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Controllable production of low molecular weight heparins by combinations of heparinase I/II/III

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ABSTRACT

Enzymatic depolymerization of heparin by heparinases is promising for production of low molecular weight heparins (LMWHs) as anticoagulants, due to its mild reaction conditions and high selectivity. Here, different heparinase combinations were used to depolymerize heparin. Heparinase I and heparinase II can depolymerize heparin more efficiently than heparinase III, respectively, but heparinase III was the best able to protect the anticoagulant activities of LMWHs. Heparinase III and heparinase I/II combinations were able to efficiently depolymerize heparin to LMWHs with higher anticoagulant activity than the LMWHs produced by the respective heparinase I and heparinase II. HepIII and HepI is the best combination for maintaining high anti-IIa activity (75.7 ± 4.21 IU/mg) at the same M_w value. Furthermore, considering both the changes in molecular weight and anticoagulant activity, the action patterns of heparinase I and heparinase II were found not to follow the exolytic and processive depolymerizing mechanism from the reducing end of heparin.

1. Introduction

Heparin, a highly sulfated, acidic, linear and complex polysaccharide that belongs to the heparin/heparan sulfate glycosaminoglycans family (Linhardt, 1991), is a polydisperse mixture with a molecular weight ranging from 5000 to 40,000 Da and an average molecular weight of 12,000–15,000 Da (Ahsan et al., 1995; Mulloy et al., 1997). Heparin was discovered by McLean and Howell in 1916 and first used for clinical trials as an anticoagulant drug in 1935 (Masuko & Linhardt, 2012). Although it has been widely used for cardiovascular surgery and the prevention of venous thromboembolism, some undesirable side effects including hemorrhagic complications and heparin-induced thrombocytopenia (HIT) have limited its application (Mackman, 2008; Masuko & Linhardt, 2012). Low molecular weight heparins (LMWHs), with molecular weights of approximately 3000–8000 Da and an average molecular weight of approximately 5000 Da, exhibit anticoagulant activity similar to heparin but have lower risks of bleeding complications and HIT compared with heparin. LMWHs are thus gradually replacing heparin in clinical applications (Mackman, 2008; Masuko & Linhardt, 2012). Furthermore, LMWHs are also used for patients with pulmonary embolism due to their more predictable pharmacodynamics and anticoagulant activities (Koch et al., 1997).

LMWHs were originally prepared by physical separation, which is unsuitable for scale production due to the limited proportion in unfractionated heparin (Ye et al., 2009). Depolymerization of heparin by a controlled chemical or enzymatic method is more feasible for mass production of LMWHs (Masuko & Linhardt, 2012). Although it is a mature industrial method, chemical depolymerization of heparin, which mostly randomly depolymerizes heparin, has many drawbacks including low selectivity, environmental pollution and difficulties in process control (Linhardt & Gunay, 1999). Enzymatic depolymerization, however, has attracted

Abbreviations: HIT, heparin-induced thrombocytopenia; LMWHs, low molecular weight heparins; HepI, heparinase I; HepII, heparinase II; HepIII, heparinase III; GlcNS6S, 6-*O*-sulfo-*N*-sulfo-α-D-glucosamine; GlcNS3S6S, 3,6-di-*O*-sulfo-*N*-sulfo-α-D-glucosamine; IdoA2S, 2-*O*-sulfo-α-L-iduronic acid; GlcNS, *N*-sulfo-α-D-glucosamine; GlcNAc, α-D-*N*-acetylglucosamine; GlcNAc6S, 6-*O*-sulfo-α-D-*N*-acetylglucosamine; IdoA, α-L-iduronic acid; GlcA, β-D-glucuronic acid; HPLC, high performance chromatography; AT-III, antithrombin III; S-2765, *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-*p*-nitroaniline-dihydrochloride; S-2238, *H*-D-phenylalanyl-L-pipecolyl-arginine-*p*-nitroaniline dihydrochloride; MBP, maltose binding protein; IPTG, isopropyl-β-D-1-thiogalactopyranoside; GPC, gel permeation chromatography; RI, refractive index; PA, photodiode array; PAGE, polyacrylamide gel electrophoresis; dp, degree of polymerization; UV, ultraviolet; M_n, number average molecular weight; M_w, weight average molecular weight; LC–MS, liquid chromatography–mass spectrometry.

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increased attention because of its high selectivity, environmental friendship and mild reaction conditions (Linhardt & Gunay, 1999; Ye et al., 2009). In recent years, there are some new heparin depolymerization methods, such as physicochemical method (Achour et al., 2013) and photochemical method (Higashi et al., 2012), but they showed either desulfation or low selectivity.

Heparinases isolated from *Pedobacter heparinus* (formerly *Flavobacterium heparinum*) can depolymerize heparin at specific 1→4 linkages between hexoamines and uronic acids via a β-elimination mechanism (Ernst, Langer, Cooney, & Sasisekharan, 1995; Lohse & Linhardt, 1992; Yang, Linhardt, Bernstein, Cooney, & Langer, 1985). Heparinases exhibit different specificities (Fig. 1); heparinase I (HepI, EC 4.2.2.7) depolymerizes heparin randomly by cleaving →4] 6-*O*-sulfo-*N*-sulfo-α-D-glucosamine (GlcNS6S)/3,6-di-*O*-sulfo-*N*-sulfo-α-D-glucosamine (GlcNS3S6S) (1→4) 2-*O*-sulfo-α-L-iduronic acid (IdoA2S) [1→ (Desai, Wang, & Linhardt, 1993a, 1993b; Xiao, Tappen, et al., 2011; Xiao, Zhao, et al., 2011), which is the most common (75–95%) disaccharide in heparin (Linhardt et al., 1988; Loganathan, Wang, Mallis, & Linhardt, 1990; Pervin, Gallo, Jandik, Han, & Linhardt, 1995). Heparinase III (HepIII, EC 4.2.2.8), however, has high selectivity compared with HepI, because it acts on a very rare disaccharide (about one in an intact heparin) in heparin, which is →4] GlcNS6S/*N*-sulfo-α-D-glucosamine (GlcNS)/α-D-*N*-acetylglucosamine (GlcNAc)/6-*O*-sulfo-α- D-*N*-acetylglucosamine (GlcNAc6S) (1→4) α-L-iduronic acid (IdoA)/β-D-glucuronic acid (GlcA) [1→, if the nonreducing end of IdoA/GlcA does not connect to a fully sulfated (six sulfo groups) tetrasaccharide residue (Desai, Wang, & Linhardt, 1993a, 1993b; Nader et al., 1990; Xiao, Tappen, et al., 2011). Heparinase II (HepII, no EC number) acts on a wider range of sites than HepI and HepIII, and accommodates many modifications to heparin (Desai, Wang, & Linhardt, 1993a, 1993b; Nader et al., 1990; Shaya et al., 2010). Importantly, the →4] GlcNAc6S (1→4) GlcA [1→ linkage at the nonreducing end of the →4] GlcNS3S6S [1→ residue, which is critical for the anticoagulant effects of heparin and LMWHs, cannot be digested by all three heparinases (Fig. 1) (Shriver et al., 2000; Yu et al., 2000; Zhao et al., 2011). Owing to their selective depolymerization of heparin, heparinases have been expected to be a promising method for producing LMWHs. HepI has been used for the production of a kind of LMWH, that is called Tinzaparin (Innohep), which is a commercialized product by Leo Pharma (Hedner, 2000). HepIII is also a good candidate for the depolymerization of heparin, as it cannot break the antithrombin III (AT-III) binding site (pentasaccharide unit), and thus can protect the anticoagulation effects of the LMWHs during the depolymerization process (Mamuwala & Venkataraman, 2011; Xiao, Tappen, et al., 2011). The disadvantage by using HepIII lies in its inefficient depolymerization, since the cleaving sites in heparin recognized by HepIII are very rare (Xiao, Tappen, et al., 2011). Due to the different site specificity of each heparinase, it is possible to produce LMWHs with high anticoagulant activities with different heparinase combinations. However, until now, to the best of our knowledge, there has been no systematic study on the depolymerization characteristics of the three heparinases, especially with respect to how to make full use of the different catalytic functions of heparinases to control the depolymerization process as well as the characteristics of LMWHs.

