ELSEVIER

Carbohydrate Polymers 41 (2000) 357–364

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Comparison of the glycosaminoglycans isolated from the skin and head cartilage of Gould's arrow squid (*Nototodarus gouldi*)

R. Falshaw[a], U. Hubl[a], D. Ofman[a], G.C. Slim[a,*], M. Amjad Tariq[a], D.K. Watt[b], S.C. Yorke[b]

[a]*Industrial Research Ltd., P.O. Box 31-310, Lower Hutt, New Zealand*
[b]*New Zealand Pharmaceuticals Ltd., P.O. Box 1869, Palmerston North, New Zealand*

Received 10 March 1999; received in revised form 28 May 1999; accepted 21 June 1999

Abstract

The glycosaminoglycans from the skin and head cartilage of the squid *Nototodarus gouldi* have been isolated and characterised by constituent disaccharide and neutral sugar analysis, ^{13}C nuclear magnetic resonance (NMR) spectroscopy, anion exchange and size exclusion chromatography. The glycosaminoglycans from both tissues are chondroitin sulphate species. The skin consists principally of unsulphated but relatively highly glycosylated material. The chondroitin sulphate from the head cartilage is more highly sulphated, predominantly C-4,6diS (chondroitin sulphate E), with a higher molecular weight than the skin derived material but somewhat less highly glycosylated. To provide a standard for the assignment of the ^{13}C NMR spectrum, C-4,6diS was chemically prepared from bovine tracheal chondroitin sulphate. This showed that it is not possible to distinguish between a mixture of the monosulphates, C-4S and C-6S, and the C-4,6diS by one-dimensional and simple two-dimensional ^{13}C NMR techniques.

Keywords: Gould's arrow squid; *Nototodarus gouldi*; Glycosaminoglycans

1. Introduction

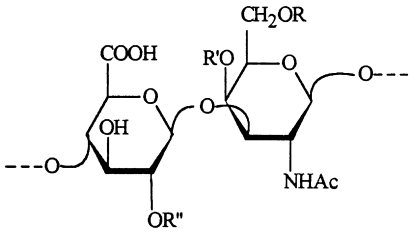
The principle glycosaminoglycan (GAG) species isolated from squid are chondroitin sulphates (CS) with varying sulphation patterns and levels of substitution with neutral sugars (Karamanos, 1992; Karamanos, Hjerpe, Aletras, Tsegenidis, Anastassiou & Antonopoulos, 1995). CS consists of a linear polysaccharide chain with a basic repeating unit of (1 → 4)-β-D-glucopyranosyluronic acid-(1 → 3)-β-D-2-acetamido-2-deoxygalactopyranose, which may be *O*-sulphated at one or more of the hexosamine 4- and 6-positions and the uronic acid 2-position (Fig. 1). In general, CS chains are connected to the protein core of a proteoglycan via a glucuronic acid–galactosyl–galactosyl–xylosyl–*O*-serine linkage (Kitagawa et al., 1995). Mammalian CS generally consists of mixtures of the monosulphated species C-4S (CS A) and C-6S (CS C), with only small amounts of other disaccharide units, and is not substituted with neutral sugars other than those in the linkage region (Fransson, 1985). The CS of lower organisms, however, such as squid, sharks (Sugahara, Shigeno, Masuda, Fujii, Kurosaka & Takeda, 1994) and sea cucumbers (Kariya, Watabe, Hashimoto & Yoshida, 1990) generally has a more varied substitution pattern involving both neutral sugar and sulphate substituents.

Traditionally C-4,6diS (CS E) has been isolated from squid skin (Seldin, Seno, Austen & Stevens, 1984). Other disulphated CS species, C-2,4diS (CS K) and C-2,6diS (CS D) along with monosulphated species, C-4S and C-6S, and low sulphated CS species, C-0S, have been identified in proteoglycans from the skin of the squid *Illex illecebrosus coidentii* (Karamanos, 1992; Karamanos, Aletras, Tsegenidis, Tsiganos & Antonopoulos, 1992) and in the cornea of the squid *Sepia officinalis* (Karamanos, Manouras, Tsegenidis & Antonopoulos, 1991). Low levels of trisulphated

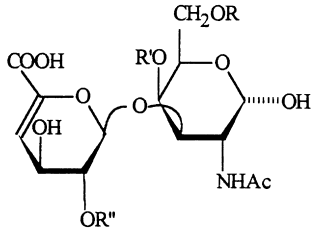
Abbreviations: ΔDi-0S, 3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-2-acetamido-2-deoxy-D-galactopyranose; ΔDi-4S, 3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-2-acetamido-2-deoxy-D-galactopyranose-4-sulphate; ΔDi-6S, 3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-2-acetamido-2-deoxy-D-galactopyranose-6-sulphate; ΔDi-4,6S, 3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-2-acetamido-2-deoxy-D-galactopyranose-4,6-disulphate; ΔDi-2,4S, 3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid-2-sulphate)-2-acetamido-2-deoxy-D-galactopyranose-4-sulphate; ΔDi-2,6S, 3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid-2-sulphate)-2-acetamido-2-deoxy-D-galactopyranose-6-sulphate; ΔDi-triS, 3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid-2-sulphate)-2-acetamido-2-deoxy-D-galactopyranose-4,6-disulphate; CS, chondroitin sulphate; DMF, dimethyl formamide; DMSO, dimethyl sulphoxide; EIMS, electron impact mass spectrometry; GLC, gas liquid chromatography; HPLC, high performance liquid chromatography; KS, keratan sulphate; NMR, nuclear magnetic resonance; SE, size exclusion

* Corresponding author. Tel.: + 64-4-569-0000; fax: + 64-4-566-6004.
E-mail address: g.slim@irl.cri.nz (G.C. Slim)

0144-8617/00/$ - see front matter
PII: S0144-8617(99)00103-4



Chondroitin sulphate



Unsaturated disaccharide

Fig. 1.

