

Studies on Glycosaminoglycans Isolated from Bivalves Molluscs *Tridacna maxima* and *Perna viridis*

M. Arumugam¹, T. Balasubramanian², M. Warda² and R.J. Linhardt²

¹Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai-608 502, India.

²Department of Chemistry, Department of Chemical and Biochemical Engineering, Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa, USA

Received: 21.01.2009, Accepted: 09.04.2009

Abstract

The glycosaminoglycans (GAGs) in two marine invertebrate molluscs such as *Tridacna maxima* and *Perna viridis* were analyzed. Both the species was found to contain variable amounts of GAGs in the form of heparin biomolecules as identified by metachromatic activity and agarose gel electrophoresis analysis. Anticoagulant property of the biomolecules was assessed by anti factor Xa activity. Their molecular weight was estimated as 15000 and 9000 Daltons through GPC-HPLC. The ¹H-NMR analysis of heparin was used to predict binding sites of the heparin. Structural characterization studies clearly demonstrated that heparin is the major GAGs constituents in the test animals.

Key words: Anticoagulant, Bivalves molluscs, Glycosaminoglycans, Heparin, Invertebrates

Introduction

Glycosaminoglycans (GAGs) have been isolated from tissues of a large number of vertebrate and invertebrate organisms. Invertebrates were first shown to contain a heparin or heparan sulfate (Burson *et al.*, 1956). An exhaustive assessment showed that molluscs are particularly rich source of the sulfated polysaccharides (Nader and Dietrich, 1989) and it amounts to 90% of the total GAGs content of the molluscs.

But the heparins isolated from molluscs are structurally different from human heparin and pharmaceutical heparins (Loganathan *et al.*, 1990). The Phylum Mollusca shows extensive species diversity and their bi-products have received much attention from the beginning of 20th century. Among the molluscs, some have pronounced bio activities and useful in the biomedical arena. It is surprising that some

of the bioactivities are attributed to the presence of polysaccharides, particularly those that are sulfated.

Heparin and heparan sulfate have been the subject of intensive study because of their well-recognized ability to bind proteins that regulate a variety of important biological processes. Heparin and heparan sulfate are comprised of alternating 1→4 linked glucosamine and uronic acid residues. Heparan sulfate is composed of primary monosulfated disaccharides of N-acetyl-D-glucosamine and D-glucuronic acid, while heparin is composed of mainly trisulfated disaccharides of N-sulfoyl-D-glucosamine and L-iduronic acid (Linhardt and Toida, 1996).

This paper reports the isolation of glycosaminoglycans and their structural

features with special reference to heparin from marine bivalve molluscs.

Materials and methods

Isolation

The whole organisms were blended and defatted with acetone, filtered and further defatted with petroleum ether. The defatted tissues were air dried at room temperature. 50 gm of dried defatted tissues were ground and mixed with 500 ml of 0.4 M Sodium Sulphate (Na_2SO_4). The mixture was incubated at 55°C for 1 hour and 30 minutes. The pH was maintained at 11.5. After incubation, Aluminium-di-Sulphate $\text{Al}_2(\text{SO}_4)_3$ crystals were added to bring down the pH to 7.7 and heated to 95°C for 1 hour. The sample was cooled and centrifuged at 2000 rpm.

After centrifugation the supernatant was collected and treated with Cetyl Pyridinium Chloride (CPC) purchased from Hi-medea, Mumbai, India for further extraction. To the collected supernatant, 70 ml of 3 % CPC in 0.8 M Sodium Chloride (NaCl) was added.

The mixture was stirred until the precipitation was completed. This suspension was incubated at 37°C for 24 hours and centrifuged at 4°C for one and half hours in a refrigerated centrifuge to collect the crude heparin complex. The precipitate was redissolved in 2M NaCl to remove pyridinium salts from heparin and centrifuged for 30 minutes at 2500 rpm in refrigerated centrifuge. The supernatant was collected and 3 volumes of 99.9% methanol were added to the precipitated crude heparin. The heparin complex was collected by centrifugation at 3000 rpm at 4°C for 30 minutes in a refrigerated centrifuge. The precipitate was also washed with 99.9%

ethanol and then kept in vacuum desiccators for drying the precipitate of crude GAGs complex.

