



# Isolation and Characterization of Glycosaminoglycans from Squid (*Loligo duvauceli*) and Cuttlefish (*Sepia pharaonis*)

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## Abstract

Glycosaminoglycans (GAGs) have wide applications in biomedical, pharmaceutical and cosmetic field. The present study was aimed at isolation and characterization of glycosaminoglycans from selected tissues of two important species of cephalopods, *Loligo duvauceli* and *Sepia pharaonis*. The isolation protocol developed was based on the stability of the GAGs at temperatures beyond ambient conditions, as well as their property as water soluble hetero-polysaccharides. Among all the tissues analyzed, the cranial cartilage of both the cephalopod species contained glycosaminoglycans with a yield of  $\sim 80 \text{ g kg}^{-1}$  dry defatted tissue. The FT-IR peak intensities confirmed that the predominant GAGs of both the cephalopod species studied were chondroitin sulphate type, with variations in their sulphation pattern. The squid and cuttlefish cranial cartilages hitherto considered as processing discards, have great potential as a source of chondroitin sulphate – GAGs.

**Keywords:** Glycosaminoglycans, FT-IR, cranial cartilage, chondroitin sulphate, *Loligo duvauceli*, *Sepia pharaonis*

## Introduction

Glycosaminoglycans (GAGs) are a group of complex heteropolysaccharides with wide therapeutic potential and is of topical. The GAGs such as hyaluronic acid, heparin and chondroitin sulphate (CS) have several applications in the medical, veterinary,

pharmaceutical and cosmetic fields. Heparin has been widely used as an injectable anticoagulant for over half a century and is also used as an inner anticoagulant surface on various experimental and medical devices. The main applications of CS and hyaluronic acid is in the treatment of osteoarthritis and are therefore regarded as nutraceuticals, in addition to their use as viscosupplements, antiviral, anti-infective and anti-inflammatory agents (Brito et al., 2008; Wang et al., 2012., Severin et al., 2012). Hyaluronan and chondroitin sulphate are found to be suitable for tissue engineering and regenerative medicine. Both these polymers are gaining popularity as a biomaterial scaffold which can significantly be improved with crosslinking of collagen, gelatin, chitosan etc producing a hydrogel, for wound healing applications.

Marine organisms are a rich source of a variety of structurally novel and biologically active metabolites (Ely et al., 2004) and own pronounced pharmacological activities and other properties useful in biomedical area. Some of these pharmacological activities are attributed to the presence of sulphated polysaccharides, particularly the glycosaminoglycans (Hovingh & Linker, 1993; Kim et al., 1996; Ulrich & Boon, 2001; Santos et al., 2002; Cesaretti et al., 2004; Pandian & Thirugnanasambandan, 2008; Saravanan & Shanmugam, 2010; Gomez et al., 2010). The structure and organization of glycosaminoglycans and proteoglycans from tissues of several vertebrates have been extensively studied. However, detailed studies on glycosaminoglycans from tissues of invertebrates are meager. This study attempts to isolate and characterize GAGs from selected tissues of squid (*Loligo duvauceli*) and cuttlefish (*Sepia pharaonis*) generated as waste during processing with a view to develop commercial products.

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## Materials and Methods

Chondroitin sulphate A (C9819, molecular weight (MW) 20-30 kD, 70% pure, 30% CS C, 15.1% sulphate), chondroitin sulphate B (C3788, MW 40-45 kD, 90% pure, 18.5% sulphate), chondroitin sulphate C (C4384, MW 50-58.8 kD, 90% pure, 18.0% sulphate) and hyaluronic acid (H1504) were obtained from Sigma-Aldrich, Saint Louis, USA. Papain, 2 x crystalline (EC 3.4.22.2) and CTAB (N-cetyl-N,N,N-trimethylammonium bromide) were from E Merck, A.G, (Darmstadt, Germany). Toluidine blue was from Fisher Scientific (USA). 1, 9 - dimethyl methylene blue (DMMB) chloride (catalogue No 03610), was from Polysciences Inc, Warrington, PA. All other chemicals used were of analytical grade. The cephalopod samples used for the study were obtained from the last haul of trawl catch landings of Vypeen Harbour, Cochin, India. Samples used for the study include mantle, tentacles, and fin that represented the edible tissues of squid and cuttlefish and skin, pen/cuttle bone, and cranial cartilage that represented the non-edible portions from squid and cuttlefish.

