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Adaptation of FACE methodology for microanalysis of total hyaluronan and chondroitin sulfate composition from cartilage

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Protocols for analyzing the fine structure of hyaluronan and chondroitin sulfate using fluorophore-assisted carbohydrate electrophoresis of 2-aminoacridone-derivatized hyaluronidase/chondroitinase digestion products were adapted for direct analysis of previously characterized cartilage-derived samples. The chondroitin sulfate disaccharide compositions for fetal and 68 year human aggrecan from FACE analyses were ΔDi4S (50%), ΔDi6S (43%), and ΔDi0S (7%); and ΔDi4S (3%), ΔDi6S (96%), and ΔDi0S (1%), respectively. The nonreducing terminal structures included predominantly 4S-galNAc with minor amounts of 6S-galNAc and Di6S for the fetal aggrecan sample and, in addition, included 4,6S-galNAc in the 68 year aggrecan sample. FACE analysis of a proteinase K digest of rat chondrosarcoma tissue gave an internal disaccharide composition for its chondroitin sulfate chains of ΔDi0S (7%) and ΔDi4S (93%) with no ΔDi6S and ΔDi4,6S detected, while ΔDiHA from hyaluronan was 5% of the total. Analysis of nonreducing terminal structures indicated the presence of 4S-galNAc (51%), galNAc (27%), and Di4S (22%) with no 4,6S-galNAc or Di6S detected. Unexpectedly, FACE analysis detected putative linkage oligosaccharide structures from the chondroitin sulfate chains including both unsulfated (85%) and 4-sulfated (15%) linkage oligosaccharides. Finally, the number averaged chain length estimated from the ratio of the molar fluorescence of the Δdisaccharides to that of the nonreducing termini or the linkage oligosaccharide structures was calculated as ~16 kDa. A tissue glucose concentration of 0.72 g/l was also measured. These results for both samples as determined by FACE analysis were similar to results previously reported, using more labor and time intensive procedures, validating the FACE protocols.

Key words: aggrecan/cartilage/chondroitin sulfate/fine structure/hyaluronan

Introduction

The biological importance of the fine structures of glycosaminoglycans has been well demonstrated for heparin and heparan sulfate by the finding that particular sulfated oligosaccharide sequences within these glycosaminoglycans can confer growth factor or anticoagulant activity (see Turnbull *et al.*, 1992; Bourin and Lindahl, 1993, for reviews). Circumstantial evidence exists that chondroitin sulfate fine structure has biological roles. For example, as the most accessible portion of chondroitin sulfate chains attached to proteoglycan core proteins, nonreducing terminal structures have been proposed to have a biologic role in tissues such as cartilage. The nonreducing termini of chondroitin sulfate chains from aggrecan purified from fetal to elderly human cartilage contain distinctly different ratios of variably sulfated galNAc residues which are characteristic and diagnostic of the age of the patient (Plaas *et al.*, 1997). Sulfated galNAc has been shown to be the predominate terminal sugar on aggrecan chondroitin sulfate synthesized *in vitro* by rat and chick cartilages (Otsu *et al.*, 1985) as well as by rat chondrosarcoma cells (Midura *et al.*, 1995). Furthermore, modification of this terminal galNAc residue during chain polymerization by specific sulfotransferases has been suggested as a mechanism for chain termination (Silbert, 1991; Midura *et al.*, 1995). In addition, antibodies raised to antigenic determinants on chondroitin sulfate chains have shown reactivity with antigens that have distinct distributions between tissues, and are variably expressed in the extracellular matrix during differentiation and development (see Hascall *et al.*, 1995, for review). Together these results suggest that unique sulfated sequences are also present on the chondroitin sulfate of proteoglycans conferring biological activities.

Therefore, an ability to elucidate the composition and fine structure of chondroitin sulfate is a necessary step in the effort to determine the role of specific structures in biologic processes. However, previous methods for analysis of chondroitin sulfate fine structure have required relatively large amounts of starting tissue for analysis, and involved time and labor intensive steps for isolation and purification of tissue proteoglycans, glycosaminoglycans, and their chondroitinase digestion products prior to analysis. Also the analyses themselves have proved time and labor intensive, normally allowing for only one sample to be analyzed at a time, usually by methods such as HPLC chromatography or capillary zone electrophoresis. We have therefore adapted the FACE protocols described in the accompanying article (Calabro *et al.*, 2000) for direct analysis of the internal disaccharide, nonreducing terminal and linkage oligosaccharide structures of chondroitin sulfate in tissues using a predominantly single tube assay system. The

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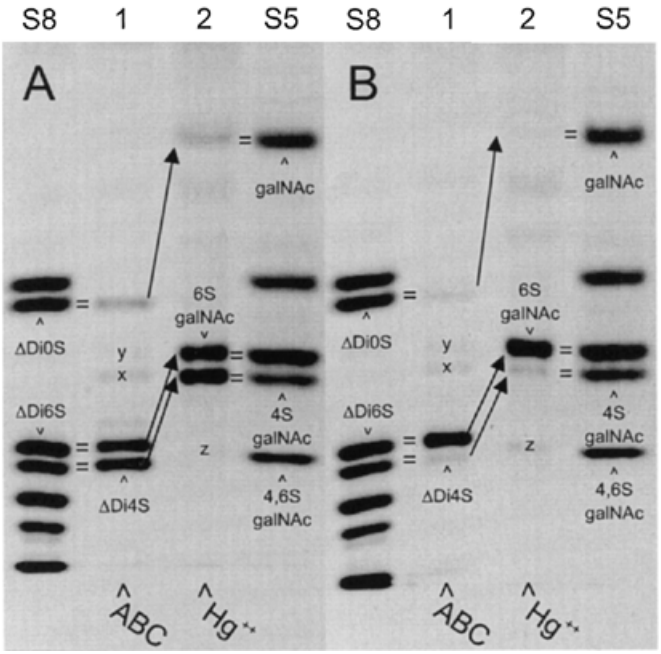

Fig. 1. FACE analyses of chondroitin sulfate chains isolated from aggrecan prepared from fetal (**A**) or 68 year (**B**) human cartilage. Samples were chondroitinase ABC digested then AMAC derivatized (ABC, lanes 1) or chondroitinase ABC digested followed by mercuric ion treatment then AMAC derivatized (Hg^{2+}, lanes 2). Analysis of the internal disaccharide composition and the nonreducing termini (x, y, z) are highlighted. A shift in mobility of a band from that of its original derivatized Δdisaccharide structure to that of its derivatized product after mercuric ion treatment is indicated by an arrow. The standard lanes, S8 and S5, contain a mixture of either eight AMAC-derivatized Δdisaccharides from top to bottom: ΔDiHA, ΔDi0S, ΔDi6S, ΔDi4S, ΔDi2S, ΔDi4,6S, ΔDi2,6S, and ΔDi2,4,6S or five AMAC-derivatized hexosamines from top to bottom: galNAc, glcNAc, 6S-galNAc, 4S-galNAc, and 4,6S-galNAc, respectively. The ΔDi2,6S and ΔDi2,4,6S standards in lanes S8 migrate together at the electrophoresis front.

sensitivity of the FACE protocols allows for analysis of hyaluronan and chondroitin/dermatan sulfate in as little as 25 μg of wet weight or 1–2 μg of dry weight of cartilage tissue which makes even topographical analysis of glycosaminoglycan composition in tissues feasible. FACE analyses of the fine structure of chondroitin sulfate chains on purified human aggrecan and in rat chondrosarcoma tissue are compared to previously published results to validate the new methodologies.

