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Fluorophore-assisted carbohydrate electrophoresis (FACE) of glycosaminoglycans

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Summary

Objective: Quantitation and analyses of the fine structure of glycosaminoglycans are increasingly important for understanding many biological processes, including those most critical for understanding skeletal biology. We have developed a novel procedure, fluorophore-assisted carbohydrate electrophoresis (FACE), for determination of glycosaminoglycan fine structure and estimation of chain length.

Design: FACE utilizes enzymes that cleave glycosaminoglycans to create products, usually disaccharides, characteristic of the enzyme specificity. Each cleavage exposes a new reducing terminus that is fluorotagged by reductive amination with 2-aminoacridone. The tagged products are then displayed by electrophoresis, identified by their characteristic migration and chemistry, and quantitated by their molar fluorescence.

Results: Each class of glycosaminoglycan and the enzymes specific for each class are discussed. Specific application of the FACE technology is shown for analysis of the glycosaminoglycans on aggrecan isolated from knee cartilage of 5- and 68-year-old patients, and assessment of hyaluronan oligosaccharides.

Conclusions: The FACE technology is a powerful tool for analysis of all four classes of glycosaminoglycans obtained from a wide variety of biologic sources. While the FACE protocols are relative simple, they provide a wealth of information including quantitation in the pmole range, determination of fine structure, and estimation of chain length. © 2001 OsteoArthritis Research Society International

Key words: FACE technology, Glycosaminoglycans.

Glycosaminoglycans

There are four general classes of glycosaminoglycans: (1) hyaluronan; (2) chondroitin sulfate and dermatan sulfate; (3) keratan sulfate; and (4) heparin and heparan sulfate. With the exception of hyaluronan, glycosaminoglycans are synthesized covalently bound to core proteins forming the class of macromolecules called proteoglycans. All cells synthesize one or more glycosaminoglycans, and the parent proteoglycans are found inside cells, on their surfaces and in extracellular matrices[1–3].

Glycosaminoglycans are synthesized as polymers of repeating disaccharides with an *N*-acetylhexosamine as one of the sugars. The alternating sugar is glucuronic acid with the exception of keratan sulfate which contains galactose instead. The synthesized polymer for hyaluronan is not modified further, whereas the other three classes are modified by: (1) the addition of *O*-sulfates on various hydroxyls (all three classes); (2) 5-epimerization of some glucuronic acid residues to form iduronic acid residues (dermatan sulfate, heparin/heparan sulfate); and (3) removal of acetyl residues from some hexosamines and replacing them with *N*-sulfates (heparin/heparan sulfate). These modifications introduce fine structures within the chains that often have major roles in a wide variety of biological processes.

Enzymes that degrade glycosaminoglycans

The reducing ends of glycosaminoglycan chains on proteoglycans are covalently attached to their core proteins and hence have no free reducing groups. It is uncertain whether or not a hyaluronan chain is substituted on its reducing end. However, the length of the polymer, often with 10 000 or more repeating disaccharides, is such that a single free reducing group would not be detectable by the procedures described below. There are two classes of enzymes that selectively degrade glycosaminoglycans: hydrolases, which add water across the bond being cleaved, and lyases (eliminases), which in effect remove water between the 4-5 carbons of hexuronic acid residues with hydrolysis of the glycoside bond from the 4-carbon (Fig. 1). This leaves a $\Delta_{4,5}$ unsaturated bond on the hexuronic acid. Each cleavage, whichever class of enzyme, creates a free reducing group that can be fluorotagged for subsequent identification. Characteristics of some of the enzymes which degrade each class of glycosaminoglycan and which are commercially available are described below.

HYALURONAN

Testicular hyaluronidase, an endo hexosaminidase, degrades both hyaluronan and chondroitin sulfate (Fig. 1).

