the FEBS Journal

Neutralizing the anticoagulant activity of ultra-low-molecular-weight heparins using *N*-acetylglucosamine 6-sulfatase

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Keywords
antidote; coagulation; heparin; sulfatase; ULMWH

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(Received 30 November 2012, revised 29 January 2013, accepted 31 January 2013)

doi:10.1111/febs.12169

Heparin has been the most commonly used anticoagulant drug for nearly a century. The drug heparin is generally categorized into three forms according to its molecular weight: unfractionated (UF, average molecular weight 13 000), low molecular weight (average molecular weight 5000) and ultra-low-molecular-weight heparin (ULMWH, average molecular weight 2000). An overdose of heparin may lead to very dangerous bleeding in patients. Protamine sulfate may be administered as an antidote to reverse heparin's anticoagulant effect. However, there is no effective antidote for ULMWH. In the current study, we examine the use of human *N*-acetylglucosamine 6-sulfatase (NG6S), expressed in Chinese hamster ovary cells, as a reversal agent for ULMWH. NG6S removes a single 6-*O*-sulfo group at the non-reducing end of the ULMWH Arixtra® (fondaparinux), effectively removing its ability to bind to antithrombin and preventing its inhibition of coagulation factor Xa. These results pave the way to developing human NG6S as an antidote for neutralizing the anticoagulant activity of ULMWHs.

Introduction

Heparin is a member of glycosaminoglycan family, consisting of a repeating disaccharide unit of iduronic acid (IdoA)/glucuronic acid (GlcA) linked with glucosamine (GlcN), with sulfo group substituents on both saccharide units [1]. Glycosaminoglycans often have distinctive structural domains associated with the presence of *N*-sulfo or *N*-acetyl glucosamine residues, including *N*-sulfo domains, mixed *N*-sulfo/*N*-acetyl domains and *N*-acetyl domains [2,3]. Heparin is primarily comprised of long blocks of NS domains that also contain a high level of *O*-sulfo groups that are responsible for its binding to proteins such as antithrombin III (AT) [4–6]. The AT-binding domain comprises a sulfated pentasaccharide sequence within heparin that has a highly conserved and well-studied sequence [7].

Heparin has been used clinically as an anticoagulant since its discovery in 1916 [2,8]. It is widely used for the treatment of deep vein thrombosis, in hip surgery, knee replacement surgery, blood transfusions and renal dialysis [9]. The drug heparin is generally classified into three forms according to its molecular weight, unfractionated (UF, average molecular weight 13 000), low molecular weight (average molecular weight 5000), and ultra-low-molecular-weight heparin (ULMWH, average molecular weight 2000) [9,10]. UF heparin is

Abbreviations
AT, antithrombin III; CHO, Chinese hamster ovary; GlcA, glucuronic acid; GlcN, glucosamine; IdoA, iduronic acid; NG6S, *N*-acetylglucosamine 6-sulfatase; UF heparin, unfractionated heparin; ULMWH, ultra-low-molecular-weight heparin.

prepared from animal tissues, such as porcine intestine, and low molecular weight heparins are prepared through controlled chemical or enzymatic depolymerization of UF heparin [11]. In 2008, a serious contamination issue of pharmaceutical heparin affected 12 countries, and was associated with an estimated 200 deaths around the world [10,12]. Unlike UF heparin and low molecular weight heparins, ULMWHs, such as Arixtra® (fondaparinux), are produced by chemical synthesis [11], avoiding the potential contamination issues associated with animal products.

The ULMWH fondaparinux has a number of advantages when compared with UF heparin. It is subcutaneously active, has a longer half-life, improving its pharmacokinetics, and exhibits a reduced incidence of heparin-induced thrombocytopaenia [13–15]. However, one advantage of UF heparin is that it's action may be reversed through administration of an antidote, protamine sulfate. Protamine sulfate is a basic polypeptide-based drug that tightly binds UF heparin, neutralizing its activity, but it does not bind the smaller ULMWH with sufficient affinity to reverse its activity [16]. An overdose of anticoagulants may lead to very dangerous bleeding in patients, so reversal of anticoagulant activity is necessary. If bleeding after an overdose of the ULMWH fondaparinux occurs, the only method to remove the heparin is through the relatively aggressive procedure of renal dialysis [16]. A more convenient and safe method for the removal of ULMWH from the blood is necessary when such overdoses occur.

N-acetylglucosamine 6-sulfatase (NG6S) is a lysosomal enzyme that is involved in the natural catabolism of glycosaminoglycans in the body [17]. NG6S is a highly glycosylated, divalent metal ion-dependent, exolytic sulfatase [18–20] that hydrolyzes a sulfo group from a non-reducing terminal 6-*O*-sulfated glucosamine residue [21,22]. In the present paper, we describe a novel approach for neutralization of fondaparinux and another ULMWH, ULMWH1 [9] (Fig. 1A), using recombinant human NG6S to remove a 6-*O*-sulfo group from their non-reducing termini. These 6-*O*-desulfated products are expected to lose their binding affinity for AT and thus their anticoagulant activity [7].

