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Extraction and quantification of sulfated glycosaminoglycan content in five different aquatic species of Malaysia

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ABSTRACT

Objective: To extract, characterize and quantify glycosaminoglycans (GAGs) from the body of cuttlefish, tennis-ball sea cucumber, shrimp, seabass and fresh water fish Nile tilapia.

Methods: The extracted crude powder was evaluated for the content of GAGs. The qualitative analysis of sulfated pattern and other important functional groups related with GAGs were explained in the form of Fourier transform infra-red spectroscopy data. Proteins and nucleic acid in the crude extract were determined by the ultraviolet spectrophotometer, while the quantification of total sulfated GAGs and estimation of *N*-sulfated and *O*-sulfated GAGs in the crude mixture were performed by using Blyscan kit.

Results: The sulfated pattern and other important functional groups related with GAGs were intercepted in Fourier transform infrared analysis. Blyscan quantification method reported that a rare variety of sea cucumber (tennis-ball sea cucumber) emerged as a rich source of GAGs with high values of both *N*-sulfated and *O*-sulfated GAGs in comparison to its other counterparts.

Conclusions: Findings in this study point out the potential of tennis-ball sea cucumber, a rare variety of sea cucumber to act as an alternative source for GAG extraction for commercial purpose.

1. Introduction

Carbohydrate, commonly known for their energy providing role in the human body in the form of its simple monosaccharide, disaccharide and polysaccharide units such as glucose, sucrose and starch, which form the major chunk of our daily meal, can also exist in the form of more dense and complex conjugates, namely, known as glycans. The science of exploring these complex molecules emerged in the last two decades and it is commonly known as glycomics studies or glycobiology[1]. One of the linear chain glycans known as glycosaminoglycans (GAGs) are large, complex sugar molecules that interact with vast varieties of proteins involved in regulating key pathological and physiological processes. Chemically, the GAGs are repeating disaccharide units of amino sugar like galactosamine or glucosamine and uronic acid such as glucoronic acid or iduronic acid[2]. GAGs are also known as mucopolysaccharide because of its viscous, lubricating properties, which are common with mucous secretion. GAGs are located in all living cell surfaces in extracellular matrix and also to some extent in the cytoplasm[3], and are mainly divided into two classes known as sulfated GAGs such as heparan sulfate/heparin, chondroitin sulfate, keratan sulfate, dermatan sulfate and non-sulfated GAG including hyaluronic acid. The chemical characteristic of sulfated GAGs is to possess a high negative charge due to deprotonation of its carboxylic acid and sulfated moiety under physiological conditions[4], making it a potential bioactive agent which can demonstrate its activity as a wound healing agent[5], an anti-coagulant[6], and potential agent to treat osteoarthritis through reforming cartilage and by inhibiting apoptosis of cartilaginous tissue[7]. The low molecular weight heparin is under scan in many studies for its novel anti-neoplastic effect and low anti-coagulant effect; apart from this there are still a lot of GAGs's roles are still under exploration around the world. These features make the GAGs a potentially vital biomolecule for coming generations[8]. The presence of GAG in different invertebrates as well as vertebrates has been studied and well documented. Conversely, till now, the presence of GAG has not been reported in plants[9]. Many studies have revealed that there is structural diversity in vertebrate GAGs. From mammals and amphibians to fish, the structure of vertebrate GAGs has extreme