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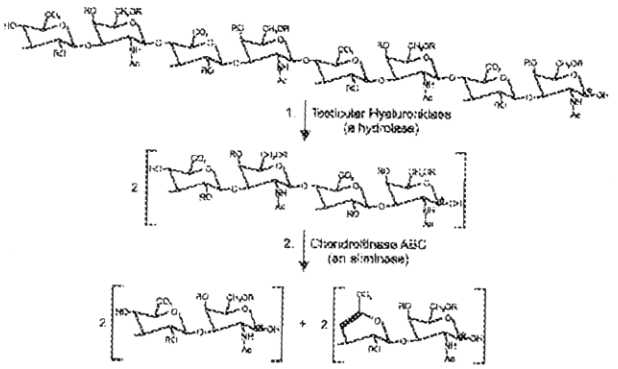

Fig. 1. Scheme showing the structure of an octasaccharide that could be derived from chondroitin sulfate. Digestion with testicular hyaluronidase, a hydrolase with specificity for both hyaluronan and chondroitin sulfate, would produce two tetrasaccharides with non-reducing glucuronic acid residues (reaction 1). Subsequent digestion of these tetrasaccharides with chondroitinase ABC, an eliminase, would produce two disaccharides with a non-reducing glucuronic acid residue and two disaccharides with a non-reducing Δ-hexuronic acid residue (reaction 2). The asterices indicate free reducing aldehydes available for fluorotagging. R: location, which may be occupied by either a sulfate or a hydroxyl group.

This enzyme is weakly reversible, and in a limit digestion produces predominantly hexa- and tetrasaccharides with non-reducing terminal glucuronic acid residues and reducing terminal *N*-acetylhexosamines[4–6]. The hyaluronidase from *Streptomyces*, an endoeliminase, is specific for hyaluronan; and, as for all eliminases, the cleavage is irreversible. The limit digestion contains hexa- and tetrasaccharides, in this case with non-reducing terminal Δ-hexuronic acid residues and reducing terminal *N*-acetylglucosamines[7]. The hyaluronidase from *Streptococcus dysIatea* is an eliminase that will also cleave un-sulfated regions in chondroitin sulfate chains. This enzyme is processive in that it has preference for cleaving disaccharides from non-reducing termini over endo activity. The limit digest contains β-disaccharides with non-reducing terminal β-hexuronic acid residues and reducing terminal *N*-acetylhexosamines.

CHONDROITIN SULFATE AND DERMATAN SULFATE

The chondroitinases (ABC, protease free ABC, AC and B) are processive eliminases (Fig. 1) with specificites for chondroitin sulfate (ABC and AC) and dermatan sulfate (ABC and B)[7,8]. Chondroitinase ABC also cleaves hyaluronan, but less effectively. The limit products are Δ-disaccharides from the cleaved regions of the chains, except in the case of protease free chondroitinase ABC which exhibits more restricted specificity, e.g. generates mixtures of β-di- and β-tetrasaccharides from the chain interior, and trisaccharides from the non-reducing termini of chains ending with sulfated GalNAc residues[9].

KERATAN SULFATE

Keratan sulfate chains are composed of β1,3-Gal-β1,4-GlcNAc repeats, that are either un-, mono-(Gal-β1,4-GlcNAc6S) or disulfated (gal6S-β1,4-GlcNAc6S). They can be depolymerized by a number of microbial endo-hydrolases[10–16], several of which are commercially available. For example, enzymes from *E. freundii* (endo-β-galactosidase) or *Pseudomonas* sp. (keratanase) hydrolyze internal β1,4-Gal linkages in regions of the glycosaminoglycan that are enriched in monosulfated disaccharide repeats, thereby releasing the disaccharide GlcNAc6S-β1,3-Gal. The *E. freundii* enzyme can additionally depolymerize unsulfated regions yielding GlcNAc-β1,3-Gal since its activity is not influenced by the sulfation of the participating glcNAc residue[15]. Extensive depolymerization of keratan sulfate into disaccharides has been achieved by keratanase II, an endo-*N*-acetylglucosaminidase purified from *Bacillus* sp.[16]. This enzyme catalyzes cleavage of β1,3 linkages involving sulfated GlcNAc residues and releases the disaccharides, Gal-β1,4-GlcNAc6S and Gal6S-β1,4-GlcNAc6S.

