Production of heparin oligosaccharides by fusion protein of MBP–heparinase I and the enzyme thermostability

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Abstract

Enzymatic depolymerization of heparin to produce LMWH, a useful anticoagulant, has attracted much attention due to its mild reaction conditions and high selectivity. In this paper, we examined the feasibility of heparin depolymerization by heparinase I fused with maltose-binding protein (MBP) (MBP–HepA), which was functionally expressed in recombinant *Escherichia coli* with high activity. Our results showed that MBP–HepA degraded heparin effectively and the LMWHs with the weight average molecular weight (M_w) less than 3000 Da and narrow polydispersity were formed by controlling the reaction time. Thermostability of the fused enzyme was studied and possible mechanism for heat inactivation was proposed. The results showed that the MBP–HepA was relatively unstable and the enzyme inactivation was dependent on a third-order kinetics at the high temperature below 45 °C.
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1. Introduction

Heparin is a highly sulphated and polydispersed linear glycosaminoglycan of alternating $1 \rightarrow 4$ linked hexuronic acid and D-glycosamine residues (M_w 3000–37,000 Da with an average M_w of 15,000 Da). For the side effects of heparin as the anticoagulant and antithrombotic agent, low molecular weight heparin (LMWH) [1] (M_w less than 8000 Da with an average M_w of around 5000 Da) capable of maintaining the anticoagulant activity while showing less side effect has been widely used and replaced heparin in clinics. Due to its lower molecular weight and less polydispersity compared with original heparin, LMWH has better pharmacokinetics on s.c. injection, longer half-lives, better bioavailability and higher safety [2]. Nowadays, LMWHs are further divided into several categories, such as ultra low molecular weight heparin ($M_w < 2500$ Da) and very low molecular weight heparin ($M_w < 3500$ Da), which are also important pharmaceuticals [3].

LMWHs can be produced by controlled chemical or enzymatic breakdown of the heparin polymer [4]. Breddin et al. [5] have reported a LMWH standard prepared by partial nitrous acid depolymerization of heparin. Another study has shown that LMWH can be produced by degrading heparin with quaternary ammonium hydroxide in non-aqueous polar solvents [6]. Although chemical depolymerization is a mature method and has been widely used in industry, it has many disadvantages, including severe pollution to the environment and decrease in the pharmaceutical activity of LMWH due to the reactions of strong oxidants with sulfate groups in heparin molecules. Compared to chemical methods, enzymatic depolymerization has been paid more and more attention due to its mild reaction conditions, high selectivity and environment friendship. Nielsen [7] has successfully produced LMWH by enzymatic depolymerization of heparin with heparinase, and also pointed out that the relatively narrow molecular weight range of LMWH can make its pharmaceutical activity higher. Yu et al. [8] have also studied the production of heparin oligosaccharides from bovine lung heparin using heparinase.

Up to now, enzymatic depolymerization of heparin has been achieved by heparinase I (EC 4.2.2.7). This enzyme was purified from *Flavobacterium heparinum* by Yang et al. in 1985 [9], but the purification process is complicated and the cost is too high to allow its application in industrial production of LMWH. Moreover, it has been shown that recombinant heparinase I is prone

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to aggregate as insoluble inclusion body. Additionally, stability of heparinase I is also one of the concerns for the enzyme application. The enzyme when once being stored for short-term period at 4 °C or frozen once can retain only 50% and 25% of its initial activity, respectively [10], while the details on the inactivation are still unknown. In our previous work, we have constructed an efficient expression system to produce soluble and active heparinase I in recombinant *Escherichia coli* for the enzymatic preparation of LMWH by fusion to maltose-binding protein (MBP), and the fused protein (MBP–HepA) can be simply purified to homogeneity by one-step affinity separation on amylose resins [11]. By this approach, the active MBP–HepA can be produced at low cost and easily used to produce LMWH from heparin. Potentially, this fusion protein can also be directly immobilized by the affinity adsorption and thus reduce the cost for LMWH production.

