ELSEVIER

Carbohydrate Research 309 (1998) 117–124

CARBOHYDRATE RESEARCH

Structure of keratan sulfate from bonefish (*Albula* sp.) larvae deduced from NMR spectroscopy of keratanase-derived oligosaccharides

Michael Peña [a], Clarrisa Williams [b], Edward Pfeiler [b,*]

[a] *Department of Chemistry and Biochemistry, Arizona State University, Tempe, AZ 85287, USA*
[b] *Department of Biology, Arizona State University, Tempe, AZ 85287, USA*

Received 20 November 1997; accepted 30 April 1998

Abstract

Structural details of keratan sulfate (KS) glycosaminoglycan, isolated from early-metamorphosing larvae (leptocephali) of bonefish (*Albula* sp.), are described. Bonefish KS was analyzed by first hydrolyzing the purified compound with KS endo-β-galactosidase (keratanase) from *Pseudomonas* spp., and then examining the resulting oligosaccharides with reversed-phase high-performance liquid chromatography (HPLC) and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy at 400 MHz. Spectral analyses were performed by COSY and HMQC. The results showed that a single oligosaccharide was produced whose structure is consistent with that of a tetrasaccharide containing two, β-linked, *N*-acetyllactosamine units. Enzymic evidence indicated that the internal galactose of the tetrasaccharide was O-sulfated at C-6, and that the reducing-end galactose was unsulfated. Spectral data for C-1 of the two galactose residues were consistent with the proposed sulfation pattern. In addition, spectral evidence confirmed that a C-6 on one of the sugars was sulfated; this sulfate was tentatively assigned to the internal galactose. Chemical studies have shown that an additional sulfate group is present, but its assignment could not be confirmed, owing to the complexity of the spectral data. The known specificities of keratanase, and the production of a single tetrasaccharide, however, require that the additional sulfate reside on C-6 of either of the two available *N*-acetylglucosamine (GlcNAc) moieties, and that it cannot alternate between the two. The inability of β-*N*-acetylglucosaminidase from beef kidney to liberate GlcNAc from the tetrasaccharide provided preliminary support for the view that this sulfate is located on the nonreducing-end GlcNAc. We conclude that the native, high molecular weight ($M_r = 55{,}000$) KS polymer from bonefish larvae consists of a disulfated disaccharide alternating with an unsulfated disaccharide in the adjacent *N*-acetyllactosamine unit, with this pattern repeating itself in a regular fashion along most, or all, of the chain. This structure could provide an explanation for the ability of bonefish KS chains to self-associate into dimers. Although the *N*-acetyllactosamine repeat is characteristic of KS in general, the sulfation pattern is different from that postulated for

* Corresponding author. Tel: 602-965-9531; fax: 602-965-8352; e-mail: auexp@asuvm.inre.asu.edu

PII S0008-6215(98)00128-1

the well-characterized KS chains of lower molecular weight obtained from mammalian cornea and cartilage. An additional difference was the inability to demonstrate sialic acid in bonefish KS.

Keywords: Keratan sulfate; Glycosaminoglycan; Bonefish; Leptocephalus

1. Introduction

Keratan sulfate (KS) glycosaminoglycan is formed of repeating units of →3)-β-D-galactose (1→4)-*N*-acetyl-β-D-glucosamine-(1→ that are usually O-sulfated at C-6 of one or both of the component sugars [1–3]. Although possessing the same *N*-acetyllactosamine repeating unit, KS chains isolated from mammalian cornea (KS I) and cartilage (KS II) show several basic differences, mainly in the content of minor sugars (e.g., galactosamine) and type of linkage to proteoglycan core protein [4,5]. In addition, KS I chains are normally larger (M_r = 7000–26,000) [6,7] than those of KS II (M_r < 6000) [8]. For both KS I and II, structural studies also indicate heterogeneity in sulfate composition, resulting in regions of unsulfated, monosulfated and disulfated disaccharide repeats [8,9]. It has been postulated that disulfated disaccharides predominate at the nonreducing end of the chain, with one or two unsulfated disaccharides located at the attachment region to the protein core [8,9].

