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Keratan sulfate disaccharide composition determined by FACE analysis of keratanase II and endo-β-galactosidase digestion products

Anna H.K. Plaas[1,2,3], Leigh A. West[2], and Ronald J. Midura[4]

[2]Shriners Hospital for Children, 12502 N. Pine Drive, Tampa, FL, 33612, USA; [3]Department of Biochemistry and Molecular Biology, University of South Florida, College of Medicine, FL, USA; and [4]The Lerner Research Institute of the Cleveland Clinic Foundation, Euclid Avenue, Cleveland, OH 44195, USA.

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Many tissues contain glycoproteins and proteoglycans, which are substituted with N-or O-linked keratan sulfate, a glycosaminoglycan in which the lactosamine (-galβ*1,4*glcNAc-) disaccharide backbone is variably modified by sulfation, fucosylation, and sialylation. We report here a rapid, sensitive, and quantitative procedure for obtaining a complete disaccharide compositional analyses for keratan sulfates after FACE separation of products generated by hydrolysis of the glycosaminoglycans with *B. fragillis* keratanase II and *E. freundii* endo-β-galactosidase. Seven digestion end products are separable in a single electrophoretic step using Monosaccharide™ composition gels. These are: the unsulfated disaccharide, glcNAcβ*1,3*gal, the fucosylated trisaccharide, galβ*1,2*[fucα*1,3*]glcNAc6S, the mono- and disulfated disaccharides, galβ*1,4*glcNAc6S or gal6Sβ*1,4*glcNAc6S from the chain interior, and the sialylated mono- and disulfated trisaccharides neuAα*2,3*galβ*1,4*glcNAc6S or neuAα*2,3*gal-6Sβ*1,4*glcNAc6S from the nonreducing terminus. FACE analyses also revealed the presence of a contaminant β-galactosidase activity in keratanase II enzyme preparations which cleaves the disaccharide, galβ*1,4*glcNAc6S to its constituent monosaccharides, gal and glcNAc6S. It was particularly prominent at enzyme concentrations > 2 mU per nmole substrate $glcNH_2$ or after prolonged digestion times (> 12 h), and was not inhibitable by thiogalactosides or N-acetyl-lactosamine. As these monosaccharide products would not be detectable using the commonly described analytical methods for KS hydrolase products, such as ^{1}H-NMR and HPLC analyses, our data illustrate that the FACE procedure represents an improved approach for accurate compositional microanalyses of corneal and skeletal keratan sulfates, especially applicable to experimentation involving small amounts (1–2 μg) of this glycosaminoglycan.

Key words: glycosaminoglycans/keratan sulfate/keratanases/proteoglycans/sulfation

Introduction

The extracellular matrix of such tissues as cornea, hyaline cartilages, and the nucleus pulposus of the intervetebral disks are a rich source of glycoproteins and proteoglycans (PGs) on which N- or O-linked oligosaccharides are extended with the glycosaminoglycan keratan sulfate (KS). Many of the basic structural characteristics of this glycosaminoglycan have been determined on KS isolated from such sources (Meyer and Anderson, 1965; Meyer *et al.*, 1953; Mathews and Cifonelli, 1965; Bavanandhan and Meyer, 1968; Choi and Meyer, 1975). It is made up of the β*1,3*-linked backbone of the repeating disaccharides (galβ*1,4*glcNAc), and chain lengths can range anywhere between 3 and ~ 40 repeats (Oeben *et al.*, 1987; Dickenson *et al.*, 1991; Plaas and Wong-Palms, 1993). Within a given chain population, the majority of the glcNAc residues and a significant proportion of adjacent gal residues are sulfated in the C6 position (Edge and Spiro, 1985; Hounsell *et al.*, 1986) giving rise to mono- or disulfated regions, respectively. In addition, fucosylation of glcNAc6S within monosulfated chain regions (Tai *et al.*, 1991, 1993, 1994), and sialic acid capping of nonreducing terminal gal or gal6S (Toda and Seno, 1970; Dickenson *et al.*, 1991, 1992) have also been reported, and such studies have further suggested tissue-specific distribution of such modifications.

A more widespread occurrence of KS-substituted molecules became apparent in the 1980s after the successful production and use of monoclonal antibodies to KS-PGs (Funderburgh *et al.*, 1982, 1987; Caterson *et al.*, 1983; SundarRaj *et al.*, 1985; Zanetti *et al.*, 1985; Keiser and Diamond, 1987). Furthermore, application of these reagents in immunoassays and immunohistochemical localizations led to the observations that KS substitution of PGs may play a role in the formation and maintenance of structural collagen networks (Quantock *et al.*, 1997; Scott and Thomlinson, 1998; Hedlund *et al.*, 1999; Svensson *et al.*, 2000) and cell-matrix adhesion processes (Burg and Cole, 1994; Wendel *et al.*, 1998; Ota *et al.*, 2000).

Despite the high sensitivity and relative specificity of such immunoreagents they are of limited use in the quantitation and fine structure analyses of KS chains, due to a common requirement for disulfated KS chain regions (Mehmet *et al.*, 1986), high epitope densities (Seibel *et al.*, 1992), and possible epitope masking by the fucose and sialic acid substitutions (Thornton *et al.*, 1989b). Thus an examination of specific aspects of the fine structure and function of KS required the development of analytical procedures for quantitation and complete disaccharide compositional analyses of this glycosaminoglycan.

A range of analytical biochemical methods for KS have been reported; they generally involve depolymerization, either by

[1]To whom correspondence should be addressed

hydrazinolysis (Shaklee and Conrad, 1986, Brown *et al.*, 1992) or enzymatic cleavage with specific hydrolases, such as endo-β-galactosidases from *E. freundii* (Fukuda, 1981; Kitamikado, 1984; Kitamikado *et al.*, 1981; Li *et al.*, 1982; Scudder *et al.*, 1984), or from *Pseudomonas* sp. (designated keratanase) (Fukuda, 1981; Kitamikado *et al.*, 1981; Li *et al.*, 1982) and the endo-β-N-acetylglucosaminidase from *Bacillus* sp. (designated keratanase II) (Nakazawa *et al.*, 1989), followed by separation and quantitation of digestion products by reverse-phase (Yamada *et al.*, 2000) or anion-exchange high-performance liquid chromatography (HPLC) (Brown *et al.*, 1995; Whitham *et al.*, 1999). Using these approaches, "compositional finger-print maps" of KS from tissues, cell cultures, and synovial fluid, can be obtained reproducibly; however, the chromatographic analyses have not been optimized for KS quantitation or disaccharide compositional analyses, and can not evaluate products liberated from unsulfated regions of KS chains. Moreover, despite the wide use of the KS hydrolases for the generation of a variety of structurally distinct oligo-saccharide sequences from the chain interior, these enzymes have not been fully described with respect to their capacity for quantitative depolymerization of KS chains of variable size, sulfation and fucosylation.

To address some of these inadequacies in KS analyses, we describe a novel application of fluorophore-assisted carbo-hydrate electrophoresis (FACE) technology to provide a simple, highly sensitive, and quantitative tool for determination of the range of unsulfated, sulfated, fucosylated, and sialylated KS hydrolase products. We discuss fine structure data obtained by FACE analyses for KS chains substituted on aggrecan from human cartilages of different ages in relation to those previously obtained using HPLC and ^{1}H-nuclear magnetic resonance (NMR) methodological approaches. We also suggest a broader applicability of this procedure for detection and characterization of KS in studies designed to determine whether different GAG fine structures may confer unique biological properties on specific extracellular matrices.

