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Characterization and Purification of Glycosaminoglycans from Crude Biological Samples

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Chondroitin sulfate (CS) is a glycosaminoglycan derived from cartilage and commonly used to treat osteoarthritis, psoriasis, and other conditions. The dimethylmethylene blue (DMMB) assay has been used often to measure glycosaminoglycan levels in relatively pure samples. In this study, we verified the accuracy of the DMMB assay in measuring CS levels in unpurified extract from bovine trachea and shark cartilage, despite potential interference from salts, proteins, and DNA. We found that the glycosaminoglycan signal obtained was due to CS and not to other glycosaminoglycan species. This was confirmed using fluorophore-assisted carbohydrate electrophoresis, which also revealed that the majority of the CS was monosulfated at the C4 or C6 position. Finally, we used anion-exchange chromatography to purify the bovine extract and obtained complete recovery of the glycosaminoglycans, with no contaminating protein. The results of this study should be very useful for future purification and analysis of this common supplement.

KEYWORDS: Glycosaminoglycan; chondroitin sulfate; bovine cartilage; DMMB assay; FACE analysis

INTRODUCTION

Cartilage contains approximately 10% by dry weight a proteoglycan (PG), known as aggrecan, along with a further 10% of collagen type II and various other proteins and glycoproteins. Aggrecan is a PG that contains a protein component to which different types of glycosaminoglycan (GAG) chains are covalently attached. GAGs are anionic heteropolysaccharide chains of repeating disaccharide units and include hyaluronan, chondroitin sulfate (CS), keratan sulfate (KS), dermatan sulfate (DS), heparan sulfate (HS), and heparin. In the case of aggrecan, this has been purified as a CS/KS PG but has also recently been shown to contain a small amount of HS (*1*). It is these GAG chains attached to the aggrecan protein core that provide the molecule with its biological activity as a "shock absorber" and without which the cartilage becomes nonfunctional, as observed in diseases such as osteoarthritis.

CS was first isolated from cartilage in 1884, and its constitutive monosaccharides and structure were described by Levene in 1925 (*2*). He revealed that CS was comprised of D-glucuronic acid (GlcA), D-galactosamine (GalNAc), and acetic and sulfuric acids in equimolar ratios. It was later shown that CS is composed of the repeating unit [→ 4Glcβ1 → 3GalNAcβ1 →]. In mammals, this is generally sulfated at the C4 and/or C6 position of GalNAc residues. The vast majority of disaccharide units in CS molecules are monosulfated (C2S, C4S, and C6S), although non- (C0S), di-, and trisulfated units are also found (*3*).

CS is extracted from various natural sources, including shark, chicken, and bovine cartilage. There is evidence that the use of CS as a supplement can ameliorate the degenerative processes involved in osteoarthritis (*4, 5*) and contribute to the preservation of articular cartilage (*6*). CS has also been used with some degree of success in ophthalmologic diseases, such as keratoconjunctivitis, and in the prevention of coronary events in susceptible patients (*7*).

The 1,9-dimethylmethylene blue (DMMB) assay is an assay that detects all types of GAGs and has been used for many years to quantify CS in semipurified samples. It is based on changes in the absorption spectrum of the DMMB dye when bound to GAGs (*8–10*). Although studies have shown that the DMMB assay is more efficient than other assays, such as toluidine blue and Alcian blue, DMMB has a number of drawbacks. For example, the results can be markedly affected by the presence of protein (*11*), DNA (*8*), or other negatively charged particles and because of the fact that the GAG−dye complex is unstable in solution and tends to precipitate (*8, 12*). It is therefore debatable whether the assay is suitable for the measurement of CS, especially in more complex systems, such as liquid cartilage extracts that contain salts, protein, DNA, and other contaminating compounds.