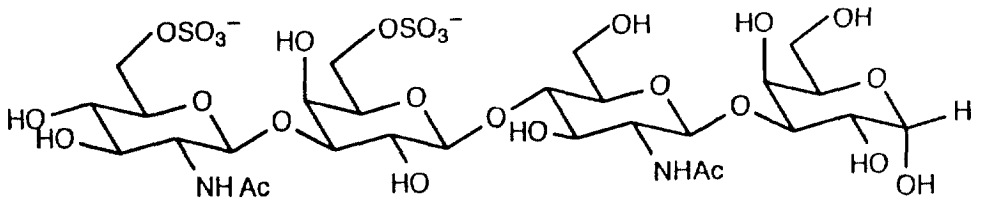
KS has been identified as the predominate glycosaminoglycan in the gelatinous body matrix of early-metamorphosing bonefish (*Albula* sp.) larvae (leptocephali), where it accounts for about 6% of the total larval dry weight [10]. A unique feature of bonefish KS is its apparent use as an endogenous source of energy during metamorphosis, the first vertebrate glycosaminoglycan thought to function as a storage polysaccharide [10,11]. Although its biochemical composition is similar to mammalian KS I [12,13], bonefish KS shows several characteristics not seen in KS I and KS II. The average molecular weight of the bonefish KS chains is much higher ($M_r \sim 55{,}000$), and these chains appear to self-associate forming a dimer of $M_r \sim 100{,}000$ as judged by Sepharose CL-6B molecular-exclusion chromatography profiles under associative conditions [12]. Keratan sulfate endo-β-galactosidase from *Pseudomonas* spp. (= keratanase) degrades these chains, with the breakdown products eluting as a single symmetrical peak close to the total volume of the column (K_{av} = 0.94), suggesting the formation of a single homogeneous oligosaccharide. This was confirmed by thin-layer chromatography (TLC) where the relative amount of the single oligosaccharide was seen to increase with time of keratanase incubation [12]. Mild acid hydrolysis of the isolated oligosaccharide, followed by TLC, indicated that it was composed of at least four sugar units, but its precise size was not determined. These results are in contrast to those obtained on bovine and porcine corneal KS, where keratanase digestion produces a series of oligosaccharides of different sizes [9,12,14], suggesting that bonefish KS differs in sulfation pattern.

Keratanase cleaves the β-(1→4)-glycosidic linkage between galactose and GlcNAc in KS but is inactive when the galactosyl residue is O-sulfated at C-6 [14]. There is evidence that the enzyme also requires that at least one of the GlcNAc residues on either side of the bond that is hydrolyzed be O-sulfated at position 6 [15,16]. Larval bonefish KS possesses about one sulfate residue per *N*-acetyllactosamine unit [12]. With this information, the sulfation pattern of the native KS polymer can be deduced once the nature of the keratanase-derived oligosaccharide has been established.

In this paper, ^{1}H and ^{13}C NMR analyses of the keratanase-produced oligosaccharide from bonefish KS is reported. The postulated sulfation pattern of the native KS polymer reveals a sequence of disulfated disaccharide alternating with unsulfated disaccharide that is repeated in a regular fashion along most of the chain. This sulfation pattern, which may be related to its novel storage function, provides a basis for explaining the ability of free KS chains from bonefish larvae to self-associate.

2. Results and discussion

Reversed-phase high-performance liquid chromatography (HPLC) of keratanase digests of purified bonefish KS (C_4 and C_{18} columns; UV [220 nm] detection; acetonitrile–water [90:10 or 50:50] mobile phase), revealed the presence of a single major peak (not shown). The HPLC data



1

supported our earlier conclusion, based on molecular-exclusion chromatography and TLC [12], that the main product of keratanase digestion is a single oligosaccharide. NMR analyses of the oligosaccharide confirmed that it was composed of a tetrasaccharide (**1**; shown as the α anomer).

Fig. 1 shows the portion of the ^{1}H NMR spectrum of the keratanase digest that was particularly informative in elucidating the structure of **1**. The ^{1}H NMR spectrum of the main peak recovered after reversed-phase HPLC (not shown) was identical to that shown in Fig. 1. The informative regions of the ^{13}C NMR spectrum are described below, but the spectrum is not illustrated.