Results

FACE analysis of chondroitinase digestion products of chondroitin sulfate chains from purified human aggrecan preparations

Figure 1 provides evaluation of the FACE protocols for two purified aggrecan samples from the knee cartilages of a human fetus and a 68 year adult. Both samples have been extensively analyzed previously using capillary zone electrophoresis, and 2-aminopyridine/AS4S anion exchange chromatography (Plaas *et al.*, 1997). In Figure 1A, the fetal aggrecan contained ΔDi4S (50%), ΔDi6S (43%), and ΔDi0S (7%) as previously detected (lane 1). The identities of the ΔDi0S, ΔDi4S, and ΔDi6S bands were confirmed after mercuric ion treatment, which yields derivatized galNAc, 4S-galNAc, and 6S-galNAc, respectively, in the appropriate molar amounts (lane 2). As expected, the major nonreducing terminus was 4S-galNAc (lane 1, band x), but a significant amount of 6S-galNAc was observed, which was not detected previously (band y). Also observed, was the presence of a minor amount of nonreducing termini that is probably Di6S, since no 4,6S-galNAc, which comigrates with Di6S, was observed in the previous analysis (lane 2, band z).

In Figure 1B, the adult sample contained almost exclusively ΔDi6S (96%) with a small amount of ΔDi4S (3%) and ΔDi0S (1%) (lane 1), the identities of which were confirmed by mercuric ion treatment (lane 2). The appearance of the 4,6S-galNAc (lane 2, band z), characteristic of the termini on the adult chondroitin sulfate chains, was apparent and was approximately equal to the 4S-galNAc terminus (lane 1, band x) as observed previously. Minor amounts of Di6S observed in the original analyses may also be present, since it comigrates with the 4,6S-galNAc. Again, the presence of 6S-galNAc, which was not detected previously, was also observed (lane 1, band y). The Di4S, which was detected previously (Plaas *et al.*, 1997), was not observed in either aggrecan preparation. No derivatized products were detected in either of the aggrecan samples that were derivatized directly without chondroitinase digestion (data not shown) indicating the absence of any preexisting saccharides with free reducing groups. As predicted from the chemistry (see Figure 1, Calabro *et al.*, 2000), the intensities of the major derivatized Δdisaccharides from both aggrecan preparations, and their respective derivatized hexosamines generated after mercuric ion treatment should be the same, and were all within 5% or less of each other.

Complete FACE analysis of the hyaluronidase and chondroitinase digestion products directly from a proteinase K digest of rat chondrosarcoma tissue

The protocols for complete FACE analysis of the fine structure for hyaluronan and chondroitin sulfate as described in the accompanying article (Calabro *et al.*, 2000) were adapted for direct analysis of tissue digests as described in the *Materials and methods*. Rat chondrosarcoma tissue was chosen to test these protocols because of the detailed information that already exists about the fine structure of the chondroitin sulfate chains on aggrecan, the predominant proteoglycan in this tissue (Shibata *et al.*, 1992; Midura *et al.*, 1995; Plaas *et al.*, 1996). Figure 2A shows a single gel image containing the complete FACE analysis for this tissue. The image was exposed to over saturate pixel intensities for the major derivatized structures allowing visualization of less abundant derivatized structures. In Figure 2B, the gel image in panel A is repeated with all the bands in each lane from top to bottom referenced by lowercase letters. Lanes 1 through 6 represent the AMAC-derivatized products from six identical aliquots of the proteinase K digest of the tissue which were each processed differently as described below and in *Materials and methods*.

One aliquot of the tissue digest was derivatized directly in order to detect any endogenous saccharides containing free reducing groups (dAMAC, lane 1). The remaining five aliquots were digested with a combination of hyaluronidase SD, an eliminase with specificity for hyaluronan, and chondroitinase ABC, an eliminase with specificity for both hyaluronan and

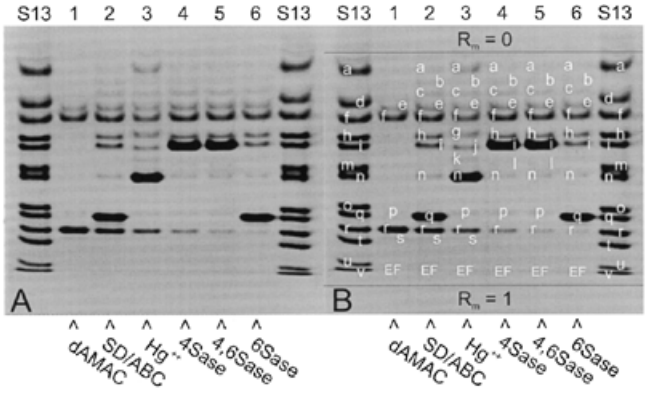

Fig. 2. (**A**) Complete FACE analyses of hyaluronan and chondroitin sulfate chains from a proteinase K digest of Swarm rat chondrosarcoma tissue. Samples were AMAC derivatized: directly (dAMAC, lane 1), after hyaluronidase SD and chondroitinase ABC digestion alone (SD/ABC, lane 2), or after subsequent mercuric ion treatment (Hg^{2+}, lane 3), chondro-4-sulfatase digestion (4Sase, lane 4), chondro-4-sulfatase and chondro-6-sulfatase digestion (4,6Sase, lane 5), or chondro-6-sulfatase digestion (6Sase, lane 6). (**B**) Assignment of lowercase letters for each of the bands in the gel from (**A**) as referenced in the text. Positions corresponding to a relative mobility (R_m) of 0 and 1 in Figure 4 are indicated. The standard lanes (S13) contain a mixture of thirteen AMAC-derivatized saccharides from top to bottom: a, galNAc, d, mannose; f, glucose; h, ΔDiHA; i, ΔDi0S; m, 6S-galNAc; n, 4S-galNAc; o, ΔDi6S; q, ΔDi4S; r, ΔDi2S; t, ΔDi4,6S; u, ΔDi2,6S; and v, ΔDi2,4,6S. Identification of the derivatized saccharide structures present in lanes 1–6 are as described in the text. This image depicts over saturated pixel intensity for the major derivatized structures in order to allow visualization of less abundant derivatized structures.

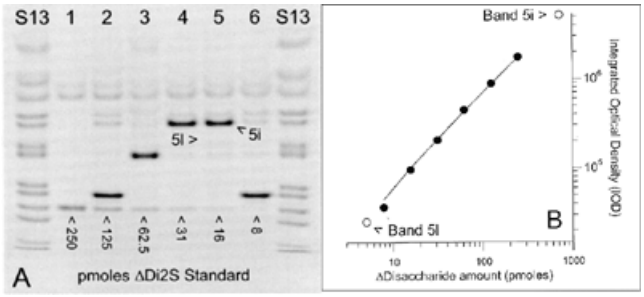

Fig. 3. (**A**) Image of the same gel shown in Figure 2, but exposed for purposes of quantitation such that all the pixels are within a linear 12-bit depth range. (**B**) Standard curve (solid circles) generated from plotting the integrated optical densities from Table I (peaks r and s) for the six ΔDi2S external standard bands shown in (**A**) versus picomoles of Δdisaccharide as determined by FACE analysis with AMAC-derivatized glucose standards (R = 0.99988, P < 0.0001). The values (open circles) for bands 5i (723 pmol) and 5l (5 pmol) in (**A**) represent the highest and lowest values quantitated in this analysis.

chondroitin/dermatan sulfate (see Figure 1; Calabro *et al.*, 2000). These enzymes were used in amounts and for a time sufficient to completely depolymerize the hyaluronan and chondroitin sulfate chains to their final digestion products. One of the aliquots digested with hyaluronidase and chondroitinase was derivatized directly (SD/ABC, lane 2). A second aliquot was mercuric ion treated prior to derivatization (Hg^{2+}, lane 3). Mercuric ion treatment quantitatively removes the Δhexuronic acid from the Δdisaccharides generated by the two eliminases, releasing the hexosamine portion (see Figure 1; Calabro *et al.*, 2000). The remaining three aliquots were further digested with

