Carbohydrate Polymers 134 (2015) 151–157

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Production of a low molecular weight heparin production using recombinant glycuronidase

Guowei Su[a,1], Linhong Li[a,1], Haichan Huang[a], Weihong Zhong[a,*], Ping Yu[b], Fuming Zhang[c], Robert J. Linhardt[c,d,e]

[a] College of Biological and Environmental Engineering, Zhejiang University of Technology, Hangzhou 310032, China
[b] Department of Biological Engineering, Zhejiang Gongshang University, Hangzhou 310018, China
[c] Department of Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA
[d] Department of Biological Science, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA
[e] Departments of Chemistry and Chemical Biology and Biomedical Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY 12180, USA

ARTICLE INFO

Article history:
Received 29 April 2015
Received in revised form 2 August 2015
Accepted 3 August 2015
Available online 6 August 2015

Keywords:
Δ4,5 unsaturated glycuronidase
Pedobacter heparinus
Heparin
Heparosan
Heparan sulfate
Depolymerization

ABSTRACT

The Δ4,5 unsaturated uronate (4-deoxy-α-L-threo-hex-4-eno-pyranosyluronic acid) residue is produced through the depolymerization of heparin, heparosan, and heparan sulfate with heparin lyases. The recovery of unsaturated uronate containing products is necessary to prepare low molecular weight heparin (LMWH) from heparin or heparosan. In this study, the gene of Δ4,5 and $\Delta4,5^{\Delta20}$ unsaturated glycuronidase (EC# 3.2.1.56) from *Pedobacter heparinus* (formerly *Flavobacterium heparinum*) was cloned into pMAL-c2x plasmid. Its fusion protein with MBP was expressed in *Escherichia coli* TB1. After purification, Δ4,5 unsaturated glycuronidase was evaluated. The $\Delta4,5^{\Delta20}$ glycuronidase showed excellent activity on the unsaturated bonds of the different depolymerized products from Hep I, Hep II, and Hep III on heparin, heparosan, and heparan sulfate.

1. Introduction

Heparin has been used widely as an anticoagulant and antithrombotic drug, and it has attracted increasing interest in anticancer and anti-inflammation therapies. About 100 tons of pharmaceutical grade heparin products are produced and used annually worldwide (Bhaskar et al., 2012). The biological safety of heparin produced from animal source has received scrutiny since the crisis of heparin contamination in 2008 (Higashi et al., 2011; Kishimoto et al., 2008; Liu, Zhang, & Linhardt, 2009). The preparation of a bioengineered heparin from a microbially produced heparosan offers a potentially safer alternative for animal sourced heparin (Lindahl et al., 2005; Laremore, Zhang, Dordick, Liu, & Linhardt, 2009; Ly et al., 2011).

Heparosan is the capsular polysaccharide of *Escherichia coli* K5 and *Pasteurella multicida* D. It serves as an important precursor in heparin biosynthesis and as an intermediate in the chemo-enzymatic synthesis of bioengineered heparin (Zhang et al., 2008). Microbial heparosan is composed of alternating *N*-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA) with a structure forum of β-GlcA(1 → [4)-α-GlcNAc(1 → 4)β-GlcA-(1 →]$_n$4)-α,β-GlcNAc (Fig. 1a), identical to animal-derived heparosan and similar to heparan sulfate (HS) and heparin in its backbone structure. However, the molecular weight of heparosan from *P. multicida* D ranges from 200 to 300 kDa, while that from *E. coli* K5 range from 10 to 20 kDa (Wang, Fu, & Zhang, 2011; Wang, Dordick, & Linhardt, 2011). The heparosan from *E. coli* K5 is more similar to heparin than that from *P. multicida* D. Thus, most studies on bioengineering heparin focused on heparosan from *E. coli* K5 (Wang et al., 2010).

In our previous study, a considerable yield of heparosan could be achieved using a glucose fed batch fermentation. It was also found that the heparosan could be removed from the cell wall of *E. coli* K5

Abbreviations: Hep I, heparin lyase I; Hep II, heparin lyase II; Hep III, heparin lyase III; HS, heparan sulfate; PAGE, polyacrylamide gel electrophoresis; Δ4,5, Δ4,5 unsaturated glycuronidase; $\Delta4,5^{\Delta20}$, Δ4,5 glycuronidase lacking the first 20 amino acids; UGL, unsaturated glycuronidase; ΔUA, unsaturated uronic acid; H, hexosamine; I, iduronic acid; subscripts 2S and 6S, 2-O-sulfo and 6-O-sulfo groups respectively; NS and NAc, N-sulfo and N-acetyl groups in the hexosamine residue.

* Corresponding author.
E-mail address: whzhong@zjut.edu.cn (W. Zhong).
[1] These authors contributed equally to this article.

http://dx.doi.org/10.1016/j.carbpol.2015.08.001
0144-8617/

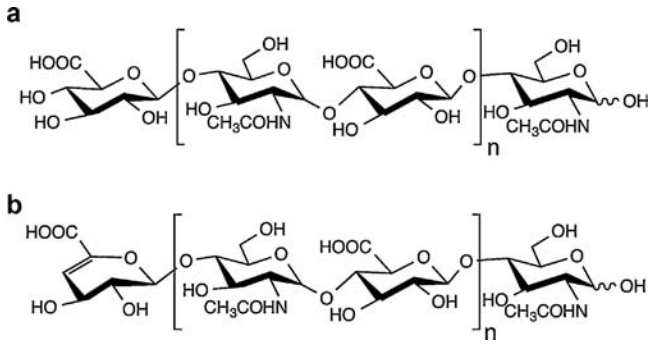

Fig. 1. Structures of (a) natural heparosan and (b) unsaturated heparosan afforded through lysase treatment.

due to both the shear force in fermenter and using heparosan lyase (Wang et al., 2010). After the lyase degradation, the structure of heparosan is converted to ΔUA(1 → [4)-α-GlcNAc(1 → 4)β-GlcA-(1 →]$_n$4)-α,β-GlcNAc (Fig. 1b, UA is the unsaturated uronate, 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid). Thus, the heparosan from K5 fermentation might be a mixture of saturated and unsaturated heparosan (Fig. 1a and b), in which unsaturated heparosan might require removal with Δ4,5 unsaturated glycuronidase (EC# 3.2.1.56).

