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Characterization of chondroitin sulfate from deer tip antler and osteogenic properties

Peraphan Pothacharoen · Kanchanok Kodchakorn · Prachya Kongtawelert

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Abstract Deer antler is a highly regenerative tissue that involves cellular differentiation, osteogenesis and ossification processes. Chondroitin sulfate is the major glycosaminoglycan contained in antler connective tissue and has been isolated from cartilaginous antler by 4 M GuHCl extraction, gradient ultracentrifugation and chromatography techniques. We examined the disaccharide composition by 2-AB labeling and anion exchange HPLC analysis of the three resultant fractions (high, medium and low density fractions). The high density fraction consists of A-unit and D-unit disaccharide in the ratio of 1:1, whereas, the CS disaccharide composition ratio of A- unit:C-unit:D-Unit:E-unit contained in medium and low density fractions are 3:4:3:1 and 2:2:2:1, respectively. The only intact CS oligosaccharides of the medium density fraction upregulated gene expression of bone-specific proteins of a human osteoblastic cell line (hFOB1.19). Thus, CS oligosaccharides from cartilaginous deer antler, with their oversulfated chondroitin sulfate composition, demonstrated the physiological properties and may be good candidates for osteogenetic agents in humans.

Keywords Chondroitin sulfate · Deer tip antler · Osteogenic properties · Osteoblast

Abbreviations

GuHCl	Guanidine hydrochloride
2-AB	2-aminobenzamide
HPLC	High performance liquid chromatography
CS	Chondroitin sulfate
PG	Proteoglycan
GlcA	Glucuronic acid
IdoA	Iduronic acid
GalNAc	*N*-acetyl-D-galactosamine
CsCl	Cesium chloride
s-GAG	Sulfated glycosaminoglycan
CS'ase	Chondroitinase
mIU	Milli international unit

Introduction

Velvet antlers are the fastest growing mammalian tissue and constitute an interesting model for cartilage and bone research. Antlers develop from pedicles that appear transiently during the fetal life of calves and grow to permanent protuberances during puberty from the lateral crests of deer frontal bone [1]. They grow in length by a continuous process of cellular differentiation, from undifferentiated progenitors in the perichondrium into chondroblasts and chondrocytes [2, 3]. The progenitor cells develop from the tip by a combination of intramembranous and chondrochondral ossification, involving both cellular differentiation and bone development processes.

Structurally, antler cartilage is similar to other hyaline cartilage but is highly vascularized, which accounts for the

Peraphan Pothacharoen and Kanchanok Kodchakorn Equal Contribution

P. Pothacharoen (✉) · K. Kodchakorn · P. Kongtawelert
Thailand Excellence Center for Tissue Engineering and Stem Cells, Department of Biochemistry, Faculty of Medicine, Chiang Mai University,
Chiang Mai, Thailand
e-mail: peraphan.pothacharoen@gmail.com

K. Kodchakorn
e-mail: tkanchanok@gmail.com

P. Kongtawelert
e-mail: prachya.kongtawelert@gmail.com

high level of metabolic processes in such a rapid regenerative tissue [4, 5]. Previous studies have reported that chondroitin sulfate is a major component of glycosaminoglycan contained in red deer antler (*Cervus elaphus*), whereas keratan sulfate, dermatan sulfate [6] and heparan sulfate are found in small amounts [7]. Antler has been used as a Chinese traditional medicine in Asian countries and is mainly used for anti ageing effects, as well as to treat sexual dysfunction in elderly men and menopause in women. Scientific research has demonstrated pharmacological effects of deer antler in aqua-acupuncture, using the water extract (DAA; Cervi Pantotricum Cornu) for treatment of bone and joint disease [8, 9].

In cartilage and bone, cellular activity largely depends on the interaction with the components of the extracellular matrix (ECM) and growth factors. Also, the composition of the matrix reflects changes in the pattern of gene expression during tissue differentiation. However, only a few components of the antler ECM have been described. Collagens have been identified in antler tissue extracts [3, 10] as well as mRNAs for collagen I, X, and both splice forms of collagen II were detected by *in situ* hybridization in the developing antler tip [4]. Aggrecan and decorin were also identified by biochemical means [6]. Recently, deer antler proteome analysis has identified biglycan and desmocollin 1 and 3 [11].