Chondroitin sulphate	Unsaturated disaccharide	R	R′	R″
C-0S	ΔDi-0S	H	H	H
C-4S, CS A	ΔDi-4S	H	SO_3^-	H
C-6S, CS C	ΔDi-6S	SO_3^-	H	H
C-2,6diS, CS D	ΔDi-2,6S	SO_3^-	H	SO_3^-
C-4,6diS, CS E	ΔDi-4,6S	SO_3^-	SO_3^-	H
C-2,4,6triS	ΔDi-triS	H	SO_3^-	SO_3^-
C-2,6diS, CS D	ΔDi-2,6S	SO_3^-	SO_3^-	SO_3^-

species, C-2,4,6triS, have also been identified in the skin of the squid *I. illecebrosus coidentii* (Karamanos et al., 1992). Recently chondroitin sulphate species sulphated on the 3-position of the glucuronic acid have been isolated from king crab (Kitagawa et al., 1997; Sugahara et al., 1996) and squid cartilage (Kinoshita, Yamada, Haslam, Morris, Dell & Sugahara, 1997).

Glucose, galactose, fucose (Karamanos, Tsegenidis & Antonopoulos, 1986), mannose and traces of xylose (Karamanos, 1992; Tsegenidis, 1992) have been found in GAGs isolated from various squid skins, with the predominant neutral sugar being glucose. The same pattern has been found for squid head cartilage (Vynios & Tsiganos, 1990). The low levels of xylose were surprising given the usual involvement of this monosaccharide in the linkage region but this may be explained by the relatively high molecular weight of these CS species (see below). The same neutral sugars have also been found in squid corneal GAGs, although xylose is present in more substantial amounts in this tissue (Karamanos et al., 1991). The neutral sugars, other than the two galactoses and xylose of the linkage region, are presumed to be pendant from the CS backbone. This has been shown for glucose, which is on the 6-position of the galactosamine of head cartilage CS of the squid *Ommastrephes sloani pacificus* (Habuchi, Sugiura & Kawai, 1977). Squid ink from *Illex argentinus*, on the contrary, contains a branched polysaccharide with the repeating unit -(1 → 3)-β-D-glucopyranosyluronic acid-[(1 → 3)-2-acetamido-2-deoxy-α-D-galactopyranose-](1 → 4)-α-D-fucopyranose (Takaya, Uchisawa, Hanamatsu, Narumi, Okuzaki & Matsue, 1994; Takaya, Uchisawa, Narumi & Matsue, 1996) where the hexosamine is pendant.

The molecular weight of the CS species isolated from squid have generally been higher than those extracted from mammalian sources. CS species with average molecular weights of 110 000 and 43 000 have been isolated from the proteoglycans of squid skin (Karamanos, 1992) and 73 000 from squid cornea (Karamanos et al., 1991), but no polydispersity data for these species was given. Mammalian CS lies in the molecular weight range of 10 000–50 000 (Fransson, 1985) with the typical bovine tracheal CS having an average molecular weight of 26 000 (Ofman, Slim, Watt & Yorke, 1997).

Squid is one of the most economically important of the fish species caught off the coast of New Zealand. Gould's arrow squid (*Nototodarus gouldi*) is a species unique to the southern oceans and makes up about half of the national squid catch (71 000 metric tonnes in 1994). Gould's arrow squid is caught by jigging in the Mainland North fishery (*Seafood New Zealand*, February 1994). In this paper we compare the GAGs from the head cartilage to the GAGs from the skin of Gould's arrow squid *N. gouldi* isolated during a survey of the GAGs available from New Zealand marine sources.

2. Methods

2.1. Isolation of CS

Tissue from the squid *N. gouldi* was provided by New Zealand Pharmaceuticals Ltd and the GAGs from the skin and head cartilage of the squid were isolated by papain digest followed by filtration and precipitation by cetylpyridinium chloride (Rodén, Baker, Cifonelli & Mathews, 1972).

2.2. HPLC

Anion exchange and size exclusion chromatography was performed on a Gilson HPLC System (John Morris Scientific Ltd, NZ) comprising 305 and 306 series pumps with associated manometric module and dynamic mixer for high pressure mixing, 231 XL sampling auto-injector and 160 Diode Array Detector, under the control of Gilson UniPoint software. Anion exchange chromatography was performed using a Mono-Q HR 5/5 column (Pharmacia) with a linear gradient of sodium chloride (0.1–2.0 M over 25 min).

SE-HPLC used Superdex 75 HR 10/30 (Pharmacia) and Biosep SEC 4000 (Phenomenex) columns in series. The mobile phase was sodium chloride (0.2 M) in potassium phosphate buffer (10 mM, pH 7.2) at a flow rate of 1 ml/min. Data was collected at 214 nm and analysed by UniPoint.