The partial assignments deduced from both the ^{1}H and ^{13}C NMR spectra of **1** are shown in Table 1. The presence of two GlcNAc residues was confirmed in the ^{13}C NMR spectrum by two clean signals at 56 ppm (56.84 and 56.86 ppm), which are distinct from the other carbons and which correspond to C-2 of glucosamine [17]. Signals in the ^{13}C NMR spectrum for the two corresponding carbonyl groups (176.22 and 176.24 ppm) of the acetyl side chain of GlcNAc also were observed. Two additional, but smaller, sets of peaks for carbonyl and methyl groups of acetyl moieties were observed, and these remain unassigned. Of the four strong signals assigned to anomeric carbons, two were distinct (93.6 and 97.7 ppm); the remaining two overlapped at 103.8 and 103.9 ppm.

Each of the anomeric carbons was correlated to the anomeric hydrogens through the HMQC

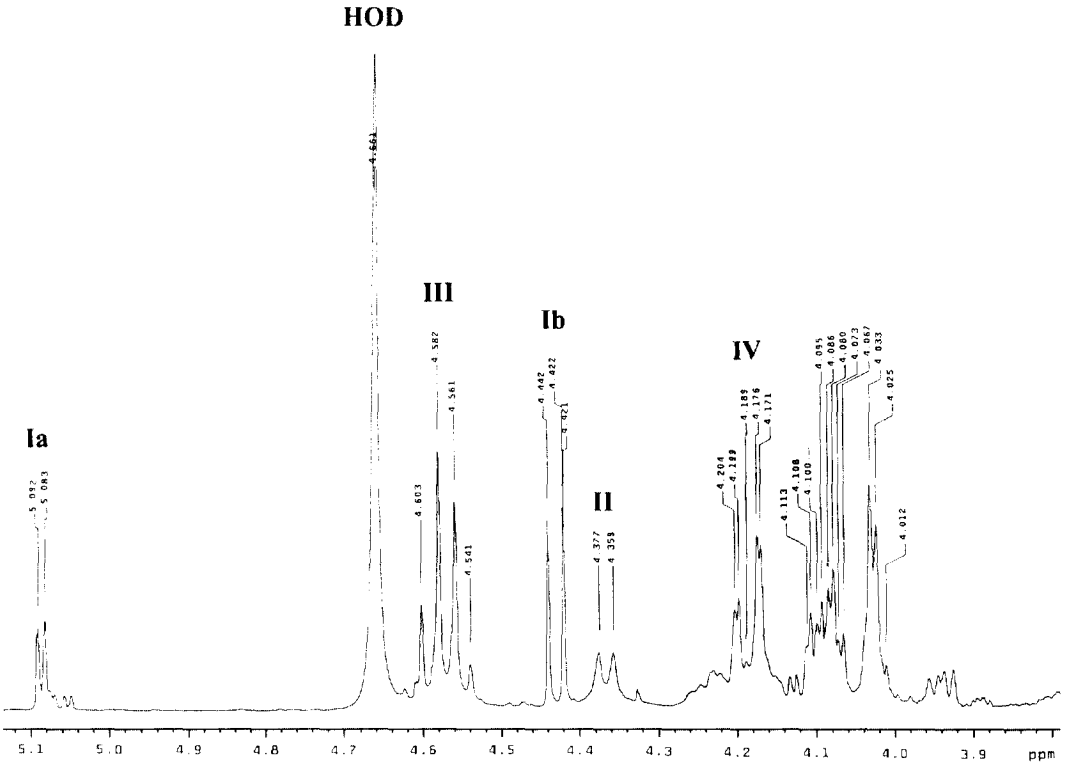


Fig. 1. Portion of the ^{1}H NMR spectrum at 400 MHz and 25 °C of the tetrasaccharide produced by keratanase treatment of bonefish keratan sulfate. Signal abbreviations are I, H-1 of reducing-end galactose (Gal-1) in anomeric forms α (Ia) and β (Ib); II, H-1 of internal β-galactose (Gal-3); III, H-1 of β-GlcNAc residues 2 and 4; IV, H-6 (one of two) of the carbon tentatively assigned to Gal-3.

spectrum (Fig. 2); the coupling constants described below are also given in Table 1. The peak at 93.6 correlated to 5.1 ppm (d, J 3.6 Hz) and 97.7 correlated to 4.4 ppm (d, J 8.0 Hz). The overlapping peaks at 103.8/103.9 correlated to 4.4 (d, J 7.2 Hz), 4.5 (d, J 8.0 Hz) and 4.6 ppm (d, J 8.4 Hz). As the tetrasaccharide obtained after keratanase digestion was analyzed in a non-reduced form, the reducing-end, unsulfated galactose should exhibit both α and β anomeric forms [18]. The ^{13}C chemical shifts at 93.6 and 97.7 ppm, and their respective coupling constants, are consistent with unsulfated α- and β-

