in osteoarthritis [11]. In addition, CS shows a dose-dependent increase in free radical scavenging [2]. This antioxidant activity, caused by the chelation of transition metals such as Cu^{2+} and Fe^{2+}, is also believed to be partially responsible for the chondroprotective effects of CS, as oxidative stress has been shown to increase the risk and effects of osteoarthritis [1,7,3,5,33]. CS is an important constituent for the preservation of corneal tissues. So far, there is no efficient treatment that could prevent the pathological process of arthropathy. Oral administration of CS was suggested to be beneficial in the treatment of osteoarthritis. To take advantage of these important functionalities, CS can be ingested as a food supplement once it has been extracted from the cartilaginous tissue. The extraction of CS requires the degradation of collagen and the core protein in the extracellular matrix.

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In this study, a combination of high hydrostatic pressure (HHP) and enzymatic hydrolysis (HHP-EH) is tested as a relatively new extraction process for isolating CS from cartilaginous tissues of antlers. HHP greater than 100 MPa increases water penetration into the protein interior and damages the cell membrane, which unfolds protein molecules and simultaneously inactivates bacteria at ambient temperatures within few minutes. This phenomenon allows HHP to be widely used in food preservation as an alternative to heat treatment, maintaining the stability and functionality of enzymes at a pressure less than 200 MPa and concurrently increasing their reaction rate [17]. For example, the catalytic activity of thermolysin increased 45-fold at 200 MPa [14] and that of α-chymotrypsin increased 7-fold at 150 MPa [19] as compared to ambient pressure.

Antler cartilaginous tissues are of relative low-value but are abundantly available in nonedible by-products rich in CS-proteoglycan, collagen and glycoprotein. There are few reports about the isolation of CS from antler cartilage and its antioxidant ability. The aim of this work was to determine the effect of high pressure, temperature and incubation time on the catalytic activity of papain for extracting CS as a potential antioxidant agent. GAGs can be directly extracted from tissues by hydrolysis with exogenous enzymes like papain or pronase [20]. Further separation of the CS in the crude extract was obtained by column chromatography, and the hyaluronic acid binding ability of CS was also examined.

2. Materials and methods

2.1. Preparation of antlers

Samples of antlers were obtained from 4-year-old wapiti stags at a local elk farm (Leduc, Alberta, Canada). The main beam of each harvested antler was skinned and divided into 4 sections (tip, upper, middle and base) as previously described [28]. Macroscopically, the tip section contains pre-chondroblast soft cartilaginous tissue with no bony structure. Most of the upper section comprises cartilaginous chondrocytes with minor osteoblasts. In contrast, bones are the major tissues found in the middle and base sections. Only the tip and upper sections were selected and transported to the laboratory on ice rinsed with cold water, dissected free of non-cartilaginous adherent connective tissues, and stored at −20 °C until extracted. Five hundred grams of frozen samples were then thawed at 4 °C, chopped into small pieces, added to 500 mL of deionised water, homogenised with a blender (Waring commercial, MX1500XTS model, Stamford, CT, USA) and then sieved through a 100-mesh screen. The unscreened particles were further liquefied using a colloid mill (Chemineer Inc., W200V model, Dayton Ohio, USA). The suspensions from blending and milling were combined and stored for further use.

