Insights into the role of 3-O-sulfotransferase in Heparan Sulfate biosynthesis

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Abstract

3-O-sulfotransferase enzyme (sHS) from *Litopeneaus vannamei* was cloned and its substrate specificity was investigated against a number of GAG structures, including modified heparin polysaccharides and model oligosaccharides. For the heparin polysaccharides, derived from porcine intestinal mucosa heparin, sulfate groups were incorporated into glucosamine residues containing both N-sulfated and N-acetylated substitution within regions of the predominant repeating disaccharide, either I-A_{NS} or I-A_{NAc}. However, the resulting polysaccharides did not stabilize antithrombin, which is correlated with anticoagulant activity. It was also shown that the enzyme was able to sulfate disaccharides, I_{2S}-A_{NS} and G-A_{NAc}. The results further illustrate that 3-O-sulfation can be induced outside of the classical heparin-binding pentasaccharide sequence, show that 3-O-sulfation of glucosamine is not a sufficient condition for antithrombin stabilization and suggest that the use of this enzyme during HS biosynthesis may not occur as the final enzymatic step.

Key words: Sulfotransferase, Biosynthesis, Heparam Sulfate, Heparin

Abbreviations: I: α-L-iduronate; I_{2S}: iduronic acid-2-O-sulfate; G: β-D-glucuronate; A*: glucosamine-3-O-sulfate; A_{NAc}: N-acetyl glucosamine; $A_{NAc,3S}$: N-acetyl glucosamine-3-O-sulfate; A_{NS}: glucosamine-N-sulfate; $A_{NS,6S}$: N-sulfated glucosamine- 6-O-sulfate; $A_{NS,6S,3S}$: N-sulfated glucosamine- 6,3-O-sulfate; NDST: N-Deacetylase/N-sulfotransferase; HS2ST: heparan sulfate 2-O-sulfotransferase; HS6ST: heparan sulfate 6-O-sulfotransferase; HS3ST: heparan sulfate 3-O-sulfotransferase; HS3ST1: heparan sulfate 3-O-sulfotransferase 1; HS3ST5: heparan sulfate 3-O-sulfotransferase 5; sHS: sulfotransferase from shrimp *L. vannamei*; hHS3ST: heparan sulfate 3-O-sulfotransferase from *Homo sapiens.*

Introduction

Heparan sulfate (HS) and heparin are sulfated glycosaminoglycans (GAG) composed of repeating disaccharide units of (1→4)-linked α-D-glucosamine and uronic acid. Whereas HS disaccharides are predominantly formed by β-D-glucuronate and α-D-glucosamine that can be either N-acetylated or N-sulfated, heparin is more sulfated, composed mainly of α-L-iduronate 2-O-sulfate and α-D-glucosamine N,6 sulfate [1]. These modifications occur in the Golgi apparatus via a series of N-deacetylases/N-sulfotransferase (NDST), sulfotransferases and a C5-epimerase. HS and heparin biosynthetic processes were first described by Lindahl in 1977 [2] and since then it has been assumed that the enzymatic processing, ultimately resulting in unique substitution patterns, occurs through a hierarchical sequence of enzymatic events [2] where NDST is followed by C5-epimarese, 2-O-sulfotransferase, 6-O-sulfotranferase and, lastly, 3-O-sulfotransferase.

The presence of HS biosynthesis enzymes in organisms is related strongly to the emergence of multicellularity and tissue organization, being a characteristic of eumetazoan lineage [3, 4]. Moreover, a correlation between the complexity of an organism and the number of HS sulfotransferase isoforms is evident. For instance, the primitive organism *C. elegans* has just one isoform for each of the five known sulfotransferases, while humans have four NDSTs, one C5-epimerase, one HS2ST, three HS6STs and seven HS3STs [3]. Interestingly, rudimentary HS biosynthesis enzymes were found in unicellular and colony-forming organism *M. brevicollis*, suggesting that GAGs could have a key role in the emergence of multicellularity through extracellular organization and establishment of cell-cell communication [3].

HS and heparin are known to regulate a wide range of physiological processes [5] and heparin is employed for its pharmacological activity in cardiovascular medicine as an anticoagulant and antithrombotic drug [6] since it modulates antithrombin (AT), the principal physiological inhibitor of the coagulation in vertebrates. The sequence in heparin which is thought principally responsible for this activity corresponds to a pentasaccharide $A_{NAc/NS,6S}$-G-$A_{NS,3S,6S}$-I_{2S}-$A_{NS,6S}$ (AGA*IA), found on average in one-third of the heparin chains [7]. This specific sequence was described following fractionation of heparin by affinity chromatography, which revealed that AT high-affinity fractions had 3-O-sulfated glucosamine [8]. From then on, this modification has been considered the key for AT-binding [9, 10], since pentasaccharide sequences lacking 3-O-sulfated glucosamine have decreased in affinity for AT [10], even though the presence of 3-O-group in central glucosamine is not essential to activate AT [9] since other GAGs and non-GAG based structures are able to activate AT [11, 12].

Despite being unique and responsible for high-affinity AT-binding, different sequences and compounds, even non-carbohydrates, can exert the same effect [12-14]. Furthermore, it is also important to highlight that under normal physiological conditions there is little circulating heparin, indicating that its biological function should be distinguished from its pharmacological use [1]. Furthermore, 3-O-sulfated glucosamine is yet to be found within commonly purified HS.

HS3ST is the largest sulfotransferase family in humans, although the simplest organism in which this enzyme has been reported is *M. brevicollis* [3] suggesting that, evolutionarily, 3-O-sulfated glucosamine could be the most ancient and has played an important role in the emergence of cellular communication. In addition, species that lack AT-mediated coagulation have heparin-like molecules containing high levels of 3-O-sulfated glucosamine with negligible anticoagulation activity [15-18]. These data bring to light questions regarding the importance of the 3-O-sulfate group in AT activation and its role regarding the biological functions of HS.

