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An improved method for the structural profiling of keratan sulfates: analysis of keratan sulfates from brain and ovarian tumors

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A previously developed method for the structural fingerprinting of keratan sulfates (Brown *et al.*, *Glycobiology,* 5, 311–317, 1995) has been adapted for use with oligosaccharides fluorescently labeled with 2-aminobenzoic acid following keratanase II digestion. The oligosaccharides are separated by high-pH anion-exchange chromatography on a Dionex AS4A-SC column. This methodology permits quantitative analysis of labeled oligosaccharides which can be detected at the sub-nanogram (~100 fmol) level. Satisfactory calibration of this method can be achieved using commercial keratan sulfate standards. Keratan sulfates from porcine brain phosphocan and human ovarian tumors have been examined using this methodology, and their structural features are discussed.

Key words: glycosaminoglycan/sulfation/fluorescence/chromatography

Introduction

The glycosaminoglycan keratan sulfate (KS) was first detected in bovine cornea (Meyer *et al*., 1953) and was later extracted from the nucleus pulposus of human intervertebral disc (Gardell and Rastageldi, 1954). Since then, KS proteoglycans have been identified in a number of tissues including aggrecan and fibromodulin in cartilage, lumican and keratocan in cornea, claustrin, abakan, phosphocan from brain and SV2 from synaptic vesicles. KS can also be found on the epithelial mucin MUC1 (Aplin *et al*., 1998) and on one isoform of CD44 (Takahashi *et al*., 1996). A number of other molecules related to KS have also been described, for example those from zona pellucida (Hokke *et al*., 1994) and L-selectin ligands from GLYCAM-1 (Hemmerrich and Rosen, 1994).

Keratan sulfates are of widespread occurrence, both in the extracellular matrix of tissues such as cartilage, cornea and brain, and on cell surfaces. However, the functions of KS are mostly poorly understood. KS in cartilage proteoglycans is clearly important in providing osmotic swelling pressure and tissue compressibility, and it is used as a marker for early osteoarthritis (e.g., Thonar *et al*., 1985, 1991). In the cornea, KS is involved in the maintenance of transparency by providing the uniform spacing between collagen fibrils (Hassell *et al*., 1983; Midura *et al*., 1990). On the surfaces of cells KS is implicated in such processes as cell migration and attachment (Fullwood *et al*., 1996) and tumor metastasis (Takahashi *et al*., 1996) (possibly via tumor cell KS interacting with selectins on vascular endothelium). There is also a significant recent report of changes in KS structure and content in the brains of Alzheimer patients (Lindahl *et al*., 1996).

The structure of KS is based upon a repeating poly-N-acetyllactosamine backbone of Galβ(1–4)GlcNAcβ(1–3). This backbone is almost always 6-O-sulfated on N-acetylglucosamine and to a variable extent on galactose (Bhavanandan and Meyer, 1968). The extent of galactose sulfation varies considerably with age, species and tissue from which the KS is isolated (e.g., Kaplan and Meyer, 1959; Roughley and White, 1980; Nieduszynski *et al*., 1990a,b). In addition to sulfation changes, the proportions and distribution of minor sugar components also show age and tissue dependence. For example, L-fucose (substituted α(1–3) onto N-acetylglucosamine residues within the main poly-N-acetyllactosamine repeat sequence) may be present in articular (e.g., femoral head) cartilage derived KS, but not in KS from nonarticular cartilages (e.g., nasal septum). Also, skeletal KS has only two major capping sequences involving α(2–3)- and α(2–6)-linked N-acetylneuraminic acid (sialic acid), whereas bovine corneal KS can terminate with over seven different sugar/linkage combinations (Tai *et al*., 1996, 1997) including N-acetyl- and N-glycolyl- neuraminic acids, N-acetylgalactosamine and α-galactose. Interestingly, KS structure is most varied at the capping end (nonreducing terminus) of the chains. It seems probable that this diversity of structure is vital for KS function in interacting with other molecules.

Any fingerprinting method of analyzing KS structure requires both a fragmentation step followed by separation of the products, usually by chromatography on a calibrated column. Several methods of fragmenting KS, either by chemical (Brown *et al*., 1992) or enzymatic treatment, have been described. The most convenient methods use one of two enzymes: keratanase or keratanase II. Keratanase cleaves at an unsulfated galactose which is flanked by sulfated N-acetylglucosamines, but fucose residues on adjacent N-acetylglucosamines interfere with enzyme action (Tai *et al*., 1993). Keratanase cleavage generally results in a large number of oligosaccharides with a wide range of sizes, making chromatographic separation difficult. Keratanase II cleaves at the majority of sulfated N-acetylglucosamines and produces oligosaccharides in the size range of 2–7 residues which are resolvable by ion-exchange chromatography.

In this study we describe the development of an improved procedure for the chromatographic profiling of keratan sulfates. This method involves digestion of the sample with keratanase II followed by fluorescent labeling of the oligosaccharide products with 2-aminobenzoic acid (2-AA). The labeled oligosaccharides are then separated by high-pH anion-exchange chromatography on a calibrated Dionex AS4A-SC column. This methodology has been applied to the analysis of brain and tumor-associated KS.