Cartilage generally contains chondroitin sulfate proteoglycans (CSPGs) that consist of one or more chondroitin sulfate (CS) chains attached to a core protein. They are increasingly implicated as important regulators of many biological processes, including the physical strength of tissues, cell adhesion, and signal transduction [12, 13]. Many reports confirm that the CSPGs of cartilage are dynamic components that play several key roles in the normal physiology of cartilage tissues and regulate biological processes such as cell migration, cytokine recognition, extracellular matrix deposition, morphogenesis, organogenesis and wound repair [14, 15]. The complexity and diversity of CS structure vary with sulfation pattern, epimerization and chain length, and are usually associated with their functions. The CS chain is an unbranched repeating disaccharide consisting of an acidic sugar, either glucuronic acid (GlcUA) or iduronic acid (IdoA), and *N*-acetylgalactosamine (GalNAc), which can be modified by *O*-sulfation reactions at various positions. Each chain is linked through a neutral trisaccharide (Gal-Gal-Xyl) to a serine residue in a proteoglycan. In mammals, common monosulfated CS-A plays a role in the canine ossification process [16], over sulfated CS-D and CS-E support neuritogenesis [17–19] and growth factor binding [20].

Antler development involves osteoblasts, progenitor cells responsible for osteogenesis and the ossification process. It thus should be of interest to analyze the CS structural details that are related to its osteogenic function. The present study focuses on the separation, characterization and biological functions of antler CS from the cartilagenous antler tip from Rusa deer.

Materials and methods

Materials

The following chemicals were purchased from Sigma-Aldrich Chemical (St. Louis, USA): CS-C from shark cartilage, chondroitinase ABC (EC 4.2.2.4) from *Proteus vulgaris,* 2-aminobenzamide (2-AB) and sodium cyanoborohydride ($NaCNBH_3$). Six unsaturated chondroitin sulfate disaccharide standards were purchased from Seikagaku Corp., Ltd. (Tokyo, Japan). Amine-bound silica PA-03 column (4.6×250 mm) was obtained from YMC Corp., Ltd. (Kyoto, Japan).

Extraction of the GAG fraction from antler cartilage

Samples of 45–60 days after casting antler were obtained from 3 month-old male Rusa (*Cervus timorensis*). The animals were bred at Rajamangala University of Technology Lanna, Lampang, Thailand. The harvested antler was skinned and macroscopically dissected out; only the tip containing cartilaginous tissue antler without bony structure was retained. The cartilaginous tip was stored at −20°C until extraction.

Preparation of antler CS

The cartilaginous tip of the antler was cut into small pieces and proteoglycans were extracted with buffer containing 0.2 M Tris–HCl (pH 8.0), 4 M guanidine HCl, 10 mM EDTA, 10 mM aminocaproic acid, 10 mM*N*-ethylmaleimide, and 2 mM PMSF, and then dialyzed against distilled water (see Fig. 1). The A_1 fraction was purified by CsCl isopyonic centrifugation [21]. After stirring overnight at 4°C, the solution was brought to a density of 1.7 g/ml with solid CsCl. A density gradient was established by centrifugation at 48,000 rpm at 4°C for 48 h. The gradient was partitioned into three fractions. The bottom fraction (Cr1), which contained most of the aggregated aggrecan was pooled and subjected to dialyze again distilled water. Each fraction (Cr1-Cr3) was subjected to β-elimination for releasing GAG polysaccharides from core proteins by 0.1 M $NaBH_4$/0.05 M NaOH solution at 45°C for 16 h. The resultant materials were then subjected to Pepclean™ C18 spin column (Pierce, USA) and strong anion exchange microspin column (Thermo Scientific, USA), respectively, for removing hydrophobic and cationic molecules from the GAG pellet as described previously [22]. Each eluted GAG mixture was dried and precipitated in 4 vol of cold ethanol.