In a previous study, a heparin-like polysaccharide from the shrimp *L. vannamei* exhibited a higher proportion of 3-O-sulfated glucosamine (A*) than mammalian heparin, however, this compound presented low anticoagulation activity [18]. Here, in order to better understand the role of 3-O-sulfotransferase in HS/heparin biosynthesis, the cloning, expression of 3-O-sulfotransferase from *L. vannamei* and an investigation into substrate recognition are reported.

Materials and Methods

1. Materials

[^{35}S]PAPS (3'Phosphoadenosine-5'phosphosulfate-[^{35}S], 2,3Ci/mmol) was purchased from Perkin Elmer NEM (Waltham, MA, USA). Hyaluronic acid sodium salt (HA, from *Strecococcus sp*) was purchased from Merck Millipore (Kenilworth, NJ, USA). Chondroitin 4-sulfate (C4S, from bovine trachea), chondroitin 6-sulfate (C6S, from shark cartilage) and dermatan sulfate (DS, from porcine mucosa) were obtained from Sigma-Aldrich Co (St, Louis, MO, USA). Heparan sulfate (HS) from bovine pancreas was prepared according to [19]. Heparin (Hep) from porcine mucosa was obtained from Bioiberica S.A. (Barcelona, Catalunya, Spain), while chemically modified heparins were prepared as described in [20]. The two oligosaccharides were synthetized as described in [21].

2. *Litopeneaus vannamei* sulfotransferase (sHS) cloning

To clone the sulfotransferase (sHS) from shrimp *L. vannamei,* the expressing sequence tag (EST), named Contig 7734-v1, from The Marine Genomics Project database (http://mgnew.clemson.edu/) was used. This EST was used because it showed similarity to human HS3ST1 and HS3ST5 by Blastx from NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Thus, a portion of the Contig 7734-v1 was used as a primer to amplify the catalytic domain of the enzyme from *L. vannamei* using the SMARTer Race 5'/3' Kit. The coding-sequence obtained was performed according to the manufacturer´s instructions. Finally, it was PCR-amplified using forward primer (5´GAAGATCTTCCGGAGGCTGCCCCAA 3´) and reverse primer (5´GAATTCGAACTTCAGCTGGCCTTAACG 3´). The PCR product was purified by agarose electrophoresis after digestion with BglII and EcoRI endonucleases enzymes, cloned in pRSET A and transformed into *E. coli* BL21 pLysS competent cells. The coding-sequence from shrimp was confirmed by Sanger sequencing in an Applied Biosystems 3130 Genetic Analyzer.

3. Protein expression and purification

After induction with 1mM IPTG, the protein expression was carried out in LB medium at 37°C for 1h and 200 rpm. The bacterial pellet was suspended in 10 mL of lysis buffer (50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, 1 mg/mL of lysozyme), incubated in ice for 30min and sonicated on ice using six 30-second bursts. Lysed cells were centrifuged (4000 *x g* for 20min, 4°C) and the supernatant was applied to a HisTrap HP column (GE Healthcare), previously equilibrated with native binding buffer (50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, 10 mM imidazole). The column was exhaustively washed with native wash buffer (50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, and 40 mM imidazole) and the recombinant protein was eluted with native elution buffer (50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, 250 mM imidazole). Fractions were pooled and buffer-exchanged to phosphate-buffered saline using PD-10 desalting column (GE Healthcare). The recombinant protein was stored at -20°C.

4. *In silico* analysis

The similarity of HS/Hep sulfotransferases from human to the cloned-sequence from the *L. vannamei* was analyzed by MUSCLE (Multiple Sequence Alignment) from EMBL-EBI (http://www.ebi.ac.uk/Tools/msa/muscle/). Furthermore, structure protein prediction was performed using PHYRE2 Protein Fold Recognition Server program (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi? id=index).

5. Recombinant sHS activity assay

5.1 Glycosaminoglycan substrate

Sulfotransferase activity was determined by incubating 40 µL of purified recombinant enzyme (0.05 µg/µL) with 100 µg of acceptor substrates and 50000 cpm [^{35}S]PAPS in 100 µL of reaction buffer (50mM Imidazole, pH 7.0, 0.025 µg/µL protamine sulfate, 1 mM dithiothreitol). Acceptor substrates were HS, heparin (Hep), modified heparin: O,N-desulfated-N-reacetylated (HepNAc), O,N-desulfated-N-resulfated (HepNSulfo), N-desulfated-N-reacetylated Heparin (HepdNSrNAc), C4S, C6S, DS and HA. Reactions were incubated at 37°C overnight and stopped by heating at 100°C for 1min. The ^{35}S-labeled products were examined by agarose gel electrophoresis.

5.2 Oligosaccharides substrates

The activity of sHS was also analyzed using two oligosaccharides (G-A_{NAc} and I_{2S}-A_{NS}) as acceptor substrates in order to confirm the specificity of recombinant enzyme to heparan sulfate/heparin sequence and whether HS/Hep biosynthesis occurs under the hierarchical model. The reaction mixture contained 50 mM MES, pH 7.0, 10 mM $MnCl_2$, 5 mM $MgCl_2$, 0.5 µg of oligosaccharide, 50000 cpm [^{35}S]PAPS and 80 µL of recombinant enzyme in a final volume of the 200 µL. After incubation at 37°C overnight, the reaction was stopped by heating at 100°C for 1min. In the negative control of each reaction, enzyme was substituted by water. ^{35}S-labeled products were chromatographed in 500 µL DEAE-Sepharose using NaCl gradient 0.02 - 2 M for 22 mL with a flow of 0.250 µL/min in an ÄKTA purifier system (GE Healthcare). Fractions (500µL) were collected and the quantity of ^{35}S-sulfated oligosaccharide was measured by liquid scintillation counting. The values for the blank reaction were subtracted from each run.

6. Agarose gel electrophoresis (PDA)

All reactions products were submitted to agarose gel electrophoresis as previously described by Dietrich and Dietrich, 1976 [22]. Briefly, 25 µg of each acceptor (5 µL) was applied to a 0.55% agarose gel in 0.05 M 1,3-diaminopropane-acetate buffer, pH 9.0 and subjected to electrophoresis at 100 V for 1h. The gels were fixed with 0.1% cetyltrimethylammoinum bromide (CETAVLON) solution for 2h, dried and stained with toluidine blue solution (0.1% toluidine blue in 1% acetic acid in 50% ethanol), destained with 1% acetic acid in 50% ethanol solution. Subsequently, the gels were exposed for three days to radiation sensitive films and developed in Cyclone Storage Phosphor System (Packard Instrument Company INC., Groningen, Netherlands).