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diversity[10]. The biosynthesis of structurally complexed GAG with diverse sulfation pattern takes place in an organ or a particular tissue during the phase of growth and development[11]. The mutational alteration in genes of enzymes involving in biosynthetic production of GAG can lead to severe consequences for an organism, highlighting that the expression of particular sulfated pattern in GAGs plays a key role in life of an organism[12]. The diversity in GAG structure is generally more in aquatic species than terrestrial animals[9]. As per the current scenario, the production of GAGs such as heparin are facing some of the key challenges that are related to its source of extraction as many communities have some reservation with its ultimate source of extraction like pork intestine or bovine lungs. It is very essential to understand these reservations and address the issue by finding alternate source of extraction[13]. In our studies, we selected five Malaysian aquatic species of different phyla including Asian seabass (*Lates calcarifer*) from Chordata, Nile tilapia (*Oreochromis niloticus*) from Chordata, tennis-ball sea cucumber (*Phyllosphorus spiculata*) from Echinodermata, shrimp (*Penaeus merguiensis*) from Crustacean and cuttlefish (*Metasepia pfefferi*) from phylum Mollusca, to evaluate them on the basis of their GAG content in their body by using specific quantification methods. In this study, extracted lyophilized powder of GAG is utilized to understand a proper sulfated distribution and other structural characteristics present in different GAGs from different species and phyla by qualitative method using Fourier transform infra-red (FTIR) spectroscopy and to understand the distribution of *N*-sulfated and *O*-sulfated GAGs within a crude mixture by using a specific detection kit Blyscan for quantification.

2. Materials and methods

2.1. Sample collection and storage

Marine species were collected from several parts of Malaysia with the local fisherman's assistance and kept in an icebox. The flesh was then sliced with a knife and washed with cold water to remove blood and other impurities. The processed sample was then kept at -80 °C.

2.2. Extraction

The samples were taken out from the freezer and treated with liquid nitrogen to facilitate easy crushing by mortar and pestle. After crushing, the sample was weighed out and blended with 3 volumes of 90% v/v ethanol. The blended suspension was then ultrasonicated at 20 KHz for 30 min to facilitate cell disruption. The suspension was then left for 14 days for maceration with ethanol to take out fat and other substances with low molecular weight. After maceration, the suspension was then filtered and collected flesh was air dried at 50 °C. The dried flesh was again weighed, extracted with 3 volumes of 5% w/v of NaOH for 6 h and centrifuged at 5000 r/min for 20 min.

The collected supernatants were concentrated to a proper volume in a rotary evaporator. The concentrated mixture was treated with 20% w/v trichloroacetic acid (TCA) for 30 min and then centrifuged at 5000 r/min for 10 min. The supernatant was collected, mixed with 4 volumes of 90% ethanol and then kept overnight in a chiller at 4 °C. To collect the precipitated GAG, the mixture was centrifuged in a refrigerated centrifuge at 8000 r/min for 30 min at 4 °C. The collected precipitate was washed with absolute ethanol, dissolved in de-ionized water and lyophilized.

2.3. FTIR spectrophotometer analysis

Infra red (IR) spectra of extracted GAG samples along with a standard heparin from pigs was obtained by mixing sample powder with potassium bromide (Merck) separately and then the mixture was compressed to obtain a salt disc of 13 mm which was subjected under Perkin Elmer FTIR spectrophotometer. The percentage of the transmittance was recorded between the range of 4000 cm^{-1} to 400 cm^{-1}[14].

2.4. Ultraviolet scanning to determine presence of protein and nucleic acid

The presence of protein and nucleic acid in the sample was determined by measuring the absorbance at 280 nm for protein and 260 nm for nucleic acid using quartz cuvette in a ultraviolet-spectrophotometer.

2.5. Total sulfated GAGs content

The total sulfated GAGs content in extracted samples was measured by a specific detection kit known as Blyscan, purchased from Biocolors, UK. The Blyscan kit consists of 1,9-dimethylmethylene blue dye, which binds specifically to sulfated GAGs and settles down in microcentrifuge tubes after centrifugation. The pellets formed light blue color in dissociating agent provided in the kit. In this test, series of concentration of chondroitin-4-sulfate was used as a standard to obtain the calibration curve, shown in Figure 1. To evaluate the GAG content in extracted fractions, 50 μL of extracted fraction from each species was used as test samples. The samples were further diluted with de-ionized water to make uniform volume of 100 μL for all samples in 1.5 mL tapered bottom microcentrifuge tubes. About 1 mL of Blyscan dye was added and the tubes were vortexed for 30 min followed by centrifugation at 12000 r/min for 10 min. The unbound dye was removed and settled pellets were dissolved in 500 μL of dissociating agent provided in the kit. The tubes were vortexed for 10 min and again centrifuged for 5 min at 12000 r/min to make the solution bubble-free. The dissociated pellet solution was divided into two wells of 96-well plate and absorbance was recorded at 656 nm. The standard plots were obtained by using chondroitin sulfate standard solution and values of the test samples were determined by using a linear equation of standard graph[15].