Another technique that has been used for GAG analysis in recent years is fluorophore-assisted carbohydrate electrophoresis (FACE) (*13–15*). This first involves the complete release of the

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GAG chain from the glycoprotein by enzymatic or chemical means, after which the GAG chain may be further enzymatically digested into its constituent disaccharides. This is followed by stoichiometric labeling of the GAG chain/disaccharides with a fluorophore 2-aminoacridone (AMAC) and resolution by polyacrylamide gel electrophoresis. This fluorophore is used for disaccharide analysis because there is no net charge compared to that of other fluorophore labels used for oligosaccharides, which is important for low-molecular-weight isomers because it does not contribute to the migration of the CS bands. The resultant fluorescent bands can then be visualized under UV light and quantified by densitometry if desired. In combination with various sulfatases, this technique can also be used to evaluate the degree and location of sulfation on the GAG chain.

In this study, we aimed to measure the concentration of (using the DMMB assay) and to characterize (using FACE) the CS in samples of liquid bovine and shark cartilage extracts, with a view to providing data that will be useful to consumers of this supplement. We also aimed to verify the DMMB assay as an appropriate assay for quantifying CS in unpurified liquid cartilage extract and to identify and quantify other GAG components, such as KS. Further, we examined purification of the cartilage extract by anion-exchange chromatography, to remove protein and other contaminants and thus provide purified CS that is ready for use as a dietary supplement.

MATERIALS AND METHODS

Quantification of Sulfated Glycosaminoglycans Using the DMMB Metachromatic Dye-Binding Assay. This is based on the method originally described by Farndale and co-authors (*9*) and modified by Melrose and Ghosh (*16*). Appropriate dilutions of uncharacterized crude liquid cartilage extract from bovine trachea or shark cartilage (Australian Cartilage Company) were prepared. A solution of 20 μg/mL was prepared from this crude liquid extract and diluted in the same manner as the standards containing 8 (A), 12 (B), 16 (C), and 20 (D) μg/mL.

Uncharacterized crude liquid cartilage extracts were also "spiked" with a known amount of the CS standard (Sigma catalog number C4384) to verify CS measurements in the crude liquid extracts. A certificate of analysis for this product showed that it contained 6.5% water, and we have adjusted the concentration of all of the CS solutions to take this into account. Crude liquid extracts (50 μL) were also digested with chondroitinase ABC (CS spiked) or keratanase I (non-CS spiked) overnight at 37 °C in Dulbecco's phosphate-buffered saline (DPBS). Samples (50 μL) were transferred in triplicate into wells of a 96-well plate. Farndale reagent (200 μL) was added to the wells, which were read immediately at $A = 535$ nm. The GAG concentration of the unknowns was calculated from the calibration curve constructed using the CS standards. The linear range of this assay is up to 0.05 mg/mL CS (50 μg/mL).

Endoglycosidase Digestion. Lyophilized chondroitinase ABC from *Proteus vulgaris* (Seikagaku catalog number 100330) and keratanase I from *Pseudomonas* sp. (Seikagaku catalog number 100810) were dissolved in MilliQ H_2O at 10 units/mL for the stock solutions. Digestion was at 50 milli-units/mL (final concentration) of chondroitinase ABC and 25 milli-units/mL keratanase I overnight at 37 °C in DPBS at pH 7.4. Lyophilized chondro-4-sulfatase from *P. vulgaris* (Seikagaku catalog number 100350), chondro-6-sulfatase from *P. vulgaris* (Seikagaku catalog number 100355), chondroitinase ACII Arthro from *Arthrobacter aurescens* (Seikagaku catalog number 100335), and chondroitinase B from *Flavobacterium heparinum* (Seikagaku catalog number 100337) were dissolved in MilliQ H_2O at 10 units/mL for the stock solutions. Digestions were at 100 milli-units/mL (final concentration) of either chondro-4-sulfatase, chondro-6-sulfatase, chondroitinase ACII, or chondroitinase B and were performed overnight at 37 °C in 0.1 M ammonium acetate in 0.025% bovine serum albumin (BSA) (pH 7.4, 7.4, and 6.2, respectively) or in 0.33 μM calcium acetate at pH 8.0 for chondroitinase B.