2.2. High hydrostatic pressure and enzymatic hydrolysis (HHP-EH) treatment

HHP-EH treatments were performed in a portable scale high hydrostatic pressure system (TFS-2 L, Toyo-Koatsu Innoway Co. Ltd., Hiroshima, Japan) with a cylindrical pressure chamber, which has a volumetric capacity of 2 L. 500 mL of the suspension (modified at pH 6.0) was then mixed with papain type 111 (4 mg/g of tissue, EC3.4.22.2, Sigma–Aldrich, USA). First, the liquid mixtures of antler samples and enzyme were poured into 5 plastic ziplock bags (10 mL per bag) and sealed. Deionised water was used as the pressurisation medium in the HHP unit. Test samples were subjected to HHP treatment at selected pressures of 0.1, 25, 50, 75 and 100 MPa for 4 h at 50 °C. Secondly, five bags were subjected to HHP treatment for different incubation times of 1, 2, 3, 4, and 8 h at 50 °C at 100 MPa. Four bags were also subjected to HHP treatment for different temperatures of 20, 30, 40 and 50 °C for 4 h at 100 MPa. Following HHP treatment, the pressure was quickly released and the extracts were boiled for 10 min to inactivate the papain. After cooling, the cooled extract was centrifuged (5000 × *g* for 10 min) and then filtered through a Whatman no.5 filter paper. The extract was stored at −20 °C until analysed. The residual tissue was further digested with papain, and uronic acid contents in both the extract and the residual tissue were determined by the carbazole reaction (see Section 2.7). These estimates enabled the proportion of uronic acid liberated to be expressed as a percentage of the total uronic acid recovered. The total extractability of uronic acid was then compared between the different extraction conditions. The preparation of each extract, which was referred to as antler papain extract, was performed in triplicate and the entire experiment was independently replicated three times to address precision.

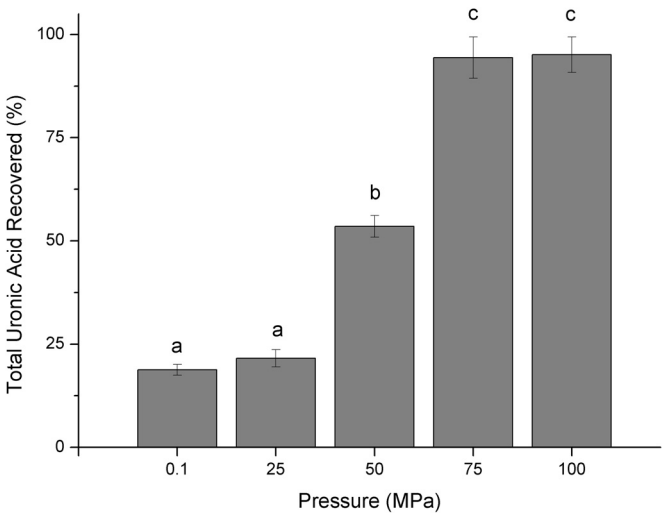

Fig. 1. Effect of pressure on the content of uronic acid by using high hydrostatic pressure and enzymatic hydrolysis at 50 °C for 4 h. Values are means of triplicate. Error bars shown are standard errors of the means ($n = 3$). Letters indicate significant differences among pressure treatments.

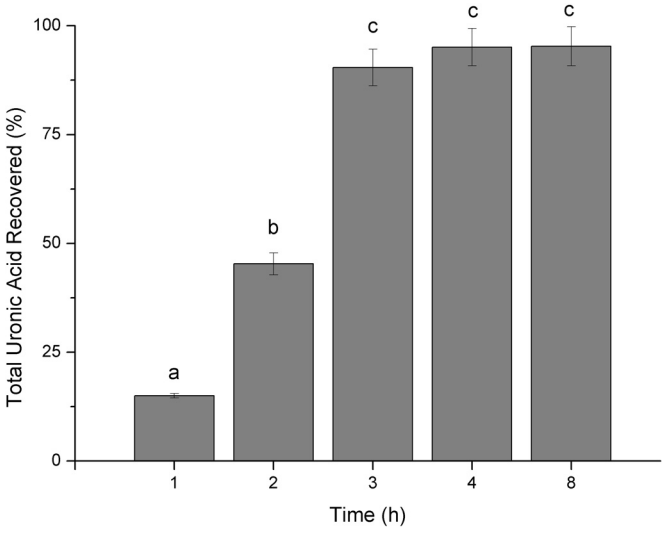

Fig. 2. Effect of incubation time on the content of uronic acid by using high hydrostatic pressure and enzymatic hydrolysis at 50 °C under 100 MPa. Values are means of triplicate. Error bars shown are standard errors of the means ($n = 3$). Letters indicate significant differences among incubation times.