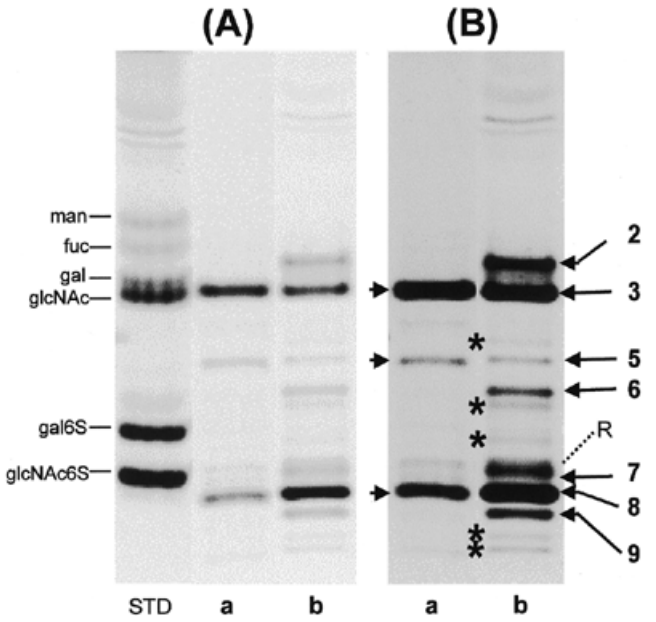

Fig. 1. FACE gel separation of KII digestion products from corneal and skeletal KS. Gel images of fluorotagged products from KII digests of bovine corneal KS (7.25 nmole $glcNH_2$) (lanes a) or human skeletal KS (11 nmole $glcNH_2$) (lanes b) were taken after either 1 min (**A**) or 1.5 min (**B**) exposures. Identified products (see Table I) are numbered in the right hand margin of **B**, reagent background bands are indicated by R, and minor oligosaccharide products, not further characterized in this study, are marked by an asterisk (*). A coelectrophoresed monosaccharide mixture consisting of 25 pmole fuc, 25 pmol man, 50 pmol gal, 150 pmol glcNAc, 300 pmol gal6S, and 600 pmol glcNAc6S is shown in STD lane, **A**.

Results

Separation and identification of KS hydrolase products from corneal and skeletal KS

Keratanase II digestion. Bovine corneal and adult human aggrecan KS were digested with 0.2 mU of KII for 4 h at 37°C, the products were fluorotagged and separated by FACE on precast Monosaccharide Composition gels (Figure 1). The corneal digest (lanes a) was resolved into three major products and the skeletal digest (lanes b) contained eight distinct products, three of which comigrated with those also present in the corneal sample. The individual species (designated 2–9, Figure 1, panel B) were identified subsequently based on size fractionation on Superdex Peptide gel filtration column, sensi-tivities to exoglycosidase digestion (Figures 2–4) and also compared to published structural information of KII products (Nakazawa *et al.*, 1989; Whitham *et al.*, 1999).

The two prominent products (bands 3 and 8) generated from both KS preparations are the disaccharides galβ*1,4*glcNAc6S and gal6Sβ*1,4*glcNAc6S, respectively. They eluted in the same positions as chondroitin sulfate (CS)-derived disaccharides after fractionation of total digests on Superdex Peptide chromatog-raphy (Figure 2, lane c) and were previously reported as the major end products after KII digestion of shark cartilage KS (Nakazawa *et al.*, 1989), bovine (Whitham *et al.*, 1999) or human corneal KS (Tai *et al.*, 1997), and bovine (Brown *et al.*, 1994) or human cartilage KS (Brown *et al.*, 1998). The human cartilage KS digest contained a product (band 2), not detected in bovine corneal KS that migrated slower than the mono-sulfated disaccharide (band 3), and in- between the fuc and gal monosaccharide standards (Figure 1A,B). In keeping with previous reports where fucose substitution of glcNAc6S in skeletal KS was detected by ^{1}H-NMR (Tai *et al.*, 1991) this products was identified as the trisaccharide galβ*1,4*[fucα*1,3*]glcNAc6S. It was eliminated when the total KII digests were pretreated with α-fucosidase and this digestion generated an equal amount of fucose and a product that comigrated with the monosulfated disaccharide band 3 (Figure 3A,B).

The less abundant KII products from both substrates (bands 5, 6, 7, and 9, Figure 1) were purified in pools a and b after the Superdex Peptide column fractionation (see Figure 2, lanes a and b) and the FACE analyses of such oligosaccharides are shown in lanes a of Figures 4 and 5. Products from corneal KS (Figure 4A,B) included two bands (5 and 7), a small amount of the disulfated disaccharide (band 8), as well as several minor products indicated by an asterisk (*). Digestion of this

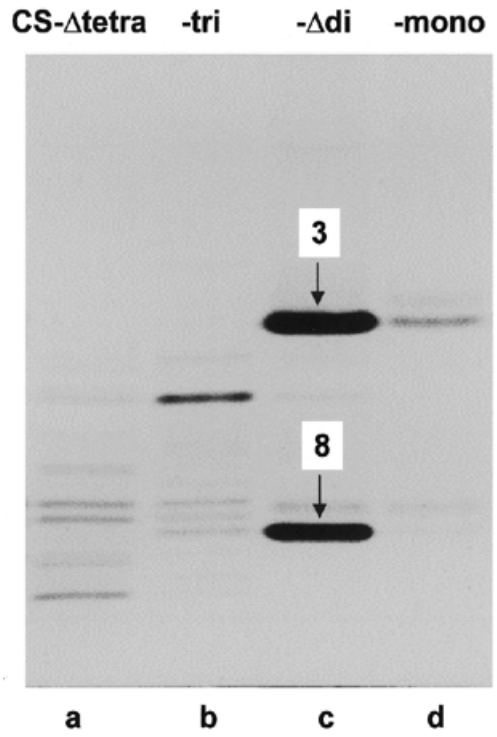

Fig. 2. FACE analyses of Superdex Peptide fractionated KII digestion products from corneal KS. Bovine corneal KS (140 nmol glcNH$_2$) was digested with 12 mU of KII for 4 h, and 95% of the digestion mixture was applied to Superdex Peptide (HR10/30), preequilibrated in 0.5 M ammonium bicarbonate, pH 7.0; chromatography was carried out at a flow rate of 0.5 ml/min, and eluant collected in 500-µl size fractions. Fractions between 26 and 31.5 ml were combined for pool a, between 32 and 37.5 ml for pool b, between 38 and 41.5 ml for pool c, and between 42 and 44 ml for pool d. This fractionation scheme had been shown previously to separate CS derived Δtetra-, tri-, Δdi-, and monosaccharides (West *et al.*, 1999).

oligosaccharide pool with β-galactosidase prior to electrophoresis resulted in increased mobility of bands 5 and 7 to positions 5′ and 7′ and the release of an equal amount of gal (lane b in Figure 4A,B). Consecutive digestion of this pool with β-galactosidase and β-N-acetyl-glucosaminidase, did not generate any additional detectable product, such as glcNAc (lane c in Figure 4A,B), consistent with a nonreducing terminal sequence, galβ*1,4*glcNAc6S in oligosaccharides 5 and 7. Hence, based on exo-glycosidase digestibility, the elution positions from the Superdex Peptide column and the electrophoretic mobilities, these represent the di- and tri-sulfated tetrasaccharides galβ*1,4*glcNAc6Sβ*1,3*galβ*1,4*glcNAc6S and galβ*1,4*glcNAc6Sβ*1,3*gal6Sβ*1,4*glcNAc6S, respectively, previously identified products after digestion of different KS preparations by KII (Whitham *et al.*, 1999).