7. NMR spectroscopy

Prior to NMR experiments, the ^{35}S-labeled HepNSulfo was purified. Briefly, to the sample was added trichloroacetic acid 90% (10% of sample volume) and, after 30 min on ice, the sample was centrifuged and the supernatant was collected. 2 volumes (v/v) of methanol were added and, after 24h at -20 °C, the precipitate formed was collected by centrifugation (10000 *x g* for 15min at 4 °C), dried and suspended in 0.5 mL deuterium oxide (D99.99%, Aldrich Chemistry, St. Louis, MO, USA) containing 0.006% TSP 3-(trimethylsilyl)proprionic -2,2,3,3-d4 acid). The spectra were obtained in the

Superconducting Fourier NMR Spectrometer AVANCE III 600 MHz, Bruker Corporation using the Triple Resonance Broadband Inverse (TBI) probe, at Instituto de Química, Unicamp, Campinas, SP, Brazil.

8. Differential scanning fluorimetry (DSF)

The capacity of ^{35}S-labeled products to stabilize antithrombin was analyzed as described by Lima et al., 2013 [11]. Human antithrombin (AT) (1 mg/mL), previously purified from citrate plasma on a heparin-Sepharose column, was incubated with different substrates in the presence of SyproOrange™ dye (Invitrogen). Firstly, the dye was diluted in water (1 sypro:50 water (v/v)) and 3.5 µL was added to the mixture reaction in PBS buffer. The dye has an excitation wavelength of 300 nm or 470 nm and emits at 570 nm when bound to hydrophobic residues. Different substrates used were 25 µg of unfractionated heparin (UFH) (10 mg/mL), 62.5 µg of Arixtra pentasaccharide ($A_{NS,6S}$-G_{2OH}-$A_{NS,6S,3S}$-I_{2S}-$A_{NS,6S}$-OMe) (12.5 mg/mL), 25 µg of each modified heparin and 25 µg of ^{35}S-labeled modified heparins, previously analyzed by PDA. Reactions were incubated at 31°C for 2 min, and, then they were subjected to a step-wise temperature gradient from 32 to 85°C in a 0.5°C steps. Between each temperature step, there was a 5s incubation period to equilibrate samples. Reactions were developed at 7500 Real Time PCR System (Applied Biosystems) in triplicate. The final curves were generated employing the first derivative of the melting curves.

Results

Phylogenetic analysis of sHS from *Litopenaeus vannamei*

After cloning and sequencing the sHS from shrimp, the amino acid sequence was predicted using Expasy software: SIB Bioinformatics Resouce Portal through the Translate tool (Figure 1A and S1). Figure 1 shows the sHS domains responsible for the carbohydrate and PAPS binding, which are shown in red in the cloned region. Furthermore, comparative analyses were performed, including all HS sulfotransferases from *Homo sapiens*. These nucleotide and amino acid sequences were obtained from PubMed-NCBI and UniProteinKB databases respectively and aligned using MUSCLE (Figure 1B and 1C). The analysis showed that the heparan sulfate 3-O-sulfotransferase family from *Homo sapiens* (hHS3ST) exhibited homology with sHS,

the isoforms 1 and 5 being the closest, and are those thought to be responsible for anticoagulant HS production. Protein 3D structure was modeled using PHYRE2 Protein Fold Recognition Server, shown in Figure 1D, which highlights the significant structural similarity between sHS and the human heparan sulfate 3-O-sulfotransferase isoform 5.

Substrate selectivity for sHS

a. Broad selectivity assays

Heparan sulfate 3-O-sulfotransferase transfers sulfate from PAPS (adenosine 3′-phosphate 5′-phosphosulfate) to the specific 3-OH position of a glucosamine to generate 3-O-sulfated heparan sulfate. The activity assay was based on the transfer of radioactive sulfate from PAP[^{35}S] to selected substrates. First, we tested the substrate selectivity of sHS towards various glycosaminoglycans. Only chemically modified heparins served as substrates for sHS (Figure 2A) whereas chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate and hyaluronic acid did not (Figure S2), indicating that the sHS modifies only HS/heparin. Nonetheless, absence of radioactivity in HS and heparin may suggest that the 3-OH sites were already modified, resulting in null sHS action. Furthermore, since N-desulfated-N-reacetylated heparin, which would be the common substrate for NDST, was not modified by sHS, the recombinant enzyme from *L. vannamei* belongs to the O-sulfotransferase family. Figure 2A shows the activity assay on modified heparins where both sHS and recombinant human HS3ST5 were able to transfer sulfur to the chemically modified substrates, O,N-desulfated-N-resulfated and O,N-desulfated-N-reacetylated heparin.

b. Determination of sulfation site by NMR

Proton and carbon NMR chemical shifts of HepNSulfo, used as control, and HepNSulfo$^{[35]}$S-sulfate, previously submitted to sHS action, were assigned by Heteronuclear Single Quantum Coherence spectroscopy (HSQC) experiments. The HepNSulfo$^{[35]}$S-sulfate exhibited signals similar to those ascribed to the control. Nevertheless, only the HepNSulfo$^{[35]}$S-sulfate showed a signal at 5.5/99.5 ppm that corresponds to $A_{NS,6X,3S}$, indicating that sHS was able to transfer the sulfate from PAPS to C3-glucosamine (Figure 2B).