Purification

After dissolving dried GAGs materials in double distilled water, followed by centrifugation in microfuge (10000 rpm for 5 min in cold room), supernatants were applied on the activated SAX column. After washing with water, elutions were made using 3% and 16% NaCl (w/v) to get low and high sulfated fractions, respectively. In both eluted fractions, Glycosaminoglycans (GAG) precipitation was made at 16% NaCl using 85% MeOH (v/v). The recovered precipitates were desalted using Ultrafree-MC spin column (5000 NMWL-amicon®) and freeze dried. To exclude the chondroitin sulfate trace, the resulting GAGs were digested by chondroitin ABC lyase (EC 4.2.2.4) from *Proteus vulgaris* (0.2 m unit/100µg in 50 mM sodium acetate, pH 8) at 37 °C for 24 h in sealed tubes. After digestion, the reactions were terminated by heating in boiling water bath for 5 minutes and the digested samples were desalted using microanalysis desalting spin column (Amika Corp®) and freeze dried. Table 1 (results) shows the yield of each fraction after the purification steps. As the 3% eluted fractions yields are very poor so we concentrated on the 16% eluted fractions for doing further characterization.

Table 1. Extractability of GAGs in different solvents from two marine molluscan species

Animals	Yield (mg)		
	Water	3% NaCl	16% NaCl
<i>Tridacna maxima</i>	2.4	0.1	3.9
<i>Perna viridis</i>	1.4	0.6	7.7

Chemical characterization

Azure-A assay was performed to estimate the level of sulfo group substitution of the purified GAGs. Metachromasia of the blue dye on addition of negatively charged GAGs results in a concentration-dependent increase in absorbance at 530 nm (Grant *et al.*, 1984). Carbazole assay was performed to determine content of uronic acid in the GAG preparation by determining the absorbance at 525 nm (Bitter and Muir, 1962). Standard Porcine intestinal heparin was used in both cases to prepare standard graphs.

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 antithrombin 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 (Teien *et al.*, 1976; Teien and Lie, 1977).

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 previously (Bianchini *et al.*, 1980).

Physico-chemical analysis

Carbon, Hydrogen and Nitrogen contents in the extracted samples were analysed by

using micro elemental analyzer Carb ERBA model 1106.

The optical rotation of both samples was measured with Rudolph Polarimeter at a concentration of 5% in double distilled water at 20°C.

The carbazole reaction which is the most satisfactory method for estimating uronic acid (Bitter and Muir, 1962) was followed for the estimation of Uronic acid. The methodology described by (Tehro and Haritiala, 1971) was followed to determine the sulfate content.

¹H-NMR analysis

NMR (Nuclear Magnetic spectroscopy) was performed on samples (~3 mg) dissolved in D₂O (99.96 atom %), filtered through a 0.45 µm syringe filter, freeze-dried twice from D₂O to remove exchangeable protons and transferred to Shigemi tubes. One dimensional (1D) ¹H-NMR experiments were performed on a Bruker DRX-400 equipped with NMR Nuts (PC computer) processing and plotting software.

Results

The yields of GAGs after SAX column elution with water, 3% NaCl and 16% NaCl for *T. maxima* and *P. viridis* are shown in the following Table 1. The net yields of 3% elution were poor than that of 16% NaCl in both cases, therefore, all the following data was represented in 16% NaCl fractions.

The yield and activity of heparin in the experimental samples by metachromatic dye method were found to be 30212 IU units per kg and 116 IU units per mg respectively in *T. maxima*. In the case of *P. viridis*, the yield and activity were estimated as 23,822 IU units per kg and 96 IU units per mg respectively. All the physico-chemical

Table 2. The physico-chemical properties of the glycosaminoglycans from *T. maxima* and *P. viridis*

Origin	Carbon %	Hydrogen %	Nitrogen %	Optical rotation $[\alpha]_D^{25}$	Sulfate %	Uronic acid %
<i>T. maxima</i>	16	2	1	+36	18	23
<i>P. viridis</i>	16	2	2	+31	11	19

Table 3. Molecular weight of the glycosaminoglycans from *T. maxima* and *P. viridis*.

Components Polydispersity	Molecular weight (Da)		
	RI	UV	UV/RI
<i>Tridacna maxima</i>	15000	-	1.11
<i>Pema viridis</i>	9000	-	1.44

properties of the isolated compounds are presented in Table 2.

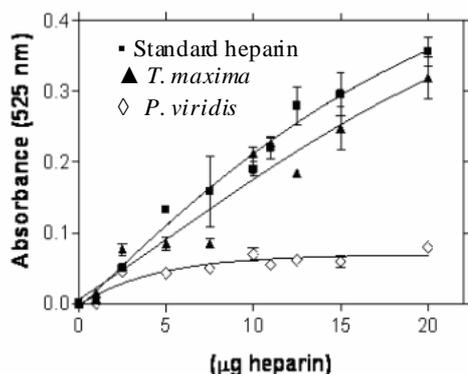


Figure 1. Azure A assay of *T. maxima* and *P. viridis* Glycosaminoglycans.