HEPARIN AND HEPARAN SULFATE

Heparan sulfate consists of block structures along the chain with regions of little or no modification interspersed between highly sulfated and modified regions, while heparin contains mostly the highly sulfated and modified regions. Heparanases are mammalian endoglucuronidases (hydrolases) that degrade chains to shorter oligosaccharides with reducing terminal glucuronic acid residues and non-reducing terminal sulfated glucosamine residues[17–19]. Class I heparanases yield products with highly sulfated domains at the non-reducing ends, while class II heparanases yield products with such residues at the reducing end[18]. The heparitinases, I, II and III, are eliminases with different specificities of cleavage depending upon the fine structure at the cleavage site[20,21]. Heparitinase I requires an unsulfated or monosulfated glucosamine (either *N*-sulfated or *N*-acetylated and 6-sulfated) in glycosidic linkage with unsubstituted glucuronic acid at the cleavage site. Heparitinase II cleaves bonds in which the glucosamine can be unsulfated or monosulfated as for heparitinase I adjacent to either glucuronic acid or iduronic acid with or without 2-sulfate. Heparitinase III cleaves between glucosamine with any degree of substitution adjacent to 2-sulfated iduronic acid. Thus, each heparitinase can yield a different pattern of products, while their combined use yields β-disaccharides.

Mercuric ion cleavage of β-hexuronic acid residues

The unsaturated, non-reducing terminal β-hexuronic acid residues generated by either the chondroitinases or the heparitinases are susceptible to treatment with mercuric ion under mild conditions, while non-reducing terminal unmodified hexuronic acid residues are not (Fig. 2). The reaction quantitatively removes the β-hexuronic acid residue while leaving the rest of the oligosaccharide (or monosaccharide in the case of β-disaccharides) intact[22].

Fluorotagging free reducing groups with 2-aminoacridone (AMAC)

The free reducing groups that are exposed by enzyme cleavage, or by enzyme cleavage followed by mercuric ion treatment, can be derivatized with 2-aminoacridone by reductive amination in the presence of cyanoborohydride (Fig. 3)[6,23,24]. Initial studies utilized 2-aminopyridine and defined conditions that achieved quantitative derivatization

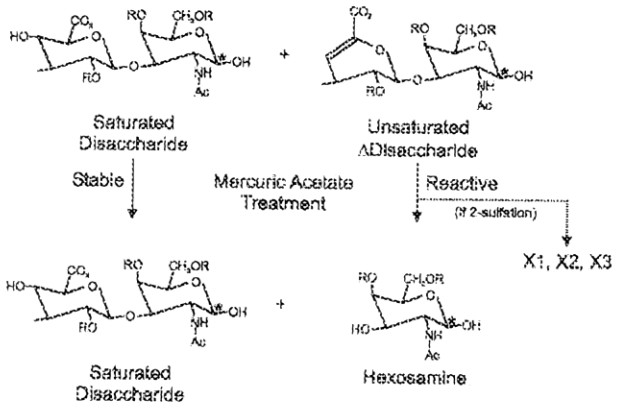

Fig. 2. Scheme showing the sensitivity of the chondroitinase ABC digestion products (reaction 2, Fig. 1) to mercuric ion treatment. The disaccharide with a non-reducing glucuronic acid residue is stable to mercuric ion treatment while the non-reducing Δ-hexuronic acid residue of the second disaccharide is removed by the mercuric ion treatment freeing the corresponding hexosamine. In the absence of 2-sulfation on the hexuronic acid no fluorotagged product is recovered from the Δ-hexuronic acid residue, while in the presence of 2-sulfation three unknown flurotagged products characteristic of 2-sulfation are observed (X1, X2 and X3, see Fig. 4A). The asterices indicate free reducing aldehydes available for fluorotagging. The Rs indicate locations, which may be occupied with either a sulfate or a proton group.