In this study, we investigated the feasibility of heparin depolymerization using MBP–HepA to produce LMWHs. The thermostability of MBP–HepA was also examined and analyzed at different conditions.

2. Materials and methods

2.1. Materials

Heparin (M_n 22,370) was a gift from Changshan Biotechnology Corporation (Hebei, China). MBP–HepA was produced and purified according to the procedure previously established by Chen et al. [11]. LMWH standard (EPCRS M_n 3700) and ultrafiltration membranes (Microcon YM-10 10,000 NMWL and Microcon YM-50 50,000 NMWL) were purchased from European Pharmocopcia and Millipore, respectively. All other reagents were of commercial grade.

2.2. Enzyme activity assay

MBP–HepA activity was measured by a UV spectrophotometer. The enzymatic reaction was carried out using heparin as substrate in Tris–HCl buffer (pH 7.4) (containing 25 g L^{-1} heparin, 40 mM NaCl, 3.5 mM CaCl$_2$ and 17 mM Tris–HCl). Degradation of heparin was detected by UV absorbance at 232 nm and the activity was calculated using a molar absorption coefficient $\varepsilon = 3800\,M^{-1}\,cm^{-1}$. One international unit (IU) was defined as the amount of protein which can form 1 μmol unsaturated uronic acid per minute at 30 °C [12].

2.3. Production of LMWHs by MBP–HepA

The LMWHs were produced through controlled degradation of original heparin by MBP–HepA. Briefly, 5 μL purified MBP–HepA (specific enzyme activity was 7.5 IU mg^{-1} and the concentration of enzyme was 0.48 mg mL^{-1}) was added to 450 μL ammonium acetate buffer (pH 7.0) containing 100 mM NH$_4$COOCH$_3$, 25 mg mL^{-1} heparin and 3.5 mM Ca(CH$_3$COO)$_2$. The mixture with a final volume of 500 μL was incubated at 15 °C for different reaction time. The reaction was monitored by UV absorbance at 235 nm (A_{235}). When A_{235}

reached predetermined values, the samples were withdrawn and boiled at 100 °C for 2 min to inactivate the enzyme. The treated samples were stored for further analysis of the molecular weight.

2.4. Measurement of molecular weight of heparin during depolymerization

The weight average molecular weight (M_w), number average molecular weight (M_n) and polydispersity index (D) of LMWH obtained by the above reaction were analyzed by a gel permeation chromatography (GPC) which is reported elsewhere [13,14]. For this analysis, a HPLC system (Shimazu Co., Japan) consisting of a computer control, a pump (LC-10ATvp), an autoinjector (SIL-10ADvp), a RI (RID-10A) and UV detectors (SPD-M10Avp) was used. The GPC column was TSK Gel G3000SW column (30 mm × 750 mm, Tosoh Co., Japan). The UV and RI detectors were linked in series in the order of the column, the UV detector and the RI detector. LMWH standard solution was prepared by dissolving in 2.84% Na$_2$SO$_4$ (pH 5.0) (the mobile phase) to give a final concentration of 10 mg mL^{-1}. The flow rate of the mobile phase was set at 0.5 mL min^{-1} and the UV detection was done at 234 nm.

2.5. Thermostability of MBP–HepA

Stability of MBP–HepA was examined at different protein concentrations and temperatures. MBP–HepA in Tris–HCl buffer (the specific enzyme activity was 16.1 IU mg^{-1} and the enzyme concentration was 0.6 mg mL^{-1}) was used for this experiment. The enzyme solutions were kept at predetermined temperatures for different time periods and then 50 μL aliquots were taken and immediately cooled on ice for 5 min prior to residual activity assay. A previous experiment for preserving the MBP–HepA solution at 4 °C confirmed that the fused heparinase I activity could be completely retained for at least a few days.