2.3. NMR spectroscopy

^{13}C and ^{1}H NMR spectroscopy were performed on a Varian Unity 500 spectrometer operating at 125.7 MHz for the collection of ^{13}C spectra and 500 MHz for ^{1}H spectra. Samples (100 mg) were lyophilised three times from 99.8 at.% D_2O (Acros) and dissolved in the same solvent (0.8 ml) in a 5 mm NMR tube for analysis. The carbon spectra were acquired at 80°C, with an acquisition time of 1.3 s, with a delay of 1.0 s between pulses and 10 000–20 000 transients were acquired until a satisfactory signal-to-noise ratio was achieved. The spectra were referenced to external DMSO in D_2O solution at 39.47 ppm. ^{1}H spectra were also collected at 80°C with an acquisition time of 4.1 s and delay between pulses of 5.0 s. Sixteen transients were collected. The HMQC-SE carbon–proton correlation spectrum was collected at 60°C.

2.4. Neutral sugar analysis

The samples of squid CS (~1 mg, accurately weighed) were analysed by the reductive hydrolysis technique of Stevenson and Furneaux (1991) with inositol (0.08 mg/sample) added at the hydrolysis stage as an internal standard and traditional hydrolysis method of Harris, Henry, Blakeney and Stone (1984).

2.5. Sulphation of CS A

The CS A was first converted to its tributylammonium salt and then sulphated with pyridine–sulphur-trioxide complex in DMF (Dace, McBride, Brooks, Gander, Buszko & Doctor, 1997; Nagasawa, Uchiyama &Wajima, 1986) as described below.

2.6. Preparation of tributylammonium salt

Bovine tracheal CS (5 g, New Zealand Pharmaceuticals) was dissolved in water (50 ml) and passed through an ion-exchange column (50 ml, Amberlite IR-120 H^+). After passing through the column the pH of the solution was 2. The pH of the solution was adjusted to 7 with tributylamine and stirred for 1 h. The excess tributylamine was removed by extracting the resulting solution with diethyl ether. The solution was dialysed (Spectra/Por membrane, MWCO = 1000) against water for 12 h to completely remove any remaining tributylamine. The retentate was concentrated on a rotary evaporator and finally lyophilised to obtain the tributylammonium salt of CS A (4.5 g).

2.7. Sulphation

The tributylammonium salt of the CS (2 g) was dissolved in dry DMF (30 ml). Pyridine–sulphur-trioxide complex (15 equivalents, 10 g or 7 equivalents, 5 g) was dissolved in dry DMF (50 ml). Both DMF solutions were cooled on an ice bath at 0°C and then the pyridine–sulphur-trioxide solution was poured into the CS solution. The resulting solution was stirred on an ice bath for 1 h. After this time the mixture was poured into ice cold water (200 ml) and the pH (initially 2) was adjusted to 7 with NaOH (2 M). The solution was dialysed (Spectra/Por membrane, MWCO = 1000) against distilled water for 12 h, then concentrated to 50 ml by rotary evaporation and eluted through a column of Amberlite IR-120 H^+ (50 ml) with water. The eluant was neutralised with NaOH (1 M) and finally lyophilised to obtain sulphated CS (1.5 g, as the sodium salt). Microanalytical data for the CS A before and after sulphation: CS A (C, 27.95%; H, 5.47%; N, 3.14%; S, 4.26%; equivalent to 0.8 sulphates/disaccharide unit), Sulphated CS (15 equivalents C, 17.05%; H, 3.00%; N, 1.95%; S, 12.01%; equivalent to 3.7 sulphates/disaccharide unit; 7 equivalents C, 24.69%; H, 4.31%; N, 2.57%; S, 7.73%; equivalent to 1.6 sulphates/disaccharide unit).

2.8. Disaccharide composition analyses

Constitutive disaccharide quantification of the squid GAG samples was carried out by digestion with chondroitinase ABC (Seikagaku) and identification of the resulting disaccharide units by anion exchange HPLC, as described by Volpi (1994b). HPLC was conducted using a Spectraphysics Model SP8700 HPLC pump, a Spectraphysics SP8750 solvent delivery system, a Spectraphysics SP8440 UV/VIS detector at 232 nm, a Waters Millennium 2010 chromatography manager, and a Spherisorb 5, SAX, 250 × 4.6 mm column with a flow rate of 1 ml/min. From 0 to 8 min the column was eluted isocratically with a NaH_2PO_4 buffer (0.001 M, pH 6.00 containing 0.15 M NaCl). From 8 to 30 min the NaCl concentration was linearly increased to 0.47 M (keeping the pH and phosphate concentration constant), and then from 30 to 35 min the NaCl concentration was linearly increased to 2.0 M. The column was then eluted isocratically for 5 min with NaH_2PO_4 buffer (0.001 M, pH 6.00 containing 2.0 M NaCl).

The disaccharides were identified by their retention times using standards obtained from Seikagaku and Sigma Chemical Company. Quantification was achieved by comparison of the response of the unknowns with known amounts of the standard disaccharides.

Constitutive disaccharide quantification of the over-sulphated CS samples was carried out by treatment with chondroitinase ABC as above followed by reduction with sodium borohydride of disaccharide standards and digest solutions before analysis by HPLC. HPLC was conducted on the Gilson system described above using a Partisil 10

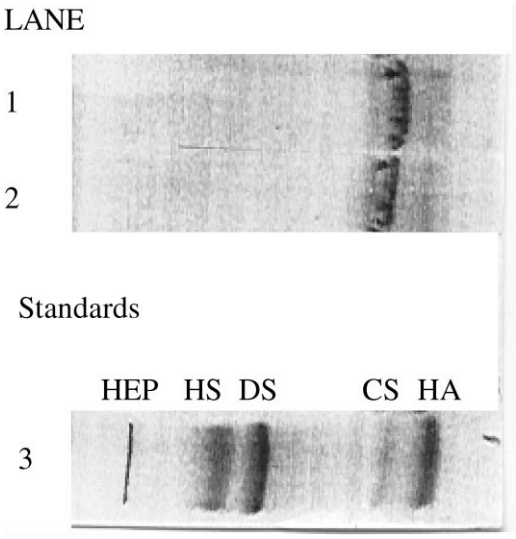


Fig. 2. Cellulose acetate electrophoresis of GAGs from squid skin (Lane 1), squid head cartilage (Lane 2) and standard GAGs (Lane 3: HEP, heparin; HS, heparan sulphate; DS, dermatan sulphate; CS, chondroitin sulphate; HA, hyaluronic acid).