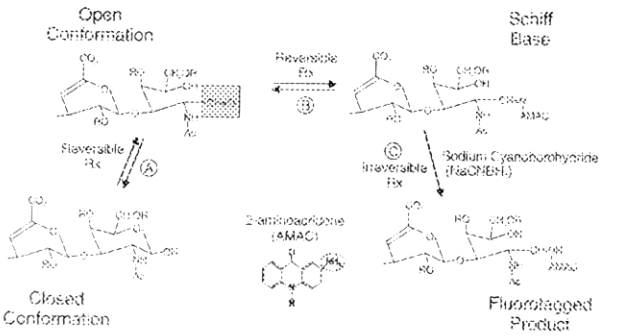

Fig. 3. Reducing sugars in solution are in equilibrium (reaction A) between a six-member closed pyranose ring conformation and an open conformation containing a free reducing aldehyde (shaded box). The amine group (dashed circle) of 2-aminoacridone (AMAC) reacts with the free reducing aldehyde to form a Schiff base (reaction B) that is stabilized by reduction with cyanoborohydride (reaction C). The final AMAC-tagged saccharide yields a fluorescent signal dependent solely on the fluorotag, not the chemistry of the saccharide. The Rs indicate locations, which may be occupied with either a sulfate or a proton group.

without desulfation[25,26]. The fluorotagging introduces a highly fluorescent label that provides an identical signal for every free reducing group.

Separation of fluorotagged standards by electrophoresis

The fluorotagged products can be separated on polyacrylamide gels (Glyko monosaccharide gels) and individual bands scanned for fluorescence[6,27]. An example of the resolution of the known β-disaccharides that would be found in a standard digestion protocol using a combination of streptococcal hyaluronidase and chondroitinase ABC for analysis of chondroitin/dermatan sulfate and hyaluronan is

