Isolation and characterization of a heparin with high anticoagulant activity from the clam *Tapes phylippinarum*: evidence for the presence of a high content of antithrombin III binding site

Marina Cesaretti, Elisa Luppi, Francesca Maccari, and Nicola Volpi1

Department of Biologia Animale, University of Modena and Reggio Emilia, Via Campi 213/D, 41100 Modena, Italy

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Heparin with high anticoagulant activity (activated partial thromboplastin time of 347 ± 56.4 and anti-Xa activity of 317 ± 48.3) was isolated from the marine clam species *Tapes phylippinarum* in an amount of ~2.1 mg/g dry animals. Agarose-gel electrophoresis showed a high content of the slow-moving heparin component (22 ± 6.8%) and 78 ± 5.4% of the fast-moving species. An average molecular mass of 13,600 was calculated by PAGE analysis, whereas a number average molecular weight Mn value of 10,700, a weight average molecular weight Mw of 14,900, and a dispersity index Mn/Mw of 1.386 were obtained by high-performance size-exclusion chromatography. Structural analysis of clam heparin, performed by depolymerizing heparin samples with heparinase (EC 4.2.2.7) and then separating the resulting unsaturated oligosaccharides by strong anion exchange-HPLC revealed the presence of large amounts (more than 130% than standard pharmaceutical heparin obtained from bovine intestine) of the oligosaccharide sequence 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 in the *T. phylippinarum* heparin, in comparison with bovine mucosal heparin and a sample of porcine mucosal heparin previously published. Furthermore, as expected from the oligosaccharide compositional analysis, due to the presence of a great mol % (80.6%) of the trisulfated disaccharide ΔUA2S (1→4)-α-D-GlcN2S6S, mollusc heparin is a more sulfated polysaccharide than bovine mucosal heparin (73.5%) and a sample of porcine mucosal heparin previously reported. To our knowledge, this is the first article describing a clam heparin having the ATIII binding site mainly identical to that of human and porcine intestinal mucosal heparins and bovine intestinal mucosal heparin but different from that found in beef lung heparin.

**Key words:** anticoagulant drug/glycosaminoglycans/heparin/molluscs/*Tapes phylippinarum*

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1To whom correspondence should be addressed; e-mail: volpi@unimo.it

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available for pharmaceutical purposes. These concerns have motivated us to look for alternative nonmammalian sources of heparin.

An enzyme-based oligosaccharide mapping has been developed (Linhardt et al., 1992a) capable of qualitatively and quantitatively detecting part of the ATIII-binding sites and ATIII-binding site precursors in heparin. In this article we describe the structural characterization of a heparin possessing high anticoagulant activity isolated from a species of marine clam, *Tapes philippinarum*, a bivalve mollusc normally cultivated as a commercial food source (Cima et al., 1998). By using the methodology developed by Linhardt and co-workers (1992a), the content of the oligosaccharide sequences bearing part of the ATIII-binding region, \( \text{UA2S(1\rightarrow4)\text{-D-GlcN2S6S(1\rightarrow4)\text{-B-D-GlcA(1\rightarrow4)\text{-A}}D-GlcN2S3S6S} \) and \( \text{UA2S(1\rightarrow4)\text{-D-GlcN2S6S(1\rightarrow4)\text{-A}}L-IdoA(1\rightarrow4)\text{-D-GlcNAc6S(1\rightarrow4)\text{-B-D-GlcA(1\rightarrow4)\text{-A}}D-GlcN2S3S6S} \) was determined. Furthermore, to our knowledge, this is the first work describing a clam heparin having the ATIII-binding site mainly identical to those of human and porcine intestinal mucosal heparins and bovine intestinal mucosal heparin, but different from that found in beef lung heparin.

**Results**

After defatting with organic solvents and extraction by proteolytic treatment, glycosaminoglycans from the body of *T. philippinarum* were fractionated on an anion-exchange resin and eluted with a linear NaCl gradient at increasing molarity. Agarose gel electrophoresis analysis (Volpi, 1993; Volpi and Maccari, 2002) 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. philippinarum* yielded \(~2.1 \text{ mg heparin/g dry animals}\) and \(~0.78 \text{ mg/g bovine intestinal mucosa}\) (data not shown).

