ISJ 2: 6-16, 2005 ISSN 1824-307X

Review

Occurrence and structural characterization of heparin from molluscs

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Accepted February 25, 2005

Abstract

Several invertebrate species contain variable amounts of one or more types of sulfated glycosaminoglycans (GAGs). At present it is well known the existence of a species-specific sulfated GAGs composition based on the relative amount and type of chondroitin sulfates, heparan sulfate and heparin. Heparin is a sulfated polysaccharide belonging to the family of GAGs with numerous important biological activities, such as anticoagulant and antithrombotic properties that derive from its interaction with diverse proteins. Unusual heparin samples for molecular mass, fine structural organization and anticoagulant activity, are isolated and characterized from molluscs. Variable presence of the trisulfated disaccharide [ΔUA2S(1->4)-α-D-GlcN2S6S] and significant modifications of the disaccharides bearing non-sulfated iduronic and glucuronic acids, [->4)-α-L-IdoA(1->4)-α-D-GlcNAc6S(1-> and ->4)-α-L-IdoA(1->4)-α-D-GlcN2S6S(1->] and [->4)-β-D-GlcA(1->4)-α-D-GlcN2S6S(1->], and oligosaccharide sequences bearing part of the ATIII-binding region, [ΔUA2S(1->4)-α-D-GlcN2S6S(1->4)-β-D-GlcA(1->4)-α-D-GlcN2S**3S**6S] and [ΔUA2S (1->4) -α-D-GlcN2S6S (1->4)-α-L-IdoA (1->4)-α-D-GlcNAc6S (1->4)-β-D-GlcA (1->4)-α-D-GlcN2S**3S**6S], are detected and measured in heparin samples derived from different clam species. This review more specifically deals with structural and biologically important aspects of heparin in invertebrates with special emphasis on the heparin from molluscs. Furthermore, the fine characterization of heparin from *Tapes phylippinarum* and *Callista chione* is reported.

Keywords: glycosaminoglycans; polysaccharides; heparin; molluscs; anticoagulant drugs

Introduction

The presence of sulfated glycosaminoglycans (GAGs) in some taxa of invertebrates is now well documented (Cassaro *et al.,* 1977; Hovingh and Linker, 1982; Pejler *et al.,* 1987; Nader *et al.,* 1984; Dietrich *et al.*, 1985; Jordan *et al.*, 1986; Chavante *et al.*, 2000; Medeiros *et al.*, 2000; Cesaretti *et al.*, 2004; Luppi *et al.*, 2005). A comprehensive survey of different classes of invertebrates has shown that heparan sulfate (HS)-like and/or heparin-like compounds, besides chondroitin sulfate, are present in many species (Medeiros *et al.*, 2000).

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Previous studies have also shown that heparin is present in several species of molluscs. A compound from the clam *Mercenaria mercenaria* (Jordan *et al.*, 1986) exhibits several structural similarities to heparin. Heparins with high anticoagulant activity have been isolated from the molluscs *Anomalocardia brasiliana* (Pejler *et al.*, 1987; Dietrich *et al.*, 1985) *Tivela mactroides* (Pejler *et al.*, 1987) and *Tapes phylippinarum* (Cesaretti *et al.*, 2004). Due to our knowledge of the sulfated polysaccharides in vertebrates and invertebrates, is now possible to draw a phylogenetic tree of the distribution of sulfated GAGs in the animal kingdom.

Heart and vascular diseases, also including thrombosis, are the leading causes of death in the United States and Europe (Arias and Smith, 2003) Even if after the introduction of antithrombotic agents, particularly heparin and its derivatives, deadly heart diseases have decreased substantially (about 30 %) when compared to malignant cancer,

they are still the main cause of death (Arias and Smith, 2003). This explains the efforts to discover and develop specific and more potent antithrombotic agents.

Commercial manufacture of heparin relies on either porcine or bovine intestinal or bovine lung tissue as raw material. The apparent link between bovine spongiform encephalopathy and the similar prion-based Creutzfeldt-Jakob disease in humans (Schonberger, 1998), has limited the use of bovine heparin. Moreover, it is not easy to distinguish bovine and porcine heparins, thus making it difficult to ensure the species source of heparin (Linhardt and Gunay, 1999). Furthermore, porcine heparin also has problems associated with religious restrictions on its use. Non-animal sources of heparin, such as chemically synthesized, enzymatically synthesized, or recombinant heparins are currently not available for pharmaceutical purposes. These concerns have motivated to look for alternative, non-mammalian sources of heparin.

Structure of heparin

Heparin is a linear polysaccharide consisting of 1->4 linked pyranosyluronic acid (uronic acid) and 2-amino-2-deoxyglucopyranose (D-glucosamine, GlcN) repeating units (Linhardt, 2003). The uronic acid usually comprises 90 % L-idopyranosyluronic acid (L-iduronic acid, IdoA) (Fig. 1a) and 10 % D-glucopyranosyluronic acid (D-glucuronic acid, GlcA). Heparin, with its high content of sulfo and carboxyl groups, is a polyelectrolyte, having the highest negative charge density of any known biological macromolecule. At the disaccharide level, a number of structural variations exist (Fig. 1b), leading to sequence microheterogeneity within heparin. GAG heparin is polydisperse with a molecular mass range of 5-40, an average molecular mass of ~12 kDa, and an average negative charge of about -75, making it an extremely challenging molecule to characterize.