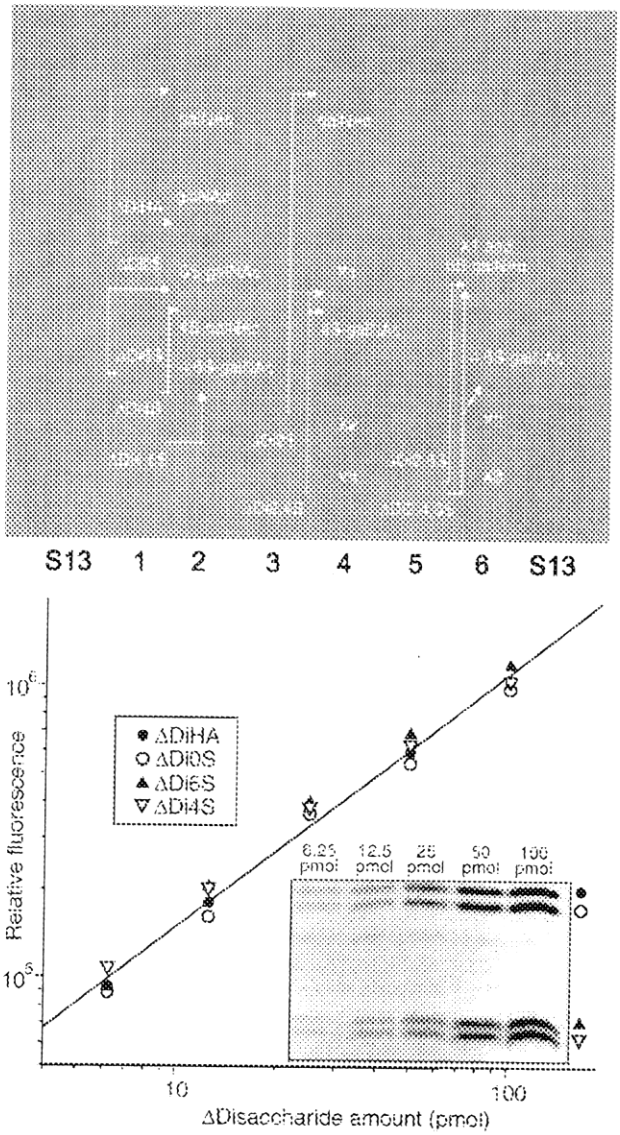

Fig. 4. Panel A: Δ-Disaccharide standards from hyaluronan and chondroitin/dermatan sulfate analysed by fluorophore-assisted carbohydrate electrophoresis (FACE) without (lanes 1, 3, 5) or with (lanes 2, 4, 6) prior mercuric ion treatment followed by derivatization with 2-aminoacridone (AMAC). Arrows indicate the major product(s) from the mercuric ion treatment of each Δ-disaccharide. AMAC-derivatized Δ-disaccharides and their mercuric ion treatment products are from different standard preparations containing several saccharide concentrations. Note the presence of the X1, X2 and X3 bands whenever 2-sulfation is present on the Δ-hexuronic acid residue. Panel B: Quantitation of AMAC-derivatized hyaluronan and chondroitin sulfate Δ-disaccharides after separation by FACE. Mixtures containing from 6.25 to 100 pmoles each of the indicated AMAC derivatives were separated by FACE. The gel (inset) was then imaged using a Quantix cooled-CCD camera, and the images analysed using a Gel-Pro Analyzer® program[6,27]. The relative fluorescence for each band is plotted vs pmoles of Δ-disaccharides as determined by hexuronic acid analysis (r=0.997, P=0.00022).

shown in Fig. 4A (lanes 1, 3, 5). Figure 4A (lanes 2, 4, 6) shows the locations of the products that would be produced when the respective β-disaccharides are treated with mecuric ion before fluorotagging, i.e. the *N*-acetylhexosamines with or without sulfates, which were

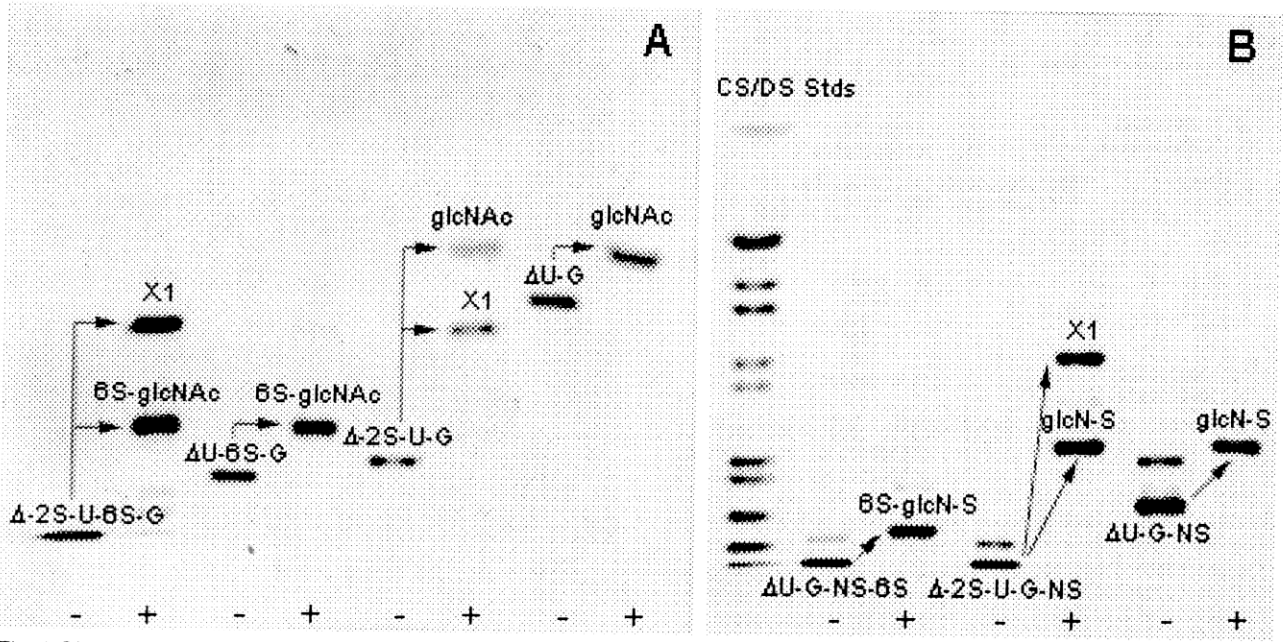

Fig. 5. Disaccharide standards from heparin/heparan sulfate analyzed by fluorophore-assisted carbohydrate electrophoresis (FACE) without (−) or with (+) prior mercuric ion treatment followed by derivatization with 2-aminoacridone (AMAC). Arrows indicate the products from the mercuric ion treatment of each Δ-disaccharide. U: hexuronic acid; G: glucosamine.