3-O-sulfotransferase in the HS biosynthesis pathway

a. Hierarchical vs non-hierarchical biosynthesis

We further tested the sHS substrate recognition using two octasaccharides (G-A_{NAc} and I_{2S}-A_{NS}) as substrates for the enzyme. As observed in Figure 3, both octasaccharides were modified by the sHS and, together with our previous findings, shows that sHS does not require either 2-O-, 6-O- or N-sulfate in heparin/HS to modify the polymer and, surprisingly, N-acetylation does not block 3-O-sulfation as anticipated by the hierarchical biosynthetic process where 3-O-sulfation would happen as the final modification step [2]. The data show that 3-O-sulfation can occur in distinct biosynthetic steps either being the last HS sulfotransferase on biosynthesis process or the first one in a non-hierarchical way, according to the oligosaccharides tested.

b. Tree structure for 3-O-sulfation

Studies employing chemoenzymatic approach have revealed that different isoforms of HS3ST could sulfate HS through different pathways in biosynthesis, since HS3ST1 can only work after the 6-O-sulfation step, while HS3ST3 must precede the 6-O-sulfation modification to generate the $A_{NS,3S,6S}$ glucosamine residue [14]. In Figure 4, different 3-O-sulfated heparin structures are shown. The classical HS biosynthetic route is illustrated by the pentasaccharide production, which is synthetized through hierarchical sequence of enzymatic events (NDST → Epimerase → HS2ST → HS6ST → HS3ST) (Figure 4, sequence 1). Indeed, the hierarchical pathway encapsulates the pentasaccharide biosynthesis yet, other 3-O-sulfated structures found within mammalian heparin cannot (Figure 4, sequences 2 and 3) [23]. Furthermore, sequences from invertebrates of higher proportions of 3-O-sulfation, within “unusual” saccharide sequences and devoid of anticoagulant properties have been found (Figure 4, sequences 4 and 5) [16, 18]. A tree structure for the biosynthesis of the major heparin and HS disaccharides has been proposed [24]. Here, this scheme is expanded and, again, shows that heparin and HS biosynthesis as a whole cannot be described fully using the original description proposed by Lindahl [2].

Thermostabilizing effects of sHS-treated heparins on AT

As described by Lima et al, 2013 [22], the anticoagulant activity of heparin is correlated with the extent to which the complex between the polysaccharide and AT is stabilised, rather than by the secondary structural changes induced in AT by heparin binding. In order to analyze whether the addition of 3-O-sulfate groups induced further AT stabilization, AT was incubated with a range of different substrates and thermostabilization was measured using differential scanning fluorimetry (DSF) (Figure 5). Only unfractionated heparin (UFH) and the pentasaccharide sequence, classical anticoagulant drugs, stabilized AT (61,75°C and 65,75°C respectively) whereas heparins[35]S-sulfate, previously modified by sHS, were unable to do so (Figure 5). This shows that a 3-O-sulfate group can be introduced into the polysaccharide but, this does not necessarily bestow stabilization on AT, or imply activity.

Discussion

The first description of 3-O-sulfated glucosamine residues in heparin were suggested by Danishefsky et al., 1969 [25] when, according to methylation analyses, the authors proposed that heparin contained small proportions of 3,6-di-O-sulfoglucosamine. Owing to the fact that 3-O-sulfation is a rare modification in heparin [26] and also in HS [27] coupled to the absence of widely available methodologies able to evaluate such a modification, it is challenging to determine the real role and extent of this modification in HS and heparin biosynthetic and biological properties.

Since the discovery of 3-O-sulfate groups in heparin with high-affinity for antithrombin [8], this modification has been considered crucial to the pharmacological role of heparin since the presence of 3-O-sulfation in the central glucosamine of the pentasaccharide (AGA*IA) increases 1000-fold the heparin binding for AT [10]. Nonetheless, besides the presence of 3-O-sulfated glucosamine, several other structural features of heparin have been reported to influence in AT activity [10, 14, 28-30].

In this study, we cloned and characterized a sulfotransferase from the shrimp *L. vannamei* similar to the isoforms 1 and 5 from *Homo sapiens*. The results suggest that this cloned enzyme is able to transfer sulfate to C3 position of glucosamine and, since these isoforms are responsible for their production [31, 32], also produce anticoagulant polysaccharides. The identity of the 3-O-sulfotransferase was confirmed by radiolabeling activity assays and NMR spectroscopy.

According to the classical HS biosynthesis [33], HS chain modification occurs hierarchically, meaning that 3-O-sulfotransferase is the last enzyme to modify the HS chains. Nevertheless, both $HepN_{Ac}$ or $G\text{-}A_{NAc}$ compounds, which show only N-acetyated glucosamine in their structure, were modified by sHS, indicating that the HS sulfotransferases could act on substrates independent of this sequential route [14, 24], which emphasizes that 3-O-sulfotransferase works at different steps of the biosynthetic process. It is important to highlight that organisms such as *M. brevicollis*, which were the first organisms to exhibit HS sulfotransferases, have only HS2ST and HS3ST. Furthermore, it has also been suggested that the epimerase enzyme appears evolutionarily later in a development [3]. Hence, 3-O-sulfated heparin/HS can be biosynthesized through different pathways.

It has been demonstrated that the high affinity of heparin towards AT requires a pentasaccharide sequence that contains a 3-O-sulfate group in a central glucosamine unit [10, 34]. However, our results show that the 3-O-sulfated chemically modified heparins were not able to further stabilize AT, compared to their untreated counterparts, suggesting that the presence or abundance the 3-O-sulfation is not, in itself, sufficient to provide anticoagulant activity [9, 18]. One may argue that the 3-O-sulfation of the studied polysaccharides is too low to promote AT stabilization but it is important to highlight that these modification themselves, even within pharmaceutical heparins, are indeed low and, in our work, they were high enough to be detected by NMR, that is, at least 2-3%. Moreover, previous studies have already shown that the presence of 3-O-sulfated groups in heparan is not essential for normal hemostasis, since $Hs3t1^{-/-}$ knockout mice did not exhibited a pro-coagulant phenotype [35], questioning the role of this modification: other heparin features are relevant for such events [18].

In summary, our data show that 3-O-sulfate groups are not solely responsible for AT stabilization and that 3-O-sulfotransferases can work in a non-hierarchical fashion during the HS/Heparin biosynthetic process.

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Conflict of Interest

None.