(4.6 mm × 25 cm) column, eluting with a linear gradient of 0–2 M NaCl in KH_2PO_4 buffer (0.01 M, pH 6.00) over 30 min.

3. Results and discussion

The GAGs from the skin and head cartilage of the squid *N. gouldi* were isolated by standard methods (Rodén et al., 1972).

An initial cellulose acetate electrophoresis (Cappelletti, Del Rosso & Chiarugi, 1979; Slim, Furneaux & Yorke, 1994) of both the head cartilage and skin GAGs showed a single major band comigrating roughly with bovine CSA and a very broad diffuse band comigrating with the hyaluronic standard (Fig. 2). The CS band was slightly denser for the head cartilage GAGs. This indicated the presence of an unsulphated species along with a more highly charged one.

The analytical anion exchange HPLC results indicated the same pattern (Fig. 3, Volpi, 1994a). Head cartilage GAGs showed a broad peak with a number of separate maxima eluting at low ionic strength, in the same region as hyaluronic acid, and another broad band eluting at higher ionic strength than bovine or shark CS. This again indicates the presence of an unsulphated species along with a more highly charged one. The skin GAGs showed a similar pattern of peaks at low ionic strength but there was no visible peak eluting at high ionic strength.

The disaccharide composition of the GAGs was determined by digestion with chondroitinase ABC, separation of the resulting unsaturated disaccharides by strong anion exchange HLPC and identification by comparison to standard compounds (Sugahara, Ohkita, Shibata, Yoshida & Ikegami, 1995; Turnbull, Lyon & Gallagher, 1995). The results are shown in Table 1. The percentage of the dry weight accounted for by all the observed disaccharides is relatively low as was also seen by Karamanos (1992) with CS from squid skin. Increasing the digestion time did not significantly increase the amount of dry weight accounted

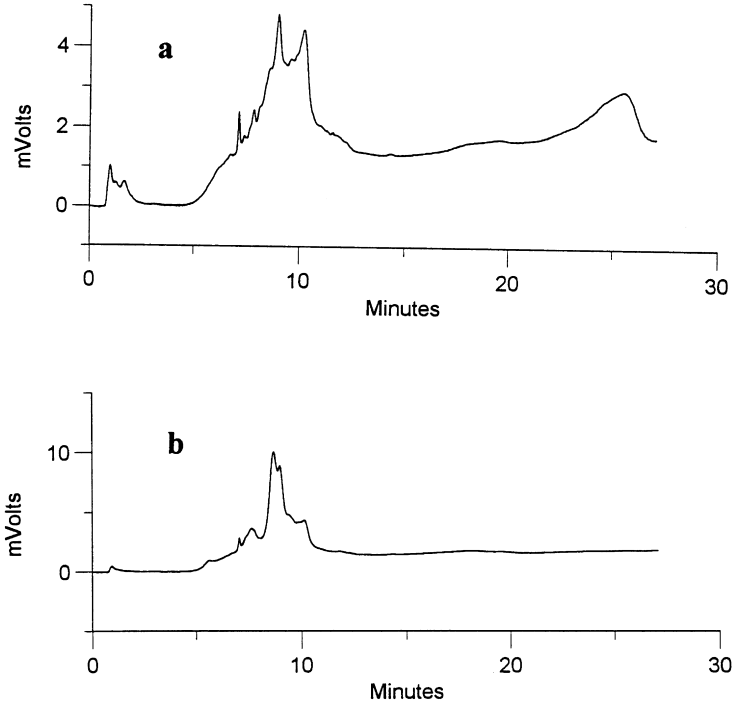


Fig. 3. Anion exchange HPLC of GAGs from: (a) squid head cartilage and (b) squid skin.

Table 1
Percentage disaccharide composition of squid head cartilage, squid skin, bovine and over-sulphated bovine GAGs. GAGs were digested with chondroitinase ABC for 4 h unless stated otherwise (n.d.: not detected)

Sample	ΔDi-0S	ΔDi-6S	ΔDi-4S	ΔDi-2,6S	ΔDi-4,6S	ΔDi-2,4S	ΔDi-triS	Dry weight accounted for (%)
Squid skin	88	6	5	1	1	n.d.	n.d.	48
Squid skin (20 h digestion)	84	7	7	1	1	n.d.	n.d	51
Squid head cartilage	19	16	27	1	36	1	n.d.	42
Bovine	n.d.	36	62	< 1	< 1	< 1	n.d.	99
Over-sulphated bovine								
7 equivalents	n.d.	35	6	n.d.	47	n.d.	12	58
15 equivalents	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0