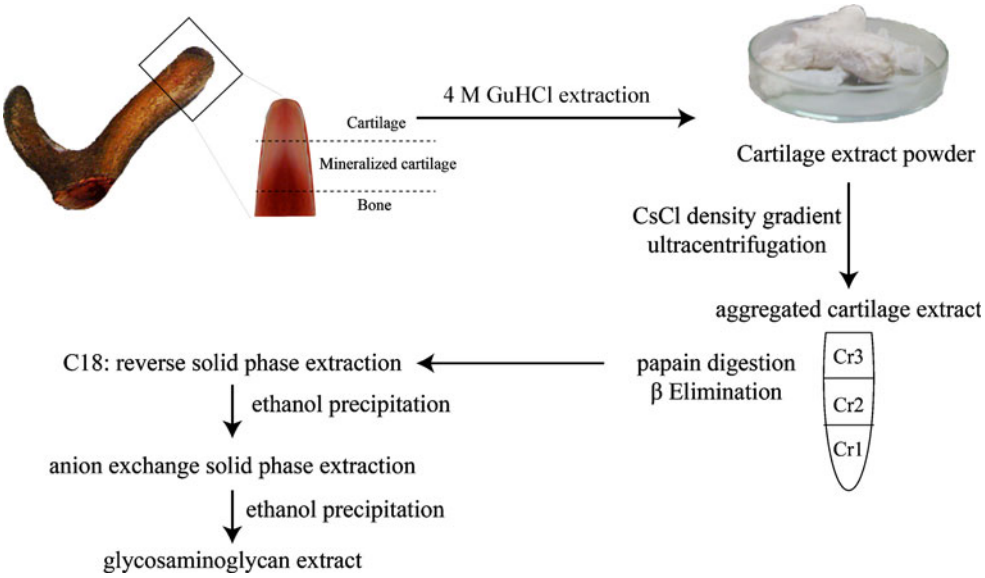

Fig. 1 Extraction and purification scheme of chondroitin sulfate from cartilaginous deer tip antler (Cr). Proteoglycans were extracted with 4 M GuHCl and sequentially isolated by CsCl density gradient ultracentrifugation. The attached proteins were removed by papain digested and β-elimination. The resultant glycosaminoglycans were further purified by C18 reverse solid phase and anion exchanged spin column as described under experiment and procedure

Analytical methods

Protein was determined by the Bradford assay [23]. Bovine serum albumin (BSA) was used as a standard.

Glucuronic acid concentrations were determined by a colorimetric assay using cabazol reagent [24] with glucuronic acid lactone as a standard. Unsaturated uronic acid was quantified spectrophotometrically based on an average millimolar absorption coefficient of 5.5 at 232 nm [25].

Sulfated glycosaminoglycan (s-GAG) was determined by the dimethylene blue (DMB) dye binding method [26]. Chondroitin sulfate C from shark cartilage was used as a standard.

Disaccharide composition analysis using 2AB labeling and HPLC

The fractions were individually incubated with 10 mIU of chondroitinase ABC (EC 4.2.2.4) in a total volume of 20 μl of 250 mM Tris–HCl buffer, pH 7.3 at 37°C for 60 min. Each digest was labeled with a fluorophore 2-AB and analyzed by anion-exchange HPLC on an amine-bound silica PA-03 column (4.6×250 mm; YMC Corp., Kyoto, Japan) eluted with a linear gradient of NaH_2PO_4 at a flow rate of 1 ml/min at room temperature for the separation of CS disaccharides [27]. Samples prepared as described above were diluted using 16 mM NaH_2PO_4 containing 5 pmol of each oligosaccharide in 50 μl. Eluates were monitored using a SpectraSYSTEM™ FL3000 fluorescence detector (Thermo Electron Corp.) with excitation and emission wavelengths of 330 and 420 nm, respectively.

2-aminobenzamide (2-AB) derivatization

The deriverization of CS disaccharide with 2-AB was performed essentially as described by Bigge *et al*. [27]. Briefly, 0.1–0.5 nmol of a given disaccharide were lyophilized in a microcentrifuge tube. An aliquot (5 μl)

Table 1 The amount total protein, sulfated glycosaminoglycan and disaccharide composition of chondroitin sulfate proteoglycan extracts

Fraction	Proteoglycan extract				Glycosaminoglycan extract	
	Density (g/ml)	Total protein (μg/mg DW)	s-GAG (μg/mg DW)	s-GAG/protein ratio[a]	s-GAG Yield[b] (μmol)	Disaccharide form[c]
Cr1	1.7342	94.1	29.6	0.31	10.57	ΔC (55.8), ΔA (49.2)
Cr2	1.6924	124.1	30.0	0.24	16.72	ΔC (27.0), ΔA (39.6), ΔD (24.1),ΔE (9.3)
Cr3	1.5851	264.1	16.7	0.06	7.57	ΔC (23.0), ΔA (35.5), ΔD (25.0),ΔE (16.0)