Isolation of the glycosaminoglycans was accomplished by a three step process involving the preparation of acetone dry powder (ADP), digestion with an exogenous proteinase (papain) and the final precipitation of the glycosaminoglycans using an azeotropic mixture of 95% ethanol in water.

ADP of the sample tissues was prepared by the method of Mathew et al. (1982) by delipidation of the homogenized samples in chilled acetone 1:5 w/v at 0°C for 72 h with change of acetone every 12 h. The samples were then extracted using diethyl ether and stored at room temperature (RT). The acetone dry powder (ADP) thus prepared were digested with papain (20 U g<sup>-1</sup> dry weight) in 0.2 M sodium acetate buffer, pH 7 containing 2mg/ml cysteine - HCl at 65°C for 72 h by the method of Roden et al. (1972). Fresh papain was added every 24 h. Appropriate controls (without any tissue) were also set.

The samples after papain digestion were subjected to centrifugation (5000 x g) for 5 min at room temperature and the clear supernatants were precipitated with 4 to 5 volumes of 95 % (v/v) ethanol at -10°C for 24 h (Albano & Maurao, 1986). The crude precipitates containing a mixture of GAGs and papain were collected by centrifugation (5000 x g) for 15 min at RT. The above residues were then dissolved in deionized MilliQ water and the

supernatants containing GAGs alone were collected by centrifugation (10000 x g) at room temperature for 10 min and finally lyophilized in a freeze dryer (Lyolab Vacuum DHPG -222, TUTC 4896).

Chemical composition analysis of the isolated glycans were performed for the content of hexuronic acid (Dische, 1947), hexosamines (Rondle & Morgan, 1955), fucose (Dische & Shettles, 1948), sialic acid (Aminoff, 1961) and sulphated GAG (Barbosa et al., 2003). The analysis of residual protein (Lowry et al., 1951) and hydroxyproline (Woessner, 1961) were determined to assess the purity of the GAG isolates. All OD (optical density) measurements were taken using a Hitachi (Model No U 2800) UV/VIS spectrophotometer.

The purity of the glycosaminoglycans was analyzed using horizontal agarose gel electrophoresis by the method of Bjornsson (1993), with modifications. The crude GAG precipitates were electrophoresed on 2.0% agarose gels using 10 mM Tris/acetate buffer, pH 8.3 at 110V for 3 hours. After electrophoresis the macromolecules were fixed with 0.1% Cetavlon (CTAB) according to the method of Pavao et al. (1995), and stained with 0.02% (w/v) toluidine blue in 3% acetic acid containing 0.5% (v/v) triton x 100 and destained using 3% acetic acid.

FT-IR spectroscopy (450 to 4000 cm<sup>-1</sup>) of purified GAG samples were performed by attenuated total reflectance (ATR) spectroscopy on a Thermo Nicolet, Avatar 370 instrument according to the method of Toida et al. (1999).

One-way analysis of variance (ANOVA) and post-hoc multiple comparison analysis was carried using Tukey's HSD test. 'p' values less than or equal to 0.05 were considered statistically significant. Data are presented as mean±standard deviation (SD).

## Results and Discussion

The percentage of yield crude GAGs isolated from the acetone dry powder of the sample tissues are shown in Fig. 1. The yield of crude GAGs in the respective tissues of squid and cuttlefish were in the order, cranial cartilage > fin > mantle > skin > tentacle and gladius/pen (Table 1). The liberation of intact GAGs from the acetone dry powder of tissues was done by papain digestion at 65°C. The extraction of intact GAGs from the connective tissues at elevated temperatures were possible owing to the stability of GAGs at temperatures

beyond ambient conditions and their property as water soluble heteropolysaccharides. An azeotropic mixture of ethanol in water (95:5) has a boiling point of 78.15°C and assist in the non – destructive precipitation of GAGs.

