Int. J. Aquat. Biol. (2021) 9(5): 344-349
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Original Article

Anticoagulant effectiveness of glycosaminoglycan extracted from the scale of Binni, *Mesopotamichthys sharpeyi* (Cyprinidae)

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Abstract: Fishery wastes are one of natural resources to extract bioactive substances such as collagen and glycosaminoglycan (GAG). The anticoagulant activity of glycosaminoglycans extracted from Binni fish, *Mesopotamichthys sharpeyi* scales was the aim of this study. The cationic salt of cetyl pyridinium chloride was used to extract the glycosaminoglycan. The structure of the isolated glycosaminoglycan was identified by ELISE glycosaminoglycan kit and compared to that of heparin. Prothrombin time (PT), and thrombin time (TT) on plasma of male mice at three concentrations of 20, 40, and 100 g/ml were used to determine the coagulant property of the extracted substance. The extracted glycosaminoglycan was calculated to be around 27.7 mg/g of dry tissue. The presence of heparin-like molecules in the glycosaminoglycan isolated from fish scales was confirmed by ELISE GAG kit. When the concentration of isolated glycosaminoglycan was increased, the time to coagulate rose. The PT and TT coagulation times were 4:1 and 2:1. Times faster than the control at 100 g/ml. When compared to synthetic anticoagulant substances like heparin, the glycosaminoglycan isolated from fish scales displayed good anticoagulation qualities.

Article history:
Received 12 July 2021
Accepted 7 September 2021
Available online 25 October 2021

Keywords:
Fish scales
Glycosaminoglycan
Prothrombin
Thrombin
Physiology parameters

Introduction

Glycosaminoglycans are polysaccharides with a negative charge (Capila and Linhardt, 2002), involving in variety of biological functions, including angiogenesis, host defense against viral infection, and blood coagulation (Zhao et al., 2013). They are divided into four families based on their disaccharide composition viz. chondroitin sulfate CS, keratan sulfate KS, hyaluronic acid HA, and heparin sulfate HS (Yamada and Sugahara, 2008). Heparin is a sulphated GAGs with anticoagulant properties utilizing in the treatment of viral infections, inflammation, and cellular proliferation in wounds and cancer, as well as in the acute coronary syndrome, lung embolism, vascular fibrillation, and deep pulmonary thumbs (Linhardt, 2003).

Glycosaminoglycans are special type of glycoprotein (Neil et al., 2005) that are prevalent in cartilages, vasculature, cell surfaces, intracellular granules, and plasma and other vertebrate's organs (Clarke, 2004; Im et al., 2013). All of these are by-products of the meat and animal industries (Volpi and Maccari, 2002) from guts and lung of cows and pigs (Linhardt and Claude, 2003). As the demand for glycosaminoglycan's rises, alternative marine resources are being used more frequently as a result, aquatic vertebrates and invertebrates have recently become increasingly important suppliers of glycosaminoglycan's (Rajapakse et al., 2005). Several researches are concentrating on the extraction of glycosaminoglycans using marine sources (Goldberg and Buckwalter, 2005). Bluefin tuna bone, jellyfish cartilaginous, and fishery wastes as potential Glycosaminoglycan's sources (Maccari et al., 2015).

Reason of mortality in the poor countries, are disorders resulting from defective clotting in the circulatory system (Zhang et al., 2014). To inhibit platelet aggregation and restore blood circulation via capillaries, medicines having anti-thrombotic, anticoagulant, and antiplatelet characteristics are utilized (Vazquez et al., 2013). The existence of heparin and heparin derivatives in marine creatures,

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DOI: https://doi.org/10.22034/ijab.v9i5. 1448

particularly squids, has been discovered (Akbulut and Akgul, 2014; Pan et al., 2017). One of significant economic fish in Iraq is the binni, *Mesopotamichthys sharpeyi* that has substantial wastes during its process especially scales, and the scales might be a promising bioactive substances resource (Tingbø et al., 2005). Therefore, in this study scales were used to extract glycol-saminoglycan (GAG) and assess their anticoagulant effectiveness *in vitro* utilizing traditional coagulation assays (World Health Organization 2008).

Materials and Methods

Sampling and preparation: Scales of the binni were obtained from a fish market in Al-Diwaniyah, Iraq. To produce the dry powder, the fish scales were cleaned thoroughly with tap water, freeze-dried, then ground in a mixer. A total of 5 g scale powder was incubated for 1 hr at 80°C in 100 ml of $MgSO_4$ 8.0 mM, then NaCl was used to raise the pH of the solution to 10. The pH was then reduced to 7 using ammonium sulfate ($2S0^4NH_4$) and heated at 50°C for 120 minutes. After filtering, the solution has been subjected to CINH 38 C21 / benzydamin (CPC). The filtrate was then treated with 20 ml of CPC (3: 0.8 M Nacl). The solution was kept at 37°C for 1 day, resulting in a white color precipitate. 5 ml NaCl before heated to 50°C was applied to extract N_5H_5C salt from of the precipitation. The sulfonated polysaccharide was precipitated with 75% ethanol and afterward the methanol was used to wash the precipitate multiple times before freeze-drying for an hour (Najjam et al., 1997; Tomatsu et al., 2005; Gui et al., 2015) (Fig. 1).