Results and Discussion

NG6S expression, purification and determination of activity

Recombinant NG6S was prepared by cloning a portion of the human NG6S gene comprising its catalytic domain (Thr44-Leu552) into a pSecTag2 vector. The cloned plasmid was transformed into Chinese hamster

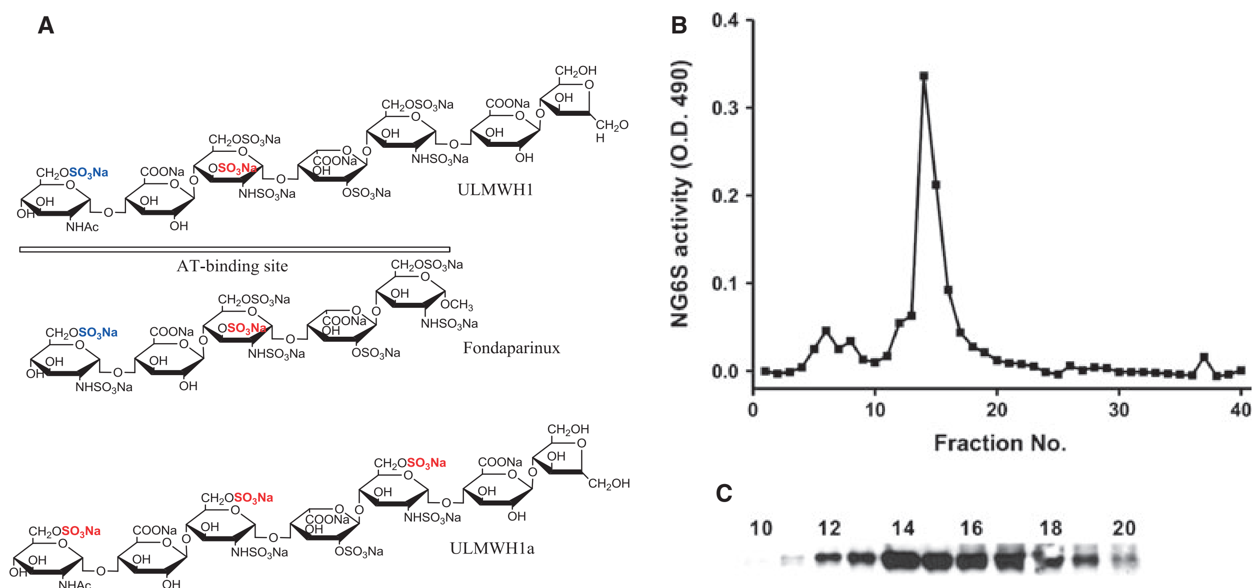

Fig. 1. NG6S purification and activity determination. (A) Structure of ULMWHs (ULMWH1 and fondaprinux) and an AT binding-negative ULMWH construct (ULMWH1a). The non-reducing end 6-*O*-sulfo group (blue) is removed by NG6S digestion. ^{35}S-labeled constructs were also used in this study. The ^{35}S label for ULMWH1 and fondaparinux was at the 3-*O*-sulfation position (red). The ^{35}S label for ULMWH1a was at the 6-*O*-sulfation position (red). (B) Activity profile of NG6S from the mono-S column. The activity was measured using 4-nitrocatecholsulfate as the substrate. (C) Western blotting of the partially purified protein in fraction collection tubes (tubes 10–20) using anti-myc serum. A protein band at 62 kDa was detected, consistent with the estimated molecular mass of recombinant NG6S.

ovary (CHO) cells, and the cells were grown in F12 medium. Supernatants containing NG6S activity were pooled and concentrated using a YM–10 filter. The concentrated solution, which is high in NG6S activity, was purified by fast protein liquid chromatography using a strong cationic exchanger (Mono–S column). The fractions containing NG6S activity were pooled and analyzed by western blotting using anti-myc serum (Fig. 1B,C).

Two ULMWHs, fondaparinux and ULMWH1 (Fig. 1A), were used in this study as substrates for NG6S. ULMWH1 was prepared as previously described [9] and fondaparinux was purchased from Cardinal Health Inc. (Dublin, OH, USA). ULMWH1 and fondaparinux both contain an AT-binding site and are terminated at their non-reducing ends by 6-*O*-sulfo-*N*-acetylglucosamine and 6-*O*-sulfo-*N*-sulfoglucosamine, respectively. Treatment at 37 °C overnight with NG6S (4 μg protein) in 100 μL of 50 mM sodium acetate pH 5.0 buffer containing 250 mM NaCl and 100 $\mu g \cdot mL^{-1}$ BSA completely removed the 6-*O*-sulfo group from the non-reducing glucosamine residue of 1 μg ULMWH.

Determination of the site desulfated by NG6S

The susceptibility of ULMWH1 to NG6S digestion was determined by measuring the retention time of ^{35}S-labeled ULMWH1 on high-resolution DEAE-HPLC. Undigested ULMWH1 eluted at 45 min (at 1000 mM NaCl), while completely digested ULMWH1 eluted at 40 min (at 900 mM NaCl) (Fig. 2A,B). This altered elution time suggests that ULMWH1 lost a single sulfo group on digestion with NG6S.

