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A Comporative Study of Anticoagulant and Antiproliferative Activity of Fast Moving Heparin From Giant Clam *Tridacna maxima* (Roeding,1798) and Green Mussel *Perna viridis* (Linnaeus,1758)

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Abstract

Heparin was isolated from two bivalve mollusks such as *Tridacna maxima* and *Perna viridis*. The isolated heparin was quantified in crude as well as purified samples and they were estimated as 2.72 & 2.2 gm / kg (crude) and 260 & 248 mg/gm (purified) in *T. maxima* and *P. viridis* respectively. Both the bivalves shown the anticoagulant activity of the crude and purified sample 20,128 USP units per kg and 7.4 USP units per mg and 39,000 USP units per kg and 75 USP units per mg and 9,460 USP units per kg and 4.3 USP units per mg and 13,392 USP units per kg and 54 USP units per mg correspondingly in *T. maxima* and *P. viridis*. The antiproliferative activity was studied with pulmonary artery smooth muscle cells (PASMC) using RPMI media reported that the result in a dose dependent manner. Among the two clams, *P. viridis* showed more antiproliferative activity than that of *T. maxima.*

Key words: antiproliferation; glycosaminoglycans; giant clam; green mussel; heparin.

INTRODUCTION

Glycosaminoglycans (GAGs) have been isolated from various tissues obtained from a large number of animal species including both vertebrates and invertebrates. Invertebrates were first shown to contain a heparin or heparan sulfate [1]. An exhaustive assessment showed that the mollusks are particularly rich source of these sulfated polysaccharides and it often corresponds up to 90% of the total GAG content these organisms [2]. Heparin and heparin-like substances have a wide range of important biological activities including inhibition of pulmonary artery smooth muscle cell (PASMC) proliferation. In the normal physiological state, the smooth muscle cells (SMCs) are entering into quiescent growth state in pulmonary arterial walls which is regulated by a balance between inhibitory and mitogenic factors [3]. The major effect of heparin on blood coagulation is to accelerate the normal rate at which antithrombin III neutralizes the proteolytic activities of several serine proteases in the coagulation sequence. The search for the new sources of heparin with low toxicity prompted many scientists to focus their attention towards marine animals. Earlier studies have shown that heparin-like compounds are also present in some invertebrates [4, 5]. A substance denoted 'mactin' with anticoagulant activity and structural similarities to mammalian heparins was isolated from the molluscs *Cyprinia islandica* and *Mactrus pussula*. Another compound from the clam *Mercenaria mercenaria* also exhibited several structural similarities to heparin. Previously, conducted detailed investigations on the heparin isolated from the clams [6] *A. braciliana* and *T. mactroides* and found that the clam heparin preparations have basically the same structure as mammalian heparins, but notable differences included the greater chain length of clam heparin. Further heparin from some mollusks and found that the difference in their anticoagulant activity [7]. In the present study was find out the source of heparin and discuss their antiproliferative and anticoagulant activity.

MATERIALS and METHODS

Isolation

The standard procedure was followed for the extraction of heparin, with suitable modification [8] for the defating and deproteinisation of *T. maxima* and *P. viridis* treated with CPC. The purification of the crude heparin complex was done by the using the anionic resin (Amberlite IRA-900 (Cl^-)). The purified glycosaminoglycans were converted into heparin sodium salts by using cationic resin (Amberlite IR-120 Na^+) and the recovered precipitate was taken for further analyses [9].

Electrophoresis

Electrophoretic analysis was performed on agarose gel plate (7.5×5.0 cm, 0.1cm thick) prepared with 0.5% agarose in 0.1M 1,2,diaminopropane acetate buffer (pH 9.0) and run as described method [10].