[a] The glycosaminoglycan and protein amounts of the cartilage proteoglycan extracts were quantified by DMB and Bradford assay

[b] The yield of extracted chondroitin sulfate oligosaccharides was quantified by the Carbazole method

[c] Disaccharide analysis was performed after digestion with chondroitinase ABC, 2AB-labeling and anion exchange HPLC. The identity of each peak was confirmed by comparing with the elution position of authentic 2AB-labeled disaccharides

of derivatization reagent mixture (0.35 M 2-AB/0.1 M $NaCNBH_4$/30% (v/v) acetic acid in dimethyl sulfoxide) was added to the disaccharide sample, and the mixture was incubated at 65°C for 2 h. The derivatized disaccharide was purified by paper chromatography using Whatman 3 MM paper in a solvent system of butanol:ethanol: water (4:1:1, v/v).

Osteogenic gene expression

Human osteoblastic cells (hFOB 1.19) were grown and treated with purified antler CS at various concentrations (10–50 μg/ml). To investigate the structural characteristics of antler CS responsible for osteogenic activity, an aliquot (0.5 mg as s-GAG disaccharide) was digested with 20 mIU of chondrotinase ABC in a total volume of 30 μl of 250 mM Tris–HCl buffer, pH 7.3 at 37°C for 2 h. Total RNA was isolated according to the manufacturer's instructions (Amersham Bioscience) after 24 h. treatment. Two microgram of total RNA were converted to cDNA using a RevertAid™ First Strand cDNA synthesis kit (MBI Fermentas, Germany). For determination of gene expression, SYBR Green detection was used and the values were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real-time quantitative polymerase chain reaction (PCR) was performed via a Chromo4™ Real-Time Detector System (Bio-Rad, USA) using SsoFast™ EvaGreen® Supermix (Bio-Rad, USA). Primer sequences (from Invitrogen) were as follows: Alkaline phosphatase (NM_001114107.2) 5′-3′ CATGGCTTTGGG CAGAAGGA; Reverse 5′-3′ CTAGCCCCAAAAA GAGTTGCAA: collagen type I (NM_000088.3) 5′-3′ CAGCCGCTTCACCTACAGC; Reverse 5′-3′ TTTTGTATT CAATCACTGTCTT GCC: osteocalcin (NM_199173.2) 5′-3′ GAAGCCCAGCGGTGCA; Reverse 5′-3′ CAC TACCTCGCTGCCCTCC: GAPDH (NM_002046.3) Forward 5′-3′ GAAGGTGAAGGTCGGAGTC; Reverse 5′-3′ GAAGATGGTGAT GGGATTTC. Relative expression levels for each primer set were normalized to the expression of GAPDH by the $2^{-\Delta CT}$ method [28].

Results

Chondroitin sulfate in deer tip antler characterization

Cartilage extract from cartilaginous antler tip was prepared by 4 M GuHCl extraction and was isolated into three fractions (from bottom to top: Cr1, Cr2 and Cr3) by density gradient ultracentrifugation. The distribution of the extracted proteoglycans after density gradient centrifugation under associative conditions is shown in Table 1. The bottom fraction (Cr1) accounted for the high molecular weight molecules with the highest density and s-GAG/protein ratio (0.31). The middle (Cr2) and the upper (Cr3) fractions contained lower molecular weight molecules with less s-GAG (s-GAG/protein ratio = 0.2 and 0.06, respectively). The peptide chains were papain digested and sequentially removed by alkaline treatment. The resultant glycosaminoglycans were further purified by Pepclean™ C18 and via a strong anion exchange spin column. Following the extraction protocol, An aliquot (500 pmol as GlcA) of the chondroitin sulfate from each fraction