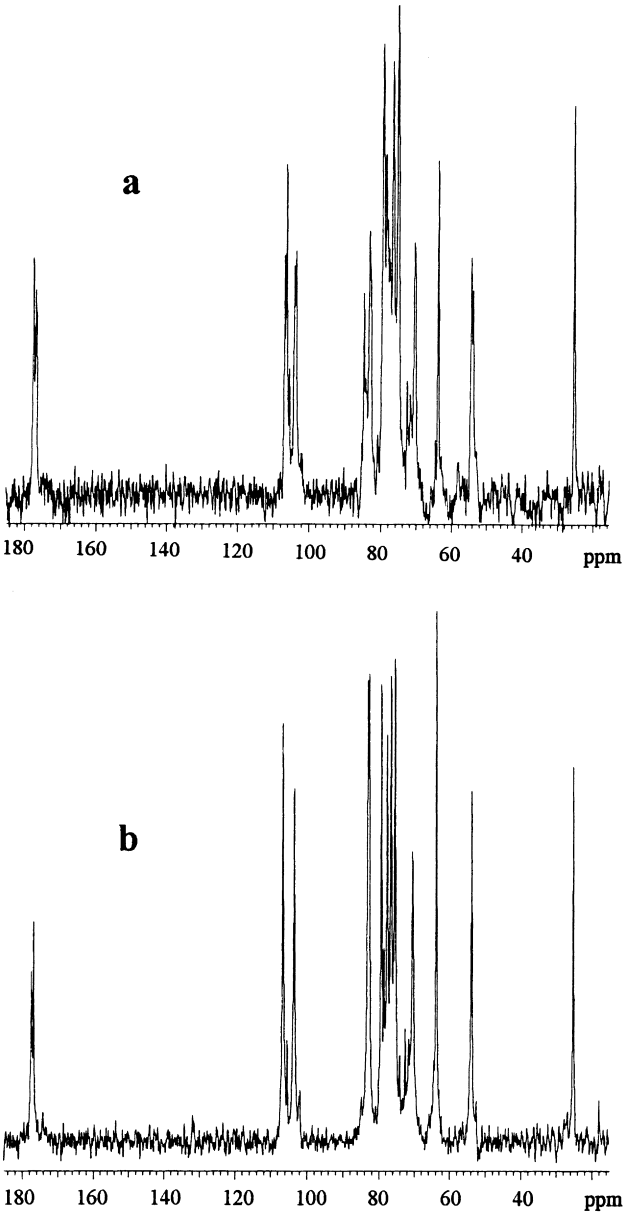


Fig. 4. ^{13}C NMR of GAGs from: (a) squid head cartilage and (b) squid skin.

for by the observed disaccharides. The limited digestion is probably due to the enzyme not being able to digest disaccharides substituted with neutral sugars (Habuchi et al., 1977; Tsegenidis, 1992). It may also have been due to the presence of large amounts of disaccharide units substituted on the 3-position of the uronic acid which are degraded by chondroitinase ABC but do not show up on the UV detector. However, there was no sign of significant amounts of this disaccharide in the NMR spectra (see below). The predominant disaccharide from skin CS was ΔDi-0S with only minor amounts of sulphated disaccharides. The disaccharides from head cartilage CS, on the contrary, were predominantly sulphated, with ΔDi-4,6S, which is traditionally associated with squid skin, being the most abundant, closely followed by ΔDi-4S and roughly equal amounts of ΔDi-6S and ΔDi-0S. There was also a small amount of the ΔDi-2,4S, but no trisulphated CS disaccharides were detected.

3.1. NMR analysis

Because of the poor recovery of disaccharides from enzyme digestion, ^{13}C NMR spectroscopy was used in an attempt to confirm the composition of the squid CS species. The ^{13}C NMR spectra are shown in Fig. 4. The spectrum of the skin CS (Fig. 4b) shows a clear set of signals for a single disaccharide repeating unit that was assigned as C-0S (Table 2) by analogy to the data for C-4S and C-6S. The assignment was confirmed by the assignment of the ^{1}H spectrum using a $^{13}C-^{1}H$ two-dimensional correlation experiment and comparison of the ^{1}H NMR data to that for the mono-sulphated chondroitins (Holme & Perlin, 1989; Ofman et al., 1997). There were also some smaller signals in the ^{13}C spectrum which could not be assigned to any known GAG structure.

The ^{13}C NMR spectrum of the head cartilage (Fig. 4a) was more complex. The signals for C-0S were assigned by comparison with the spectrum for the skin CS and all remaining signals could be assigned to C-4S or C-6S from literature data (Holme & Perlin, 1989; Ofman et al., 1997) (Table 2), except for two signals at 72.6 and 78.5 ppm. The absence of a recognisable set of signals for the C-4,6diS disulphate was a surprise. In an attempt to distinguish the signals for C-4,6diS from the un- and mono-sulphated disaccharide signals the disulphated material was synthesised.

Table 2
NMR data (in ppm) for C-0S from squid

	Squid	Skin	Head
	δ ^{13}C	δ ^{1}H	δ ^{13}C
Uronic acid			
1	106.8	4.47	106.2
2	75.5	3.37	75.2
3	76.7	3.56	76.6
4	82.8	3.78	82.7
5	79.4	3.69	79.4
6	176.9		176.9
Hexosamine			
1	103.6	4.58	103.6
2	53.9	4.00	53.8
3	84.9	3.78	84.7
4	70.6	4.12	70.5
5	77.7	3.69	77.7
6	63.8	3.73, 3.73	63.8
CH*3*	25.3	2.00	25.3
C=O	177.6		177.6