2.3. Column chromatography

Antler CS fractions were isolated and examined for molecular size using Sephacryl S-300 chromatography (Pharmacia Biotech Inc., Quebec, Canada). A portion of the antler papain extract was fractionated using a 1×110 cm column equilibrated and eluted with 0.05 M NaCl buffer, pH 5.8, at a flow rate of 3 mL/h. Blue dextran and tritiated water were used to determine void volume (V_o) and total volume (V_t) of the column, respectively. The partition coefficient was calculated from the formula: $K_{av} = (V_e - V_o)/(V_t - V_o)$, in which V_e represents the volume of the peak fraction. The eluates (1 mL) were analysed for protein at 280 nm absorbance, hydroxyproline, sulfated GAG and uronic acid content as explained in Section 2.7. Antler CS fractions were pooled and freeze-dried for further study. All chromatography data presented in this paper are means of 3 experiments.

2.4. Electrophoresis and western blot

Electrophoresis was performed in 0.6% acrylamide in agarose in Tris buffer, pH 6.8. Samples were dissolved in deionised water. Two slabs were generally run at the same time, one for staining with toluidine blue and the other for western blot with monoclonal antibodies to chondroitin sulfate (CS-56) (Sigma–Aldrich, USA). Electrophoretic transfer to nitrocellulose was accomplished in Tris–borate (gel electrophoresis buffer) without sodium dodecyl sulfate at 40 V for 2 h. Nitrocellulose sheets were then soaked in 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.2 for 1 h at room temperature. After washing in PBS (three times for 5 min each), nitrocellulose sheets were incubated with anti-CS monoclonal antibodies (in PBS containing 1% BSA) for 1 h at room temperature. The incubation was followed by washing in PBS and 1 h incubation with rabbit anti-mouse IgM conjugated with horseradish peroxidase. Colour was developed by incubating in 0.05% diaminobenzidine tetra-hydrochloride in PBS containing hydrogen peroxide (0.01% w/v) and cobalt chloride (0.033% w/v) for 5 min. Stained blots were then washed several times in water and dried.

2.5. Interaction of antler CS fractions with hyaluronic acid

Hyaluronic acid from human umbilical cord (Sigma–Aldrich, USA) was dissolved in 0.5 M sodium acetate buffer, pH 6.8, and added to the selected antler CS fraction in the same buffer at a weight ratio of hyaluronic acid to proteoglycan of 1:100. After allowing the solution to stand at 4 °C for 12 h, the extent of aggregation was examined by chromatography on Sepharose CL-2B by monitoring fractions with the uronic acid assay. A bovine articular aggrecan (A1960, Sigma–Aldrich, USA) was used as a reference.

2.6. Measurement of DPPH radical scavenging activity

A previously described standard procedure was used for the measurement of 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity [21]. Briefly, 1 mL of DPPH (100 μM, Sigma–Aldrich, USA) in ethanol and 1 mL of antler CS fraction at different concentrations of CS (0.625–10 mg/mL on) in 100 mM Tris–HCl buffer (pH 7.4) were mixed. This reaction mixture was shaken and incubated for 20 min in the dark at room temperature. The absorbance was measured at 517 nm against a blank control (100 mM Tris–HCl buffer). Measurements were performed in triplicate over a 60-s period for each sample. The DPPH radical scavenging activity, namely the inhibitory ratio, was calculated using the following equation: scavenging activity (%) = $(1 - A_{sample}/A_{blank}) \times 100$, where A_{blank} is the absorbance of the blank. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Ascorbic acid and butylated hydroxytoluene (BHT) (Sigma–Aldrich, USA) were used as positive controls. Two CS from bovine cartilage (C6737, Sigma–Aldrich, USA) and shark cartilage (C4384, Sigma–Aldrich, USA) were used as reference CS. The results were presented as the means of experiments performed in triplicate ± standard deviation.

2.7. Analytical methods

Moisture content in antler cartilaginous tissue was estimated from the loss of sample weight by heating at 110 °C overnight. Uronic acid contents were determined by the original [8] and the carbazole reaction [13], using D-glucuronolactone as a standard. Sulfated GAG was analysed using the dimethylmethylene blue dye binding method [9]. A CS from shark cartilage was used as a standard GAG. The content of hydroxyproline (reflecting that of collagen) was determined by hydrolysis in 6 N HCl at 110 °C for 20 h [26]. The content of collagen was calculated by multiplying the content of hydroxyproline by 7.46 (collagen contains 13.4 percent hydroxyproline). Sialic acid content was determined by the periodate-thiobarbituric acid reaction [31] after hydrolysis of samples in 0.1 N sulphuric acid at 80 °C for 1 h. Protein was determined using the Lowry method [16] using BSA as a standard. Analysis of amino acids of purified CS fraction was performed by HPLC after hydrolysis with 6 N HCl at 110 °C for 24 h as previously described [28].