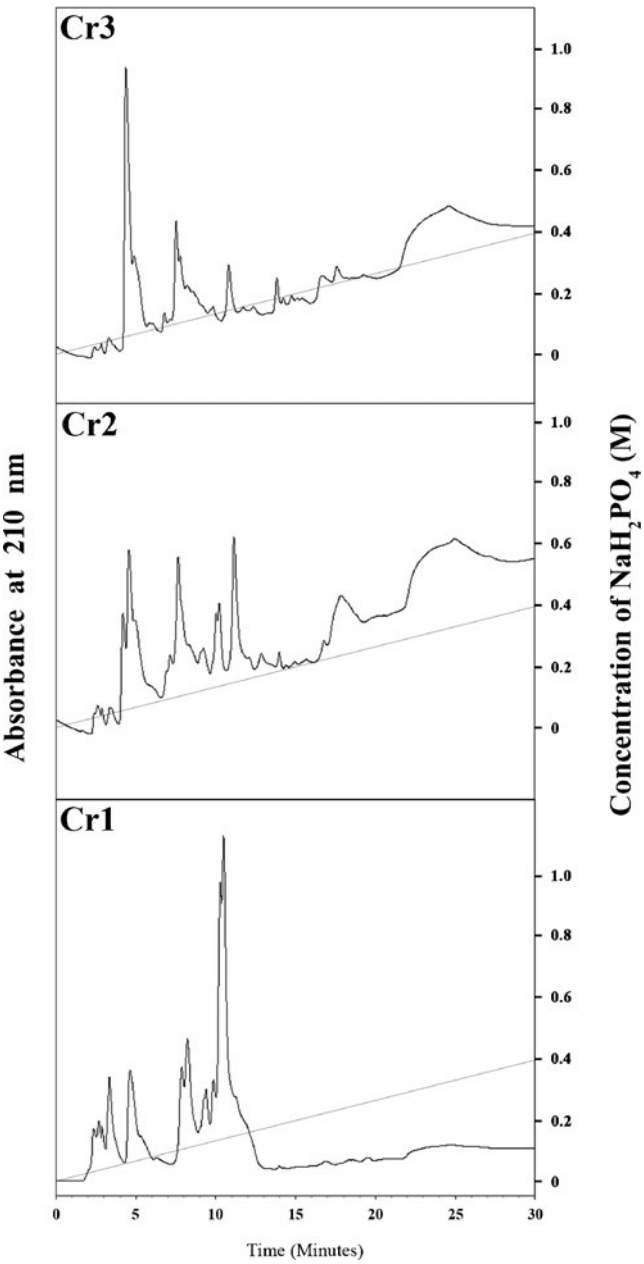

Fig. 2 Chondroitin sulfate oligosaccharide extract profiles. Purified chondroitin sulfate oligosaccharide from fraction *Cr1–Cr3* were individually analyzed on anion exchange HPLC on an amine bound silica PA-03 column using linear gradient of NaH_2PO_4 as indicated. Eluted fractions were monitored with absorbance 210 nm

were subjected to characterization by anion-exchange HPLC and determined by absorbance at 210 nm as shown in Fig. 2.

Analysis of disaccharide composition of deer antler chondroitin sulfate

To further analyze the disaccharide composition of fractionated chondroitin sulfate, the purified chondroitin sulfate oligosaccharide was exhaustively digested with chondroitinase ABC (CS'ase ABC), labeled with a 2-AB and analyzed by anion-exchange HPLC. The analysis for disaccharide composition is shown in Table 1 and Fig. 3. The Cr1 fraction contained common monosulfated chondroitin sulfate, 55% A-Unit ($\Delta^{4,5}$HexAβ1-3GalNAc(4*S*)) and 45% C-Unit ($\Delta^{4,5}$HexAβ1-3GalNAc(6*S*)) with the A-Unit/C-Unit ratio is 1:1. Interestingly, in addition to common monosulfated disaccharide units, there were significant amounts of oversulfated chondroitin sulfated D-Unit ($\Delta^{4,5}$HexA(2*S*)β1-3GalNAc(6*S*)) and E-Unit ($\Delta^{4,5}$HexAβ1-3GalNAc (4*S*, 6*S*)) found in Cr2 (24.1, 9.3%, respectively) and Cr3 (25.0, 16.0%, respectively) fraction.

