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Anticoagulant Effects of Glycosaminoglycan Extracted from Fish Scales

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Abstract

Introduction: Fishery wastes are the unique sources of natural products, and structural and chemical characteristics of their extracted compounds are different from those of terrestrial animals. They are known as rich sources of bioactive molecules, including collagen and glycosaminoglycan (GAG). In the present study, we extracted and analysed the anticoagulant activity of GAG from fish (*Rutilus frisii kutum*) scales.

Methods: The GAG compounds were extracted using cationic salt of cetylpyridinium chloride (CPC). Fourier-transform infrared spectroscopy (FTIR) was used to identify and compare the structure of the extracted GAG with that of heparin. Anticoagulant property of extracted material was measured by prothrombin time (PT), activated partial thromboplastin time (aPTT), and thrombin time (TT) on human plasma at three concentrations of 50, 100, and 200 µg/mL.

Results: The mass of the extracted GAG was estimated approximately 23.8 mg/g of dry tissue. FTIR analysis confirmed the presence of heparin-like compounds in fish scales-extracted GAG. Human plasma coagulation times significantly increased with increasing the extracted GAGs concentration. At the concentration of 200 µg/mL, coagulation time of aPTT was 4.3 times as that of control reaching 138.6 seconds.

Conclusion: The results of the current study showed that the GAG extracted from fish scales had valuable anticoagulant properties compared to synthetic anticoagulant compounds such as heparin.

Keywords: Fish, Glycosaminoglycan, Anticoagulant, Cetylpyridinium chloride

Introduction

Glycosaminoglycans (GAGs) are negatively charged polysaccharides[1,2]. These compounds play an important role in many cellular activities, angiogenesis as well as host defence against viral infection, and blood coagulation.[3-5] Based on their disaccharide composition, GAGs can be categorized into chondroitin sulphate (CS), keratan sulfate (KS), hyaluronic acid (HA) and heparin sulfate (HS) families.[6] Heparin is a sulphated GAG with anticoagulant property used in the conditions such as acute coronary syndrome, lung embolism, vascular fibrillation, and deep pulmonary thumbs, even in the treatment of infectious diseases, inflammation, and cellular growth in wounds and cancer.[7-9]

GAGs are particularly abundant in various mammalian organs such as cartilages, blood vessels, cell surfaces, intracellular granules and plasma.[10-12] These are also by-products of the meat and livestock industry, especially bovine and porcine wastes such intestines and lungs.[13] An increasing demand for GAGs has resulted in the use of alternative marine resources; so recently aquatic vertebrates and invertebrates have become more important sources of GAGs.[14]

Currently many studies have been focused on the extraction of GAGs from marine origins.[12,14] Shark fin cartilage, squid cartilage and fisheries discards have been considered as valuable resources for GAGs.[15-17] The causes of death in developing countries are almost the diseases raised by undesirable function of the circulatory system resulted from coagulation abnormalities.[18] Drugs with anti-thrombotic, anticoagulant, and antiplatelet properties are used as remedies for preventing platelet aggregation and restoring blood circulation through vessels.[19] Studies on aquatic organisms, particularly squids, have shown the presence of heparin and heparin compounds which can suppress

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blood coagulation and prevent vein hardening.[19]
Rutilus frisi kutum is one of the most important commercial and economic fisheries in the north of Iran. Due to its high consumption, large quantities of wastage particularly scales are produced, which could have great potential to be applied as bioactive compounds.[20] Therefore, in this study, the fish by-products were used to extract GAG compounds and to evaluate their in vitro anticoagulant activity using classical coagulation assays.

Methods

Preparing Fish Scales

Fish (*Rutilus frisi kutum*) scales were prepared from the fishery market in Rasht city, Iran. The fish scales were washed well with tap water and freeze-dried and grounded in the blender to obtain the dried powder.[21]

Extraction of GAGs From Fish Scales

The fish scale powder (2.5 g) was incubated with 50 mL of sodium sulphate 4.0 mM for 1 hour at 60°C. The solution pH was adjusted by NaOH to 11.5. In the next step, aluminium sulphate was used to reduce the pH to 7.7, and it was heated at the temperature of 95°C for 1 hour. The sample was filtered and exposed to cetylpyridinium chloride (CPC). Then, 10 mL of CPC (3% in 0.8 M sodium chloride) was added to the collected filtrate. Suspension was incubated at 37°C for 18 hours, and white precipitate was formed. To remove pyridinium salt from the precipitate, 2 mM sodium chloride (pre-heated to 40°C) was used. Afterwards, ethanol (95%) was added to precipitate the sulphated polysaccharides. The precipitate was washed twice by methanol and freeze-dried for 1 hour.[22]