2.8. Statistical analysis

All analyses were performed in triplicate, unless otherwise specified. The values were averaged and standard deviations (SD) were calculated. All data were analysed by one-way analysis of variance and Duncan's multiple range tests using SPSS software (version 10 SPSS, Chicago, IL, USA). The results were considered significant at $P < 0.05$.

3. Results

3.1. Antler tissue composition

The moisture content of antler cartilaginous tissues was 76.2 ± 1.4% (mean ± SD in triplicates) of wet weight. The concentrations of protein, hydroxyproline, sialic acid and uronic acid, expressed as milligrams per gram of dry tissue, were 724.8 ± 9.3, 35.5 ± 1.2, 6.7 ± 0.2 and 41.2 ± 0.9, respectively.

3.2. Effect of high hydrostatic pressure

Fig. 1 illustrates that the content of uronic acid liberated from antler cartilaginous tissues with papain under the fixed conditions of pH 6.0, 50 °C and 4 h incubation time was dependent on increased hydrostatic pressure. Increased pressure, by increasing the solubility of CS, was one of the most important variables in the HHP-EH process. The content of released uronic acid was highest at 75 MPa (94.4 ± 2.9% of total uronic acid recovered) and at 100 MPa (95.1 ± 2.5% of total uronic acid recovered). This value was 2 and 5 times higher ($P < 0.05$) than values obtained at 50 MPa (53.5 ± 3.0%) and 25 MPa (21.6 ± 1.1%), respectively. The extractability of uronic acid was less than 19 ± 1.1% at ambient pressure (0.1 MPa). As a result, higher pressure at 100 MPa led to a higher extraction yield.

3.3. Effect of incubation time

Fig. 2 illustrates that the content of uronic acid liberated from antler cartilaginous tissues with papain under the fixed conditions

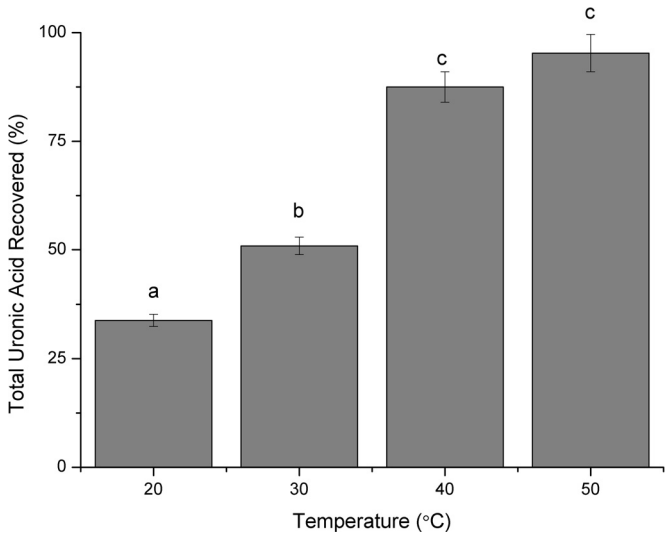

Fig. 3. Effect of temperature on the content of uronic acid by using high hydrostatic pressure and enzymatic hydrolysis at 100 MPa for 4 h. Values are means of triplicate. Error bars shown are standard errors of the means ($n = 3$). Letters indicate significant differences among temperatures.

of pH 6.0, 50 °C and 100 MPa was dependent on the incubation time. The liquid mixtures of antler tissue and papain were hydrolysed in the high-pressure chamber machine for 1–4 and 8 h. The results show that the yield of total uronic acid significantly increased ($P < 0.05$) between 1 and 3 h incubation time and then increased slightly from 3 to 4 h. Papain demonstrated significant increases in the uronic acid yield during the initial 3 h incubation. However, the effect of the incubation time between 4 h and 8 h was not significantly different in papain treatment ($P > 0.05$). The result indicated that incubating for longer than 4 h was likely unnecessary because the yield did not significantly increase thereafter.