The chemical composition analyses of the crude GAGs indicated that polyanionic glycans are widely distributed in both the species (Tables 2 and 3). The traces of hexosamines ( $0.46 \pm 0.01$  and  $0.18 \pm 0.01$  mg g<sup>-1</sup>) in the gladius/pen of squid and cuttlefish respectively can be attributed to the amino sugars in the chitin residues that might have precipitated during extraction. The presence of fucose positive results ( $0.36 \pm 0.01$  µg g<sup>-1</sup>) in GAG isolates of the cranial cartilage of squid requires further investigation. Fucose containing GAGs in invertebrates have been reported by several authors (Kariya et al., 1990; Vieira et al., 1991; Kariya et al., 1997). Evidence for the presence of neutral monosaccharides have also been found as branches in oversulphated chondroitin sulphate E from squid (*Illex illecebrosus coidentii*) cranial cartilage (Habuchi et al., 1977; Vynios & Tsiganos, 1990), in chondroitin sulphate from sea cucumber (Vieira et al., 1991) and in chondroitin from squid skin (Karamanos et al.,

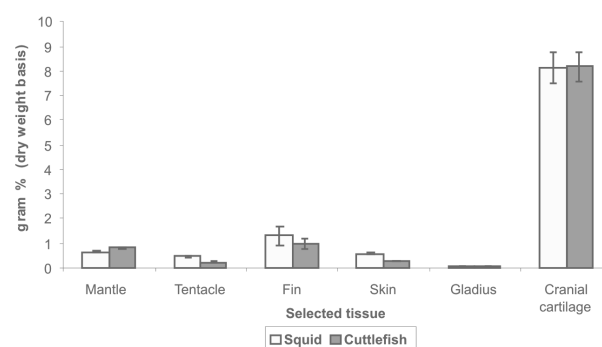


Fig. 1. Histogram showing the percentage yield of glycosaminoglycans obtained from the different tissues of squid and cuttlefish.

1990). Unlike invertebrates, neutral monosaccharides residues have not been found in galactosaminoglycans from mammalian tissues (Karamanos et al., 1992). None of the GAG isolates contained sialic acid in contrast to those from vertebrates as reported by Karamanos et al. (1990). Hydroxyproline and protein were not detected in any of the samples indicating that the GAG preparations were free of collagen or any other residual protein.

Table 1: Yield (gram %) of acetone dry powder (ADP) from tissues of squid and cuttlefish

|            | Edible Tissue           |                          |                          | Non Edible Tissue        |                         |                         |
|------------|-------------------------|--------------------------|--------------------------|--------------------------|-------------------------|-------------------------|
|            | Mantle                  | Tentacle                 | Fin                      | Skin                     | Gladius/<br>Pen*        | Cranial<br>cartilage    |
| Squid      | 17.54±0.92 <sup>b</sup> | 17.63±0.79 <sup>b</sup>  | 16.47±0.96 <sup>b</sup>  | 16.08±1.39 <sup>b</sup>  | 82.45±2.53 <sup>a</sup> | 14.82±0.55 <sup>b</sup> |
| Cuttlefish | 20.78±1.57 <sup>b</sup> | 20.21±0.71 <sup>bc</sup> | 17.56±1.22 <sup>cd</sup> | 19.28±0.62 <sup>bc</sup> | 76.95±2.43 <sup>a</sup> | 15.64±0.42 <sup>d</sup> |

All values expressed as mean + standard deviation, n = 4. Different superscripts in the same row indicate significant differences (p<0.05).