Heparin's complexity extends through multiple structural levels. At the proteoglycan level, different numbers of GAG chains (possibly having different saccharide sequences) can be attached to the various serine residues present on the core protein. Heparin chains are biosynthesized attached to a unique core protein, serglycin, found primarily in mast cells. On mast cell degranulation, proteases act on the heparin core protein to release peptidoglycan heparin, which is further processed by a β-endoglucuronidase into GAG heparin (Linhardt, 2003). The chemical, physical, and biological properties of heparin are primarily ascribed to GAG structure (or sequence), saccharide conformation, chain flexibility, molecular weight, and charge density.

HS, while structurally related to heparin, is much less substituted with sulfo groups and has a more varied structure (or sequence) (Fig. 1b) (Gallagher *et al.*, 1992). D-glucuronic acid predominates in HS, and it is polydisperse, having an average molecular mass of ~30 ranging from 5 to 50. HS chains also often contain domains of extended sequences having low or high sulfation (Gallagher *et al.*, 1992).

Interaction of heparin with proteins

With the discovery of increasing numbers of heparin-binding proteins (Capila and Linhardt, 2002), there was a need to characterize the molecular properties, within the proteins and heparin, responsible for specific recognition (Table 1). The biological activities of heparin and HS primarily result from their interaction with hundreds of different proteins. By using modelling, Cardin and Weintraub (1989) demonstrated that some heparin binding proteins had defined motifs corresponding to consensus sequences, giving the first evidence for the general structural requirements for GAG-protein interactions. Their results suggested that if the XBBBXXBX (B is a basic and X is a hydropathic amino acid residue) sequence was contained in an α-helical domain, then the basic amino acids would be displayed on one side of the helix with the hydropathic residues pointing back into the protein core. Heparin binding sites, commonly observed on the external surface of proteins, correspond to shallow pockets of positive charge. Thus, the topology of the heparin binding site is also an important factor in heparin binding consensus sequences. Structural analysis of the heparin binding sites in acidic fibroblast growth factor (FGF-1), basic FGF (FGF-2), and transforming growth factor β-1 (TGFβ-1) implicated a TXXBXXTBXXXTBB motif (T defines a turn) (Hileman *et al.*, 1998).

By screening peptide libraries the conservation of amino acids in heparin binding domains and the importance of spacing between basic amino acids in heparin binding was demonstrated. Peptides enriched in arginine and lysine and polar hydrogen-bonding amino acids were observed to bind heparin with highest affinity (Capila and Linhardt, 2002). Studies on the role of the pattern and the spacing of the basic amino acids in heparin binding domains showed that heparin interacted more tightly with peptides containing a complementary binding site of high positive charge density while the less sulfated heparan sulfate interacted more tightly with a complementary site on a peptide that had more widely spaced basic (Linhardt, 2003).

Anticoagulant activity

The anticoagulant action of pharmaceutical heparin (120-180 USP U/mg) is the most thoroughly studied of its activities. Anticoagulation occurs when heparin binds to ATIII, a serine protease inhibitor (serpin). ATIII undergoes a conformational change and becomes activated as an inhibitor of thrombin and other serine proteases in the coagulation cascade (Fig. 2).

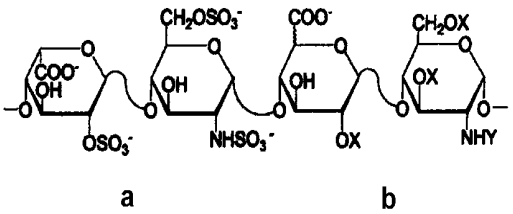

Fig. 1 Structure of heparin: a) major trisulfated dlsaccharide repeating unit (X = sulfo or H, Y = sulfo, Ac or H); b) undersulfated structural variants (modified from Linhardt, 2003).

Table 1. Characteristics of selected heparin-binding proteins (modified from Linhardt, 2003). [a] HS: high sulfation; IS: intermediate sulfation; LS: low sulfation.

Heparin-binding protein	Physiological/Pathological role	Kd	Oligosaccharide size	Sequence features [a]	Function
Proteases/Esterases					
AT III	Coagulation cascade serpin	ca. 20 nM	5-mer	GlcNS6S3S	Enhances
SLPI	Inhibits elastase and cathepsin G	ca. 6 nM	12-mer to 14-mer	IS	Enhances
C1 INH	Inhibits C1 esterase	ca 100 nM	-	HS	Enhances
VCP	Protects host cell from complement	nM	-	-	Unclear
Growth factors					
FGF-1	Cell proliferation, differentiation, Morphogenesis and angiogenesis	nM	4-mer to 6-mer	IdoA2S-GlcNS6S	Activates signal transduction
FGF-2	Same as FGF-1	nM	4-mer to 6-mer	IdoA2S-GlcNS	Same as FGF-1
Chemokines					
PF-4	Inflammation and wound healing	nM	12-mer	HS/LS/HS	Inactivates heparin
IL-8	Pro-inflammatory cytokine	ca 6 μM	18-mer to 20-mer	HS/LS/HS	Promotes
SDF-1α	Pro-inflammatory mediator	ca 20 nM	12-mer to 14-mer	HS	Localizes
Lipid-binding proteins					
Annexin II	Receptor for TPA and plasminogen, CMV and tenascin C	ca. 30 nM	4-mer to 5-mer	HS	unclear
Annexin V	Anticoagulant activity; Influenza and hepatitis B viral entry	ca. 20 nM	8-mer	HS	Assembles
ApoE	Lipid transport; AD risk factor	ca. 100 nM	8-mer	HS	Localizes
Pathogen proteins					
HIV-1 gp120	Viral entry	0.3 μM	10-mer	HS	Inhibits
CypA	Viral entry	-	-	-	Inhibits
Tat	Transactivating factor, primes cells for HIV infection	ca. 70 nM	6-mer	HS	Antagonizes
HSV gB and gC	Viral entry into cells	-	-	-	Inhibits
HSV gD	Viral entry and fusion	-	-	$GlcNH_2$3S	Inhibits
Dengue virus envelope protein	Viral localization	ca. 15 nM	10-mer	HS	Inhibits
Malaria CS protein	Sporozoite attachment to hepatocytes	ca. 40 nM	10-mer	HS	Inhibits
Adhesion proteins					
Selectins	Adhesion, inflammation and metastasis	μM	> 14-mer	HS with $GlcNH_2$	Blocks
Vitronectin	Cell adhesion and migration	μM	-	-	Removes
Fibronectin	Adhesion	μM	8-mer to 14-mer	HS with GlcNS	Reorganizes
HB-GAM	Neurite outgrowth in development	ca. 10 nM	16-mer to 18-mer	HS	Mediates
AP	In amyloid plaque	μM	4-mer	HS	Assembles