3.4. Effect of temperature

The effect of different temperatures is illustrated in Fig. 3, when conditions are fixed at a constant pressure of 100 MPa for 4 h incubation time. The result showed that the HHP-EH demonstrated significant increases ($P < 0.05$) in total uronic acid yield from 20 to 30 °C, and then again significantly increased from 30 to 40 °C. However, the effect of the temperature between 40 and 50 °C was not significantly different in the HHP-EH treatment ($P > 0.05$). The results indicated that incubating at below 40 °C was not fully activating the papain to liberate CS from the samples.

3.5. Isolation of antler CS fractions after HHP-EH treatment

The CS uronic acid extracted from antler cartilaginous tissues hydrolysed with papain at 50 °C for 4 h in 100 MPa accounted for ~94% of total uronic acid recovered (Fig. 1). The hydrolysed antler papain extracts were applied to the Sephacryl S-300 chromatography column to isolate antler CS fractions. The majority (94%) of antler CS fractions eluted at peaks of K_{av}, 0.15 in a single fraction (Fig. 4). Other fractions in the Sephacryl S-300 gel chromatography showed that broader peaks ranging from 0.25 to 0.95 K_{av} contain hydroxyproline. Thus, major antler CS-containing eluates (0.1–0.2 K_{av}) were collected and examined by amino acid analysis and electrophoresis followed by western blot with the monoclonal antibody to identify CS.

3.6. Characterisation of CS

Toluidine blue-stained gel electrophoresis of antler CS fractions from gel chromatography on Sephacryl S-300 (Fig. 4) is shown in Fig. 5a. The molecular size of the antler CS fraction eluted (Fig. 5a, lane 1) is apparently smaller than bovine cartilage CS (Fig. 5a, lane 2). Both the antler CS fraction and bovine cartilage CS were stained with a monoclonal antibody (anti-CS56) specific to CS (Fig. 5b, lane 1). The result of western blot shows that the presence of the epitopes can be recognised by anti-CS56, confirming that the collected fraction contained CS. The antler CS fraction possessed a small amount of amino acids (approximately 23.5 mg per gram by dry weight, Table 1).

3.7. Interaction of the antler CS fraction and hyaluronic acid

The antler CS fraction was then examined for its capability to interact with hyaluronic acid and form high molecular weight aggregates by using Sepharose CL-2B chromatography (Fig. 6). Sepharose CL-2B chromatography with and without prior

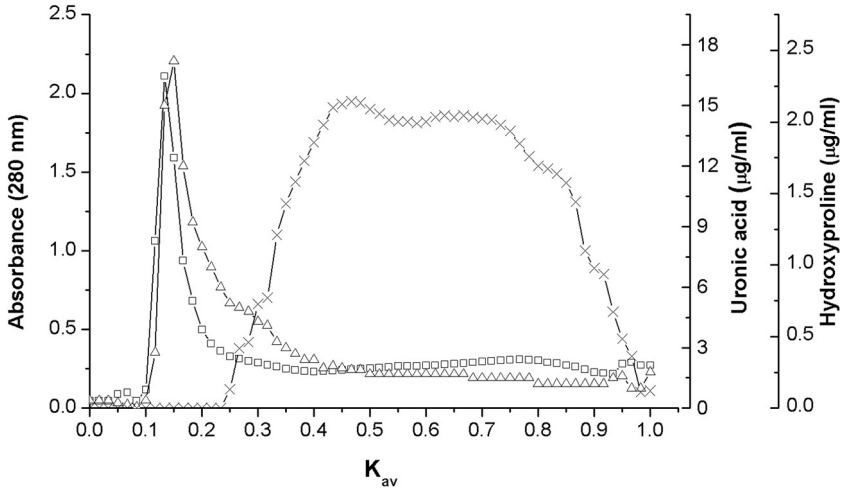

Fig. 4. Chromatography of CS extracted from antler cartilage after the HHP-EH treatment on Sephacryl S-300 in phosphate buffered saline, pH 7.0. Fractions (1 mL) collected at a flow rate of 3.0 mL/h at 21 °C were determined for absorbance at 280 nm (—□—), uronic acid content (—Δ—) and hydroxyproline (—×—). The partition coefficient (K_{av}) of the CS peak was calculated from the formula: $K_{av} = (V_e - V_o)/(V_t - V_o)$, in which V_e represents the volume of the peak fraction. Void volume (V_o) and total column volume (V_t) were determined using blue dextran (Pharmacia Biotech Inc.) and tritiated water, respectively.