In the present study, the depolymerizations of the three heparinases were compared and the action patterns of HepI and HepII were discussed. We also studied the depolymerization of heparin by utilizing different combinations of the heparinases, and a novel combinatorial enzymatic method for production of LMWHs was established and elucidated.

2. Materials and methods

2.1. Materials

Pharmaceutical grade heparin (M_n = 22,370, anti-factor Xa activity = 187 ± 21 IU/mg, anti-factor IIa activity = 177 ± 6 IU/mg) was provided as a gift from the Changshan Biotechnology Corporation (Hebei Province, China). Heparin low-molecular-mass calibration CRS (Chemical Reference Substance) (H0190000, M_n = 3800, for molecular weight detection) and heparin low-molecular-mass for assay BRP (Biological Reference Preparation) (H0185000, for detection of anti-factor Xa activity and anti-factor IIa activity) were purchased from EDQM (European Directorate for the Quality of Medicines). The high performance chromatography (HPLC) system (Waters Co., USA) that was utilized consisted of a computer control, binary HPLC pump (Waters 1525), autosampler (Waters 2707), GPC column (TSK Gel G2000SW$_{XL}$, 7.8 mm × 300 mm, Tosoh Co., Japan), and a Refractive Index (RI) Detector (Waters 2414) linked after a Photodiode Array (PA) Detector (Waters 2998). This HPLC system was used to detect the molecular weight distribution of the depolymerized products. AT-III, chromophore substrate *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-*p*- nitroaniline-dihydrochloride (S-2765), *H*-D-phenylalanyl-L-pipecolyl-arginine-*p*-nitroaniline dihydrochloride (S-2238) and bovine factor Xa were purchased from Chromogenix (Sweden). All the other reagents were purchased from Sigma–Aldrich (USA).

2.2. Methods

2.2.1. Production and purification of HepI, HepII and HepIII

HepI, HepII and HepIII were solubly expressed with high activities by fusion with maltose binding protein (MBP) in *E. coli* according to the procedure established by Chen, Xing, and Lou (2005). The host for expression of HepI, HepII and HepIII was *E. coli* TB1, *E. coli* TB1 and *E. coli* TOP10, respectively. The cells were grown in a M9YE medium supplemented with 100 μg/mL ampicillin at 15 °C (Chen, Xing, Ye, Kuang, & Luo, 2007). The cells were harvested 24 h after induction with 0.24 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and disrupted by a high-pressure homogenizer (JuNeng biology & Technology Co., Ltd., Guangzhou, China). The soluble fraction of cell crude extract was obtained from the supernatant after centrifugation and the fusion enzymes were purified by MBP-Trap purification (MBPTrap HP, GE Healthcare, Chalfont St Giles, BUCKS, UK) according to the manufacturer's instruction manual. The K_m values of HepI, HepII and HepIII used in the present study were 85.3, 46.8 and 292 μM, respectively. The V_m values of HepI, HepII and HepIII were 4.56, 4.83 and 12.11 mM min^{-1} mg^{-1}, respectively.

2.2.2. Enzymatic activity assay

The activity assay of HepII and HepIII was performed according to the 232 nm UV method as previously described for HepI (Ye et al., 2009). In brief, 500 μL of 25 g/L heparin solution (25 g/L heparin, 40 mM NaCl, 3.5 mM $CaCl_2$ and 17 mM Tris–HCl, pH 7.4) was mixed with 950 μL reaction buffer (200 mM NaCl, 3.5 mM $CaCl_2$ and 20 mM Tris–HCl, pH 7.4) and 50 μL enzyme solution with proper dilution. The entire system was kept at 30 °C for its 2 min reaction. Heparin degradation was monitored by UV absorbance at 232 nm and the activity was calculated from the increase in absorbance at 232 nm during the detection time, using a molar extinction coefficient of 3800 M^{-1} cm^{-1}. One international unit (IU) was defined as the amount of enzyme that can form 1 μmol unsaturated uronic acid per minute at 30 °C.

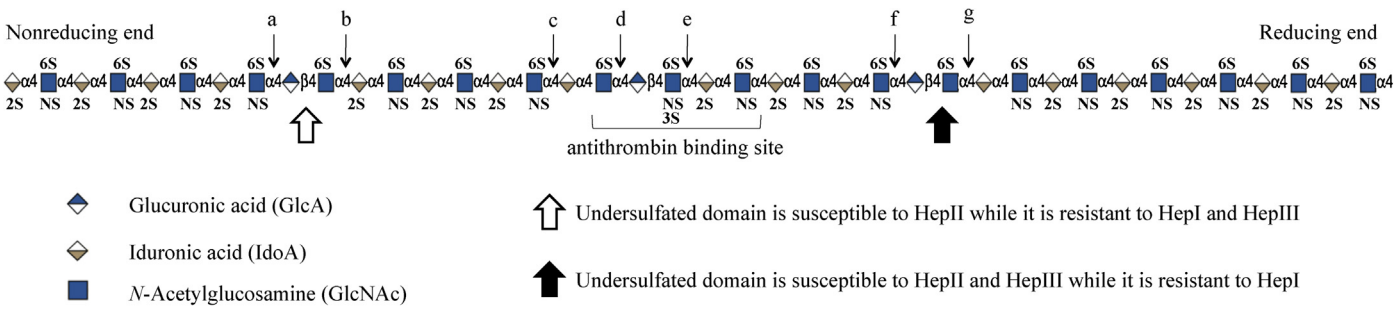

Fig. 1. Schematic representation of heparin (degree of polymerization (dp) 40, saccharide with a molecular weight of about 12,000) and its linkages that are susceptible to heparinase cleavage. Linkages a, b, c, d, f and g are resistant to HepI, linkage d is resistant to HepII, linkages a, f and g are susceptible to HepIII. The antithrombin binding site (containing linkages d and e) is susceptible to HepI and HepII while it is resistant to HepIII. HepII can cleave more undersulfated domains than HepIII, and HepI cannot cleave undersulfated domains.

2.2.3. Depolymerization of heparin by heparinases

Depolymerized products were produced through the controlled depolymerization of heparin by heparinases.

For the depolymerization of heparin by HepI, 1 mL of HepI solution with an enzyme activity of 15 IU/mL was added to a 100 mL reaction solution containing 50 g/L heparin, 20 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$, pH 7.4, adjusted with 1 M NaOH. For the depolymerization of heparin by HepII, 1 mL of HepII solution with an enzyme activity of 17.5 IU/mL was added to a 100 mL reaction solution containing 50 g/L heparin, 20 mM Tris, 100 mM NaCl, 20 mM $CaCl_2$, pH 7.6, adjusted with 1 M NaOH. For the depolymerization of heparin by HepIII, 1 mL of HepIII solution with an enzyme activity of 2.5 IU/mL was added to a 100 mL reaction solution containing 50 g/L heparin, 20 mM Tris, 50 mM NaCl, 20 mM $CaCl_2$, pH 7.6, adjusted with 1 M NaOH.

For the depolymerization of heparin by the combination of HepIII and HepI, heparin was exhaustively depolymerized by HepIII overnight until the absorbance at 232 nm did not change, and then we adjusted the concentration of NaCl as well as the pH for the HepI depolymerization, which was achieved by adding 1 mL of HepI solution with an enzyme activity of 15 IU/mL.

For depolymerization of heparin by the combination of HepIII and HepII, heparin was exhaustively depolymerized by HepIII overnight until the absorbance at 232 nm did not change, and then we adjusted the concentration of NaCl as well as the pH for the HepII depolymerization, which was achieved by adding 1 mL of HepII solution with an enzyme activity of 17.5 IU/mL.

All of these enzymatic reactions were carried out at 30 °C in shake flasks at 170 rpm. 10 mL aliquots were taken every 20 min and the reaction was immediately terminated by submerging each aliquot in a 100 °C water bath for 10 min. The precipitant was removed by centrifugation at 10,000 × *g* for 30 min. The depolymerized products were obtained as a precipitate after six additions of ethanol, and the precipitate was then dried by lyophilization for the subsequent analysis.