We next tested whether NG6S acts on ULMWH1 in the presence of AT. AT is known to tightly bind

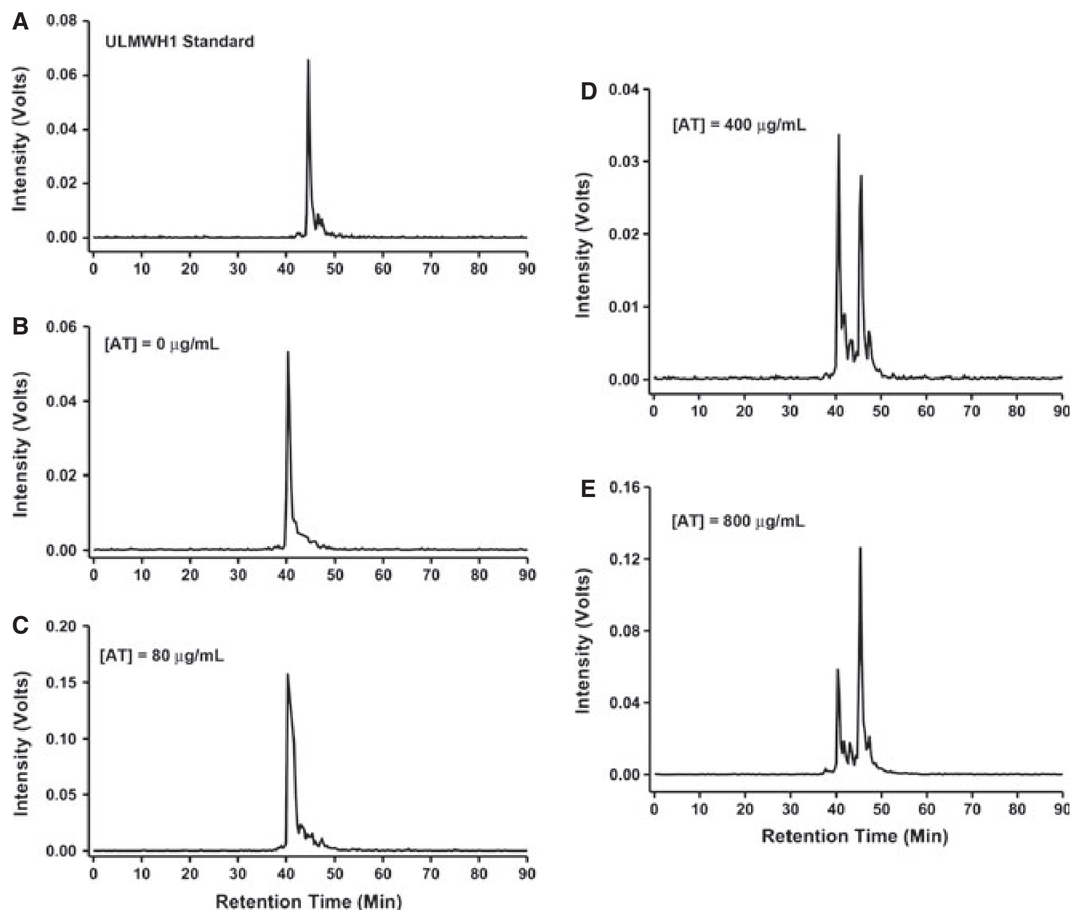

Fig. 2. DEAE-HPLC profiles of ULMWH1 treated with NG6S in the presence of AT. (A–E) ULMWH1 was incubated with various amounts of AT for 30 min in the reaction buffer at room temperature, then NG6S was added. After overnight digestion, the sample was purified using a DEAE-Sepharose column eluted with an NaCl gradient (0–1.0 M NaCl, pH 7.0) at a flow rate of 0.4 $mL \cdot min^{-1}$. ULMWH1 mixed with < 80 $\mu g \cdot mL^{-1}$ AT is completely digested, and more than 50% of ULMWH1 mixed with 400 $\mu g \cdot mL^{-1}$ AT is digested.

ULMWHs with nanomolar affinities [9], initiating anticoagulation. The plasma concentration of AT is ~ 2 μg·mL^{-1} [23], suggesting that, in order for NG6S to reverse ULMWH anticoagulant activity, it must drive the AT-bound ULMWH towards the free form. Various concentrations of AT (0–800 μg·mL^{-1}) were incubated with ULMWH1 for 30 min at room temperature before adding NG6S to initiate the reaction. The results clearly demonstrate that NG6S efficiently acts on ULMWH1 even at AT concentrations as high as 80 μg·mL^{-1} (Fig. 2C). Significant amounts of ULMWH1 were even 6-*O*-desulfated at AT concentrations as high as 400 and 800 μg·mL^{-1} (Fig. 2D,E). A time course for digestion of UMLWH1 by NG6S is shown in Fig. 3.