Furthermore, low molecular weight heparin (LMWH, MWavg ~ 6000) or ultra low molecular weight heparin (ULMWH, MWavg < 2000) might need to be prepared from heparosan (Xu et al., 2011). Thus, a low molecular weight heparosan would first need to be prepared from heparosan using heparin lyase III (Hep III). The resulting unsaturated low molecular weight heparosan could then be converted to a saturated low molecular weight heparosan using Δ4,5 unsaturated glycuronidase. Thus, we undertook to evaluate the microbial production Δ4,5 unsaturated glycuronidase in large scale and its potential application in the preparation of ULMWH heparins.

In the past decades, only several reports focus on the study on heparin/heparan sulfate Δ4,5 unsaturated glycuronidase. Warnick and Linker (1972) first isolated and purified an unusual α-glycuronidase from *Flavobacterium heparinum*. The enzyme acts on degradation products of heparin or heparan sulfate, and its activity decrease rapidly with increasing molecular weight of oligosaccharides. Myette et al. (2002) cloned the heparin/heparan sulfate Δ4,5 unsaturated glycuronidase gene from *F. heparinum*. After construction of a recombinant pET 28b expression plamid, the soluble express of Δ4,5 unsaturated glycuronidase was also achieved in *E. coli* BL21 (DE3). Meanwhile, it was found that the deletion of first 60 bp for 20 amino acids (Δ4,5$^{\Delta 20}$ unsaturated glycuronidase) resulted in improved solubility of Δ4,5 unsaturated glycuronidase (Myette et al., 2002). Kuberan, Lech, Beeler, Wu, and Rosenberg (2003) reported that antithrombin III binding heparan sulfate pentasaccharide was synthesized using a 6 steps enzymatic method, in which Δ4,5 unsaturated glycuronidase was utilized to recover the unsaturated glycuronide produced by heparinase I (Hep I). Nakamichi, Mikami, Murata, and Hashimoto (2014) cloned *P. heparinus* UGL genes into a T7-based expression plasmid (pET21b) and constructed overexpression systems for *P. heparinus* UGLs (pET21b-Phep_2830). After purified by gel filtration chromatography and anion exchange chromatography, its crystal structure was evaluated and exhibited a special pocket-like structure and lid loop, which are involved in heparin disaccharide recognition.

The pET-series plasmid is a commonly selected vector for recombinant protein expression. However, it needs carefully controlled induction conditions to obtain the target as a soluble protein. Additional plasmids, such as pMAL, have been developed for the expression of soluble fusion proteins. For examples, Kapust and Waugh (1999) found that the soluble fusion partner maltose-binding protein (MBP) is a far more effective solubilizing agent than the other two fusion partners (glutathione S-transferase, GST; and thioredoxin, TRX) and MBP could inhibit the aggregation of six diverse proteins that normally accumulate in an insoluble form. Lee, Tsang, Cheng, and Luk (2006) found that the expression vectors pMAL-cRI with MBP tag enhanced the yield and solubility of protein at room temperature incubation. Wang, Fu, et al. (2011) and Wang, Dordick, et al. (2011) also concluded that the purity, specific activity of the purified expressed pectate lyase gene (Pcpel2) expressed by the pMAL system were higher than that by pET system.

In the present study, the plasmid pMAL-c2x was selected to clone and express Δ4,5 unsaturated glycuronidase. The harvested Δ4,5 unsaturated glycuronidase was further purified and utilized to evaluate its activity on the conversion of unsaturated lyase-depolymerized products from heparason, heparin, and HS to their saturated forms.

2. Materials and methods

2.1. Strains and plasmids

P. heparinus ATCC 13125, *E. coli* DH5α and TB1 were purchased from ATCC. PMD19-T was purchased from TaKaRa Biotech Co. Ltd (Dalian, China), and pMAL-c2x was purchased from Beijing Ding Guo Chang Sheng Biotechnology Co. Ltd.

2.2. Chemicals and reagents

Taq DNA polymerase used in this research was purchased from Sigma (USA). Kits used in extracting genome and plasmids of bacteria were purchased from OMEGA. Gel extraction kit, heparin sodium from porcine intestine (MW 6000–20,000 Da), IPTG and ampicillin was purchased from Shanghai Sangon Biotech (Shanghai, China). T4 DNA ligase was purchased from TaKaRa Biotech Co. Ltd (Dalian, China). DNA markers and protein markers were purchased from TransGen Biotech Co. Ltd (Beijng, China). Biowest agarose was purchased from Gene Company Ltd. (Hongkong). Amylose resin was purchased from New England Biolabs (Ipswich, MA, USA).

2.3. Gene cloning and expression of the Δ4,5 unsaturated glycuronidase in E. coli

The genomic DNA of *P. heparinus* was isolated from a 3–4.5 mL culture using the E.Z.N.A. DNA Kit (Omega Bio-tek) according to the manufacturer's instructions for Gram-negative bacteria. Following purification, genomic DNA was precipitated in ethanol. After harvest by centrifugation, the genomic DNA was suspended in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) for the following

Table 1
Activity assay method for Δ4,5 and Δ4,5$^{\Delta 20}$ unsaturated glycuronidase.

Reagents	Test group	Control group
[a]Reagent A	0.23 mL	0.23 mL
Reagent B	0.05 mL	0.05 mL
Mixed and kept at 25 °C for 3 min, then add the following		
Reagent D	0.02 mL	0.02 mL
Reagent E	0.2 mL	–
Mixed rapidly and kept at 25 °C for 30 min, then add the following		
Reagent C	0.5 mL	0.5 mL
Reagent E	–	0.2 mL
1 mL mixture was transferred to quartz cuvette to measure $A_{235\,nm}$ with the control group as zero		

[a] Reagent A: 20 mM TrisHCl, 50 mM NaCl, 4 mM $CaCl_2$; Reagent B: 1% heparin sodium solution; Reagent C: 50 mM HCl; Reagent D: Hep III solution (Hep III was mixed in Reagent A); Reagent E: Δ4,5 and 4,5$^{\Delta 20}$ unsaturated glycuronidase solution (was mixed in Reagent A).