A major breakthrough in the study of heparin-catalyzed anticoagulation resulted from the separation of distinct heparin fractions differing markedly in affinity for ATIII. Low-affinity ATIII binding heparin comprises about two-thirds of porcine intestinal heparin and has a low anticoagulant activity (typically <20 U/mg). In contrast, high ATIII affinity heparin comprises the remaining third of porcine intestinal heparin and has a high anticoagulant activity (typically ~300 U/mg). Rosenberg *et al.* (1979) and Lindahl *et al.* (1979) examined the ATIII binding site by performing a partial chemical and enzymatic depolymerization of heparin and then purified the products using affinity chromatography on immobilized ATIII. The isolation of 3-O-sulfatase from human urine, capable of desulfonating 3-O-sulfoglucosamine residues provided the crucial clue to the structure of the ATIII binding site (Fig. 3).

NMR studies by several groups proved the presence of the 3-O-sulfo group within the ATIII binding site, and chemical synthesis of a pentasaccharide containing the 3,6-di-O-sulfo group substantiated these findings (Torri *et al.*, 1985). The ATIII binding sequences found in certain HSs are partially responsible for the blood compatibility of the vascular endothelium (Marcum and Rosenberg, 1987).

The ATIII pentasaccharide is sufficient to catalyze the ATIII-mediated inhibition of factor Xa,

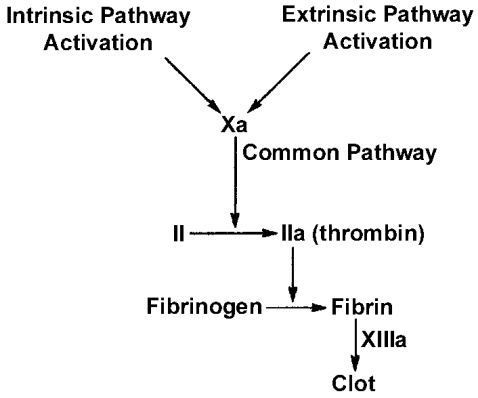

Fig. 2 Convergence of intrinsic/extrinsic pathways of the coagulation cascade leading to fibrin generation.

a critical serine protease in the coagulation cascade. To catalyze the ATIII-mediated inhibition of thrombin, 16-18 saccharide units are required (Sinay, 1999). Thus, the structure-activity relationship of thrombin inhibition has been more difficult to establish because it relied on the synthesis of oligosaccharides substantially larger than ATIII for pharmacological evaluation. Recent studies show that a relatively nonspecific but highly charged thrombin binding domain in heparin, localized on the nonreducing side of the heparin's ATIII binding site, is required to form a ternary complex. Success in understanding the structure-activity relationship of heparin's inhibition of thrombin has resulted in a new class of potent, synthetic, but still experimental thrombin inhibitors. Other heparin binding proteins, such as tissue factor pathway inhibitor (TFPI) and annexins, can also play important roles in anticoagulation.

Occurrence of heparin in vertebrates and invertebrates

An updated phylogenetic tree of the distribution of sulfated GAGs in the animal kingdom is shown in Fig. 4 (Medeiros *et al.*, 2000). Whereas HSs are ubiquitous components of all tissue-organized metazoan, heparin has shown a very peculiar distribution in mammalian and other vertebrate tissues as well as invertebrates. Chondroitin sulfate also has a widespread distribution (Fig. 4). Since the earlier studies from a variety of mammals, it has been found that lung, intestine, and liver were the organs richest in heparin (Nader *et al.*, 2004) (Table 2). Except for rabbit tissues, heparin's presence was demonstrated in lung, skin, ileum, lymph nodes, thymus, and appendix of all species studied. The absolute content of heparin varied depending on different tissues. The lack of heparin in rabbits was correlated with the absence of mast cells in the species (Nader *et al.*, 1980). A large variation of the concentration of heparin among species is evident. Thus, bovine and dog tissues contain the highest amounts of heparin. Generally, in non-mammalian vertebrate tissues the amount of heparin is considerably lower (Nader *et al.*, 2004).

In invertebrates, heparin is found in few taxa, namely molluscs, crustacean, annelida, echinoderma and

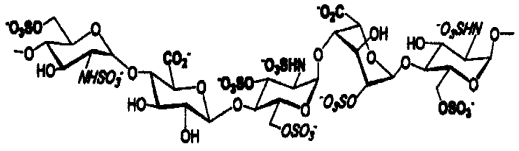

Fig. 3 Antithrombin III pentasaccharide binding site. The anionic groups in bold are critical (95 % loss in binding energy on removal), and those in italics are important (25-50 % loss in binding energy on removal) for interaction with ATIII (modified from Linhardt, 2003).

cnidaria (Table 3). As observed for vertebrate heparin samples, the anticoagulant activity and molecular mass varied according to the species analyzed. Furthermore, no correlation between molecular mass and anticoagulant activity of the heparins is evident. All these results imply that heparins have a large structural variation depending on their origin (Nader *et al.*, 2004).