Table 1. Protein (BCA Method) and GAG (DMMB Assay) Levels in Bovine (**A**) and Shark (**B**) Cartilage Extract Samples

	protein (mg/mL, mean ± SD)	sulfated GAG (mg/mL, mean ± SD)
A		
sample 1	35.3 ± 2.7	16.1 ± 0.6
sample 2	59.8 ± 5.6	32.9 ± 1.1
sample 3	96.4 ± 4.0	25.5 ± 0.1
B		
sample 1	68.0 ± 3.3	10.8 ± 0.3
sample 2	79.0 ± 2.2	6.0 ± 1.8

Protein Estimation Using the Bicinchoninic Acid (BCA) Assay. This method was originally described by Smith and co-workers (*17*) and is based on the reduction of copper (from Cu^{2+} to Cu^{1+}) in alkali conditions, similar to the principle of the Lowry method.

FACE Analysis. This method has been adapted from the methods of Lehrman and Gao (*18*), Calabro et al. (*19*), and a patented method (*20*). It is useful for analyzing disaccharides of CS, HS, and KS origin.

For FACE labeling (derivation) of standard CS disaccharides (Δdi-0S, Δdi-4S, Δdi-6S, Sigma-Aldrich) with 2-aminoacridone hydrochloride (AMAC; Fluka), each standard Δ-disaccharide (50 nmol in 0.1 M ammonium acetate) was completely evaporated in a 1.5 mL microcentrifuge tube using a speed vacuum (no/low heat setting). After electrophoresis, gels were imaged by UV light using the Gel Doc system (BioRad) while still in their glass support plates. Analysis of bands was performed by comparing their migration and pixel intensity to those of the standard Δ-disaccharides, run on the same gel.

Anion-Exchange Chromatography. The pH of the bovine cartilage extract was adjusted to approximately 7 with 4 M NaOH, and 5 mL of this extract was added to 5 mL of DPBS. Using the BioRad Biologic LP system, the resultant solution was loaded onto a column containing approximately 2 mL of Q-Sepharose Fast Flow resin (Sigma Aldrich), after pre-equilibration of the column with 10 mL of DPBS. The flow through was collected, and after washing the column with 15 mL of DPBS, the bound fraction was eluted with 10 mL of 1 M NaCl in DPBS. Samples were concentrated via precipitating the glycosaminoglycans with 4 volumes of ethanol followed by centrifugation at 14000*g*.

RESULTS AND DISCUSSION

Measurement of GAG and Protein Concentration in Extracts. It was important to determine the GAG concentration in each of the sample extracts, as well as the protein concentration, particularly because protein has been reported to negatively interfere with the results of the DMMB assay (*11*). GAG levels (DMMB assay) and protein levels (BCA method) varied substantially between the extract samples analyzed, as indicated in **Table 1**. The pH for each of the samples was in the range of 4.5−5.5. The presence of substantial levels of protein was anticipated, because this is an extract obtained directly from protease treatment of cartilage, with no purification at this stage, except for the manual removal of fatty material and other solids. Note that the GAG levels did not necessarily correlate with those of protein, with sample 3 (**Table 1A**), for example, having the highest protein levels but not GAG levels. The reason for this is likely to be due to variability in both the starting cartilage tissue and in the extraction process. There is no logical reason, however, that this influences the conclusions of this study.

Measurement of Sulfated Glycosaminoglycans: Verification of the DMMB Assay on Crude Cartilage Extracts. Because we were planning to measure CS in crude biological samples that are ill-defined, it was important to rigorously verify the DMMB assay and demonstrate that the signal from the assay was actually derived from CS and not a mixture of CS, KS, and DS or derived from non-PG contaminants, such as DNA. The low pH