Figure 1A illustrates the agarose gel electrophoresis of the mollusc heparin showing the two components, the slow-moving heparin (1) species and the fast-moving heparin (2) species was performed by specific calibration curves of oligosaccharide standards of known molecular mass and sulfate groups amount (Volpi, 1993; Volpi and Maccari, 2002). Figure 1B illustrates the densitometric scanning of *T. philippinarum* heparin showing \(22 \pm 6.8\%\) of the slow-moving component and \(78 \pm 5.4\%\) of the fast-moving species.

Figure 2A illustrates the polyacrylamide gel electrophoresis (PAGE) analysis of *T. philippinarum* heparin showing an average molecular mass of 13,600 calculated on a calibration curve of oligosaccharide standards of known molecular mass prepared from bovine mucosal heparin. Figure 2B shows the high-performance size-exclusion chromatography (HPSEC) profile of the clam heparin obtained by a refractive index detector, and the third-grade polynomial calibration curve used to determine the size-exclusion chromatography parameters. The number average molecular weight (Mn) was 10,700, the weight average molecular weight (Mw) was 14,900, the dispersity index (Mn/Mw) was 1.386, and the viscosity average molecular weight (Mv) was calculated to be 14,900.

The \(^1\text{H}-\text{nuclear magnetic resonance (NMR)}\) analysis of *T. philippinarum* heparin (Figure 3) revealed the presence of signals corresponding to the residue of IdoA2S, \(85.23\) (I1, H-1), \(4.37\) (I2, H-2), \(4.16\) (I3, H-3), \(4.02\) (I4, H-4), and \(4.75\) (I5, H-5), to the residue of GlcN2S, \(85.37\) (G1, H-1), \(3.29\) (G2, H-2), \(3.58\) (G3, H-3), \(3.70\) (G4, H-4), and \(3.94\) (G5, H-5), to the residue of GlcNAc, \(82.01\) (Ac, COCH\(_3\)). *T. philippinarum* heparin had an optical rotation of \([\alpha]_D^\circ = +42^\circ\) (c = 1 mg/ml) quite similar to the optical rotation observed for bovine mucosal heparin (\(+40^\circ\)) and porcine mucosal heparin (Linhardt et al., 1992b). The value of optical rotation and the \(^1\text{H}-\text{NMR} \) spectrum, the agarose gel, and the PAGE, showed *T. philippinarum* heparin free from impurities.

Qualitative and quantitative oligosaccharide maps (Linhardt et al., 1988, 1989, 1992a, Loganathan et al.,
Fig. 2. (A) PAGE analysis of the molecular mass of the \textit{T. phylippinarum} heparin (Hep). 20 \mu g of the purified polysaccharide 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: 7560; 3: 6300; 4: 4560; 5: 3640; 6: 2820; 7: 1620. (B) HPSEC analysis of the \textit{T. phylippinarum} heparin (Hep). 50 \mu g of the purified polysaccharide were injected in the column. HPLC mod. LC-1500 was from Jasco (pump mod. PU-1580, UV detector mod. UV-1570, Refractive Index detector Mod. RI-2031, software Jasco-Borwin rel. 1.5). The mobile phase was composed of a 125 mM Na$_2$SO$_4$ and 2 mM NaH$_2$PO$_4$ adjusted to pH 6.0 with 0.1 N NaOH. Flow rate was 0.9 ml/min with a back pressure of 40 kg/cm$^2$. Standards were solubilized in the mobile phase at a concentration of 5 mg/ml. 10 \mu l (50 \mu g) were injected in HPLC. Columns were Protein Pak 125 (Waters, cod. 84601, 7.8 mm $\times$ 30 cm) and Protein Pak 300 (Waters, cod. T72711, 7.5 mm $\times$ 30 cm) assembled in series. The retention times were plotted against the logarithm of molecular mass for standard heparins. The curve that fits the experimental data is a third-grade polynomial with the formula $y(x) = -ax^3 + bx^2 - cx + d$ performed by the Jasco Borwin program (upper panel). The number average molecular weight (Mn), the weight average molecular weight (Mw), the viscosity average molecular weight (Mv), and the polydispersity index (Mw/Mn) were calculated by the Jasco Borwin GPC software.
1990; Rice and Linhardt, 1989) were performed by depolymerizing *T. phylippinarum* heparin (Figure 4A) with heparinase (EC 4.2.2.7) and then separating the resulting unsaturated oligosaccharides by strong anion exchange (SAX)-high-performance liquid chromatography (HPLC) in comparison with the bovine mucosal heparinase-depolymerized heparin (Figure 4B). Mass balance closure of 91–95% (Table I) was excellent for both *T. phylippinarum* and bovine heparins, confirming their purity. Linhardt et al. (1992a) obtained a value of 93% for porcine mucosal heparin. Important differences between bovine and mollusc heparins were the higher content of the \( \Delta UA2S(1\rightarrow4)\alpha-D-GlcN2S6S \) disaccharide (see also Table II) and the lower percentage of the \( 3b, 4, \) and \( 6 \) oligosaccharide sequences in the clam heparin (Table I). On the contrary, a very strong increase (more than 130% than standard pharmaceutical heparin obtained from bovine intestine) was calculated for the oligosaccharide sequence \( 8 \) bearing part of the ATIII-binding region, \( \Delta UA2S(1\rightarrow4)\alpha-D-GlcN2S6S(1\rightarrow4)\alpha-L-IdoA(1\rightarrow4)\alpha-D-GlcNAc6S(1\rightarrow4)\beta-D-GlcA(1\rightarrow4)\alpha-D-GlcN2S3S6S \) in the *T. phylippinarum* heparin (Table I). This unusual sequence contains sulfation at position 3 of the glucosamine residue, characteristically found in the ATIII-binding site (Linhardt et al., 1992a) of porcine mucosal heparin in a concentration very close (2.2 mol %) to that calculated in this study for bovine mucosal heparin.

