Carbohydrate Polymers 90 (2012) 348–352

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Expression of the heparinase gene from *Flavobacterium heparinum* in *Escherichia coli* and its enzymatic properties

Ping Yu*, Yan Wu

College of Food Science and Biotechnology, Zhejiang Gongshang University, 149 Jiaogong Road, Hangzhou 310035, People's Republic of China

ARTICLE INFO

Article history:
Received 29 March 2012
Received in revised form 15 May 2012
Accepted 16 May 2012
Available online 26 May 2012

Keywords:
Heparinase
Expression
Purification
Escherichia coli
Enzymatic properties

ABSTRACT

Heparinase has an important application in the preparation of low-molecular-weight heparins by heparin enzymolysis. A heparinase gene from *Flavobacterium heparinum* was cloned and expressed in *Escherichia coli* BL21 in order to enhance its activity. The expressed heparinase was purified to homogeneity by a metal chelating affinity column and its enzymatic properties were evaluated. A maximal heparinase activity of 1061 IU/L toward the substrate heparin was achieved when the recombinant strain was induced with 0.5 mM isopropyl-β-D-thiogalactoside at 28 °C for 9 h. The optimal temperature and pH of heparinase were 30 °C and 7.0, respectively. The recombinant heparinase was heat-unstable and had a higher stability at pHs from 7.0 to 10.0. Observed activities of heparinase were the highest in the presence of Ca^{2+} and Cu^{2+} and the lowest in the presence of Mn^{2+} and Pb^{2+}. These results lay a good foundation for the preparation of LMWHs by heparin enzymolysis.

1. Introduction

Heparin, a class of glycosaminoglycans characterized by a linear polysaccharide with repeating units of D-glucosamine, is used widely as an injectable anticoagulant to treat and prevent thrombosis (Banga & Tripathi, 2009; Ernst et al., 1996; Shpigel et al., 1999; Silva & Dietrich, 1975). However, it has some side effects. For example, it often affects the stability of platelet (Chen, Ye, Kuang, & Xing, 2007; Coppell et al., 2006). Moreover, its true physiological roles remain somewhat unclear in the body until today (Chen et al., 2007; Hyun, Lee, & Kim, 2010). Low-molecular-weight heparins (LMWHs), degradable products of the heparin through the depolymerization reaction, can also be used as an injectable anticoagulant without side effects, and so heparin is replaced gradually by LMWHs that become a preferred drug in the clinical application (Durila, Kalincik, Cvachovec, & Filho, 2010; Weitz, 1997).

Low-molecular-weight heparins can be prepared by heparin depolymerization with two ways: chemical and enzymatic methods (Linhardt & Gunay, 1999; Shaya et al., 2010; Shi, Ji, Chi, & Zhang, 2003). Although the chemical depolymerization to produce LMWHs is a mature industrialized method, it has some disadvantages, such as heavy environmental pollution and low pharmaceutical activity because sulfate groups are often oxidized during this process (Shi et al., 2003). Therefore, the enzymatic depolymerization to produce LMWHs has been given an increasing attention due to its excellent reaction conditions, such as a high rate and selectivity, a low energy requirement, the moderate interaction with the substrate and the environmental friendship, etc. (Linhardt & Gunay, 1999; Weitz, 1997). LMWHs can be prepared by heparinase digestion. Three heparinases have been separated and purified from *Flavobacterium heparinum* so far. Isolation, purification and function of these heparinases have been studied in detailed (Kim, Kim, Kim, Linhardt, & Kim, 2000; Lohse & Linhardt, 1992; Watanabe et al., 1998). At present, the commercial heparinase is mainly from *F. heparinum* (Chen et al., 2007). The yield of heparinase from this strain, however, is still low (Galliher, Cooney, Langer, & Linhardt, 1981; Sasisekharan, Moses, Nugent, Cooney, & Langer, 1994; Silva & Dietrich, 1975; Su et al., 1996). Moreover, the addition of heparin is essential for the production of the heparinase by this strain and this makes the production cost too high (Bohmer, Pitout, Steyn, & Visser, 1990; Lohse & Linhardt, 1992). It is therefore very interesting and attractive to increase the yield of heparinase and investigate its enzymatic properties in order to better prepare LMWHs by enzymatic depolymerization.