Table 1
Partial assignments for ^{13}C and ^{1}H NMR chemical shifts for tetrasaccharide (**1**)

Residue and ring position	Chemical shift (ppm)		Coupling constant (Hz)
	^{13}C	^{1}H	
GlcNAc-4 (nonreducing end)			
1	103.8/103.9	4.4/4.6	d, J 8.0/8.4
2	56.84/56.86		
CH_3	23.500/23.507	1.89/1.90	
CO	176.22/176.24		
Gal-3			
1	103.8/103.9	4.4	d, J 7.2
6	~69	4.2 (6,6′)	dd, J 2, 11.2
GlcNAc-2			
1	103.8/103.9	4.5/4.6	d, J 8.0/8.4
2	56.84/56.86		
CH_3	23.500/23.507	1.89/1.90	
CO	176.22/176.24		
Gal-1 (reducing end)			
1 (α anomer)	93.6	5.1	d, J 3.6
1 (β anomer)	97.7	4.4	d, J 8.0

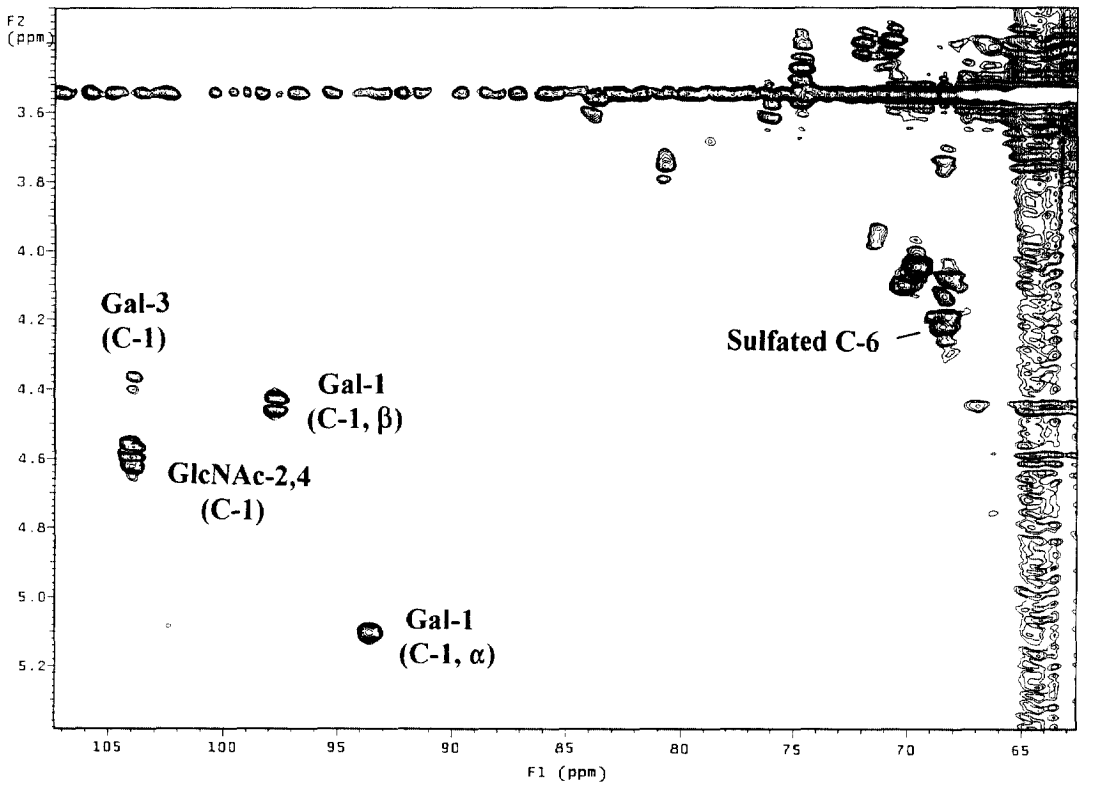


Fig. 2. Details of the two-dimensional HMQC spectrum of the keratanase-derived tetrasaccharide of bonefish keratan sulfate.