2.6. Change in MBP–HepA size during the thermal inactivation

To examine the inactivation mechanism of MBP–HepA, changes in molecular size of the fusion enzyme were analyzed by GPC with TSK G4000SW$_{XL}$ column (30 mm × 750 mm, Tosoh Co., Japan) and dynamic light scattering (DLS) instrument (DynaPro DLS with a laser of 781.8 nm wavelength). Prior to GPC analysis, MBP–HepA in Tris–HCl buffer (200 mM NaCl, 20 mM Tris–HCl, pH 7.4) (specific enzyme activity was 9.9 IU mg^{-1} and the concentration of enzyme was 0.2 mg mL^{-1}) before and after the preservation at certain temperatures for a predetermined time was treated by UF membrane with cut-off molecular weight of 50,000 Da to remove the low molecular weight substances possibly present in the initial enzyme solution. The flow rate of the eluting buffer (0.1 M H$_3$PO$_4$, 0.1 M Na$_3$PO$_4$, pH 6.5) was set at 0.5 mL min^{-1} and UV detector was set at 280 nm.

For DLS analysis, the fluctuations in light intensity dependent on the Brownian motion of the molecules were measured by a

photodiode at 90°. Before DLS analysis, the enzyme solution before and after the heat treatment was centrifuged at $12,000 \times g$ for 15 min at 4 °C.

3. Results and discussion

3.1. Preparation of LMWHs by MBP–HepA

Heparin depolymerization by MBP–HepA was carried out at 15 °C for the preparation of LMWHs throughout the experiment since our previous results showed that the fused enzyme was unstable at above 25 °C, while retained the full activity below 15 °C for at least 58 h (data not shown).

After 0.5 h and 3 h degradation of heparin by MBP–HepA, the A_{232} value, reflecting the formation of LMWHs, reached 0.20 and 0.41, respectively. The profiles of the molecular weight distribution of heparin-degraded products are shown in Fig. 1A and B. With the reaction time elapsing, the peak area of the lower molecular weight products (longer retention time) increased. Changes in the relative area of the various oligosaccharides indi-

cated by different peaks in Fig. 1A and B are shown in Fig. 1C. Obviously, some oligosaccharides with higher molecular weight (peak 1) were produced early during the degradation of heparin, but were then gradually transformed into lower molecular weight oligosaccharides (such as peak 6) with the reaction progressing. This result indicated that the reaction time was one of the key factors in controlling the molecular weight of the products at a certain enzyme concentration.