Fourier-Transform Infrared Spectroscopy Analysis

Fourier-transform infrared spectroscopy (FTIR) of GAGs from fish scale was performed using FTIR spectrophotometer (FTIR, 8400S spectrophotometer, Japan). The amount of 10 mg of the samples (extracted GAGs and heparin) was mixed with 100 mg of dried potassium bromide (KBr) and compressed to prepare a salt disc (10 mm diameter) for reading the spectrum. The spectra were taken between wave numbers in 4000 and 450 cm^{-1}, and automatic signals were collected in 32 scans at a resolution of 4 cm^{-1}. The transmission spectrum and standard were documented. All assays were performed at room temperature in a dry atmosphere.[17]

Clotting Assays

Anticoagulation measurement was performed in a laboratory with citrated human platelet poor plasma (ppp). Human plasmas were collected from healthy individual donors (11 different donors) and mixed with 2.5% sodium citrate solution (9: 1 v/v blood to sodium citrate). The plasma was obtained by centrifugation at 5400 g for 30 minutes, afterwards the plasma was maintained at -60°C until use.[23]
The anticoagulant activity of the extracted GAG was determined by classical coagulation assays: activated partial thromboplastin time (aPTT), prothrombin time (PT), and thrombin time (TT). The sample (GAG) was dissolved in normal saline. All tests were conducted in triplicate.

Activated Partial Thromboplastin Time Assay

The 10 µL extracted GAGs of different concentrations (50, 100, and 200 µg/mL) was mixed with 90 µL of human pooled plasma, and incubated at 37°C for 3 minutes according to Tingbo method.[17] After that, 100 µL of aPTT reagent (kaolin bovine phospholipid reagent) was added to the samples and incubated at 37°C for 10 minutes. Then, the clotting time was measured after adding 100 µL $CaCl_2$ (25 mM) using a coagulometer (SY-B032, SUNNYMED).[17] Heparin (1 IU/mL) in different concentrations and NaCl (50 µg/mL) were used as the positive and negative controls, respectively.
In coagulometer, the time of clot formation is measured by vibrating probe which is sensitive to blood viscosity changes. The probe is put in the sample and a digital display shows the time elapsed from the probe insertion until the clot is formed.

Prothrombin Time Assay

To determine the PT, the human pooled poor plasma (PPP) (90 µL) was mixed with 10 µL of the heparin (different concentrations), NaCl (50 µg/mL) and different concentrations of the extracted GAGs (50, 100, and 200 µg/mL). The mixtures were incubated at 37°C for 10 minutes (Diagnostica Stago, Asnières, France). Then, 200 µL of pre-incubated (37°C) PT reagent (thromboplastin reagent) was added and the the clotting time was again recorded using a coagulometer.[17]

Thrombin Time Assay

In the TT assay, 90 µL of human PPP was mixed with 10 µL of different concentrations of GAGs (50,100, and 200 µg/mL) and incubated at 37°C for 2 minutes. Then, 100 µL of thrombin was added and the TT was recorded. Positive and negative controls were determined by replacing the 10 µL of GAGs with heparin and NaCl respectively.[17]

Statistical Analysis

The Kolmogorov–Smirnov test was applied to identify the normal distribution of data. Because of the normal distribution of data, for comparing the groups in the variables studied, two-way analysis of variance (ANOVA) was used. In addition, the Duncan multiple-range test was conducted as the post hoc test. All data were expressed as mean ± standard deviation (SD). The significance of differences was defined at $P < 0.05$. Statistical procedures were performed using SPSS software version 19.0.

Results

The yields of GAGs were 23.8 mg/g of dry tissue. This value was obtained from 2.5 g dry weight of fish scales. FTIR spectroscopy was applied to confirm the composition and the structural integrity of extracted GAGs (Figure 1a). The FTIR analysis of the GAGs from fish scales revealed a pattern similar to the heparin (Figure 1b), and proved a strong OH band at 3200–3500 cm^{-1} and a strong OH bend at 1620–1660 cm^{-1}. More absorbance at 1239 cm^{-1} and at 821 cm^{-1} was assigned to the stretching of S=O (sulphate group).