Table 2
Method for measure the activity of Δ4,5 unsaturated glycuronidase on the depolymerization products prepared using Hep I, Hep II or Hep III.

Reagents	Test group for Δ4,5	Control group for Δ4,5	Test group for Hep I/II/II	Control group for Hep I/II/II
[a]Reagent A	0.23 mL	0.23 mL	0.23 mL	0.23 mL
Reagent B	0.05 mL	0.05 mL	0.05 mL	0.05 mL
Mixed and kept at 25 °C for 3 min, then add the following				
Reagent C	–	0.5 mL	–	0.5 mL
Reagent D	0.02 mL	0.02 mL	0.02 mL	0.02 mL
Reagent E	0.2 mL	0.2 mL	–	0.2 mL
Mixed rapidly and kept at 25 °C for 30 min, then add the following				
Reagent C	0.5 mL	–	0.5 mL	–
Reagent E	–	–	0.2 mL	–
$A_{235\,nm}$ of the mixture was measured with the control group as zero				

[a] Reagent A: 20 mM TrisHCl, 50 mM NaCl, 4 mM $CaCl_2$; Reagent B: 1% heparin sodium/HS/heparosan solution; Reagent C: 50 mM HCl; Reagent D: Hep I/II/III solution (Hep I/II/III was mixed in Reagent A); Reagent E: Δ4,5 and 4,5$^{\Delta 20}$ unsaturated glycuronidase solution (was mixed in Reagent A).

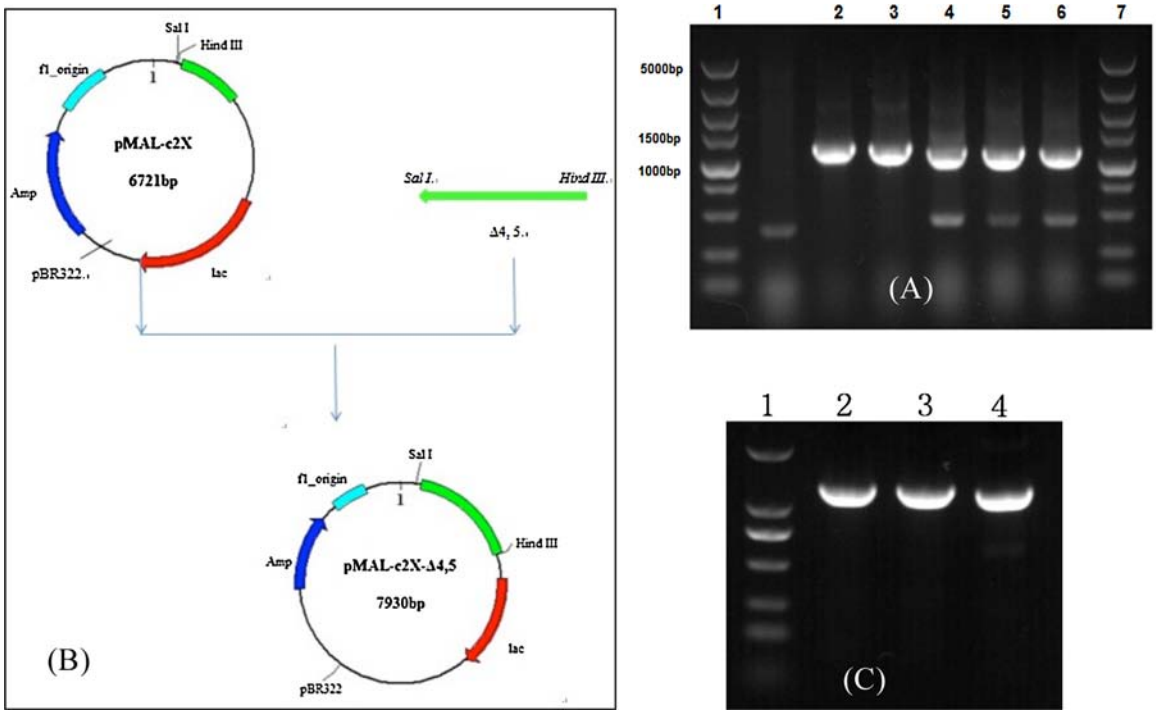

Fig. 2. Construct of expression vector pMAL-c2X-Δ4,5 and pMAL-c2X-Δ4,5$^{\Delta 20}$ (A), touchdown PCR products for Δ4,5 and 4,5$^{\Delta 20}$ unsaturated glycuronidase gene from the genomic DNA of *Pedobacter heparinus* ATCC 13125 (B), and colony PCR confirmation for the positive transformant colonies (C). In (B), Lane 1, 7, DNA molecular weight marker; Lane 2, 3, Δ4,5; Lane 4–6: Δ4,5$^{\Delta 20}$. In (C), Lane 1, DNA molecular weight marker; Lane 2, 3, *E. coli* TB1/pMAL-c2X-Δ4,5; Lane 4, *E. coli* TB1/pMAL-c2X-Δ4,5$^{\Delta 20}$.

experiment. The quality of genomic DNA was measured by spectrophotometer at 260/280 nm and by electrophoresis on a 1% agarose gel.

The full-length gene of Δ4,5 unsaturated glycuronidase was directly amplified from the genomic DNA using a pair of primers (forward 45UG-F, cag**gtcgac**atgaaatcactactcagtg and reverse 45UG-R, ccg**aagctt**ttaagactgattaattgttttca) with *Sal* I and *Hin*d III restriction sites denoted in bold using Touchdown PCR method (an initial denaturation at 95 °C for 5 min, 20 cycles consisted of the denaturation at 94 °C for 50 s, annealing at 56 °C for 45 s, and declining 0.5 °C every cycle, and extension at 72 °C for 90 s, then 10 cycles consisting of 94 °C for 50 s, 46 °C for 45 s, 72 °C for 90 s). Overhangs of dA were generated in a final 10 min extension at 72 °C using Taq DNA polymerase. PCR products were gel purified, ligated into the pMD19-T cloning vector, and transformed into chemically competent cells of *E. coli* DH5α. Positive clones were identified by blue/white colony selection and confirmed by colony PCR screening. The 1.2 kb gene of Δ4,5 unsaturated glycuronidase was subcloned into expression plasmid pMAL-c2x as a *Sal I-Hind III* cassette. Final expression clones were confirmed by plasmid DNA sequencing.