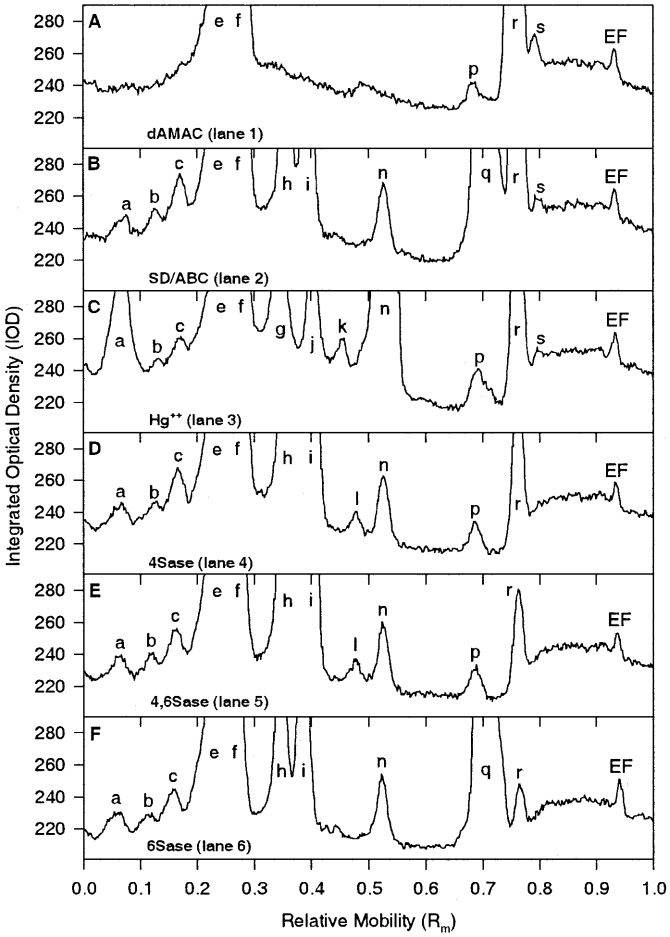

Fig. 4. (**A–F**) represent line plots of initial integrated optical density versus relative mobility from lane 1 (dAMAC), lane 2 (SD/ABC), lane 3 (Hg^{2+}), lane 4 (4Sase), lane 5 (4,6Sase), and lane 6 (6Sase) of the image in Figure 3A, respectively. Peak designations correspond to the bands in Figure 2B.

chondro-4-sulfatase alone (4Sase, lane 4), chondro-6-sulfatase alone (6Sase, lane 6) or chondro-4-sulfatase and chondro-6-sulfatase together (4,6Sase, lane 5).

Figure 3A shows an image of the same gel as shown in Figure 2, but taken with a shorter exposure time such that all pixels were within a linear 12-bit depth range. This image was analyzed using the Gel-Pro Analyzer program version 3.0 from Media Cybernetics using the "join valley" method of base line correction set at either 1 or 2% slope change. The line plots of integrated optical density versus relative mobility (R_m) generated from this analysis for lanes 1 through 6 are shown in Figure 4, panels A through F, respectively. The peak designations in each line plot correspond to the band designations in Figure 2B. The positions at the top (R_m = 0) and bottom (R_m = 1) of each lane in Figure 2B, which correspond to the same relative mobility in the line plots of Figure 4, are indicated. The values of integrated areas for select peaks in Figure 4 are listed in Table I.

Prior to electrophoresis, each of the six samples in the FACE analysis were mixed 1:1 with one of six different AMAC-derivatized ΔDi2S standards (see Figure 3A). ΔDi2S was chosen as a standard for the rat chondrosarcoma tissue since it contains

Table I. Normalized integrated optical densities for select peaks of FACE analysis in Figure 4

Peak[b]	Saccharide structures	Integrated optical density (IOD)[a]					
		Lane 1 dAMAC	Lane 2 SD/ABC	Lane 3 Hg^{2+}	Lane4 4Sase	Lane 5 4,6Sase	Lane 6 6Sase
a	galNAc		33,170	388,300	43,070	46,390	51,750
e	Unknown	211,200	244,000	284,800	251,500	233,800	257,800
f	Glucose	730,800	698,000	657,200	690,500	708,200	684,200
g	glcNAc			276,900			
h	ΔDiHA		303,000		295,700	316,300	304,700
i	ΔDi0S, unS-LO_{ABC}		404,000		4,892,000	5,103,000[c]	363,900
j	unS-LO_{Hg}			148,900			
k	4S-LO_{Hg}			27,850			
l	4S-LO_{4Sase}				26,180	25,390[c]	
n	4S-galNAc		83,780	4,536,000	91,940	74,190	85,430
p	Unknown, Di4S	37,910		80,180	41,560	50,110	
q	ΔDi4S, 4S-LO_{ABC}, p		4,910,000				5,011,000
r/s[d]	ΔDi2S	1,750,000	883,000	448,000	205,000	96,260	36,780

[a]Values in lanes 1 and 3-6 were normalized to lane 2 based on the sum of the unknown e/glucose values.
[b]Peak designations correspond to those in Figure 4.
[c]Peaks 5i (723 pmol) and 5l (5 pmol) represent the highest and lowest values measured (see Figure 3).
[d]Values for the external ΔDi2S standard were not normalized, and represent their initial values.

no endogenous 2-sulfated structures (see Figure 5A). The values of integrated optical density for the ΔDi2S standards listed in Table I (peaks r and s; The r band represents the AMAC-derivatized ΔDi2S structure while the s band is a trace contaminant in the standard as a result of storage at 4°C) were used to generate the standard curve shown in Figure 3B (solid circles). After calculating the integrated optical densities for each hyaluronan and chondroitin sulfate structure identified as described in the following sections and listed in Table II, this standard curve was used to determine the picomoles represented by each peak in Figure 4. The highest (5i = 723 pmol) and lowest (5l = 5 pmol) values measured in this analysis are plotted on the standard curve in Figure 3B (open circles). They represent a linear range of detection for this analysis of over 100-fold.

Identification of glucose and other endogenous saccharides with free reducing groups

In most tissues, the major endogenous saccharide with a free reducing group is expected to be glucose. This is the case for the rat chondrosarcoma tissue. As seen in Figures 2 (lane 1) and 4 (panel A), the major AMAC-derivatized structure in the dAMAC sample is glucose (band/peak f), which migrates at the same position as the D-glucose band in the standard lane (S13; AMAC-derivatized L-glucose migrates in the same position as AMAC-derivatized D-glucose). The calculated glucose concentration in the tissue based on FACE analysis (Table II) is 0.72 g/l, which is close to the physiologic concentration of blood glucose of 1 g/l. In addition to glucose, a second endogenous saccharide with a free reducing group was detected in the dAMAC sample (band/peak e). Derivatized unknown e migrates at the same position as a derivatized fucose standard (data not shown), which is just behind the derivatized glucose. The calculated concentration of unknown e in the tissue, based on FACE analysis (Table II) using the formula weight for fucose, is 0.24 g/l. One other fluorescent compound, observed in the dAMAC sample in Figures 2 and 4, is band/peak p. This is a contaminant in the AMAC reagent, and is observed in sham samples, which contained no tissue sample, but only the buffer and reagents (data not shown). This contaminant migrates at the same position as AMAC-derivatized 4,6S-galNAc and Di6S, and its fluorescent value is subtracted from this peak in analyses of the other lanes.

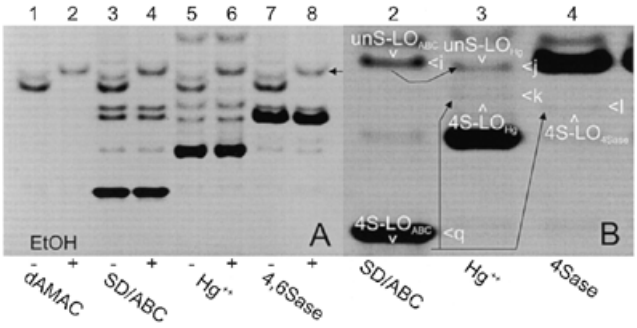

Fig. 5. (**A**) Comparison of FACE profiles from chondrosarcoma tissue samples processed immediately after proteinase K digestion (-, lanes 1, 3, 5, and 7) or after subsequent ethanol (EtOH) precipitation (+, lanes 2, 4, 6, and 8). Samples were AMAC derivatized: directly (dAMAC, lanes 1 and 2), after hyaluronidase SD and chondroitinase ABC digestion (SD/ABC, lanes 3 and 4), after hyaluronidase SD and chondroitinase ABC digestion followed by mercuric ion treatment (Hg^{2+}, lanes 5 and 6), or after hyaluronidase SD, chondroitinase ABC, chondro-4-sulfatase and chondro-6-sulfatase digestion (4,6Sase, lanes 7 and 8). (**B**) An enlargement of a portion of the image in Figure 2 (lanes 2–4) which highlight those bands (i, j, k, l, and q) associated with the linkage oligosaccharide (LO) derived structures described in the text.