galactopyranose [19]. The chemical shifts at 103.8/103.9 and 4.4 ppm in the ^{13}C and ^{1}H spectra, and the corresponding coupling constant, are consistent with C-1 of a sulfated, β-linked internal galactose [20]. The other two correlations at 103.8/103.9 (J 8.0 and 8.4 Hz) are assigned to C-1 of the two glucosamines. We assumed that the two anomeric carbons of each glucosamine would have similar chemical shifts in the ^{13}C and ^{1}H spectra, and this was confirmed. The coupling constants implied a tetrasaccharide with three β-glycosidic links. The H-2 of the reducing-end α-galactose can be assigned to a location in the ^{1}H spectrum from correlation of the anomeric H in the COSY spectrum (Fig. 3); it was, however, buried in the region from 3.5 to 3.7 ppm. A pair of doublets at 4.2 ppm (J 2, 11.2 Hz) appeared to correspond to one hydrogen of a methylene (at a C-6 position) of one of the sugars. The coupling values indicated a coupling to the other methylene H at C-6, and to H-5. These values are consistent with the Karplus correlation and the geometry about the C-5/C-6 position [21]. The pair of doublets correlated to a carbon at ~69 ppm (Fig. 2). This value is larger than a typical C-6 of either a free (unsulfated) glucosamine or galactose (about 62 ppm for each). This would indicate that this particular H is associated with a C-6 methylene that bears a sulfate group, tentatively assigned to the internal galactose. Our partial assignments for ^{1}H chemical shifts are in general agreement with those seen at 500 MHz for a similar, β-linked, trisulfated tetrasaccharide obtained from KS I [18].

The spectral data confirm an earlier suggestion that the oligosaccharide produced by keratanase treatment of bonefish KS was composed of at least four sugars units [12]. In addition to showing approximately equimolar amounts of galactose and GlcNAc, chemical analyses show about one sulfate group per hexosamine [12], indicating that each tetrasaccharide should have two sulfate groups. We were, however, only able to detect one of these; the presence of the second sulfate group could not be confirmed due to the complexity of the spectral data resulting from the presence of both nonreducing-end and internal GlcNAc residues, and reducing-end and internal galactosyl residues.

Because keratanase is unable to cleave the β-glycosidic linkage when galactose is O-sulfated at C-6 [14], the internal galactose must bear a sulfate group, and the reducing-end galactose must be