For the expression of Δ4,5 glycuronidase beginning with M21 (Δ4,5$^{\Delta 20}$), the forward primer 45UG20-F (ctggtcgacatgacagttacgaaagg, also containing a *Sal* I restriction site near its 5′ terminus) was used in place of primer 45UG-F (above). The original expression plasmid pMAL-c2x/Δ4,5 was used as the DNA template in PCR reactions involving a total of 30 cycles.

Both pMAL-c2x-Δ4,5 and pMAL-c2x-Δ4,5$^{\Delta 20}$ plasmids were constructed and transformed into *E. coli* TB1 for expression of MBP fusion Δ4,5 unsaturated glycuronidase. The solo colonies of strains *E. coli* TB1/pMAL-c2x-Δ4,5 and *E. coli* TB1/pMAL-c2x-Δ4,5$^{\Delta 20}$ were inoculated into 50 mL LB media respectively and incubated at 37 °C overnight. Then 2 mL cultures were inoculated into 50 mL LB media containing 50-μg/mL ampicillin. After 2–3 h culture at 37 °C until the A_{600} of the broth reached 0.6–0.8, 0.8 mM IPTG was then added and the culture incubated at 20 °C for 16–18 h.

2.4. Purification of recombinant Δ4,5 glycuronidase

Bacterial cells in 1 mL broth were harvested by centrifugation at 3000 × *g* and 4 °C for 20 min and suspended in 10 mL buffer A (20 mM Tris–HCl (pH 7.4), 0.2 M NaCl, and 1 mM EDTA (pH 8.0),

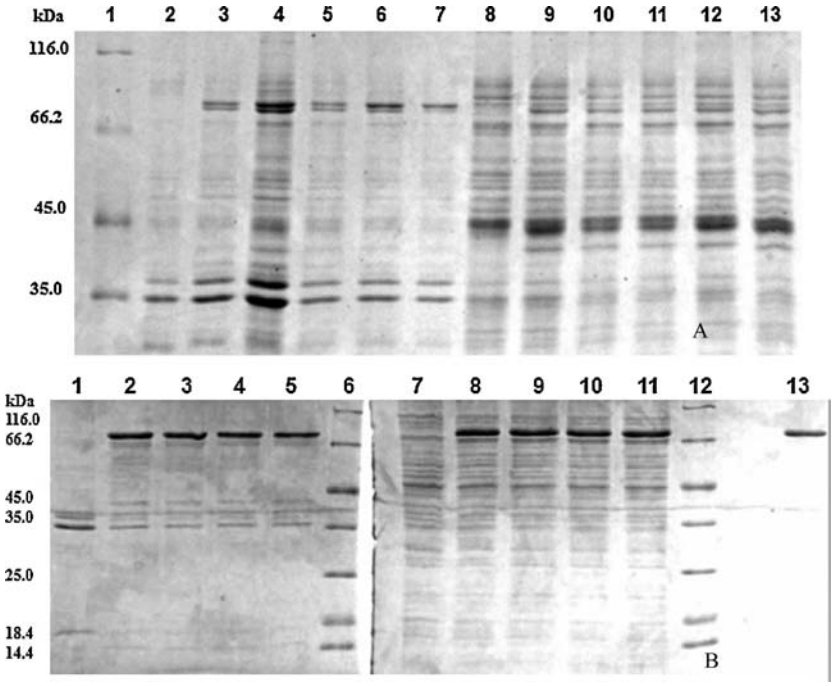

Fig. 3. SDS-PAGE results from the expression and purification of Δ4,5 (A) and $\Delta4,5^{\Delta20}$ (B) glycuronidase via a recombinant strain *E. coli* TB1/pMAL-c2X-$\Delta4,5^{\Delta20}$. (A) Lane 1, protein molecular weight markers; Lane 2–7, cell debris with IPTG 0, 0.4, 0.5, 0.6, 0.7, and 0.8 mmol/L; Lane 8–13, soluble fraction of crude cells lysate with IPTG 0, 0.4, 0.5, 0.6, 0.7 and 0.8 mmol/L; (B) Lane 1–5, cell debris with IPTG 0, 0.8, 0.9, 1.0, and 1.1 mmol/L; Lane 6, 12, protein molecular weight markers; Lane 7–11, soluble fraction of crude cells lysate with IPTG 0, 0.8, 0.9, 1.0, and 1.1 mmol/L; Lane 13, amylose resin column chromatography eluent.

Table 3
Purification of $\Delta4,5^{\Delta20}$ using column with MBP resin.

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification-fold	Recovery (%)
Crude enzyme	21.97	884	40.23	–	–
Purified enzyme	1.90	618	325.47	8.09	69.91

1 mM DTT). Cell suspension solution was intermittent sonicated in an ice-water bath using a sonicator at 40% output. The crude lysate was fractionated by centrifugation (8000 × *g*, 4 °C, 30 min). The supernatant was soluble recombinant protein while the cell debris was suspended in buffer A. After filtration through a 0.45 μm filter, the MBP fused Δ4,5 glycuronidase was purified by MBP affinity for maltose on a 10 mL column precharged with 2–3 mL amylose resin and subsequently equilibrated with binding buffer. Column washes included an intermediate step with binding buffer. The Δ4,5 enzyme was eluted from the column in 5–10 mL fractions using elution buffer (20 mM Tris–HCl, pH 7.4, 0.2 M NaCl, and 1 mM EDTA, pH 8.0, 1 mM DTT, and 10 mM maltose). Protein concentrations were determined by the Shanghai Sangon Biotech protein assay and confirmed by UV spectroscopy. Protein purity was assessed by SDS-PAGE followed by Coomassie Brilliant Blue staining.