Table I. Keratan sulfate oligosaccharide elution times on a Dionex AS4A-SC column

Oligosaccharide	Code[a]	Elution time (min)	Relative elution time[b]
Galβ$^{1-4}$(Fucα$^{1-3}$)GlcNAc(6S)±2AA	F1	4.88	0.094
Galβ$^{1-4}$GlcNAc(6S)-2AA	R1	9.79	0.189
Galβ$^{1-4}$(Fucα$^{1-3}$)GlcNAc(6S)β$^{1-3}$Galβ$^{1-4}$(Fucα$^{1-3}$)GlcNAc(6S)-2AA	F2	13.31	0.257
NeuAcα$^{2-6}$Galβ$^{1-4}$GlcNAc(6S)-2AA	C6T	15.00	0.290
Galβ$^{1-4}$GlcNAc(6S)β$^{1-3}$ Galβ$^{1-4}$(Fucα$^{1-3}$)GlcNAc(6S)-2AA	F3	15.22	0.294
Galβ$^{1-4}$(Fucα$^{1-3}$)GlcNAc(6S)β$^{1-3}$Galβ$^{1-4}$GlcNAc(6S)-2AA	F4	17.23	0.333
NeuAcα$^{2-6}$Galβ$^{1-4}$GlcNAc(6S)β$^{1-3}$ Galβ$^{1-4}$(Fucα$^{1-3}$)GlcNAc(6S)-2AA	C6F	17.99	0.347
NeuAcα$^{2-3}$Galβ$^{1-4}$GlcNAc(6S)β$^{1-3}$ Galβ$^{1-4}$(Fucα$^{1-3}$)GlcNAc(6S)-2AA	C3F	18.55	0.358
Galβ$^{1-4}$GlcNAc(6S)β$^{1-3}$Galβ$^{1-4}$GlcNAc(6S)-2AA	R2	19.25	0.372
NeuAcα$^{2-6}$Galβ$^{1-4}$GlcNAc(6S)β$^{1-3}$Galβ$^{1-4}$GlcNAc(6S)-2AA	C1	21.78	0.421
NeuAcα$^{2-3}$Galβ$^{1-4}$GlcNAc(6S)β$^{1-3}$Galβ$^{1-4}$GlcNAc(6S)-2AA	C2	22.37	0.432
Gal(6S)β$^{1-4}$GlcNAc(6S)-2AA	R3	24.86	0.480
Gal(6S)β$^{1-4}$GlcNAc(6S)β$^{1-3}$ Galβ$^{1-4}$(Fucα$^{1-3}$)GlcNAc(6S)-2AA	F5	26.73	0.516
Gal(6S) β$^{1-4}$GlcNAc(6S)β$^{1-3}$Galβ$^{1-4}$GlcNAc(6S)-2AA	R4	31.39	0.606
Galβ$^{1-4}$(Fucα$^{1-3}$)GlcNAc(6S)β$^{1-3}$Galβ(6S)$^{1-4}$GlcNAc(6S)-2AA	F6	34.25	0.662
Galβ$^{1-4}$GlcNAc(6S)β$^{1-3}$Gal(6S)β$^{1-4}$GlcNAc(6S)-2AA	R5	37.00	0.715
NeuAcα$^{2-6}$Galβ$^{1-4}$GlcNAc(6S)β$^{1-3}$Gal(6S)β$^{1-4}$GlcNAc(6S)-2AA	C3	38.43	0.742
NeuAcα$^{2-3}$Galβ$^{1-4}$GlcNAc(6S)β$^{1-3}$Gal(6S)β$^{1-4}$GlcNAc(6S)-2AA	C4	39.09	0.755
Gal(S6)β$^{1-4}$GlcNAc(6S)β$^{1-3}$Gal(6S)β$^{1-4}$GlcNAc(6S)-2AA	R6	51.77	1.000
NeuAcα$^{2-3}$Gal(S6)β$^{1-4}$GlcNAc(6S)β$^{1-3}$Gal(6S)β$^{1-4}$GlcNAc(6S)-2AA	C5	52.80	1.020

[a] Notation used by Brown *et al.* (1994a,b): F (fucose-containing), R (repeat region), C (capping oligosaccharide).

[b] Relative elution position compared to the tetrasulfated tetrasaccharide (R6).

Results

Partial calibration of the profiling method was achieved using pure, characterized KS oligosaccharides labeled with 2-AA. Retention times were determined in this manner for the repeat unit disaccharides and tetrasaccharides, R1–R6, and two sialic acid capping oligosaccharides C3 and C4 containing α(2–3)- and α(2–6)-linked NeuAc, respectively. The remainder of the major peaks obtained when profiling KS from cartilage sources have been assigned by careful examination of KS profiles before and after glycosidase digestion. The retention times of the most abundant oligosaccharides obtained from skeletal KS, together with the codes and structures of the oligosaccharides, are given in Table I. It should be noted that linkage region oligosaccharides are not identified by this methodology. The GalNAc involved in the attachment of the KS chain to the protein core has been previously reduced in the process of chain release and therefore cannot subsequently be labeled with 2-AA.