Biological role of heparin in molluscs

The biological function of the clam heparins and their apparently specific ATIII-binding regions is unclear at the moment. Molluscs do not possess any blood coagulation system similar to that of mammals, yet their heparins are capable of dramatically accelerating the inactivation of mammalian coagulation enzymes by the mammalian protease inhibitor, ATIII. It is possible that the bivalve heparin is designed to interact with an endogenous antithrombin-like protease inhibitor acting on serine protease target enzymes. The existence and function of such an enzyme system remain to be established.

In mammals heparin is released from the mast cells in response to specific inflammatory agents such as IgE antibodies or complement fragments (anaphylatoxins). Since the discovery of mast cells by Paul Ehrlich (1879) and after the demonstration that their metachromatic properties when stained with basic dyes was related to heparin, the question whether this compound was only confined to the mast cells or not has been a matter of controversy. Studies on the concentration of heparin and its content in different fetal and adult bovine tissues (Nader *et al.*, 1982) have shown that a good correlation between the mast cell number and heparin concentration could be obtained in all tissues analyzed.

Studies on mast-cell deficient mice of the genotypes W/W^v and $S1/S1^d$ established that mast cells originate from hematopoietic stem cells. These experiments also demonstrated that heparin is present in appreciable amounts in the skin of the breeders and of the normal progeny. On the other hand, no heparin was detected in the skin of the W/W^v genotype, which are deficient in mast cells. No significant differences in the relative amounts of

Table 2. Distribution of heparin in mammalian and other vertebrates (modified from Nader *et al*., 2004). Bov = bovine.

Tissue µg/g dry tissue	**Rabbit**	**Guinea Pig**	**Rat**	**Dog**	**Cat**	**Pig**	**Bov**	**Human**	**Bony Fish**	**Shark**
Lung	<1	70	67	217	63	211	300	8	0.03	
Liver	<1	<1	<1	141	1	<1	50	<1	1.32	0
Ileum	<1	27	1	400	87	113	1015	32	0	0
Kidney	<1	4	<1	2	6	<1	26	<1		0.29
Aorta	<1	<1	9	102	<1	2	150	<1		
Brain	<1	<1	<1	<1	<1	<1	<1	<1	0	0
Muscle	<1	<1	36	9	<1	5	2	<1	0	0
Spleen	<1	<1	<1	11	<1	<1	19	<1		11.9
Skin	<1	<1	175	15	63	2	108	39	0	0
Lymph	<1	11	5	160	74	242	180	41		
Thymus	<1	112	20	20		10	286	35		
Appendix	<1			17	38		20	47		
Branchia									0.03	0

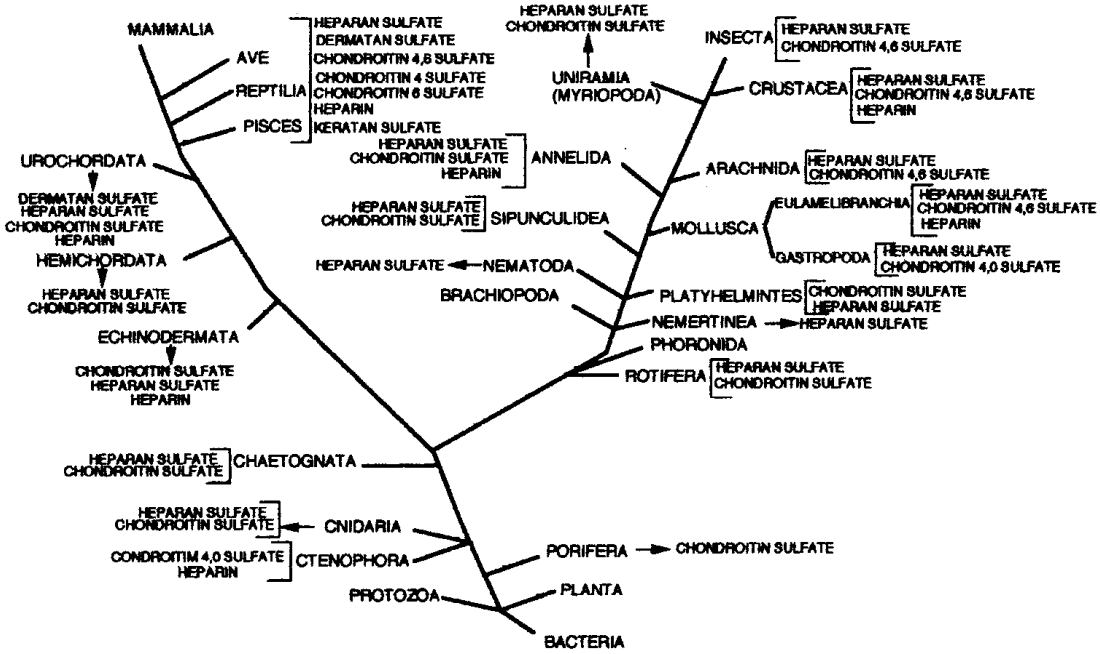

Fig. 4 Distribution of sulfated glycosaminoglycans in the animal kingdom (Modified from Medeiros *et al.,* 2000).