freed by the mercuric ion treatment. While unsulfated β-hexuronic acid gives no identifiable fluorotagged product after mercuric ion treatment, the 2-sulfated β-hexuronic acid does (band X1). The presence of this band, then, is diagnostic of 2-sulfated hexuronic acid in the original chondroitin/dermatan sulfate chain. The graph in Fig. 4B shows the fluorescent response for increasing concentrations of four of the β-disaccharides, and shows that: (1) the molar fluorescence is the same for each; and (2) the level of detection extends down to the pmol range for the β-disaccharides.

A similar strategy can be used for heparin/heparan sulfate. An example of the resolution of several standard fluorotagged β-disaccharides derived from enzyme digestion of heparin/heparan sulfate is shown in Fig. 5 (lanes −) as well as fluorotagged products after mercuric ion treatment (lanes +). In this case a Glyko oligosaccharide gel was used, which gives better resolution for the more highly sulfated species [Wang, A., unpublished observations]. Again, band X1 is characteristic of β-disaccharides with 2-sulfated β-hexuronic acid.

The enzymes that cleave keratan sulfate are hydrolases and do not generate unsaturated sugars at the non-reducing ends of the digestion products. Thus, the products are not altered by mercuric ion treatment. An example of the use of these enzymes followed by fluorotagging for analysis of keratan sulfate on aggrecan is given below.

Applications

ANALYSES OF CHONDROITIN SULFATE AND KERATAN SULFATE ON AGGRECAN

Aggrecan purified from human knee cartilages of a 5- and a 68-year-old donor were analyzed for chondroitin sulfate by digesting 2 μg (as glycosaminoglycan determined by dimethylmethylene blue binding assay) for 4 h with 2 milli-units of chondroitinase ABC or for keratan sulfate by digesting 20 μg consecutively with 2 milli-units of keratanase II (*Bacillus* sp.) (4 h) and 2 milli-units of endo-beta-galactosidase (*E. freundii*) (18 h). Products from both digests were fluorotagged before separation and quantitation by FACE (Fig. 6 and Table 1). The FACE analyses readily display the distinct glycosaminoglycan compositions of the two aggrecan populations. Aggrecan from young cartilage contains predominantly chondroitin sulfate (~92%

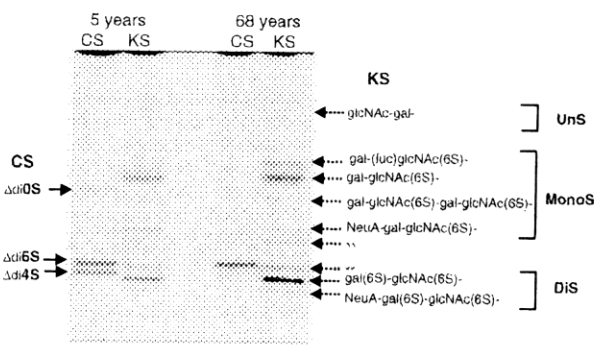

Fig. 6. Aggrecan samples from human knee cartilages of a 5- and a 68-year-old donor [see Ref. 26 for sample preparation details] were digested with chondroitinase ABC or keratanase II plus endo-beta-galactosidase. The products were fluorotagged and analysed by FACE. The gel image shows the separation of chondroitin lyase products from 200 ng of aggrecan (lanes denoted CS) and keratan sulfate hydrolase products from 2 μg of aggrecan (lanes denoted KS). The migration positions of the chondroitin lyase products (Δ-Di0S, Δ-Di4S and Δ-Di6S) are indicated in the left hand margins. The migration positions of the identified keratan sulfate hydrolase products, as well as two unidentified minor products (denoted **) that are most likely hexasaccharide intermediates from keratanase II digests, are shown in the right hand margins. The three sets of digestion products used for calculation of the keratan sulfate disaccharide compositions [0S=glcNAc-gal; MS=gal-glcNAc(6S); DiS=gal(6S)-glcNAc(6S), see Table I] are also given.