2.5. Assay for enzyme activity of unsaturated glycuronidase

Standard reactions were carried out at 25 °C, pH 7.0 for 30 min with the procedures in Table 1. Hydrolysis of unsaturated polysaccharides was determined by measuring the loss of the Δ4,5 chromophore by a spectrophotometer at 235 nm. The activity unit was determined as: one unit is equal to enzyme required for hydrolysis of 0.10 μmoL unsaturated uronate per h at pH 7 and 25 °C. The enzyme activity was calculated using the following molar extinction coefficients and formula: Units/mL enzyme = ($A_{235\,nm}$ Control − $A_{235\,nm}$ Test) × 2 × 10 × 3 × df/(5.50 × 0.2). In which, 2 is conversion coefficient from 30 min to 1 h; 10 is conversion coefficient from 1 μmoL to 0.10 μmoL; 3 is total volume of reaction mixture (mL); df is dilution fold of enzyme solution; 5.50 is extinction coefficient of unsaturated uronic acid at 235 nm and μmoL level; 0.2 is volume of enzyme solution (mL).

2.6. Application of $\Delta4,5^{\Delta20}$ glycuronidase

Heparin, HS, and heparosan were used as the substrates Hep I, Hep II, and Hep III (prepared in our lab, Huang et al., 2013, 2014) to test the activity of the $\Delta4,5^{\Delta20}$ glycuronidase on their different depolymerization products. After lyase digestion, the depolymerization products were treated with $\Delta4,5^{\Delta20}$ glycuronidase. The A_{235} change was measured to evaluate the reverse effect on unsaturated bonds. According to our previous report (Huang et al., 2014), all the reaction conditions and the procedure are provided in Table 2.

2.7. Nuclear magnetic resonance analysis

The depolymerized and the recovery products of heparin and heparosan were analyzed by ^{1}H nuclear magnetic resonance (NMR) respectively. All the samples filtered through 0.22-μm membrane after boiling and then desalted by 3-kDa MWCO membrane. The final sample was lyophilized for ^{1}H NMR analysis on a Bruker 500 MHz NMR spectrometer.

3. Results and discussion

3.1. Gene cloning and expression vector construction of Δ4,5 glycuronidase

The genes of Δ4,5 and $\Delta4,5^{\Delta20}$ were isolated from the genomic DNA of *P. heparinus* ATCC 13125 using PCR reaction. Fig. 2A shows that the 1.2 kb genes were successfully obtained. The genes

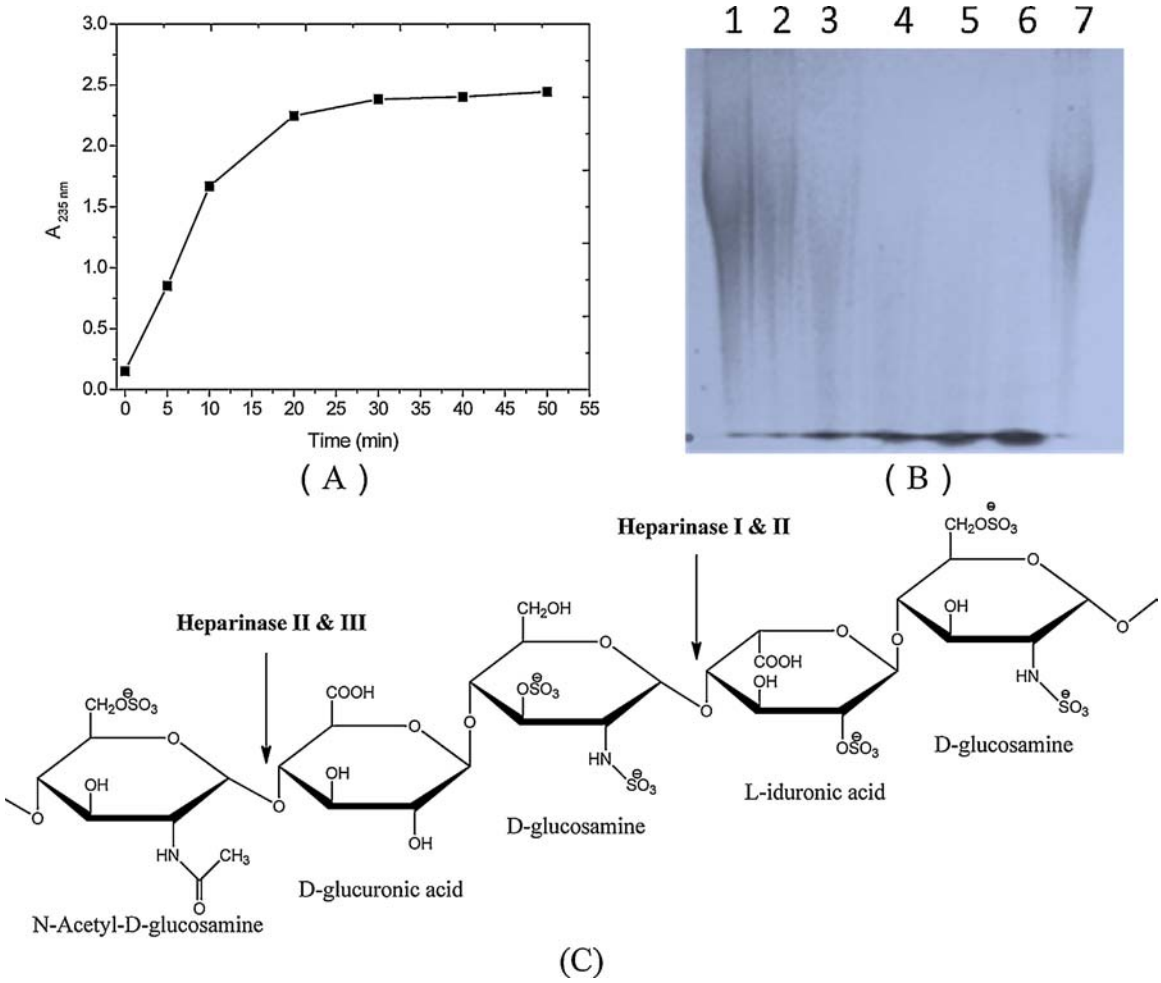

Fig. 4. Change of A_{235} (A) and molecular weight (B) of heparin digested by Hep II with activity selectivity (C), in (B) Line 1, 7, heparin sodium; Line 2–6, heparin sodium digested by Hep II in 0, 5, 10, 20, 50 min, respectively.

were then amplified with pMD19-T cloning vector in DH5α. The expression of the genes was performed by expression plasmid pMAL-c2x in TB1. The recombinant plasmids pMAL-c2X-Δ4,5 and pMAL-c2X-$\Delta4,5^{\Delta20}$ were transformed into TB1 and screened in LB medium containing ampicillin (50 μg/mL). The correct clone was confirmed by PCR (Fig. 2B and C) and DNA sequencing. All the results indicate that the expression vector is constructed successfully.