Effects of deer antler chondroitin sulfate on the bone matrix protein marker gene expression

The bone matrix markers (alkaline phosphatase, collagen type I and osteocalcin) gene expression was used to investigate the osteogenic activity of deer antler chondroitin sulfate. The human osteoblastic cell line (hFOB 1.19) was treated with chondroitin sulfate oligosaccharides or chondroitinase ABC digested chondroitin sulfate oligosaccharide from fraction Cr1-Cr3 at various conditions (10–50 μg s-GAG/ml). After 24 h. treatment, total RNA was extracted and converted to cDNA. Quantitative real time PCR was performed to analyze mRNA expression (Fig. 4). Low doses (10–25 μg/ml) of common monosulfated component in Cr1 showed significant increase only with

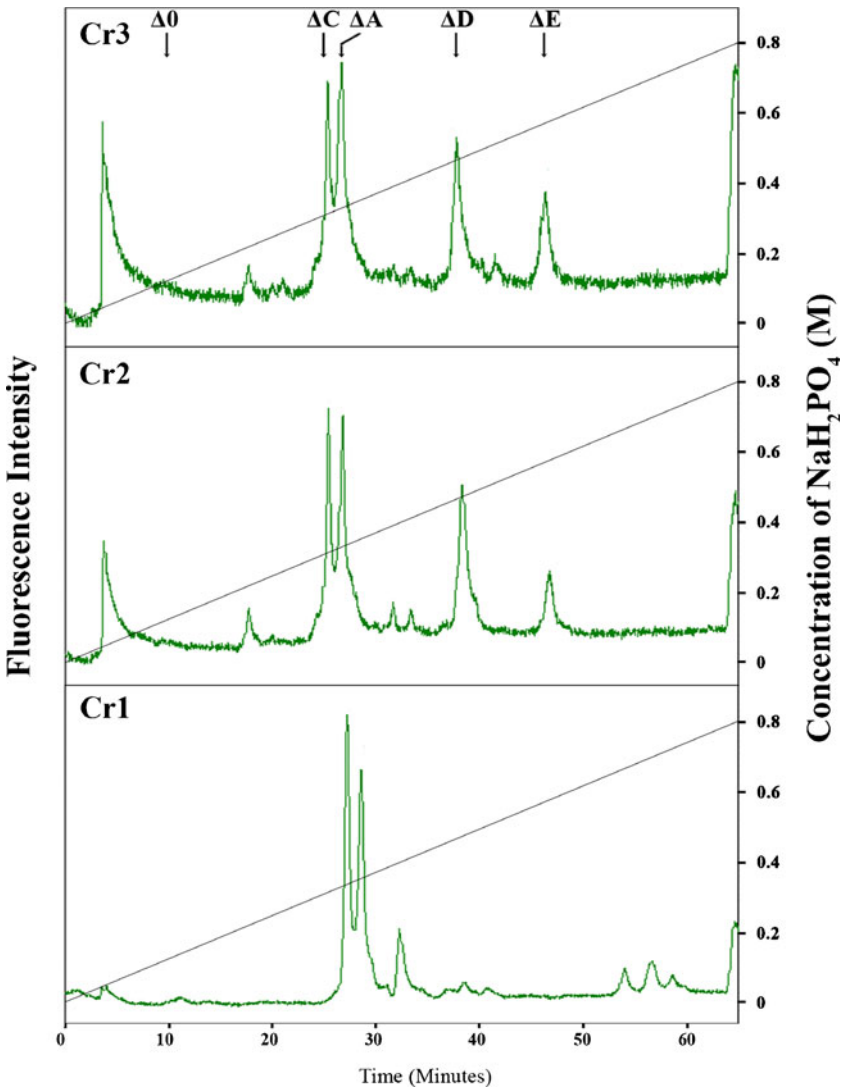

Fig. 3 Disaccharide composition of chondroitin sulfate oligosaccharide extract. Purified chondroitin sulfate oligosaccharides from fraction *Cr1–Cr3* were individually exhaustive digested with chondroitinase ABC, labeled with 2AB and subjected to anion exchange HPLC on an amine bound silica PA-03 column using linear gradient of NaH_2PO_4. The elution positions of standard 2-AB labeled unsaturated disaccharides are indicated: ΔO, ($\Delta^{4,5}$HexAβ1-3GalNAc); ΔA, ($\Delta^{4,5}$HexAβ1-3 GalNAc(4*S*); ΔC, ($\Delta^{4,5}$HexAβ1-3GalNAc (6*S*); ΔD, ($\Delta^{4,5}$HexA(2*S*)β1-3 GalNAc(6*S*); ΔE, ($\Delta^{4,5}$HexA β1-3GalNAc(4*S*, 6*S*))