FACE analyses of the Superdex Peptide pool from the skeletal KS digests (Figure 5) showed bands 5 and 7 and two additional products, designated bands 6 and 9. Digestion of this oligosaccharide pool with the broad specificity α-neuraminidase from *Vibrio cholerae* (Figure 5, lane b) or an α*2,3*-specific neuraminidase (Figure 5, lanes b and c, respectively) resulted in selective and essentially quantitative removal of bands 5 and 7 with appearance of the mono- and disulfated disaccharides (bands 3 and 8) as well as neuA. NeuA released during these digestions was resolved into three bands following FACE separation on Monosaccharide gels, and this also was observed when products from a neuraminidase digest of the standard oligosaccharides, 3′-SLN or 6′-SLN were analyzed by FACE (Figure 5, lanes e and g). This identified the two bands as the nonreducing terminal trisaccharides neuAα*2,3*galβ*1,4*glcNAc6S and neuAα*2,3*gal6Sβ*1,4*glcNAc6S. These trisaccharide products were essentially absent in corneal KII digests (Figures 1 and 4A), showing that corneal KS chains used in this study were essentially devoid of nonreducing terminal neuA. Other minor oligosaccharide size products present in these Superdex Peptide pools (*, in Figures 4 and 5A) were not further identified here, but these most likely include previously described tri- and tetrasulfated and/or fucosylated oligosaccharides generated as intermediates during the endolytic cleavage pattern of KII.

Endo-β-galactosidase products. Published methodologies for KS fine structure analyses have primarily established chain sulfation sequences but were not optimized to determine products from unsulfated regions of the glycosaminoglycan. Such regions can be depolymerized by the endo-β-galactosidase (EB) from *E. freundii* (Fukuda, 1981), which cleaves the galβ*1,4* linkages to either glcNAc or glcNAc6S (Scudder *et al.*, 1984, 1986), thus generating the non- and monosulfated disaccharides glcNAcβ*1,3*gal and glcNAc6Sβ*1,3*gal, respectively.

Products generated by EB from corneal and skeletal KS were analyzed by FACE (Figure 6A, lanes a and c, respectively). The major product that migrated between the glcNAc and gal6S monosaccharide standards (band 4) was identified as the monosulfated disaccharide glcNAc6Sβ*1,3*gal, as it was recovered in virtually identical yields to the equivalent KII product galβ*1,4*glcNAc6S, band 3 (Table I), it eluted from Superdex Peptide in the disaccharide position (data not shown), and it was also obtained when corneal and skeletal KS were digested with *Pseudomonas* sp. keratanase (Figure 6A, lanes b and d, respectively), an endo-galactosidase with a strict requirement for a sulfated glcNAc6S adjacent to susceptible galβ*1,4* linkages (Nakazawa *et al.*, 1989). EB digests of corneal KS included an additional species (band 1), which migrated very slowly on the monosaccharide gel (Figure 6A, lane a). This species was identified as the unsulfated disaccharide glcNAcβ*1,3*gal since it comigrated with such a product prepared by β-galactosidase digestion of the trisaccharide galβ*1,4*glcNAcβ*1,3*gal (Figure 6B, lane b and c).

Both endo-galactosidases generated other products (marked ODS, Figure 6A lanes a–d) that migrated faster than the disaccharides, and were in distinct arrays for each KS preparation. For skeletal KS, two of these (bands 10 and 11) were identified as nonreducing terminal tetrasaccharides neuAα*2,3*galβ*1,4*glcNAc6Sβ*1,3*gal and neuAα*2,3*gal6Sβ*1,4*glcNAc6Sβ*1,3*gal, respectively, based on their complete digestibility with *V. cholera* neuraminidase (data not shown). The remainder of the ODS products in both digests were chain portions containing disulfated or fucosylated disaccharides undigestible by both endo-galactosidases (Fukuda, 1981; Scudder *et al.*, 1983, 1984) but depolymerized to the di- and trisaccharides gal6Sβ1,4glcNAc6S and galβ1,4[fucα1,3]glcNAc6S by KII (data not shown).

The recovery of all identified hydrolase products and their relative abundance was calculated from the gel images shown

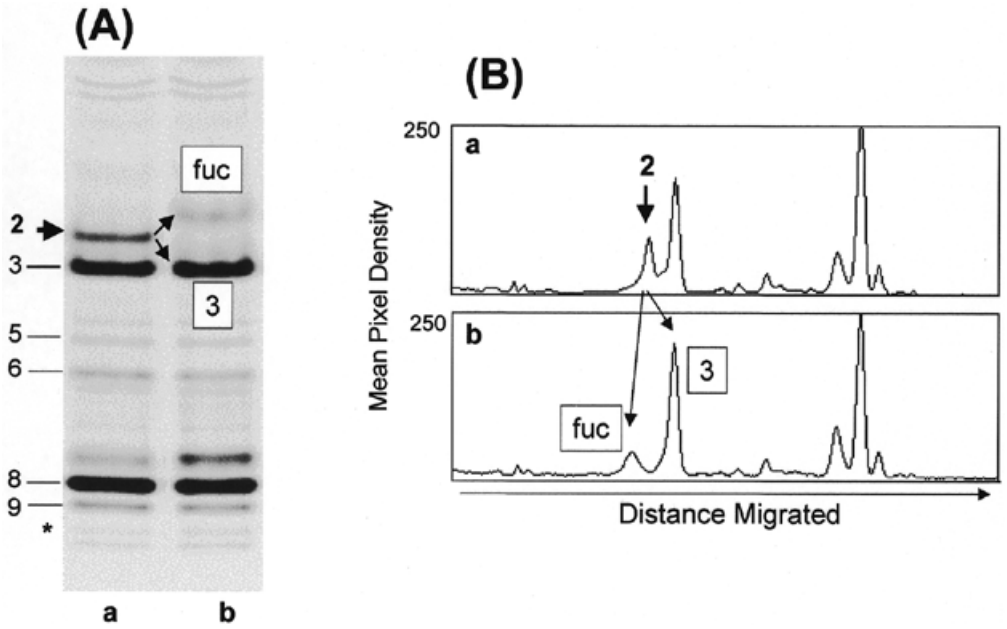

Fig. 3. Identification of fucosylated products in KII digests of skeletal KS. Products generated after digestion of human skeletal KS (5 nmole $glcNH_2$) with either 2.5 mU KII (lane a) or 2.5 mU KII followed by 10 μU α-fucosidase (lane b) were separated by FACE. **A** shows the gel image taken at 0.5 min exposure time, **B** shows a scan of the gel image used for product quantitation. Band 2 (= 135 mean pixel density in lane a) was converted to fuc (= 112 mean pixel density) and also gave an increase of 127 mean pixel densities in band 3 (lane b).