Chemical characterization by Azure A and carbazole assays (Figures 1-2) showed that *T. maxima* contained approximately more than the half of negative charge found in a standard porcine heparin. The uronic acid content of the isolated glycosaminoglycans from both the clam and mussel estimated as 23 and 19 in *T. maxima* and *P. viridis* respectively.

The electrophoretic pattern of heparin in the two species indicates the presence of

fast moving heparin, as identified with standard (Figure 3). The molecular weight was obtained in the GPC-HPLC system by using the UV and RI detectors with column TSK G2000 SW and TSK G3000 SW joined series are presented in Table 3. The molecular weight for *T. maxima* and *P. viridis* was determined as 15000 Da and 9000 Da respectively when using RI detectors. But at the same time, no peaks have been detected in the UV detectors were used.

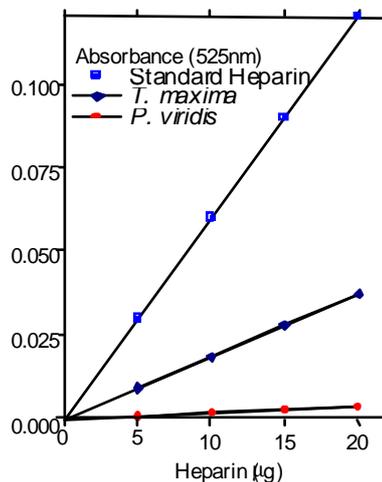


Figure 2. Carbazole assay for GAGs from two molluscs (*T. maxima* and *P. viridis*).

Anticoagulant activity of *T. maxima* and *P. viridis* as measured by factor Xa amidolytic assay, showed significantly ($P \leq 0.05$) lower activity (20.6 ± 3.2 and 12.05 ± 2.6 IU/mg) in comparison with heparin standard from porcine mucosa (120.5 ± 23.5 IU/mg) was presented in Figure 4.

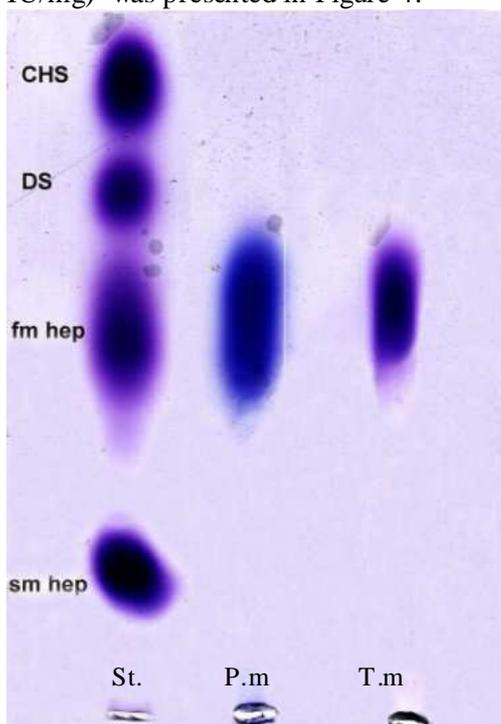


Figure 3. Agarose gel electrophoresis with 1, 2-diaminopropane buffer (St.= GAGs standards, P.m=*P. viridis*, T.m=*T. maxima*, sm= Slow moving heparin, fm= Fast moving heparin, DS= Dermatan sulphate, CHS= Chondroitin sulphate).

$^1\text{H-NMR}$ analysis (Figures 5-6) revealed the presence of signals corresponding to GlcNAc (N-acetyl methyl at 1.99 ppm and H-1 at 5.4 ppm) in both samples, which is more prominent in *P. viridis* than *T. maxima*. At 4.48 and 3.35 ppm, there are very clear H-1 and H-2 signals of GlcA respectively in *T. maxima*. These two

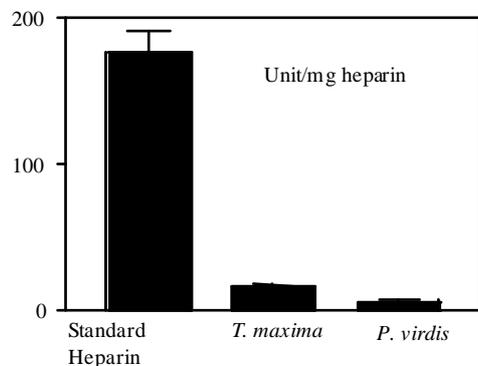


Figure 4. Comparison of anti-factor Xa activities of heparin from porcine intestine (Standard), *Tridacna maxima* and *Perna viridis*. (Values of bars are mean \pm SEM of at least 5 times determinations).