\* Air dried samples

Table 2: Composition of the GAG isolates in the edible and non-edible tissues of squid.

| Chemical composition analysis of squid crude GAGs (mg g <sup>-1</sup> dry tissue) |               |           |            |                   |                  |                      |
|-----------------------------------------------------------------------------------|---------------|-----------|------------|-------------------|------------------|----------------------|
| Sample tissue                                                                     | Edible Tissue |           |            | Non Edible Tissue |                  |                      |
|                                                                                   | Mantle        | Tentacle  | Fin        | Skin              | Gladius/<br>Pen* | Cranial<br>cartilage |
| Hexosamines                                                                       | 3.25±0.65     | 3.5±0.11  | 6.59±0.67  | 3.09±0.87         | 0.46±0.01        | 41.8±0.55            |
| Uronic acid                                                                       | 4.27±0.55     | 1.98±0.28 | 8.42±1.03  | 3.22±0.98         | 0.018±0.00       | 38.03±0.92           |
| Sulphated GAG                                                                     | 7.65±0.24     | 4.23±0.07 | 12.73±0.52 | 5.28±0.02         | ND               | 78.15±0.62           |

ND: not detected

Table 3: Composition of the GAG isolates in the edible and non-edible tissues of cuttlefish.

| Sample tissue | Chemical composition analysis of cuttlefish crude GAGs (mg/g dry tissue) |           |           |                   |                  |                      |
|---------------|--------------------------------------------------------------------------|-----------|-----------|-------------------|------------------|----------------------|
|               | Edible Tissue                                                            |           |           | Non Edible Tissue |                  |                      |
|               | Mantle                                                                   | Tentacle  | Fin       | Skin              | Gladius/<br>Pen* | Cranial<br>cartilage |
| Hexosamines   | 4.11±0.88                                                                | 0.79±0.11 | 3.88±0.94 | 0.84±0.15         | 0.18±0.01        | 36.8±0.45            |
| Uronic acid   | 2.98±0.35                                                                | 1.02±0.66 | 5.21±0.39 | 0.59±0.008        | 0.042±0.00       | 32.08±0.95           |
| Sulphated GAG | 6.11±0.98                                                                | 1.91±0.01 | 8.49±1.21 | 1.47±0.09         | ND               | 69.19±1.36           |

ND: not detected

The present study reveals that among all the tissues analyzed, the cranial cartilage of both the cephalopod species contained appreciable amount ( $\sim 80 \text{ g kg}^{-1}$  dry defatted tissue) of GAGs and the results are comparable with other sources as well (Rodrigues et al., 2005; Garnjanagoonchorn et al., 2007). The GAG extraction protocol and the analytical techniques employed in this study can be a reliable method for the extraction and identification of GAGs from other potential sources as well.

Techniques employing horizontal agarose gel electrophoresis for the characterization of acidic polysaccharides were described by many authors (Bjornsson, 1993; Pavao et al., 1998; Theocharis et al., 1999; Pandian & Thirugnanasambandan, 2008). Plates 1 and 2 shows the toluidine blue stained agarose gel pattern of the GAGs extracted from the tissues of squid and cuttlefish respectively. The relative molecular weight of the standard chondroitin sulphate A and chondroitin sulphate C were 40,000 Da and 60,000 Da respectively. Agarose gel electrophoresis of the glycans revealed the presence of GAGs having higher molecular weight than that of standard GAGs. Thus the difference in electrophoretic mobility of the various GAGs is a first indication of distinctive structure of these polysaccharides.

The IR spectra represents a quick and simple method of obtaining information on the composition of the GAGs isolated. Among all the tissues analyzed the results of IR spectra of the cranial cartilage GAG isolates of both the squid and cuttlefish (Fig. 2 and 3) showed closer peak intensities of similar configuration to the chondroitin sulphate standard (Fig. 4), than that of heparin standard (Fig. 5). The IR spectra of the all the GAG samples were characterized by a broad band above  $3000 \text{ cm}^{-1}$  and intense absorption

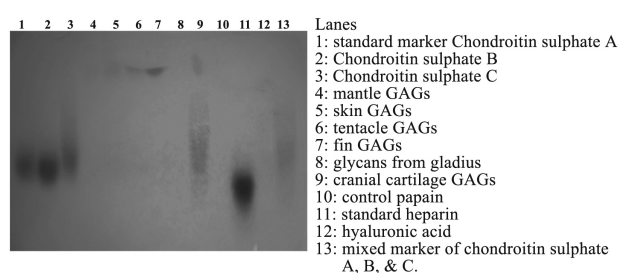


Plate 1: Agarose gel electrophoretic pattern of the glycosaminoglycans isolated from selected tissues of squid.