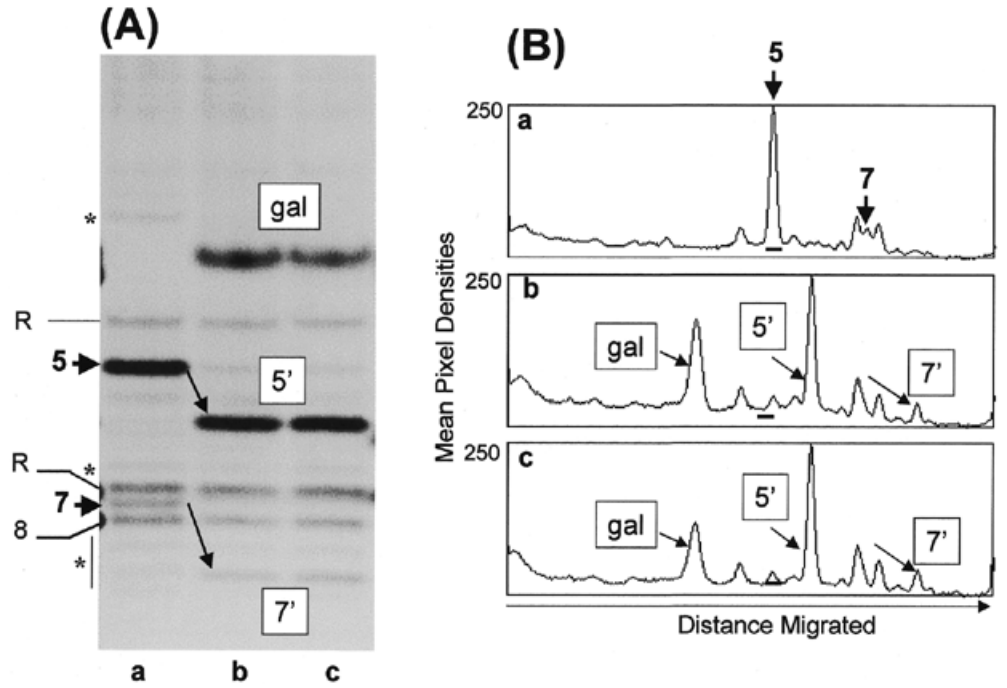

Fig. 4. Identification of oligosaccharides in KII digests of corneal KS. Bovine corneal KS (72 nmole $glcNH_2$) was digested with 2.5 mU of KII for 24 h in 50 μl of 0.1 M ammonium acetate, pH 6.0, the digestion mixture fractionated by Superdex Peptide chromatography, and oligosaccharides eluting between 26 and 38 ml (pools a and b, see **Figure** 2) analyzed by FACE (**A**) without further treatment (lane a) or after digestion with 1 mU of β-galactosidase (lane b), or 1 mU of each, β-galactosidase and β-N-acetyl-glucosaminidase (lane c). **B** shows the gel image used for product quantitation, as bands 5 (= 250 mean pixel densities) and 7 (= 75 mean pixel densities) were converted to bands 5′ (=250 mean pixel densities) and 7′ (=72 mean pixel densities) following exoglycosidase digestion.

in Figures 1 and 6 (see *Materials and methods* for detail) and data are summarized in Table I. Following a 4-h incubation with 1 mU KII per nmole $glcNH_2$, the proportion of substrate $glcNH_2$ recovered in digestion products was ~ 91% and ~79% for corneal or skeletal KS, respectively. Mono- and disulfated disaccharides (bands 3 and 8) constituted ~ 91% of the products,

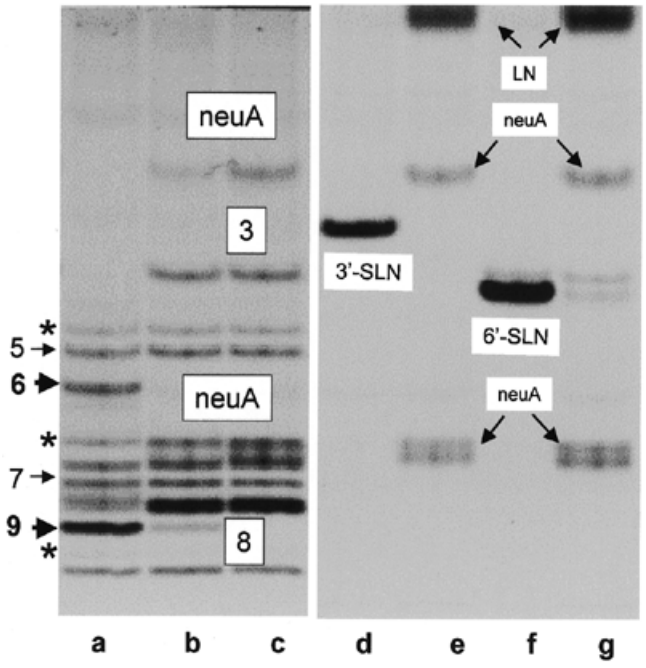

Fig. 5. Identification of oligosaccharides in KII digests of skeletal KS. Human skeletal KS (110 nmole $glcNH_2$) was digested with 5 mU of KII for 24 h in 50 µl of 0.1 M ammonium acetate, pH 6.0, the digestion mixture fractionated by Superdex Peptide chromatography, and oligosaccharides eluting between 26 and 38 ml (pools a and b, see Figure 2) analyzed by FACE without further treatment (lane a), after digestion with 10 mU α2,3-neuraminidase (lane b) or 10 mU neuraminidase (*V. cholerae*) (lane c). Sialylated trisaccharides, 3′SLN (neuAα*2,3*galβ*1,4*glcNAc), and 6′SLN (neuAα*2,6*galβ*1,4*glcNAc), were incubated without (lanes d and f) or with (lanes e and g) neuraminidase (*V. cholerae*).

in corneal KS digests, and the remainder were di-and trisulfated tetrasaccharide intermediates. In digests of skeletal KS, the mono- and disulfated disaccharides (bands 3 and 8) and the fucosylated trisaccharide (band 2) were the major products (~ 80%), nonreducing terminal neuA capped trisaccharides accounted for ~10%, and the rest (~10%) was recovered as di- and trisulfated tetrasaccharide intermediates.

Limit digestion of skeletal KS with EB and KII

Skeletal KS was incubated with increasing amounts (0.1, 0.25, and 0.5 mU per nmole $glcNH_2$) of either EB or KII for 4 or 24 h, to optimize quantitate recoveries for all identified hydrolase digestion products. At all EB concentrations and both incubation times, about one-third of the substrate $glcNH_2$ (~3 nmol) was recovered as the monosulfated disaccharide product glcNAc6Sβ*1,4*gal and ODS were also produced with the same efficiency under all digestion conditions. There was, however, no evidence for the presence of unsulfated disaccharide products, even with high amounts of enzyme or prolonged incubation times, consistent with previous analyses of human aggrecan KS digests using NMR spectroscopy where unsulfated glcNAc residues were also not detected in the chain interior parts (Brown *et al.*, 1998).

Digestion of skeletal KS with increasing amounts of KII (Figure 7) showed that mono- and disulfated disaccharides, the fucosylated trisaccharide derived from the chain interior, and the two sialylated trisaccharides from the nonreducing termini were readily detectable at all enzyme concentrations after 4 or 24 h incubation. The tetrasaccharides (bands 5 and 7) and other unidentified larger oligosaccharides (designated by *, see also Figure 1B) were detected only in 4-h incubations using lower concentrations (0.1 and 0.25 mU) of enzyme as short-lived

Table I. Identity and yield KS hydrolase digestion products

Hydrolase product[a]	nmole product (bovine cornea)[b,c]	nmol product (human skeletal)[b,d]
KII		
2, galβ*1,4*[fucα*1,3*]glcNAc6S	ND	0.8 ± 0.2 (9.5)
3, galβ*1,4*glcNAc6S	3.5 ± 0.2 (53)[e]	2.0 ± 0.3 (24)
5, galβ*1,4*glcNAc6Sβ*1,3*galβ*1,4*glcNAc6S[f]	0.4 ± 0.1 (6)	0.3 ± 0.1 (3.6)
6, neuAα*2,3*galβ*1,4*glcNAc6S	ND	0.3 ± 0.05 (3.6)
7, galβ*1,4*glcNAc6Sβ*1,3*gal6Sβ*1,4*glcNAc6S[f]	0.2 ± 0.02 (3)	0.6 ± 0.1 (7.1)
8, gal6Sβ*1,4*glcNAc6S	2.5 ± 0.3 (38)	3.9 ± 0.4 (46)
9, neuAα*2,3*gal6Sβ*1,4*glcNAc6S	ND[g]	0.5 ± 0.05 (6)
Product yield (%)	91	79
EB		
1, glcNAcβ*1,3*gal	0.1 ± 0.02	ND
4, glcNAc6Sβ*1,3*gal	3.7 ± 0.2	2.2 ± 0.3
10, neuAα*2,3*galβ*1,4*glcNAc6Sβ*1,4*gal	ND	0.18 ± 0.03
11, neuAα*2,3*gal6Sβ*1,4*glcNAc6Sβ*1,4*gal	ND	0.32 ± 0.03
Product yield (%)	52	24

[a]Number correspond to the separated products shown in Figures 1 and 8.
[b]Values represent the mean + SD from from independent digestions followed by FACE analyses.
[c]Substrate $glcNH_2$ contents per incubation was 7.25 nmole (determined by FACE, see *Materials and methods*).
[d]Substrate $glcNH_2$ contents per incubation was 11 nmole (determined by FACE, see *Materials and methods*).
[e]Numbers in parentheses represents the abundance of each product as a percentage of the sum of the total products (2, 3, 5, 6, 7, 8, and 9).
[f]Oligosaccharides contain 2 nmol/glcNH2 per nmole of product.
[g]ND = not detectable.