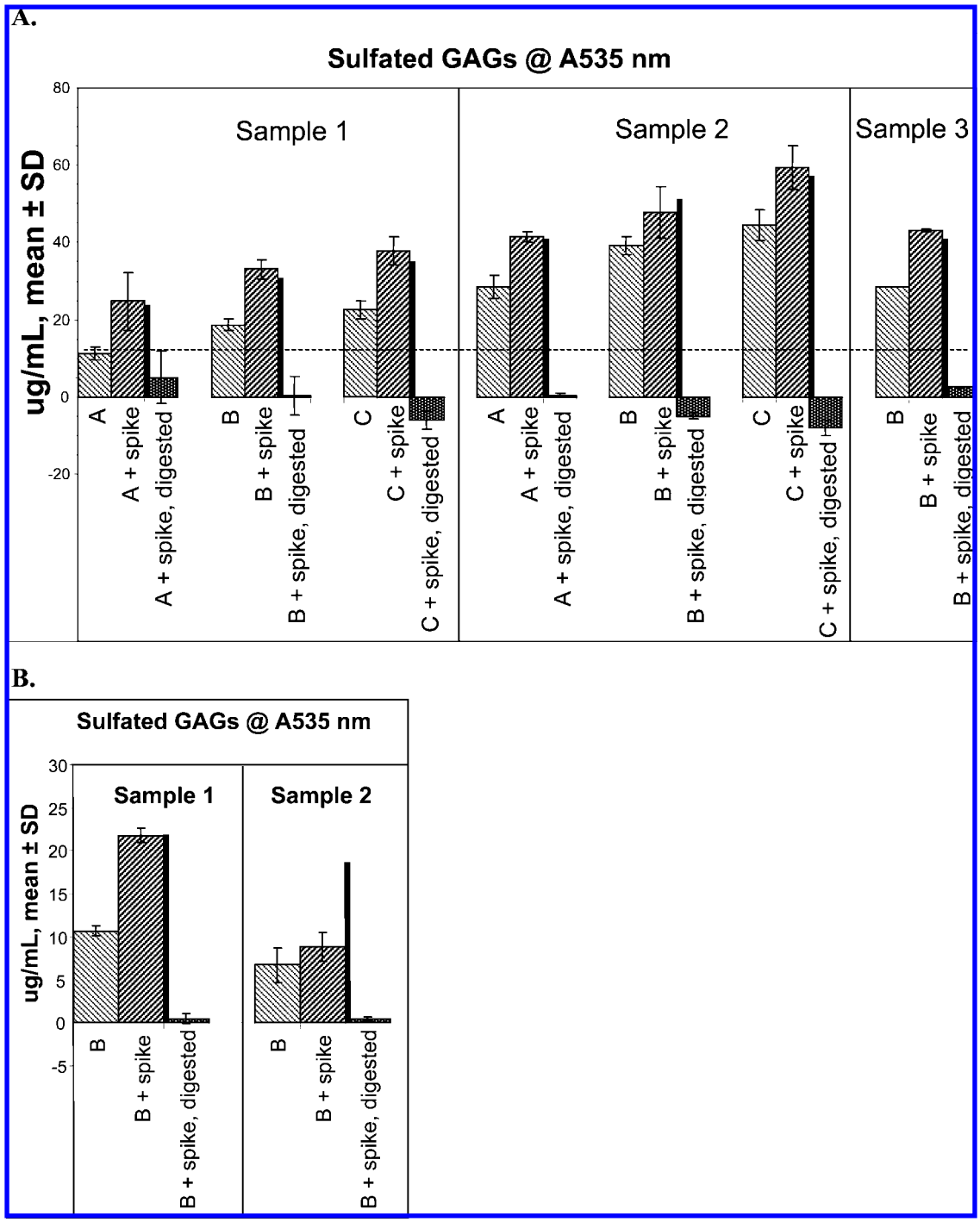

Figure 1. DMMB assay of sulfated GAG levels in bovine and shark cartilage extract before and after spiking with CS and digestion with chondroitinase ABC. Bovine cartilage extract (samples 1−3) (**A**) and shark cartilage extract (samples 1 and 2) (**B**) were "spiked" with a known amount of the standard CS (12 μg/mL) to verify CS measurements in the crude cartilage extracts. The narrow solid black bar indicates the predicted GAG level after CS spiking. Samples (with CS spike) were also digested with chondroitinase ABC overnight at 37 °C in DPBS. Samples (50 μL) were then transferred in triplicate into wells of a 96-well plate. Farndale reagent (200 μL) was added to the wells, which were read immediately at $A = 535$ nm. The GAG concentration of the unknowns was calculated from the calibration curve constructed using CS standards. The dotted line indicates the minimum concentration reliably measurable in this assay.

and high salt concentration of the reagent used in the assay results in a negligible signal from hyaluronan and DNA (*21*). Our approach to verify the assay was 2-fold. First, we spiked each of the samples with a known amount of purified CS and measured the amount of CS before and after the spike (**Figure 1**). This is an approach used previously to analyze extracts from skin and muscle (*22*). Second, we digested the samples with chondroitinase ABC (this included the CS-spiked samples) and keratanase I (bovine cartilage samples 1 and 2 only, non-CS spiked; **Figure 2**) to ascertain how much of the signal obtained from the DMMB assays was due to the signal provided by each of the sulfated GAG types (i.e., CS, DS, or KS). It was also important to perform multiple dilutions of the sample, because the DMMB assay is only linear over a small concentration range (0–0.05 mg/mL of CS).