Anticoagulant activity

Heparin readily catalyzes the inactivation of factor Xa by antithrombin III. Factor Xa inactivation was used in this study to assess the anticoagulant activity of the GAGs prepared from both mussels using a Heparin Assay Kit (Sigma). In this assay, when both factor Xa and antithromin III is present in excess the inhibition of factor Xa is directly proportional to the limiting concentration of heparin. Thus, residual factor Xa activity, measured with factor Xa-specific chromogenic substrate, is inversely proportional to the heparin concentration [11, 12].

Antiproliferative activity

The isolated bovine pulmonary artery smooth muscle cells were seeded at 1.5×10^4 cells/well into a 6-well tissue culture plate, grown for 2 days; then growth was arrested at the end of 48 hrs by reducing the serum concentration of the medium from 0.1 to 10 percent. The media was then changed to the experimental samples which contained either standard media (RPMI – 1640 with 10% fetal bovine serum (FBS) (Sigma, St. Louis, Mo), growth arrest media (RPMI with 0.1% FBS) or standard media with oligomers/heparin (5µg/ml). All media contained streptomycin (100µg/ml), Penicillin (100U/ml) and amphotericin B (1.25µg/ml). After 4 to 5 days of growth, the cells were lifted with trypsin / EDTA and then counted using a Coulter Counter. Results are presented as mean ± standard error of the mean. Comparisons among groups were made with a factorial analysis of variance (ANOVA), using the STATE VIEW software package (Brainpower, In., to Calabases, CA.) for Macinotosh computers.

RESULTS

The amount of heparin (heparin complex) was estimated as 2.72gm per kg of dry tissue in *T. maxima* and 2.2gm/kg in *P.viridis* (crude). After purification by using the amberlite anion exchange resin, the yield was found as 260mg/gm and 248mg/gm respectively in *T. maxima* and *P. viridis* were presented in Table 1.

Table 1. The yield of crude and purified heparin complex of T. maxima and P. viridis.

S. No.	Source	Net Yield	
		Crude (gm/kg)	**Purified(mg/gm)**
1.	Tridacna maxima	2.72	260
2.	*Perna viridis*	2.2	248

The electrophoretic pattern of heparin in the two species indicates the presence of fast moving heparin, as identified with standard (Fig.1).

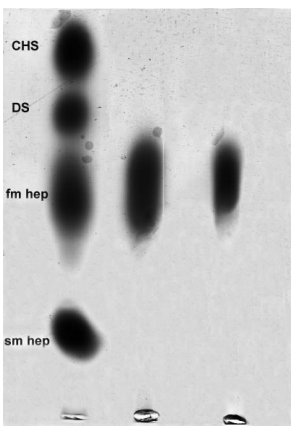

Figure 1. Agarose gel electrophoresis with 1,2-diaminopropane buffer.
St. – GAGs standards **P.m-** *P. viridis*; **Tm-** *T. maxima*
sm – Slow moving heparin, **fm** – Fast moving heparin, **DS** – Dermatan sulphate **CHS** – Chondroitin sulphate

Table 2. The anticoagulant activities of the heparin extracted from T. maxima and P. viridis

S. No.	Origin	Anticoagulant assay (USP method)			
		Crude		Purified	
		Activity IU/mg	**Yield IU/kg**	**Activity USP units/mg**	**Yield USP units/kg**
1.	*Tridacna maxima*	7.4	20,128	75	39,000
2.	*Perna viridis*	4.3	9,460	54	13,392

In the anticoagulant assay (Table. 2) the yield and anticoagulant activity of the *T.maxima* crude sample were reported to be 20,128 USP units per kg and 7.4 USP units per mg; whereas the purified sample showed, 39,000 USP units per kg and 75 USP units per mg of the sample respectively. Likewise in the case of *P. viridis*, the yield and anticoagulant activity were estimated as 9,460 USP units per kg and 4.3 USP units per mg in the crude sample and 13,392 USP units per kg and 54 USP units per mg in the purified sample respectively.