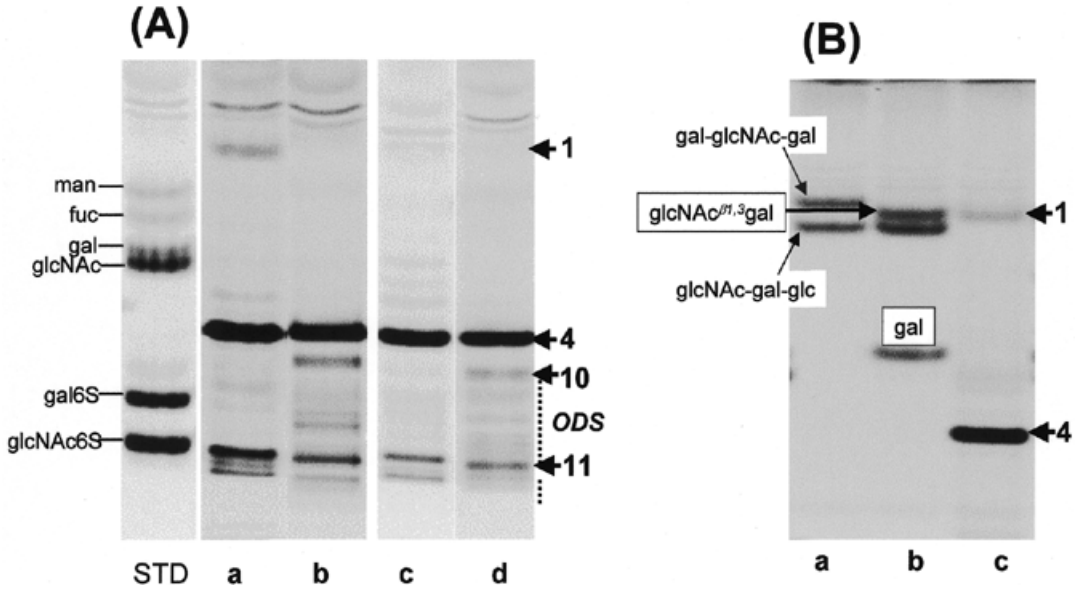

Fig. 6. FACE gel separation of products generated by digestion of corneal and skeletal KS with *E. freundii* EB or *Pseudomonas* sp. keratanase. Gel images of fluorotagged products from EB (lanes a and b) or keratanase (lanes c and d) digests of bovine corneal KS (7.25 nmole $glcNH_2$) (lanes a and c) or human skeletal KS (11 nmole $glcNH_2$) (lanes b and d) are shown. Products (see Table I) are numbered in the right-hand margin of panel, and ODS signifies the range of disulfated or fucosylated oligosaccharides that are resistant to enzymatic cleavages. A coelectrophoresed monosaccharide mixture consisting of 25 pmole fuc, 25 pmol man, 50 pmol gal, 150 pmol glcNAc, 300 pmol gal6S, 600 pmol glcNAc6S, is shown in STD lane in **A**. By comparison with the EB digest (lane b), the keratanase digest of skeletal KS had a lower recovery of the neuA capped products 10 and 11 and is likely due to a contaminating sialidase (Dickenson et al., 1991) in such enzyme preparations. **B** shows the identification of band 1 as the unsulfated disaccharide (glcNAcβ*1,3*gal) after digestion of (galβ*1,4*glcNAcβ*1,3*alβ*1,4*glcNAcβ*1,3*alβ*1,4*glc) first with EB (lane a) and then with β-galactosidase (lane b) where the product (glcNAcβ*1,4*gal) comigrated with band 1 in the EB digestion from corneal KS (lane c).

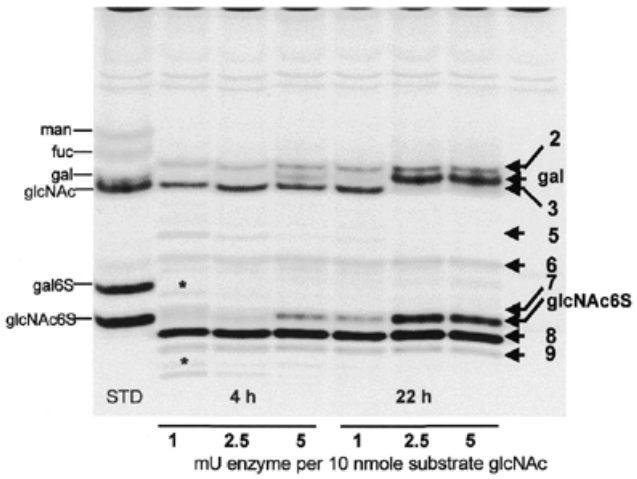

Fig. 7. EB and KII digestion products from human aggrecan KS generated after 4 or 22 h using increasing enzyme concentrations. Human aggrecan (11 nmol $glcNH_2$) was digested with different amounts of KII (1, 2.5, and 5 mU) for 4 or 22 h and products (see Table I) are identified in the right-hand margin of the gel. The gal and glcNAc6S products, generated by a contaminant exo-galactosidase activity in KII are also shown in the right-hand margins of the gel image..

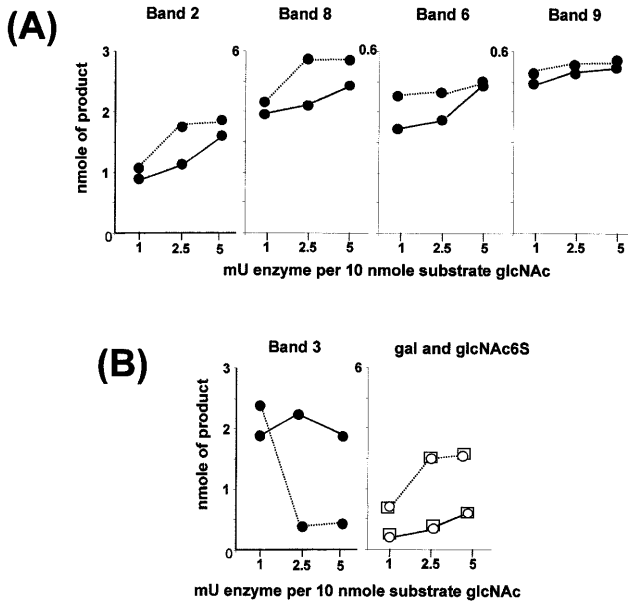

Fig. 8. Molar recoveries of products generated after KII digestion of human aggrecan. The molar abundance of each separated digestion product shown in gel image in Figure 7 was calculated as outlined in *Materials and methods*. Band 2 = galβ*1,4*[fucα*1,3*]glcNAc6S, Band 8 = gal6Sβ*1,4*glcNAc6S, Band 6 = neuAα*2,3*galβ*1,4*glcNAc6S, Band 9 = neuAα*2,3*gal6Sβ*1,4*glcNAc6S and Band 3 = galβ*1,4*glcNAc6S.

intermediates. Calculation of the molar yields of each KII end product (Figure 8A) showed that with 2.5–5.0 mU of enzyme after 4 or 24 h maximum yields of the fucosylated-monosulfated trisaccharide (band 2), the disulfated disaccharide (band 8) and the neuA-capped terminal trisaccharides (bands 6 and 9) were obtained. In contrast, the recovery of the monosulfated disaccharide (band 3) reached a maximum of ~2 nmole after a 4 h, at both enzyme concentrations and then

decreased after 22 h, especially when higher amounts of enzyme was used (Figures 7 and 8B). Concurrent with this decreased yield of monosulfated disaccharide, two new products, gal and glcNAc6S (Figure 7) were generated. This suggested the presence of a contaminate exo-galactosidase activity in the KII preparation that cleaves the monosulfated disaccharide galβ*1,3*glcNAc6S. This was also supported by the finding that the monosaccharide products were always recovered in essentially equimolar amounts and their yields corresponded to proportional deficit in recoverable monosulfated disaccharide products.