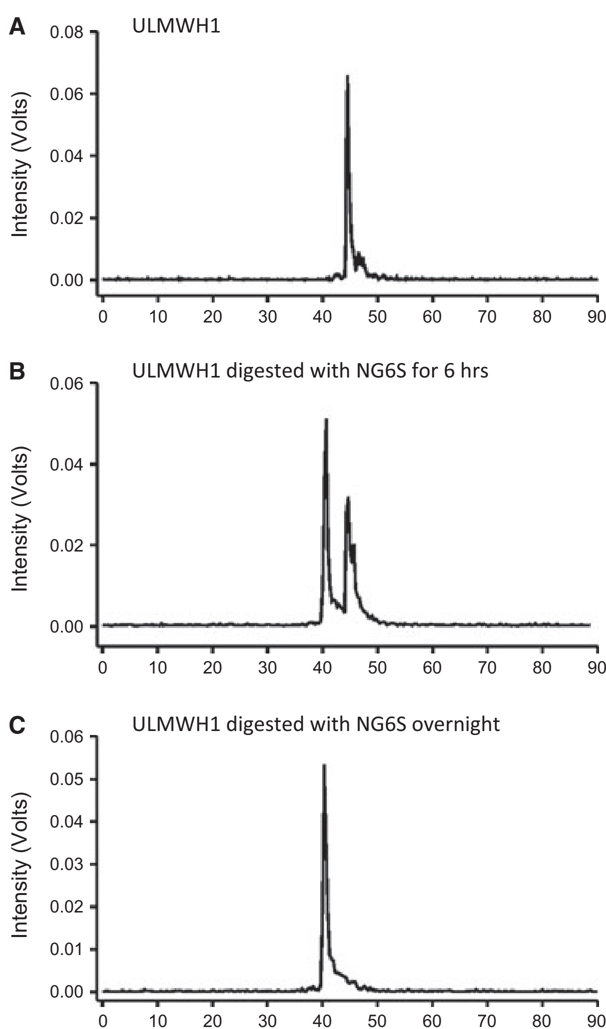

Fig. 3. Time course of ULMWH1 digestion by NG6S. (A) DEAE-HPLC profile of undigested ULMWH1 (45 min). (B) DEAE-HPLC profile of ULMWH1 digested with NG6S for 6 h, showing a new peak at 40 min. (C) DEAE-HPLC profile of ULMWH1 completely digested with NG6S overnight.

The structure of the NG6S-treated ULMWH1 was next analyzed by MS to confirm the number and position(s) of sulfo groups that had been hydrolyzed. Molecular mass measurement of untreated and NG6S-treated ULMWH1 indicated a decrease in mass by 79.9544 Da (4 × (459.7487 − 439.7601) = 79.9544), corresponding to the loss of a single sulfo group from ULMWH1 after NG6S treament (Fig. 4). MS/MS analysis was next used to determine which sulfo group had been lost. Comparison of the MS/MS fragmentation patterns of untreated and NG6S-treated ULMWH1 demonstrates that the 6-*O*-sulfo group at the non-reducing end of ULMWH1 is lost (Fig. 5). The MS/MS signal at *m*/*z* 198.9916 in the untreated ULMWH1 confirmed the presence of a 6-*O*-sulfo group ($^{0,2}A_1{}^{1-}$ fragment [24]) on the non-reducing end. After NG6S treatment, the peak at *m*/*z* 198.9916 disappeared, unambiguously demonstrating that this 6-*O*-sulfo group had been lost from the saccharide residue at the non-reducing end. NG6S treatment also resulted in the disappearance of the B1 and C1 ions. The $(Y_5{-}2H + 6Na)^{2-}$ fragment was detected in both NG6S-treated and untreated ULMWH1, clearly demonstrating that there was no additional sulfo group loss from any of the other saccharides after NG6S treatment. Thus, as expected, NG6S removes a single 6-*O*-sulfo group from the non-reducing end *N*-acetylglucosamine 6-*O*-sulfate (GlcNAc6S) residue of ULMWH1.

NG6S treatment of ULMWHs eliminates AT binding and reverses anticoagulant activity as measured by anti-factor Xa assay

ULMWH binds to AT and results in AT undergoing a conformational change, causing AT to become a potent inhibitor of coagulation factor Xa [25]. We next examined whether NG6S treatment of ULMWHs, ULMWH1 and fondaparinux, eliminated AT binding and reversed their anti-Xa activities. First, we examined the binding of NG6S-treated and untreated ULMWHs to AT. ^{35}S-labeled ULMWH1 and ^{35}S-labeled ULMWH1a (ULMWH1 lacking a 3-*O*-sulfo group, Fig. 1A) and ^{35}S-labeled fondaparinux were prepared. ULMWH1a serves as a negative control as it does not bind to AT [26]. These samples were subjected to AT affinity chromatography to determine the percentage of bound radiolabel. The results show that AT binding of ^{35}S-labeled ULMWH1 decreased from 50% to 5% after removing the terminal 6-*O*-sulfo group, and AT binding of ^{35}S-labeled fondaparinux decreased from 60% to < 5% (Fig. 6). The AT-binding level of the negative control using ^{35}S-labeled ULMWH1a was ~ 5% (Fig. 6). These