2.2.4. Average molecular weight measurement of depolymerized products

The measurements of the M_w, M_n and polydispersity index (P) of the depolymerized products were performed using an HPLC method with gel permeation chromatography (GPC) according to European Pharmacopoeia (http://online.edqm.eu, 2011). Briefly, a 28.4 g/L solution of anhydrous sodium sulfate that was adjusted to pH 5.0 using dilute sulfuric acid was utilized as the mobile phase. 20 mg of the lyophilized depolymerized products to be examined was dissolved in 2 mL of the mobile phase as the test solution. 20 mg of heparin low-molecular-mass for the calibration CRS (chemical reference sample) was dissolved in 2 mL of the mobile phase to be used as the reference solution. The GPC column was a TSK Gel G2000SW_{XL} (7.8 mm × 300 mm, Tosoh Co., Japan). The operating conditions were as follows: the flow rate was 0.5 mL/min, RI detector connected in series to a PA monitor set to 234 nm such that the PA monitor is connected to the column outlet, and the RI detector to the PA monitor outlet, the column temperature was 35 °C, and both the reference solution and test solution injections were 25 μL. The GPC chromatograms were recorded on a computer with the Breeze solution Version 2 software and analyzed with its "GPC Postrun" function using heparin low-molecular-mass for the calibration CRS (chemical reference sample) as the calibrant.

2.2.5. Anti-factor Xa and anti-factor IIa activity assay

The detection of anti-factor Xa and anti-factor IIa activities was performed according to European Pharmacopoeia (http://online.edqm.eu, 2011). Briefly, 50 μL of AT-III solution (1 IU/mL for anti-factor Xa detection and 0.5 IU/mL for anti-factor IIa detection) and 50 μL of the diluted sample or reference solution were added and mixed. Tubes were equilibrated in a 37 °C water bath for 1 min before adding 100 μL of bovine factor Xa solution (0.426 IU/mL) for anti-factor Xa detection or thrombin solution (5 IU/mL) for anti-factor IIa detection. After incubation for 1 min, 250 μL of a chromophore substrate containing 175 mM NaCl, 8 mM EDTA, 50 mM Tris–HCl, 0.5 mM S-2765 for anti-factor Xa or S-2238 for anti-factor IIa detection was added. The reaction was stopped after 4 min by adding 375 μL of acetic acid (5.25 M) and the absorbance at 405 nm was measured. Regression of the absorbance against log concentrations of the sample or reference was used to calculate the potency, using standard statistical methods for parallel-line assays.

2.2.6. Polyacrylamide gel electrophoresis (PAGE) analysis

Depolymerized products were analyzed by PAGE using a mini-gel apparatus (Bio-Rad, Hercules, CA) according to the procedure described by Xiao, Tappen, et al. (2011), Xiao, Zhao, et al. (2011) and Yang et al. (2009). Briefly, pharmaceutical grade heparin, LMWHs marker and depolymerized products were prepared to a final concentration of 20 mg/ml in water. Each sample (6 μL) was mixed with 20 μL of 50% (w/v) sucrose, and then 5 μL of these mixtures were loaded into a stacking gel of 5% (w/v total acrylamide) and fractioned on a 22% resolving gel. Electrophoresis was performed at 100 V for 10 min and then at 200 V for 90 min. Gels were visualized with 0.5% (w/v) alcian blue in 2% (v/v) acetic acid solution for 30 min.

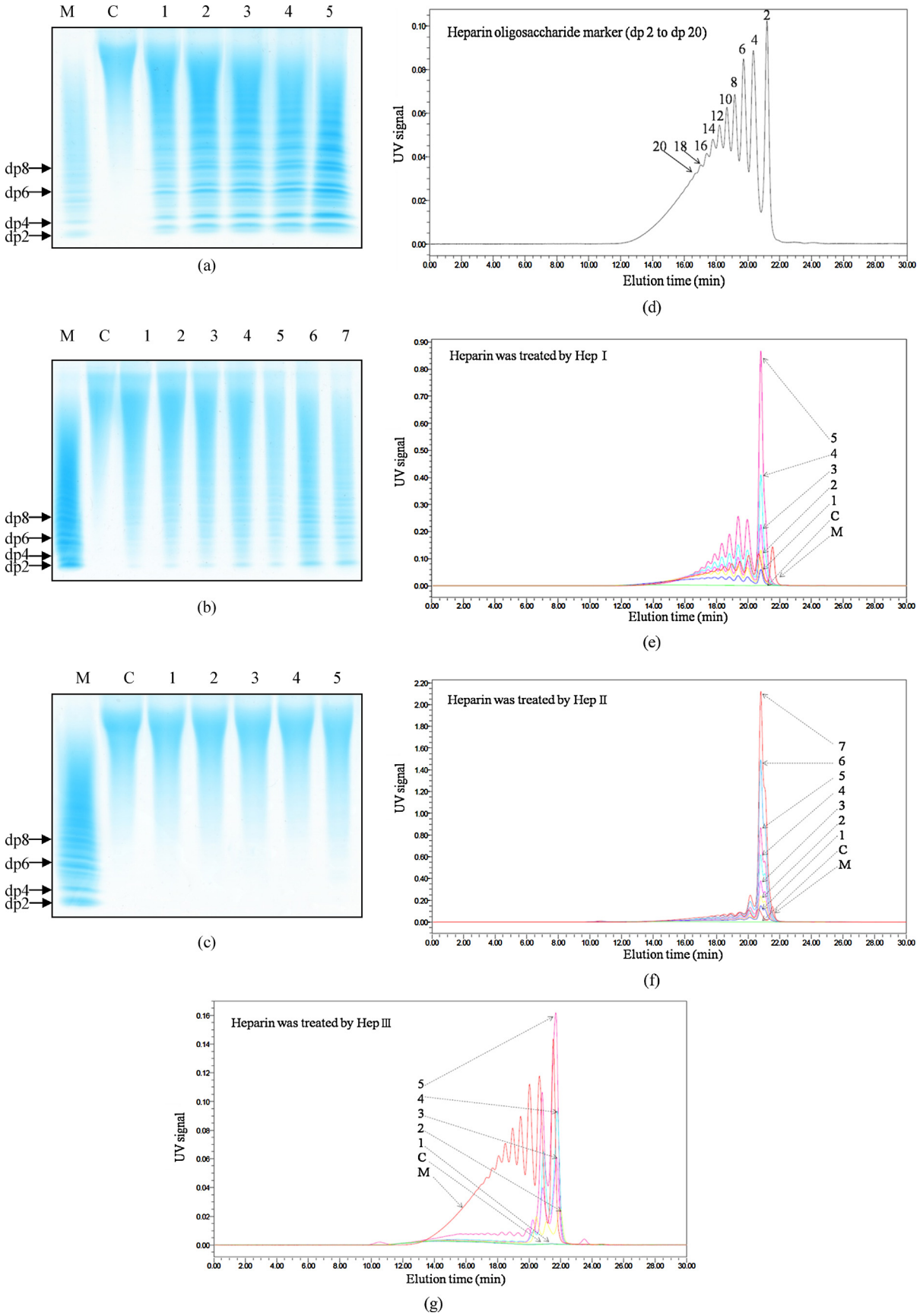

Fig. 2. PAGE (22% total acrylamide) analysis of products depolymerized by HepI (a), HepII (b), HepIII (c) and GPC analysis of heparin oligosaccharide marker (d), products depolymerized by HepI (e), HepII (f), HepIII (g). M: heparin oligosaccharide marker; C: heparin. Numbers in (d) refer to different degree of polymerization. Numbers in (a), (b) and (c) refer to different time-points which correspond to numbers in (e), (f) and (g), respectively.