A chromatographic profile of KS derived from bovine nasal septum is shown in Figure 1a. Previous studies (Nieduszynski *et al*., 1990a,b) have demonstrated that this type of KS contains α(2–3)-linked sialic acid at the nonreducing terminus of the chains, but no α(2–6)-linked sialic acid or α(1–3)-linked fucose (as is found in KSs from older articular cartilage and cornea). The profile is relatively simple, with the 2-AA–derivatised repeat unit disaccharides and tetrasaccharides comprising the major proportion. The peak at 39.1 min corresponds to the α(2–3)-NeuAc-containing oligosaccharide C4. The remaining peaks at 22.4 min and 52.8 min (a very minor component) can therefore be assigned to the other α(2–3)-NeuAc–containing oligosaccharides C2 and C5, respectively. This is confirmed by sialidase digestion of the labeled oligosaccharides which results in the disappearance of C2, C4, and C5 and an increase in the peaks corresponding to their non-sialylated counterparts, R2, R5, and R6 (see Figure 1b).

A profile of a 2-AA–labeled keratanase II digest of bovine articular cartilage (femoral head) is shown in Figure 2a. It can be seen that this fingerprint is somewhat more complex than that obtained from the nasal septum, with this extra complexity arising from the presence of oligosaccharides containing α(2–6)-linked sialic acid and α(1–3)-linked fucose. Comparison of this profile with that obtained for the bovine nasal septum (BNS) KS sample enables the identification of the α(2–6)-NeuAc-containing oligosaccharides C1 and C3 at 21.8 and 38.4 min, respectively. These elute slightly earlier (~0.6 min) than their respective α(2–3)-linked counterparts (as is also the case for unlabeled, reduced oligosaccharides; Brown *et al*., 1995). Treatment of the labeled bovine femoral head (BFH) KS digest with neuraminidase (Figure 2b) confirms this assignment, with these peaks disappearing and a resultant increase in peaks R2 and R5. In addition to these major peaks it was observed that a further three minor peaks are also removed following sialidase treatment of BFH-KS. A small peak at ~18.6 min completely disappeared and, from its retention time, is probably the fucosylated hexasaccharide cap NeuAcα(2–3)Galβ(1–4)GlcNAc(6S)β(1–3)Galβ(1–4)[Fucα(1–3)]GlcNAc(6S) (C3F), which has been previously identified in articular cartilage KS as a minor component (Brown *et al*., 1996). There is also a smaller peak that elutes fractionally earlier at ~18.0 min which is possibly the isomer NeuAcα(2–6)Galβ(1–

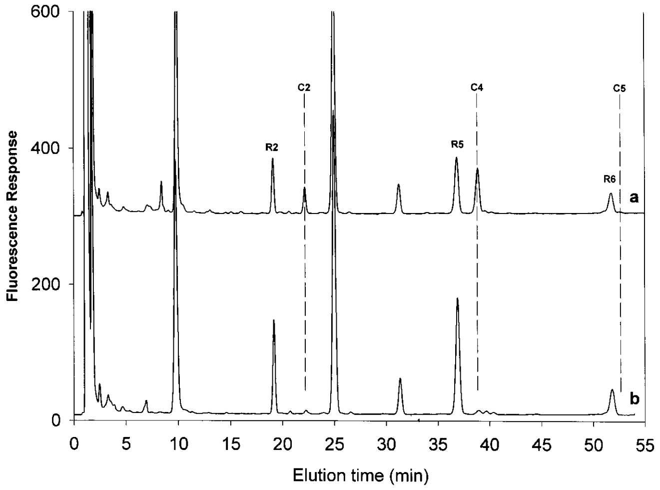

Fig. 1. Dionex AS4A-SC ion-exchange chromatograms of oligosaccharides produced by keratanase II digestion/2-AA labeling of bovine nasal septum KS; (a) control and (b) sialidase treated. The column (25 × 0.4 cm) was eluted at a flow rate of 2 ml/min. The gradient program was as follows: 5 min of 150 mM NaOH followed by a linear gradient from 5–65 min of 600 mM NaCl/150 mM NaOH. Peaks are labeled to show oligosaccharide identity as in Table I.