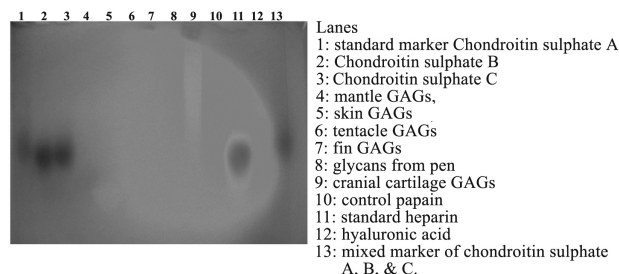


Plate 2: Agarose gel electrophoretic pattern of the glycosaminoglycans isolated from selected tissues of cuttlefish.

around  $1650$  and  $1050 \text{ cm}^{-1}$ . In the case of standard chondroitin sulphate C, the stretching and deformation vibrations of  $\text{-C-O-H-}$  bands at  $1637.63 \text{ cm}^{-1}$  and  $1420.03 \text{ cm}^{-1}$  corresponds to the presence of combined carboxylate with amine and sulphate. The peak intensities at  $1629.80 \text{ cm}^{-1}$  and  $1411.75 \text{ cm}^{-1}$  in the cranial cartilage GAG isolates of squid as well as prominent peaks at  $1643.77 \text{ cm}^{-1}$  and  $1405.30 \text{ cm}^{-1}$  in the case of cuttlefish GAGs corresponds to the of  $\text{-C-O-H-}$  bands indicating the presence of combined carboxylate with amine and sulphate. The broad band above  $3000 \text{ cm}^{-1}$  was assigned to hydroxyl-stretching vibrations of polysaccharide and water involved in hydrogen bonding. The sulphate band in the case of standard chondroitin sulphate (Fig. 4)