Table II. Calculated data for saccharide structures determined by FACE analysis

	Saccharide	Formula weight[a]	IOD/peak[b]	pmol/peak[c]	pmol/100 mg tissue[d]	g/l or mg/ml[e]	Percent of total
Hyaluronan	ΔDiHA	401	276,900	41	162,400	0.65	
Chondroitin sulfate							
	Internal disaccharides:						
	ΔDi4S	503	4,630,000	656	2,625,000	13.20	93%
	ΔDi0S	401	344,700	50	200,700	0.81	7%
	Nonreducing termini:						
	4S-galNAc	306	83,840	13	53,190	0.16	51%
	galNAc	204	43,600	8	30,430	0.06	27%
	Di4S	504	36,990	7	26,690	0.13	22%
	Linkage oligosaccharides:						
	unS-LO	1072	148,900	22	89,990	0.96	85%
	4S-LO	1276	26,470	5	20,730	0.26	15%
	Other saccharides:						
	Glucose	180	694,800	100	398,800	0.72	74%
	Unknown e	164	247,200	36	145,600	0.24	26%
	Contaminants:						
	From AMAC		43,190	8			

[a]Formula weights are for the sodium salt were applicable.
[b]Calculated from valued in Table I as described in the text.
[c]Calculated based on the standard curve in Figure 3B.
[d]Calculated based on a dilution factor of 4000.
[e]Calculation assumes a tissue density equal to water such that 1 mg = 1 µl.

the sample aliquots analyzed in lanes 1 through 6 of Figure 2 were initially the same independent of subsequent sample treatment. Therefore, these saccharides could serve as internal standards for differences in samples as a result of processing and gel loading. The initial integrated optical densities obtained from all the peaks in Figure 4 were therefore normalized based on the combined values for the glucose plus unknown e peaks with the value for the SD/ABC sample set at 100%. These normalized values are reported in Table I. Values for the glucose plus unknown e peaks were all within 10% of each other except for the value for the Hg^{2+} sample, which was 63% of the SD/ABC sample. This is due to selective loss of neutral structures on the Dowex H^+ resin used to remove the Hg^{2+}, as compared to the quantitative recovery normally expected for the negatively charged Δdisaccharides and sulfated galNAc structures. When the total fluorescence for all derivatized structures in the SD/ABC lane was compared to the total fluorescence in the Hg^{2+} lane after adjusting for the observed loss of neutral saccharides, the values are within 2% of each other. Therefore, for the Hg^{2+} treated sample only, values for uncharged structures (i.e., glucose, unknown e, galNAc, and glcNAc) were normalized based on the relative amounts of glucose plus unknown e (63%), while negatively charged structures (i.e., 4S-galNAc and Di4S) were normalized based on the relative total fluorescence adjusted as described above (2%).

In some samples, the high amount of glucose in the sample relative to the amount of hyaluronan and chondroitin/dermatan sulfate requires removal of the glucose prior to processing. Excessive amounts of glucose can deplete the AMAC reagent during derivatization, causing incomplete derivatization of the hyaluronidase and chondroitinase digestion products. Culture medium with 1 g/l of glucose contains ~50 nmol of reducing equivalents in a 10 µl aliquot, the desired upper limit in the derivatization reaction. Further, the fluorescent signal from excessive amounts of derivatized glucose is sufficient to prevent accurate detection of neighboring structures such as ΔDiHA and ΔDi0S. Glucose (and unknown e) can be selectively removed from proteinase K digested samples after ethanol precipitating the glycosaminoglycans. The small molecular weight glucose partitions into the supernatant fraction while the high molecular weight hyaluronan and chondroitin/dermatan sulfate precipitate. Since most of the protein in the sample has been digested to amino acids and small peptides by the proteinase K (data not shown), the resulting precipitate containing the glycosaminoglycans is normally easily resuspended in the ammonium acetate buffer. In Figure 5A, the results of ethanol precipitation of the proteinase K digested rat chondrosarcoma tissue are shown with the samples in lanes 1 (dAMAC), 3 (SD/ABC), 5 (Hg^{2+}) and 7 (4,6Sase) having been processed the same as the samples in lanes 1, 2, 3, and 5 of Figure 2, respectively, except that no ΔDi2S standard was added. The samples in lanes 2, 4, 6, and 8 were processed the same as those in lanes 1, 3, 5, and 7, respectively, but after ethanol precipitation as described in *Materials and methods* to remove selectively glucose and unknown e. Mannose (arrow)

was added as an internal standard upon resuspension of the precipitates. For all four precipitated samples, the glucose and unknown e were removed, while the patterns of derivatized hyaluronidase and chondroitinase digestion products were unchanged with recoveries of 94% or better. Note the absence of any ΔDi2S in any of the samples validating its use as a standard for this tissue.

Identification of nonreducing terminal structures

As seen in Table II, the three nonreducing terminal structures on chondroitin sulfate chains from rat chondrosarcoma tissue as measured by these FACE analyses were 4S-galNAc (51%), galNAc (27%), and Di4S (22%). The galNAc and 4S-galNAc nonreducing terminal structures were measured directly from peaks a and n, respectively, of the SD/ABC (panel B), the 4Sase (panel D), the 6Sase (panel F) and the 4,6Sase (panel E) samples in Figure 4. It is the averages of these four measurements for both galNAc and 4S-galNAc from Table I that are reported as calculated values in Table II. The presence of Di4S as a nonreducing terminal structure was deduced by comparing peak p in the Hg^{2+} sample (panel C) to those in the dAMAC (panel A), the 4Sase (panel D), and the 4,6Sase (panel E) samples of Figure 4. Peak p in the Hg^{2+} sample contains a shoulder on the front of the peak compared to peak p in these other three samples. Since the mercuric ion treatment removes any ΔDi4S or ΔDi6S from this position in the gel, the shoulder potentially contains Di4S, Di6S and/or 4,6S-galNAc. However, all of the shoulder was removed by chondro-4-sulfatase digestion alone (Figure 4D) indicating that it was all Di4S with no Di6S or 4,6S-galNAc. The remaining peak p in the 4Sase and 4,6Sase samples are similar to that in the dAMAC sample, and therefore represent only the contaminant in the AMAC reagent as described above. The calculated value for Di4S as reported in Table II was therefore determined by subtracting the average of the values in Table I for peak p in the dAMAC, 4Sase, and 4,6Sase samples (the value for the AMAC contaminant) from the value in Table I for peak p in the Hg^{2+} sample. Neither Di0S nor 6S-galNAc was detected as nonreducing terminal structures.

Identification of presumptive linkage oligosaccharide (LO) structures

There are several peaks in Figure 4, which do not migrate at known positions. We deduce that three of these peaks (j, k, and l) are derived from the linkage oligosaccharide (LO) region of chondroitin sulfate chains based on the following reasoning. Chondroitinase ABC digestion leaves an unsaturated hexuronic acid adjacent to galNAc (with or without 4-sulfation) at the nonreducing terminus of linkage oligosaccharides from chondroitin sulfate chains from the rat chondrosarcoma (Figure 6, solid inverted triangles) (Shibata *et al.*, 1992). Endo-galactosidase would expose a new reducing end on the linkage oligosaccharides after cleavage at either galactose residue, which could then be derivatized with AMAC (Figure 6, open inverted triangles). Therefore, chondroitinase ABC containing a contaminating endo-galactosidase activity would generate the proposed linkage oligosaccharide derived structures (LO_{ABC}) with (4S-) or without (unS-) sulfate as illustrated in Figure 6. Cleavage by endo-galactosidase may be selective for one or the other galactose residue generating two potential structures as indicated in Figure 6. The specific galactose