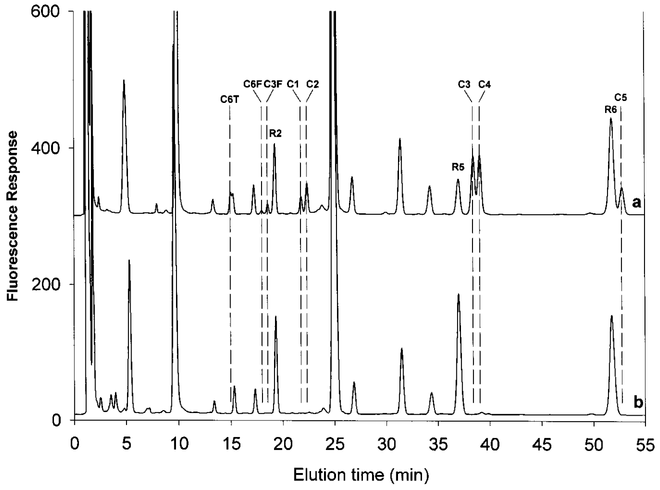

Fig. 2. Dionex AS4A-SC ion-exchange chromatograms of oligosaccharides produced by keratanase II digestion/2-AA labeling of bovine femoral head cartilage KS; (a) control and (b) sialidase treated. The column (25 × 0.4 cm) was eluted at a flow rate of 2 ml/min. The gradient program was as follows: 5 min of 150 mM NaOH followed by a linear gradient from 5–65 min of 600 mM NaCl/150 mM NaOH. Peaks are labeled to show oligosaccharide identity as in Table I.

4)GlcNAc(6S)β(1–3)Galβ(1–4)[Fucα(1–3)]GlcNAc(6S) (C6F). This has not been isolated from articular cartilage in previous studies; however, this structure probably does occur and it is possible that previous structural studies have failed to isolate it because of its very low abundance. The third minor component removed by sialidase treatment is present in the leading edge of a double peak at ~15.0 min. The retention time is consistent with the presence of one sulfate and one carboxylate group (in sialic acid) within this oligosaccharide, eluting as it does ~5 min after the monosulfated disaccharide. Therefore, it is likely that this peak is the trisaccharide NeuAcα(2–6)Galβ(1–4)GlcNAc(6S) (C6T) identified previously in studies of corneal keratan sulfates (Tai *et al*., 1996).

The remaining major unassigned peaks in the profile for BFH-KS can be attributed to fucose-containing oligosaccharides. Fucosidase digestion of the samples and comparison of the elution order with that obtained for reduced oligosaccharides (Brown *et al*., 1995) was used to confirm the assignment, which was assumed to be broadly equivalent to the elution order of the unlabeled oligosaccharides on this column (Brown *et al*., 1995). Figure 3 shows expansions of BFH-KS fingerprints of fucosidase digested material predigestion (i.e., intact KS chains) and post-digestion/labeling (i.e., oligosaccharides). It can be seen from Figure 3c that fucosidase treatment of the intact KS chains results in the reduction of all the putative fucose-containing peaks. However, when fucosidase treatment is carried out after digestion and labeling (Figure 3b) only a subset of peaks is affected (namely F2, F4, and F6). The peaks which are unaffected (F1, F3, and F5) correspond to oligosaccharides where the fucose residue is substituted onto the terminal GlcNAc(6S) which is labeled. Fucose in this environment, i.e., adjacent to label, is evidently not susceptible to digestion. Complete elimination of the peaks was not achieved due to the relatively low activity of this fucosidase upon these substrates, possibly due to an inhibitory effect caused by the presence of the sulfate groups.

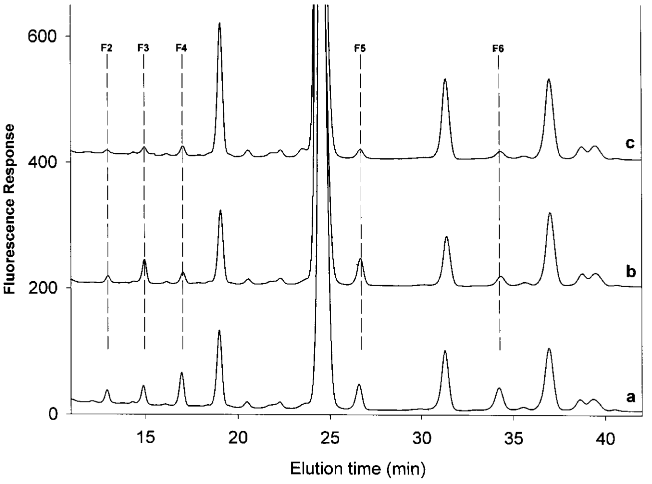

Fig. 3. Dionex AS4A-SC ion-exchange partial chromatograms of oligosaccharides produced by keratanase II digestion/2-AA labeling of bovine femoral head cartilage KS; (a) control, (b) fucosidase treated post digestion and labeling, and (c) fucosidase treated prior to digestion. The column (25 × 0.4 cm) was eluted at a flow rate of 2 ml/min. The gradient program was as follows: 5 min of 150 mM NaOH followed by a linear gradient from 5–65 min of 600 mM NaCl/150 mM NaOH. Peaks are labeled to show oligosaccharide identity as in Table I.