By comparing the concentrations measured after spiking the samples with the known concentrations of CSA with the predicted concentrations (**Figure 1**), we observed that the two

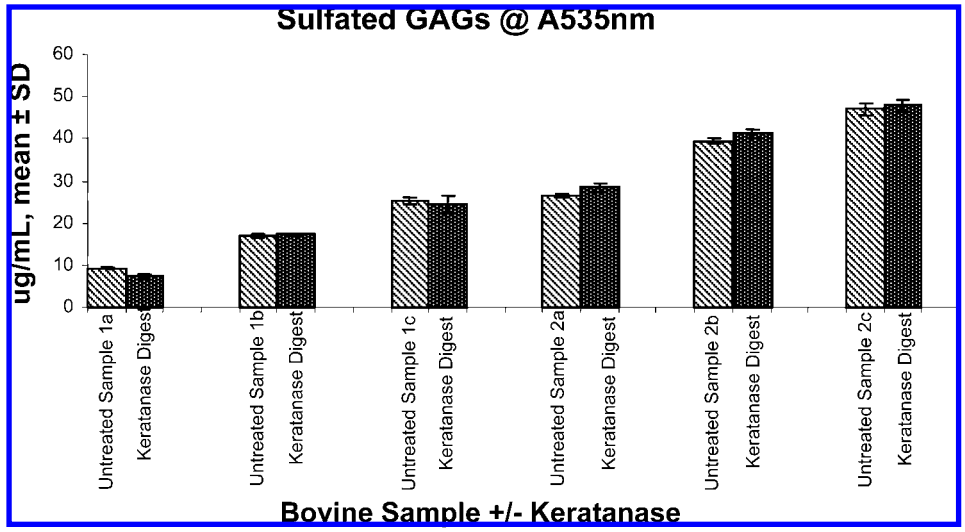

Figure 2. DMMB assay of GAG levels in bovine cartilage extract (samples 1 and 2) before and after digestion with keratanase I. Cartilage extract samples were digested with keratanase I overnight, and a DMMB assay for GAG levels was conducted before and after keratanase I digestion. Experimental details are as indicated in the caption for **Figure 1**.

values were within the error of the assay for seven of the nine measurements (with only sample 2, **Figure 1B**, showing a substantial difference between the predicted and measured results). This suggested that overall we were able to accurately and confidently measure the amount of CS added to each of the biological samples and that the protein present in the extract (**Table 1**) did not, under these conditions, have a marked inhibitory effect on the assay that has been previously noted for protein contaminants (*11*), and our results here are in agreement with studies performed previously on skin and muscle extracts (*22*).

In support of this data, we performed chondroitinase ABC digestion of the samples (both spiked and nonspiked) and compared the concentration of CS before and after the digestions. The use of chondroitinase ABC digestion is an important additional step, which was not performed by previous investigators. Digestion with chondroitinase ABC abolished the GAG signal (**Figure 1**), indicated by a signal of <0.01 mg/mL (below the limit of detection of the assay). In contrast, keratanase I digestion (**Figure 2**, bovine samples 1 and 2 only, non-CS spiked) had no effect on the signal obtained in the DMMB assay. When these results were taken together, they provided strong evidence that the signal obtained in the assay was from CS and not from KS or contaminating macromolecules that are also capable of producing a signal in the DMMB assay, such as DNA (*8*).

FACE Analysis. Samples of the three different types of disaccharides that make up the bulk of CS in cartilage were analyzed by FACE. The FACE methodology enables us to estimate the composition of chondroitin-0-sulfate (unsulfated, C0S), chondroitin-4-sulfate (C4S, CSA), and chondroitin-6-sulfate (C6S, CSC) within a sample, giving the relative percentage of the C4S and C6S disaccharides. As can be seen in **Figure 3A**, the migration rates through the gel are different for each of the disaccharides, with the unsulfated disaccharide migrating the slowest.