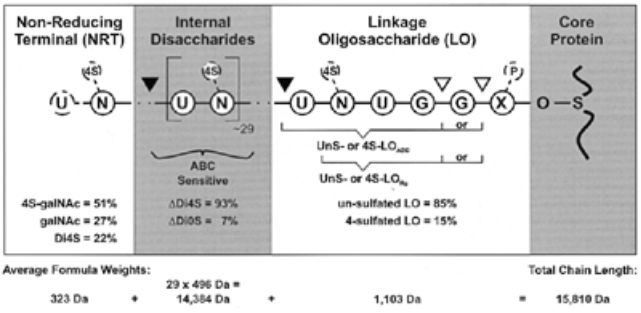

Fig. 6. Schematic diagram showing the saccharide composition of the chondroitin sulfate chains determined directly from FACE analyses of a proteinase K digest of Swarm rat chondrosarcoma tissue. The three regions that comprise a chondroitin sulfate chain are shown and include nonreducing termini (NRT, far left panel), internal disaccharides (left center panel), and linkage oligosaccharides (LO, right center panel). Other abbreviations used are U, glucuronic acid; N, N-acetylgalactosamine; G, galactose; X, xylose; S, serine residue; 4S, 4-sulfation and P, phosphorylation. Dashed lines indicate sites of heterogeneity of structure within these three regions. A list of saccharides identified by FACE analysis in each region and their percentage of the total saccharide from that region are listed (see Table II). The solid inverted triangles indicate the sites of cleavage for chondroitinase ABC. The open inverted triangles indicate proposed cleavage sites for the suspected contaminating endo-galactosidase activity. The proposed structures of linkage oligosaccharides generated by digestion with chondroitinase containing a contaminating galactosidase activity (unS- or 4S-LO_{ABC}), and subsequent treatment with mercuric ion (unS- or 4S-LO_{Hg}) are indicated. The calculation of number averaged chain length is described in the text.

residue cleaved may be affected by steric hindrance of the attached peptide, by sulfation on the galNAc residue or by phosphorylation of the xylose residue as previously reported (Shibata *et al.*, 1992).

Novel peaks, j and k, appear after mercuric ion treatment (Figure 4C), and therefore originally contained nonreducing terminal unsaturated hexuronic acid residues. If derived from the proposed unS-LO_{ABC} and 4S-LO_{ABC} species, respectively, peaks j and k would have the proposed unS-LO_{Hg} and 4S- LO_{Hg} structures, respectively, shown in Figure 6. As there are no bands migrating at novel locations in the SD/ABC sample which are sensitive to mercuric ion treatment (Figure 4B), the postulated unS-LO_{ABC} and 4S-LO_{ABC} structures would migrate at positions of known derivatized Δdisaccharide structures (see below). The appearance of peak l only after chondro-4-sulfatase digestion indicates that it is a desulfated form of the 4S-LO_{ABC} structure (4S-LO_{4Sase}; Figure 4D). Cleavage at alternate galactose residues to generate the unS-LO_{ABC} and 4S-LO_{ABC} structures may account for why the desulfated form of the 4S-LO_{ABC} structure (i.e., 4S-LO_{4Sase}) migrates at the position of band l, and not at the proposed position of the unS-LO_{ABC} (i.e., with ΔDi0S) as described below. The proposed migration patterns of the linkage oligosaccharide-derived structures described above are shown in Figure 5B, which is an enlargement of the area of lanes 2–4 of the gel in Figure 2.

The quantitation supports the qualitative evaluation described above. The calculated value for the unS-LO in Table II was taken directly from the value in Table I for peak j or the unS-LO_{Hg} structure in the Hg^{2+} sample (Figure 5B, lane 3). The derivatized unS-LO_{ABC} structure, which gives rise to peak j after mercuric ion treatment, comigrates with derivatized ΔDi0S in peak i of the SD/ABC sample (Figure 5B, lane 2).

This is supported by the fact that the sum of the calculated values in Table II for ΔDiHA, ΔDi0S and the unS-LO, and the sum of the values for peaks h (i.e., ΔDiHA) and i (i.e., ΔDi0S and unS-LO) in Table I of the SD/ABC sample are within 4%. The appearance of peak j, near the position of ΔDi0S after mercuric ion treatment, might suggest that it was derivatized Di0S, which is not affected by this treatment. However, peak j has shifted position relative to peak i following mercuric ion treatment indicating the presence of a Δhexuronic acid residue in the parent compound. Importantly, the sum of the calculated values in Table II for the three identified nonreducing terminal structures is within 3% of the sum of the calculated values for the two proposed linkage oligosaccharide structures. The molar equivalence of these two values is necessary since chondroitin sulfate chains contain one nonreducing terminus and one linkage oligosaccharide per chain. As expected both the linkage oligosaccharides and nonreducing terminal values predict similar number averaged chain lengths for the chondroitin sulfate chains (see below).

The derivatized 4S-LO_{ABC} structure which gives rise to peak k (i.e., 4S-LO_{Hg}) after mercuric ion treatment and peak l (i.e., 4S-$LO4_{Sase}$) after chondro-4-sulfatase digestion probably migrates near ΔDi4S in peak q of the SD/ABC sample (Figure 5B, lane 2). In fact, a minor band just ahead of band q can be seen in Figure 2, lanes 2 and 6, which appears as a shoulder on peak q in Figure 4B,F. The large quantity of ΔDi4S present in the tissue makes it impossible to detect this structure directly in the SD/ABC sample. Therefore, the calculated value for the 4S-LO in Table II was determined by averaging the values in Table I for peak k (the 4S-LO_{Hg} structure in the Hg^{2+} sample) and peaks l (the 4S-LO_{4Sase} structure in both the 4Sase and 4,6Sase samples) (Figure 4C–E). The relationship between peaks k and l is inferred from the fact that the value for peak k and the two values for peak l in Table I are all within 5% of their average value as listed in Table II.

Identification of internal Δdisaccharide structures

As seen in Table II, the two internal Δdisaccharides on chondroitin sulfate chains from rat chondrosarcoma tissue as determined by these FACE analyses were ΔDi4S (93%) and ΔDi0S (7%). In addition, FACE analysis showed that rat chondrosarcoma tissue contained 5% more internal Δdisaccharide in the form of ΔDiHA from hyaluronan. The ΔDi4S was measured by five independent measurements. The amount of ΔDi4S was calculated by subtracting the sum of the calculated values in Table II for the Di4S, 4S-LO and AMAC contaminant from the value in Table I for peak q of the SD/ABC and 6Sase samples (see Figure 4B,F, respectively). Peak q in the SD/ABC and 6Sase samples contains predominantly derivatized ΔDi4S, but also minor amounts of these other three derivatized structures, which migrate at similar positions (see above). Alternatively, the amount of ΔDi4S was calculated by subtracting the calculated value in Table II for 4S-galNAc as a nonreducing terminal structure from the value for peak n in Table I of the Hg^{2+} sample (see Figure 4C). Peak n in the Hg^{2+} sample contains the 4S-galNAc originally present as a nonreducing terminal as well as 4S-galNAc as a result of mercuric ion treatment of ΔDi4S. Finally, the amount of ΔDi4S was calculated by subtracting the sum of the calculated values in Table II for the ΔDi0S, ΔDiHA, Di4S, and unS-LO structures from the values for the sum of peaks h and i in Table I of the 4Sase and 4,6Sase samples (see Figure 4D,E, respectively). Digestion with chondro-4-sulfatase quantitatively converts ΔDi4S and Di4S to ΔDi0S and Di0S, which then both migrate at the position of peak i where the ΔDi0S and unS-LO in the original chondroitinase digest migrate. Peak h, which contained only ΔDiHA, was included since it does not completely resolve from peak i in the 4Sase and 4,6Sase samples. These five independent measurements for ΔDi4S were all within 6% of their average value, which was reported as the calculated value for ΔDi4S in Table II.