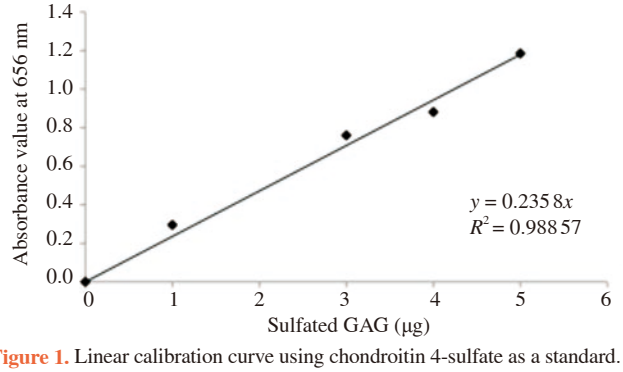

Figure 1. Linear calibration curve using chondroitin 4-sulfate as a standard.

2.6. Nitrous acid cleavage by utilizing Blyscan kit

In a microcentrifuge tube, 100 μL of the test sample was mixed with 100 μL of sodium nitrite reagent following addition of 100 μL of 33% sodium acetate solution and vortexed. The reaction was allowed to proceed for 60 min at room temperature, with occasional mixing. The solution turned pale yellow with formation of gas bubbles. A 100 μL ammonium sulfamate reagent was added after the completion of the reaction, which resulted in vigorous bubbling when nitrous acid was removed. The content was further mixed for 10 min and then 100 μL of the sample was used to quantify the GAG content with the same method described above for total sulfated GAG. The absorbance obtained due to the presence of the *O*-sulfated GAGs, which cannot be digested by nitrous acid, was deducted with total GAG absorbance reading to obtain *N*-sulfated GAG content.

3. Results

The qualitative characterization of extracted crude samples was performed by using FTIR spectrophotometer. The FTIR spectra in Figure 2 shows the peak in the range of 3 100 cm^{-1} to 3 700 cm^{-1} in all samples, suggesting the stretching of hydroxyl group. The minor strenches at range of 2 900 cm^{-1} to 2 300 cm^{-1} suggested H-C-H stretching, while intensities recorded at the range of 1 600 cm^{-1} to 1 700 cm^{-1} in all the samples strongly pointed towards the presence of N-H bend, and meanwhile can also be utilized to understand the C═O stretch as the peak is at 1 673.00 cm^{-1} with its shoulder at 1 630.08 cm^{-1} in the sample of Nile tilapia, suggesting the presence of amide group. The peak at the range of 1 200 cm^{-1} to 1 250 cm^{-1} mainly pointed towards the strong sulfation pattern, which can be easily seen in heparin showing sharp peak at 1 232 cm^{-1} while all other samples had just minor signatures at this region. Peak at the range of 1 200 cm^{-1} to 1 000 cm^{-1} is a possible signature of pyranose ring[16]. The peak range below 1 000 cm^{-1} can be used to differentiate between the same type of GAG such as peak in Nile tilapia at 835 cm^{-1} with its shoulder at 748 cm^{-1} suggesting the presence of chondroitin-4-sulfate in the crude mixture[17].