3.2. Expression and purification of Δ4,5 glycuronidase

We optimized the induction condition to obtain the high level expression of enzymes. The SDS-PAGE (Fig. 3) shows the expression of enzymes at different inducer concentration. Large differences in the protein expression pattern, enzyme expression level, cell debris and soluble fraction in crude cells lysate was observed for Δ4,5 and $\Delta4,5^{\Delta20}$. This indicates that $\Delta4,5^{\Delta20}$ glycuronidase has better soluble expression characteristics and its optimal inducer concentration was 0.8 mmol/L.

The MBP-fused Δ4,5 glycuronidase was purified by amylose–agarose chromatography. The result of purification was shown in Fig. 3B, lane 13. Only a single protein band was shown in the SDS-PAGE gel demonstrating that the glycuronidase obtained was pure. The specific activity of $\Delta4,5^{\Delta20}$ unsaturated glycuronidase could be improved by ~8-fold after purification (Table 3).

Table 4
Action of $\Delta4,5^{\Delta20}$ glycuronidase on the depolymization products.

Enzymes		A_{235} in the reaction mixture		
Lyase	$\Delta4,5^{\Delta20}$ glycuronidase	Heparin sodium	HS	Heparosan
Hep I	–	0.903	0.288	0.058
	+	0.898	0.227	−0.023
	A_{235} decrease	*0.05*	*0.061*	*0.081*
Hep II	–	1.47	0.827	0.152
	+	0.836	0.478	−0.008
	A_{235} decrease	*0.634*	*0.349*	*0.160*
Hep III	–	0.222	0.239	0.345
	+	−0.192	−0.241	0.023
	A_{235} decrease	*0.414*	*0.48*	*0.322*

3.3. Application of $\Delta4,5^{\Delta20}$ glycuronidase

The activity of $\Delta4,5^{\Delta20}$ glycuronidase working on the products of depolymerized heparin, HS, and heparosan from heparin lyases (Hep I, II and III) digestion was evaluated. The results (Figs. 4A, B and 5, Table 4) showed that the $\Delta4,5^{\Delta20}$ glycuronidase has significant effect on the removal of the unsaturated uronate residue of the depolymerized products of glycosaminoglycan prepared through lyase digestion.

As shown in Table 4, the ability of Hep I/II/III to depolymize heparin/HS/heparosan was different. Hep II showed the highest activity on heparin sodium while Hep III showed the lowest activity. Hep II

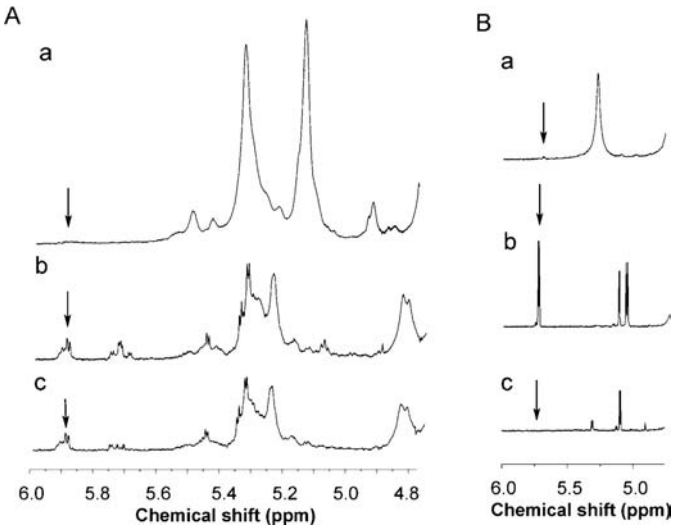

Fig. 5. The partial ^{1}H NMR spectra (4.8–6.0 ppm) of heparin and heparosan samples. (A) Heparin samples include: (a) untreated heparin with no peak corresponding to the 4-proton in of the ΔUA_{2S} residue at 5.9 ppm (indicated by arrow); (b) Hep II treated heparin affords a peak corresponding to the 4-proton in of the ΔUA_{2S} residue at 5.9 ppm (indicated by arrow); (c) Hep II treated heparin that was subsequently treated with Δ4,5 glycuronidase still shows a peak corresponding to the 4-proton in of the ΔUA_{2S} residue at 5.9 ppm (indicated by arrow) demonstrating the resistance of the ΔUA_{2S} residue to Δ4,5 glycuronidase. (B) Heparosan samples include: (a) untreated heparosan with no peak corresponding to the 4-proton in of the ΔUA_{2OH} residue at 5.7 ppm (indicated by arrow); (b) Hep III treated heparosan affords a peak corresponding to the 4-proton in of the ΔUA_{2OH} residue at 5.7 ppm (indicated by arrow); (c) Hep III treated heparosan that was subsequently treated with Δ4,5 glycuronidase shows the disappearance of the peak corresponding to the 4-proton in of the ΔUA_{2OH} residue at 5.9 ppm (indicated by arrow) demonstrating the sensitivity of the ΔUA_{2OH} residue to Δ4,5 glycuronidase.

also exhibited higher activity on HS than Hep I and Hep III. Hep III showed higher activity on heparosan than Hep II and Hep I.