References

1. M. C. Meneghetti, A. J. Hughes, T. R. Rudd, H. B. Nader, A. K. Powell, E. A. Yates and M. A. Lima, *J R Soc Interface*, 2015, **12**, 0589.
2. U. Lindahl, *Ups J Med Sci*, 1977, **82**, 78-79.
3. A. Ori, M. C. Wilkinson and D. G. Fernig, *J Biol Chem*, 2011, **286**, 19892-19904.
4. G. F. Medeiros, A. Mendes, R. A. Castro, E. C. Baú, H. B. Nader and C. P. Dietrich, *Biochim Biophys Acta*, 2000, **1475**, 287-294.
5. J. R. Bishop, M. Schuksz and J. D. Esko, *Nature*, 2007, **446**, 1030-1037.
6. L. B. Jaques, *Pharmacol Rev*, 1979, **31**, 99-166.
7. U. Lindahl, G. Bäckström, M. Höök, L. Thunberg, L. A. Fransson and A. Linker, *Proc Natl Acad Sci U S A*, 1979, **76**, 3198-3202.
8. U. Lindahl, G. Bäckström, L. Thunberg and I. G. Leder, *Proc Natl Acad Sci U S A*, 1980, **77**, 6551-6555.
9. B. Richard, R. Swanson and S. T. Olson, *J Biol Chem*, 2009, **284**, 27054-27064.
10. D. H. Atha, J. C. Lormeau, M. Petitou, R. D. Rosenberg and J. Choay, *Biochemistry*, 1985, **24**, 6723-6729.
11. M. A. Lima, A. J. Hughes, N. Veraldi, T. R. Rudd, R. Hussain, A. S. Brito, S. F. Chavante, I. I. Tersariol, G. Siligardi, H. B. Nader and E. A. Yates, *MedChemComm*, 2013, **4**, 870-873.
12. B. L. Henry, J. Connell, A. Liang, C. Krishnasamy and U. R. Desai, *J Biol Chem*, 2009, **284**, 20897-20908.
13. A. S. Brito, R. S. Cavalcante, L. C. Palhares, A. J. Hughes, G. P. Andrade, E. A. Yates, H. B. Nader, M. A. Lima and S. F. Chavante, *Carbohydr Polym*, 2014, **99**, 372-378.
14. Z. Wang, P. H. Hsieh, Y. Xu, D. Thieker, E. J. Chai, S. Xie, B. Cooley, R. J. Woods, L. Chi and J. Liu, *J Am Chem Soc*, 2017.
15. A. H. Straus, O. A. Sant'anna, H. B. Nader and C. P. Dietrich, *Biochem J*, 1984, **220**, 625-630.
16. G. Pejler, A. Danielsson, I. Björk, U. Lindahl, H. B. Nader and C. P. Dietrich, *J Biol Chem*, 1987, **262**, 11413-11421.
17. C. P. Dietrich, H. B. Nader, J. F. de Paiva, E. A. Santos, K. R. Holme and A. S. Perlin, *Int J Biol Macromol*, 1989, **11**, 361-366.
18. S. F. Chavante, A. S. Brito, M. Lima, E. Yates, H. Nader, M. Guerrini, G. Torri and A. Bisio, *Carbohydr Res*, 2014, **390**, 59-66.
19. C. P. Dietrich and H. B. Nader, *Biochim Biophys Acta*, 1974, **343**, 34-44.
20. E. A. Yates, F. Santini, M. Guerrini, A. Naggi, G. Torri and B. Casu, *Carbohydr Res*, 1996, **294**, 15-27.
21. R. Liu, Y. Xu, M. Chen, M. Weïwer, X. Zhou, A. S. Bridges, P. L. DeAngelis, Q. Zhang, R. J. Linhardt and J. Liu, *J Biol Chem*, 2010, **285**, 34240-34249.
22. C. P. Dietrich and S. M. Dietrich, *Anal Biochem*, 1976, **70**, 645-647.
23. U. Lindahl, K. Lidholt, D. Spillmann and L. Kjellén, *Thromb Res*, 1994, **75**, 1-32.
24. T. R. Rudd and E. A. Yates, *Mol Biosyst*, 2012, **8**, 1499-1506.
25. I. Danishefsky, H. Steiner, A. Bella and A. Friedlander, *J Biol Chem*, 1969, **244**, 1741-1745.
26. B. E. Thacker, D. Xu, R. Lawrence and J. D. Esko, *Matrix Biol*, 2014, **35**, 60-72.
27. I. L. Tersariol, T. M. Ferreira, M. G. Medeiros, M. A. Porcionatto, C. T. Moraes, L. R. Abreu, H. B. Nader and C. P. Dietrich, *Braz J Med Biol Res*, 1994, **27**, 2097-2102.

28. M. Guerrini, S. Guglieri, B. Casu, G. Torri, P. Mourier, C. Boudier and C. Viskov, *J Biol Chem*, 2008, **283**, 26662-26675.
29. M. Guerrini, S. Elli, P. Mourier, T. R. Rudd, D. Gaudesi, B. Casu, C. Boudier, G. Torri and C. Viskov, *Biochem J*, 2013, **449**, 343-351.
30. M. Guerrini, P. A. Mourier, G. Torri and C. Viskov, *Glycoconj J*, 2014, **31**, 409-416.
31. P. Datta, G. Li, B. Yang, X. Zhao, J. Y. Baik, T. R. Gemmill, S. T. Sharfstein and R. J. Linhardt, *J Biol Chem*, 2013, **288**, 37308-37318.
32. M. B. Duncan, J. Chen, J. P. Krise and J. Liu, *Biochim Biophys Acta*, 2004, **1671**, 34-43.
33. U. Lindahl, M. Kusche, K. Lidholt and L. G. Oscarsson, *Ann N Y Acad Sci*, 1989, **556**, 36-50.
34. M. Petitou and C. A. van Boeckel, *Angew Chem Int Ed Engl*, 2004, **43**, 3118-3133.
35. N. W. Shworak, S. HajMohammadi, A. I. de Agostini and R. D. Rosenberg, *Glycoconj J*, 2002, **19**, 355-361.
36. M. Biasini, S. Bienert, A. Waterhouse, K. Arnold, G. Studer, T. Schmidt, F. Kiefer, T. Gallo Cassarino, M. Bertoni, L. Bordoli and T. Schwede, *Nucleic Acids Res*, 2014, **42**, W252-258.