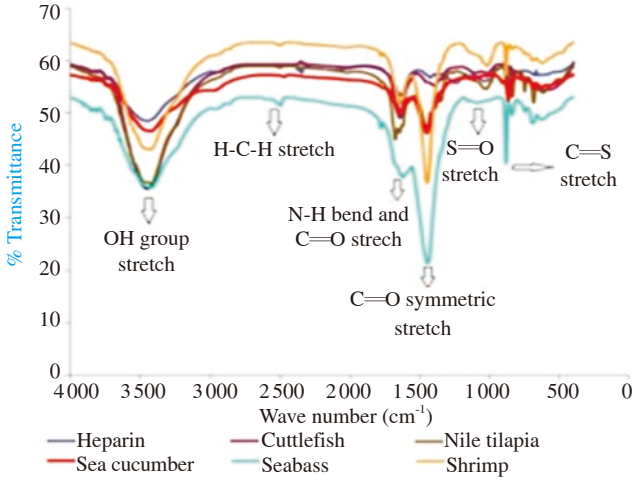

Figure 2. FTIR analysis of extracted samples compared with standard heparin scanned in a range of 4 000 cm^{-1} to 400 cm^{-1}.

The data presented in Table 1 demonstrate that the peak obtained in samples is related to heparin (standard) with the reported range in many studies. The crude extract was also analyzed for the presence of residual protein and nucleic acid after extraction by using ultraviolet spectrophotometer at the range of 200 nm to 280 nm.

The results shown in Figure 3 justify the effect of NaOH and TCA in degradation and removal of protein and nucleic acid from the samples. While in some samples like sample B (cuttlefish) and sample D (Nile tilapia), almost all proteins and nucleic acids were found to be decimated, samples like A (tennis-ball sea cucumber), C (seabass) and E (shrimp) were found to have slight absorbance at 260 nm and 280 nm, signifying the traces of proteins and nucleic acids left after TCA treatment.

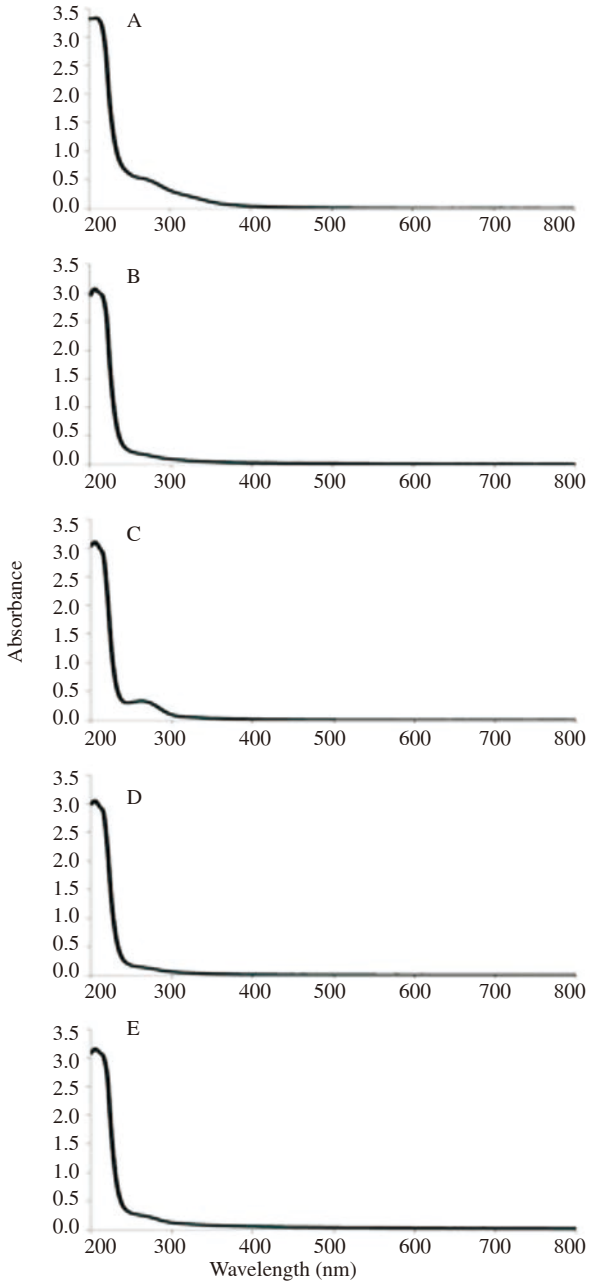

Figure 3. Ultraviolet spectrophotometer scanning of extracted samples, sample A (tennis-ball sea cucumber), sample B (cuttlefish), sample C, (seabass), sample D (Nile tilapia) and sample E (shrimp).