In order to examine the feasibility of LMWH production with narrow polydispersity, the depolymerized mixtures at different reaction time of 0.5 h, 4 h, 11 h, 24 h, 33 h and 48 h were collected, respectively, followed by UF filtration with cut-off molecular weight of 10,000 to remove the enzyme, high molecular weight heparin and other impurities prior to the GPC analysis. The chromatograms of the filtrates are shown in Fig. 2. Heparin was obviously degraded into LMWHs with the enzymatic reaction progressing. Only three main components appeared at 48 h when the enzyme depolymerization was completed (Fig. 2B). Based on the results shown in Fig. 2B, all the parameters (M_w, M_n and D) of the depolymerized mixtures at the different reac-
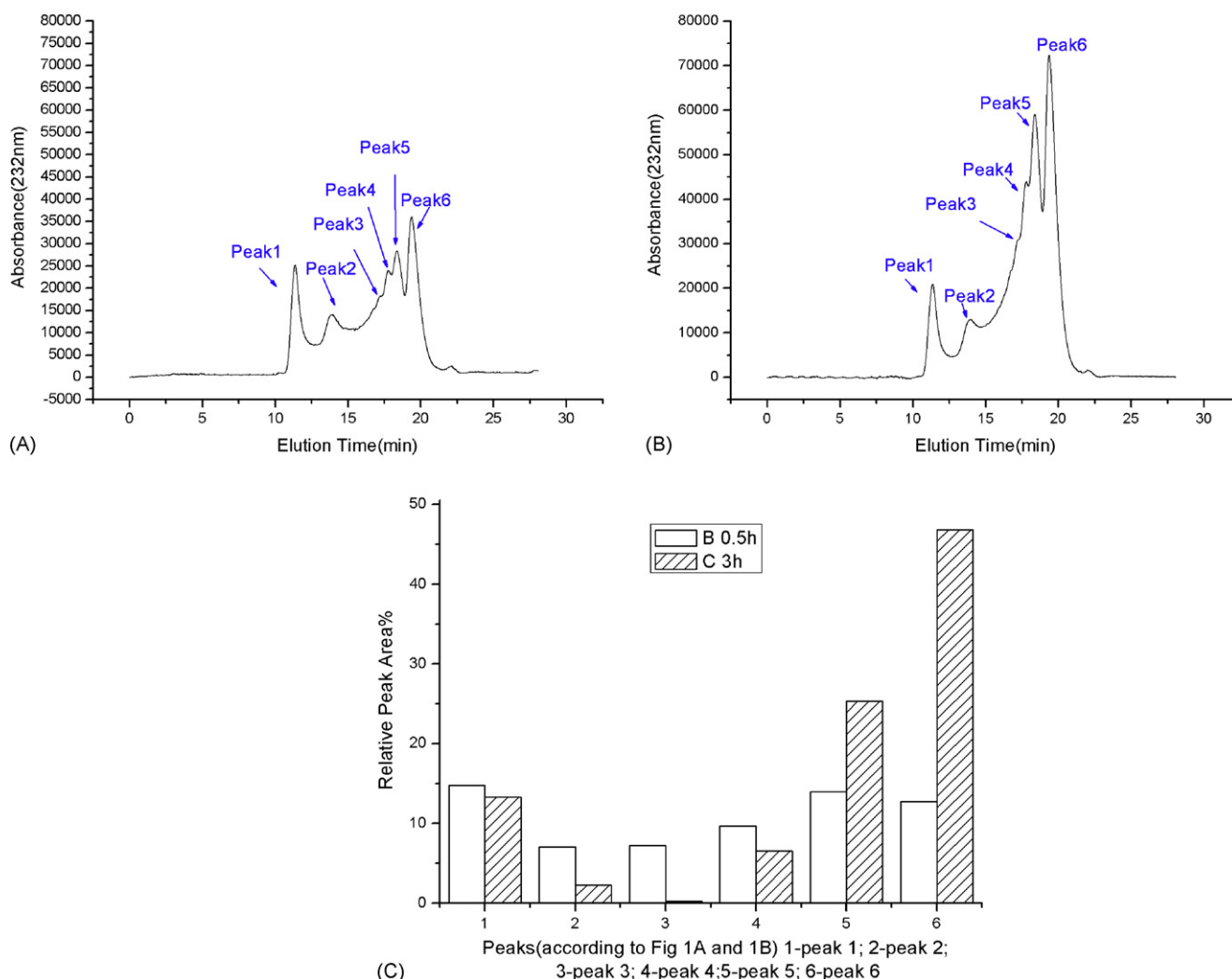