The ΔDi0S and ΔDiHA were measured from the galNAc (peak a) and glcNAc (peak g) peaks, respectively, in the mercuric ion treated (Hg^{2+}) sample (Figure 4C). Both these peaks show baseline separation from the peaks adjacent to them. The value for ΔDiHA in Table II was taken directly from peak g in the Hg^{2+} sample of Table I. However, the value for ΔDi0S in Table II was the result of subtracting the calculated value in Table II for galNAc as a nonreducing terminal from the value for peak a in the Hg^{2+} sample of Table I. The reason that peaks i and h in the SD/ABC sample (Figure 4B) were not used directly to measure the ΔDi0S and ΔDiHA was because they contained the derivatized unS-LO_{ABC} structure as described above.

The large quantity of ΔDi4S in chondroitin sulfate from rat chondrosarcoma tissue makes it difficult to detect ΔDi6S directly from the SD/ABC sample (Figure 4B). Chondro-4-sulfatase digestion was used to uncover any minor amounts of ΔDi6S present (Figure 4D) by shifting the position of the Di4S and ΔDi4S to that of Di0S and ΔDi0S, respectively. Only the contaminant from the AMAC reagent in peak p as described above was uncovered indicating that no detectable ΔDi6S was present. As previously discussed, no ΔDi2S was detected (see Figure 5A) in the chondrosarcoma tissue. No peaks between a relative mobility of 0.8 and the electrophoresis front (EF) were detected in Figure 4 that would indicate the presence of AMAC-derivatized ΔDi4,6S or ΔDi2,6S both of which clearly resolved in this analysis (see Figure 2, lane S13). Finally, although a small peak of fluorescent material is detected at the electrophoresis front, this peak is present in the dAMAC sample (Figure 4A) and is unaffected by any subsequent treatments (Figure 4B–F). Therefore, no ΔDi2,4S, or ΔDi2,4,6S, which both run at the electrophoresis front were detected.

Determination of number averaged chain size from FACE analysis

The number averaged chain size for the chondroitin sulfate chains from rat chondrosarcoma tissue was determined based on the information in Table II. First, the average number of internal disaccharides per chondroitin sulfate chain was calculated based on the fact that there is only one nonreducing terminus and one linkage oligosaccharide per chain. The total fluorescence for all the internal disaccharides (i.e., ΔDi4S and ΔDi0S) was divided by either the total fluorescence for all the nonreducing termini (i.e. 4S-galNAc, galNAc, and Di4S) or for all the linkage oligosaccharides (i.e., unS-LO and 4S-LO). Based on this calculation the average number of internal disaccharides per chondroitin sulfate chain was either 30 or 28 depending on whether the nonreducing termini or linkage oligosaccharide values, respectively, were used. An average value of 29 internal disaccharides per chain was then multiplied by an average formula weight for the internal disaccharides of

496 Da, which was calculated from the percent composition and formula weights for the ΔDi4S and ΔDi0S listed in Table II. To the resulting calculated number averaged weight of the internal disaccharides was then added the average formula weight of one nonreducing terminus and one linkage oligosaccharide (calculated from the percent composition and molecular weights listed in Table II). These calculations are illustrated in Figure 6, which shows a schematic of the structure of chondroitin sulfate chains from rat chondrosarcoma tissue as determined by FACE analysis. The final estimation of chain size based on the FACE analysis was ~16,000 Da. The weight averaged chain size for these chains, as determined by Superose 6 chromatography, was 24,000 Da (data not shown).

Important considerations

Since tissue glucose concentrations can be measured by the FACE protocols, the tissue was kept on ice after harvesting until the proteinase K digestion step to minimize glucose utilization by the chondrocytes during tissue processing, and therefore to prevent underestimation of tissue glucose concentrations. Only extracellular glucose is measured by this assay since the free reducing group of glucose is normally blocked by phosphorylation upon entering the cell. Unlike with purified proteoglycan or glycosaminoglycan preparations, tissue samples must first be solubilized to release glycosaminoglycan chains regardless of their location within cells, on cell surfaces or in extracellular matrices. This allows for sampling of tissue aliquots which contain the same initial glycosaminoglycan content, and which can therefore be directly compared after the various enzymatic and chemical treatments used in our protocols.

Proteinase K digestion was chosen for solubilizing tissues because of its following properties. Proteinase K is a nonspecific serine protease, so that it is predicted to work equally well on all proteins independent of their amino acid composition. It is stable over a wide pH range (4–12.5) with optimal activity at a pH of 6.5–9.5. The activity of proteinase K is increased by denaturing agents, and is stable to metal ions, chelating agents, sulfhydryl reagents, or trypsin/chymotrypsin inhibitors. This provides many options when dovetailing these analyses with already existing protocols. Proteinase K has a temperature optimum of 65°C, with digestion at this elevated temperature facilitating the solubilization of cellular components. Proteinase K is rapidly denatured at temperatures above 65°C, and therefore can be easily inactivated by boiling prior to steps involving other enzymatic treatments. Finally, while autolysis of proteinase K occurs increasingly at alkaline pH, the enzyme is not inactivated by autolysis, thereby minimizing the amount of enzyme required.

There are also some general advantages to protease digestion. Protease digestion removes most of the macromolecular protein, which can prevent complete solubilization of glycosaminoglycans in an ethanol precipitate for those samples, which may require such treatment (see Figure 5A). Protease digestion removes noncovalently bound proteins such as aggrecan and link protein from hyaluronan, which allows for complete digestion of the hyaluronan by hyaluronidase and/or chondroitinase. Protease digestion also releases individual glycosaminoglycan chains from proteoglycan core proteins containing multiple chains (i.e., aggrecan). Finally protease digestion, particularly at 65°C, potentially inactivates endogenous enzymatic activities, which might show specificity for the glycosaminoglycans and complicate the analysis.

The hyaluronidase and chondroitinase digestions can be done on separate sample aliquots to simplify quantitation of hyaluronan. Hyaluronidase SD digestion alone yields only ΔDiHA with minor amounts of ΔDi0S, and no ΔDi4S or ΔDi6S. Presumably, the ΔDi0S released is from unsulfated regions of two or more disaccharides in the chondroitin sulfate chains. Digestion with chondroitinase ABC alone normally underestimates the amount of hyaluronan. Samples digested with both enzymes are digested first with the hyaluronidase for 1 h prior to chondroitinase digestion, because incomplete hyaluronan digestion was observed when both enzymes were added at the same time. It may be that the ΔDi0S generated by the chondroitinase inhibits the hyaluronidase activity. Two unknown AMAC-derivatized structures, peaks b and c, which migrate to positions between derivatized galNAc and glucose were observed in all the samples which were hylauronidase and chondroitinase digested (Figures 2 and 4). These minor structures probably arise from the action of contaminating enzyme activities in the hyaluronidase and/or chondroitinase on N- or O-linked oligosaccharides in the proteinase K preparation, since these products are not observed in the ethanol precipitated samples (Figure 5A) and the purified aggrecan samples (Figure 1).

Finally, the derivatized ΔDi2S standard in Figures 2 and 4 contains a minor peak (peak s), which runs slightly ahead of the major ΔDi2S peak (peak r). This is a break-down product as a result of storage of the standard at 4°C rather than –70°C, and which becomes more prominent with time. A similar break down product running just ahead of the major peak is observed for all of the derivatized Δdisaccharide standards with storage at 4°C.

Discussion

Plaas *et al.* (1997) reported that the internal disaccharide compositions for the chondroitin sulfate chains on fetal versus 68 year human aggrecan to be ΔDi4S (51%), ΔDi6S (38%) and ΔDi0S (11%) versus ΔDi4S (4%), ΔDi6S (94%) and ΔDi0S (2%), respectively. These results are similar to those determined by FACE analysis for the human fetal, ΔDi4S (50%), ΔDi6S (43%) and ΔDi0S (7%), and 68 year, ΔDi4S (3%), ΔDi6S (96%), and ΔDi0S (1%), aggrecan preparations shown in Figure 1. Unlike the FACE protocols reported here, the previous methodology, using AS4S anion exchange chromatography of 2-aminopyridine derivatized products, was unable to detect unsulfated disaccharides such as ΔDi0S and ΔDiHA, and therefore required separate analysis by capillary zone electrophoresis to determine these structures. Plaas *et al.* (1997) also reported the identification of the nonreducing terminal structures on human aggrecan, which included 4S-galNAc, and minor amounts of Di4S and Di6S for the fetal aggrecan samples, and in addition included 4,6S-galNAc approximately equal to the 4S-galNAc in the 68 year aggrecan samples. Our results for the nonreducing terminal structures as determined by FACE analysis are similar, except for detection of minor amounts of 6S-galNAc in both aggrecan preparations, and the lack of detection of Di4S in either aggrecan sample.