In general the retention times on the column are highly reproducible, for experiments carried out within a single day with the same eluant stock the retention times are within 0.5%. However, the retention times are sensitive to inconsistencies in preparing eluants of precise concentrations and thus may vary from day to day. For this reason it is advantageous to analyze profiles in terms of the relative elution times of the oligosaccharides when compared to the tetrasulfated repeat region tetrasaccharide (R6), designated as 1.000 (see Table I). This oligosaccharide elutes late in the profile and is generally clearly identifiable, although its abundance is naturally dependent upon the sulfation level of the sample being studied. The relative

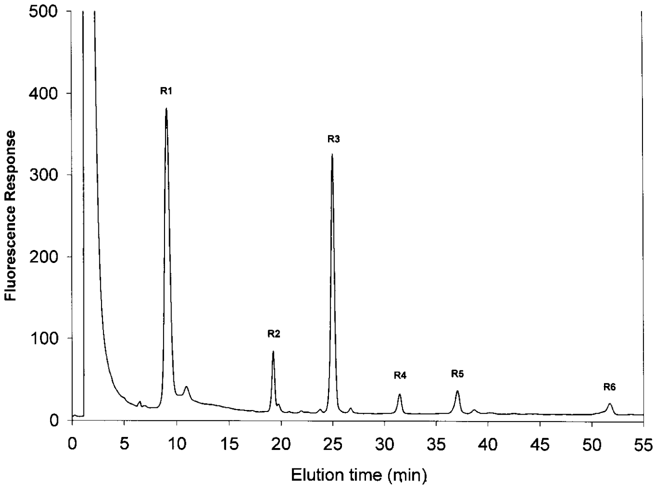

Fig. 4. Dionex AS4A-SC ion-exchange chromatograms of oligosaccharides produced by keratanase II digestion/2-AA labeling of commercial bovine corneal KS (Sigma Chemical Co.). The column (25 × 0.4 cm) was eluted at a flow rate of 2 ml/min. The gradient program was as follows: 5 min of 150 mM NaOH followed by a linear gradient from 5–65 min of 600 mM NaCl/150 mM NaOH. Peaks are labeled to show oligosaccharide identity as in Table I.

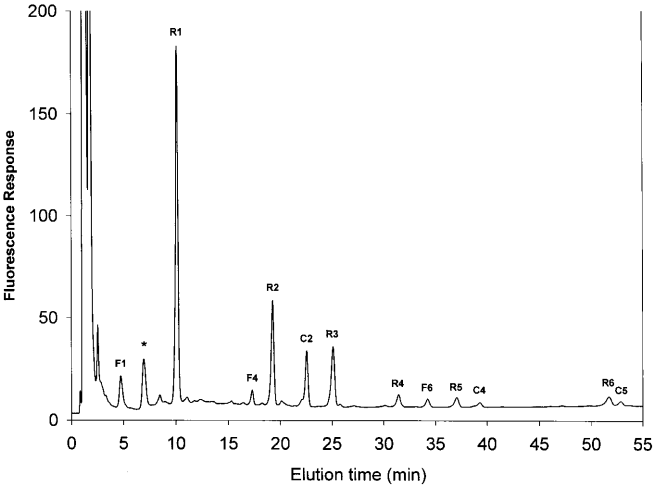

Fig. 5. Dionex AS4A-SC ion-exchange chromatograms of oligosaccharides produced by keratanase II digestion/2-AA labeling of phosphacan KS isolated from porcine brain. The column (25 × 0.4 cm) was eluted at a flow rate of 2 ml/min. The gradient program was as follows: 5 min of 150 mM NaOH followed by a linear gradient from 5–65 min of 600 mM NaCl/150 mM NaOH. Peaks are labeled to show oligosaccharide identity as in Table I.

elution positions of the oligosaccharides are essentially insensitive to variability in buffer concentration and provide an accurate and convenient method for comparing profiles.

Calibration of this method in laboratories lacking KS standards can be achieved using commercially available KS. Figure 4 shows the profile of bovine corneal KS purchased from the Sigma Chemical Co. (UK). It can be seen that this profile is very simple with the oligosaccharides from the poly-N-acetyllactosamine repeat sequence as the predominant components. Sialic acid- and fucose-containing peaks are of very low abundance and are evidently lost during the preparation of this material. This is somewhat advantageous, as the resultant profile is easy to interpret without reference to data from other calibrants. Oligosaccharide R6 is clearly present and the relative elution positions of the repeat region oligosaccharides can be compared with those presented here.

It should be noted that this methodology focuses entirely on those KSs which are susceptible to keratanase II, i.e., those which contain predominantly sulfated N-acetylglucosamine residues. Any KS sample containing a high proportion of unsulfated N-acetylglucosamine will not be degraded by the enzyme and is therefore not appropriate for analysis by this procedure.

Examples of applications of this technique in the study of KS samples available in very small quantities are shown in Figures 5 and 6. Figure 5 shows the profile of KS from the brain KS-proteoglycan phosphacan derived from porcine material. It can be seen that the profile is relatively simple with the monosulfated disaccharide (R1) as the principal component. Analysis of peak areas suggests that this sample is ~12% sulfated on galactose. The principal chain capping structure is α(2–3)-linked NeuAc (see oligosaccharides C2, C4, and C5) however a small proportion of α(2–6)-linked NeuAc is also present (less than 10% of the total NeuAc) as evidenced by the shoulders on the left-hand side of the C2 and C4 peaks. Oligosaccharides containing α(1–3)-linked fucose are also present (see oligosaccharides F1, F4, and F6), albeit at low abundance. Interestingly, the profile demonstrates the presence of a significant component at 6.96 min (labeled with an asterisk on Figure 5) which is currently unidentified.