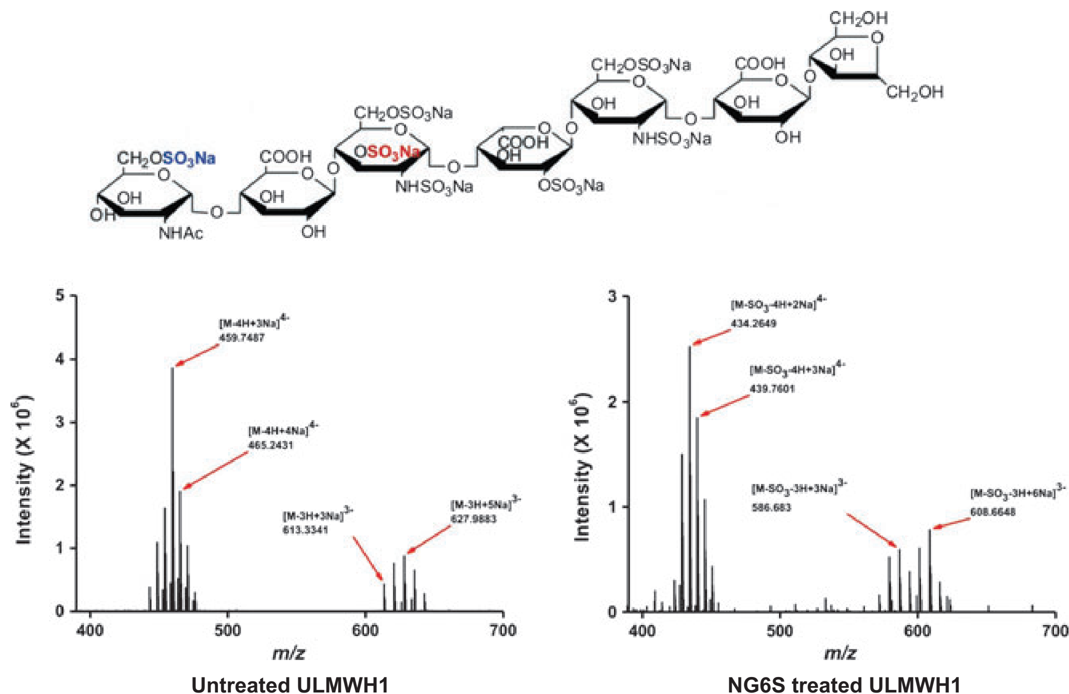

Fig. 4. MS analysis of ULMWH1. NG6S removes one sulfo group from ULMWH1. Accurate MS analysis indicated that, after NG6S treament, the mass decreased by 79.9544 Da, corresponding to loss of a single sulfo group from ULMWH1. The sodium adducts were observed as clusters, which is common for highly negatively charged ULMWH.

results confirm that NG6S treatment of ULMWHs greatly reduced their binding affinity for AT.

Nanomolar concentrations of ULMWH1 and fondaparinux strongly inhibit factor Xa activity in the presence of AT, as determined by the anti-Xa activity assay. As expected based on their loss of AT-binding affinity, neither ULMWH displayed anti-Xa activity following NG6S removal of the 6-*O*-sulfo group (Fig. 7). In this experiment, heparan sulfate obtained from bovine kidney was used as a negative control as it has no anti-Xa activity [27]. These data clearly demonstrate that NG6S effectively eliminates binding of two ULMWHs by hydrolyzing the 6-*O*-sulfo group from the non-reducing terminal glucosamine residues, resulting in oligosaccharides that have no anti-Xa activity.

The activity of NG6S at various pH

We determined the sulfatase activity of NG6S using the synthetic substrate, 4-nitrocatecholsulfate, at various pH (Fig. 8A). As expected, the optimal pH for NG6S is pH 5, consistent with the general properties of lysosomal proteins. We then compared the susceptibility of ULMWH1 to NG6S digestion at pH 5.0 and 7.0 (Fig. 7B,C). As expected, complete digestion of ULMWH1 was observed when the digestion was performed at pH 5.0, but only 15–20% of ULMWH1 was digested at pH 7.0. A lower digestion efficiency was observed for NG6S at physiological pH.

Conclusions

The widely used anticoagulants, UF heparin, low-molecular-weight heparin and the ULMWH, fondaparinux, have a worldwide market size of several billion US dollars/year [28]. ULMWHs are unique among this group of anticoagulants as they are synthesized as homogenous compounds using chemical or chemoenzymatic approaches [9,29]. Arixtra® was approved by the US Food and Drug Administration in 2001 and generic fondaparinux was approved in 2011. An overdose of UF heparin, and, to a lesser degree, an overdose of low molecular weight heparin, may be reversed through administration of the antidote protamine sulfate. However, a major problem associated with the use of fondaparinux and other ULMWHs is the lack of a similar antidote. Thus, developing an antidote for ULMWHs is very important for improving the safety of this class of heparin-based anticoagulant drugs. In the present paper, we describe a novel approach to remove the anti-Xa activities of ULMWH1 and