Blyscan kit was utilized for accurate quantification of total sulfated GAGs in the extracted crude samples and further estimation of *N*- and *O*-sulfated GAGs in those total quantified GAGs (Figure 4). The high presence of sulfated GAG in tennis-ball sea cucumber was reported in comparison to other aquatic species. While all the samples except for

tennis-ball sea cucumber were found to have less than 1.0% of GAG content, the tennis-ball sea cucumber was estimated to have around 2.2% of GAG content, which was 4 fold high to its respective counterparts.

Table 1
Comparison of FTIR frequencies of extracted samples with reported ranges in previous studies. cm^{-1}.

Functional groups	Samples	Peaks	Peaks in standard (Heparin)	Reported range
OH (Hydroxyl group) stretch	Cuttlefish	3446	3446	3100-3700[18,19]
	Nile tilapia	3419		
	Sea cucumber	3420		
	Seabass	3418		
	Shrimp	3420		
N-H bend	Cuttlefish	1638	1634	1600-1700[18]
	Nile tilapia	1639		
	Sea cucumber	1638		
	Seabass	1620		
	Shrimp	1638		
Sulfate group	Cuttlefish	1018, 838	1235, 1029, 1148.57, 940.32, 894, 816	1250-700[14,20,21]
	Nile tilapia	1018, 881, 835, 748		
	Sea cucumber	865.92		
	Seabass	1093.62, 881.12, 841, 748		
	Shrimp	1015.69, 881.25		

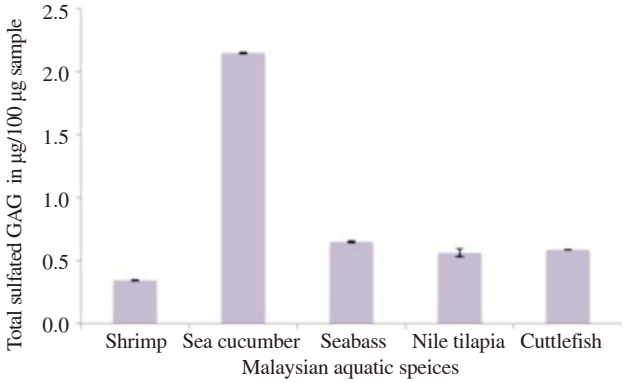

Figure 4. Total sulfated GAGs content (µg/100 µg) in different samples of Malaysian marine species by using Blyscan kit.

The data in Figure 5 are the quantification of *O*-sulfated GAGs in the extracted samples. Tennis-ball sea cucumber was reported to have a high content of GAG in a crude mixture, which accounted for about 1.6% of total estimated GAG. The *O*-sulfated GAG reported in other species was under 0.5% of total estimated GAG. On the *N*-sulfated GAG estimation (Figure 6), tennis-ball sea cocumber was found to have the highest presence of *N*-sulfated GAG with around 0.7%, followed by seabass and cuttlefish with 0.5% and 0.4% respectively. Nile tilapia was found to have the lowest (about 0.1%) and shrimp to have almost negligible amount of *N*-sulfated GAG.

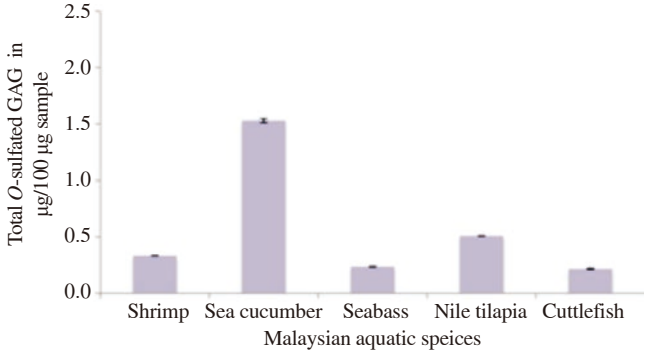

Figure 5. Total *O*-sulfated GAG content in (µg/100 µg) five different samples of Malaysian aquatic species.