3. Results

3.1. Depolymerization of heparin by different heparinases

Firstly, HepI, HepII and HepIII were used separately to achieve controlled digestion of heparin into LMWHs. Polyacrylamide gel electrophoresis (PAGE) and gel permeation chromatography (GPC) have been widely used to analyze the size distributions of depolymerized products during the depolymerization of heparin in many literatures (Achour et al., 2013; Edens et al., 1992; Higashi et al., 2012; Linhardt & Gunay, 1999; Xiao, Tappen, et al., 2011; Xiao, Zhao, et al., 2011; Ye et al., 2009), and the results obtained by PAGE are consistent well with the LC–MS analysis (Higashi et al., 2012). Here, we combined PAGE and GPC analysis to analyze the size distributions of depolymerized products during the depolymerization by heparinases (Fig. 2). The bands of depolymerization products moved closer to the foot of PAGE as the depolymerization times of HepI and HepII increased, indicating that the heparin became smaller oligosaccharide fragments. The bands corresponding to disaccharide, tetrasaccharide, hexasaccharide and octasaccharide products became thicker as the HepI and HepII depolymerization times increased (Fig. 2a and b). Interestingly, HepI produced less disaccharide products and more tetrasaccharide products than HepII (Fig. 2a and b), which was consistent with the previous study (Xiao, Tappen, et al., 2011) and the GPC analysis (Fig. 2e and f) according to the heparin oligosaccharide marker (Fig. 2d). However, the bands of depolymerization products by HepIII were significantly different (Fig. 2c), they were slightly below the unfractionated heparin even though it was exhaustively depolymerized by HepIII (Fig. 2c lane 5), and there were very few bands corresponding to low degree of polymerization products which can not be seen on PAGE (Fig. 2c lane C). But we can see very small peaks corresponding to disaccharide and tetrasaccharide on GPC (Fig. 2g), which was consistent with the previous study (Xiao, Tappen, et al., 2011). These observations indicated that both HepI and HepII could efficiently depolymerize heparin to LMWHs, while HepIII could only slightly degrade the heparin molecules.

We further determined the molecular weights and the anticoagulant activities of these depolymerized products (Fig. 3). Both the anti-factor Xa and anti-factor IIa activities decreased linearly as the M_w decreased during the depolymerization process with the three individual heparinases. Interestingly, the products produced by HepI showed almost the same linear relationship between the M_w and anti-factor Xa activity as the products produced by HepII (the slope was 3.997×10^{-2}), while the slope for the products produced by HepIII was much lower (the slope was 2.178×10^{-2}). But for the linear relationships between the M_w and anti-factor IIa activity, the slopes for products produced by HepI, HepII and HepIII were 2.930×10^{-2}, 2.850×10^{-2}, and 7.310×10^{-3}, respectively (Fig. 3a and b). These results indicated that although HepI and HepII could efficiently depolymerize heparin to LMWHs, the anticoagulant activities decreased very fast with the decrease of M_w during the depolymerization process. On the other hand, HepIII showed different characteristics; it could not efficiently depolymerize heparin to LMWHs, but the anticoagulant activities decreased more slowly with the decrease of M_w during the depolymerization. From these observations, it was reasonable to speculate that the combinations of HepIII and HepI/HepII, that is, exhaustive depolymerization with HepIII followed by the controlled depolymerization with HepI/HepII, can protect the advantageous anticoagulant activities resulting from the use of HepIII during the depolymerization, while enabling the HepI/HepII to efficiently depolymerize heparin to ideal LMWHs. Thus, we studied the depolymerization characteristics of heparin after utilizing combinations of HepIII and HepI/HepII.

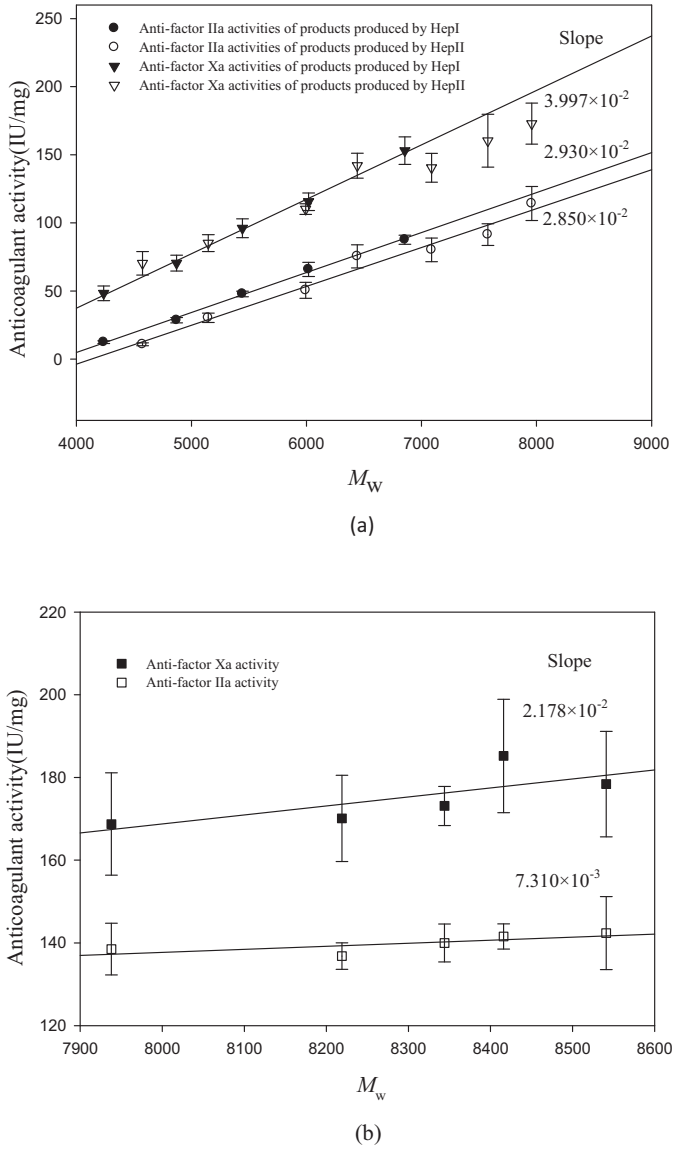

Fig. 3. Relationship between M_w and anticoagulant activity during the heparin depolymerization by HepI and HepII (a), and HepIII (b) ($n = 3$).

3.2. Depolymerization of heparin with a combination of HepIII and HepI

The PAGE analysis of the products produced by the combination of HepIII and HepI is shown in Fig. 4a and b. The bands of oligosaccharides moved closer to the foot of the PAGE gel as the depolymerization time by HepI increased, and the bands corresponding to disaccharide, tetrasaccharide, hexasaccharide and octasaccharide products became thicker, indicating that the oligosaccharide fragments became smaller as the depolymerization proceeded. These results were consistent well with the GPC analysis result (Fig. 4b), and the results of products produced by only HepI (Fig. 2a and e), indicating the combination of HepIII and HepI could efficiently depolymerize heparin to produce LMWHs.

The anticoagulant activities of products produced by combining of HepIII and HepI were analyzed, and changes of the anticoagulant activities with the M_w of the depolymerized products due to the depolymerization are shown in Fig. 4c. The anti-factor Xa and anti-factor IIa activities changed linearly with M_w, and the slopes were 3.955×10^{-2} and 2.720×10^{-2}, respectively. Interestingly, the anticoagulant activities of the products produced by HepI