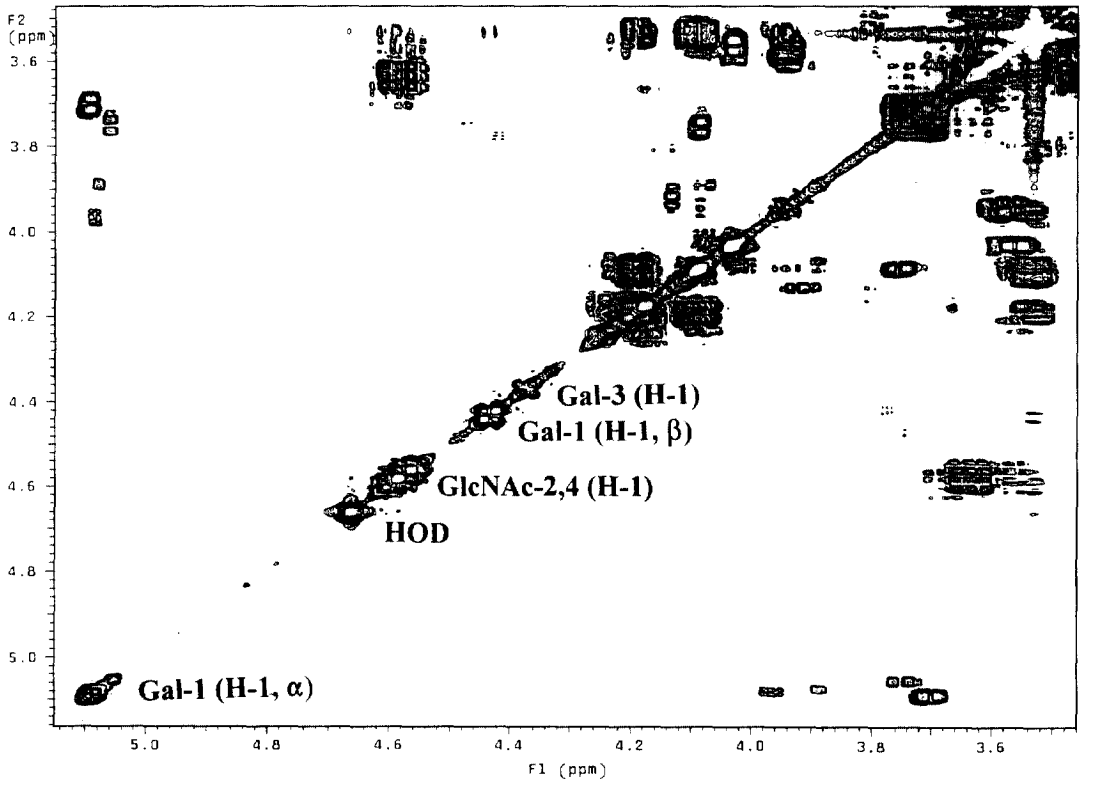


Fig. 3. Details of the two-dimensional COSY spectrum of the keratanase-derived tetrasaccharide of bonefish keratan sulfate.

unsulfated, in order for a tetrasaccharide to be produced. Therefore, only two possibilities exist for assigning the remaining sulfate; it must reside on C-6 of the GlcNAc either immediately preceding (a) or following (b) the sulfated galactose as shown.

(a) β-D-GlcNAc6S-(1→3)-β-D-Gal6S-(1→4)-β-D-GlcNAc-(1→3)-D-Gal

(b) β-D-GlcNAc-(1→3)-β-D-Gal6S-(1→4)-β-D-GlcNAc6S-(1→3)-D-Gal

We attempted to distinguish between these two possibilities by incubating the tetrasaccharide with bovine kidney β-*N*-acetylglucosaminidase (EC 3.2.1.52; from Sigma Chemical Co., St. Louis, MO) for 90 min at 37 °C and then analyzing the digestion mixture by TLC. No free GlcNAc or 6-*O*-sulfoGlcNAc was detected. If the nonreducing GlcNAc was unsulfated as in (b), it would have been liberated. The inability to produce free 6-*O*-sulfoGlcNAc suggests that, unlike one form of the enzyme [22], the β-*N*-acetylglucosaminidase used here cannot hydrolyze the β-glycosidic linkage if GlcNAc is O-sulfated. These results support tetrasaccharide (a), shown also in **1**. We feel, however, that the evidence for the assignment for the second sulfate is still preliminary and requires confirmation.

Either tetrasaccharide (a) or (b), however, satisfies the requirement for GlcNAc sulfation at C-6 on either side of the bond hydrolyzed [15], but because keratanase digestion apparently yields only a single class of oligosaccharide, the position of the sulfated GlcNAc must be fixed and cannot vary along the chain. Any alternation of sulfated GlcNAc would, after keratanase treatment, result in oligosaccharides of different sizes. This implies that the sulfation pattern of the intact KS polymer consists of a disulfated disaccharide alternating with an unsulfated disaccharide in the adjacent *N*-acetyllactosamine unit, with this pattern repeating itself in a regular fashion along the most, or all, of the chain (Fig. 4). Based on the molecular weights of the intact polymer and the tetrasaccharide, the average number of tetrasaccharide repeats is estimated at about 50. This sulfation pattern differs dramatically from that postulated for mammalian KS I and KS II [8,9,16]. The regular, alternating sequence of unsulfated regions may provide appropriate contact zones for noncovalent interactions between free KS chains, providing a possible explanation for the ability of these chains to self-associate [12].

Fig. 4. Schematic representation of a portion of the postulated structure of keratan sulfate from bonefish larvae demonstrating the tetrasaccharide repeats obtained after keratanase digestion (arrows indicate β-glycosidic linkage attacked by keratanase) and the regular, repeating sequence of negative charges along the backbone of the polymer. Solid squares = GlcNAc; open squares = galactose; negative charge = O-sulfate linked to C-6 of corresponding hexose.

