



## RESEARCH ARTICLE

### Extraction and Biochemical Characterization of Sulphated Glycosaminoglycans from Chicken Keel Cartilage

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#### ABSTRACT

The present study was conducted to explore the potential and cheaper source of major and abundantly found sulphated glycosaminoglycans (GAGs) in chicken keel cartilage. Chicken is comparatively readily accessible to all the communities of Pakistan and its cartilages are the rich source of sulphated GAGs. The GAGs were extracted from prewashed and ground keel cartilages (n=3) of chicken using 3 M MgCl<sub>2</sub>, dialyzed, digested with papain, precipitated with three volumes of ethanol, and finally lyophilized to dry powder. The dry products were used for proximate analysis (carbohydrates 65.49±0.10, crude protein 12.82±0.26, ash 11.12±.56, moisture 9.88±0.32 and fat 0.69±0.14%). Dimethylmethylene blue binding (DMMB) assay was performed to determine the quantity of total GAGs in each group of product and protein contents were estimated by Bradford method. Identification of extracted samples of GAGs was performed with FTIR spectrometer using KBr disc and purity of the samples was determined by SDS-PAGE. Quantity of total GAGs in extracted samples was 70.77±2.27% and estimated amount of protein was 4.64±0.29%. FTIR spectra of standard and samples of CS showed identical and characteristic peaks in finger print region. Finger print region revealed the presence of C-O-S, S=O, -COO, -C-C, R-SO<sub>2</sub>-R, -CONH<sub>2</sub> and R-SO<sub>2</sub>-NH<sub>2</sub> molecules. SDS-PAGE analysis revealed the presence of 77.8 and 50.5 kDa proteins in all extracted samples of GAGs. It can be concluded that chicken keel cartilage is the potential and cheap source of GAGs. Analysis by SDS-PAGE revealed that most of the non-collagen protein can be removed by three volumes of solvent extraction and FTIR is an advance technique for identification of GAGs in mid infrared region (400-4000 cm<sup>-1</sup>).

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#### INTRODUCTION

Sulphated glycosaminoglycans are major components in various mammalian organs such as cartilages, blood vessels, cell surfaces, intracellular granules and plasma (Hochberg *et al.*, 2000). Clinically, it is used as a chondro-protective drug in all types of arthritis in human or companion animals. The GAGs have ability to absorb large amount of water due to its negative charge and thus produce gel like matrix. This matrix produces slippery effect in joints, rebuilds cartilage due to mechanical and pathological breakdown and facilitates the mobility (Haq *et al.*, 2005). In addition, this property contributes to the

resilience of synovial fluid of joints and vitreous humor of the eye (Cooper and Coggon, 1999). Various sources of GAGs particularly chondroitin sulphate include whale cartilage, porcine skin, shark cartilage, shark fin cartilage, and squid cartilage (Mizumoto *et al.*, 2012). Other sources which had been explored are muscle tissue in Atlantic cod and spotted wolffish (Tingbø *et al.*, 2005). Many methods are routinely used for extraction and segregation of GAGs (Ben Mansour *et al.*, 2009; Yanagishita *et al.*, 2009). Several clinical studies have demonstrated favorable therapeutic effects of GAGs in treatment of osteoarthritis (Neil *et al.*, 2005). GAGs contents decrease with aging/disease condition particularly in hyaline cartilage.

GAGs as therapeutic agents reduce radiographic progression in juvenile idiopathic arthritis and is considered as surrogate marker for cartilage loss (Nielsen *et al.*, 2008). Several techniques are being employed for qualitative and quantitative analysis of GAGs. In the present study chicken keel cartilages were treated with  $MgCl_2$ , dialyzed, digested with papain, precipitated with ethanol and finally lyophilized to dry powder. The dry product was weighed and total GAGs contents in each sample were determined by DMMB assay and protein contents were determined by Bradford method. Fourier transform infrared spectroscopy (FTIR) was performed to identify the GAGs in extracted semi-purified samples on mid IR region ( $400-4000\text{ cm}^{-1}$ ) while SDS-PAGE was performed to assess purity of the semi-purified samples (Sharma *et al.*, 2007).