Digestion of the purified aggrecan standard, unpurified bovine, or shark cartilage extract with chondroitinase ABC results in a band in FACE analysis that comigrates with the C4S and C6S standards [**Figures 3B** (lane 4), **4** (lane 4), and **5** (lane 5)]. Chondroitinase ABC will digest hyaluronan inefficiently at pH 7.4, and as such, this might be a minor component of the digested extract, comigrating with the C0S standard. Treatment

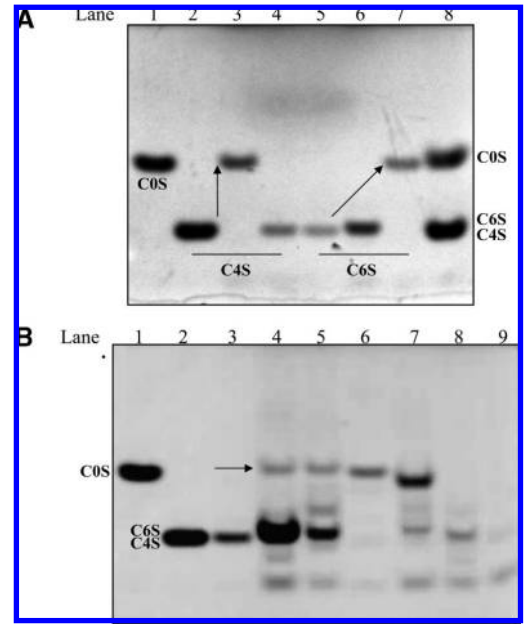

Figure 3. FACE gel analysis of CS Δ-disaccharide and aggrecan standards. CS disaccharides (**A**) and aggrecan (**B**) standards were analyzed by FACE. The samples in each lane were chondroitin-0-sulfate (unsulfated standard C0S, Δdi-0S, lane 1), chondroitin-4-sulfate (C4S, Δdi-4S, lane 2), and chondroitin-6-sulfate (C6S, Δdi-6S, lane 5). Digestion of Δdi-4S with C4 sulfatase (lane 3) and digestion of Δdi-6S with C6 sulfatase (lane 7) both resulted in a shift in mobility of the bands (arrowed in each case). Lanes 4 and 6 show the treatment of Δdi-4S with C6 sulfatase or Δdi-6S with C4 sulfatase, respectively. Lane 8 shows the CS standards run together. Purified aggrecan standard (3B) from bovine articular cartilage (Sigma-Aldrich, catalog number A1960) was analyzed by FACE. The samples in each lane were C0S (Δdi-0S) in lane 1, C4S (Δdi-4S) in lane 2, and C6S (Δdi-6S) in lane 3. Digestion of aggrecan with chondroitinase ABC (lane 4). Digestion of aggrecan with both chondroitinase ABC and C4 sulfatase (lane 5) or chondroitinase ABC and C6 sulfatase (lane 6). The arrow in lane 4 indicates an increase in intensity of the band migrating with the C0S standard. Lanes 7 and 8 show digestion with either keratanase I or chondroitinase ACII, respectively. Lane 9 shows undigested aggrecan.

of this chondroitinase ABC-digested cartilage extract with C4 sulfatase [**Figures 3B** (lane 5), **4** (lane 5), and **5** (lane 6)], which