Fig. 1. LMWHs formed during the MBP–HepA catalyzed depolymerization of heparin in 500 μL depolymerization buffer: (A) chromatograms at the depolymerization time 0.5 h; (B) chromatograms at the depolymerization time 3 h; (C) changes in relative peak areas after different reaction time.
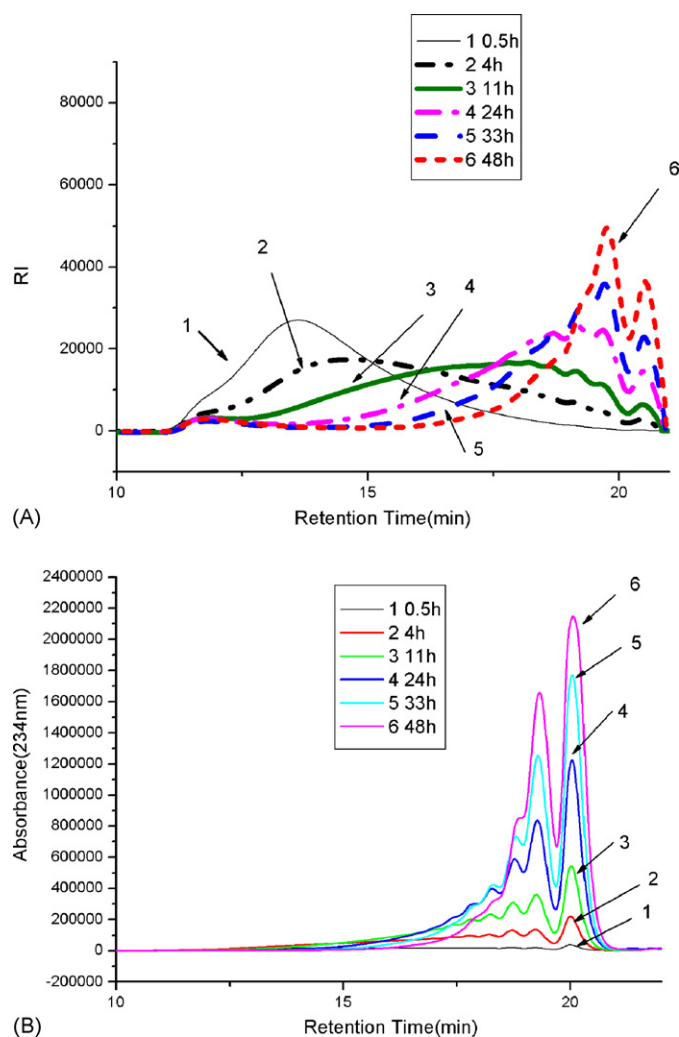
Fig. 2. Chromatograms of depolymerized heparin after different reaction times: (A) RI chromatograms and (B) UV chromatograms. The numbers 1–6: reaction time of 0.5 h, 4 h, 11 h, 24 h, 33 h and 48 h, respectively.

tion times were obtained (Table 1). The M_w of the products after 33 h and 48 h reaction time was calculated to be 2767 Da and 1871 Da, respectively, and their respective polydispersities were 1.7 and 1.4. This result indicated that LMWH and ultra low molecular heparin with a narrow polydispersity could be produced by controlling the reaction time.

In the depolymerization by the non-fusion heparinase I with a total activity of 62.8×10^{-3} IU (while it was 18.0×10^{-3} IU in our study) at 30 °C reported by Nielsen [7], LMWH with the average M_w of 1405 was formed after 22 h reaction time.

Table 1
Comparison of M_n, M_w and D of the depolymerized mixtures obtained at different reaction time

	1[a]	2[a]	3[a]	4[a]	5[a]	6[a]
Reaction time (h)	0.5	4	11	24	33	48
M_w	33799	20893	10055	8301	2767	1871
M_n	14674	6117	3434	2289	1646	1315
D	2.3	2.9	2.9	3.6	1.7	1.4

[a] Line no. in Fig. 1.

However, the reaction temperature (15 °C) and the total enzyme activity used in our study were much lower, which led to the longer reaction time. From the above discussion and the fact that MBP–HepA with high activity can be easily produced by recombinant *E. coli* and its affinity separation can be easily realized [10], we concluded that MBP–HepA can be a useful enzyme for the production of desired LMWHs.

3.2. Thermostability of MBP–HepA

The stability of MBP–HepA at 30 °C, 35 °C and 45 °C (pH 7.4) was examined (Fig. 3A). The inactivation rate of MBP–HepA was accelerated with the increase of temperature. The half-life of the MBP–HepA at 30 °C, 35 °C and 45 °C was about 10 min, 3 min and 1.3 min, respectively. These results showed that the stability of the fused MBP–HepA decreased with the increase of temperature, which was similar to other heparinases [10]. However, the fused enzyme used in our study was rather stable at 15 °C. The enzymatic activity could be maintained completely when stored at 15 °C even for more than 58 h (data not shown).