## MATERIALS AND METHODS

**Extraction and semi-purification:** Chicken keel cartilages (100g each) were collected from retail broiler meat shops, rinsed with fresh water and boiled in hot water at  $90-95^\circ\text{C}$  for five minutes. GAGs were extracted by modified method as described by Luo *et al.* (2002). Chicken cartilages (10g) were chopped, ground in blender, suspended in 50mL of  $3M\text{ MgCl}_2 \cdot 6H_2O$  ( $60.9\text{g}/100\text{mL}$ ) and incubated at  $4^\circ\text{C}$  for 48 hrs. The extracts were dialyzed against de-ionized water using tubing with an exclusion Mol. wt. of 3,500, flat width 55mm, length 15m, volume  $9.62\text{mL}/\text{cm}$  (Cellu•Sep®, cat #5015-55) at  $4^\circ\text{C}$  for 48 hrs and lyophilized. Semi-purification of lyophilized extracts was carried out in accordance with Karamanos *et al.* (1992).

**Qualitative analysis of semi-purified polysaccharides:** Composition of extracted semi-purified samples for qualitative determination of GAGs was performed following procedure described by Foot and Mulholland (2005). Identification of major GAGs component in chicken cartilage i.e. chondroitin sulphate (CS) was done with the help of Fourier Transform IR instrument (Thermo Nicolet IR 200 spectrometer, WI, USA) by comparing standard spectrum of CS (Sigma, cat # 27042) with samples' spectra.

**Quantitative analysis of semi-purified polysaccharides:** GAGs contents in the dried extracted samples were estimated by DMMB assay (Farndale *et al.*, 1986). Standard curve was plotted between known concentrations ( $1\mu\text{g}-6\mu\text{g}$ ) of standard CS as major component of total GAGs from bovine origin (Sigma, cat # 27042) in de-ionized water with dye reagent solution ( $10.7\text{mg DMB}$  in  $55\text{mol}/\text{l}$  formic acid, pH 3.3) and their respective absorbance values recorded at  $535\text{nm}$  using spectrophotometer UV-ALB 280 (BioCom, Ltd). Protein contents in dried semi-purified samples were quantified by method described earlier (Bradford, 1976).

**Determination of proteins by SDS-PAGE:** SDS-PAGE was performed for determination of proteins in GAGs (Sharma *et al.*, 2007) under denaturing conditions. Proteins were resolved on 10% separating gel and 5% stacking gel. Perfect protein marker™ catalog number 69079-3 (Novagen) was used as reference with known molecular weight proteins. Samples were mixed with loading buffer

(Bromophenol Blue 1%; 2 drops), glycerol 2 ml,  $\beta$ -Mercaptoethanol 1ml) to prepare working dilutions (10ml) for gel electrophoresis. Samples were gently but thoroughly mixed using vortex and heated in a boiling water bath for 5 minutes to denature proteins. A volume of  $30\mu\text{L}$  of each sample was loaded into the wells of the gel such that final concentration of the protein in each well was about  $5-10\mu\text{g}/\mu\text{L}$ . Electrophoresis tank was filled with running buffer (3g Tris, 14.4g glycine and 1g SDS per liter distilled water, pH 8.4) and technique was performed for about 8-12 hrs at a current supply of 170V in a cooling chamber maintained at  $4^\circ\text{C}$ . On completion of electrophoresis run gels were transferred to stain with Coomassie Brilliant Blue stain and placed on a shaker with constant agitation for 4 hours. Stained gels were de-stained until background became transparent and the image was captured by digital camera.

## RESULTS

Extracted semi-purified polysaccharides from chicken keel cartilages were used for determination and estimation of sulphated GAGs contents biochemically. By proximate analysis the highest percent contents observed were of carbohydrate ( $65.49\pm 0.10$ ) in nature followed by crude protein ( $12.82\pm 0.26$ ), ash ( $11.12\pm 0.56$ ), moisture ( $9.88\pm 0.32$ ) and fat ( $0.69\pm 0.14$ ). Fiber contents were not observed in any of the processed samples.

