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Blue-spotted Stingray: A promising source of beneficial Glycosaminoglycans (GAGs)

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Abstract

Background: Glycosaminoglycans (GAGS) are polysaccharide composed of repeating disaccharide units either sulfated or non-sulfated monosaccharides of uronic acids and amino sugars which are structured alternately [1, 2]. As the sources of GAGs are mainly from the non-halal source and less extracted from halal source especially from marine lives, thus this invention is likely conducted in order to find the new halal source of GAGs. Objective: The objectives of the study are to extract the GAGs from Blue-spotted Stingray and characterize the extracted GAGs by using Blyscan assay, FTIR and NMR. Results: The Blue-spotted stingray shows high sulfated GAG content which is 7.58% from crude obtained. Carboxylate with sulfate and amine presende in FTIR spectra indicate that the sample is chondroitin sulfate. The presence of O-sulfated group and methyl group of GalNAc and GlcNAc in NMR spectra indicate the presence of chondroitin sulfate. Conclusion: From the results, it can be concluded that GAGs contained in Blue-spotted Stingray.

Keywords— Glycosaminoglycan (GAGs), extraction, chondroitin sulfate, Blyscan, Fourier-Transform Infrared (FTIR), Nuclear Magnetic Resonance (NMR)

INTRODUCTION

GAG is a beneficial polysachharide that can be found naturally in human cell surface and extracellular matrix. It is a naturally unbranched polysaccharide that composed from repeating disaccharides unit of hexoamine and uronic acid or galactose[3] and they are structured alternately [1, 2]. GAG is said can be a good antioxidant, anti-

aging, anti-coagulant, anti-inflammatory and probably a promising anticancer [4-6]. Previously, GAGs were mainly extracted from bovine cartilage as its extraction yield is higher compared to other sources [7-9]. However, people nowadays especially in Muslim country are concern about the halalness of the GAGs sources, thus the extraction of GAGs from halal source is going to be a novelty study. Furthermore, [10] in his previous research stated that the GAGs can be found more in fibrocartilagenous tendon of stingray . The structure of the GAG can be characterised by using rapid established phsyco-chemical methods such as Fourier Transform Infrared (FTIR) and Nuclear Magnetic Resonance (NMR) spectroscopy.. This is a complimentary approach as a result of the particular absorption of the infrared radiation via resonance associated with vibration from non-centrosymmetric modes [11]. The infrared absorption spectrum is resulted from the vibrations that describing the fingerprint characteristics of biochemical compounds such as carbohydrates, amino acids, fatty acids, lipids, proteins and polysaccharides simultaneously [13-15]. [12] has stated that NMR is a powerful technique to determine the structure of molecules and the most common NMR analysis for biological compounds is proton NMR (^{1}H NMR). The chemical shift values in units of parts per million (ppm) will be expressed to obtain the resonances that related to the reference compound. The signals produced at the different chemical shift values present the spectra and they are depend on type of solvent, its concentration and pH [16]and the molecular structure [17].

MATERIALS AND METHOD

A. Extraction

Blue-spotted stingray (*Dasyatis kuhlii*) was collected from Wildlife Handler Resource, Selangor, Malaysia. The samples were sliced and chopped into small pieces then macerated in ethanol for a week. After a week, the sample was weighed and blended with a mixture of urea and NaOH for deproteinization. The sample then centrifuged and the supernantant collected were lyophilized by freeze drying.

B. Blyscan test

The crude GAGs were characterized by doing Blyscan test to obtain percentage of GAG. Total sulfated GAG was carried out by using Blyscan kit manual provided.

C. FTIR and NMR analysis

FTIR and NMR spectroscopy were done in order to confirm the structure of GAGs obtained. FTIR was done by taking a bit of the solid crude of GAG and mixed with Potassium Bromide (KBr). The sample mixture was placed in press and then pressed at 5000-10000 psi. FTIR program was launched and run. The data collected was saved and analysed. For NMR analysis, the GAG crude was dissolved in distilled water with 10mg/ml concentration then submitted to University Malaysia Pahang's Central Laboratory for Proton NMR analysis.

RESULTS AND DISCUSSION

A. Extraction

In this project, the Blue-spotted stingray was used. This type of stingray usually found in the entire continental waters of Asia. A whole part of the stingray is used in the extraction. Ethanol can dissolve a larger portion of polar compounds, most of the polar compounds are easily eluted by ethanol which is bioactive responsible for their activity. On the other side, some non polar groups may also be dissolved in ethanol. For deproteinization, the mixture solution of NaOH and urea with specific molarity were used. The mixture solution is not only practical in removing protein, but also can remove DNA. Sodium hydroxide is used in the last step of precipitation. As GAG is organic disaccharides with acidic sugar which is not really solule in water, treatment with NaOH will produces corresponding sodium carboxylate salt. The ionic condition of the salt makes the GAG compound soluble in water. The supernatant collected from the last extraction step then sent for freeze dry. The crude weight of the sample after freeze drying was taken. The crude weight obtained is about 5280.40mg from 300g of raw sample used.