Even though MBP–HepA was inactivated at 45 °C by the first-order kinetics, the inactivation of MBP–HepA at 30 °C and 35 °C (pH 7.4) clearly displayed a non-first-order kinetics (Fig. 3A). By changing the initial concentrations of MBP–HepA, the enzyme inactivation profiles at 35 °C (pH 7.4) were also obtained (Fig. 3B). The inactivation rate was obviously dependent on the initial concentration of MBP–HepA, and the rapid inactivation of MBP–HepA was observed at the high concentration, also indicating the non-first-order kinetics pattern. By curve-fitting with the experimental data shown in Fig. 3A, the inactivation of MBP–HepA at 30 °C and 35 °C was found to be expressed by third-order kinetics (Fig. 3C).

3.3. Change in MBP–HepA size during thermal inactivation

The changes in molecular size of MBP–HepA during thermal inactivation were examined by GPC (Fig. 4). The heat treatment interestingly appeared to decrease the molecular weight of MBP–HepA. Many studies have shown that the heat treatment will lead to the conformational changes for many proteins [15,16]. Generally, an enzyme at a high temperature can gradually become unfolded or denatured. Once the enzyme has been denatured, the exposed hydrophobic surfaces tend to avoid interaction with the aqueous solvent, then the unfolded enzyme become insoluble and form aggregates [17,18]. It is known that all enzymes have to be in higher order structures such as quaternary state for the catalytic activity, and the dissociation to either dimmer or monomer will inevitably lead to the inactivation [16]. Lin et al. [16] have reported that the thermal inactivation of D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is caused by the enzyme dissociation followed by its aggregation.