Figure 6 shows a KS profile from a human ovarian tumor. In this sample the principal component is the disulfated disaccharide (R3) which is slightly more abundant than the monosulfated disaccharide (R1). Analysis of peak areas suggests the galactose sulfation level of this sample is ~55%. Oligosaccharides containing α(2–3)-linked NeuAc (C2 and C4) are present representing chain caps, however α(2–6)-linked NeuAc appears to be totally absent, as is α(1–3)-linked fucose. Several significant but unknown peaks (labeled with asterisks in Figure 6) are also present in the profile. For example the two peaks 17.05 and 20.84 min. elute in the region corresponding to oligosaccharides with two sulfate groups. These unknown oligosaccharides are not sensitive to digestion with either sialidase or fucosidase, suggesting the presence of hitherto unidentified structures. These oligosaccharides may be new capping structures, a distinct possibility as the α(2–3)-linked NeuAc oligosaccharides appear to be relatively low in abundance, suggesting that either the chains are very long or they are not all terminated with NeuAc.

Discussion

The methodology reported here represents a development of the existing KS profiling method described by Brown *et al.* (1995) and has several significant advantages. First, sensitivity using the 2-AA label is increased ~50-fold over pulsed electrochemical detection, allowing as little as 100 fmol of an oligosaccharide to be detected. The profiles do not suffer significantly from baseline drift, a major problem with pulsed electrochemical detection which is somewhat sensitive to salt gradients and buffer impurities. Second, the 2-AA label can only be incorporated into a reducing sugar, produced by the action of the enzyme keratanase II. Therefore, the method only detects products of digestion, i.e., KS oligosaccharides, and is insensitive to impurities, for example other glycosaminoglycans, which do not get labeled. Finally, in contrast to pulsed electrochemical detection, relative peak areas of individual oligosaccharides represent molar ratios which allows parameters such as galactose sulfation, fucosylation

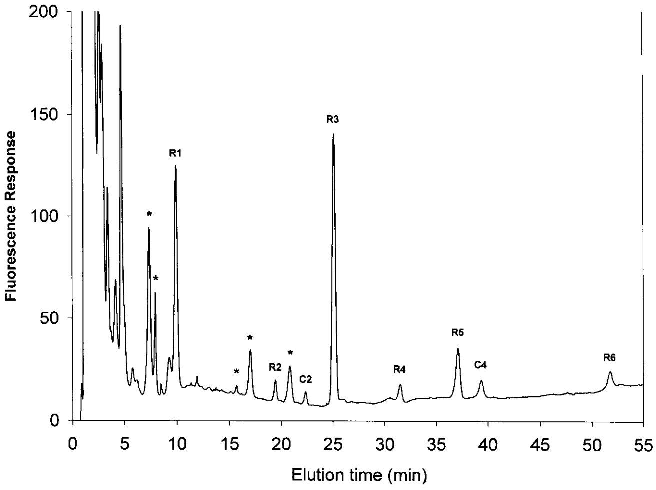

Fig. 6. Dionex AS4A-SC ion-exchange chromatograms of oligosaccharides produced by keratanase II digestion/2-AA labeling of KS isolated from human ovarian tumor. The column (25 × 0.4 cm) was eluted at a flow rate of 2 ml/min. The gradient program was as follows: 5 min of 150 mM NaOH followed by a linear gradient from 5–65 min of 600 mM NaCl/150 mM NaOH. Peaks are labeled to show oligosaccharide identity as in Table I.

levels, and average chain length to be calculated. Experiments using pure oligosaccharides have shown that the labeling procedure does not result in any detectable loss of either sulfate groups or acid-sensitive components such as sialic acid or fucose. Complete separation of labeled KS oligosaccharides can still be achieved in less than 1 h and retention time reproducibility is generally within 0.5%, except during the isocratic phase of the separation prior to the sodium chloride gradient where high concentrations of salts in the sample can significantly affect oligosaccharide retention times. However, for typical KS samples there are only two oligosaccharides eluting in this region, F1 and R1, the latter of which (the monosulfated repeat-region disaccharide) is generally clearly identifiable due to its high abundance.

The addition of the label, itself carrying a negatively charged carboxylate group, does not significantly affect the elution order of the oligosaccharides from that of the previous method. The contribution of the sulfate group to oligosaccharide retention on the column still dominates over the effect of a carboxylate group. By comparison with nearby peaks, the elution position of an unknown oligosaccharide can yield clues to its identity, especially when combined with glycosidase digestion of the original KS sample and/or the labeled oligosaccharides. The basic methodology described here is fully calibrated for skeletal KS; however, it is not capable of fully resolving the many oligosaccharides derived from the highly complex KS from bovine cornea (Tai *et al*., 1996). It can be seen in Figure 5 and 6 that unknown oligosaccharide peaks are present in the profiles for both the brain and tumor KS and clearly new structural elements are present in both samples which remain to be identified. This is currently the focus of ongoing studies in our laboratory.