High KII enzyme:substrate ratios were required for quantitative depolymerization of skeletal KS, but because this condition also results in marked β-galactosidase cleavage of the monosulfated disaccharide product, we explored the possibility to inhibit the exoglycosidase activity during digestion. Skeletal KS (10 nmole $glcNH_2$) was digested with 5 mU KII in the absence or presence of N-acetyl-lactosamine (galβ*1,4*glcNAc), a well-defined β-galactosidase substrate and products analyzed by FACE (data not shown). The disaccharide was not cleaved by KII, and its presence had no inhibitory effect on the generation of gal and glcNAc6S monosaccharides during the KII digestion. Similarly, addition of thiogalactosides (Huber *et al.*, 1991), at concentrations which completely prevented N-acetyllactosamine cleavage by testicular β-galactosidase during KII digestion of KS, had no effect on the secondary cleavage of the monosulfated disaccharide. Together, these observations suggest that the KII-associated galactosidase activity is different from other known β-galactosidases in that it only cleaves the galβ*1,4* linkage to glcNAc6S and is not inhibited by thiogalactosides. It is, however, not possible to conclude from the current data if this activity resides in the KII enzyme protein itself, or if it is due to a copurified contaminate enzyme produced by *B. fragillis* similar to the exo-β-galactosidase from *Charonia lampas* that cleaves the disaccharide galβ*1,4*glcNAc6S) (Nakazawa *et al.*, 1989).

Aggrecan KS chain compositional analyses after KII and EB digestions

Portions of purified aggrecan preparations were sequentially digested with KII and EB (2.5 mU for 18 and 4 h, respectively) and products analyzed by FACE (Figure 9). The recovery of substrate $glcNH_2$ as digestion products (including the monosaccharides, gal, and glcNAc6S) was approximately 77, 72, 75, and 92% for aggrecan from the 5-, 13-, 15-, and 68-year-old tissues, respectively, the higher recovery in the older donor consistent with a more extensive KS substitution of aggrecan

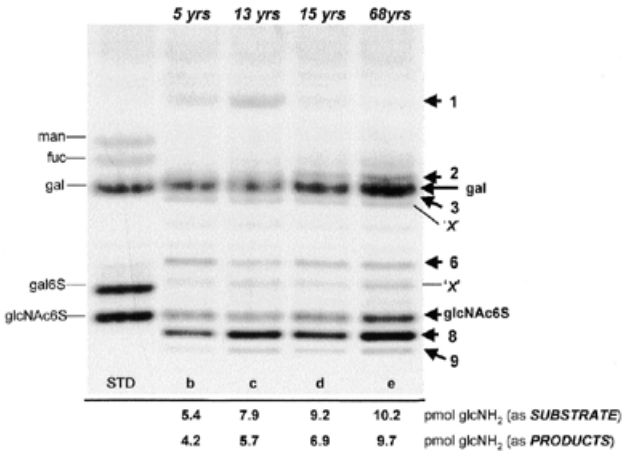

Fig. 9. FACE analyses of KII and EB digestion products of human aggrecan. Portions (20 μg of DMMB) of human aggrecan prepared from femoral condylar cartilage of 5-, 13-, 15-, and 68-year-old donors were sequentially digested with 5 mU KII (22 h) and 2.5 mU EB (4 h). Hydrolase products are identified in the right-hand margin, together with an additional minor band (designated x) that was generated during the EB digestions and not further characterized.

core bound O-linked oligosaccharides in mature human articular cartilage (Santer *et al.*, 1982).

Disaccharide compositional analyses of KS substituted on the four aggrecan preparations are summarized in Table II. First, unsulfated disaccharides generated by EB accounted for only 1–4 % of the total products in the growth and adolescent cartilage aggrecan and were essentially undetectable in the adult cartilage aggrecan. The proportion of monosulfated disaccharides, (galβ*1,4*glcNAc6S) was ~ 50% for the 5-year sample, somewhat lower in the adolescent sample (~ 40%), and decreased further to ~ 30% in the 68-year-old sample. This age-associated decrease in monosulfated disaccharides was balanced to a small extent by a concomitant increase in the proportion of disulfated disaccharides (gal6Sβ*1,4*glcNAc6S). In addition, however, presence of the fucosylated, monosulfated trisaccharide galβ*1,4*(fucα*1,3*)glcNAc6S markedly increased on aggrecan KS after 13 years of age, and this modification accounted for much of the age-associated decrease in the recovery of (galβ*1,4*glcNAc6S).

Quantitation of the sialic acid containing trisaccharides derived from the nonreducing termini in all digests also provided information on the degree of gal sulfation at the nonreducing end of such capped chains. About 50% of the

Table II. Aggrecan keratan sulfate disaccharide composition (% of total disaccharide products)

Age (years)	glcNAcβ*1,3*gal	galβ*1,3*glcNAc6S[c]	galβ*1,3* [fuc]glcNAc6S	gal6Sβ*1,3*glcNAc6S[d]
5[a]	3 ± 0.2	49 ± 1	2 ± 0.5	46 ± 3
13[a]	4 ± 0.5	42 ± 1	5 ± 1	49 ± 2
15[a]	1 ± 0.3	41 ± 2	16 ± 2	42 ± 2
68[b]	Not detectable	32 ± 4	18 ± 4	50 ± 3

[a]Numbers represent the mean ± SD from duplicate digests.
[b]Numbers represent the mean ± SD from triplicate digests.
[c]Includes disaccharides from the chain internal and the nonreducing terminal sialic acid capped trisaccharide (band 6).
[d]Includes disaccharides from the chain internal and the nonreducing terminal sialic acid capped trisaccharide (band 9).

terminal gal residues in the 13-, 15-, and 68-year-old KS samples analyzed here were sulfated in this position, and this represents essentially the same proportion as that determined for gal sulfation in the chain interior. In contrast, in the 5-year-old sample, the degree of gal sulfation was lower at the terminal position than in the chain interior, with only ~10% of the neuA recovered on the disulfated disaccharide gal6Sβ*1,4*glcNAc6S and ~90% on the monosulfated disaccharide galβ*1,4*glcNAc6S. There were no completely unsulfated chain terminal tri- or tetrasaccharides (neuAα*2,3*galβ*1,4*glcNAcβ*1,3*gal) in EB digests, suggesting that chain sulfation of KS on articular cartilage aggrecan is throughout the length of the chain and also common near the nonreducing terminus.

Assuming that neuA capping of aggrecan KS chains is almost complete (Dickenson *et al.*, 1991; Thornton *et al.*, 1989a, 1989b), the molar ratio of neuA to chain internal $glNH_2$ was used to calculate the number-averaged chain length and molecular weights of each KS population (Table III). These computed values showed a gradual but substantial increase in the average length of KS chains with increasing age of cartilage tissue. The chains on 5-year-old aggrecan were composed of about 7 disaccharides; those on the 13- and 15-year-old aggrecan of 8–10 disaccharides, with the longest chains composed of ~14 disaccharides on the 68-year-old sample. Number-averaged molecular weights for these KS chains, computed from the number and composition of internal disaccharides, neuA cap, and the contribution of the oligosaccharide linkage region, ranged from about 4.7 kDa in the 5-year sample to about 9 kDa in the 68-year-old sample. These size ranges are in very close agreement previously reported size ranges of aggrecan KS chains estimated by gel filtration chromatography (Theocharis, 1985; Brown *et al.*, 1998).