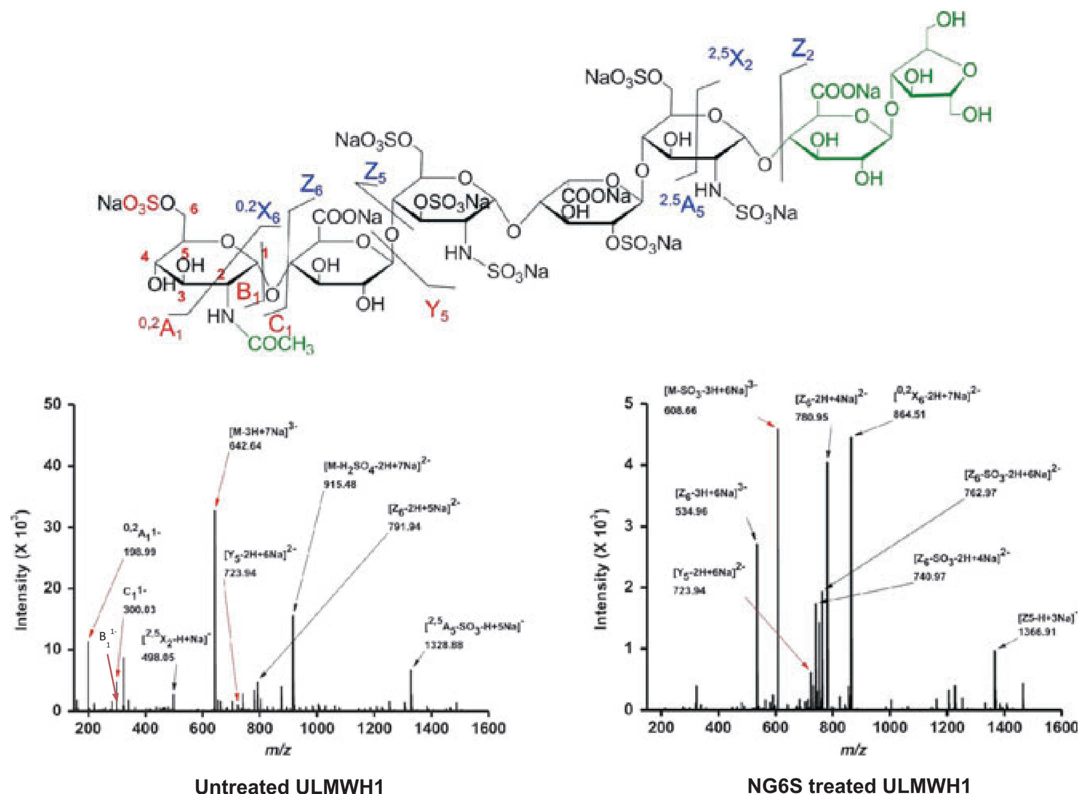

Fig. 5. MS/MS analysis of ULMWH1. Specific loss of the 6-*O*-sulfo group at the non-reducing end of ULMWH1 is confirmed by MS/MS analysis. The MS/MS signal of 198.9916 ($^{0,2}A_1^{1-}$ fragment) in the untreated ULMWH1 spectrum confirms the presence of a 6-*O*-sulfo group (red) at the non-reducing end. After NG6S treatment, the peak at 198.9916 disappeared, as did the C_1 ion. The $(Y_5-2H + 6Na)^{2-}$ fragments, detected in both NG6S-treated and untreated ULMWH1, indicate the absence of loss of non-specific sulfo groups from the other sugars in response to NG6S treatment.

fondaparinux using NG6S. Although this method may one day be implemented as an antidote for fondaparinux and other ULMWHs, there are certain limitations. First, the structure of ULMWH is critically important for its susceptibility to NG6S neutralization, requiring the 6-*O*-sulfoglucosamine residue of the AT-binding site to reside at the non-reducing terminus of the ULMWH. Second, as NG6S is a lysosomal enzyme, its pH optimum is ~ 5.0 [30]. At a physiological pH of 7, the activity of NG6S is significantly reduced. Therefore, either a substantial amount of NG6S will be required for *in vivo* neutralization of ULMWH, or protein engineering will be required to shift the pH optimum of NG6S as demonstrated in other enzymes [31].

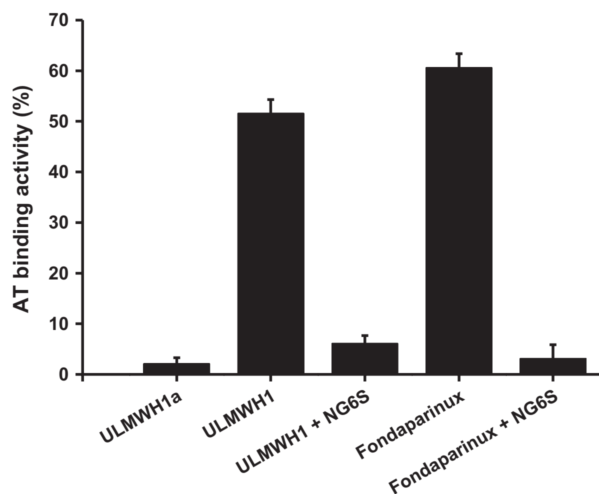

Fig. 6. AT binding of NG6S-treated fondaparinux and ULMWH1. ^{35}S-labeled oligosaccharides were digested with NG6S and purified using a DEAE-Sepharose column. ^{35}S-labeled ULMWH1a without AT-binding affinity was used as a negative control. After NG6S treatment, the levels of AT binding in ULMWH1 and fondaparinux were reduced to that of the negative control.

Experimental procedures

Protein expression and purification

Full-length human *N*-acetylglucosamine 6-sulfatase NG6S (clone ID# 4515104) was purchased from Open Biosystems (Huntsville, AL, USA). The catalytic domain