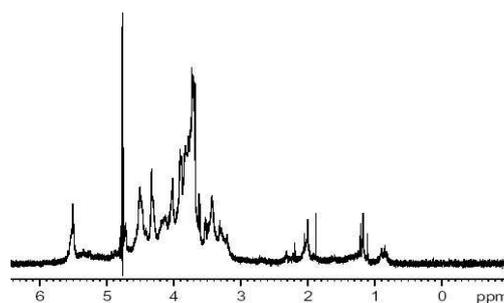


Figure 5. $^1\text{H-NMR}$ spectroscopy of GAGs from *Tridacna maxima*

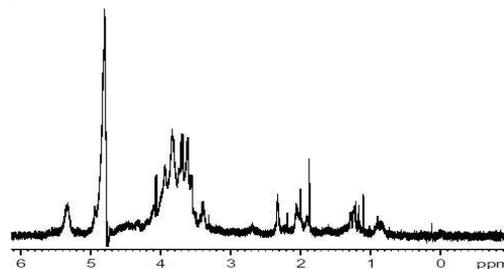


Figure 6. $^1\text{H-NMR}$ spectroscopy of GAGs from *Perna viridis*

signals are not apparent in *P. viridis*. The presence of H-1 and H-2 signals of GlcN S at 5.5 and 3.25 ppm, respectively, and the small signal of N-acetyl methyl signal of

GlcNAc show that the glycosaminoglycans isolated from *T. maxima* is more sulfated than that of *P. viridis*.

Discussion

In any compound, the structural variations is ascertained by the concentration of three major elements viz., Carbon, Hydrogen and Nitrogen because the bonding pattern of these elements are responsible for the structural conformation and properties.

Carbon, Hydrogen and Nitrogen percentage in glycosaminoglycans is 16.50 (C), 2.93 (H) and 1.11 (N) in *T. maxima* and 16.02 (C), 2.08 (H) and 2.10 (N) in *P. viridis*, as against 13.0-24.6% (C), 2.0-5.3% (H) and 0.6-4.5% (N) in (Kavanagh and Jaques, 1973). From the above results, it could be seen that the carbon, hydrogen and nitrogen levels of *T. maxima* and *P. viridis* are lying within the range of elements in the commercial heparin. This is further substantiated in the anticoagulant activity of heparin complex isolated from molluscs, similar to the commercial heparin.

Previous reports have recorded different percentage of elements-C,H,N are 23.5, 3.7 and 2.4 per cent respectively in *Katelysia opima* (Somasundaram, 1990) and they are 23.5, 4.67 and 2.4 in *Hemifusus pugilinus* (Benny, 1996).

The seaweed *Acanthophora spicifera* showed intermediate values of 12.8, 2.1 and 0.5 per cent respectively. The variation in the C, H and N content may be due to the variation of disaccharide pattern present in the heparin complex isolated from different sources.

Optical rotation is a tool for the identification of different sulfated polysaccharides. The common glycosaminoglycans, heparin and heparan

sulphate show positive optical rotation whereas other group of galactoaminoglycans viz., Chondroitin sulphate and Dermatan sulphate exhibit the negative optical rotation (Bartolucci *et al.*, 1995). Further, heparin which has positive optical rotation is capable of forming salts with metals and has uronic acid, sulfate and glucosamine components (Jorpes, 1939; Jaques, 1967). This is the reason why the measurement of optical rotation and the estimation of uronic acid, sulfate and glucosamine have been attempted in this study.

In the present study the optical rotation of heparin and heparin sulfate in both the molluscan samples is as +36° and +31° in *T. maxima* and *P. viridis* respectively. This rotation is lesser than that of porcine mucosal heparin (+53°) (Kim *et al.*, 1996), the lobster *Homon americanus* (+46° to +80°) and that of the commercial beef liver heparin (+51°) (Hovingh and Linker, 1982). Similarly positive rotation value is +50° for the unfractionated heparin and low molecular heparin and +48° for oligo-heparin produced from opocrin (Bianchini *et al.*, 1995). Thus the positive optical rotation as shown by the heparin and heparin-like glycosaminoglycans has been recorded with heparin components from molluscan samples in the present study. Further the heparan sulphate can be distinguished from other glycosaminoglycans by their saccharide composition and optical rotation (Griffin *et al.*, 1995; Kim *et al.*, 1996).