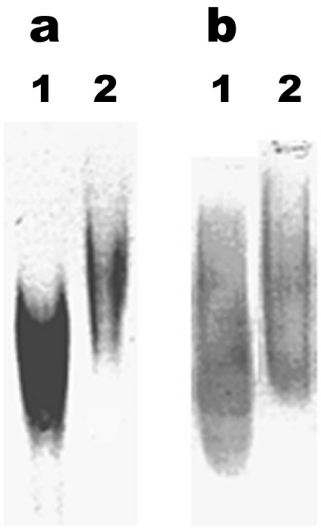

Fig. 5. Agarose-polyacrylamide gel electrophoresis and western blot of CS isolated from Sephacryl S-300 chromatography (Fig. 5). (a) Stained with toluidine blue; (b) stained with the monoclonal antibody to chondroitin sulfate (CS-56). Lanes 1 and 2 represent CS isolated from antler cartilage tissues and from bovine cartilage, respectively.

Table 1
Amino acid composition of isolated CS fraction (mg/g).

Aspartic acid	1.62	Tyrosine	0.37
Glutamic acid	3.73	Methionine	0.11
Serine	2.40	Valine	1.10
Histidine	0.43	Phenylalanine	0.82
Glycine	4.91	Isoleucine	0.33
Threonine	1.47	Leucine	1.61
Arginine	0.97	Lysine	0.82
Alanine	2.70	Cysteic acid	0.10

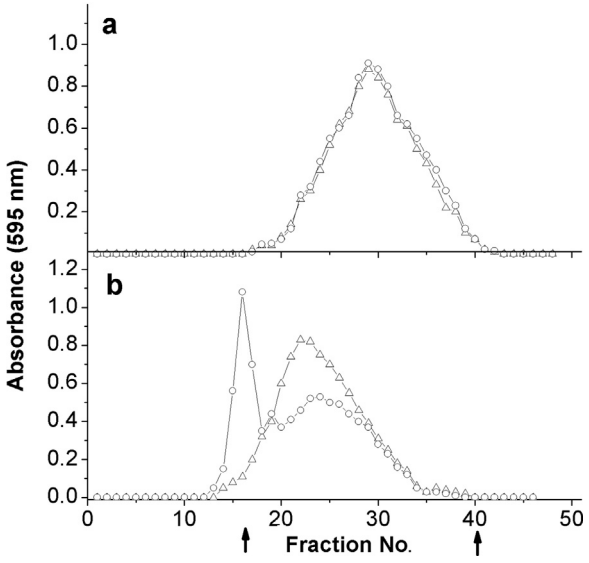

Fig. 6. Chromatography on Sepharose CL-2B of CS isolated from antler cartilaginous tissues (a) and aggrecan from bovine nasal cartilage (b) with (◯) and without (Δ) prior incubation with hyaluronic acid. Each sample was applied to a 0.8 × 68 cm column equilibrated and eluted with 0.5 M sodium acetate buffer, pH 5.8. Fractions (0.6 ml) collected at a flow rate of 1.0 mL/h were monitored for GAG by the dimethyl-methylene blue dye-binding method. The left and right arrows in each chromatogram show the void volume and total column volume, respectively.

incubation with hyaluronic acid showed that there was no interaction of the antler CS fraction with exogenous hyaluronic acid. In contrast, the aggrecan from bovine articular cartilage interacted with hyaluronic acid, which was observed as the appearance of a peak excluded from Sepharose CL-2B (Fig. 6b). In the present study, the result suggested that the present preparation of the antler CS fraction most likely lacked the G1 domain containing the hyaluronic acid binding region as compared to the aggrecan from bovine articular cartilage that contained the functional peptide.