The results of these analyses were used to calculate the disaccharide composition (Table II). 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 \( \Delta UA2S(1\rightarrow4)\alpha-D-GlcN2S6S \), with respect to bovine mucosal heparin (Table II) 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 (Linhardt et al., 1992b). 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 (Table II). This increased number of ATIII-binding sites in *T. phylippinarum* heparin is accompanied by increased anticoagulant activity. Higher activated partial thromboplastin time (APTT) (~+140%) and ATIII-mediated antifactor Xa (~ more than 150%) activities were observed for the mollusc heparin in comparison with bovine mucosal heparin (Table III).

**Discussion**

Pharmaceutical heparin is typically prepared from bovine or porcine intestinal mucosa or beef lung (Casu, 1985; Linhardt et al., 1992b). Invertebrates also show the presence of various types of glycosaminoglycans (Cassaro and Dietrich, 1977), and heparin has been purified from several species of molluscs (Cassaro and Dietrich, 1977; Dietrich et al., 1985; Pejler et al., 1987). Heparin from the clam *T. phylippinarum* was recovered at about 2.1 mg/g dry tissue, having a large percentage of the more sulfated and
high-molecular-mass slow-moving heparin component, and an average molecular mass of ~14,000 calculated by PAGE and HPSEC analyses. Due to the presence of a high percentage of the slow-moving species (Bianchini et al., 1985; Volpi, 1993), heparin from T. phylippinarum is more sulfated, owing to the presence of a great mol % of the trisulfated disaccharide $\alpha-L$-IdoA$\alpha-L$-D-Glc$\beta$-GlcN2S6S(1→80%), than in mammalian heparins. Some
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Table I. Oligosaccharide analysis of heparins

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>T. phylippinarum heparin</th>
<th>Bovine mucosal heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>3.3</td>
<td>5.3</td>
</tr>
<tr>
<td>3</td>
<td>71.0</td>
<td>62.2</td>
</tr>
<tr>
<td>3a</td>
<td>2.0</td>
<td>2.6</td>
</tr>
<tr>
<td>3b</td>
<td>0.4</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>0.9</td>
<td>3.7</td>
</tr>
<tr>
<td>4a</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>5</td>
<td>7.4</td>
<td>6.5</td>
</tr>
<tr>
<td>6</td>
<td>2.8</td>
<td>4.0</td>
</tr>
<tr>
<td>6a</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>7</td>
<td>1.1</td>
<td>1.9</td>
</tr>
<tr>
<td>8</td>
<td>5.1</td>
<td>2.2</td>
</tr>
<tr>
<td>Total (1–8)</td>
<td>94.9</td>
<td>91.5</td>
</tr>
</tbody>
</table>

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 (Linhardt et al., 1992a). The 2.5 (T. phylippinarum heparin) and 1.5 (bovine mucosal heparin) mol % correspond to unknown oligosaccharides. ND = not detected.