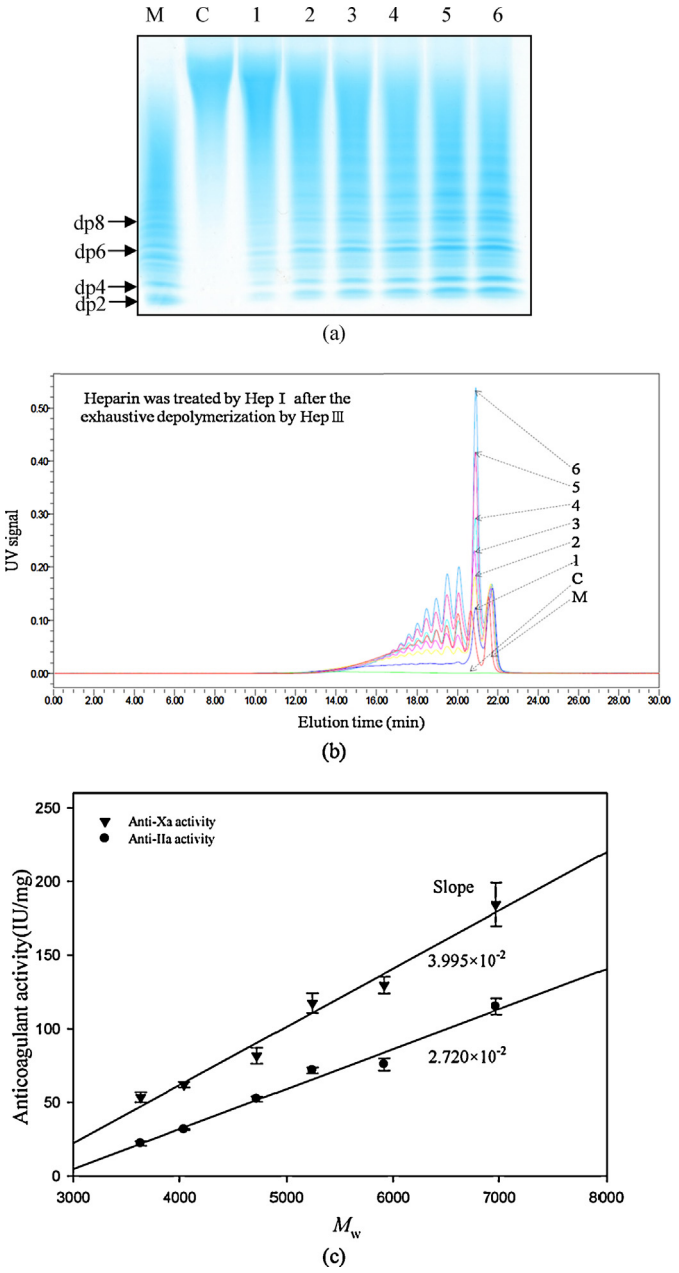

Fig. 4. (a) PAGE (22% total acrylamide) analysis of products depolymerized by HepI after the exhaustive depolymerization by HepIII. (b) GPC analysis of products depolymerized by HepI after the exhaustive depolymerization by HepIII. M: heparin oligosaccharide marker; C: heparin; Lanes 1–6: heparin was treated by HepI from time-point 1 to time-point 6. (c) Relationship between M_w and anticoagulant activity during the heparin depolymerization by HepI after exhaustive depolymerization by HepIII ($n = 3$).

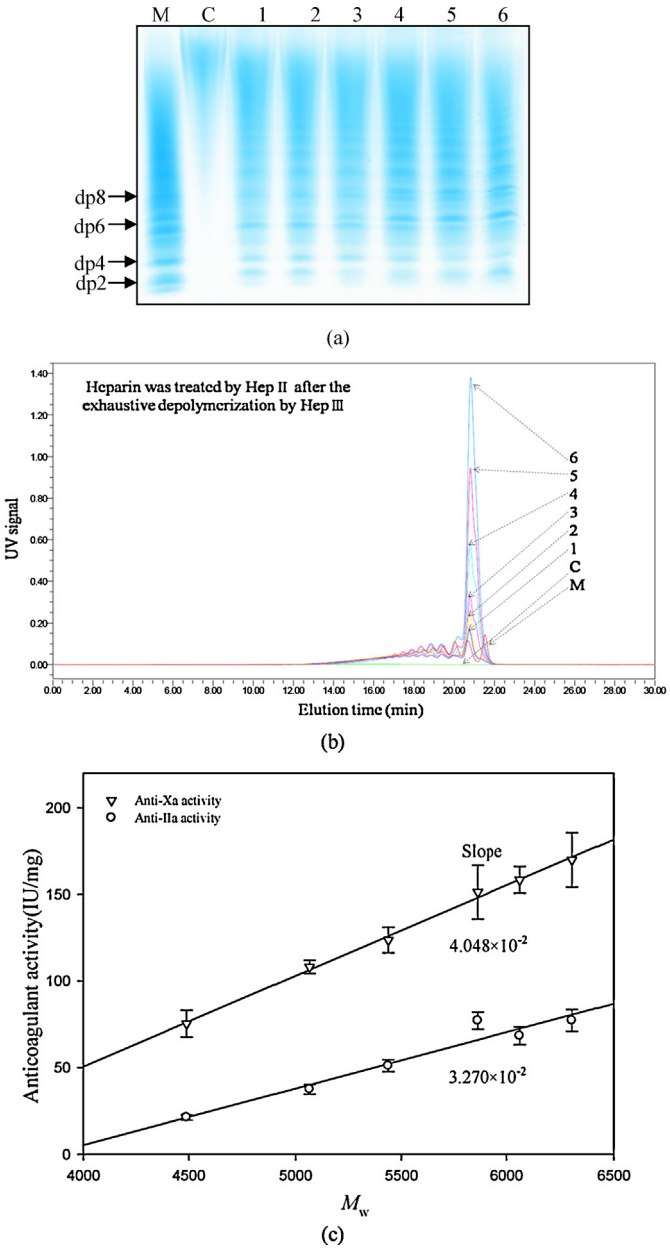

Fig. 5. (a) PAGE (22% total acrylamide) analysis of products depolymerized by HepII after the exhaustive depolymerization by HepIII. (b) GPC analysis of products depolymerized by HepII after the exhaustive depolymerization by HepIII. M: heparin oligosaccharide marker; C: heparin; Lanes 1–6: heparin was treated by HepII from time-point 1 to time-point 6. (c) Relationship between anticoagulant activity and M_w of LMWHs prepared by HepII after exhaustive depolymerization by HepIII ($n = 3$).

after the exhaustive depolymerization by HepIII were higher than that produced by only HepI at the same M_w value, especially for the anti-factor IIa activity (Figs. 3a and 4c).

These results demonstrated that the combination of HepIII and HepI could effectively depolymerize heparin to LMWHs with higher anticoagulation activity than the single HepI biocatalysis.

3.3. Depolymerization of heparin with a combination of HepIII and HepII

Fig. 5a shows the PAGE analysis of products produced by the combination of HepIII and HepII. The bands corresponding to disaccharide, tetrasaccharide, hexasaccharide and octasaccharide products became thicker and more bands of oligosaccharides were observed as the increase of depolymerization time by HepII increased. These observations were consistent with the GPC analysis (Fig. 5b) and were similar to the depolymerization of heparin by single HepI (Fig. 2a and e), single HepII (Fig. 2b and f) and the combination of HepIII and HepI (Fig. 4a and b), indicating the combination of HepIII and HepII also could efficiently depolymerize heparin to prepare LMWHs.

Fig. 5c shows the relationship of anticoagulant activities of these products with the M_w. The linear slopes for anti-factor Xa and anti-factor IIa activities vs. M_w were 4.048×10^{-2} and 3.270×10^{-2}, respectively. The anticoagulant activities of the products produced by HepII after the exhaustive depolymerization by HepIII were

higher than that produced by only HepII at the same M_w value, especially for the anti-factor IIa activity (Figs. 3b and 5c).

These results indicated that the combination of HepIII and HepII could also depolymerize heparin effectively to LMWHs with higher anticoagulation activity than the single HepII biocatalysis.

Furthermore, the products produced by combinations of HepIII and HepI/HepII showed almost the same linear relationship between the M_w and anti-factor Xa activities, while they showed different linear relationships between the M_w and anti-factor IIa activities (Figs. 4c and 5c). The LMWHs produced by combining HepIII and HepI exhibited higher anti-factor IIa activities than those produced by combining HepIII and HepII at the same M_w (Figs. 4c and 5c).

4. Discussion

The present study demonstrates that HepI and HepII can effectively depolymerize heparin to LMWHs, while HepIII can only generate high molecular weight heparin. However, as the M_w decreased, the anticoagulant activities of the products decreased very quickly for those produced by the depolymerization of heparin by HepI and HepII, while the anticoagulant activities decreased more slowly for the HepIII biocatalysis. The combinations of HepIII with HepI/HepII were able to depolymerize heparin effectively to LMWHs with higher anticoagulation activities than those produced by only HepI or HepII.