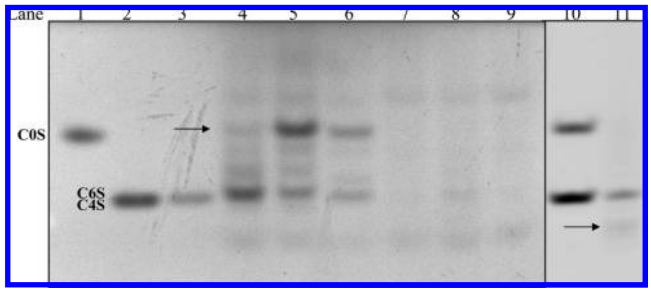

Figure 4. FACE gel analysis of crude and anion-exchange-purified bovine cartilage extract. A sample of bovine cartilage extract was analyzed by FACE. Lane 1 contains the C0S (Δdi-0S) standard, while lane 2 contains the C4S (Δdi-4S) standard and lane 3 contains the C6S (Δdi-6S) standard. Lane 4 shows digestion with chondroitinase ABC, while lanes 5 and 6 show digestion with both chondroitinase ABC and C4 sulfatase or chondroitinase ABC and C6 sulfatase, respectively. The arrow in lane 4 indicates an increase in intensity of the C0S disaccharide. Lanes 7−9 show digestion with keratanase I, chondroitinase ACII, or the undigested control, respectively. The inset containing lanes 10 and 11 shows CS standards run together (lane 10) alongside chondroitinase-ABC-digested material purified by anion-exchange chromatography (lane 11). The arrow in lane 11 indicates a potential artifact from labeling (*23*), which can also be seen in the unpurified extract (lanes 4−9).

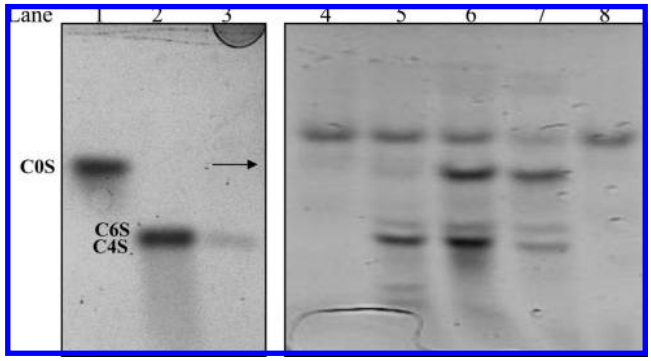

Figure 5. FACE gel analysis of crude shark cartilage extract. A sample of shark cartilage extract was analyzed by FACE. Lane 1 contains the C0S standard (Δdi-0S), while lane 2 contains the C4S (Δdi-4S) and lane 3 contains the C6S (Δdi-6S) standards. The arrow indicates the position of the C0S standard. Lane 4 shows digestion with chondroitinase B. Lanes 5−7 show digestion with chondroitinase ABC, both chondroitinase ABC and C4 sulfatase, and both chondroitinase ABC and C6 sulfatase (lane 7). Lane 8 shows the undigested control.

removes the sulfate group at the C4 position of GalNAc residues, results in diminution of the band comigrating with the C4S and C6S disaccharides for purified aggrecan and unpurified bovine cartilage extract but not for the unpurified shark cartilage extract. Concurrently, a shift in the mobility of this band is observed (indicated by an arrow), so that it now migrates with the C0S standard. This indicates that a large proportion of the CS in the cartilage extract sample, particularly in the unpurified bovine cartilage extract, is sulfated at the C4 position of GalNAc residues. This effect is also observed when C6 sulfatase is used [**Figures 3B** (lane 6), **4** (lane 6), and **5** (lane 7)]. The shift in the mobility of the band for the bovine extract is not as intense as when C4 sulfatase is employed; conversely, when using the purified aggrecan or shark cartilage extract, the shift in the mobility of the band from C6 sulfatase is observed in parallel with the diminution of the band comigrating with the C4S and C6S disaccharides. This indicates that, in aggrecan and the shark extract, a larger proportion of the CS disaccharides are sulfated at the C6 position of the GalNAc residues, while in the bovine extract, a larger proportion are sulfated at the C4 position.

Keratanase I overnight digestion of the purified aggrecan standard (lane 7 in **Figure 3B**) results in a band that represents GlcNAc6S1 → 3Gal (a keratanase-I-specific product), whereas keratanase I digestion of unpurified bovine cartilage extract (lane 7 in **Figure 4**) had no apparent effect. This may reflect the differing amounts of KS from these cartilage sources, because the purified aggrecan is from articular cartilage, whereas the unpurified bovine extract is from tracheal cartilage.