Table II. Disaccharide composition of T. phylippinarum and bovine mucosal heparins

<table>
<thead>
<tr>
<th>Disaccharide sequence</th>
<th>Tapes</th>
<th>Bovine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ 4)-α-L-IdoA2S(1→4)-α-D-GlcNAc6S(1→</td>
<td>80.6</td>
<td>73.5</td>
</tr>
<tr>
<td>Δ 4)-β-D-GlcA(1→4)-α-D-GlcNAc6S(1→</td>
<td>4.0</td>
<td>4.4</td>
</tr>
<tr>
<td>Δ 4)-α-L-IdoA2S(1→4)-α-D-GlcNAc6S(1→</td>
<td>6.0</td>
<td>10.7</td>
</tr>
<tr>
<td>Δ 4)-α-L-IdoA1(1→4)-α-D-GlcNAc6S(1→</td>
<td>1.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Δ 4)-β-D-GlcA(1→4)-α-D-GlcNAc6S(1→</td>
<td>2.3</td>
<td>1.7</td>
</tr>
<tr>
<td>Δ 4)-α-L-IdoA1(1→4)-α-D-GlcNAc6S(1→</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Total mol % (measured in oligosaccharides 1–8)</td>
<td>94.9</td>
<td>91.5</td>
</tr>
</tbody>
</table>

The mol % of each oligosaccharide was % total absorbance corresponding to each peak. The oligosaccharides each contain a ΔUA2S at their reducing termini that is used for their detection. The ΔUA2S residue is assumed to arise from an α-L-IdoA2S residue on the basis of the specificity of heparinase.

quantitative differences between heparins from different sources are worth discussing. The T. phylippinarum heparin has more than twice the amount of anticoagulant activity (see later discussion) and shows an average molecular mass and a content of slow-moving species higher than the commercial mammalian heparin preparations (Dietrich et al., 1985; Edens et al., 1992; Linhardt et al., 1992b). These do not seem to be relevant structural differences, because heparin populations with these features have been isolated from the commercial preparations by a variety of methods

Table III. Anticoagulant activities of T. phylippinarum and bovine mucosal heparins

<table>
<thead>
<tr>
<th>Heparin samples</th>
<th>APTT</th>
<th>anti-Xa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine mucosal</td>
<td>145 ± 24.6</td>
<td>125 ± 18.7</td>
</tr>
<tr>
<td>T. phylippinarum</td>
<td>347 ± 56.4</td>
<td>317 ± 48.3</td>
</tr>
</tbody>
</table>

The APTT is a global clotting assay used to measure inhibition of coagulation factors in the intrinsic pathway and is commonly used to monitor heparin therapy. One hundred microliters of sample were added to 100 μl APTT reagent and incubated for 5 min at 37°C. Clotting time was measured with a Fibrometer after the addition of 100 μl preswelled 25 mM CaCl2. Measurement of clotting time was stopped at 300 s. Anti-factor Xa activity of the samples was quantitated using a Helios B Spectronic Unicam spectrophotometer. Twenty-five microliters of the samples were incubated in 375 μl Tris buffer for 1 min at 37°C, followed by an addition of 50 μl bovine factor Xa. Following a 2-min incubation at 37°C, 50 μl of Spectrozyme factor Xa (2.5 mM, American Diagnostica, Greenwich, CT) were added, and the change in optical density at 405 nm was measured for 1 min. Percentage inhibition was determined in relation to the baseline using the following formula: %I = ([ΔODbaseline − ΔODsample]/ΔODbaseline) × 100.

(Bianchini et al., 1982, 1985; Sache et al., 1982). Furthermore, studies are in progress to know whether T. phylippinarum heparin is linked to a core protein during its biosynthetic processes.