The tributylammonium salt of a 45:55 mixture of C-6S and C-4S derived from bovine tracheal cartilage (Ofman et al., 1997) was treated with 7 and 15 equivalents of pyridine sulphur trioxide complex per disaccharide unit in dry DMF at 0°C (Nagasawa et al., 1986). Disaccharide analysis (Table 1) showed that the material produced using 7 equivalents of sulphating reagent was principally a mixture of unreacted C-6S and C-4,6diS from sulphation of the free hydroxyl of the 4-sulphate. Unfortunately, the ^{13}C NMR spectrum of this mixture was indistinguishable from a mixture of C-4S and C-6S except for the loss of the signal for the free primary hydroxyl at 61 ppm. This was disappointing as it was expected that the close proximity of the two sulphate groups in C-4,6diS would have caused a change in conformation of the galactosamine ring giving significant shifts in the ^{13}C NMR spectrum. The overlap of signals for the sulphated 6-position and unsulphated 4-position in both ^{1}H and ^{13}C NMR makes the assignment particularly difficult even with a ^{13}C–^{1}H two-dimensional correlation experiment. Sulphation with 15 equivalents of the sulphating reagent produced material that was completely resistant to chondroitinase ABC digestion and with a complex ^{13}C NMR spectrum that could not be readily assigned.

3.2. Neutral sugar analysis

The samples of GAGs were analysed for neutral sugar using the traditional hydrolysis method of Harris et al. (1984). Fucose, arabinose, xylose, mannose, galactose and glucose were detected, and in addition a species identified as tetritol tetraacetate (characteristic fragments in the EIMS spectrum, *m/z* 103, 115, 128, 145, 217) was also observed. This may have arisen from the degradation of the acidic monosaccharides (Anderson & Cree, 1966; Hallen, Nasir-Ud-Din & Jeanloz, 1989). An alternative reductive hydrolysis procedure for neutral sugar content was therefore employed. This involves in situ reduction of monosaccharides with 4-methylmorphine borane as they are generated by acid hydrolysis (Stevenson & Furneaux, 1991). This technique stabilises the acid labile sugars by conversion to the corresponding alditols as they are produced. No tetritol tetraacetate was observed after hydrolysis by this method. Inositol was added at the reductive hydrolysis stage as an internal standard and used to calculate the percentage by weight of each neutral sugar in the total sample. The results are shown in Table 3. From the amount of xylose found in each sample, assuming that there is only one xylose per CS chain in the linkage region (Deutsch, Midura & Plaas, 1995), the average molecular weight of the squid skin CS is approximately 16 000 and the squid head CS is approximately 30 000. For each CS there is slightly more galactose than can be accounted for by the linkage region. Glucose is the predominant neutral sugar, as found previously for other squid-derived GAGs (Habuchi et al., 1977; Karamanos, 1992; Karamanos et al., 1986; Tsegenidis, 1992; Vynios & Tsiganos, 1990) with approximately one glucose for every six CS disaccharide units in the squid skin CS and one in seven in the head cartilage CS.

Size exclusion high performance chromatography (SE-HPLC, Fig. 5) using molecular weight standards derived from bovine CS by free radical degradation (Ofman et al., 1997) showed that the squid head cartilage contained a high molecular weight species eluting in the exclusion volume of the column (>45 000 molecular weight) and a low molecular weight species (MW < 3000). Both peaks were of similar intensity. The skin CS gave four broad overlapping peaks of approximately equal intensity centring on molecular weights of 45 000, 30 000, 7000 and <3000. The average molecular weights indicated by SE-HPLC agree roughly with those estimated from the xylose contents (see above), which shows that the CS has not been significantly degraded by the isolation procedure. Because the peaks were either in the exclusion volume of the column or overlapping, it was not possible to calculate molecular dispersities.

Table 3
Percentage composition of neutral sugars in GAGs from sqiud skin and head cartilage

	Neutral sugar (wt.%)					
	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose
Skin	2.3	0.3	0.8	0.4	3.2	5.8
Head cartilage	1.6	0.2	0.5	0.5	2.1	4.1

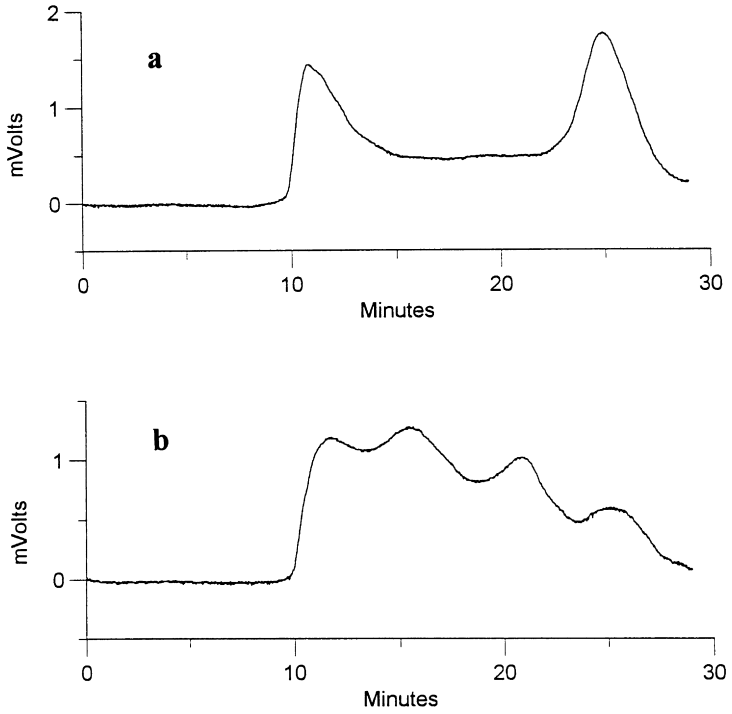


Fig. 5. SE-HPLC of GAGs from: (a) squid head cartilage and (b) squid skin.