In the present study, a heparinase gene from *F. heparinum* was cloned and overexpressed in *Escherichia coli* BL21. The expressed heparinase with a 6× His-tag fusion at its N-terminal was purified to homogeneity by a Hi-Trap metal chelating affinity column loaded with 100 mM nickel sulfate. Enzymatic properties of the expressed heparinase and the effect of metal ions on its activity were also investigated.

* Corresponding author. Tel.: +86 571 88071024; fax: +86 571 28891365.
E-mail address: yup9202@yahoo.com.cn (P. Yu).

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http://dx.doi.org/10.1016/j.carbpol.2012.05.050

2. Materials and methods

2.1. Strains, plasmids and reagents

The strain *F. heparinum* DSM 2366 was purchased from Germany Collection of Microorganisms and Cell Cultures. The strain *E. coli* BL21 (DE3) and the plasmid pET-28a (+) were purchased from the Novagen Co. Ltd and used as the host strain and the primitive expression vector, respectively. PCR reagents, restriction endonucleases, the Hi-Trap metal chelating affinity column and the Bacterial DNA extraction kit were purchased from the TaKaRa Biotech Co. Ltd, Japan. Kanamycin and heparin were purchased from the Sigma, Co. Ltd. All other reagents were analytical grade and used as the routine method.

2.2. Construction of the plasmid pET-Hep and its transformation into E. coli BL21

The strain *F. heparinum* was inoculated into a B_1 medium (10 g/L peptone, 5 g/L beef extract, 5 g/L yeast extract, 5 g/L NaCl, pH 6.5) and grew with a vigorous agitation at 30 °C for 3 d. Cells were harvested by centrifugation at 12,000 rpm for 10 min. The genomic DNA from this strain was isolated using the Bacterial DNA extraction kit. The primer set, F_1 5′-TAGAATTCCAGCA A AAAAAATCCG-3′ and R_1 5′-GCAAGCTTGTCTGGCAGTTTCGCTGTA-3′, was designed for the amplification of the heparinase gene with the genomic DNA as the template. Restriction sites *Eco*RI and *Hind* III were added to the upstream from the primer F_1 and the downstream from the primer R_1, respectively. PCR conditions consisted of an initial denaturation at 94 °C for 5 min, 35 cycles of the amplification consisted of the denaturation at 94 °C for 1 min, annealing at 58 °C for 1.5 min, and extension at 72 °C for 1 min. Then the further extension at 72 °C was performed for 10 min. The resultant PCR products were purified, digested with *Eco*RI and *Hind* III, and ligated into the *Eco*RI–*Hind* III digested vector pET-28a (+) to construct the recombinant plasmid pET-Hep. The resultant plasmid pET-Hep was transformed into the *E. coli* strain DH5α by $CaCl_2$-heat shock method (Sambrook, Fritsch, & Maniatis, 1989). The recombinant plasmid was isolated from the positive transformant using a High Pure Plasmid Isolation Kit (Roche, Germany). The presence and the correct orientation of the insert sequence were confirmed by PCR and DNA sequencing.

The plasmid having a correct insert sequence was transformed into the *E. coli* BL21 competent cells by $CaCl_2$-heat shock method (Sambrook et al., 1989). Cells were streaked onto LB plates containing 50 μg/mL of kanamycin and incubated at 37 °C until the single colony appeared. Single colony from culture plates was picked up separately with a sterile toothpick and resuspended into LB media containing 50 μg/mL of kanamycin and incubated at 37 °C until OD_{600} reached 0.6. 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) was added to induce the expression of the heparinase gene. At the induced stage, the culture temperature was set as 28 °C.

2.3. SDS-PAGE analysis of the heparinase expression

Cell cultures were taken after being induced for 0, 3, 5, 7 and 9 h and centrifuged at 12,000 rpm for 20 min to obtain the cells. Cells were resuspended in a 100 μL Na_2HPO_4–NaH_2PO_4 buffer (0.025 M, pH 6.8) for being subject to the ultrasonication treatment (work 6 s, pause 6 s, 300 W) to obtain the supernatant. 20 μL of the supernatant was taken for the SDS-PAGE analysis. The SDS-PAGE analysis was performed on a 15% running gel. Resolved proteins were visualized by staining with Coomassie Brilliant Blue R250.