Table 3. Distribution of heparin in invertebrates (from Nader *et al.,* 2004 updated). The molecular mass and the anticoagulant activity is reported.

Class and species	Average M.M. (kDa)	Anticoagulant activity (USP)
Molluscs		
Ciprinia islandica	nd	95
Mactrus pussula	nd	100
Mercenaria mercenaria	18	348 (Anti-IIa)
Anomalocardia brasiliana	32	320
Donax striatus	20	220
Tivela mactroides	25	180
Tapes phylippinarum	14	350
Callista chione	11	97
Crustacea		
Ucides chordatus	nd	60
Dedrocephalus brasiliensis	10	52
Penaeus brasiliensis	9	60
Annelida		
Aphrodite longicornis	nd	nd
Hermodice carunculata	nd	nd
Echinoderma		
Mellita quinquisperforata	12	50
Cnidaria		
Physalia sp.	nd	nd
Mnemiopsis sp.	nd	nd
Sipuncula nudu	nd	nd

the other sulfated GAGs, namely HS, dermatan sulfate and chondroitin sulfate were observed among the breeders analyzed. This suggests that heparin is not replaced by other sulfated GAGs in the animals that lack heparin. These results clearly indicate that heparin is related to the presence of mast cells (Kitamura and Go, 1979; Marshall *et al.*, 1994). Furthermore, in mammals, the heparin-containing mast cells are accumulated in lymphoid organs and in tissues exposed to the external milieu (skin, lungs, intestine) and one suggested role for this polysaccharide in mammalian is to fight external parasites (Nader *et al.*, 2004).

Heparin and other sulfated GAGs as well as histamine were found and quantified in various organs of the mollusc *Anomalocardia brasiliana*. The heparin was present in granules inside the cytoplasm of mast-like cell (Pejler *et al.*, 1987; Dietrich *et al.*, 1985). A good correlation between heparin and histamine content was found in the labial palp, intestine, ctenide, mantle, and foot tissues.

Some conclusions can be derived from these studies: 1) heparin seems to be present exclusively in mast cells of vertebrates or mast-like cells in the case of molluscs; 2) its primary biological activity is not related with antithrombotic activity since molluscs, which do not possess a coagulation system, contain heparin and rabbits that possess this system are devoid of heparin; 3) many indirect evidences suggest that in mammals and molluscs heparin and its mast cells are involved in defense mechanisms independently of the immune system and support the hypothesis that this macromolecule could function as a mechanism for the surveillance of these organisms against certain pathogens.

Extraction, purification and characterization of heparin from *Tapes phylippinarum*

GAGs extracted from *T. phylippinarum* by defatting and proteolytic treatments, were successively fractionated on an anion-exchange resin and eluted with a linear NaCl gradient at increasing molarity. Agarose-gel electrophoresis analysis of single fractions eluted from the anion-exchange resin showed low amounts of chondroitin sulfate besides heparin. After treatment with endonuclease and chondroitinase, and precipitation with organic solvent, *T. phylippinarum* yielded approx. 2.1 mg heparin/g of dry animals (Volpi, 1993; Volpi and Maccari, 2002).

Fig. 5A illustrates the agarose-gel electrophoresis of the mollusc heparin showing the two components, the slow moving species having high-molecular mass and charge density and the fast moving heparin, possessing a lower molecular mass and sulfate groups amount. The densitometric scanning of *T. phylippinarum* heparin showed 22 ± 6.8 % of the slow moving component and 78 ± 5.4 % of the fast moving species (Cesaretti *et al.*, 2004).

Fig. 6A illustrates the PAGE analysis of *T. phylippinarum* heparin showing an average molecular mass of 13.600 calculated on a calibration curve of oligosaccharide standards of

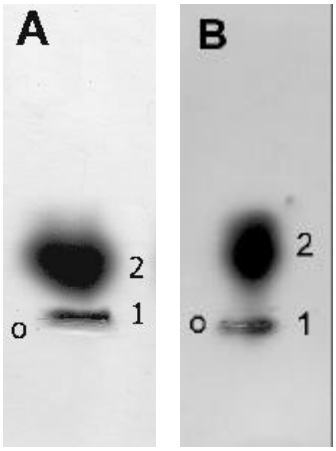

Fig. **5.** Agarose-gel electrophoresis of heparin (4 µg) purified from A) *Tapes phylippinarum* (from Cesaretti ***et al.,*** 2004) and B) *Callista chione* (from Luppi ***et al.,*** 2005). The gel was dried and stained according to the sequential procedure by using toluidine blue (Volpi and Maccari, 2002). The quantitative analysis of the (1) slow moving heparin component and of the (2) fast moving heparin species was performed by specific calibration curves obtained from purified heparin species. O = origin

known molecular mass prepared from bovine mucosal heparin.