As the crystal structure of heparinase I has not been reported until now, it is difficult to predict the structure and the initial form of MBP–HepA before the thermal inactivation. However, Song et al. [19] have fused MBP to PAZ (one of the domains of
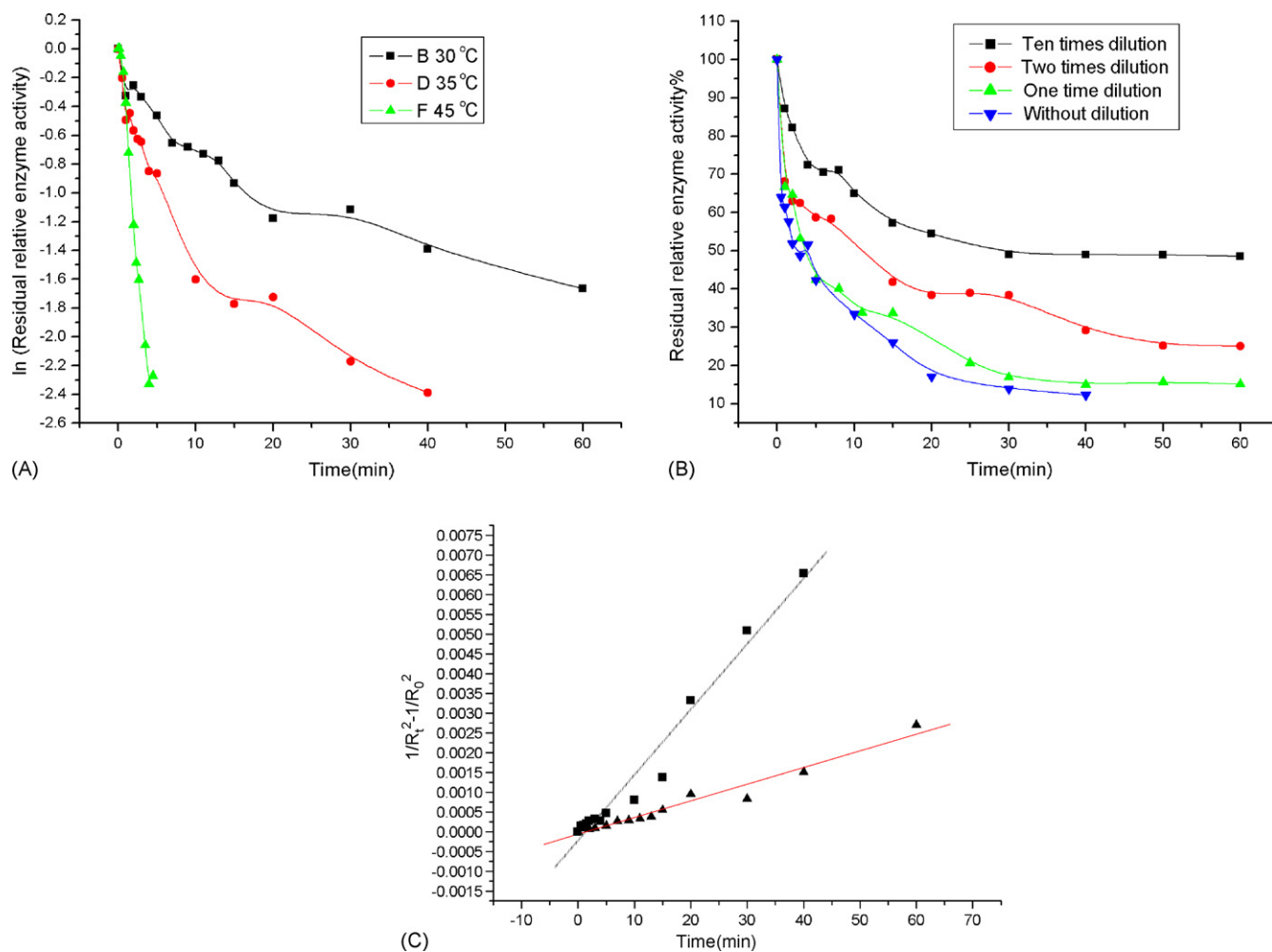
Fig. 3. Thermostability of MBP–HepA at different temperatures (A) and different initial fusion protein concentrations (35 °C) (B). Curve-fitting of the MBP–HepA inactivation data at 30 °C and 35 °C which in (A) was carried out by a third-order kinetics ($dR/dt = k_d R^3$) (▲, 30 °C, ■, 35 °C) (C), where R_t and R_0 represent the enzyme residual relative activity at time t and time 0, respectively.
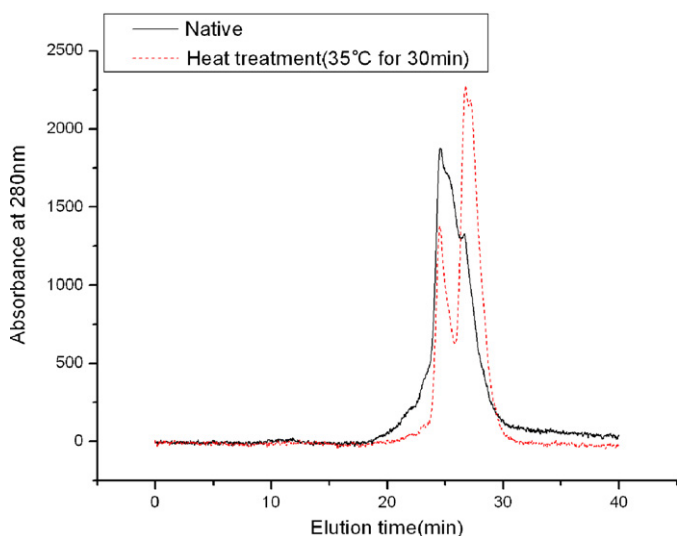