Glycolsaminoglycans (GAGs) detection: GAGs are extensively dispersed and, based on the particular GAG, are linked to physiological and pathological functions. As a result, the development of precise, quick, sensitive, and specific assays of certain GAGs is critical. GAGs are used not only for diagnosis and therapeutic efficiency, but for many other abnormalities in which are down or up regulated, such as mucolipidoses (Tomatsu et al., 2005), cancer (Yip et al., 2006), osteoarthritis (Plaas et al., 1998), rheumatoid arthritis (Wang and Roehrl, 2002),

Figure 1. *Mesopotamichthys sharpeyi* (1), Fish scales (2) demineralization of scales (3) and filtration and extraction glycolsaminoglycans (4).

diabetes (Olczyk et al., 1997), infectious diseases (Iaci et al., 2007), and spinal cord damage (Aydin, 2015; Engvall, 2010).

In this study, glycolsaminoglycans assay kit (Colorimetric method) we used and for this purpose enzyme-linked immunosorbent assay (480 nm) bought from Chondrex Company (Woodinville, WA 98072, USA) and using the company protocol, the antigen is bound to an antibody that is coupled to an enzyme, and antigen is detected by hydrolysis of a substrate by the linked enzymes (Mucci et al., 2000; Shute, 2012).

Coagulant test: The anticoagulation test was performed using citrated ICR mice platelet deficient plasma. The plasma was taken from 36 male mice (with 20±2 g weight) after dividing into 3 groups as control, glycolsaminoglycans and Heparin treatments each containing 12 mice. The plasma was mixed with 0.5% $Na_3C_6H_5O_7$ solution as 99:1 blood to sodium citrate. The plasma was prepared by centrifuging at 5400 g for 25 min and then filtered and kept at -50°C until to use (Krylov et al., 2011). Standard clotting assays were used to determine the anticoagulant action of the isolated prothrombin time (PT), and thrombin time (TT). Sodium hydroxide was used to dissolve the sample glycolsaminoglycans. All of the procedures had been done three times (Majdoub et al., 2009).

Evaluating prothrombin time (PT): 70 pl of mice plasma was mixed with 30 pl heparin, 30 g/mL NaCl, and different concentrations of isolated GAGs (20, 50,

Table 1. Relationship between protheombin time (PT) and thrombin (TT) with glycosaminoglycan as PT at various concentrations (/ml).

PT	20	40	100	*P*
Glycolsaminoglycans			20.4±0.4	<0.05
Heparin			23.6±0.7	<0.05
Control	10.2±0.3	10.2±0.3	10.2±0.3	
Control	12.7±0.5	12.7±0.5	12.7±0.5	
Glycolsaminoglycans	20.5±0.4	23.5±1.2	24.7±0.2	<0.05

The results were expressed as means ±SE (n = 12/group)

and 100 pg/l) to measure the PT. For 15 minutes, the mixes were incubated at 50°C (Chondrex, USA). Then 100 pl of thromboplastin reagent before incubating at 50°C PT was added, and the clotting time was measured using a coagulometer (Garnjanagoonchorn et al., 2007).

Evaluating thrombin time (TT): 70 pl of mice plasma were mixed 10 s of various GAG levels (20, 40, and 100 pg/mL) and incubated at 50°C for 5 minutes. The TT was then recorded when 100 pl of thrombin was added. Heparin and NaCl were used as control sample, consequently, in place of the 10 pl of glycolsaminoglycans (Pomin and Mulloy, 2015).

Data analysis: Due to the normal distribution, two-way analysis of variance ANOVA was employed to compare the groups. In addition, as a post hoc test, the Duncan multiple-range test was used. The mean and standard deviation were used to express all of the data (SE). $P<0.05$ was used to determine the significance of the difference. SPSS software version 20.0 was used for data analysis.