The improved methodology described here now makes it possible to analyze min quantities of KS. Although the samples described in this study have been extensively purified prior to analysis, this is not strictly necessary and acceptable profiles can be obtained from relatively crude samples, for example protease digested tissue or ethanol precipitates. This technique has widespread application in many areas of KS and KS-related research including brain development (particularly neurite outgrowth and axon targeting studies; Seo and Geisert, 1995; Hemming and Saxod, 1997), cell-adhesion processes (Fullwood *et al*., 1996), studies of selectin ligands (Hemmerich and Rosen, 1994; Green *et al*., 1995), and osteoarthritis and cartilage catabolism (Liepold *et al*., 1989; Carroll *et al*., 1991).

Materials and methods

Materials

Keratanase II lyase (*bacillus* sp.) was obtained from ICN Biomedicals Ltd. (High Wycombe, bucks. UK.). 2-Aminobenzoic acid (as a 2-AA labeling kit), sialidase *(Clostridium perfringens sp.*) and α1→3,4-fucosidase (almond meal) were purchased from Oxford Glycosystems (Oxford, UK). Sodium hydroxide (A.R. 46/48%) was from Fisons Scientific Equipment (Loughborough, Leicestershire, UK). Sodium chloride (ACS reagent) was from Sigma Chemical Co. (Poole, UK). All other chemicals were analytical grade.

The HPLC system

A Dionex chromatography system comprising an eluant de-gas module, an advanced gradient pump, a rheodyne 9125 injection valve, an IonPac AS4A-SC column (4.0 × 250 mm) with an AG4A-AS guard column (4.0 × 50 mm), an advanced computer interface and AI-450 software were from Dionex (Camberley, Surrey, UK). Fluorescence was monitored on-line using a SP-920 fluorescence detector from Jasco (Essex, UK).

Preparation of keratan sulfate standards for column calibration

KS chains from bovine femoral head cartilage and bovine nasal septum cartilage were prepared as described previously (Dickenson *et al*., 1990). Bovine corneal KS was also purchased from Sigma Chemical Co. (Poole, UK, product number K3001). KS samples (0.1 mg) were digested with 1 mU of keratanase II in 10 μl 100 mM ammonium acetate (pH 6.5). Digestion was carried out at 37°C for 24 h.

Characterized oligosaccharides were produced as described by Brown *et al*. (1994a,b). Briefly KS chains from bovine articular cartilage were digested with keratanase II and separated on a Nucleosil 5SB strong anion-exchange column. Pure oligosaccharides were pooled and desalted on a column of Bio-Gel P2, and their structures were determined using a combination of one- and two-dimensional 600 MHz NMR spectroscopy.

Oligosaccharide labeling

Samples were lyophilized to remove all traces of water and labeled with the fluorophore 2-aminobenzoic acid using the 2-AA labeling kit from Oxford Glycosystems according to the manufacturer's instructions. Briefly, this involved the addition of 2 μl of a solution containing DMSO, glacial acetic acid, 2-aminobenzoic acid and sodium cyanoborohydride to the digested KS chains (<50 μg). Labeling was carried out for 2 h at 65°C. Adequate labeling of samples can be obtained by preparing the labeling mixture from pure reagents as follows: (1) add 150 μl glacial acetic acid to 350 μl DMSO, (2) add 120 μl of this mixture to 8.8 mg 2-AA, then (3) add 100 μl of this mixture to 11.8 mg $NaCNBH_3$. Labeling is performed with 2 μl of this mixture for 2 h at 65°C as before. For routine analysis this labeling mix is satisfactory, however, for small amounts of important samples the commercial 2-AA kit was seen to produce fewer contaminant peaks eluting at low salt concentrations during chromatographic separation.

Glycosidase treatment

*Neuraminidase (*Vibrio cholerae*).* Aliquots (10 µl) of labeled oligosaccharides or BFH/BNS standards were lyophilized, resuspended in 40 µl neuraminidase digestion buffer (50 mM sodium acetate/4 mM $CaCl_2$, pH 5.5 containing 100 µg/ml BSA) and 0.005 U neuraminidase added. The samples were digested for 16 h at 37°C.

Fucosidase (Almond meal). Aliquots (10 µl) of the BFH standard, both prior to keratanase II digestion and after digestion/fluorescent labeling, were lyophilized, and resuspended in 10 µl digestion buffer (50 mM sodium acetate, pH 5.0), and 0.01 units α(1–3/4)-fucosidase was added. The samples were digested for 48 h at 37°C.

Anion-exchange chromatography

Labeled samples were diluted to 250 µl with distilled water. Aliquots (2.5 µl) were applied to a Dionex AS4A-SC column (250 × 4 mm) equilibrated with 2 ml/min 150 mM NaOH and eluted with a linear gradient of 2 ml/min 0–600 mM NaCl / 150 mM NaOH from 5 to 65 min. The column was maintained at a constant temperature of 50°C and the eluate monitored on line using a fluorescence detector. The excitation and emission wavelengths were 315 nm and 400 nm, respectively. These conditions had previously been established to give the optimum resolution of oligosaccharides and reproducibility of results.