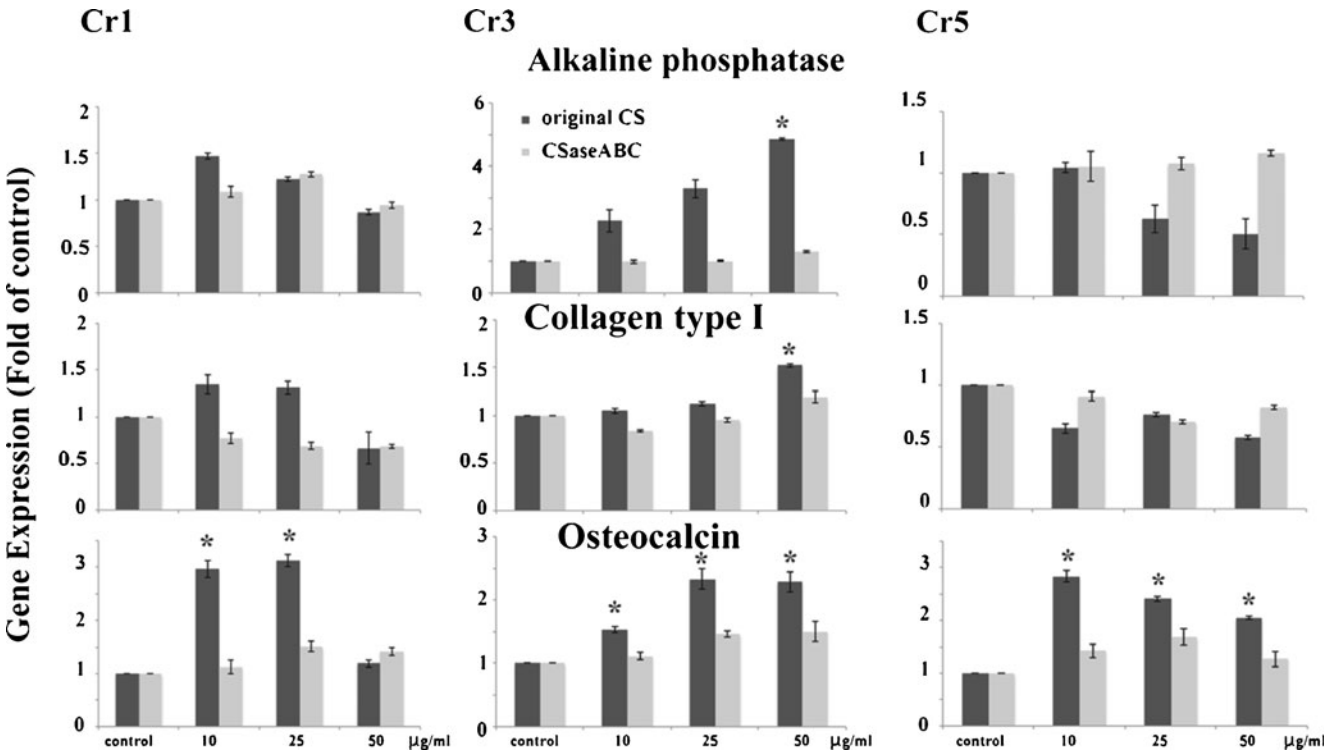

Fig. 4 Effects of antler cartilaginous chondroitin sulfate extract (*Cr1–Cr3*) on gene expression level of *alkaline phosphatase*, *collagen type I* and *osteocalcin*. Human osteoblastic cell line (hFOB1.19) was cultured in serum free DMEM at various concentration of original chondroitin sulfate or chondroitinase ABC digested chondroitin sulfate for 24 h. The mRNA concentrations were quantified and normalized to GAPDH mRNA. Values are mean ± SD. (n=3), Mean with different *superscript asterisk* within a histogram are significantly different at $p \leq 0.05$ by Man Whitney Test

osteocalcin mRNA at 2.9 and 3.1 fold, respectively. Interestingly, alkaline phosphatase, collagen type I and osteocalcin mRNA levels significantly increased when cells were treated with chondroitin sulfate extract from Cr2 with respective increases of 4.8, 1.5 and 2.3 fold at the high dose (50 μg/ml), compared with the control. In addition, the mRNA induction effects were completely abolished after exhaustive digestion with chondroitinase ABC. Chondroitin sulfate from Cr3 showed significant increase in osteocalcin mRNA level at the low dose (10–25 μg/ml).