Table I
Calculated from FACE gel

	Glycosaminoglycan composition (mg/mg total S-GAG)		Disaccharide composition						
	CS	KS	CS*			KS†			
			0S	4S	6S	0S	MS	MS(fuc)‡	DS
5 years	0.89 (92%)	0.08 (8%)	8	40	52	4	52	nd¶	44
68 years	0.52 (68%)	0.26 (32%)	2	4	94	nd¶	40	12	48

CS: Chondroitin sulfate.
KS: Keratan sulfate.
*Percent of total Δ-disaccharides (0S+4S+6S).
†Percent of total disaccharides (0S+MonoS+DiS).
‡Monosulfated and fucosylated disaccharide.
¶Not detected.

of total glycosaminoglycan), while aggrecan from adult cartilage contains more keratan sulfate (~32% of the total glycosaminoglycan). Previously reported differences in sulfation of both the glycosaminoglycans are also readily visualized. Aggrecan chondroitin sulfate from the young donor is composed of 4 and 6-sulfated disaccharides, whereas chondroitin sulfate from the adult donor is almost exclusively of 6-sulfated disaccharides. Keratan sulfate from both young and adult cartilages is extensively sulfated at the C6 position of GlcNAc residues. However, in the adult, an increase in the C6-sulfation of Gal residues and in the fucosylation of GlcNAc6S residues is seen.

NON-REDUCING TERMINI

Previous work used fluorotagging with 2-aminopyridine and anion exchange on PA1 columns to resolve chondroitinase digestion products of purified human aggrecan samples from knee cartilage of individuals differing in age[26]. The fluorotagged non-reducing termini were identified and quantitated. This allowed the number average molecular weights of the chondroitin sulfate chains to be estimated; i.e. the total mass of digestion products divided by the number of moles of non-reducing termini. The results also showed that most chains, ~85%, terminated with *N*-acetylgalactosamine, either 4-sulfated or 4,6 disulfated. The latter was only found on aggrecan isolated from adult individuals, i.e. after reaching skeletal maturity. Similar analyses of aggrecan from osteoarthritic cartilage revealed non-reducing termini and internal sulfation patterns more characteristic of normal cartilage from individuals immediately before skeletal maturity[2,28].

The FACE procedure in combination with selective sulfatases and mercuric ion treatment can also resolve and quantitate the low abundant non-reducing termini[6,27]. The analysis in this case requires less time and experimental manipulation. With the exception of the mercuric ion step, in which a cation exchange step is used to remove the mercuric ion, all of the steps are done in the same reaction tube. Further, the enzymes and reagents do not enter the gel and hence are not removed prior to analysis. In the previous procedure, the unsulfated Δ-disaccharide is lost during the step required to remove the fluorotagging reagent, and quantitation of the non-reducing terminal 6-sulfated *N*-acetylgalactosamine was obscured by a minor side-reaction involving desulfation of the 6-sulfated β-disaccharide[25].

QUANTITATION OF HYALURONAN

The β-disaccharide derived from digestion of hyaluronan by streptococcal hyaluronidase or chondroitinase ABC is not sulfated and therefore was not recovered in the previous method[25]. Thus, the FACE procedure provides an ideal method for analysis of this glycosaminoglycan. The advantages include: (1) sensitivity (in the ng range); (2) rapidity (typically within 1 day); and (3) multiple samples per analysis (typically 6–8 per gel).