It was reported that Hep II catalyzes the eliminative scission of the glycosidic linkages between *N*-sulfo or *N*-acetyl glucosamine and glucuronic or iduronic acid residues. However, Hep I and Hep III have selective substrate specificities that can be exploited for more detailed characterization of the molecular structure and intra-chain sequences in heparin and HS, respectively. Hep III catalyzes the eliminative scission of glycosidic linkages between the GlcNS or GlcNAc and GlcA (Fig. 4C). Hep III tolerates the C-6 sulfation of amino sugars and acts on HS in the low sulfation regions, but has little activity against heparin (Godavarti et al., 1996; Linhardt et al., 1990; Merry et al., 1999; Wei, Lyon, & Gallagher, 2005). The preferred substrates for Hep III are GlcNS or GlcNAc linked to GlcA, such as commonly found in HS and heparosan. In heparin, Hep I, II, and III also show specific selectivity on bond cleaved and the disaccharides formed (Wu, Zhang, Mei, Li, & Xing, 2014).

The activity of $\Delta 4,5^{\Delta 20}$ glycuronidase on the unsaturated depolymerization products formed using Hep I, Hep II and Hep III on the substrate types was also different. For heparin sodium, $\Delta 4,5^{\Delta 20}$ glycuronidase exhibited higher activity on the depolymerized products formed using Hep II than on those formed using Hep III and no significant $\Delta 4,5^{\Delta 20}$ glycuronidase activity was observed on those formed using Hep I. For HS, $\Delta 4,5^{\Delta 20}$ glycuronidase showed higher activity on the depolymerization products formed using Hep III than on those formed using Hep II and no significant $\Delta 4,5^{\Delta 20}$ glycuronidase activity was observed on those formed using Hep I. For heparosan, $\Delta 4,5^{\Delta 20}$ glycuronidase also exhibited higher activity on the depolymerized products by Hep III than on those formed using Hep II and no significant $\Delta 4,5^{\Delta 20}$ glycuronidase activity was observed on those formed using Hep I.

In ^{1}H NMR spectra of heparin samples (Fig. 5A), a peak at ~5.9 ppm (Fu et al., 2014), corresponding to the H4 of ΔUA_{2S} is formed on treating with Hep II, but is not diminished on treating with Δ4,5 unsaturated glycuronidase. This clearly demonstrates that the ΔUA_{2S} residue is not substrate for the Δ4,5 unsaturated glycuronidase. In contrast, in the ^{1}H NMR spectra of heparosan samples (Fig. 5B), the peak at ~5.7 ppm, corresponding to the H4 of ΔUA_{2OH} appearing after digestion heparin by Hep III, completely disappears on treating with Δ4,5 unsaturated glycuronidase. This clearly demonstrates that the ΔUA_{2OH} residue is a substrate for the Δ4,5 unsaturated glycuronidase. These results are consistent with the changes of A_{235} nm reported in Table 4.

These results were similar to those presented in previous publications. For example, Myette et al. (2002) reported that Δ4,5 glycuronidase discriminates on the basis of both the glycosidic linkage and the sulfation pattern within its saccharide substrate. The glycuronidase displays a strong preference for 1→4 linkages, making this enzyme specific to heparin/heparan sulfate rather than 1→3 linked glycosaminoglycans such as chondroitin/dermatan sulfate or hyaluronan. Furthermore, for disaccharide selectivity, ΔUAH_{NAc} and $\Delta UAH_{NAc,6S}$ were the best substrates of Δ4,5 glycuronidase. $\Delta UAH_{NH2,6S}$ and ΔUAH_{NS} were relatively poor substrates of Δ4,5 glycuronidase. $\Delta UAH_{NS,6S}$ falls roughly in the middle between these two boundaries and the Δ4,5 glycuronidase have no activity on $\Delta UA_{2S}H_{NS}$.

The depolymerization products afforded by Hep I acting on heparin mainly include $\Delta UA_{2S}H_{NS}$ disaccharide units, on which Δ4,5 glycuronidase showed no activity. Thus, it is reasonable that Δ4,5 glycuronidase showed no significant activity on the depolymerized products of Hep I. However, the depolymerization products of Hep III mainly include ΔUAH_{NAc} and $\Delta UAH_{NAc,6S}$ disaccharide units, on which Δ4,5 usually show activity. Thus, it is also reasonable that Δ4,5 showed best effect on the depolymerized products of Hep III and it is reasonable that the peak at ~5.7 ppm (H4 ΔUA_{2OH}, Fig. 5B) vanished completely. As for the depolymerized products of Hep II, which include more types of disaccharide units including $\Delta UA_{2S}H_{NS}$ and ΔUA_{2OH}, similar to the depolymerized products of Hep III or Hep I. Thus, it is also reasonable that Δ4,5 showed some activity toward on the depolymerization products of Hep II. However, Δ4,5 glycuronidase have no activity on $\Delta UA_{2S}H_{NS}$, so the peak at ~5.9 ppm (H4ΔUA_{2S}, Fig. 5A) showed no significantly change.

4. Conclusions

From *P. heparinus*, the gene of Δ4,5 and $\Delta 4,5^{\Delta 20}$ unsaturated glycuronidase fused with MBP was successful expressed in *E. coli* and purified by MBP affinity column. The $\Delta 4,5^{\Delta 20}$ glycuronidase showed excellent effect on the reverse of unsaturated bonds of the different depolymerized products via Hep I, Hep II, and Hep III on heparin, heparosan, and heparan sulfate.

Acknowledgements

This study was financially supported by the Natural Science Foundation of China (Grant no. 21076195), the Science Technology Department of Zhejiang Province (Grant no. 2011C24007), and the Specialized Research Fund for the Doctoral Program of Higher Education (Grant no. 20113317110002). The authors would like to thank Professor Jian Liu (The University of North Carolina at Chapel Hill) for supplying *E. coli* BL21 (carrying the plasmid containing the Hep A, B, C gene, respectively) used in this study.

References

Bhaskar, U., Sterner, E., Hickey, A. M., Onishi, A., Zhang, F., Dordick, J., et al. (2012). Engineering of routes to heparin and related polysaccharides. *Applied Microbiology and Biotechnology*, *93*, 1–16.

Fu, L., Zhang, F. M., Li, G. Y., Onish, A., Bhaskar, U., Sun, P. L., et al. (2014). Structure and activity of a new low-molecular-weight heparin produced by enzymatic ultrafiltration. *Journal of Pharmaceutical Siences*, *105*, 1375–1383.