Table 1. Extraction yield (gram) and protein absorbance reading. The table shows the raw weight and crude extraction yield of blue spotted stingray with its protein absorbance after deproteinization.

Sample	Raw weight (g)	Crude weight (g)	Protein absorbance (after deproteinization)
Blue Spotted Stingray	300	5.2804	0.0180

B. Blyscan test

The yield of sulfated GAG from blue spotted stingray was extrapolated from the calibration curve where chondroitin sulfate is used as a standard. From the given concentration, 1.0, 2.0, 3.0, 4.0

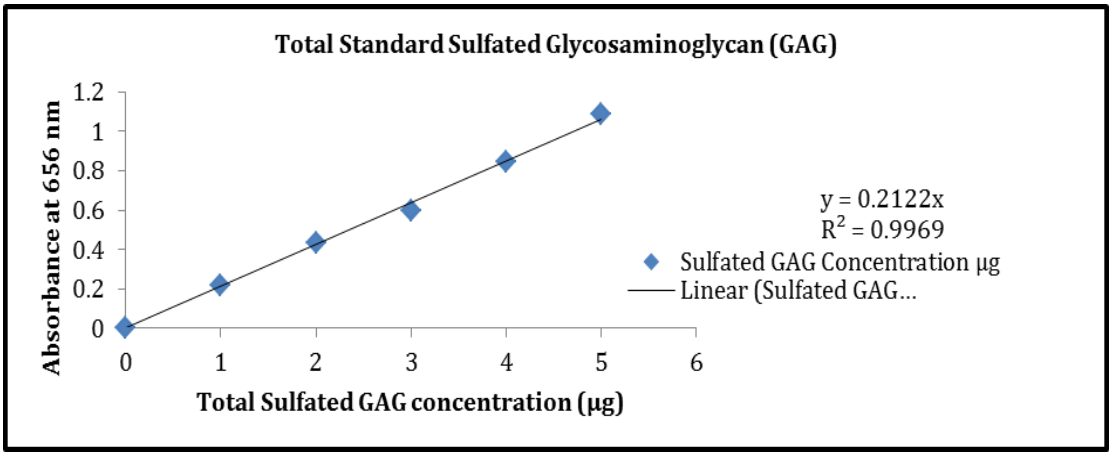

Figure 1. The Blyscan sulfated GAG standard calibration curve. The graph shows the sulfated GAG standard calibration curve determine from Blyscan assay with different concentration of 1, 2, 3, 4, 5 µg/ml read under wavelength 525nm.

and 5.0µg/ml were prepared and the standard curve then plotted as in Fig 1. For samples preparation, samples with 1mg/ml concentration were prepared. From the concentration, only 50µl of samples were taken to be tested as in procedure. The mean absorbance reading for Blue-spotted stingray is 0.8033. For 1mg/ml concentration, 50µl contains 50µg sample. By using the standard sulfated GAG calibration curve, 3.7891µg of sulfated GAG was found. Total crude of GAG extracted is 5280.4mg, thus that makes the total percentage of sulfated GAG from the crude is 7.58%.

C. FTIR analysis

[18]stated that FTIR spectroscopy is one of the simple and reliable constant analytical techniques that analyse a broad range of sample types rapidly. The absorption spectra were read within the range of 450 – 4000^{cm-1}. The IR spectrum of the GAG sample was characterized by a broad band above 3000^{cm-1} and its intense absorption was about 1650 to 1020^{cm-1}. Corresponding to the presence of the combination of carboxylate with sulfate and amine, the stretching and deformation vibrations of –C-O-H- bands were at 1600cm^{-1} and 1400^{cm-1}. The peak intensities of GAG sample at 1654.54^{cm-1} and 1404.06^{cm-1} showed the presence of carboxylate with sulfate and amine. The particular hydroxyl-streching vibration of water and polysaccharide mixed up in hydrogen bonding was represent by the broad band above 3000^{cm-1}. In the GAG sample spectrum, the similar peak intensities were found at 1100^{cm-1} to 600^{cm-1}. The IR spectra data for the GAG sample confirms that the condroitin sulfate is the type of GAG in the Blue-spotted Stingray.