Heparin and heparan sulphate glycosaminoglycans have a more heterogeneous structure due to sulfated regions distributed along the chain. They are composed of disaccharides of $\alpha(1\rightarrow4)$ -Linked-L-iduronic acid -2 sulphate-D-

glucosamine -N, 6-disulphate (Casu, 1988). This is to clarify that the heparin or heparan sulphate are mainly composed of iduronic acid/glucuronic acid, sulfate and glucosamine. The concentration or percentage of such contents in this prevailing disaccharide varies depending on heparin type (Linhardt *et al.*, 1988). In the case of heparin, the structural characterization and their properties depend on the concentrations uronic acid and sulfate. Hence, their biological activities vary with concentrations.

The sulfated acidic mucopolysaccharide is entirely accounted for the uronic acid, sulfate and other elements (Cassaró and Dietrich, 1977). The quantitative determination of the small amounts of sulfate present in glycosaminoglycans is important in polysaccharide chemistry (Terho and Hartiala, 1971).

In the present findings, the uronic acid and sulfate content are 23.0 % and 18.2 % in *T. maxima* and 19.0 % and 11.0% in *P. viridis* respectively. In the glycosaminoglycans of *Hemifusus pugilinus* the sulfate content is 9.91% and uronic acid, 26.9% (Benny, 1996), whereas in the glycosaminoglycans (heparan sulfate) from the snail *Helix aspersa*, the uronic acid and sulfate contents vary from 22 to 53% and 7 to 10% respectively (Hovingh and Linker, 1988) and the leech glycosaminoglycans also shows the uronic acid content ranging from 14 to 23% and sulfate from 1.5 to 6.5%.

Further in the glycosaminoglycans of *Anodonta californiensis*, the sulfate content in mantle and gills is in the range from 8 to 43% (Hovingh and Linker, 1993). This proves that the uronic acid and sulphate content vary with species and anticoagulant activity.

The present electrophoresis observation in the two species reveals migration of the heparin is closely similar to that of the fast moving heparin. In the case of anticoagulant activity both the clams showed the limited activity it may due to the starting material. The differences found in anticoagulant activity for the two mollusc heparins and mammalian heparin seems to be related to the different molecular weight of the compounds. But both the clams showed the intermediate molecular weight.

In the ^1H NMR (Nuclear Magnetic Spectroscopy) analysis the isolated heparin and heparin-like glycosaminoglycans from the species of bivalves showed the same basic structure consisting of separate disaccharides units- GlcUA, IdoA and GlcNAc- comparable to standard heparin and heparan sulfate. However, further differentiation between heparin and heparan sulfate is difficult, since the analyses made for both the structural and functional criteria are inadequate to separate the two forms of glycosaminoglycans in this study.

Acknowledgements

The authors are thankful to the authorities of Annamalai University for providing the facilities. We are thankful to Dr. Bruna parma, Opocrin Research Institute, Brazil for useful suggestions for designing this work.

References

- Bartolucci, C., L. Cellai, MA. Iannelli, D. Lamba, L. Liverani, G. Mascellani and E. Perola 1995. Inhibition of human leukocyte elastase by chemically and naturally oversulfated galactosaminoglycans. *Carb. Res.* **276**:401-408.
- Benny, A. 1996. *Studies on potential anticoagulant (Glycosaminoglycan) from the Marine Neogastropod Hemifusus pugilinus*, C.A.S in Maine Biology,