The effect of heparin (GAGs) isolated from *P. viridis* & *T. maxima* and Upjohn heparin grown in RPMI medium containing 0.1% and 10% FBS is depicted in Fig. 2. The heparin and heparin-like GAGs isolated from *P. viridis* recorded increasing inhibition over the growing cells when the concentration increased from 1.0µg to 100µg i.e., the percentage cell mean in the case of 1.0µg was found as 61.764±3.660, in 10µg it was 33.064±3.507 and 100µg concentration it was 15.071±4.609 showing an increasing influence over the cell growth. The inhibition of *P. viridis* is comparatively more even than that of Upjhon heparin, the standard used in the present study, which showed the percentage mean cell growth as follows: 1µg-72.533±11.200, 10µg-8.809±7.940 and 100µg -19.168±5.921 respectively.

But on the contrary, the heparin and heparin-like glycosaminoglycans extracted from *T. maxima* was found to promote the cell growth in lower concentrations i.e., 1µg -

134.939±26.468% and 10µg - 123.934±25.325%. But at the same time, at higher concentration it was also found to be reducing the growth (at 100µg concentration the mean cell growth was only 77.429±18.923%).

DISCUSSION

In the present investigation, the yield of crude heparin complex was found as 2.72gm/kg and 2.2gm/kg in *T. maxima* and *P. viridis* respectively. Whereas quantified the heparin yield as 2.2 to 2.8gm/kg, 1.8 to 2.5gm/kg and 2.7 to 3.8 gm/kg in *A. brasiliana*, *D. striatus* and *T. mactroides* respectively [7]. At the same time the yield of heparin and other sulphated mucopolysaccharides from thymus as 274µg/kg only [13]. Likewise, the sulfated mucopolysaccharides isolated by using quaternary ammonium salts [4] and reported the yield as 170, 174, 843, 307 and 1,090 µg/kg dry tissue in different molluscan species such as *Aulocombia ater*, *Perna perna*, *Mesodesma donacium*, *Loligo brasiliense* and *Octopus* species respectively.

The present electrophoretic observation in the two species reveals migration of the heparin and heparin-like

glycosaminoglycans is closely similar to that of the fast moving heparin. The previous study in the invertebrates [4] also reported the same migratory pattern of bands for the glycosaminoglycans. Particularly the *Octopus* sp. showed a polydisperse sulfated mucopolyaccharides migrating in the region corresponding to all the sulfated mucopolysaccharide standards.

In the present study, the crude sample of *T. maxima* and *P. viridis* reported 7.4 USP units/mg and 4.3 USP units/mg of anticoagulant activity respectively. But in the purified sample of *T. maxima* and *P. viridis,* the anticoagulant activity was found to be higher (75 USP units/mg and 54 USP units/mg respectively) than that of the crude sample which is further higher than that of *K. opima* (39.7 BP units /mg), Sheep heparin (59 USP/mg), fin back whale *Balaenoptera physalus* (40 to 70 USP units/mg), *H. pugilinus* (26 USP units/mg) [14] and heparan sulfate anticoagulant activity [15] in *Pomacea* sp., *T. gibbus*, *A. brasiliana*, beef pancreas (<5 IU/mg) and lobster heparan sulfate (16 IU/mg) respectively [16].

The anticoagulant assay in the purified sample of *T. maxima* (39,000 USP units/kg) is lower than that of the *K. opima* [17] (2,83,370 USP units/kg) and *A. rhombea* (74,140 USP units/kg). But at the same time, it is higher than that of *C. madrasensis* [16] (14,125 USP units/kg) and *O. vulgaris* [17] (12,960 USP units/kg). Whereas in the case of *P. virdis* the yield in the anticoagulant assay was reported as 9,460 USP units/kg and 13,392 USP units/kg in crude and purified sample respectively. This variation might be due to the presence of non-anticoagulant substance in the samples since the activity of heparin depends upon the amount of impurity carried over in the isolated products [18].