KS also has been isolated from other non-mammalian sources, including adult fish (Pacific mackerel) skin [23]. Although biochemical composition of larval bonefish and mackerel skin KS is very similar [12,23], with identical sulfate contents, mackerel skin KS shows a molecular weight profile and heterogeneity in sulfate composition typical of corneal KS [8,9]. Although the nature of the linkage of bonefish KS chains to core protein of the proteoglycan [12] has not been determined, the almost complete absence of galactosamine [13] strongly suggests an N-linkage to an asparagine residue, typical of KS I and mackerel skin KS. Both mannose and fucose are postulated to reside near the core protein linkage region in KS I [8], and minor amounts of these two sugars are found in bonefish KS [13]. *N*-acetylneuraminic acid (sialic acid) is also found in both KS I and KS II [8,24]. The C-3 carbon of sialic acid and its two hydrogens show characteristic chemical shifts in both the ^{1}H and ^{13}C NMR spectra [25,26], which we did not observe during spectral analyses of the keratanase-derived tetrasaccharide of bonefish KS. Commercial keratanase from the same source as used here sometimes contains sialidase activity [24]. Sialidase contamination of keratanase will degrade sialic acid in oligosaccharides derived from KS II [16], suggesting that this may have occurred in our experiments. We failed, however, to detect sialic acid in purified bonefish KS using the thiobarbituric acid procedure [27].

3. Experimental

Keratan sulfate extraction and purification.—KS was extracted from whole-body homogenates of early-metamorphosing larvae (leptocephali) of bonefish (*Albula* sp.) using a preparative extraction described previously [12]. In this procedure, larval homogenates are first treated with protease (Sigma

type III from papaya) for 72 h, followed by treatment with 10% trichloroacetic acid and low-speed centrifugation to remove precipitated protein. The supernatant is then treated with 1.25 volumes ethanol/1% sodium acetate which produces a precipitate consisting of chondroitin sulfate and undersulfated chondroitin sulfate [28]. The remaining supernatant is made to 3 volumes ethanol/1% sodium acetate which produces a precipitate of keratan sulfate peptidoglycan. KS was isolated by centrifugation, washed twice with 80% ethanol, dissolved in water and lyophilized.

Preparation of keratan sulfate oligosaccharides.—Purified KS was dissolved in 0.05 M Tris-HCl (pH 8.0) at a concentration of 11–38 mg×mL^{-1}. Keratanase (EC 3.2.1.103; from *Pseudomonas* spp.; Sigma) was then added (0.02–0.08 units of keratanase per mg KS), and the solution was incubated for 24 h at 37 °C. The reaction mixture was heated for 5 min at 100 °C to stop the reaction and then centrifuged. The supernatant fraction containing the KS oligosaccharides was recovered and lyophilized.

^1H and ^{13}C NMR analyses.—About 90–100 mg of oligosaccharide was dissolved in 6 mL of 99.98% atom-% D_2O (Cambridge Isotopes) and lyophilized. The procedure was repeated eight times to completely exchange the hydrogens of the hydroxyl functional groups with deuterium. The material was isolated as a hygroscopic white powder. The oligosaccharide was dissolved in 1.5 mL of D_2O and examined by high-field ^1H and ^{13}C NMR on a Varian 400 MHz NMR Spectrometer at 25 °C. Chemical shifts were referenced to the methyl groups of the *N*-acetyl chains of GlcNAc (1.9 ppm ^1H and 23.50 ppm ^{13}C) [17]. Two-dimensional COSY and HMQC spectra were obtained using software provided by the manufacturer.

Acknowledgements

The authors thank Steve Velarde, Ron Nieman, and Michael Williams for technical assistance with the NMR analyses, and Therese A. Markow for use of laboratory facilities. This work was supported by a Minority Access to Research Careers (MARC) training grant (T34 GM 08491) from the National Institutes of Health to Dr. Markow, and a National Science Foundation Career Award (CHE-9503260) to M. Peña. C. Williams is a National Institutes of Health MARC trainee.