- Annamalai University, Portonovo, India. (Ph.D. Thesis)
- Bianchini, P., G.L. Bergonzini, B. Parma and B. Osima 1995. Relationship between plasma antifactor xa activity and the antithrombotic activity of heparin of different molecular mass. *Haemostasis* **25**: 288-298.
- Bianchini, P., H.B. Nader, H.K. Takahashi, B. Osima, A.H. Straus and C.P. Dietrich 1980. Fractionation and identification of heparin and other acidic mucopolysaccharides by a new discontinuous electrophoretic method. *J. Chromt.* **196a**: 455-462.
- Bitter, T. and H.M. Muir 1962. A modified uronic acid carbazole reaction. *Anal. Biochem.* **4**: 330-334.
- Burson, S.L., M.J. Fahrenbach, L.H. Fomnhagen, B.A. Riccardi, R.A. Brown, J.A. Brockman, H.V. Lewry and E.L.R. Stockstad 1956. Isolation and purification of mactins, heparin-like anticoagulants from mollusca. *J. Amer. Chem. Soc.* **78**: 5874-5879.
- Cassaro, C.M. and C.P. Dietrich 1977. Distribution of sulfated mucopolysaccharides in invertebrates. *J. Biol. Chem.* **252**: 2256-2261.
- Casu, B. 1988. Structure and biological activity of heparin. *Adv. Carbohydr. Chem. Biochem.* **43**: 51-134.
- Grant, A.C., R.J. Linhardt, G.L. Fitzgerald, J.J. Park and R. Langer 1984. Metachromatic activity of heparin and heparin fragments. *Anal. Biochem.* **137**: 25-32.
- Griffin, C.C., R.J. Linhardt, C.L. Van, T. Toida, R.E. Hileman, R.C. Schubert and S.E. Brown 1995. Isolation and characterization of heparan sulfate from crude porcine intestinal mucosa peptidoglycan heparin. *Carbohydr. Res.* **276**: 183-197.
- Hovingh, P. and A. Linker 1982. An unusual heparan sulfate isolated from lobsters (*Homarus americanus*). *J. Biol. Chem.* **257**: 9840-9844.
- Hovingh, P. and A. Linker 1988. Glycosaminoglycans in two molluscs *Aplysia californica* and *Helix aspersa* and in the leech, *Nephelopsis obscura*. *Comp. Biochem. Phys.* **119B**: 691-696.
- Hovingh, P. and A. Linker 1993. Glycosaminoglycans in *Anodonta californica* a fresh water mussel. *Biol. Bull.* **183**: 263-276.
- Jaques, L.B. 1967. The pharmacology at heparin and heparinoids. In *Progress in medicinal chemistry*. (Eds. G.P. Ellis and G.B. Wet butterworths), London. **5**: 138-198.
- Jorpes, J.E. 1939. *Heparin; its chemistry, physiology and application in medicine*. Oxford University Press, London.
- Kavanagh, L.W. and L.B. Jaques 1973. Comparative of analyzed values from commercial heparin. *Arznxim Rorsch, Drug Res.* **23**: 605-611.
- Kim, Y.S., Y.Y. Jo, M. Chang, T. Toida, Y. Parks and R.J. Linhardt 1996. A new glycosaminoglycans from the Giant African snail *Achantina fulica*. *J. Biol. Chem.* **271(20)**: 11750-11755.
- Linhardt, R.J. and T. Toida 1996. Heparin oligosaccharide-new analogs. In *Development and application in carbohydrates as Drugs* (Eds. Z.B. Wite zale and K.A. Nie forth). Marker Dekker, New York, NY. pp. 277-341.
- Linhardt, R.J., K.G. Rice, Y.S. Kim, D.L. Lohse, H.M. Wang and D. Loganathan 1988. Mapping and quantification of the major oligosaccharide components heparin. *J. Biochem.* **254**: 781-787.
- Loganathan, D., H.M. Wang, L.M. Mallis and R.J. Linhardt 1990. Structural variation in the antithrombin III binding site region and its occurrence in heparin from different sources. *Biochem.* **29**: 4362-4368.
- Nader, H.B. and C.P. Dietrich 1989. Natural occurrence possible biological role of heparin. In *Heparin, chemical and biological properties, clinical application* (Eds. D.A. Lane and U. Lindhal), London-Melbourne, Auckland, Edward Arnold. pp. 81-84.
- Petitou, M. and C.A.A. Van Boeckel 1997. Heparin: from the original "soup" to well-designed heparin mimetics. *Pure and Appl. Chem.* **69(9)**: 1839-1846.
- Somasundaram, S.T. 1990. *Heparin-like (glycosaminoglycans) from Katelysia opima*. Centre of Advanced study in marine Biology, Annamalai University, Portonovo, India. (Ph.D. Thesis)
- Teien, A.N. and M. Lie 1977. Evaluation of an amidolytic heparin assay method: increased sensitivity by adding purified antithrombin III. *Thromb Res.* **10**: 399-410.
- Teien, A.N., M. Lie and U. Abilguard 1976. Assay of heparin in plasma using a chromogenic substrate for activated factor. *Xa. Thromb. Res.* **8**: 413-416.
- Terho, T. and K. Hartiala 1971. Method for determination of the sulfate content of glycosaminoglycans. *Analytical Biochem.* **41**: 471-471.