Results

The anticoagulant properties of the extracted glycolsaminoglycans are shown in Table 1. The mean of PT and TT for mice plasmas were 20.4±0.4, 16.0±0.3 seconds, respectively. There was a significant difference in PT and TT between concentrations of the extracted glycolsaminoglycans ($P<0.05$). The anticoagulant indices of glycols-aminoglycans of fish scale increased as the concentrations of glycolsaminoglycans elevated. The prolonged coagulation times were observed in the presence of the extracted glycolsaminoglycans at the same concentrations, compared to control. At a concentration of 3 100 g/mL, the PT and TT glycols-

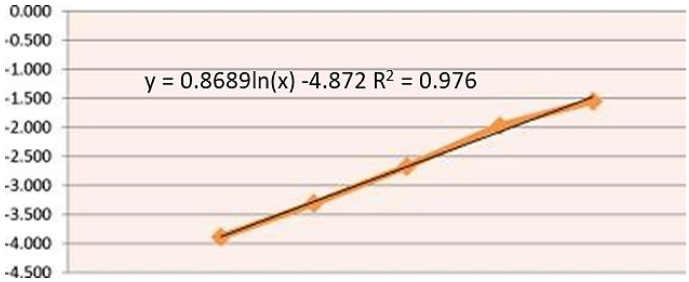

Figure 1. Stander curve of glycolsaminoglycans assay kit from normal values.

aminoglycans of were 20.4±0.4 and 19.4±0.6 seconds, respectively which was 4 and 2 times slower than the control group. Although, in the PT ($P<0.05$), there was significant variance between glycolsaminoglycans and heparin compared with control group.

Discussions

Anticoagulant properties are found in heparin and heparin-like substances such as Glycolsaminoglycans and keratan sulphate in marine fishes (Athukorala et al., 2006). The quantity of glycolsaminoglycans found in fish scale of the binni fish was 27.7mg/g of tissue. ***Salmo salar, Somniosus microcephalus, Galeus melastomus, Deania calcea, Amblyraja hyperborea,*** and ***Acipenser sinensis*** had 10.31, 13.96, 7.53, 6.66, and 15.51g of the sulphated Glycolsaminoglycans from their cartilage in pervious researches, respectively (Plaas et al., 1998; Tomatsu et al., 2005; Maccari et al., 2015). Furthermore, the amount of glycolsaminoglycans extracted from 100 g of squid shell was estimated to be around 2 g /100 g dry weight (Yang et al., 2015).

Coagulation analyzes of PT, and TT were performed in this work to assess anticoagulant activity. Intrinsic and extrinsic coagulation cascades are assessed using the PT test and the TT test assesses the fibrin polymerization process. The findings of the

TT showed that scale glycolsaminoglycans can extend the coagulation time by 4:1 to 2: 1 times. There were significant variations in PT in the influence of glycolsaminoglycans. Anticoagulant action of chondroitin sulphate isolated from various fish species, i.e. shark and sturgeon cartilage, has also been observed (Pan et al., 2017). The glycolsaminoglycans extracted from sea cucumber produced similar results, with longer coagulation times as the content of glycolsaminoglycans elevated (Yip et al., 2006).

As a result, scale glycolsaminoglycans seem to have effect on the activity of extrinsic clotting factors. The increased TT levels imply that scale glycolsaminoglycans can influence the action of intrinsic coagulation factors and thrombin (Engvall, 2010). Heparin, for example, is a similar glycolsaminoglycans molecule with a wide range of biological functions. Several factors, including species, structural composition, and sulphating patterns of glucose amines, might alter the anticoagulation function of glycolsaminoglycans substances (Goldberg and Buckwalter, 2005; Tomatsu et al., 2005; Maccari et al., 2015).

The size of the scales' mono-sulfated disaccharides, which are found in glycolsaminoglycans, play an important role in anticoagulation and anti-thrombin activities. The interaction of glycolsaminoglycans with the factors involved in the coagulation pathway results in a decrease in factor activity and an increase in clotting times (Majdoub et al., 2009; Pomin and Mulloy, 2015). When compared to heparin, glycolsaminoglycans compounds isolated from fish scales demonstrated reduced coagulant activity. It is probably the amount of pollutants in the extracted glycolsaminoglycans molecules is to blame (Plaas et al., 1998; Iaci et al., 2007). The CPC method could extract a large amount of glycolsaminoglycans compound from fish scales. The coagulation tests PT, and TT revealed that the glycolsaminoglycans molecules might delay coagulation periods. Finally, non-usable scales of fish can be a useful source of biological active chemicals (Engvall, 2010; Aydin, 2015). As conclusion, glycolsaminoglycans from fish scales were found to have coagulant properties by delayed prothrombin and thrombin times in the plasma of male mice and it can be used as a heparin alternative, at least in experimental studies.

Acknowledgements

We would like to express our sincere thanks to people who took part in the experiments. The author also expresses gratitude to the Research Clinical science laboratory staff in Pharmacy collage, University of Al-Qadisiyah, for supporting and completion of this work.

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