3.8. DPPH radical scavenging activity

The DPPH radical scavenging activity of the antler CS fraction after HHP-EH treatment was measured at various concentrations. As shown in Fig. 7, DPPH radical scavenging activities of antler CS fraction, bovine cartilage CS and shark cartilage CS at a concentration of 5 mg/mL were measured as 50.9 ± 1.1%, 7.6 ± 0.1%, and 4.8 ± 0.1%, respectively. The scavenging effect of the antler CS fraction increased with increasing concentrations up to 10 mg/mL, indicating that the highest DPPH radical scavenging activity was 61.9 ± 1.4%. The DPPH radical scavenging activity of the antler CS fraction was significantly higher ($P < 0.05$) than that of CS from bovine or shark cartilage but lower than that of either ascorbic acid or BHT.

4. Discussion

Proteoglycans present in the bone matrix help in bone mineralization and calcium accumulation. Chondroitin sulfate is reported to have functional roles in cell proliferation and wound healing [23]. Antler CS is one of the natural GAG composed of the alternating sugars GlcA and GalNAc. CS, an important component of the extracellular matrix, can be extracted from cartilaginous tissue and is available as a food supplement. Although CS functions as a physiologically active component, there is little information on the extraction of CS in animal tissues by using chemicals, which are not suitable for food supplements. In the present study, we used the HHP-EH process, which has several advantages such as higher extraction yield, user friendly, low energy consumption, low temperature, and no use of chemicals. The results obtained in the present study indicate that the highest extractability of CS in the antler cartilage is related to papain digestion under HHP (100 MPa). The extractability of CS liberated from the antler tissues was estimated from

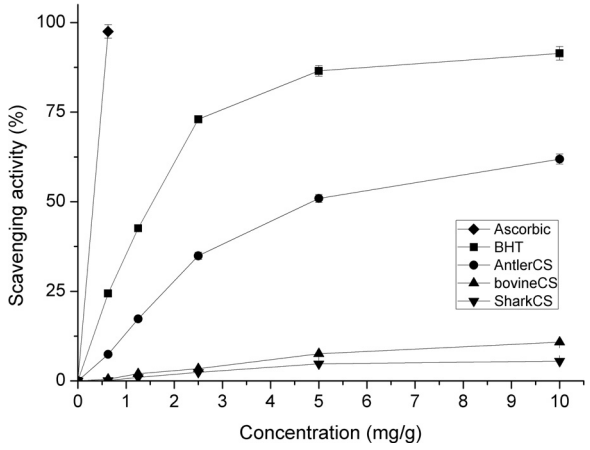

Fig. 7. The scavenging effect of chondroitin sulfate isolated from antler cartilage, bovine cartilage and shark cartilage on DPPH radicals. Values are means of triplicate. Error bars shown are standard errors of the means ($n = 3$).

the amount of uronic acid recovered from the papain digest. The estimated extractability under hydrostatic pressure was 6-fold higher at 100 MPa than that obtained at ambient pressure (0.1 MPa) at 50 °C during the 4 h incubation time (Fig. 1). The results show that the catalytic effect of papain is accelerated by HHP, indicating that the optimal conditions of pressure, incubation time and temperature are obtained at 100 MPa for 4 h at 50 °C, respectively. As a result, the HHP-EH process shows that the extractability of CS is approximately 95% of total uronic acid in antler cartilage tissue as compared to less than 20% extractability from a previous report, which used papain for 24 h at ambient pressure on the 0.5 M sodium acetate soluble fraction from antlers [30]. The low extractability was mainly due to the multiple steps involved in isolating CS from antler cartilage with a high risk of CS loss. In the present study, the high extractability of CS indicates that the mild pressure (100 MPa) is not only directly related to water penetration into the structure of collagen, proteoglycan and other proteins found in extracellular matrix but also, more importantly, to accelerate the present process of papain treatment. Meersman et al. (2006) [18] reported that the high pressure increases the rate of mass transfer, enhances water penetration into the solid material and disrupts cell membranes to release intracellular products. The rationale behind HHP effects has three main factors: the energy, the densification effect and the chemical reactivity [24]. Due to compressibility, the difference between final and initial volumes under high pressure is always negative (ΔV value <0), leading to low energy and a densification effect. However, this does not give any prediction of the volume changes of chemical reactions in relation to the equilibrium between the states (reaction volume) or the activation volume of the chemical reaction. In addition, the chemical reactivity may be improved by high pressure, inducing an increase in solubility and consequently, the concentration of the solvated species. This phenomenon (electrostriction) leads to the reduction of the average distance between the solvated species, inducing an increase in the kinetic rate of the reaction. For example, the stability and functionality of most enzymes are not altered at pressures lower than 100 MPa [17], while pressure-induced denaturation occurs when pressures exceed 300 MPa [32]. Reversible pressure denaturation occurs at pressures below 300 MPa, and higher pressures are needed to cause irreversible denaturation of the protein. High pressure also causes deprotonation of charged groups and the disruption of salt bridges and hydrophobic bonds, resulting in conformational changes and protein denaturation under high pressure >300 MPa [32]. Most enzymes also lose their catalytic activities with pressure exceeds 300 MPa, resulting in changes in the substrate property or producing rate-limiting conformational changes. In this study, therefore, we have examined the optimal conditions of HHP treatment (<100 MPa) combined with enzymatic hydrolysis to extract CS from fresh antler cartilage. A high pressure (100 MPa) used in this study noticeably accelerates papain catalytic activity. Because HHP technology has been commercially available for many years for industrial-scale applications, it is worthwhile to investigate other enzymes for digesting various sources of cartilage components.