Fig. 4. GPC profiles of MBP–HepA before and after thermal deactivation.

argonaute proteins) using the modified pMAL_c2x vector which is the same as we used. The crystal structure of MBP–PAZ has indicated that the active form of this fused enzyme is a trimer with three maltose binding protein associated together and three PAZ arrayed toward outside, giving pairwise superposition rms deviations of 2.16–4.22 Å. Based on this report, we presumed that the dissociation of the oligomeric MBP–HepA might have occurred by the heat treatment under the conditions in our study, leading to the non-first-order inactivation of the fusion protein as described above and decrease in the molecular weight.

The dissociation of MBP–HepA at the high temperature was also supported by using DLS analysis (Table 2). After 60 min pretreatment of MBP–HepA solution at 30 °C (pH 7.4), the average diameter of the fusion protein in solution decreased, probably pertaining to the formation of lower molecular weight protein. However, at the initial stage (0–30 min), the average size of the enzyme solution changed slightly, while the relative enzyme activity decreased significantly. It should be pointed out that the average size of initial MBP–HepA of 38.20 nm seemed too large even taking the MBP–HepA oligomer into consideration. The reason still remained unclear. Presumably, it was

Table 2
Changes in the average diameters of MBP–HepA under different conditions

Conditions	Incubation time (min) (30 °C, pH 7.4)				SDS ($10\,\mathrm{g\,L^{-1}}$) treatment
	0	10	30	60	
Average diameter (nm)	38.20	39.27	39.96	28.89	7.76
Residual relative activity (%)	100	67.59	35.18	4.45	2.41

attributed to the denaturation of some enzyme molecules caused during the purification process. So the original enzyme solution of MBP–HepA might have been the mixture of the active oligomer and probably inactive MBP–HepA aggregates. Incidentally, the enzyme solution remained clear with no appearance of turbidity under all the conditions in the experiment, indicating absence of protein precipitation.

In addition, the average diameter of the enzyme solution decreased dramatically (from 38.20 nm to 7.72 nm) when $10\,\mathrm{g\,L^{-1}}$ anionic detergent SDS was added to the MBP–HepA solution, indicating that SDS significantly dispersed the MBP–HepA oligomer or the aggregates already formed in initial enzyme solution to reduce the fusion protein size. Liu et al. [20] have compared the mechanisms of aggregation of α-amylase and β-amylase in aqueous solutions by SDS treatment, and found that average diameter of α-amylase aggregates can be reduced from 40 nm to 1.0 nm by adding SDS at less than $10\,\mathrm{g\,L^{-1}}$. As a strong cationic surfactant, SDS can denature the structures of proteins by breaking the interactions and by interfering hydrophobic interactions among the protein molecules. SDS is also capable of binding to the proteins' backbones and imparting a negative charge to the proteins such as β-amylase [19], whose electrostatic repulsion can destroy the aggregates.

From the above discussion, the inactivation process of MBP–HepA seemed rather complicated, both the dissociation and aggregation of the fusion protein could cause the inactivation of the enzyme during the heat treatment. Even the dissociation and aggregation of a protein will affect the change in the protein sizes during the thermal inactivation, the dissociation of MBP–HepA in the present study might have played a main role in the inactivation of the fusion enzyme under the conditions examined.

4. Conclusions

MBP–HepA produced by recombinant *E. coli* enabled heparin to be effectively degraded into LMWH. Our result suggested that the depolymerization time in the bioreaction system was a key factor to obtain the desired LMWH with weight average molecular weight of less than 3000 Da and narrow polydispersity.

The thermal inactivation of MBP–HepA activity at the temperature less than 45 °C was expressed by a non-first-order reaction kinetics, implying that the dissociation of the oligomeric enzyme presumably pertained to the inactivation process. Improvement of the thermostability of MBP–HepA can thus be expected by using some chemicals such as trehalose or polymers, which is being investigated now.

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