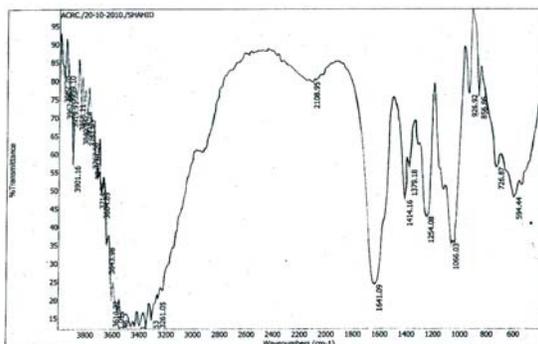
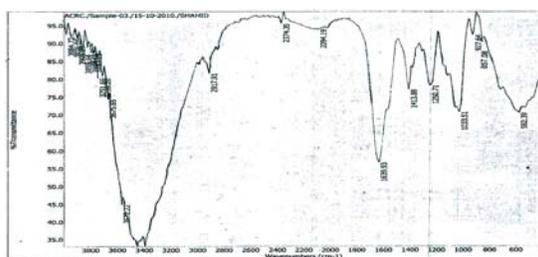
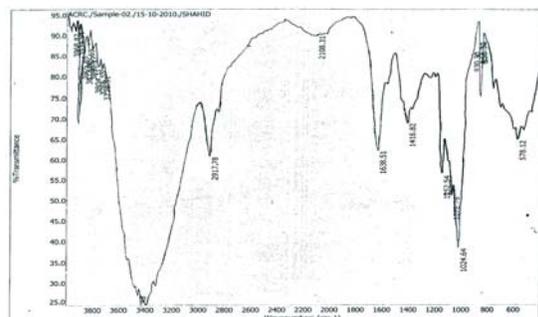
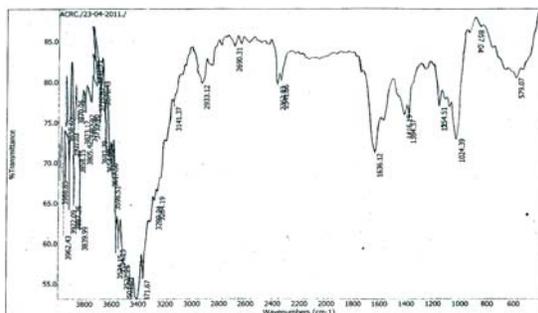
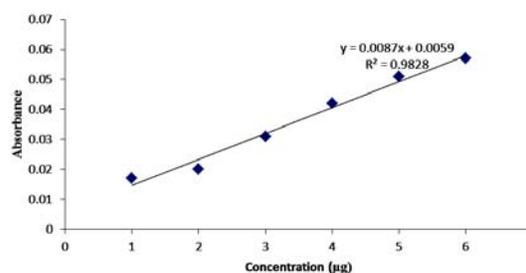
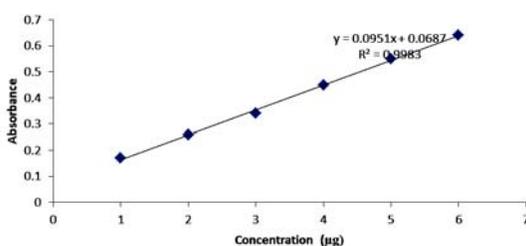
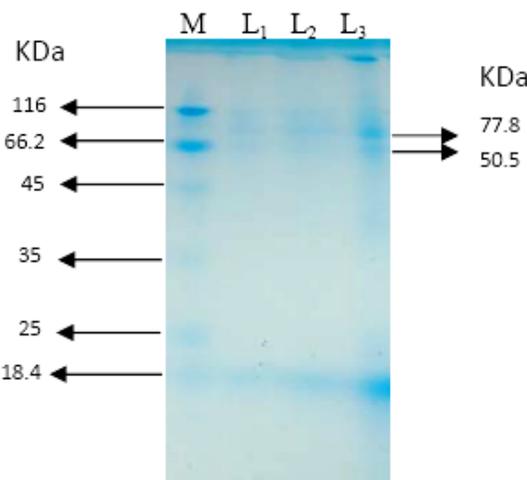
**Qualitative analysis:** GAGs in extracted semi-purified polysaccharides samples ( $n=3$ ) was identified by FTIR spectroscopy technique using potassium bromide pellet and by comparing with standard CS, major component of total GAGs. Characteristic peaks of CS (standard) and samples extracted from chicken keel cartilages at mid infra red region at wavelength of  $400 - 4000\text{cm}^{-1}$  are presented at Table 1. Characteristic Peak of C-O-S was observed at  $856\text{cm}^{-1}$  in standard CS and same was recorded in extracted samples at  $853$  and  $854\text{cm}^{-1}$  by FTIR spectroscopy. Spectra of standard and extracted samples 01, 02 and 03 are shown in Figs. 1, 2, 3 and 4, respectively.

**Quantitative analysis of extracted samples:** Quantity of GAGs contents in extracted samples from chicken keel cartilages were determined by DMMB assay. Calibration curve was plotted between known concentrations (1, 2, 3, 4, 5 and  $6\mu\text{g}/\text{ml}$ ) of standard CS and respective absorbance values (0.017, 0.020, 0.031, 0.042, 0.051 and 0.057) at  $535\text{nm}$  showed linear relationship (Fig. 5). Statistical mean percent concentrations of GAGs in extracted semi-purified polysaccharides from cartilages calculated from standard curve were  $73.44\pm 1.02$ ,  $68.34\pm 0.89$  and  $70.54\pm 0.92$  for samples 1, 2 and 3, respectively. Mean percent concentration of GAGs calculated was  $70.77\pm 2.35$ .