Antler CS fractions treated by HHP-EH process were examined for their capabilities to interact with hyaluronic acid to form high molecular weight aggregates (Fig. 6). The chromatography of the antler CS fraction following incubation with exogenous hyaluronic acid showed an absence of the peak from the column (Fig. 6a). However, the bovine articular cartilage aggrecan interacted with hyaluronic acid, which is evidenced by the appearance of a peak excluded from Sepharose CL-2B (Fig. 6b), indicating an interaction of the CS fraction with hyaluronic acid. The binding ability shows that aggrecan possesses the G1 domain containing the hyaluronic acid binding region, which is located at the N-terminus [22], and constitutes about one-quarter to one-third of the total core protein [15]. The antler CS fraction shows a lack of the G1 domain specific to hyaluronic acid with the formation of macromolecular aggregates [22].

Table 2
Chemical analyses of high hydrostatic pressure and papain digests from velvet antler[a].

Uronic acid	32.5 ± 1.6
Sulphated glycosaminoglycan	51.6 ± 2.9
Sialic acid	19.5 ± 1.2
Hydroxyproline	1.2 ± 0.1
Protein	339 ± 22.1

[a] μg/mg of dry weight of high hydrostatic pressure and papain digest.

Although antlers have been used as a Chinese medicine for many years, only limited information is available on the chemical compositions, bioactive ingredients, extraction methods and pharmacological effects [28,30]. We have also showed the chemical analyses of high hydrostatic pressure and papain digests from antler cartilage (Table 2). Increasing evidence indicates that acidic polysaccharides, which are widely distributed in animals, possess potential antioxidant activity by scavenging free radicals [2]. Although the antler CS fraction was not superior to ascorbic acid and BHT for DPPH scavenging activity, its antioxidative activity was much higher than that of bovine and shark CS, indicating greater potential as antioxidant components, because much attention has been given to antioxidants in preventing free radical-induced damage. The difference in the DPPH radical scavenging activity of the HHP-EH-treated antler CS from bovine and shark CS requires further investigation. We are also conducting biological functional studies to investigate the osteogenic properties of extracted CS using our method. Preliminary results indicate potential applications as osteogenic candidates (unpublished data).

5. Conclusions

Chondroitin sulphate is an acidic polysaccharide of potential importance with wide applications. However, not much attention has been given to the economical production of CS abundant in antler cartilage. With the method described in this paper, the CS uronic acid extracted from antler cartilaginous tissues accounted for ~94% of total uronic acid recovered by using a combination of high hydrostatic pressure (100 MPa) and papain enzymatic hydrolysis digests. Highest yields of CS extracts were obtained by the HHP-EH process at 50 °C in 100 MPa for 4 h incubation time. The yields of CS found in the present study are much higher than those previously reported [30]. The antler CS fraction has no capability to form aggregates with hyaluronic acid and shows DPPH radical scavenging activity as a potential antioxidant constituent. This extraction technique may be useful to isolate CS from other cartilaginous tissues as an efficient and cost-effective method.

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