Discussion

In this study, we characterized and investigated the biological function of chondroitin sulfate oligosaccharide purified from rusa deer (*Cervus timorensis*) antler tip. Cartilaginous antler tip mainly contains chondroitin sulfate [6], while hyaluronan, keratan sulfate, dermatan sulfate and heparan sulfate [7] are also found in smaller amounts.

The highest density gradient ultracentrifugation fraction, Cr1, accounted for the most highly aggregated major proteoglycan, which was aggrecan [29], and thus had a high content of sulfated glycosaminoglycan (s-GAG) (s-GAG/protein ratio = 0.31). The common monosulfated disaccharide A-Unit and C-Unit were detected from 2-AB labeling and HPLC analysis in a ratio of 1:1 (49.2% and 50.8%, respectively). The ratio of C-Unit/A-Unit found in antler aggrecan chondroitin sulfate is similar to that of human fetal aggrecan chondroitin sulfate, which is about 0.96 [29, 30]. The medium and low density gradient ultracentrifugation fraction, Cr2 and Cr3 contained more protein with s-GAG/protein ratios 0.24 and 0.06, respectively. Interestingly, for the first time we report the presence of oversulfated chondroitin sulfate disaccharide unit contained in antler cartilage. We found that there were D-Units and E-Units together with common monosulfated chondroitin sulfate disaccharides in the middle and low density fractions after ultracentrifugation. The chondroitin sulfate disaccharide composition for Cr2 and Cr3 were A-Unit (27.0%), C-Unit (39.6%), D-Unit (24.1%), E-Unit (9.3%) and A-Unit (23.5%), C-Unit (35.5%), D-Unit (25.0%), E-Unit (16.0%), respectively.

Bone matrix proteoglycans play roles in bone mineralization and calcium accumulation [31]. Glycosaminoglycans in addition to proteoglycan have high affinity for hydroxyapatite [32]. Chondroitin sulfate is reported to be

involved in regenerative biological process such as cell proliferation [33, 34] and wound healing [34, 35].

The development of antler begins with mesenchymal condensations and further differentiation to chondrocytes forming cartilage anlagen. Chondrocytes then form a growth plate and undergo maturation and hypertrophy, followed by the formation of growth plate, vascular invasion, and osteoprogenitor cell differentiation. During bone formation, osteoblasts produce and secrete cytokines and bone matrix proteins, which play a role in cell proliferation, mineralization, bone matrix formation and finally become compact bone [36]. In addition, it is suggested that CS-GAG side chains play a role facilitating interaction between cytokines to cell surface receptors on osteogenic potential of osteoprogenitor cells [37]. Thus, in the present study, we examined further the osteogenic activity of the antler CS oligosaccharide on human fetal osteoblastic cells line (hFOB 1.19) by measuring mRNA expression of the bone specific proteins alkaline phosphatase, collagen type I and osteocalcin, which characterise osteogenic differentiation [38–40].

It was found that only Cr2, which contained the specific oversulfated oligosaccharide motif, but not Cr3, significantly induced mRNA expression of the bone specific proteins mentioned above. This revealed that a selected oversulfated CS motif had the ability to enhance osteogenic differentiation. In a previous study it was concluded that oversulfated CS-E oligosaccharides enhanced osteoblast differentiation driven by BMP-4 [41]. Our study has now shown that, oversulfated antler CS affected osteoblast differentiation directly by upregulating collagen type I, alkaline phosphatase and osteocalcin, genes all associated with the osteoblast phenotype and required for bone mineralization.

The structure of the active CS oligosaccharide was clearly very important, as the activity was destroyed by chondroitinase digestion and furthermore, inspite of different activities, Cr2 and Cr3 fractions had similar disaccharide compositions. These finding therefore show that the bone specific mRNA inducing activity of Cr2 is dependent on the specific pattern of sulfation within the active CS oligosaccharide sequence, rather than any general activity of CS with a high oversulfated disaccharide content.

In conclusion, we reported for the first time the presence of oversulfated chondroitin sulfate: D-Units and E-Units in CS from the cartilaginous antler tip for the first time. The direct osteogenic activity of this antler oversulfated CS was demonstrated and this may play a significant role in antlerogensis. However, as the chondroitin sulfate oligosaccharides investigated are heterogeneous in composition and sequence, further studies are needed to define the specific sulfated sequence within the oversulfated chondroitin sulfate in cartilaginous antler hat form the motifs with this biological activity.

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