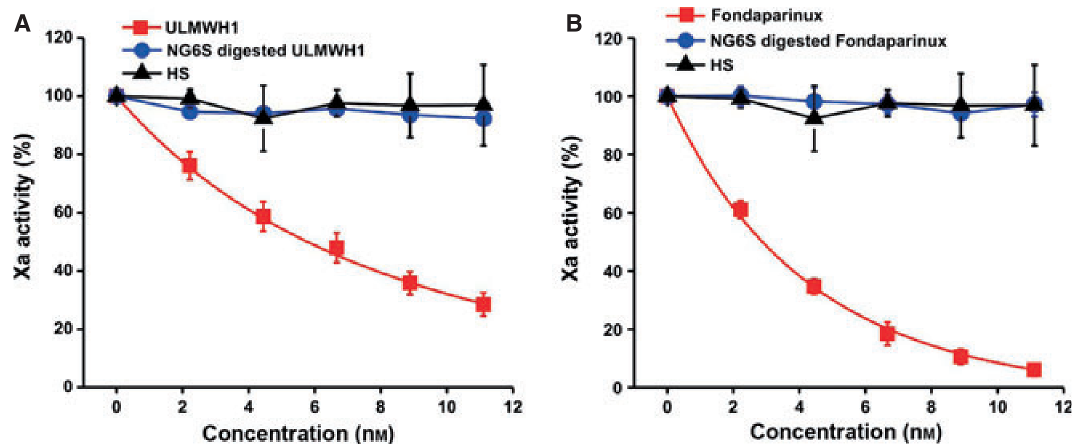

Fig. 7. Anti-Xa activity of NG6S-treated ULMWH1 and fondaparinux. Oligosaccharide was treated with NG6S and purified using a DEAE-Sepharose column. Bovine kidney heparan sulfate without anti-Xa activity was used as a negative control. NG6S digestion removes the anti-Xa activity of ULMWH1 and fondaparinux.

(Thr44-Leu552) was cloned into the pSecTag2 vector (Invitrogen, Carlsbad, CA, USA) using *Hin*dIII and *Xho*I sites. The expression plasmid pSecTag2-NG6S was transfected into wild-type CHO cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The cells were grown in F12 medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 units of penicillin and 100 μg of streptomycin per ml) at 37 °C under 5% CO_2 for 2–3 days. The supernatants were pooled and subjected for protein purification.

NG6S was partially purified as described previously [22]. Briefly, the supernatant was concentrated to ~ 5 mL using a YM–10 filter (Millipore, Billerica, MA, USA). Ion-exchange chromatography was performed using an FPLC system on a strong cationic exchanger Mono–S pre-packed column (column i.d. 4.6 mm, bed height 100 mm, GE Healthcare, Piscataway, NJ, USA) equilibrated with 0.1 M sodium acetate, pH 4.0. The bound proteins were eluted using a linear gradient from 0 to 1.0 M NaCl in 0.1 M sodium acetate, pH 4.0. NG6S activity from various fraction collection tubes was determined using 4-nitrocatechol-sulfate as substrate, and the samples with high NG6S activity were pooled and stored at −80 °C until use.

Western blot analysis of purified NG6S

Eluants (10 μL) from each fraction collection tube with NG6S activity were analyzed by 15% SDS/PAGE. ECL-protein molecular weight markers (2 μL) were used. After electrophoresis, protein was transferred to nitrocellulose membrane (Amersham Biosciences, Amersham, UK) and detected using mouse anti-myc serum (Invitrogen) followed by horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Amersham Biosciences). ECL western blotting detection reagents (Amersham Biosciences) was used to induce chemiluminescence, and the blot was exposed to X-ray film for 15 min.

Degradation of ULMWH by NG6S

The degradation of ULMWH by active NG6S was performed in a reaction buffer comprising 50 mM sodium acetate, pH 5.0, 250 mM NaCl and 100 $\mu g{\cdot}mL^{-1}$ BSA) at 37 °C. When 2 mM of 4-nitrocatecholsulfate was used as substrate instead of ULMWHs, absorbance was read at 490 nm.

HPLC analysis

The product was purified using a DEAE-Sepharose column eluted with an NaCl gradient (0–1.0 M NaCl, pH 7.0) at a flow rate of 0.4 $mL{\cdot}min^{-1}$, and was resolved using a TSKgel DNA-NPR HPLC column (column i.d. 4.6 mm, bed height 75 mm, Tosoh Bioscience, Tokyo, Japan) with radioisotope detection. The elution conditions for the HPLC analysis were as described previously [26]. Briefly, the column was eluted with NaCl as follows: 0 M NaCl for 10 min followed by a gradient of NaCl (0–1 M) for 30 min, followed by 1 M NaCl for 15 min, followed by 0 M NaCl for 10 min, in a solution containing 20 mM Tris/HCl, pH 7.0.

MS analysis

A Thermo Scientific (Waltham, MA, USA) LTQ Orbitrap XL FT mass spectrometer with a standard, factory-installed nano-spray ion source was used in these experiments. ULMWH1 and NG6S-treated ULMWH1 (~ 2 μM) in 50 : 50 methanol/water with 1.0 mM NaOH were used for analysis [32]. Negative-ion mode electrospray ionization was used to