Preparation of phosphacan from porcine brain

Whole brains from freshly slaughtered pigs were finely chopped and placed in 2 volumes of extraction buffer (4 M guanidine hydrochloride, 50 mM sodium acetate, pH 5.8, containing the protease inhibitors 100 mM aminohexanoic acid, 10 mM EDTA, 20 mM benzamidine hydrochloride, 0.5 mM phenylmethanesulfonylfluoride, and 5 mM N-ethylmaleimide. The extract was left at 4°C for at least 48 h after which time the extract was dialyzed against 7 volumes of water plus protease inhibitors in order to reduce the guanidine concentration to 0.5 M The extract was adjusted to 1.4 g/ml by the addition of solid cesium chloride and centrifuged at 88,500 × g_{av} for 48 h at 12°C. The bottom third of each centrifuge tube, corresponding to a density range of 1.43–1.55 g/ml, was collected using a peristaltic pump, dialyzed against distilled water and lyophilized.

The sample was dissolved in 0.2 M sodium acetate and the CS/DS and HS proteoglycans removed by precipitation using 1.25 volumes of ethanol followed by centrifugation at 2500 r.p.m. for 30 min. KS-containing proteoglycans were then precipitated from the supernatant using 4 volumes of ethanol, followed by centrifugation at 2500 r.p.m. for 1 h. The extract was subjected to ion-exchange chromatography on a Q-Sepharose column (1.5 × 15 cm) equilibrated with 0.15 M NaCl/6 M urea/TrisHCl, pH 6.8, at a flow rate of 1 ml/min. The KS proteoglycans were eluted using a linear gradient of 2 M NaCl/6 M urea/TrisHCl, pH 6.8. The eluate was monitored on line by absorbance at 280 nm and fractions were analyzed by ELISA using the anti-KS antibody 5D4 and the anti-phosphacan antibody 3F8. The fractions containing phosphacan were pooled, desalted, and lyophilized. Final purification was achieved by gel-permeation chromatography on a Superose 6 column eluted with 0.15 M NaCl/6 M urea/TrisHCl, pH 6.8, at a flow rate of 0.3 ml/min. The eluate was monitored on line by absorbance at 280 nm and fractions were analyzed by ELISA using 5D4 and 3F8. The fractions containing phosphacan were pooled, desalted, and lyophilized.

Preparation of keratan sulfates from human ovarian tumors

Clinical samples, obtained on the day of excision, were finely chopped and placed in 5 volumes of extraction buffer (4 M guanidine hydrochloride, 2% Triton X-100, 50 mM sodium acetate, pH 5.8, containing the protease inhibitors 100 mM aminohexanoic acid, 10 mM EDTA, 20 mM benzamidine hydrochloride, 0.5 mM phenylmethanesulfonylfluoride, and 5 mM N-ethylmaleimide. The extract was left at 4°C for at least 24 h. The extract was adjusted to 1.5 g/ml by the addition of solid cesium chloride and the extract centrifuged at 88,500 × g_{av} for 48 h at 12°C. The bottom third of each centrifuge tube, corresponding to a density range of 1.55–1.65 g/ml, was collected using a peristaltic pump, dialyzed against distilled water, and lyophilized. The sample was subsequently treated with 80 Kunitz units DNase I and 5 Kunitz units RNase A for 1 h at 37°C to remove nucleic acid contaminants.

The digested material was chromatographed on a Q-Sepharose column (2.5 × 25 cm) equilibrated with 6 M urea/50 mM TrisHCl/0.15 M NaCl, pH 6.8, at a flow rate of 1 ml/min. Bound material was eluted with a linear gradient of 0.15–2.0 M NaCl/6 M urea/50 mM TrisHCl. Fractions were analyzed for KS by ELISA with 5D4 and immunopositive fractions were pooled, dialyzed against distilled water, and lyophilized. The material was resuspended in 100 µl of 0.1 M sodium acetate/0.1 M hexanoic acid/5 mM benzamidine hydrochloride, pH 7.0 and 0.05 U chondroitin ABC lyase and 1 U heparin lyase III added, and the sample was left at 37°C for 24 h. The digest was subsequently chromatographed on a Q-Sepharose column (1.5 × 15 cm) equilibrated with 6 M urea/50 mM TrisHCl/0.15 M NaCl, pH 6.8, at a flow rate of 2 ml/min. Bound material was eluted with a gradient of 0.15–2.0 M NaCl (0.15–1.0 M over 60 min and 1.0–2.0 M over 5 min). Fractions were analyzed for KS by ELISA with 5D4 and immunopositive fractions were pooled, dialyzed against distilled water, and lyophilized. This procedure yielded KS proteoglycans of high purity.

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Abbreviations

KS, keratan sulfate; 2-AA, 2-aminobenzoic acid (2-aminoanthranilic acid); ELISA, enzyme-linked immunosorbent assay; BFH, bovine femoral head; BNS, bovine nasal septum.

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