Qualitative and quantitative oligosaccharide mapping (Linhardt ***et al.***, 1988, 1989, 1990, 1992; Rice and Linhardt, 1989) was performed by depolymerizing *T. phylippinarum* heparin (Fig. 7A) with heparinase (EC 4.2.2.7) and then separating the resulting unsaturated oligosaccharides by SAX-HPLC. Mass balance close to 91-95 % (Table 4) was excellent for *T. phylippinarum* heparin, confirming its purity. Important differences between pharmaceutical bovine and mollusc heparin were the higher content of the [ΔUA2S(1->4)-α-D-GlcN2S6S] disaccharide and the lower percentage of the **3b**, **4** and **6** oligosaccharide sequences in the clam heparin (Table 4). In contrast, a striking higher amount (more than 130 % than standard pharmaceutical heparin) was calculated for the oligosaccharide sequence **8** bearing part of the ATIII-binding region, [ΔUA2S (1->4) -α-D-GlcN2S6S (1->4) -α-L-IdoA (1->4) -α-D-GlcNAc6S (1->4)-β-D-GlcA (1->4)-α-D-GlcN2S**3S**6S] in the *T. phylippinarum* heparin (Table 4). This unusual sequence contains sulfation at position 3 of the glucosamine residue, characteristically found in the ATIII-binding site. The results of these analyses were used to calculate the disaccharide composition. As expected from the oligosaccharide compositional analysis, heparin from *T. phylippinarum* is a more sulfated polysaccharide, as also indicated by the presence of a greater mol % of the trisulfated disaccharide [ΔUA2S(1->4)-α-D-GlcN2S6S], with respect to bovine mucosal heparin (73.5 %), and, very interestingly, also with respect to porcine mucosal (72.8 % of the trisulfated disaccharide) and human heparin (71.0 % of the trisulfated disaccharide) calculated with the same methodological approach. Furthermore, there is a significant increase (+ 35 %) of the disaccharide bearing the sulfate group in position 3 of the N-sulfo-glucosamine 6-sulfate part of the ATIII-binding region. The increased number of ATIII binding sites in *T. phylippinarum* heparin is accompanied by an increased anticoagulant activity. Higher APTT (350 ± 56 IU/mg) and ATIII-mediated antifactor Xa (320 ± 48 IU/mg) activities were observed for the clam heparin in comparison with pharmaceutical bovine mucosal heparin (Linhardt ***et al.***, 1992).

Isolation and fine structural characterization of heparin from *Callista chione*

Fig. 5B illustrates the agarose-gel electrophoresis of the mollusc heparin (approx. 1.9 mg heparin/g of dry animals) showing the two components, the slow moving species (15 ± 1.3 %) and the fast moving heparin (85 ± 7.6 %).

Figure 6B illustrates the PAGE analysis of *C. chione* heparin showing an average molecular mass of 10.950. The ^{1}H-NMR spectrum, the agarose-gel and the PAGE, showed *C. chione* heparin free from impurities (Luppi ***et al.***, 2005).

C. chione heparin was treated with heparinase (EC 4.2.2.7) and the resulting unsaturated oligosaccharides then separated by SAX-HPLC (Figure 7B). Mass balance close to 88 % (Table 4) was calculated for *C. chione* heparin mainly due to the presence of approximately 10 % not identified oligosaccharides signed in the Fig. 7B with X. Important differences between pharmaceutical grade and mollusc heparin were the lower (approximately 37 %) content of the [ΔUA2S(1->4) -α-D-GlcN2S6S] disaccharide and the lower percentage of the **4** and **6** oligosaccharide sequences in the *C. chione* heparin (Table 4). On the contrary, a strong increase was calculated for the oligosaccharides **1**, **2**, **3b**, **5** and **6a**. No substantial quantitative modifications were found for the sequence **8** bearing part of the ATIII-binding region, [ΔUA2S (1->4)-α-D-GlcN2S6S (1->4)-α-L-IdoA (1->4)-α-D-GlcNAc6S (1->4) -β-D-GlcA (1->4) -α-D-GlcN2S**3S**6S] in the *C. chione* heparin (Table 4). The results of these analyses were used to calculate the disaccharide composition. As expected from the oligosaccharide compositional analysis, heparin from *C. chione* is a less sulfated polysaccharide, due to the presence of a lower mol % of the trisulfated disaccharide [ΔUA2S(1->4)-α-D-GlcN2S6S]. Furthermore, there is a significant decrease of the specific sulfatation in position 2 of the iduronic acid unit with the strong increase of the disaccharides bearing non-sulfated iduronic acid, [->4)-α-L-IdoA(1->4)-α-D-GlcNAc6S(1->] and [->4)-α-L-IdoA(1->4)-α-D-GlcN2S6S(1->], and a strong decrease of the sulfatation in postion 2 of the gluronic acid with a greater percentage of the disaccharide [->4)-β-D-GlcA(1->4)-α-D-GlcN2S6S (1->]. No significant quantitative modification of the disaccharide bearing the sulfate group in position 3 of the N-sulfo-glucosamine 6-sulfate part of the ATIII-binding region (Table 4) was evident.

The anticoagulant activity of the clam heparin was next evaluated. Heparin isolated from *C.*