Antiproliferative activity of isolated heparin from *P. viridis* could also be compared with that of (Dahlberg et al., 1996) in which they reported such inhibition over the cell growth in Elkins-Sinn heparin on PASM cells. But at the same time, they also observed a stimulatory effect on PASM cell growth (1.4% increase) at 1.0µg/ml concentration of Choay heparin which has also reported less antiproliferative activity of 29±5% at 10µg/ml concentration like the heparin GAGs extracted from *T. maxima* (Fig. 2).

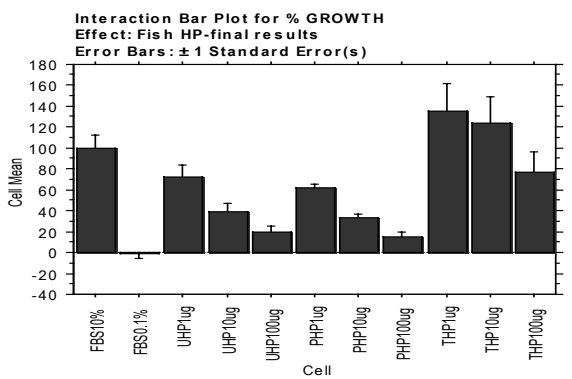

Figure 2. Shown the antiproliferative effect of heparin s from P. viridis (PHP) andT. maxima (THP) and Upjohn heparin (UHP).

Further it is interesting to note that the same commercial standard Upjohn heparin showed varying antiproliferative activity on the PASM cells in a medium containing 0.1% FBS. In the present study, it produced 27.36% and 61.19% of inhibition on the PASM cells grown in RPMI medium containing 0.1% and 10% FBS; whereas it showed an antiproliferative activity of 48±5 and 80±4 at the concentration of 1 or 10µg/ml [19].

The results reveal that there is a dose dependant decrease in the percentage of viable cells when treated with the heparin and heparin-like GAGs isolated from *T. maxima* & *P. viridis* and standard heparin. There was a significant reduction in the number of live cells in the case of all the concentrations of heparin GAGs from *P.viridis* in an ascending order with increasing concentration. But, at the same time, the isolated compound from *T. maxima* did not show any inhibitory effect in 1µg and 10µg concentration and it showed some inhibitory effect in 100µg concentration. From the above results, it is quite evident that the heparin GAGs isolated from *P. viridis* has more antiproliferative effect than even the commercial standard Upjohn heparin and that of *T. maxima*.

Apart from the above, the previous investigations have shown variable effects of heparin on cell growth. Heparin did not inhibit endothelial cell prolilferation [20, 21] demonstrated the inhibition over the aortic smooth muscle cell growth. When using the rat aortic smooth muscle cells, reported varying antiproliferative activity in 10 different commercial heparins [22]. Among them, two lots of heparin stimulated the cell growth at low concentration as the heparin GAGs isolated from *T. maxima*. Therefore, both the cell type to be studied and the source of heparin are important in determining the antiproliferative activity of heparins. Further, for as yet uncertain chemical reasons, not all lots of heparin have the antiproliferative activity to the same degree [19]. So the foregoing account may shed light on the previous paradoxes in which different commercial heparin GAGs isolated from different sources have had variable antiproliferative effects even on the same cell types.

Further the inhibiting effect of heparin on smooth muscle cell growth in vitro has been shown to be independent of its anticoagulant activity [23, 24]. This concept is very well supported by the results of the present study also. In the present investigation though the purified heparin and heparin-like GAGs extracted from *T. maxima* reported more anticoagulant activity (30,212 IU/kg) than that of *P. viridis* (16,144IU/kg), the heparin GAGs from *P. viridis* showed more antiproliferative activity than that of *T. maxima* which showed stimulatory effect also in lower concentrations (1µg and 10µg) studied. This suggests that the anticoagulant activity does not correlate with the ability of a given heparin GAGs to inhibit the cell growth. From the foregoing account it could be concluded that the heparin GAGs isolated from *P. viridis* and *T. maxima* has an anti-cell proliferaative component which needs to be further studied for using it as an anticancer drug.

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