Compositional analysis of the nonreducing terminal, internal disaccharide and linkage oligosaccharide structures of the chondroitin sulfate chains of aggrecan from rat chondrosarcoma cultures have been made previously (Shibata *et al.*, 1992; Midura *et al.*, 1995; Plaas *et al.*, 1996). Some of the methods previously used in these analyses to isolate and purify aggrecan, its chondroitin sulfate chains and their chondroitinase digestion products prior to analysis included dissociative isopycnic density gradient centrifugation (i.e., cesium chloride, guanidine HCl), ultrafiltration (i.e., Centricon 30, Microcon 3), size exclusion chromatography (i.e., Sephadex G-50, Superose 6, Sephacryl S-1000, Toyopearl HW40S, Progel-TSK G2500PWXL) and anion exchange chromatography (i.e., Q-Sepharose, CarboPak PA1, AS4S Ion Pac). We have adapted the FACE protocols as described in the accompanying article (Calabro *et al.*, 2000) for direct analysis of the fine structure of hyaluronan and chondroitin sulfate in proteinase K digests of tissues with a minimum of sample processing. All sample processing, except mercuric ion treatment, takes place in one tube with the mercuric ion treatment taking place after the hyaluronan and chondroitin sulfate chains have been completely digested to their final digestion products. This single tube approach avoids potential losses of small or undersulfated fractions of chondroitin sulfate chains that are possible when samples are processed by size exclusion (i.e. Sephadex G-50) or ion exchange chromatography (i.e., Q-Sepharose), respectively.

FACE analysis of the glycosaminoglycans in the proteinase K digest of rat chondrosarcoma tissue showed that the proportion of ΔDiHA from hyaluronan was 5% of the total of ΔDi0S and ΔDi4S from chondroitin sulfate consistent with the stoichiometry of hyaluronan and aggrecan containing complexes normally found in cartilage (Morales and Hascall, 1988). The internal disaccharide compositions for the chondroitin sulfate chains on rat chondrosarcoma aggrecan as previously measured were ΔDi0S (5–10%), ΔDi4S (90–95%), ΔDi6S (0.2–0.5%), and ΔDi4,6S (0.5%) (Shibata *et al.*, 1992; Midura *et al.*, 1995; Plaas *et al.*, 1996). Our result determined by FACE analysis of the chondroitin sulfate chains in the proteinase K digest of rat chondrosarcoma tissue shown in Table II indicate the presence of only ΔDi0S (7%) and ΔDi4S (93%) with no ΔDi6S and ΔDi4,6S detected. The nonreducing terminal structures for the chondroitin sulfate chains on rat chondrosarcoma aggrecan as previously measured were 4S-galNAc plus 4,6S-galNAc (85–87%), and Di4S plus Di6S (13–15%) (Midura *et al.*, 1995; Plaas *et al.*, 1996). Our results as determined by FACE analysis of the chondroitin sulfate chains in the proteinase K digest of rat chondrosarcoma tissue indicate the presence of 4S-galNAc (51%), galNAc (27%), and Di4S (22%) with no 4,6S-galNAc or Di6S detected (see Table II).

An unexpected consequence of our FACE analysis following chondroitinase digestion was the detection of putative linkage oligosaccharide structures from the chondroitin sulfate chains as a consequence of a proposed contaminating endo-galactosidase activity in the chondroitinase preparation. Results of our FACE analysis of the chondroitin sulfate chains in the proteinase K digest of rat chondrosarcoma tissue strongly suggest the presence of an unsulfated linkage oligosaccharide (unS-LO, 85%) and a 4-sulfated linkage oligosaccharide (4S-LO, 15%). These values are similar to those previously reported by Shibata *et al.* (1992) of 64% and 36%, respectively. One major difference in our analysis versus previous analyses was that we have measured the bulk mass of aggrecan chondroitin sulfate as synthesized by the tissue *in vivo* as a subcutaneous tumor while the previous analyses measured only newly synthesized and metabolically labeled ([^{35}S]sulfate and [^{3}H]glucosamine) chondroitin sulfate from primary chondrosarcoma chondrocyte cultures. The difference between the availability of sulfate to the chondrocytes for chondroitin sulfate synthesis under *in vivo* versus culture conditions may account for the slightly higher level of unsulfated saccharide structures measured for the aggrecan from the tissue versus cultures. This may also explain the absence of any 6-sulfation in the tissue, which may require saturating concentrations of phosphoadenosinephosphosulfate (PAPS) for even the minor amount of 6-sulfation detected in the cultures to be synthesized.

Finally the number averaged chain length estimated by the ratio of the molar amount of the total Δdisaccharides to either the total nonreducing terminal or the total linkage oligosaccharide structures from these FACE analyses was ~16 kDa. This is consistent with previous estimates of chain length for chondroitin sulfate from rat chondrosarcoma aggrecan estimated either by monosaccharide analysis (~18 kDa) or by number averaged chain size calculations using the 2-aminopyridine/AS4S anion exchange chromatography method (~17 kDa) (Midura *et al.*, 1995; Plaas *et al.*, 1996). A weight averaged chain size of ~24 kDa (K_d = 0.56) was determined by Superose 6 chromatography for chondroitin sulfate chains from the proteinase K tumor digests used for the FACE analyses described here, and from chondrosarcoma chondrocyte cultures as described previously (Midura *et al.*, 1995).

In summary, we have developed a fast, simple, and sensitive method for detection and quantitation of the hyaluronidase and chondroitinase digestion products of hyaluronan and chondroitin/dermatan sulfate chains using 2-aminoacridone (AMAC) derivatization and fluorophore-assisted carbohydrate electrophoresis (FACE). The FACE protocols described here and in the accompanying article (Calabro *et al.*, 2000) have several important features that make them useful for a wide variety of applications. The amount of the fluorescence from the AMAC is independent of the chemistry of the saccharide to which it is attached. This means that the fluorescence is a direct measure of the molar amount of a derivatized structure, which is a powerful tool in elucidating the nature of unexpected chemistries such as the linkage oligosaccharides identified in these analyses. The sensitive nature of the assay (in the nanogram/picomole range) allows for detection of hyaluronan and chondroitin sulfate from small tissue samples. The hyaluronan and chondroitin sulfate digestion products visualized in each lane of the FACE gel in Figure 2 is from 25 μg of rat chondrosarcoma tissue based on wet weight. Assuming the tissue is 95% water, that amounts to 1–2 μg of tissue by dry weight. This level of sensitivity when combined with the protocols described in this paper for detection of hyaluronan and chondroitin sulfate directly from a proteinase K tissue digest brings experiments such as topographic analysis of glycosaminoglycans in tissues like cartilage into the realistic and manageable range, especially considering the single tube nature of the assay and the ability to process and image a number of samples simultaneously. As shown in Figure 2 complete characterization of a sample can be accomplished with a single gel in less

than a week using the combination of direct fluorescent derivatization, hyaluronidase and chondroitinase digestion, mercuric ion treatment, and sulfatase digestions.

Materials and methods

Materials used in these experiments were as described in the accompanying article (Calabro *et al.*, 2000), with the following additions. D-Glucose and D-mannose were purchased from Aldrich-Sigma. Proteinase K was from Gibco. Hyaluronidase SD was purchased from Seikagaku, America. Superose 6 (HR 10/30) column was obtained from Pharmacia.