Quantification of protein contents in semi-purified polysaccharides ( $n=3$ ) samples from chicken keel cartilages was carried out by Bradford's method. Linear relation was revealed between BSA concentrations (1, 2, 3, 4, 5 and  $6\mu\text{g}/\text{ml}$ ) and absorbance values (0.17, 0.26, 0.34, 0.45, 0.55 and 0.64) at  $595\text{nm}$  (Fig. 6). The standard curve was used to determine amount of proteins in extracted GAGs samples from chicken keel cartilages. Mean concentration (%) of protein moiety in extracted samples were  $5.0\pm 0.11$

**Table 1:** Characteristic Peaks of extracted samples from chicken keel cartilages at mid IR region (400–4000 cm<sup>-1</sup>)

| Characteristics  | Wave numbers (cm <sup>-1</sup> ) |      |      |      |
|--|----------------------------------|------|------|------|
|  | Standard                         | S1   | S2   | S3   |
| -CONH vibration of amide group   | 1641                             | 1639 | 1638 | 1636 |
| coupling of C-O stretching vibration, OH variable angle vibration and existence of free COO <sup>-</sup> | 1414                             | 1413 | 1416 | 1416 |
| S=O stretching vibrations  | 1254                             | 1250 | 1252 | 1254 |
| -C-O-S   | 1066                             | 1033 | 1024 | 1024 |
| -C-C vibrations  |                                  |      | 1078 |      |
| -COO; -C-C   | 926                              | 927  | 930  | ---  |
| C-O-S  | 856                              | 854  | 853  | 854  |
| R-SO2-R; R-SO2-NH2; R-O-SO2-R  | 594                              | 582  | 578  | 579  |

**Fig. 1:** Spectrum of standard CS by FTIR**Fig. 2:** Spectrum of sample (01) by FTIR**Fig. 3:** Spectrum of sample (02) by FTIR**Fig. 4:** Spectrum of sample (03) by FTIR**Fig. 5:** Standard curve of CS (concentrations verses absorbance)**Fig. 6:** Standard curve of protein using bovine serum albumin (concentrations verses absorbance)**Fig. 7:** Segregated protein moieties found in extracted samples with their molecular weights

4.56±0.12 and 4.36±0.08 (µg/ml) for sample 1, 2 and 3, respectively. Overall statistical mean protein contents in samples were 4.64±0.29.

**Determination of proteins by SDS-PAGE:** Protein moieties in extracted GAGs samples were characterized by SDS-PAGE under de-natured conditions using 10% polyacrylamide resolving gel. Protein marker used as standard was composed of proteins with known molecular weights that segregated along with samples. Segregation pattern of proteins as impurities in extracted GAGs samples align with known protein marker is presented (Fig. 7). Segregation pattern of protein moieties from extracted samples was similar. Two protein impurities were observed in resolving gel having R<sub>F</sub>-values of 0.146 and 0.208. Molecular weights of impurities calculated from standard curve were 77.8 and 50.5 kDa, respectively.

## DISCUSSION

Glycosaminoglycans are linear, complex and polydisperse natural polysaccharides. Main component of GAGs is chondroitin sulfate comprised of repeating disaccharide units of N-acetylgalactosamine and glucuronic acid. Other predominant proteoglycan in cartilage tissues is aggrecan (Nakano *et al.*, 2010). These are natural polymers used for regeneration of soft tissues (Silva *et al.*, 2010). Glycosaminoglycans are commonly extracted from bovine and shark cartilages. Proteoglycans can efficiently be isolated from broiler chicken by-products including crushed bones, skin, adipose tissue and cartilage (Nakano *et al.*, 2010). Chicken keel cartilage has the highest proportion of sulphated GAGs and can act as potential raw material source. Chicken keel cartilage collected from retail broiler sale outlets in Lahore city were analyzed both quantitatively and qualitatively. Selection of cartilages is in accord with the report of Nakano *et al.* (2010) being the richest source of GAGs. Major techniques carried out for this purpose were extraction, semi-purification, proximate analysis, DMMB assay, Bradford's method, FTIR and SDS-PAGE.