Linhardt and co-workers (1992a) developed an oligosaccharide mapping strategy able to correlate the anticoagulant properties of heparins with the quantitative presence of oligosaccharides bearing part of the ATIII-binding region. When porcine mucosal heparin is treated with heparin lyase I, six major oligosaccharides (2, 3–6, 8) are observed on SAX-HPLC, accounting for 85–90 mol % of the oligosaccharide products (Linhardt et al., 1988, 1992a; Rice and Linhardt, 1989). The structures of additional minor oligosaccharides (1, 3a, 3b, 4a, 6a, and 7) have also been reported (Linhardt et al., 1990, 1992a; Loganathan et al., 1990). Furthermore, specific oligosaccharides 3-O-sulfated corresponding to part of the ATIII-binding site have been identified as the tetrasaccharide 7 [ΔUA2S(1→4)-α-D-GlcN2S6S(1→4)-β-D-GlcA(1→4)-α-D-GlcN2S3S6S] and the hexasaccharide 8 [ΔUA2S(1→4)-α-D-GlcN2S6S(1→4)-β-D-GlcA(1→4)-α-D-GlcN2S3S6S] (Linhardt et al., 1992a). By using the same methodological approach used by Linhardt et al. (1992a) to characterize the oligosaccharide pattern of porcine mucosal heparin, we analyzed the structure of a heparin sample purified from the clam T. phylippinarum in comparison with bovine mucosal heparin. T. phylippinarum heparin was found to be composed of a greater mol % (5.1) of the hexasaccharide 8 than bovine mucosal heparin (2.2) and porcine mucosal heparin (2.2) (Linhardt et al., 1992a) whereas the mol % of the tetrasaccharide 7 was calculated to be lower (1.1) in comparison with bovine heparin (1.9) but greater than porcine heparin (0.6) (Linhardt et al., 1992a).

Porcine mucosal heparin samples with high (antifactor Xa activity of 457 U/mg) and low (antifactor Xa activity of 24 U/mg) ATIII affinity were prepared by Linhardt et al. (1992a). The high-ATIII-affinity sample yielded a high mol % of the hexasaccharide 8 (7.2 mol %)
after treatment with heparin lyase I, whereas low-ATIII-affinity heparin showed little or no 3-O-sulfation. *T. phylippinarum* heparin is enriched in this ATIII-binding site portion showing 130% more ATIII-binding sites than bovine mucosal and porcine mucosal heparin (Linhardt et al., 1992a) calculated from the mole % of the hexasaccharide 8. This increased number of ATIII-binding sites in clam heparin is accompanied by increased anticoagulant activity. The anticoagulant activity of *T. phylippinarum* heparin expressed as APTT is ~140% more than that of bovine mucosal heparin (this study and Dietrich et al., 1985). By considering anti-Xa activity, *T. phylippinarum* heparin (317 U/mg) had 120–150% greater potency than did porcine (145 U/mg) (Linhardt et al., 1992b) and bovine heparin (125 U/mg), and it showed ~30% lower activity than a high-ATIII-affinity heparin sample (Linhardt et al., 1992a).

As defined by Linhardt and colleagues (1992a), oligosaccharide 5 has the same structure as 7 except for the lack of the 3-O-sulfate group, and hexasaccharide 6a shows the same sequence of 8 without possessing the 3-O-sulfate group. For this reason, oligosaccharides 5 and 6a represent the precursors of the ATIII-binding site. Porcine mucosal heparin possesses a more abundant precursor oligosaccharide 5 (6.9 mol %) than the 3-O-sulfated parent tetrasaccharide 7 (0.6 mol %), whereas the precursor 6a is present in lower amounts (6.9 mol %) than the hexasaccharide 8 containing the ATIII-binding site (2.2 mol %) (Linhardt et al., 1992a). Interestingly, the same trend of the oligosaccharide precursors and the 3-O-sulfated parent oligosaccharides have been found in this study for bovine mucosal and *T. phylippinarum* heparins, confirming that the 3-O-sulfation of precursor 5, contrary to precursor 7, may be limiting not only in mammalian species but also in molluscs.