The anticoagulant properties of extracted GAG are shown in Tables 1-3. The mean values of the aPTT, PT, and TT for healthy human plasmas were recorded at 32±0.6, 13.1±0.4 and 14.7±0.2 seconds. APTT and TT times showed a significant difference between various concentrations of extracted GAG and control ($P<0.05$). With increasing the concentrations of GAGs, the anticoagulant indexes of GAGs from fish scale also increased. In the presence of extracted GAGs, the longer coagulation times were observed rather than those observed for the control at the same concentration.

The aPPT and TT of scale GAGs were prolonged to 138.6±1.8 and 19.4±0.6 seconds at concentration of 200 µg/mL respectively, which were 4.3 and 1.3 times as much time as the control group. However, there was no significant difference between treatments and controls in the PT ($P>0.05$) (Table 2).

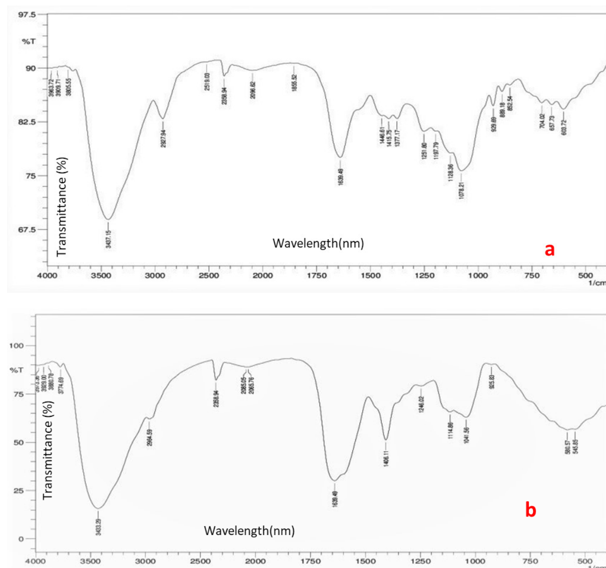

Figure 1. (a) FT-IR Spectrum in KBr Pellet of the Glycosaminoglycans From Fish (*R. frisi kutum*) Scales in the Wavenumber Ranged From 4000 to 450 cm^{-1}. (b) FTIR Spectrum of Standard Heparin (Sigma Co. USA).

Discussion

Heparin and heparin like compounds such as GAGs, and keratan sulphate extracted from marine fish have been indicated to represent anticoagulant activities.[23] Here, GAGs which were present in fish scale were estimated as 23.8 mg/g of tissue. In previous studies, the amount of sulphated GAG from their cartilage were about 10.31, 13.96, 7.53, 6.66, and 15.51g of dry weight in *Salmo salar*, *Somniosus microcephalus*, *Galeus melastomus*, *Deania calcea*, *Amblyraja hyperborea*, and *Acipenser sinensis* respectively.[12,22,24] In addition, the amount of GAG obtained from 100 g of squid shell was reported about 2 g per 100 g dry weight.

In the FTIR analysis, the presence of peaks in the range of 3160-3640 cm^{-1} is related to the OH stretching band which was stronger in the heparin, being equal to 3441/63 cm^{-1}.[25-27] The peak observed in the range of 1500 cm^{-1} to 1750 cm^{-1} was related to C = O stretching groups. The presence of peaks in the range of 979.77 cm^{-1} and 1242.07 cm^{-1} is related to the stretching groups of C-O and bending groups of C-OH in heparin structure. FTIR spectroscopy of GAGs scale showed the major peak at 1650.9 cm^{-1} which is a feature of GAGs. In FTIR spectrum the strong absorbance band at 1646 cm^{-1} (C=O stretching) and two medium size bands at 1378 and 1412 cm^{-1} (O-C=O bending) are related to uronic acids.[24] The sulphating polysaccharide was assigned to the peak in the range of 1100-1300 cm^{-1}. This peak represents asymmetric vibrations of S=O, and was shown at 1300 cm^{-1} in the FTIR of GAGs extracted from fish scale. The FTIR analysis showed that the extracted GAG had functional groups and bands similar to heparin. This result of FTIR showed that a good agreement was obtained with previous studies

Table 1. Anticoagulant Activity of the Glycosaminoglycans From Fish (*R. firisi kutum*) Scales at Different Concentrations Evaluated by the Measurement of the Activated Partial Thromboplastin Time

Sample	aPTT(s) at Different Concentrations (µ/mL)			*P* Value
	50	100	200	
Negative control	32±0.6	-	-	-
Scale GAGs	34.2±0.4	61.8±0.9	138.6±1.8	<0.05
Heparin	34.5±1.6	57.5±3.4	163.2±0.3	<0.05
P values	<0.05	<0.05	<0.001	

Results were expressed as means ±SD (n = 3) and all measurements were done in triplicate.