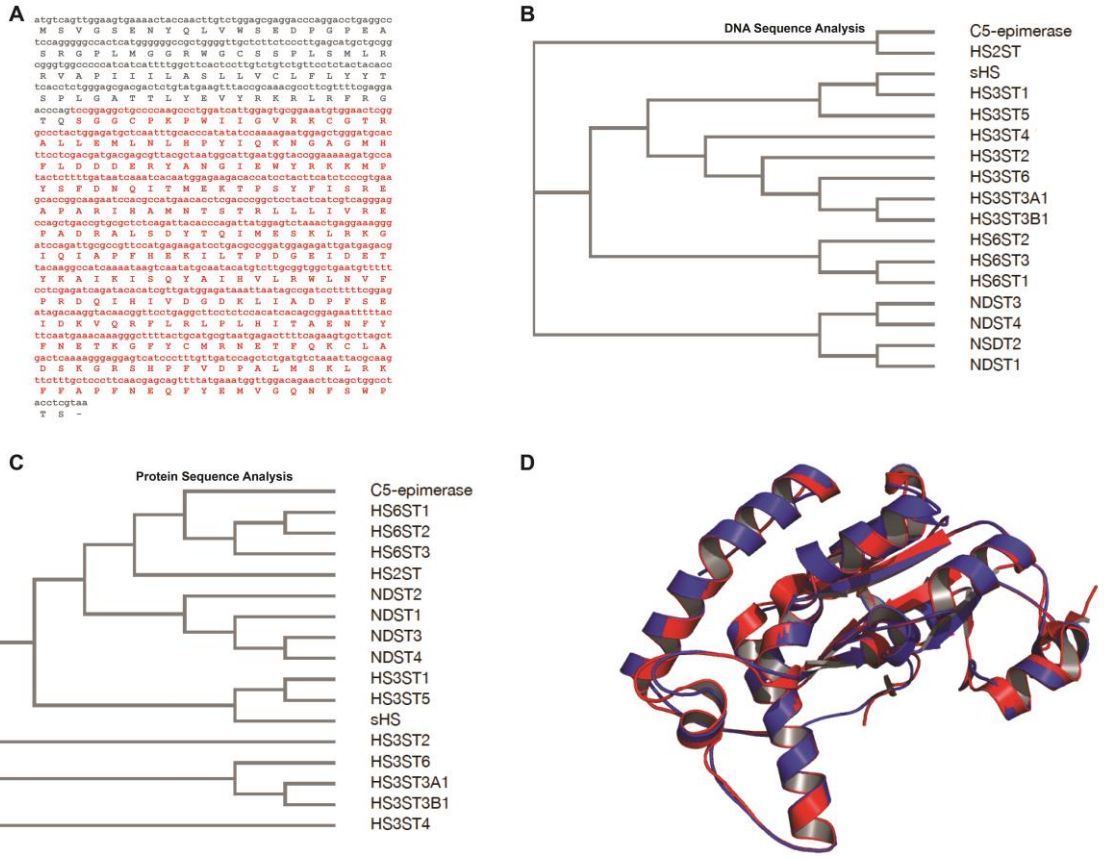


Figure 1: Composite DNA, predicted amino acid sequences and structural model for the shrimp *Litopeneaus vannamei* sulfotransferase. (A) The nucleotide fragment as well as the amino acid sequence in red are related to enzymatic domains responsible for the carbohydrate and PAPS binding. This cDNA sequence (red) was cloned in pRSET A. Amino acid sequence was predicted using by Expasy (http://web.expasy.org/translate/). (B) Cladogram analysis of DNA sequences demonstrate that the isoforms 1 and 5 of 3-O-sulfotransferase (HS3ST) from *Homo sapiens* display higher identity to sHS. (C) Cladogram analysis of amino acid sequences confirms the highest similarity is among isoforms 1 and 5 of HS3ST to sHS. (D) Comparison between tertiary structures of sHS from shrimp *L. vannamei* (red) and HS3ST5 from *Homo sapiens* (blue). The 3D structure of sHS was modeled based on the human 3-O-sulfotransferase isoform 5 crystal structure (PDB #3BD9) using SWISS-MODEL [36]. Structural alignment was performed on The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC. Abbreviations: NDST: N-Deacetylase/N-sulfotransferase; HS2ST: 2-O-sulfotransferase; HS3ST: 3-O-sulfotransferase; the isoforms of each enzyme correspond to numbers from 1 to 6.