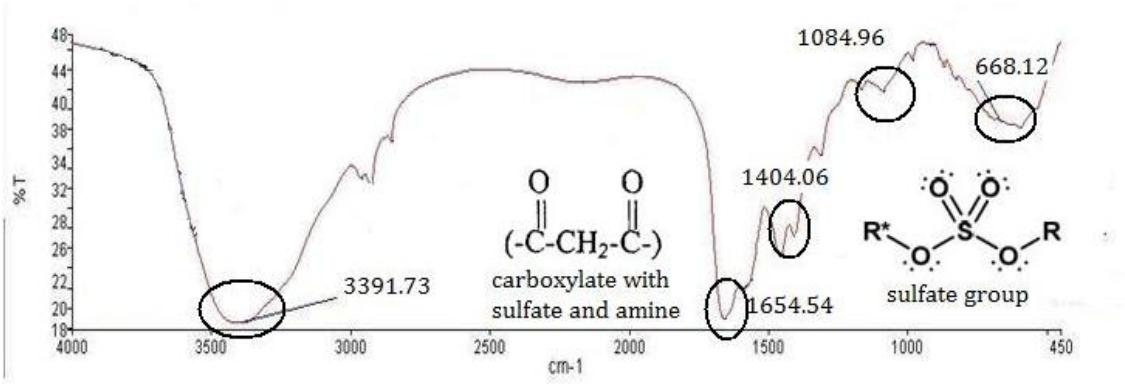

Figure 2. Blue-spotted stingray FTIR spectra. The peaks at 3391, 1654, 1404, 1084 and 668 in FTIR spectrum shown above strongly suggesting the chemical entity as chondroitin-4-sulfate (CS).

D. NMR analysis

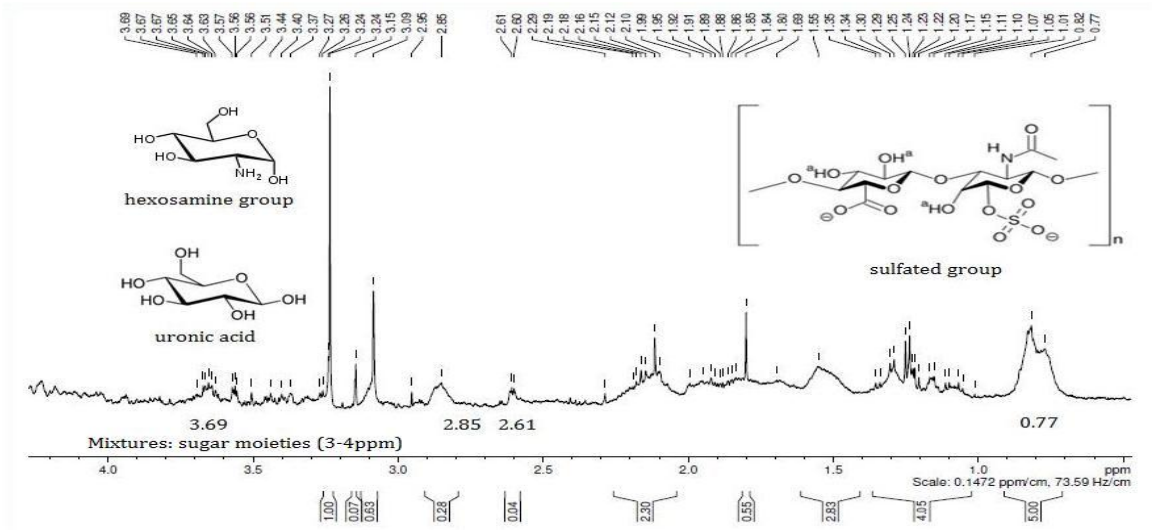

Figure 3. Blue-spotted stingray NMR spectra. The identification of the GlcUA (H1) & (H2) proton resonance at 3.37ppm and 3.57ppm were confirmed by decoupling experiment. Proton NMR result shows the presence of chondroitin sulfate. Proton NMR was run by Central Laboratory with crude concentration 50 mg/ml.

GAG crude then was analysed by using ^{1}H NMR. The sample spectra analysed from ^{1}H NMR is in the range of 0.20 to 4.0 ppm as the GAGs peaks located within this range. The standard chondroitin sulfate was analysed in order and compared with the sample spectra in order to confirm the presence of GAGs. Fig 4 represents the spectra for Blue-spotted stingray. As can be seen, the spectra can be divided into different

ranges which are from 0.77 – 2.61 ppm and 2.85 – 3.69 ppm, indicates the presence of sugar ring protons and methyl protons important in GAGs characterization. In the range of 2.85 – 3.69 ppm, the narrow spectral range make it is difficult to differentiate for the signals of several ring hydrogens which are H-2, H-3, H-4, H-5 and H-6 of hexosamine and hexuronic acids. However, it can be said that there were some information related to Glycosaminoglycan characteristics. O-sulfated carbon atoms can be found in the range of 0.4 – 0.7 ppm. The differences between 1.91 – 2.19 ppm show the presence of methyl group in GalNAc and GlcNAc.

CONCLUSION

The Blue-spotted stingray shows high sulfated GAG content which is 7.58% from crude extracted. Carboxylate with sulfate and amine presende in FTIR spectra indicate that the sample is chondroitin sulfate. The presence of O-sulfated group and methyl group of GalNAc and GlcNAc in NMR spectra indicate the presence of chondroitin sulfate. It can be concluded that GAGs contained in Blue-spotted Stingray**.**

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