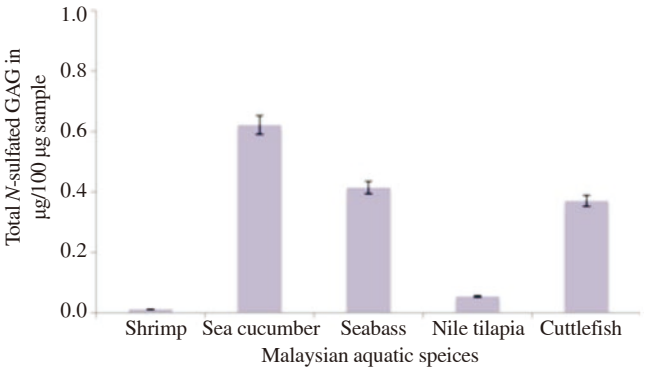

Figure 6. Total *N*-sulfated GAG content in (µg/100 µg) five different samples of Malaysian aquatic species.

4. Discussion

Extraction of pharmacologically and biologically active biomolecule, GAGs has been performed from many vertebral and invertebral species in the past. Out of many species, only a few have been found to have commercial importance. The scarcity of commercial resources to extract important GAG such as heparin, has created a serious problem for many communities to accept it in its present form, although people know it can be extracted from pork intestine and bovine lungs. The country like Malaysia is surrounded by Andaman Sea and Indian Ocean in west and by South China Sea in the east, making it highly rich in marine resources.

Apart from coastal areas, the fresh water bodies of Malaysia also provide a lot of aquatic species that can be utilized for extraction of key biomolecules like GAGs. Some of the important GAGs like chondroitin sulfate are mainly used as a dietary supplement to treat cartilage related disorders like rheumatoid disease and osteoarthritis, and they are mainly extracted from the cartilage of various organisms like shark. It has been well established and documented that GAGs like chondroitin sulfate coexist with some extracellular proteins like collagen. Previous studies found that some organs of fish like seabass are rich source of collagen and also the body of sea cucumbers have been reported to have high amount of collagen.

Based on this knowledge, we selected five different aquatic species of Malaysia to figure out their GAGs content and their potential to replace classical sources of extraction currently being used in the industries.

The FTIR studies not only provided qualitative characterization to confirm the presence of GAGs in extracted lyophilized powder, but also explained the sulfated pattern of extracted GAGs from different sources. FTIR study suggested that seabass and Nile tilapia had high sulfated pattern, though not as high as heparin, they can be utilized for testing of anti-coagulant activity. The least sulfated pattern in tennis ball-sea cucumber can be assumed to have low-molecular-weight GAG, which can be isolated by purification, to be used for anti-metastatic effect in further studies.

The ultraviolet studies used to identify the residuals of proteins and nucleic acids in the extracted crude samples proved the effectiveness of extraction methodology utilized in this study, as most of the samples were found to have either minor or almost no proteins or nucleic acids left after extraction.

The most possible reason for the presence of protein traces in

these samples even after deproteinization with 20% w/v TCA is the abundance of collagen in these samples, especially in seabass and sea cucumber[22,23], which is difficult to be completely removed. Collagen is most abundantly present in fiber form, accounting for 30% of total proteins in vertebrates[24]. Increase in strength of TCA to completely decimate the collagen in crude GAG powder can also result in loss in percentage yield, as the increased TCA strength can also precipitate the GAG in the form of proteoglycan mixture[25]. The traces can also be effectively removed by eluting the crude mixture through an anion exchange column, which can bind effectively to the highly negative charged GAGs.

The results revealed that the new species of sea cucumbers (tennis-ball sea cucumber) can act as a replacement for classical sources of GAGs extraction such as pork intestine, because quantification study has suggested high content of both *N*-sulfated and *O*-sulfated GAGs. The present need is to explore more organisms like these for their GAG content to finally find out a better alternative for GAGs extraction. Besides that, there is also a need to perform bioactivity studies on these extracted GAGs to explore their pharmacological potentials.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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