4. Conclusion

The GAGs from both the tissues examined were shown to be chondroitin sulphate species. The skin consists principally of C-0S but with a relatively high level of glycosylation, particularly with glucose as has been observed before for squid CS (Karamanos et al., 1986). This high level of glycosylation makes it resistant to chondroitinase digestion. It has a relatively low molecular weight with a broad distribution. This is in contrast to CS isolated from other species of squid which have generally been C-4,6diS (Seldin et al., 1984) or high molecular weight C-0S (Karamanos, 1992; Karamanos et al., 1992). Overall, the CS from the head cartilage is more highly sulphated, consisting largely of disulphated C-4,6diS, with a higher molecular weight than the skin derived material but less highly glycosylated. From the electrophoresis and anion exchange HPLC data it is likely that the head cartilage has two separate species of CS, one consisting of unsulphated disaccharide repeat units, similar to that from the skin, and one which is predominantly CS E. The electrophoresis data also indicates that the skin contains small amounts of a CS with mono- and di-sulphated disaccharide units. Neither sample showed any trisulphated disaccharide units.

The ^{13}C NMR spectra of the squid head GAGs and material produced by chemical sulphation of bovine derived CSA shows that this technique cannot be readily used to distinguish between a mixture of monosulphates C-4S and C-6S and the disulphate C-4,6S.

Acknowledgements

This work was funded by the New Zealand Foundation for Science Research and Technology, contract numbers C08603 and NZP501. Thanks are due to Dr H. Wong for NMR spectra.

References

Anderson, D. M. W., & Cree, G. M. (1966). Studies on uronic acid materials. Part XIV. Methylation with the sodium hydride–methyl iodide dimethyl sulphoxide system. *Carbohydrate Research*, *2*, 162–166.

Cappelletti, R., Del Rosso, M., & Chiarugi, V. P. (1979). A new electrophoretic method for the complete separation of all known animal glycosaminoglycans in a monodimensional run. *Analytical Biochemistry*, *99*, 311–315.

Dace, R., McBride, E., Brooks, K., Gander, J., Buszko, M., & Doctor, V. M. (1997). Comparison of the anticoagulant action of sulfated and phosphorylated polysaccharides. *Thrombosis Research*, *87*, 113–121.

Deutsch, A. J., Midura, R. J., & Plaas, A. H. K. (1995). Structure of chondroitin sulfate on aggrecan isolated from bovine tibial and costochondral growth plates. *Journal of Orthopaedics Research*, *13*, 230–239.

Fransson, L. A. (1985). Mammalian glycosaminoglycans. In G. O. Aspinall (Ed.), *The polysaccharides*, (pp. 337–415). Orlando: Academic Press.

Habuchi, O., Sugiura, K., & Kawai, N. (1977). Glucose branches in chondroitin sulphates from squid cartilage. *Journal of Biological Chemistry*, *252*, 4570–4576.

Hallen, A., Nasir-Ud-Din, A., & Jeanloz, R. W. (1989). Chemical structure of shark cartilage chondroitin sulphate. *Journal of the Chemical Society of Pakistan*, *11*, 168–177.

Harris, P. J., Henry, R. J., Blakeney, A. B., & Stone, B. A. (1984). An improved method for the methylation analysis of oligosaccharides and polysaccharides. *Carbohydrate Research*, *127*, 59–73.

Holme, K. R., & Perlin, A. S. (1989). Nuclear magnetic resonance spectra of heparin in admixture with dermatan sulphate and other glycosaminoglycans. 2-D spectra of the chondroitin sulphates. *Carbohydrate Research*, *186*, 301–312.

Karamanos, N. K. (1992). Two squid skin proteoglycans each containing chondroitin sulfates with different sulfation patterns. *Biochemistry and Cell Biology*, *70*, 629–635.

Karamanos, N. K., Tsegenidis, T., & Antonopoulos, C. A. (1986). Study of the glycosaminoglycans from squid skin. *Comparative Biochemistry and Physiology B–Comparative Biochemistry*, *85*, 865–868.

Karamanos, N. K., Manouras, A., Tsegenidis, T., & Antonopoulos, C. A. (1991). Isolation and chemical study of the glycosaminoglycans from squid cornea. *International Journal of Biochemistry*, *23*, 67–72.

Karamanos, N. K., Aletras, A. J., Tsegenidis, T., Tsiganos, C. P., & Antonopoulos, C. A. (1992). Isolation characterization and properties of the oversulphated chondroitin sulphate proteoglycan from squid skin with peculiar glycosaminoglycan sulphation pattern. *European Journal of Biochemistry*, *204*, 553–560.

Karamanos, N. K., Hjerpe, A., Aletras, A., Tsegenidis, T., Anastassiou, E. D., & Antonopoulos, C. A. (1995). Antibodies to three chondroitin sulfate-containing proteoglycans in squid skin recognize hexa- or longer chondroitin oligosaccharides as major antigenic determinants. *Archives of Biochemistry and Biophysics*, *316*, 100–109.

Kariya, Y., Watabe, S., Hashimoto, K., & Yoshida, K. (1990). Occurrence of chondroitin sulphate E in glycosaminoglycan isolated from body wall of sea cucumber *Stichopus japonicus*. *Journal of Biological Chemistry*, *265*, 5081–5085.

Kinoshita, A., Yamada, S., Haslam, S. M., Morris, H. R., Dell, A., & Sugahara, K. (1997). Novel tetrasaccharides isolated from squid cartilage chondroitin sulfate E contain unusual sulfated disaccharide units GlcA(3-*O*-sulfate)β1-3GalNAc(6-*O*-sulfate) or GlcA(3-*O*-sulfate)β1-3GalNAc(4,6-*O*-disulfate). *Journal of Biological Chemistry*, *272*, 6–19 665.