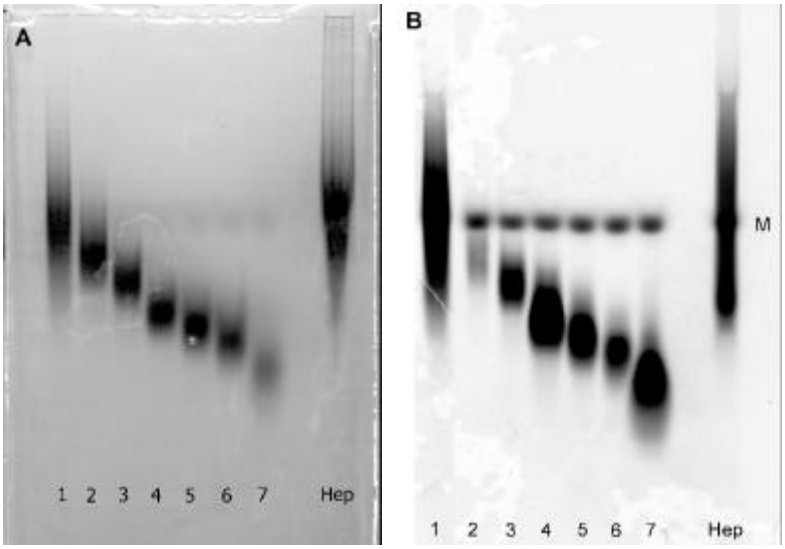

Fig. 6 PAGE analysis of the molecular mass of the A) *Tapes phylippinarum* (from Cesaretti *et al.,* 2004) and B) *Callista chione* (from Luppi *et al.,* 2005) heparins (Hep). 40 μg of the purified polysaccharides were layered on the gel and the calibration curve was constructed by using oligosaccharide standards of known molecular mass prepared from bovine mucosal heparin. 1. 13,500; 2. 7,560; 3. 6,300; 4. 4,560; 5. 3,640; 6. 2,820; 7. 1,620. M = bromophenol blue.

Table 4. Oligosaccharide analysis of clam heparins. Total mol % of the oligosaccharides is calculated by summing the mol % for the oligosaccharides in each column. An error of ± 0.1 mol % is possible in the measurement of each oligosaccharide. The 2.5 (*Tapes phylippinarum* heparin, from Cesaretti *et al*., 2004) and 10.0 (*Callista chione* heparin, from Luppi *et al*., 2005) mol % correspond to unknown oligosaccharides.

Oligosaccharide	*T. phylippinarum*	*C. chione*
1	n.d.	11.8
2	3.3	9.5
3	71.0	33.7
3a	2.0	1.7
3b	0.4	6.4
4	0.9	1.9
4a	0.5	n.d.
5	7.4	13.5
6	2.8	1.8
6a	0.4	4.3
7	1.1	1.4
8	5.1	2.0
Total (**1**-**8**)	94.9	88.0

n.d. = not detected.

1, ΔUA(1->4)-α-D-GlcN2S6S;
2, ΔUA2S(1->4)-α-D-GlcN2S;
3, ΔUA2S(1->4))-α-D-GlcN2S6S;
3a, ΔUA2S(1->4)-α-D-GlcN2S(1->4)-α-L-IdoA2S(1->4)-α-D-GlcN2S;
3b, ΔUA2S(1->4)-α-D-GlcN2S(1->4)-β-D-GlcA(1->4)-α-D-GlcN2S6S;
4, ΔUA2S(1->4)-α-D-GlcN2S6S(1->4)-α-L-IdoA2S(1->4)-α-D-GlcN2S;
4a, ΔUA2S(1->4)-α-D-GlcN2S6S(1->4)-α-L-IdoA(1->4)-α-D-GlcN2S6S;
5, ΔUA2S(l->4)-α-D-GlcN2S6S(1->4)-β-D-GlcA(1->4)-α-D-GlcN2S6S;
6, ΔUA2S(1->4)-α-D-GlcN2S6S(1->4)-α-L-IdoA2S(1->4)-α-D-GlcN2S6S;
6a, ΔUA2S(1->4) -α- D-GlcN2S6S(1->4) -α- L-IdoA(1->4)-α-D- GlcNAc6S (1->4) -β-D-GlcA(1->4) -α- D-GlcN2S6S;
7, ΔUA2S(1->4)-α-D-GlcN2S6S(1->4)-β-D-GlcA(1->4)-α-D-GlcN2S**3S**6S;
8, ΔUA2S(1->4) -α-D-GlcN2S6S (1->4)-α-L-IdoA(1->4)-α-D-GlcNAc6S(1->4)-β-D-GlcA(1->4)-α-D-GlcN2S**3S**6S.