References

[1] S. Hirano, P. Hoffman, and K. Meyer, *J. Org. Chem.*, 26 (1961) 5064–5069.
[2] V.P. Bhavanandan and K. Meyer, *J. Biol. Chem.*, 242 (1967) 4352–4359.
[3] V.P. Bhavanandan and K. Meyer, *J. Biol. Chem.*, 243 (1968) 1052–1059.
[4] M.B. Mathews and J.A. Cifonelli, *J. Biol. Chem.*, 240 (1965) 4140–4145.
[5] N. Seno, K. Meyer, B. Anderson, and P. Hoffman, *J. Biol. Chem.*, 240 (1965) 1005–1010.
[6] J.R. Hassell, D.A. Newsome, and V.C. Hascall, *J. Biol. Chem.*, 254 (1979) 12346–12354.
[7] H.W. Stuhlsatz, F. Hirtzel, R. Keller, S. Cosma, and H. Greiling, *Hoppe-Seyler's Z. Physiol. Chem.*, 362 (1981) 841–852.
[8] H.W. Stuhlsatz, R. Keller, G. Becker, M. Oeben, L. Lennartz, D.C. Fischer, and H. Greiling, *Structure of Keratan Sulphate Proteoglycans: Core Proteins, Linkage Regions, Carbohydrate Chains*, in H. Greiling and J.E. Scott (Eds.), *Keratan Sulphate*, The Biochemical Society, London, 1989, pp 1–11.
[9] M. Oeben, R. Keller, H.W. Stuhlsatz, and H. Greiling, *Biochem. J.*, 248 (1987) 85–93.
[10] E. Pfeiler, *Mar. Biol. Lett.*, 5 (1984) 241–249.
[11] E. Pfeiler, *Fish Physiol. Biochem.*, 15 (1996) 359–362.
[12] E. Pfeiler, *Fish Physiol. Biochem.*, 4 (1988) 175–187.
[13] E. Pfeiler, *Leptocephalous Larval Contents of Glycosaminoglycans: An Endogenous Source of Energy During Metamorphosis?*, in B.T. Walther and H.J. Fyhn (Eds.), *Physiological and Biochemical Aspects of Fish Development*, University of Bergen, Norway, 1993, pp 341–345.
[14] K. Nakazawa and S. Suzuki, *J. Biol. Chem.*, 250 (1975) 912–917.
[15] K. Nakazawa, M. Ito, T. Yamagata, and S. Suzuki, *Substrate Specificity of Keratan Sulphate-Degrading Enzymes (Endo-β-Galactosidase, Keratanase and Keratanase II) from Microorganism*, in H. Greiling and J.E. Scott (Eds.), *Keratan Sulphate*, The Biochemical Society, London, 1989, pp 99–110.
[16] T.N. Huckerby, J.M. Dickenson, G.M. Brown, and I.A. Nieduszynski, *Biochim. Biophys. Acta*, 1244 (1995) 17–29.
[17] D.R. Bundle, H.J. Jennings, and I.C.P. Smith, *Can. J. Chem.*, 51 (1973) 3812–3819.
[18] E.F. Hounsell, J. Feeney, P. Scudder, P.W. Tang, and T. Feizi, *Eur. J. Biochem.*, 157 (1986) 375–384.
[19] P.M. Collins and R.J. Ferrier, *Monosaccharides: Their Chemistry and Their Roles in Natural Products*, John Wiley and Sons, New York, 1995.

[20] G.H. Cockin, T.N. Huckerby, and I.A. Nieduszynski, *Biochem. J.*, 236 (1986) 921–924.
[21] R.M. Silverstein, G.C. Bassler, and T.C. Morrill, *Spectrometric Identification of Organic Compounds*, John Wiley and Sons, New York, 1981.
[22] H. Kresse, W. Fuchs, J. Glössl, D. Holtfrerich, and W. Gilberg, *J. Biol. Chem.*, 256 (1981) 12926–12932.
[23] M. Ito, M Kitamikado, and T. Yamagata, *Biochim. Biophys. Acta*, 797 (1984) 221–230.
[24] J.M. Dickenson, T.N. Huckerby, and I.A. Nieduszynski, *Biochem. J.*, 282 (1992) 267–271.
[25] L.W. Jaques, E.B. Brown, J.M. Barrett, W.S. Brey, and W. Weltner, *J. Biol. Chem.*, 252 (1977) 4533–4538.
[26] D. Bailey, M.J. Davies, F.H. Routier, C. Bauer, J. Feeney, and E.F. Hounsell, *Carbohydr. Res.*, 300 (1997) 289–300.
[27] L. Warren, *J. Biol. Chem.*, 234 (1959) 1971–1975.
[28] E. Pfeiler, *Fish Physiol. Biochem.*, 12 (1993) 143–148.