Preparation of purified aggrecan samples for fluorescent derivatization

Aggrecan samples from fetal and 68-year-old human cartilage were prepared by guanidine HCl extraction and cesium chloride gradient centrifugation as previously described (Plaas *et al.*, 1996). The aggrecan preparations were generous gifts of Dr. Anna Plaas (Shriners Hospital for Crippled Children, Tampa, FL). Aggrecan preparations (in ultrapure water) were processed for derivatization as follows. Three identical aliquots from each preparation were frozen on dry ice, and lyophilized until dry on a vacuum concentrator. Each aliquot contained no more than 50 nmol of potential disaccharide reducing equivalents. Each aliquot was resuspended in 100 µl of 0.0005% phenol red, 100 mM ammonium acetate, pH 7.0. One aliquot was immediately frozen on dry ice, and lyophilized until dry on a vacuum concentrator prior to derivatization (dAMAC). The remaining two aliquots were digested with chondroitinase ABC (100 mU/ml) for 3 h at 37°C, and then frozen on dry ice, and lyophilized until dry on a vacuum concentrator. One chondroitinase digest was derivatized directly (ABC). The other chondroitinase digest was treated with mercuric ion prior to derivatization (Hg^{2+}) as described in the accompanying article (Calabro *et al.*, 2000).

Preparation of proteinase K digest of Swarm rat chondrosarcoma tissue

The Swarm rat chondrosarcoma tissue was propagated in rats as described previously (Calabro and Hascall, 1994). Briefly, one tumor-laden rat was sacrificed by CO_2 asphyxiation. The tumor and surrounding facia were excised, and immediately placed on ice. The tumor was quickly pressed through a wire mesh sieve to mince the cartilage tissue and to remove the surrounding facia. The minced cartilage tissue (~3 g) was immediately transferred to a preweighed tube on ice. A 100 mg aliquot of this cartilage tissue was transferred to a 1.5 ml screw cap tube on ice. The tissue was assumed to have a density equal to water (1 mg/µl) per its high water content, and the tissue sample was diluted to a final volume of 900 µl containing 0.0005% phenol red, 100 mM ammonium acetate, pH 7.0. A 50 µl aliquot of a fresh solution of proteinase K (2.5 mg/ml) in 0.0005% phenol red, 100 mM ammonium acetate, pH 7.0 was added, and the tissue digested for 2 h at 60°C with mixing every 30 min. A second 50 µl aliquot of a fresh solution of proteinase K was added, and the tissue digested for an additional 2 h at 60°C with mixing every 30 min. The sample was then boiled for 10 min to inactivate the proteinase K, and centrifuged at 10,000 × *g* for 15 min at room temperature to pellet any undigested material. An enzyme/buffer sham sample containing only the buffer was also digested with proteinase K as described above. A 200 µl aliquot of the proteinase K digested tissue was eluted on a Superose 6 column in 0.5 M ammonium acetate, pH 7, to obtain an estimate of the weight averaged chain size of the chondroitin sulfate chains.

Processing of proteinase K digested tissue for fluorescent derivatization

One 100 µl aliquot of the supernatant from the proteinase K digested tissue sample was diluted to 1 ml with 0.0005% phenol red, 100 mM ammonium acetate, pH 7.0. Six 100 µl aliquots of the diluted sample were transferred to new microfuge tubes. One aliquot remained untreated (dAMAC). The remaining five aliquots were digested for 1 h at 37°C with 100 mU/ml of hyaluronidase SD followed by 3 h at 37°C with the addition of 100 mU/ml of chondroitinase ABC. One digested aliquot received no further treatment (SD/ABC). Three digested aliquots were further incubated during the last 2 h of the chondroitinase ABC digestion with 100 mU/ml each of chondro-4-sulfatase alone (4Sase), chondro-6-sulfatase alone (6Sase) or the two sulfatases together (4,6Sase). Finally, one digested aliquot was further treated with mercuric ion (Hg^{2+}) as described in the accompanying article (Calabro *et al.*, 2000). To each of the six processed aliquots from the diluted tissue sample, 1 ml of -20°C absolute ethanol was added, the samples mixed, and stored a minimum of 2 h at -20°C. The samples were centrifuged at 10,000 × *g* for 15 min at 4°C to pellet any macromolecular material, and the supernatants containing the hyaluronidase and chondroitinase digestion products were transferred to new microfuge tubes. Both the supernatant and pellet fractions were dried on a vacuum concentrator prior to derivatization with 2-aminoacridone (AMAC) as described in the accompanying article (Calabro *et al.*, 2000). The pellet fractions contained less than 1% of the total derivatized digestion products (data not shown). After derivatization an aliquot of each supernatant sample was mixed 1:1 with one AMAC-derivatized ΔDi2S standard solution containing 16, 31, 62.5, 125, 250, or 500 pmol of derivatized ΔDi2S per 5 µl. The ΔDi2S standards were calibrated by FACE analysis using AMAC-derivatized glucose standards. Samples were run with (Figures 2, 3) and without (Figure 5A) ΔDi2S standards on FACE gels as described below. Enzyme/buffer shams for all six treatments were prepared similarly for derivatization.

Preparation of ethanol precipitated proteinase K digested tissue for fluorescent derivatization

One 100 µl aliquot of the supernatant from the proteinase K digested tissue sample was transferred to a new microfuge tube. One milliliter of -20°C absolute ethanol was added, the sample mixed, and stored a minimum of 2 h at -20°C. The sample was centrifuged at 10,000 × *g* for 15 min at 4°C to pellet any macromolecular material including the hyaluronan and chondroitin sulfate chains, and the supernatant containing the small molecular weight molecules such as glucose and salts were aspirated to waste. The pellet was washed with 1 ml of -20°C absolute ethanol by inverting, and centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatant was aspirated to waste, and the precipitate dried for 5 min on a vacuum concentrator. The precipitate was resuspended in 1 ml of 20 nmol/ml

mannose, 0.0005% phenol red, 100 mM ammonium acetate, pH 7. The concentration of mannose was determined by FACE analysis using AMAC-derivatized glucose standards, and it served as an internal standard in place of the endogenous glucose removed by the ethanol precipitation. Six 100 µl aliquots of the precipitated tissue sample were transferred to new microfuge tubes, and then processed as described above for the direct tissue samples (i.e., dAMAC, SD/ABC, Hg^{2+}, 4Sase, 6Sase, and 4,6Sase).

Gel electrophoresis, gel imaging, and data analysis

Samples were run on MONO® composition gels with MONO® gel running buffer as described in the accompanying article (Calabro *et al.*, 2000). The gels were illuminated with UV light (365 nm) from an Ultra Lum Transilluminator, and imaged with a Quantix cooled CCD camera from Roper Scientific/ Photometrics as described in the accompanying article (Calabro *et al.*, 2000). Digital images for each gel were taken at two exposures. One exposure over saturated pixel intensity for the major derivatized structures in order to allow visualization of less abundant derivatized structures as seen in Figures 1, 2, and 5. The second exposure had all pixels within a linear 12-bit depth range, and was used for quantitation as seen in Figure 3A. The images were analyzed using the Gel-Pro Analyzer program version 3.0 from Media Cybernetics. Base line was determined using the "join valleys" method set at either 1 or 2%.

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Abbreviations

Abbreviations are as described in the accompanying article (Calabro *et al.*, 2000) with the following additions: unS-LO, unsulfated linkage oligosaccharide; 4S-LO, 4-sulfated linkage oligosaccharide; LO_{ABC}, linkage oligosaccharide structures derived from chondroitinase and endo-galactosidase digestion; LO_{Hg}, linkage oligosaccharide structures derived from mercuric ion treatment of LO_{ABC}; LO_{4Sase}, linkage oligosaccharide structure derived from chondro-4-sulfatase digestion of 4S-LO_{ABC}; dAMAC, sample derivatized directly with AMAC; ABC, sample digested with chondroitinase ABC prior to derivatization; SD/ABC, sample digested with hyaluronidase SD and chondroitinase ABC prior to derivatization; Hg^{2+}, SD/ABC sample that was mercuric ion treated prior to derivatization; 4Sase, SD/ABC sample that was digested with chondro-4-sulfatase prior to derivatization; 6Sase, SD/ABC sample that was digested with chondro-6-sulfatase prior to derivatization; 4,6Sase, SD/ABC sample that was digested with both sulfatases prior to derivatization; EF, electrophoresis front; R_m, mobility of a band relative to the top of the gel and the electrophoresis front; and PAPS, phosphoadenosinephosphosulfate.

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