Table 2. Anticoagulant Activity of the Glycosaminoglycans From Fish (*R. firisi kutum*) Scales as Expressed by Prothrombin Time (PT)

Sample	PT (s) at Different Concentrations (µ/mL)			*P* Value
	50	100	200	
Negative control	13.1±0.4	-	-	-
Scale GAGs	13.2± 0.3	13.5± 0.4	14.6±0.5	<0.05
Heparin	11.2± 0.3	11.7± 0.9	163.2±0.3	<0.05
P values		11.1±1.0	<0.001	

Results were expressed as means ±SD (n = 3) and all measurements were done in triplicate.

Table 3. Anticoagulant Activity of the Glycosaminoglycans From Fish (*R. firisi kutum*) Scales as Expressed by Thrombin Time (TT)

Sample	PT (s) at Different Concentrations (µ/mL)			*P* Value
	50	100	200	
Negative control	14.7±0.2	-	-	-
Scale GAGs	16.5±0.8	17.7±0.6	20.4±0.6	<0.05
Heparin	20.5±0.4	23.5±1.2	26.7±0.2	<0.05
P values	<0.05	<0.05	<0.05	

Results were expressed as means ±SD (n = 3) and all measurements were done in triplicate.

on GAGs extracted from some bony fishes.[12,23,27]

In the present study, to evaluate the anticoagulant activity, coagulation assays of aPTT, PT, and TT were used. The aPTT and PT assays are used to evaluate intrinsic and extrinsic coagulation cascades, respectively. Meanwhile, TT assay evaluates the process of the fibrin polymerization. The results of TT and aPTT assays revealed that scale GAGs are able to prolong the coagulation time about 1.4 to 4.3 times as much as the control. However, there was no significant differences in PT recorded in the presence of GAGs and controls. The similar results were documented about anticoagulant activity of chondroitin sulphate extracted from some fish species particularly shark and sturgeon cartilage.[16,28] The same results were reported for the GAG obtained from sea cucumber which prolonged coagulation times with increasing concentration of GAGs.[23]

Therefore, the activity of extrinsic coagulation factor seems to remain unchanged by scale GAGs. In contrast, increasing values of aPPT and TT suggest that scale GAGs have ability to modulate the activity of intrinsic coagulation factors and thrombin.[23,29]

The GAG compounds like heparin possess various biological activities. The anticoagulant property of GAG compounds could be influenced by several factors such as species, structural composition and sulphating patterns of glucose amines.[13,30] The scale GAGs contain mono-sulfated disaccharides which play a significant role in anticoagulation and antithrombin properties. The interaction between GAGs and the factors involved in coagulation pathway lead to the reduction in the activities of factors, and elevation of coagulation times.[28,31]

GAG compounds extracted from fish scales showed less anticoagulant property compared to heparin. The presence of some impurities in the extracted GAG compounds could be the reason.[28] In general, high amount of GAG compound could be extracted from fish scales by the CPC method. FTIR analysis revealed structural similarity of the extracted GAG and standard heparin. The results of anticoagulant measurements (PT, aPTT, and TT) indicated that the GAG compounds were able to prolong coagulation times. Finally, due to increased consumption of this fish in the north of Iran, non-usable scales of this fish can be a good source to extract biological active compounds. It seems that further purification of the GAGs is necessary to produce more potential anticoagulant compounds. Further investigations are imperative, and after preclinical and clinical studies, the fish scale GAG extract can be used in pharmaceutical industries.

Conclusion

GAGs were extracted from fish by-products by cationic salt. These extracted GAGs had a comparable structure with heparin in functional groups, characterized by FTIR analysis. GAGs from fish scale showed the anticoagulant property. Therefore, it can be a substitute for heparin, at least in laboratory investigations.

Ethical Approval

This study was approved by the Ethics Committee of Islamic Azad University, Branch of Lahijan, Iran, and a written informed consent was obtained from the participants.

Competing Interests

The author declares that she has no competing interests.

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