Although the site specificities of HepI, HepII and HepIII are well known, the sequence of events during the enzymatic depolymerization of heparin is still controversial. HepI and HepII reportedly show an exolytic bias in their action patterns on oligosaccharide substrates (Xiao, Tappen, et al., 2011), which is in contrast to the observed endolytic action patterns on polysaccharide substrates (Jandik, Gu, & Linhardt, 1994; Linhardt, Fitzgerald, Cooney, & Langer, 1982). Furthermore, for an octasaccharide, HepI is reported to act predominantly from the nonreducing end by an exolytic and processive depolymerization mechanism (Ernst, Rhomberg, Biemann, & Sasisekharan, 1998), while HepII shows an endolytic and nonrandom action pattern (Rhomberg, Shriver, Biemann, & Sasisekharan, 1998). But in the previous studies on the enzymatic depolymerization mechanism of heparin, no anticoagulant activity has been reported. In this study, we systematically analyzed the change in anticoagulant activity during the depolymerization of heparin and showed that the activity ratio (anti-factor Xa activity/anti-factor IIa activity) increased significantly with the decrease of M_w in the reaction system with both single HepI and HepII (Fig. 6). This result indicated that the anti-factor IIa activities of depolymerized products decreased relatively faster than the anti-factor Xa activities. This observation gave us valuable information about the action patterns of HepI and HepII on heparin. Heparin needs the AT-III binding site to initiate its anti-factor Xa activity and another five to six disaccharides (mostly trisulfated) on the nonreducing end to initiate its anti-factor IIa activity (Dementiev, Petitou, Herbert, & Gettins, 2004; Masuko & Linhardt, 2012; Petitou, Casu, & Lindahl, 2003; Petitou et al., 1999; Wei, Johnson, Esmon, & Huntington, 2004), so if HepI and HepII catalyzed heparin by the exolytic and processive degrading mechanism from the reducing end of heparin, it would lose its anti-factor Xa and anti-factor IIa activities to the same extent (Fig. 7), but the present study showed a trend that was not in agreement with this hypothesis. Thus, the enzymatic action pattern on heparin, i.e., exolytic and processive degrading mechanism from the reducing end of heparin, could be excluded at a minimum for both HepI and HepII. But the other reported action patterns, such as the endolytic action pattern (Jandik, Gu, & Linhardt, 1994; Linhardt, Fitzgerald, Cooney, & Langer, 1982; Rhomberg, Shriver, Biemann, & Sasisekharan, 1998),

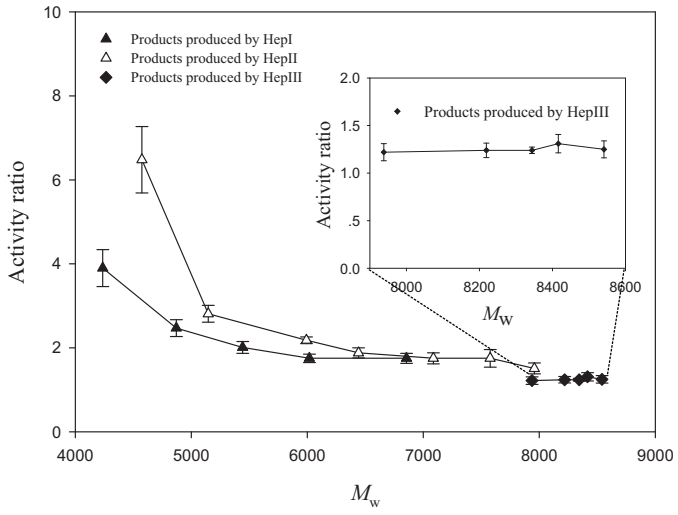

Fig. 6. The effects of the depolymerization by different heparinases on the anticoagulant activity ratio (anti-factor Xa activity/anti-factor IIa activity) of LMWHs ($n = 3$).

exolytic and/or processive degrading mechanism from the nonreducing end of heparin (Ernst, Rhomberg, Biemann, & Sasisekharan, 1998; Xiao, Tappen, et al., 2011), could not be confirmed by the present study, so this aspect requires further study.

Heparin contains a significant level of sequence heterogeneity. The contained repeating disaccharide units have 48 different structures due to the numbers and positions of sulfo groups and acetyl groups, as well as due to the configuration of uronic acid. The most common trisulfated disaccharide repeating unit (75–95%) is the →4] IdoA2S (1 → 4) GlcNS6S [1→ unit (Linhardt et al., 1988; Loganathan et al., 1990; Pervin et al., 1995). Notably, a specific pentasaccharide sequence, →4] GlcNAc6S (1 → 4) GlcA (1 → 4) GlcNS3S6S (1 → 4) IdoA2S (1 → 4) GlcNS6S [1→, is the AT-III binding site which can interact directly with AT-III and inhibit the coagulation cascade primarily through coagulation factor Xa (Masuko & Linhardt, 2012; Petitou et al., 2003). Heparin can effectively bind thrombin to form a heparin-AT-III-thrombin ternary complex that inhibits the formation of insoluble fibrin clots if the pentasaccharide is flanked by another five to six disaccharides (mostly trisulfated) (Masuko & Linhardt, 2012; Petitou et al., 2003). The AT-III binding site in heparin can be cleft by HepI and HepII, while this cannot be done by HepIII according to their site specificities, that is →4] GlcNAc6S (1 → 4)GlcA[1→ linkage at the nonreducing end of →4] GlcNS3S6S [1→ is resistant to these three heparinases (Shriver et al., 2000; Yu et al., 2000; Zhao et al., 2011) and the other linkage, →4] IdoA2S (1 → 4) GlcNS6S [1→, can be cleft by HepI and Hep II, but not by HepIII (Desai, Wang, & Linhardt, 1993a, 1993b; Nader et al., 1990; Xiao, Tappen, et al., 2011; Xiao, Zhao, et al., 2011) (Fig. 1). In addition, the most common (75–95%) trisulfated disaccharide, →4] IdoA2S (1 → 4) GlcNS6S [1→, which flanks the AT-III binding site, can be cleft by HepI and HepII, but it is resistant to HepIII (Fig. 1). However, the undersulfated domain flanking the AT-III binding site can be cleft by HepII and HepIII, while it is resistant to HepI, the possibility that the undersulfated domain can be cleft by HepII is much larger than that of HepIII because of the broader site specificity of HepII (Fig. 1). It is thus understood that heparin can be effectively depolymerized to LMWHs when HepI or HepII is involved in the depolymerization reaction and the anticoagulant activity changes of the LMWHs were caused during the depolymerization process (Table 1 and Fig. 6). The combination of HepIII and HepI is the best combination for maintaining high anticoagulant activity at the same M_w value.