A structural variability in heparins from various species within and immediately adjacent to the ATIII-binding site has been reported (Loganathan et al., 1990). The ATIII-binding sites in human and porcine intestinal mucosal heparins (Linhardt et al., 1992b) and in bovine intestinal mucosal heparin (this study) are identical having the predominant structure (4)-α-L-IdoA2S(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(1→4)-α-L-IdoA2S(1→4)-α-D-GlcN2S6S(1→4)-β-D-GlcA(1→4)-α-D-GlcN2S6S(1→4)-α-L-IdoA2S(1→4)-α-D-GlcN2S6S(1→4)-β-D-GlcA(1→4)-α-D-GlcN2S6S(1→4)-α-L-IdoA2S(1→4)-α-D-GlcN2S6S(1→4). The oligosaccharide 8 contains a portion of the ATIII-binding site as it is missing of the (4)-α-L-IdoA2S(1→4)-α-D-GlcN2S6S(1→4)-β-D-GlcA(1→4)-α-D-GlcN2S6S(1→4)-α-L-IdoA2S(1→4)-α-D-GlcN2S6S(1→4)-α-D-GlcN2S6S(1→4). The oligosaccharide 8 is present in low amounts (~2 mol %) than the hexasaccharide 5 containing the ATIII-binding site (2.2 mol %) (Linhardt et al., 1992a).

The biological function of the clam heparins and their apparently specifically 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 clam 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. Heparin is released from the mast cells in response to specific inflammatory agents, such as IgE antibodies or complement fragments (anaphylatoxins). Indeed, a series of observations suggest that heparin may serve as a modulator of cellular immunological reactions or other defense mechanisms. 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 (Straus et al., 1982). The findings that heparin is present in molluscs, which apparently have no immune response, leads us to support the hypothesis that this macromolecule could function as a mechanism for the surveillance of these organisms against certain pathogens.

### Materials and methods

#### Materials

Heparin sodium salt from bovine intestinal mucosa was from Sigma (St. Louis, MO). This sample was further purified on a QAE Sephadex A-25 anion-exchange resin with precipitation in the presence of 80% methanol, dialysis, and freeze-drying as reported shortly. Heparin lyase I, heparinase, from *Flavobacterium heparinum* (EC 4.2.2.7), specific activity of 1.5 U/mg protein and heparan sulfate lyase, from *F. heparinum* (EC 4.2.2.8), specific activity of 1.5 U/mg protein, were from Seikagaku (Tokyo). Heparin lyase II, heparinase II, from *F. heparinum* (no EC number), 100–300 U/mg solid, was from Sigma. Papain from papaya latex (EC 3.4.22.2), specific activity of 16–40 U/mg protein, was from Sigma. DNase I from bovine pancreas (EC 3.1.21.1), specific activity of 10,000 U/ml and chondroitinase ABC, chondroitin ABC lyase, from *Proteus vulgaris* (EC 4.2.2.4), specific activity of 0.5–2 U/mg, were from Sigma. Unsaturated heparan/heparin disaccharides were from Seikagaku. QAE Sephadex A-25 anion-exchange resin was from Pharmacia Biotech (Uppsala, Sweden). Spectrapore dialysis tubing (Mr 1,000 cutoff) were from Spectrum (Rancho Dominguez, CA).

#### Preparation of *T. phylippinarum* heparin

Adult specimens of the bivalve mollusc *T. phylippinarum* were procured from a local market and immediately killed and the shell removed. The body (~112 g) was defatted by grinding with 10 volumes of acetone, filtration, and drying at 60°C for 24 h. The pellet (~14 g) was solubilized (1 g/20 ml) in 100 mM Na-acetate buffer, pH 5.5, containing 5 mM ethylenediamine tetra-acetic acid and 5 mM cysteine. Papain (100 mg per g of
Glycosaminoglycans were eluted with a linear gradient of NaCl from 50 mM to 1.2 M from 0 to 150 min using low-pressure liquid chromatography (Biologic LP chromatography system from BioRad, Hercules, CA) at a flow of 1 ml/min. Two volumes of ethanol were added to thecol- 

erated with 1 U chondroitinase ABC at 37°C for 12 h. After boiling for 5 min, NaCl concentration was brought to 16%, and the glycosaminoglycans were precipitated by adding 80% methanol. The dried precipitate was dissolved in 50 mM Tris-Cl buffer, pH 7.9, and treated with 1 U chondroitinase ABC at 37°C for 12 h. After boiling for 5 min, NaCl concentration was brought to 16%, and the glycosaminoglycans were precipitated by adding 80% methanol. The recovered precipitate was solubilized in 20 ml bidistilled water, dialyzed overnight at 4°C, and freeze-dried. Approximately 30 mg of heparin were recovered.