Godavarti, R., Davis, M., Venkataraman, G., Cooney, C., Langer, R., & Sasisekharan, R. (1996). Heparinase III from *Flavobacterium heparinum*: Cloning and recombinant expression in *Escherichia coli*. *Biochemical and Biophysical Research Communications*, *225*, 751–758.

Higashi, K., Ly, M., Wang, Z. Y., Masuko, S., Bhaskar, U., Sterner, E., et al. (2011). Controlled photochemical depolymerization of K5 heparosan, a bioengineered heparin precursor. *Carbohydrate Polymers*, *86*, 1365–1370.

Huang, H. C., Lv, S. C., Zhao, L., Yan, Z. Q., Zhong, W. H., & Zhang, F. M. (2013). Capsule treatment by soluble expressed recombinant heparinase II enhance the foreign gene transformation to *Escherichia coli* K5. *Journal of Pure and Applied Microbiology*, *7*, 3009–3015.

Huang, H. C., Zhao, Y. Y., Lv, S. C., Zhong, W. H., Zhang, F. M., & Linhardt, R. J. (2014). Quantitation of heparosan with heparin lyase III and spectrophotometry. *Analytical Biochemistry*, *447*, 46–48.

Kapust, R., & Waugh, B. D. S. (1999). *Escherichia coli* maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. *Protein Science*, *8*, 1668–1674.

Kishimoto, T. K., Viswanathan, K., Ganguly, T., Elankumaran, S., Smith, S., Pelzer, K., et al. (2008). Contaminated heparin associated with adverse clinical events and activation of the contact system. *New England Journal of Medicine*, *358*, 2457–2467.

Kuberan, B., Lech, M. Z., Beeler, D. L., Wu, Z. L., & Rosenberg, R. D. (2003). Enzymatic synthesis of antithrombin III binding heparin sulfate pentasaccharide. *Nature Biotechnology*, *21*, 1343–1346.

Laremore, T. N., Zhang, F., Dordick, J. S., Liu, J., & Linhardt, R. J. (2009). Recent progress and applications in glycosaminoglycan and heparin research. *Current Opinion in Chemical Biology*, *13*, 633–640.

Lee, N. P. Y., Tsang, S., Cheng, R. H., & Luk, J. M. (2006). Increased solubility of integrin beta A domain using maltose-binding protein as a fusion tag. *Protein and Peptide Letters*, *13*, 431–435.

Lindahl, U., Li, J. P., Kusche-Gullberg, M., Salmivirta, M., Alaranta, S., Veromaa, T., et al. (2005). Generation of "neoheparin" from *E. coli* K5 capsular polysaccharide. *Journal of Medicinal Chemistry*, *48*, 349–352.

Linhardt, R. J., Turnbull, J. E., Wang, H. M., Loganathan, D., & Gallagher, J. T. (1990). Examination of the substrate specificity of heparin and heparan sulphate lyases. *Biochemistry*, *29*, 2611–2617.

Liu, H. Z., Zhang, Z. Q., & Linhardt, R. J. (2009). Lessons learned from the contamination of heparin. *Nature Product Reports*, *26*, 313–321.

Ly, M., Wang, Z. Y., Laremore, T. N., Zhang, F. M., Zhong, W. H., Pu, D., et al. (2011). Analysis of *E. coli* K5 capsular polysaccharide heparosan. *Analytical and Bioanalytical Chemistry*, *399*, 737–745.

Merry, C. L. R., Lyon, M., Deakin, J. A., Hopwood, J. J., & Gallagher, J. T. (1999). Highly sensitive sequencing of the sulphated domains of heparan sulphate. *Journal of Biological Chemistry*, *274*, 18455–18462.

Myette, J. R., Shriver, Z., Kiziltepe, T., McLean, M. W., Venkataraman, G., & Sasisekharan, R. (2002). Molecular cloning of the heparin/heparan sulfate Δ4,5 unsaturated glycuronidase from *flavobacterium heparinum*, its recombinant expression in *Escherichia coli*, and biochemical determination of its unique substrate specificity. *Biochemistry*, *41*, 7424–7434.

Nakamichi, Y., Mikami, B., Murata, K., & Hashimoto, W. (2014). Crystal Structure of a bacterial unsaturated glucuronyl hydrolase with specificity for heparin. *Journal of Biological Chemistry*, *289*, 4787–4797.

Wang, H. Z., Fu, L., & Zhang, X. G. (2011). Comparison of expression, purification and characterization of a new pectate lyase from Phytophthora capsici using two different methods. *BMC Biotechnology*, *11*, 32.

Wang, Z., Dordick, J. S., & Linhardt, R. J. (2011). *Escherichia coli* K5 heparosan fermentation and improvement by genetic engineering. *Bioengineered Bugs*, *2*, 63–67.

Wang, Z. Y., Ly, M., Zhang, F. M., Zhong, W. H., Suen, A., Hickey, A. M., et al. (2010). *E.coli* K5 fermentation and the preparation of heparosan, a bioengineered heparin precursor. *Biotechnology and Bioengineering*, *107*, 964–973.

Warnick, T. C., & Linker, A. (1972). Purification of an unusual glycuronidase from flavobacteria. *Biochemistry*, *11*, 568–572.

Wei, Z., Lyon, M., & Gallagher, J. T. (2005). Distinct substrate specificities of bacterial heparinases against N-unsubstituted glucosamine residues in heparan sulphate. *Journal of Biological Chemistry*, *280*, 15742–15748.

Wu, J. J., Zhang, C., Mei, X., Li, Y., & Xing, X. H. (2014). Controllable production of low molecular weight heparins by combinations of heparinase I/II/III. *Carbohydrate Polymers*, *101*, 484–492.

Xu, Y., Masuko, S., Takieddin, M., Xu, H. M., Liu, R. P., Jing, J., et al. (2011). Chemoenzymatic synthesis of structurally homogeneous ultra-low molecular weight heparins. *Science*, *334*, 498–501.

Zhang, Z. Q., McCallum, S. A., Xie, J., Nieto, L., Corzana, F., Jimenez-Barbero, J., et al. (2008). Solution structures of chemoenzymatically synthesized heparin and its precursors. *Journal of the American Chemical Society*, *130*, 12998–21300.