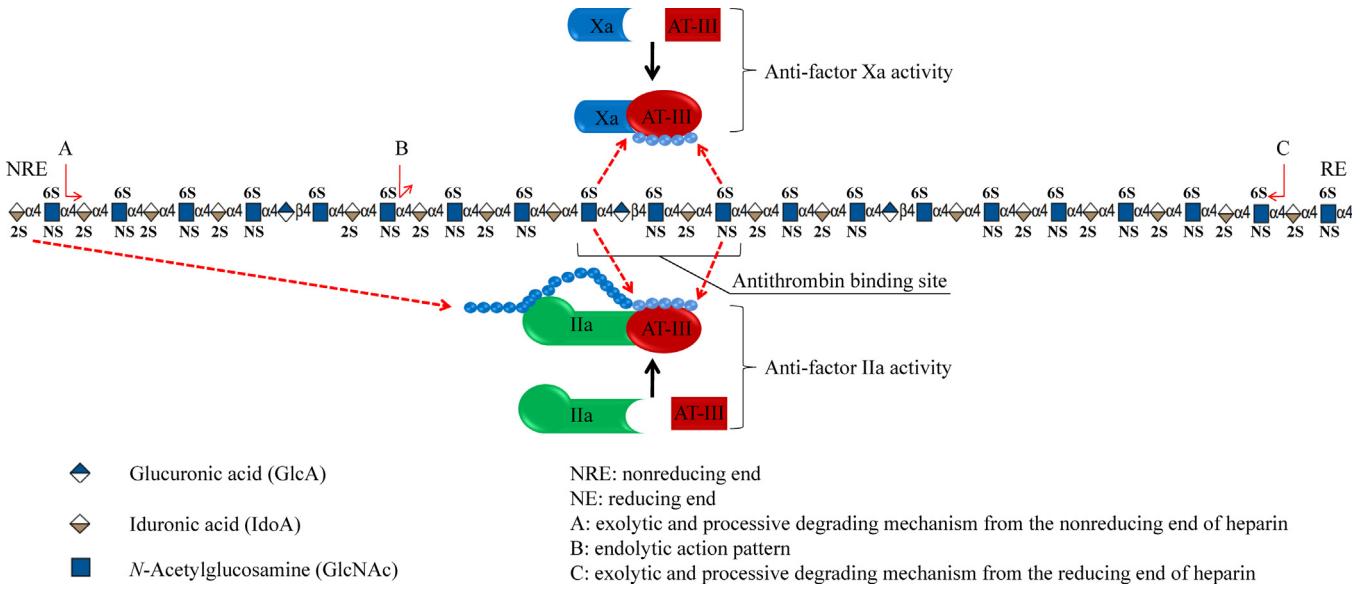

Fig. 7. Schematic representation of the mechanism of heparin induced anticoagulant activities (anti-factor Xa activity and anti-factor IIa activity) and the effects of the depolymerization by HepI and HepII on the anticoagulant activity ratios (anti-factor Xa activity/anti-factor IIa activity) of LMWHs. Heparin needs the AT-III binding site to initiate the anti-factor Xa activity and another five to six disaccharides (mostly trisulfated) on the nonreducing end to initiate the anti-factor IIa activity. If HepI and HepII follow the action patterns of A and B, then the anti-factor IIa activities of the LMWHs decrease relatively faster than the anti-factor Xa activities. If HepI and HepII follow the action pattern of C, the LMWHs will lose the anti-factor Xa and anti-factor IIa activities to the same degree.

Table 1
Summary of different depolymerization methods used in this study ($n = 3$).

Enzyme	Method	Slope		Examples of oligosaccharides produced by different methods						
		Anti-factor Xa (10^{-3})	Anti-factor IIa (10^{-3})	M_w	M_n	P[a]	M_w < 8000 (%)	Anti-Xa (IU/mg)	Anti-IIa (IU/mg)	AR[b]
I	C[c]	39.97	29.3	6018	3901	1.54	82.00	115.58 ± 6.41	65.86 ± 5.16	1.75
II	C[c]	39.97	28.5	5993	3875	1.55	84.00	110.12 ± 3.95	50.48 ± 5.86	2.18
III	E[d]	21.78	7.31	7938	5227	1.52	52.72	168.74 ± 12.38	138.51 ± 6.26	1.22
III I	EC[f]	39.55	27.2	5915	3127	1.90	78.05	129.66 ± 5.68	75.7 ± 4.21	1.71
III II	EC[f]	40.48	32.7	6063	3959	1.53	81.00	158.39 ± 7.69	68.41 ± 5.16	2.32

[a] Polydispersity index.
[b] Activity ratio (anti-factor Xa activity/anti-factor IIa activity).
[c] Controlled depolymerization by single HepI or HepII.
[d] Exhaustive depolymerization by single HepIII.
[f] Exhaustive depolymerization by HepIII followed by HepI or HepII.

According to European Pharmacopoeia (http://online.edqm.eu, 2011), LMWHs having an average M_w of less than 8000 Da and for which at least 60% of the total mass has a M_w of less than 8000 Da, their potency should not be less than 70 IU of anti-factor Xa activity per milligram, as calculated in reference to the dried substance. In addition, the ratio of anti-factor Xa activity to anti-factor IIa activity should not be less than 1.5. Through the combination of these three heparinases, we can easily prepare LMWHs meeting the standards of the European Pharmacopoeia (http://online.edqm.eu, 2011) (Table 1). Indeed, to meet different clinical needs (Fareed, Hoppensteadt, & Bick, 2000), we can also produce LMWHs with different characteristics through the combination of these three heparinases.

5. Conclusions

Different enzymatic reactions for the depolymerization of heparin were studied in this research. HepI and HepII were able to depolymerize heparin efficiently to achieve LMWHs, while HepIII was only able to generate the high molecular weight of heparin. The anticoagulant activities of depolymerized products showed a linear relationship with M_w during the depolymerization, while the slopes for HepI and HepII were larger than the slope for HepIII, indicating that HepIII biocatalysis could maintain higher anticoagulant activities of the depolymerized products. The LMWHs produced by HepI exhibited higher anti-factor IIa activities than those generated by HepII, while the LMWHs produced by the two enzymes exhibited the same anti-factor Xa activity at the same values of M_w. Compared with the reactions by single HepI or HepII, the combinations of HepIII and HepI/HepII were able to depolymerize heparin effectively to LMWHs while maintaining higher anticoagulation activity. The anticoagulant activities of the LMWHs produced by HepI or HepII after exhaustive depolymerization by HepIII were higher than those produced by single HepI and HepII in the M_w range studied. Exhaustive depolymerization by HepIII followed by controlled depolymerization by HepI seemed to be the best combinatorial enzymatic method for the production of LMWHs with higher anticoagulant activity and the LMWHs were able to meet the standards of European Pharmacopoeia (http://online.edqm.eu, 2011). Furthermore, based on the changes in the ratio of anti-factor Xa activity to anti-factor IIa activity and M_w, the action patterns of HepI and HepII did not follow the exolytic and processive degrading mechanism from the reducing end of heparin, which was found for the first time in this study. Further study is needed to elucidate the exact action patterns of HepI and HepII during the depolymerization of heparin, which will be important for understanding of

heparin structure and rational design of depolymerization strategies of heparin to produce more favorable LMWHs.

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References

Achour, O., Bridiau, N., Godhbani, A., Le Joubioux, F., Bordenave, J. S., Sannier, F., et al. (2013). Ultrasonic-assisted preparation of a low molecular weight heparin (LMWH) with anticoagulant activity. *Carbohydrate Polymers*, *97*(2), 684–689.

Ahsan, A., Jeske, W., Hoppensteadt, D., Lormeau, J. C., Wolf, H., & Fareed, J. (1995). Molecular profiling and weight determination of heparins and depolymerized heparins. *Journal of Pharmaceutical Sciences*, *84*(6), 724–727.

Chen, Y., Xing, X. H., Ye, F. C., Kuang, Y., & Luo, M. F. (2007). Production of MBP-HepA fusion protein in recombinant *Escherichia coli* by optimization of culture medium. *Biochemical Engineering Journal*, *34*(2), 114–121.

Chen, Y., Xing, X. H., & Lou, K. (2005). Construction of recombinant *Escherichia coli* for over-production of soluble heparinase I by fusion to maltose-binding protein. *Biochemical Engineering Journal*, *23*(2), 155–159.

Dementiev, A., Petitou, M., Herbert, J. M., & Gettins, P. (2004). The ternary complex of antithrombin–anhydrothrombin heparin reveals the basis of inhibitor specificity. *Nature Structural & Molecular Biology*, *11*(9), 863–867.

Desai, U. R., Wang, H. M., & Linhardt, R. J. (1993a). Substrate specificity of the heparin lyases from *Flavobacterium heparinum*. *Archives of Biochemistry and Biophysics*, *306*(2), 461–468.

Desai, U. R., Wang, H. M., & Linhardt, R. J. (1993b). Specificity studies on the heparin lyases from *Flavobacterium heparinum*. *Biochemistry*, *32*(32), 8140–8145.

Edens, R. E., Al-Hakim, A., Weiler, J. M., Rethwisch, D. G., Fareed, J., & Linhardt, R. J. (1992). Gradient polyacrylamide gel electrophoresis for determination of molecular weights of heparin preparations and low-molecular-weight heparin derivatives. *Journal of Pharmaceutical Sciences*, *81*(8), 823–827.