Discussion

Enzymatic depolymerization of KS coupled with FACE separation of products has been adapted as a rapid, high-throughput procedure for analyzing the fine structural details of corneal and skeletal KS. This gel separation procedure was first introduced by Jackson and others (Jackson, 1994; Hu, 1995; Starr *et al.*, 1996) for analyses of monosaccharides, and recently modified (Calabro *et al.*, 2000a,b; Plaas *et al.*, 2001) for separation and quantitation of enzymatic degradation products from CS and HA. The results obtained in this study illustrate the suitability of the FACE procedure for analyses of KS hydrolase products to obtain accurate quantitative disaccharide compositional analyses for this glycosaminoglycan.

The KS hydrolase products identified here (Table I) corresponded to those detected by previous investigations employing size exclusion or ion exchange HPLC separation procedures and/or ^{1}H-NMR spectroscopy. Each disaccharide product from the KS chain interior had a distinct electrophoretic mobility on the monosaccharide gels, and these were in the following ascending order: the EB-generated unsulfated disaccharide (glcNAcβ*1,3*gal), the KII-derived monosulfated disaccharide (galβ*1,4*glcNAc6S), the EB-derived monosulfated disaccharide (glcNAc6Sβ*1,3*gal), and the KII-derived disulfated disaccharide (gal6Sβ*1,4*glcNAc6S). The KII-derived fucosylated trisaccharide (galβ*1,3*4[fucα*1,3*]-glcNAc6S) migrated slower than its unmodified monosulfated disaccharide (Figure 3), confirming fluorescent product separation is determined by both the charge and size characteristics of each saccharide.

Similarly, the sialic-capped trisaccharides (neuAα-*2,3*galβ*1,4*glcNAc6S, neuAα*2,3*gal6Sβ*1,4*glcNAc6S) and tetrasaccharides (neuAα*2,3*galβ*1,4*glcNAc6Sβ*1,3*gal, neuAα*2,3*-gal6Sβ*1,4*glcNAc6Sβ*1,3*gal) generated from the nonreducing terminus by KII and EB, respectively, had distinct mobilities (Figures 5 and 9) and were thus readily identified and quantitated. Other nonreducing terminal oligosaccharide products such as α*2,6*neuA, α*2,3*gal- or β*1,3*galNAc-capped oligosaccharides, had been detected previously as minor components in KII digests of human skeletal or bovine corneal KS (Dickenson *et al.*, 1991; Tai *et al.*, 1992, 1996). These were not identifiable here, even when samples enriched in tri- and tetrasaccharides were analyzed separately (see Figures 4 and 5) and may be explainable if such termination sequences are on subpopulations of KS chains that were enriched as a result of additional steps for KS purification, that were used in other studies (Brown *et al.*, 1996; Huckerby *et al.*, 1998; Tai *et al.*, 1992, 1996).

When KS was digested with low amounts of KII (e.g., < 0.1 mU of enzyme per/nmole substrate) and for a short time (4 h), several larger oligosaccharide species derived from the chain interior were detected after FACE analyses (Figures 1 and 7B), but their absence following longer incubation times and with higher amounts of enzyme suggests that they are intermediate cleavage products that accumulate only when the endolytic/exolytic depolymerization of the chain interior does not proceeded to completion. Complete digestion of all sulfated and unsulfated regions of KS with KII and EB to identifiable end products (Table I) was therefore considered a prerequisite for quantitative compositional disaccharide analyses of this glycosaminoglycan and can be achieved by increasing enzyme concentrations to 0.25–0.5 mU per nmol $glcNH_2$. It is of importance to note, however, that under such digestion conditions, the disaccharide galβ*1,4*glcNAc6S is cleaved to its constituent monosaccharide products, gal and glcNAc6S, by a contaminating exoglycosidase in the KII preparations (Figures 8 and 9). Such a finding may explain the discrepancy between our data obtained for the disaccharide compositional analyses (Table III) and previously published reports on changes in gal sulfation of KS on aggrecan from human cartilages of different ages. The FACE analyses performed here included the monosaccharides generated by KII and the data obtained showed only a marginal increase (from ~44 to ~50%) in gal sulfation in the interior regions of

Table III. Human aggrecan KS chain sizing

Age (years)	glcNAc:neuA (mol:mol)	Mol weight average[a]
5	7:1	4700
13	8:1	5300
15	10:1	6600
68	14:1	9000

[a]Calculated as the sum of all recovered disaccharides (Table I) + 1 neuA cap, + 1 O-linked oligosaccharide linkage region [$(galNAc)_1(glcNAc)_1(gal)_2(neuA)_1$].

aggrecan KS during tissue maturation and thus did not confirm the substantial increases in this parameter between 4 and 18–20 years of age, reported by Brown and colleagues (1998). It is likely that the products from the contaminating galactosidase in the KII were also generated in such studies, but they were not detected during the HPLC separation of digestion products, thus leading to underestimation of the monosulfated disaccharide contents of the samples. It should be noted that a reasonably accurate estimation of the monosulfated disaccharide contents can be obtained after digestion with EB or *Pseudomonas* keratanase, because these enzymes give good depolymerization efficiencies for monosulfated regions of KS and lack contaminate activities (Figure 6 and Table II). Alternatively, chemical depolymerization of KS using the hydrazinolysis procedure (Shaklee and Conrad, 1986; Brown *et al.*, 1992) may be optimized for use in combination with FACE to include cleavage of linkages adjacent to fucose substituted galNAc6S for quantitative depolymerization of fucosylated KS chains, such as those abundant in several cartilages.

Individually purified standards for KS hydrolase digestion products are not commercially available, but the FACE runs can be readily calibrated by inclusion of purified monosaccharide standards (such as shown in Figure 1) and/or hydrolase digestion products obtained from bovine corneal KS obtained from commercial sources. Furthermore, as shown here, the identity of fucosylated and sialylated oligosaccharides can be verified by digestion of hydrolase products by specific exoglycosidases as both the released monosaccharides and the modified oligosaccharides products (Figures 2–5) are separable and quantifiable on a MonoComposition gels.

Altogether, the FACE procedure described here provides a sensitive, rapid separation and quantitation method for the full range of unsulfated, sulfated, fucosylated, and sialylated products generated in sequential incubation of KS with KII and EB. The approach represents an inexpensive and basic laboratory method for routine identification of KS hydrolase products, KS quantitation, and chain fine structure analyses, that can be used as an alternative to the more commonly described applications of ^{1}H-NMR spectroscopy and HPLC.

Materials and methods

Sodium cyanoborohydride (95% pure), sodium bicarbonate, acetic anhydride, dimethylsulfoxide, and HCl (highest purity grade available), and phenylethyl-β-D-thiogalactoside was from Sigma Aldrich (St. Louis, MO); isopropyl-β-D-thiogalactoside was from Roche Molecular Biochemicals (Indianapolis, IN); 2-aminoacridone was from Molecular Probes (Eugene, OR); proteinase K (fungal) was from Life Technologies (Grand Island, NY); Dowex AG50 WX-8 (H^+ form) was from BioRad (Hercules, CA); the prepacked Superdex Peptide Column (HR10/30) was from Amersham Pharmacia Biotech (Piscataway, NJ); Ultrafree-MC® (0.45 µm) and MicroCon3 (3000 mwt cut-off) centrifugal filtration devices were from Millipore Corporation (Bedford, MA); precast Monosaccharide™ gels, Mono Gel Buffer, and the gel electrophoresis apparatus were from Glyko (Novata, CA). Human aggrecan was extracted and purified from normal knee cartilage obtained from 5-, 13-, 15-, and 68-year-old donors as described (West *et al.*, 1999).