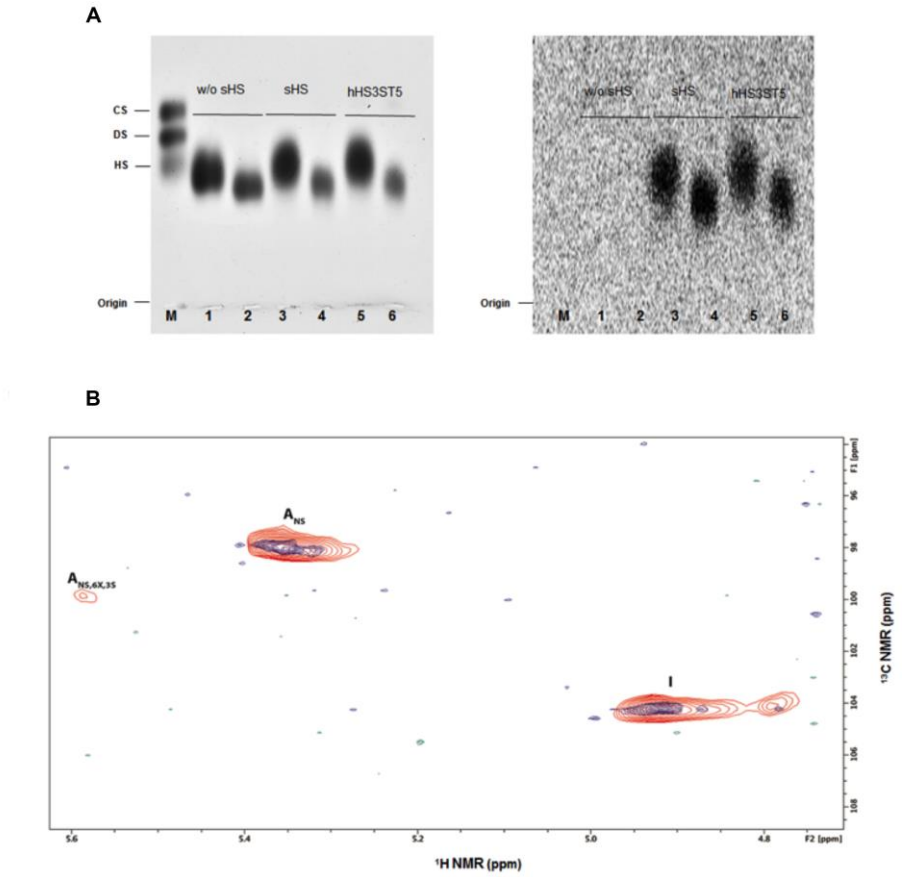


Figure 2: sHS substrate selectivity. (A) Activity assay on modified heparins. 1, 3 and 5: Substrate used was O,N-desulfated-N-resulfated heparin (25 µg of uronic acid); 2, 4 and 6: using as the substrate O,N-desulfated-N-reacetylated heparin (25 µg of uronic acid). In 1 and 2, the reaction mixture did not have the recombinant enzyme in their preparations. Left: PDA gel stained with toluidine blue. Right: PDA gel exposed for three days to radiation sensitive films. Both sHS enzyme as well as recombinant human HS3ST5 were able to transfer $^{[35]}$S-sulfate to chemically modified heparins. M: Mixture of standard glycosaminoglycans containing chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS) (5 µg each). (B) Description of compound HepNSulfo$^{[35]}$S-sulfate, previously modified by sHS, by two-dimensional heteronuclear (HSQC) NMR. The HepNSulfo control (blue) and the HepNSulfo$^{[35]}$S-sulfate (red) spectra displayed similiar components, whereas the HepNSulfo$^{[35]}$S-sulfate showed the presence of 3-O-sulfated glucosamine ($A_{NS,6X,3S}$), indicating that sHS is indeed a 3-O-sulfotransferase. Abbreviations: I: iduronic acid, A_{NS}: glucosamine N-sulfated and $A_{NS,6X,3S}$: glucosamine N,3-sulfated, where 6X could be 6OH or 6S.

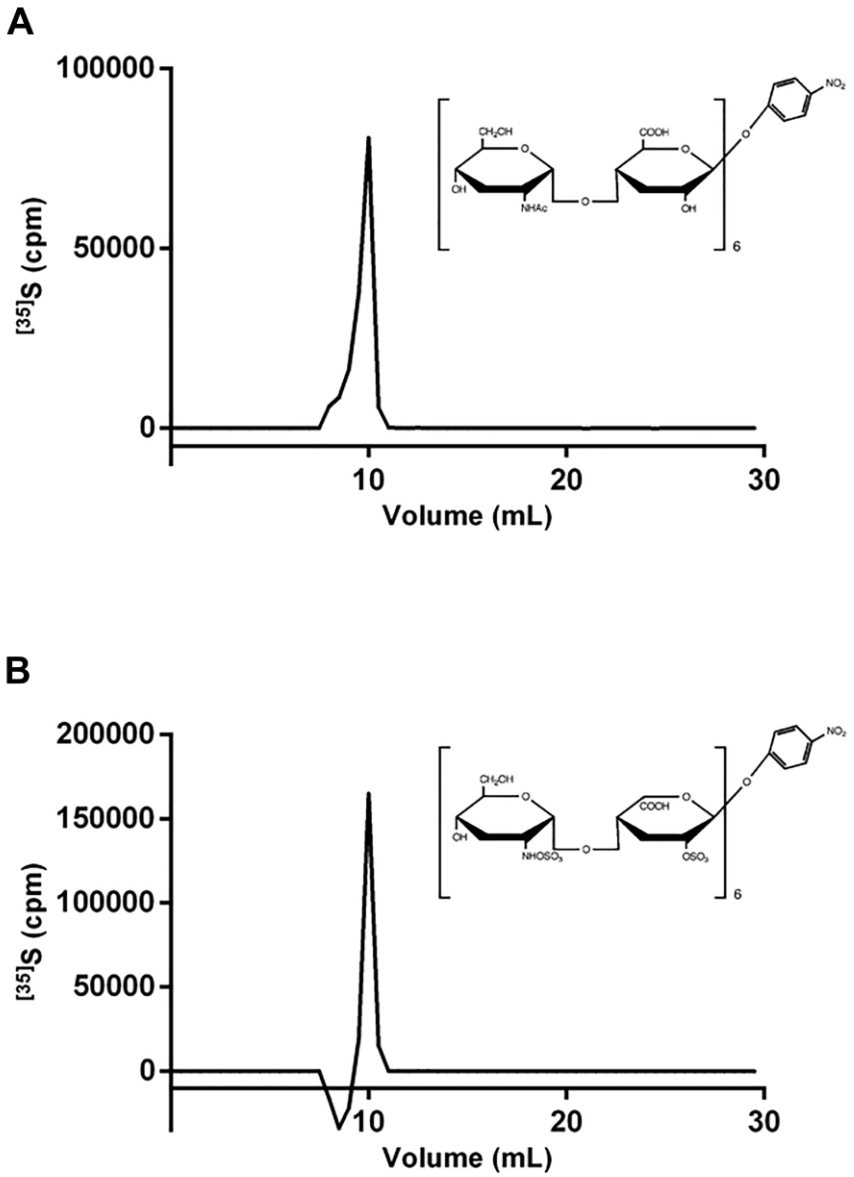


Figure 3: Analysis of 3-O-sulfation oligosaccharides by sHS. [35]S-sulfate products were analyzed using DEAE-Sepharose column and the ^{35}S radioactivity was measured by liquid scintillation counting. (A) The oligosaccharide Glc-Glc_{NAc} was used as substrate for the reaction. (B) The oligosaccharide $IdoA_{2S}$-Glc_{NS} was acceptor for the sHS activity reaction. The blank run value was subtracted from each test compound.