Ernst, S., Langer, R., Cooney, C. L., & Sasisekharan, R. (1995). Enzymatic degradation of glycosaminoglycans. *Critical Reviews in Biochemistry and Molecular Biology*, *30*(5), 387–444.

Ernst, S., Rhomberg, A. J., Biemann, K., & Sasisekharan, R. (1998). Direct evidence for a predominantly exolytic processive mechanism for depolymerization of heparin-like glycosaminoglycans by heparinase I. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(8), 4182–4187.

Fareed, J., Hoppensteadt, D. A., & Bick, R. L. (2000). An update on heparins at the beginning of the new millennium. *Seminars in Thrombosis and Hemostasis*, *261*, 5–21.

Hedner, U. (2000). Development of tinzaparin: A heparinase-digested low-molecular-weight heparin. *Seminars in Thrombosis and Hemostasis*, *261*, 23–29.

http://online.edqm.eu (2011). European Pharmacopoeia 7.0.

Higashi, K., Hosoyama, S., Ohno, A., Masuko, S., Yang, B., Sterner, E., et al. (2012). Photochemical preparation of a novel low molecular weight heparin. *Carbohydrate Polymers*, *67*(2), 1737–1743.

Jandik, K. A., Gu, K. A., & Linhardt, R. J. (1994). Action pattern of polysaccharide lyases on glycosaminoglycans. *Glycobiology*, *4*(3), 289–296.

Koch, A., Bouges, S., Ziegler, S., Dinkel, H., Daures, J. P., & Victor, N. (1997). Low molecular weight heparin and unfractionated heparin in thrombosis prophylaxis after major surgical intervention: Update of previous meta-analyses. *British Journal of Surgery*, *84*(6), 750–759.

Linhardt, R. J. (1991). Heparin: An important drug enters its 7th decade. *Chemistry & Industry*, *2*, 45.

Linhardt, R. J., Fitzgerald, G. L., Cooney, C. L., & Langer, R. (1982). Mode of action of heparin lyase on heparin. *Biochimica et Biophysica Acta*, *702*(2), 197–203.

Linhardt, R. J., Rice, K. G., Kim, Y. S., Lohse, D. L., Wang, H. M., & Loganathan, D. (1988). Mapping and quantification of the major oligosaccharide components of heparin. *Biochemical Journal*, *254*(3), 781–787.

Linhardt, R. J., & Gunay, N. S. (1999). Production and chemical processing of low molecular weight heparins. *Seminars in Thrombosis and Hemostasis*, *253*, 5–16.

Loganathan, D., Wang, H. M., Mallis, L. M., & Linhardt, R. J. (1990). Structural variation in the antithrombin-III binding-site region and its occurrence in heparin from different sources. *Biochemistry*, *29*(18), 4362–4368.

Lohse, D. L., & Linhardt, R. J. (1992). Purification and characterization of heparin lyases from *Flavobacterium heparinum*. *Journal of Biological Chemistry*, *267*(34), 24347–24355.

Mackman, N. (2008). Triggers, targets and treatments for thrombosis. *Nature*, *451*(7181), 914–918.

Mamuwala, Z. S., Venkataraman, G. (2011). Methods of making low molecular weight heparin compositions. U.S. Patent 2011076729A1, March 31.

Masuko, S., & Linhardt, R. J. (2012). Chemoenzymatic synthesis of the next generation of ultralow MW heparin therapeutics. *Future Medicinal Chemistry*, *4*(3), 289–296.

Mulloy, B., Gee, C., Wheeler, S. F., Wait, R., Gray, E., & Barrowcliffe, T. W. (1997). Molecular weight measurements of low molecular weight heparins by gel permeation chromatography. *Thrombosis and Haemostasis*, *77*(4), 668–674.

Nader, H. B., Porcionatto, M. A., Tersariol, I., Pinhal, M., Oliveira, F. W., Moraes, C. T., et al. (1990). Purification and substrate specificity of heparitinase I and heparitinase II from *Flavobacterium heparinum*. Analyses of the heparin and heparan-sulfate degradation products by C-13 NMR spectroscopy. *Journal of Biological Chemistry*, *265*(28), 16807–16813.

Pervin, A., Gallo, C., Jandik, K. A., Han, X. J., & Linhardt, R. J. (1995). Preparation and structural characterization of large heparin derived oligosaccharides. *Glycobiology*, *5*(1), 83–95.

Petitou, M., Casu, B., & Lindahl, U. (2003). 1976–1983, a critical period in the history of heparin: The discovery of the antithrombin binding site. *Biochimie*, *85*(1-2), 83–89.

Petitou, M., Herault, L. P., Bernat, A., Driguez, P. A., Duchaussoy, P., Lormeau, J. C., et al. (1999). Synthesis of thrombin-inhibiting heparin mimetics without side effects. *Nature*, *398*(6726), 417–422.

Rhomberg, A. J., Shriver, Z., Biemann, K., & Sasisekharan, R. (1998). Mass spectrometric evidence for the enzymatic mechanism of the depolymerization of heparin-like glycosaminoglycans by heparinase II. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(21), 12232–12237.

Shaya, D., Zhao, W. J., Garron, M. L., Xiao, Z. P., Cui, Q. Z., Zhang, Z. Q., et al. (2010). Catalytic mechanism of heparinase II investigated by site-directed mutagenesis and the crystal structure with its substrate. *Journal of Biological Chemistry*, *285*(26), 20051–20061.

Shriver, Z., Sundaram, M., Venkataraman, G., Fareed, J., Linhardt, R., Biemann, K., et al. (2000). Cleavage of the antithrombin III binding site in heparin by heparinases and its implication in the generation of low molecular weight heparin. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(19), 10365–10370.

Wei, L., Johnson, D. J. D., Esmon, C. T., & Huntington, J. A. (2004). Structure of the antithrombin-thrombin-heparin ternary complex reveals the antithrombotic mechanism of heparin. *Nature Structural & Molecular Biology*, *11*(9), 857–862.

Xiao, Z. P., Tappen, B. R., Ly, M., Zhao, W. J., Canova, L. P., Guan, H. S., et al. (2011). Heparin mapping using heparin lyases and the generation of a novel low molecular weight heparin. *Journal of Medicinal Chemistry*, *54*(2), 603–610.

Xiao, Z., Zhao, W., Yang, B., Zhang, Z., Guan, H., & Linhardt, R. J. (2011). Heparinase 1 selectivity for the 3,6-di-O-sulfo-2-deoxy-2-sulfamido-alpha-D-glucopyranose (1,4) 2-O-sulfo-alpha-L-idopyranosyluronic acid (GlcNS3S6S-IdoA2S) linkages. *Glycobiology*, *21*(1), 13–22.

Yang, B., Yu, G., Zhao, X., Jiao, G., Ren, S., & Chai, W. (2009). Mechanism of mild acid hydrolysis of galactan polysaccharides with highly ordered disaccharide repeats leading to a complete series of exclusively odd-numbered oligosaccharides. *FEBS Journal*, *276*(7), 2125–2137.

Yang, V. C., Linhardt, R. J., Bernstein, H., Cooney, C. L., & Langer, R. (1985). Purification and characterization of heparinase from *Flavobacterium heparinum*. *Journal of Biological Chemistry*, *260*(3), 1849–1857.

Ye, F. C., Kuang, Y., Chen, S., Zhang, C., Chen, Y., & Xing, X. H. (2009). Characteristics of low molecular weight heparin production by an ultrafiltration membrane bioreactor using maltose binding protein fused heparinase I. *Biochemical Engineering Journal*, *46*(2), 193–198.

Yu, G. L., LeBrun, L., Gunay, N. S., Hoppensteadt, D., Walenga, J. M., Fareed, J., et al. (2000). Heparinase I acts on a synthetic heparin pentasaccharide corresponding to the antithrombin III binding site. *Thrombosis Research*, *100*(6), 549–556.

Zhao, W. J., Garron, M. L., Yang, B., Xiao, Z. P., Esko, J. D., Cygler, M., et al. (2011). Asparagine 405 of heparin lyase II prevents the cleavage of glycosidic linkages proximate to a 3-O-sulfoglucosamine residue. *FEBS Letters*, *585*(15), 2461–2466.