Figure 7 shows an example of the application of FACE for analyses of purified and partially purified hyaluronan oligosaccharides prepared by molecular sieve chromatography of a testicular hyaluronidase digest (kindly provided by Dr Markku Tammi, University of Kuopio). The white arrowheads identify the band originally indicated for each sample. While the smaller oligosaccharides, such as HA_{12} (6 disaccharide repeats), contain a single dominant hyaluronan band, the larger oligosaccharides contain

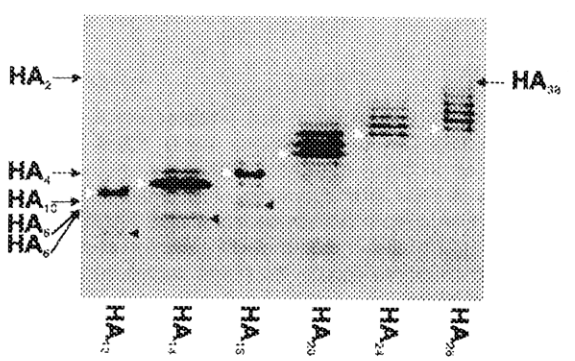

Fig. 7. FACE analyses of 2-aminoacridone (AMAC) derivatized testicular hyaluronidase products from hyaluronan (HA) recovered by molecular sieve chromatography. Column fractions corresponding to specific HA oligomer sizes were collected, AMAC-derivatized, and separated by fluorophore-assisted carbohydrate electrophoresis (FACE) to confirm size distribution and purity. The size for each preparation (HA_{12}, HA_{14}, HA_{16}, HA_{20}, HA_{24} and HA_{26}) as originally determined by molecular sieve chromatography is indicated at the bottom of each lane with the band corresponding to that size indicated by a white arrowhead. The relative positions of saturated hyaluronan oligomer standards containing one (HA_2), two (HA_4), three (HA_6), four (HA_8), five (HA_{10}) and nineteen (HA_{38}) disaccharides are indicated by arrows. The black arrowheads indicate contaminating chondroitin sulfate oligosaccharides.

additional bands from other oligosaccharides, predominantly higher than the originally indicated component. For example, the HA_{24} sample shows bands extending up to at least HA_{38}, reflecting both the resolution of the FACE method and the difficulty in purifying the higher oligosaccharides by molecular sieve chromatography. While not shown, the arrows at the side indicate the positions where oligosaccharides smaller than HA_{12} would run. Thus, the oligosaccharides exhibit a ladder based on molecular size until HA_6, which migrates nearly the same as HA_8, while HA_4 and HA_2 migrate to anomalous positions higher in the ladder. Additional bands, indicated by the black arrowheads, are present in the analyses of the smaller oligosaccharides. These are contaminating oligosaccharides derived from chondroitin sulfate as indicated in subsequent analyses (not shown) in which the samples were treated with chondroitinase and analyzed by FACE. In each case these bands disappeared with the appearance of disaccharides characteristic of chondroitin sulfate. Thus, the original sample of hyaluronan used for preparing the oligosaccharides contained a small amount of chondroitin sulfate that was converted to oligosaccharides by the testicular hyaluronidase; and these co-purified with the respective hyaluronan oligosaccharides.

TISSUE SAMPLES

The high resolution and sensitivity of the FACE procedure makes it feasible to analyse small pieces of tissue (in the mg range). An example of such an analysis for hyaluronan and chondroitin sulfate in tissue from a rat chondrosarcoma is described in detail elsewhere[27]. In this case, proteinase K was used to solubilize the tissue, and after heat inactivation of the enzyme, a portion was fluorotagged directly and analyzed. This reveals the presence of sugars, primarily glucose, with free reducing groups that are present in the tissue. If the amounts are high relative to the amounts of glycosaminoglycans expected, the latter can be precipitated with 70% ethanol before resolubilization and further analysis.

Conclusions

The methodology outlined in this chapter, once refined, will make it feasible to quantitate each class of glycosaminoglycan in small samples, and to determine many of the details of their fine structures. For example, microdissection of tissue from frozen sections and FACE analysis might reveal details of regional differences, such as in normal or pathological articular cartilage, which reflect cell and matrix dynamics.

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