Digestion of purified aggrecan standard and the unpurified bovine cartilage extract (lane 8 in **Figures 3B** and **4**) with chondroitinase ACII, which has similar specificity to chondroitinase ABC but does not cleave DS, results in a band that comigrates with the C4S and C6S standards. This confirms that this band contains CS and is not exclusively DS. Digestion of the unpurified shark cartilage extract (lane 4 in **Figure 5**) with chondroitinase B, which cleaves DS only, had no apparent effect.

These results for the bovine extract correlate with those of Lammi et al. (*23*), who used FACE analysis to examine the sulfation levels in chondroitin sulfate in bovine patellae and found that C4S and C6S were the most abundant isoforms, with C0S comprising only about 2–8%. Along with previous reports that CS is generally monosulfated in mammals (*3*), this indicates that the CS in the cartilage extract that we examined is present in its naturally sulfated form. This suggests that any beneficial effects arising from ingestion of supplemental CS would be obtained when using CS derived from this extract. We also observed fluorescent material below the position of the C4S and C6S bands (indicated by the arrow in the inset of **Figure 4**). This is thought to represent small amounts of C2S and CSE (a disulfated isoform), as well as labeling artifacts (*23*).

Anion Exchange. After processing sample bovine cartilage extract on the Q-Sepharose column, the recovery rate of GAGs as measured by DMMB assay was 101.3 ± 5.7% (mean ± standard deviation, $n = 2$); meaning that 101.3% of the GAG recorded in the original cartilage extract was present in the "sample" fraction. Negligible levels of GAGs (below the limit of detection of the assay) were recorded in the flow-through and wash fractions.

There was negligible protein (below the limit of detection of the BCA technique) present in the sample fraction after running through the Q-Sepharose column; thus, the fraction containing the GAGs contained almost no contaminating protein. In contrast, 100% of the protein in the original sample was recovered in the column flow through, which, as mentioned above, did not contain detectable levels of GAGs.

A total of 10 mL of the purified sample was ethanol-precipitated, dried, and dissolved in H_2O to a final concentration of 100 mg/mL. We found that there was no loss of GAG material in this process, as measured by the DMMB assay. A sample of the resultant solution was then digested with chondroitinase ABC, AMAC-labeled and electrophoresed on a FACE gel (inset of **Figure 4**). A band can be seen that comigrates with the C4S and C6S standards, as observed for FACE analysis of the unpurified bovine extract (lane 4 in **Figure 4**), with low levels of C0S, because hyaluronan does not bind to the anion-exchange column under the conditions that we used in these studies. When these results are taken together, they demonstrate the efficiency of anion-exchange techniques for the purification of CS-containing bovine cartilage extract and indicate that CS purified using this method should be ready for use as a dietary supplement.

In the study described here, we have validated the use of the DMMB assay as a reliable and accurate measure of the levels of soluble CS in crude cartilage extracts. We have confirmed this by, first, spiking samples with known concentrations and then, second, degrading the CS specifically with the endoglycosidase, chondroitinase ABC. We have found that the concentration of CS varies among different samples of liquid cartilage extract, by as much as a factor of 2. We have also found that the levels of protein present in the liquid cartilage extracts are variable, with them being between 35 and 96 mg/mL. It is important to note that even though the protein contaminated the original liquid extracts, it was removed with the use of a further purification step involving anion-exchange chromatography. This gave fractions that contained almost exclusively CS GAG.

We have also confirmed that the FACE gel technology is a highly useful technique for analysis and identification of the CS disaccharides present in these same crude biological samples. The CS present in the cartilage extracts was found to contain either predominantly C4S or C6S, with low levels of C0S. These proportions were related to the species used to source the tissue, with the shark tissue giving predominantly C6S, while the bovine tissue contained mostly C4S disaccharides. This indicates that this method will be suitable to supply information on these types of products that will be suitable for manufacturers, consumers, and regulators.

ABBREVIATIONS USED

CS, chondroitin sulfate; GAG, glycosaminoglycan; DMMB, dimethylmethylene blue; FACE, fluorophore-assisted carbohydrate electrophoresis; PG, proteoglycan; KS, keratan sulfate; DS, dermatan sulfate; HS, heparan sulfate; GlcA, D-glucuronic acid; GalNAc, D-galactosamine; DPBS, Dulbecco's phosphate-buffered saline; BSA, bovine serum albumin.

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