Chemical and spectroscopic characterization

The percentage of slow-moving and fast-moving heparin species was determined by agarose-gel electrophoresis (Volpi, 1993; Volpi and Maccari, 2002) and polydispersity of the heparin were determined by PAGE (Edens et al., 1992) and HPSEC by using heparin of known molecular mass (Volpi and Bolognani, 1993).

The chemical properties of heparin were recorded with a Bruker AMX400 Wb spectrometer operating at 400.13 MHz. The sample was previously lyophilised three times with D₂O (99.96 atom %). Heparin samples were at 1.0 mM, and all spectra were obtained at 29°C. The chemical shifts (δ) were quoted with respect to external sodium 4,4-dimethyl-4-silapentene-1-sulfonate (0.0 ppm). The assignment of the signals was made according to Casu et al. (1996) and Linhardt et al. (1992b).

The optical rotation ([α]D) of T. phylippinarum heparin was measured by polarimetry at c = 1 mg of heparin/ml of water and l = 10 cm.

Oligosaccharide mapping

T. phylippinarum heparin (0.1 mg in 100 μl of 50 mM pH 7.3 acetate buffer containing 25 mmol calcium acetate) was treated for 12 h at 37°C with 15 mU heparinase, after which it was frozen and stored at −70°C.

Oligosaccharides defined 1–8 according to Linhardt et al. (1992a) produced by the action of heparinase were separated and quantified by SAX-HPLC separation at 232 nm using a 5-μm Spherisorb SAX column (150 × 4.6 mm from Phase Separations, Deeside Industrial Park, Deeside Clwyd, UK). Isocratic separation was run from 0 to 5 min with 50 mM NaCl, pH 4.0, and linear gradient separation was from 5 to 90 min with 100% 50 mM NaCl, pH 4.0, to 100% 1.2 M NaCl, pH 4.0, at a flow of 1.2 ml/min, as previously reported (Volpi, 2003). The structures of 1–8 have been fully established (Linhardt et al., 1989, 1992a; Loganathan et al., 1990; Rice and Linhardt, 1989) and are as follows:

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(1→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-GlcN2S6S;ΔUA2S(1→4)-α-D-GlcN2S6S(1→4)-α-L-IdoA(1→4)-α-D-GlcN2S6S;ΔUA2S(1→4)-β-D-GlcA(1→4)-α-D-GlcN2S6S;ΔUA2S(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-GlcN2S3S6S;
8. ΔUA2S(1→4)-α-D-GlcN2S6S(1→4)-α-L-IdoA(1→4)-α-D-GlcN2S3S6S.

Major species were identified and quantified on the basis of their comigration with oligosaccharide standards prepared according to Linhardt (Linhardt et al., 1989, 1992a; Loganathan et al., 1990; Rice and Linhardt, 1989) and the structure established by comparison with disaccharide standards and SAX-HPLC separation. The major peaks were first assigned by the coinjection of oligosaccharide standards pre-

Anticoagulant properties

The APTT and the amidolytic anti-factor Xa assay of T. phylippinarum heparin in comparison with bovine mucosal heparin were determined by methods previously described (Bianchini et al., 1982; Dietrich et al., 1985). A standard curve was prepared for each test by using the 3rd International Heparin Standard, and the heparin samples
to be tested were diluted so that their activities fell within the standard curve range. Specific activities were calculated as units per milligram. The concentration of heparin used in these bioassays was estimated by carbazole (Bitter and Muir, 1962), agarose-gel electrophoresis (Volpi and Maccari, 2002), and HPSEC (Volpi and Bolognani, 1993) assays.

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Abbreviations

APTT, activated partial thromboplastin time; ATIII, antithrombin III; HPLC, high-performance liquid chromatography; HPSEC, high-performance size-exclusion chromatography; NMR, nuclear magnetic resonance; PAGE, polyacylamide gel electrophoresis; SAX, strong anion exchange.

References