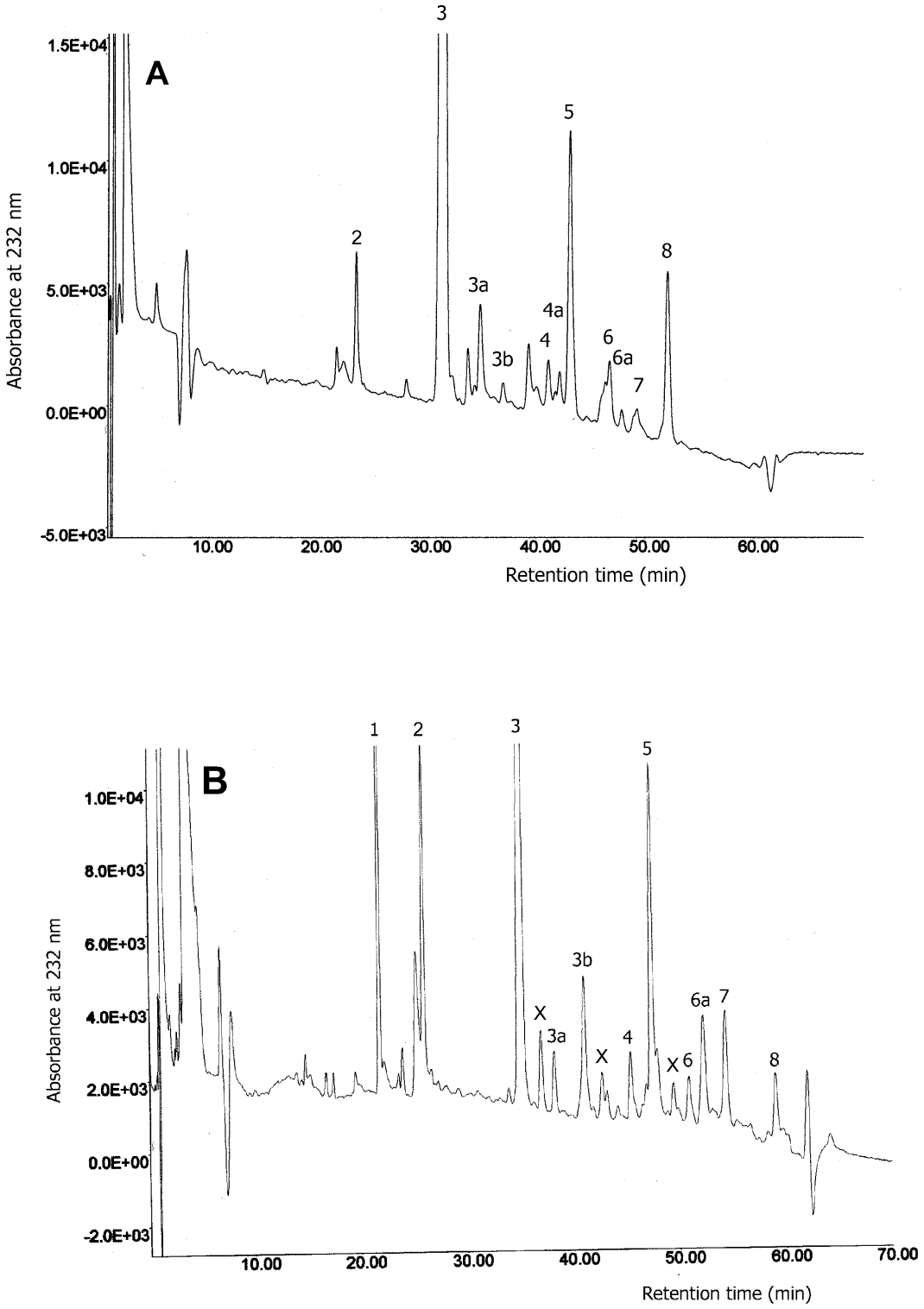

Fig. 7 SAX-HPLC chromatograms of heparin lyase-treated A) *Tapes phylippinarum* (from Cesaretti *et al.,* 2004) and B) *Callista chione* (from Luppi *et al.,* 2005) heparin samples. 10 μg of polisaccharides were analyzed. The major peaks corresponding to oligosaccharides 1-8 are indicated and they were assigned by coinjection with standards (see their structures in Table 4).

chione in our laboratory showed a lower anti-factor Xa activity of approximately 60 % (52 ± 7.4 UI/mg) and a decrease of the APTT activity of 33 % (97 ± 12.1 UI/mg) in comparison with standard pharmaceutical grade heparin.

Conclusions

The study of heparin will certainly extend well into this new century with so many questions left still unanswered. Some focal points in the near future include: a) improved preparation and synthesis of heparins; b) new heparin-based anticoagulants with improved properties; c) new therapeutic applications for heparins; d) new heparin mimetics; (e) new biomaterials and (f) development of an improved understanding of physiology and pathophysiology through glycomics.

The preparation of heparin from mammalian tissues creates concern particularly after the recent appearance of bovine spongiform encephalopathy in Europe. Bovine tissues are now rarely used in heparin production, and there are growing concerns about porcine tissues. Heparins prepared by alternative routes, such as defined, recombinant mammalian cell lines capable of being cultured in large-scale fermentations, or non mammalian tissues, offer an exciting alternative to the present preparations.

New anticoagulants based on heparin's structure might offer enhanced specificity targeting one or selected groups of coagulation proteases and avoiding undesired interactions with other proteins, thus decreasing the side effects associated with heparins use. Furthermore, new therapeutic applications might include the use of heparin to treatment of infectious diseases, inflammation, and control of cell growth in wound-healing and cancer. These new activities will require the elimination of heparin's anticoagulant activity, the engineering of appropriate pharmacokinetics and pharmacodynamics, and optimally oral bioavailability. A concern about the application of heparins to promote wound healing is that they might simultaneously promote cancer. Thus, wound healing applications will probably require localization of the drug at the site of action possibly through the application of polymers or gels. These new potential applications of heparin may be strictly associated with molecules possessing peculiar and unusual structure, as those isolated from invertebrates.

Finally, one of the most important future directions of heparin research is driven by the recent sequencing of the human genome and the field of genomics. While much attention is currently focused on the proteome encoded by the genome and the rapidly developing field of proteomics, the glycome has garnered little attention. Glycomics is the study of the structure and function of the glycome, the most important and complex of the post-translation modifications that proteins undergo. An improved understanding of the glycome should be beneficial in better understanding genetic diseases, offering new therapeutic approaches to treat these very serious pathologies. Moreover, improved knowledge of glycomics, in vertebrate and invertebrate species, should lead to a better understanding of physiology and pathophysiology, offering new approaches to drug development.

Acknowledgments

Research partially supported by the "Cassa di Risparmio di Modena" (2003) to N Volpi.

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The abbreviations used are: Ac, acetate; APTT, activated partial thromboplastin time; ATIII, antithrombin III; FGF, fibroblast growth factor; GAGs, glycosaminoglycans; GlcA, β-D-glucopiranosyluronic acid; GlcN, 2-deoxy-2-amino-α-D-glucopyranose; HPLC, high-performance liquid chromatography; HPSEC, high-performance size-exclusion chromatography; HS, heparan sulfate; IdoA, α-L-idopyranosyluronic acid; PAGE, polyacrylamide gel electrophoresis; S, sulfate; SAX, strong anion exchange; TFPI, tissue factor pathway inhibitor; TGFβ, transforming growth factor β; UA, uronic acid.