Proximate analysis of dry extracted samples revealed the highest percent contents carbohydrates (65.49±0.10) and then crude protein (12.82±0.26). Extracting compound used was 3M MgCl<sub>2</sub> and non-significant differences were observed among samples collected on different days. In accord values of carbohydrates (88.4±2.6%) had been documented using MgCl<sub>2</sub> by Luo *et al.* (2002). Reason to use magnesium chloride for extraction is that the finished products (GAGs) are to be used in human preparations and this compound is safe. This technique of extraction was preferred by Arcanjo *et al.* (1994) working with extraction of uronic acid. Nakano *et al.* (2010) preferred extraction by proteolysis activity of papain from broiler chicken by-products. Zhao *et al.* (2012) used sodium hydroxide for extraction of CS from Chinese sturgeon cartilage. There are many animals as well as birds which can be used as potential source of GAGs.

Percent GAGs contents estimated by DMMB assay in extracted samples from chicken keel cartilages at 535 nm were 70.77±2.35. Comparable value of GAGs (75.5±4.2%) was reported by Luo *et al.* (2002). GAGs have been extracted from a number of species from different sources and variable quantities obtained. GAGs contents in cartilage of crocodile sternum, trachea and rib were 11.55, 9.51 and 5.56 percent, respectively. Riemer *et al.* (2012) fragmented biopolymers in extracellular matrix and GAGs under acidic conditions in tissue samples. Sufficient quantity of glucosamine and galactosamine were revealed from fragmentation of GAGs. These are obtained from marine as natural product (Blunt *et al.*, 2011). GAGs contents differ with species and source of cartilage used for extraction. Non-significant differences are observed if species and source for extraction is same as is evident in present experiments. Differences in percent yield of GAGs are attributed to materials used for extraction and techniques applied for analysis (Tolar *et al.*, 2009; Dalchau *et al.*, 2011).

Non-significant differences in quantities of protein fractions were observed in extracted dry chicken keel cartilage samples by Bradford method. Percent value of

proteins measured by this technique calculated from standard curve of bovine serum albumin was 4.64±0.29. A quantity of 1.8±0.6% had been reported by Luo *et al.* (2002) as determined by Lowry method. So, protein contents differ with species and source used as well as technique involved for analysis of proteins.

Identification of functional groups in extracted samples from chicken keel cartilage was carried out by FTIR following Colthup chart of characteristic group infrared absorption. Non significant differences were noted among characteristic peaks for different functional groups of standard and extracted samples (n=3). In wave numbers for standard CS and extracted samples were similar (856 cm<sup>-1</sup> for standard; 853, 854 cm<sup>-1</sup> for samples, respectively). FTIR has been used as molecular biological tool for detection of collagen in healthy and glioma tumors of brain tissues (Noreen *et al.*, 2011). Olkowski *et al.* (2011) recognized etiology of lameness in broilers and determined changes associated with femoral degeneration using synchrotron-based imaging technique.

Multiple factors affect the quantity of Chondroitin-4-Sulphate calculated by FTIR. In present experiment this technique was used for qualitative detection only and DMMB as quantitative analysis.

Proteins in extracted GAGs samples were identified on the basis of molecular weights of polypeptides revealed in separating polyacrylamide gel (10%). Pattern of protein electrophoresis was similar in all the extracted samples (n=3) in polyacrylamide gel. Two polypeptide bands were observed in each sample having molecular weights of 77.8 and 50.5 kDa. Arcanjo *et al.* (1994) obtained extracts from chicken cartilage by magnesium chloride (1M MgCl<sub>2</sub>) and reported two polypeptides under reducing conditions with molecular weights of 125 and 54 kDa. In the absence of beta-mercaptoethanol polypeptides of 160 and 200 kDa were revealed whereas under reducing conditions a poly component of 70-90 kDa along with a striking peptide of 57 kDa were observed (Rodriguez-Boulan *et al.*, 2005). Migration pattern of 50.5 kDa protein under reduced conditions was similar to that of fibromodulin as reported by Gomes *et al.* (1996) by SDS-PAGE. Peptide band of 77.8 kDa observed in all extracted samples correlated with decorin (70-90 kDa) which is not altered in the presence of reducing agent. Presence of protein in nature impurities in different species and source of cartilage differ significantly. Differences in the same species and source of cartilage samples differ with the technique used for extraction of GAGs. Differences in number and molecular weights of protein impurities have been observed under reduced and non reduced conditions of SDS-PAGE.

**Conclusion:** Present study revealed that chicken keel cartilage is an economical and easily available source of GAGs. Method of extraction using magnesium chloride is safe and this compound is preferred to be used as therapy in osteoarthritis patients.

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