Glycosidases

Keratanase II (*Bacillus* sp.), endo-β-galactosidase (*E. freundii*), and keratanase (*Pseudomonas* sp.) were from Seikagaku (America Inc.). N-acetyl-β-D-glucosaminidase (*D. pneumoniae*) and β-galactosidase (bovine testis) were obtained from Roche Molecular Biochemicals. Neuraminidase type II (*V. cholerae*) was from Sigma-Aldrich, and almond meal α-fucosidase was from Glyko. All enzymes were dissolved in water and stored in small aliquots at –80°C. All reconstituted enzymes showed no notable losses in activity when used within 3 months of preparation.

Saccharide and GAG preparations

Bovine corneal keratan sulfate, para-lacto-N-neo-hexaose (gal*β1,4*glcNAc*β1,3*gal*β1,4*glcNAc*β1,3*gal*β1,4*glc), man, fuc, gal, gal6S, glcNAc, galNAc, glcNAc6S, and the 3′-fucosyllactose were from Sigma-Aldrich. 3′- and 6′-Sialyl-N-acetyllactosamine was from V-Labs (Covington, LA). All saccharides were suspended in water and stored in aliquots at –80°C.

Human aggrecan was extracted and purified from normal knee cartilage obtained from a 68-year-old donor as described (West *et al.*, 1999). For the preparation of KS peptides, 100-µg portions of aggrecan (based on S-GAG as determined by the dimethylmethylene blue dye binding assay; Farndale *et al.*, 1986) were dissolved in 100 µl 50 mM sodium acetate, pH 7.0, and digested with 12.5 µg of proteinase K at 60°C for 18 h. The enzyme was inactivated at 100°C for 10 min and insoluble materials removed by centrifugation at 15,000 x *g* for 10 min at 4°C. Oligosaccharide peptides, amino acids, and buffer salts were separated from the GAG peptides by centrifugation through MicroCon3 devices at 9000 x *g* for 15 min at room temperature. The retained GAG peptides were washed with 100 µl water, recentrifuged, and then recovered from the filter in 200 µl of water for storage at –80°C. Using this procedure, recovery of > 95% of the S-GAG from aggrecan was routinely obtained, as determined by the dimethylmethylene blue assay.

Hexosamine composition of GAG substrates

Five micrograms of proteoglycans (as determined by dimethylmethylene blue assay) or 2, 5, 10 nmole of glcNAc and galNAc standards were dispensed into 500-µl polypropylene Eppendorf tubes and dried by speedvac lyophilization. These were resuspended in 100 µl 6 N HCl and heated for 2 h at 100°C. Acid was evaporated *in vacuo*, and hydrolysates washed twice with 50 µl of water followed by speedvac lyophilization. Reacetylation of hexosamine sugars was carried out essentially as described (Patel and Parekh, 1994), by suspending samples in 50 µl of water, adding 12.5 µl each of 1 M sodium bicarbonate and 5% acetic anhydride (both freshly made in water) and maintaining the mixture at room temperature for 10 min. An additional 12.5 µl of acetic anhydride was added, and the reaction continued for another 20 min at room temperature. Removal of reagent was performed by ion exchange on Dowex H^+ by adding the reaction mixture to a 100-µl packed bed volume of washed resin in 0.45-µm Ultrafree MC filtration units, and briefly centrifuged (for 1 min at 5000 × *g*) to collect the desalted monosaccharides into the filtrate. These were immediately mixed with 2 nmole of glucose, speedvac lyophilized, and fluorotagged for quantitation after FACE separation on

Monosaccharide™ composition gels. The yield of fluorotagged glcNAc and $galNH_2$ after the hydrolysis and reacetylation was 65 ± 3% and 58 ± 5%, respectively, relative to the internal glucose standard. Based on this, bovine corneal KS contained 1.45 ± 0.11 nmol $glcNH_2$ per µg of glycosaminoglycan, proteinase generated aggrecan glycosaminoglycan chains contained 1.12 ± 0.18 nmole $glcNH_2$ per µg of sulfated glycosaminoglycan and intact human aggrecan preparation contained between 0.54 and 1.02 nmole of $glcNH_2$ per µg of sulfated glycosaminiglycan.

Enzymatic depolymerization of KS

Bovine corneal KS or aggrecan KS peptides were suspended in 0.1 M ammonium acetate, pH 6.0, at concentrations typically ranging from 5–12 nmole $glcNH_2$c per 75 µl buffer. Hydrolase digestions were carried out at 37°C using enzyme:substrate concentrations indicated in the figure legends for each experiment, and terminated after 4 or 22 h by heating to 100°C for 10 min. Undigested GAGs and enzyme proteins were precipitated after addition of 900 µl of ice-cold absolute ethanol at –20°C for 2 h and pelleted by centrifugation (15,000 × *g*, 20 min, at 4°C). The hydrolase products were quantitatively recovered in the supernatants and dried by speedvac lyophilization prior to the fluorotagging procedure described below. In selected experiments, KS hydrolase–generated oligosaccharides were further digested with exoglycosidases. For this, dried products were resuspended in 50 mM ammonium acetate buffer adjusted to the pH optimum of a given exoglycosidase (as recommended by the supplier). Exoglycosidases were added singly or in combination, and incubations were routinely carried out for 22–48 h, after which enzymes were inactivated at 100°C for 10 min, buffer removed by speedvac lyophilization, and the products fluorotagged as described.

Fluorotagging and FACE analyses of sacccharides

Fluorotagging was carried out essentially as described (Jackson, 1994). Briefly, monosaccharide standards (man, 0.25 nmole; fuc, 0.25 nmole; gal, 0.5 nmole; glcNAc 1.5 nmole; gal6S, 3 nmole; glcNAc6S, 6 nmole) and glycosidase products (containing between 1 and 10 nmol reducing sugar) were dried by speedvac lyophilization in 500-µl Eppendorf tubes. They were then mixed with 10 µl 0.1 M 2-amino-acridone (dissolved in glacial acetic acid:dimethylsulfoxide, 3:17, v/v) and incubated for 15 min at room temperature. A 10-µl portion of 1 M sodium cyanoborohydride (freshly prepared in water) was added; samples were mixed again and incubated at 37 °C for 16 h to complete the reductive amination reaction. Forty microliters of diluted glycerol (25% v/v in water) was added to each sample and a portion (6 µl = 10%) was removed immediately for FACE separation on Monosaccharide Composition gels. Electrophoresis was carried out for 60–80 min at 4°C, as described in Calabro *et al*. (2000a).

Gel imaging and quantitation of fluorotagged products

After completion of the electrophoretic run, gel cassettes were removed from the running tank, excess electrophoresis buffer washed off the outside of the glass plates, and cassettes placed on a trans-illuminator light box fitted with a 312-nm light source (Model 3–3000, from Photodyne, New Berlin, WI). The fluorescent images were displayed using an Eagle Eye II gel documentation system (Stratagene Cloning Systems) and recorded as TIFF files. To display major (150–600 pmole) and minor (20–100 pmole) bands within the linear range of pixel densities (0 = white, 250 = black), short (30 s) and long (1.2 min) exposures were recorded for each gel. The mean pixel density (= the sum of all pixels per band divided by the number of pixels per band) for each product band was measured using Scion Image Analyses Software. Quantitation of molar yields of enzyme products at each exposure time, was determined from the pixel density per pmole values obtained for the standard monosaccharide bands and accurate product quantitation was achieved between 20 and 400 pmole of product.

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Abbreviations

EB, endo-b-galactosidase (*E. freundii)*; CS, chondroitin sulfate; FACE, fluorophore-assisted carbohydrate electrophoresis; HA, hyaluronan; HPLC, high-performance liquid chromatography; KS, keratan sulfate; KII, keratanase II (*B. fragillis*); NMR, nuclear magnetic resonance; PG, proteoglycan.

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