Kitagawa, H., Tanaka, Y., Tsuchida, K., Goto, F., Ogawa, T., Lidholt, K., Lindahl, U., & Sugahara, K. (1995). *N*-acetylgalactosamine (GalNAc) transfer to the common carbohydrate–protein linkage region of sulfated glycosaminoglycans—identification of UDP-GalNAc:chondro-oligosaccharide a-*N*-acetylgalactosaminyltransferase in fetal bovine serum. *Journal of Biological Chemistry*, *270*, 22 190–22 195.

Kitagawa, H., Tanaka, Y., Yamada, S., Seno, N., Haslam, S. M., Morris, H. R., Dell, A., & Sugahara, K. (1997). A novel pentasaccharide sequence GlcA(3-sulfate)(β1-3)GalNAc(4-sulfate)(β1-4)(Fucα1-3)-GlcA(β1-3)GalNAc(4-sulfate) in the oligosaccharides isolated from King Crab cartilage chondroitin sulfate K and its differential susceptibility to chondroitinases and hyaluronidase. *Biochemistry*, *36*, 3998–4008.

Nagasawa, K., Uchiyama, H., & Wajima, N. (1986). Chemical sulphation of preparations of chondroitin 4-and 6-sulphate, and dermatan sulphate, preparation of chondroitin sulphate E-like materials from chondroitin 4-sulphate. *Carbohydrate Research*, *158*, 183–190.

Ofman, D., Slim, G. C., Watt, D. K., & Yorke, S. C. (1997). Free radical induced oxidative depolymerisation of chondroitin sulphate and dermatan sulphate. *Carbohydrate Polymers*, *33*, 47–56.

Rodén, L., Baker, J. R., Cifonelli, J. A., & Mathews, M. B. (1972). Isolation and characterisation of connective tissue polysaccharides. *Methods in Enzymology*, *28*, 73–140.

Seldin, D. C., Seno, N., Austen, K. F., & Stevens, R. L. (1984). Analysis of polysulfated chondroitin disaccharides by high-performance liquid chromatography. *Analytical Biochemistry*, *141*, 291–300.

Slim, G. C., Furneaux, R. H., & Yorke, S. C. (1994). A procedure for the analysis of glycosaminoglycan mixtures based on digestion by specific enzymes. *Carbohydrate Research*, *255*, 285–293.

Stevenson, T. T., & Furneaux, R. H. (1991). Chemical methods for the analysis of sulphated galactans from red algae. *Carbohydrate Research*, *210*, 277–298.

Sugahara, K., Shigeno, K., Masuda, M., Fujii, N., Kurosaka, A., & Takeda, K. (1994). Structural studies on the chondroitinase ABC-resistant sulfated tetrasaccharides isolated from various chondroitin sulfate isomers. *Carbohydrate Research*, *255*, 145–163.

Sugahara, K., Ohkita, Y., Shibata, Y., Yoshida, K., & Ikegami, A. (1995). Structural studies on the hexasaccharide alditols isolated from the carbohydrate–protein linkage region of dermatan sulfate proteoglycans of bovine aorta. Demonstration of iduronic acid-containing components. *Journal of Biological Chemistry*, *270*, 7204–7212.

Sugahara, K., Tanaka, Y., Yamada, S., Seno, N., Kitagawa, H., Haslam, S. M., Morris, H. R., & Dell, A. (1996). Novel sulfated oligosaccharides containing 3-*O*-sulfated glucuronic acid from king crab cartilage chondroitin sulfate K—unexpected degradation by chondroitinase ABC. *Journal of Biological Chemistry*, *271*, 26 745–26 754.

Takaya, Y., Uchisawa, H., Hanamatsu, K., Narumi, F., Okuzaki, B., & Matsue, H. (1994). Novel fucose-rich glycosaminoglycans from squid ink bearing repeating unit of trisaccharide structure (-6GalNAc-α-1-3GlcA-β-1-3Fuc-α-1-)-n. *Biochemical and Biophysical Research Communications*, *198*, 560–567.

Takaya, Y., Uchisawa, H., Narumi, F., & Matsue, H. (1996). Illexins A, B, and C from squid ink should have a branched structure. *Biochimica Biophysica Research Communications*, *226*, 335–338.

Tsegenidis, T. (1992). Influence of oversulphation and neutral sugar presence on the chondroitinases ac and abc actions towards glycosaminoglycans from ray raja-clavata and squid illex-illecebrosus-coidentii skin. *Comparative Biochemistry and Physiology B–Comparative Biochemistry*, *103*, 275–279.

Turnbull, J. E., Lyon, M., & Gallagher, J. T. (1995). Structural analysis of glycosaminoglycans. In M. A. Haralson & J. R. Hassell (Eds.), *Extracellular matrix macromolecules: a practical approach*, (pp. 199–220). Oxford: Oxford University Press.

Volpi, N. (1994). Structural analysis and physico-chemical properties of charge-fractionated dermatan sulphate samples. *Carbohydrate Research*, *260*, 159–167.

Volpi, N. (1994). Dermatan sulphate from beef mucosa: structure, physicochemical and biological properties of fractions prepared by chemical depolymerisation and anion exchange chromatography. *Carbohydrate Research*, *255*, 133–144.

Vynios, D. H., & Tsiganos, C. P. (1990). Squid proteoglycans: isolation and characterisation of three populations from cranial cartilage. *Biochimica Biophysica Acta*, *1033*, 139–147.