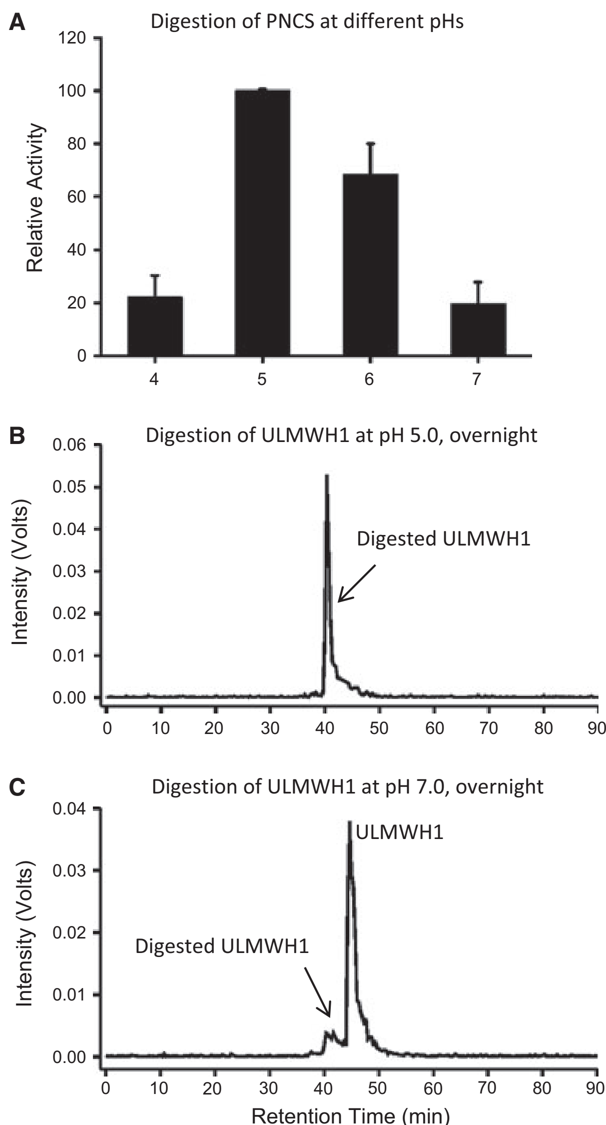

Fig. 8. The activity of NG6S at various pH. (A) The activity of NG6S toward a synthetic substrate, 4-nitrocatecholsulfate, at various pH. (B) DEAE-HPLC profile of ULMWH1 digested with NG6S at pH 5. (C) DEAE-HPLC profile of ULMWH1 digested with NG6S at pH 7. The elution positions of ULMWH1 and digested ULMWH1 are indicated.

ionize the sample. The optimized parameters, used to prevent in-source fragmentation, included a spray voltage of 1.2 kV, a capillary voltage of −40 V, a tube lens voltage of −50 V, and a capillary temperature of 250 °C. External calibration of mass spectra routinely produced a mass accuracy of better than 3 ppm. All Fourier transform (FT) mass spectra were acquired at a resolution of 60 000 with a 350–1500 Da mass range. MS/MS product ions are generated by collisionally induced dissociation fragmentation. Peaks were assigned on the basis of their accurate mass measurement values using the software package GLYCOWORKBENCH 2.0 [33].

Preparation of ^{35}S-labeled oligosaccharides

The preparation method for ^{35}S-labeled ULMWH1 and ULMWH1a has been described previously [9]. For ULMWH1, the ^{35}S label is present at the 3-*O*-sulfo group, and for ULMWH1a, the ^{35}S label is present at the 6-*O*-sulfo group. The preparation of ^{35}S-labeled fondaparinux was performed by incubating 3-*O*-sulfotransferase 1 enzyme, [^{35}S]3′-Phosphoadenosine-5′-phosphosulfate and fondaparinux-3-OH substrate (a generous gift from M. Petitou at Sanofi-Synthelabo, Toulouse, France) [34]. A disaccharide analysis was performed to ensure the appropriate sulfation as described previously [35].

AT binding

AT binding assays were based on a previously published method [27]. Approximately 1 × 10^5 cpm [^{35}S]-labeled ULMWHs were incubated with 5 μg AT in 50 μL reaction buffer containing 10 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 μM dextran sulfate, 0.02% sodium azide and 0.0004% Triton X–100 for 30 min at room temperature. A 60 μL 1 : 1 slurry of pre-treated concanavalin A/Sepharose (Sigma, St. Louis, MO, USA) was added, and the reaction was agitated for 1 h at room temperature on an orbital shaker. The beads were washed three times at room temperature for 1 min with the reaction buffer, and eluted with buffer containing 10 mM Tris/HCl (pH 7.5), 1 M NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 μM dextran sulfate, 0.02% sodium azide and 0.0004% Triton X–100.

Determination of anti-Xa activity

Anti-Xa activity assays were based on a previously published method [27,36]. Briefly, bovine factor Xa (Sigma) was diluted to 5 U·mL^{-1} (~ 80 nM) with NaCl/P_i containing 1 mg·mL^{-1} BSA. Human AT (Cutter Biological, Berkeley, CA, USA) was diluted with NaCl/P_i containing 1 mg·mL^{-1} BSA to give a stock solution at a concentration of 0.4 μM. The chromogenic substrate S–2765 was obtained from Diapharma (West Chester, OH, USA) and made up to 1.3 mM in water. The oligosaccharide (fondaparinux, ULMWH) was dissolved in NaCl/P_i at various concentrations (0–100 nM). The reaction mixture, which consisted of 80 μL of AT stock solution and 15 μL of the solution containing the sample, was incubated at 37 °C for 2 min. Factor Xa (10 μL) was added. After incubating 37 °C for 4 min, 30 μL of S–2765 was added. The absorbance of the reaction mixture was measured at 405 nm continuously for 10 min. The absorbance values were plotted against the

reaction time. The initial reaction rates at varying ULMWH concentrations were converted to an activity percentage based on the initial rate of the reaction without ULMWH. The ratios were plotted against ULMWH concentrations.

Acknowledgements

This work is supported by US National Institutes of Health grants HL094463 (to J.L.) and HL096972 (to R.J.L.).

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