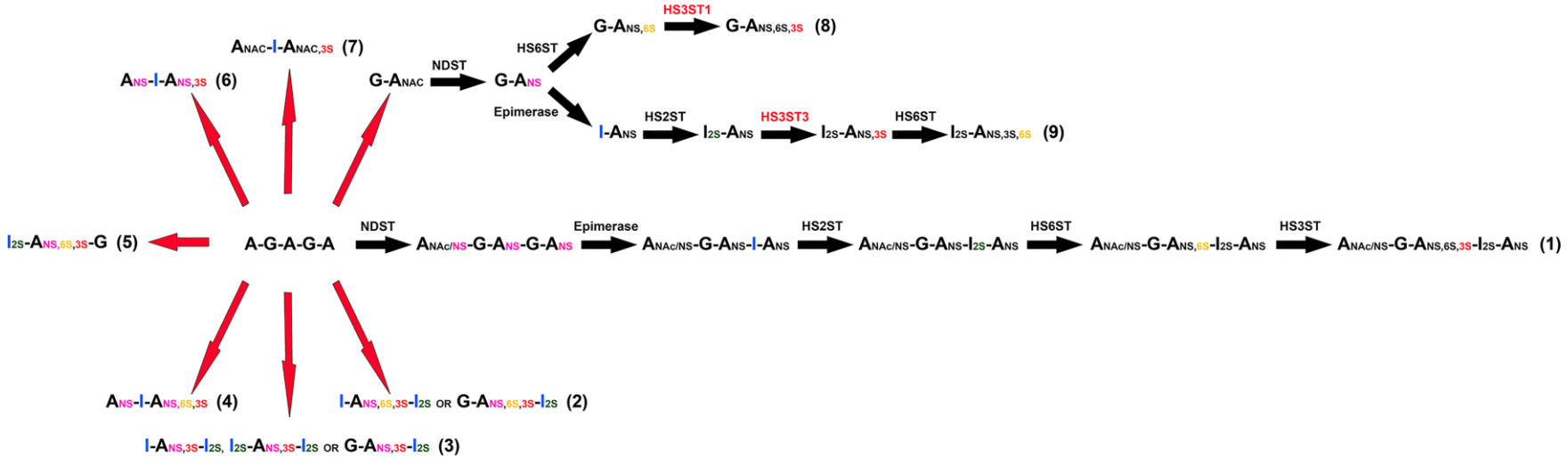
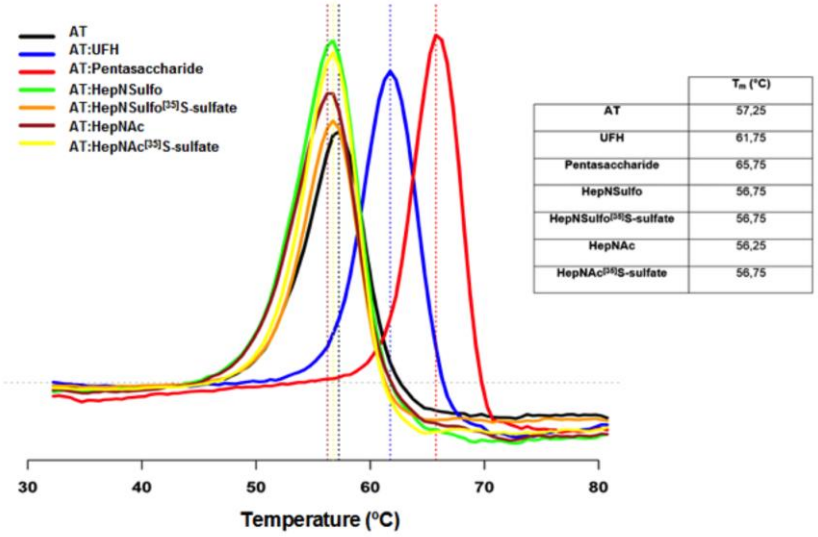


Figure 4: Different 3-O-sulfated heparin sequences found in animals and produced chemoenzymatically. (1) A minimum sequence of heparin (pentasaccharide) involved in AT-binding. It has been proposed that the pentasaccharide is synthesized according to the classical biosynthetic pathway, in which the enzymatic events happen through a hierarchical sequence, as described by Lindahl, 1977 [2]. (2 and 3) Sequences present in heparin described in Lindahl, 1994 [23]. (4) Heparin sequence found in clams that does not correlate with affinity for AT [16]. (5) Sequence described in shrimp *L. vannamei* that has negligible anticoagulant activity despite its high affinity for AT and unusually higher proportion of 3-O-sulfated residues [18]. (6 and 7) Scheme for 3-O-sulfated heparin sequences described by this study. The starting material for chemical modifications were porcine intestinal mucosa heparin, the schemes show iduronate rather than glucuronate once heparin does have significant higher proportions of iduronate. (8 and 9) Different oligosaccharide substrates required by HS3ST isoforms. While HS3ST3 must precede the 6-O-sulfation to generate the I_{2S}- $A_{NS,3S,6S}$, HS3ST1 can only work after the 6-O-sulfation step [14].



	T_m (°C)
AT	57,25
UFH	61,75
Pentasaccharide	65,75
HepNSulfo	56,75
HepNSulfo[35]S-sulfate	56,75
HepNAc	56,25
HepNAc[35]S-sulfate	56,75

Figure 5: Antithrombin stabilization assay with substrates modified by sHS. UFH (unfractionated heparin) and pentasaccharide (Arixtra™) were used as positive control, since they are already described as anticoagulant drugs. AT was incubated with different substrates and subjected to a step-wise temperature gradient. The melting temperatures for each condition are shown. Only the UFH and the pentasaccharide were able to stabilize AT, whereas none of the compounds (HepNSulfo$^{[35]}$S-sulfate and HepNAc$^{[35]}$S-sulfate) modified by the sHS and their counterpart (HepNSulfo and HepNAc) did.