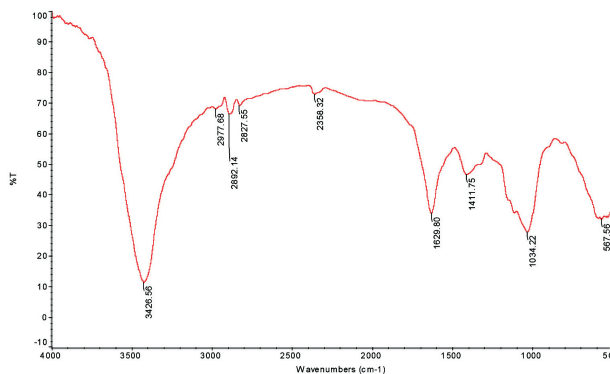


Fig. 2. FT-IR spectra of GAG isolates from squid head cartilage

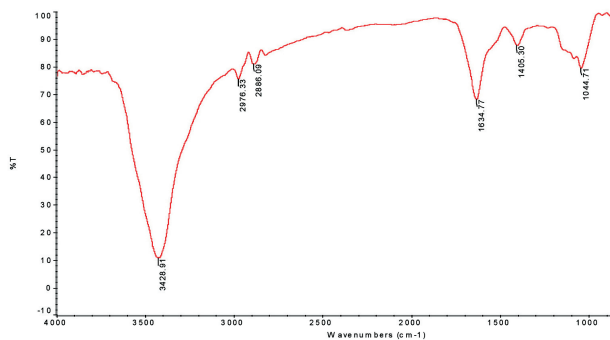


Fig. 3. FT-IR spectra of GAG isolates from cuttlefish head cartilage

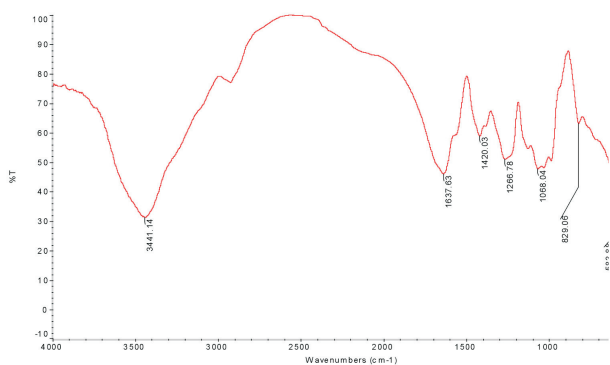


Fig. 4. FT-IR spectra of standard Chondroitin sulphate C

started from 1266.78  $\text{cm}^{-1}$  and down to 829.06  $\text{cm}^{-1}$ . Similar peak intensities were found at the 1034.22  $\text{cm}^{-1}$  and at 1044.71  $\text{cm}^{-1}$  in the cranial cartilage GAGs of squid and cuttlefish respectively, indicating heavily sulphated groups. FT-IR spectral studies of chondroitin sulphate glycosaminoglycans conducted by several workers reveal peak intensities characteristic of hydroxyl-stretching, combined carboxylate amine

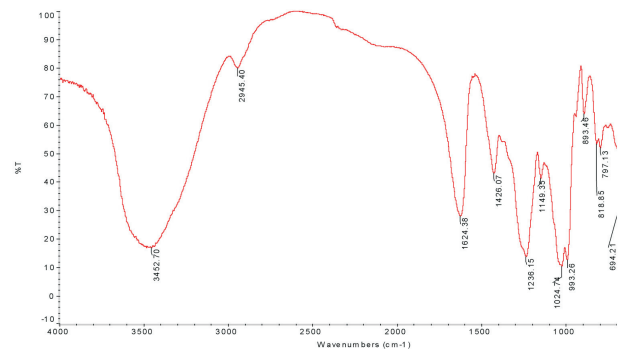


Fig. 5. FT-IR spectra of standard Heparin

and sulphate, compared to the characteristic peak C-O-S compared to that of sample peak intensities at 1629.80  $\text{cm}^{-1}$  and 1411.75  $\text{cm}^{-1}$  for squid and in the range 1634.77  $\text{cm}^{-1}$  and 1405.30  $\text{cm}^{-1}$  for cuttlefish (Wang et al., 2007). A similar FT-IR spectrum was also recorded in chondroitin sulphate from chicken keel cartilage as compared to that of standard chondroitin sulphate C observed at 856  $\text{cm}^{-1}$ , with characteristic sulphation patterns (Khan et al., 2013). The IR spectral data confirms that the prominent GAG types in the cranial cartilages of both cephalopods are of the chondroitin sulphate type. Because no absorption band at 1540  $\text{cm}^{-1}$  was detected in the IR spectrum, it was confirmed that neither the purified GAGs from the cranial cartilages of squid and cuttlefish nor the GAGs from all other representative tissues (data not shown) in both the species, contained residual protein contaminants, highlighting the suitability of the extraction protocol employed.

In conclusion, the results of the present study indicate that the cranial cartilage GAGs of both the cephalopod species are of chondroitin sulphate type. The specific modification in the sulphation pattern and the possibility of the presence of neutral monosaccharides or fucosylated derivatives requires further investigation. Most of the commercially available GAGs have so far been extracted from higher vertebrates, which often points to the recent health concern issues on interspecies viral infections as well as some lower vertebrates *viz.*, sharks, rays etc that are potentially endangered. An important waste reduction strategy for the processing industry is the recovery of marketable by-products from processing discards. Since marine natural products continue to be viewed as one of the few *de novo* sources of bioactive substances, the utilization of

cephalopod processing discards for the extraction of GAGs will be quite encouraging so as to meet demands without over exploitation of other endangered resources.

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