2.4. Heparinase purification

Cells having the highest heparinase activity were cultured in a LB medium (50 μg/mL kanamycin) at 37 °C until OD_{600} reached 0.6. 0.5 mM IPTG was added to the cultures and cells were cultured for another 9 h at 28 °C with a vigorous agitation. Cultures were centrifuged at 12,000 rpm for 20 min and resuspended in a 5 mL of the precooled buffer (20 mM Tris, 500 mM NaCl, 50 mM imidazole, pH 8.0), followed by the ultrasonication treatment (work 6 s, pause 6 s, 300 W) for 15 min to disrupt the cells. After the cells were centrifuged for 20 min at 12,000 rpm at 4 °C, the supernatant was filtered using a 0.45 μm filter membrane and then loaded onto a Hi-Trap metal chelating affinity column loaded with 100 mM nickel sulfate. The column was eluted with a buffer (20 mM Tris, 500 mM NaCl, 50 mM imidazole, pH 8.0) to obtain the purified heparinase (His-hepA). The purity of heparinase was detected by SDS-PAGE analysis. The analysis of the heparinase activity was performed according to the method as described by Lohse and Linhardt (1992). The relative activity was calculated as the ratio of the heparinase activity under a specific condition to that of the control sample.

2.5. Enzymatic properties of heparinase

Optimal temperatures for the heparinase activity were investigated by incubating 0.5 mL reaction mixture for 10 min. Reaction mixtures contained a 0.2 mL of 20 mM phosphate buffer (pH 7.0), 0.2 mL heparin as the substrate and 0.1 mL of the enzyme, over the range of 15–50 °C. The effect of pH on the heparinase activity was studied using 0.5 mL reaction mixture containing a 0.2 mL of the buffer and 0.2 mL heparin as the substrate at 37 °C for 10 min. Buffers used were a 20 mM phosphate buffer pH 6–7.5 and a 20 mM Tris–HCl buffer pH 7.5–8.5. To study the heparinase stability, enzyme solutions were preincubated for 30 min at various temperatures from 15 to 75 °C and pHs from 3 to 12 without the substrate, and were immediately cooled to 4 °C. The residual activity of heparinase was determined and the relative activity was calculated. To investigate the effect of metal ions on the activity of heparinase, the enzyme was preincubated with various metal ions dissolved in a 20 mM phosphate buffer pH 7.0 at 37 °C for 30 min. The residual activity was then measured. Controls were enzyme solutions without metal ions.

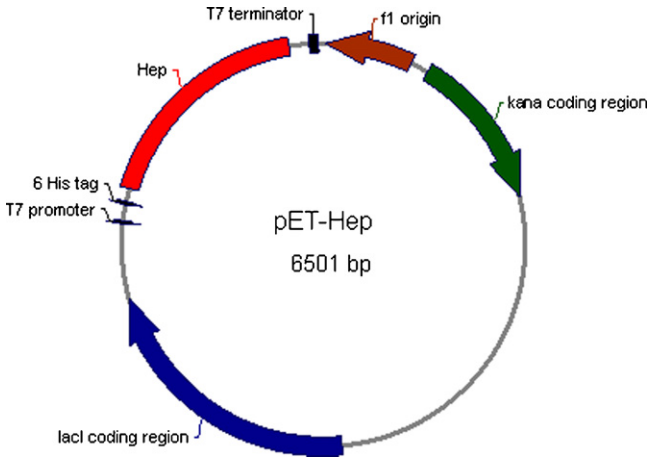

Fig. 1. The schematic map of the constructed expression vector pET-Hep bearing a heparinase gene sequence from *F. heparinum*. 6 His tag: 6× His tag coding region, Hep: heparinase gene sequence, Kan coding region: kanamycin coding sequence, *Lac*I coding region: *Lac*I coding sequence.

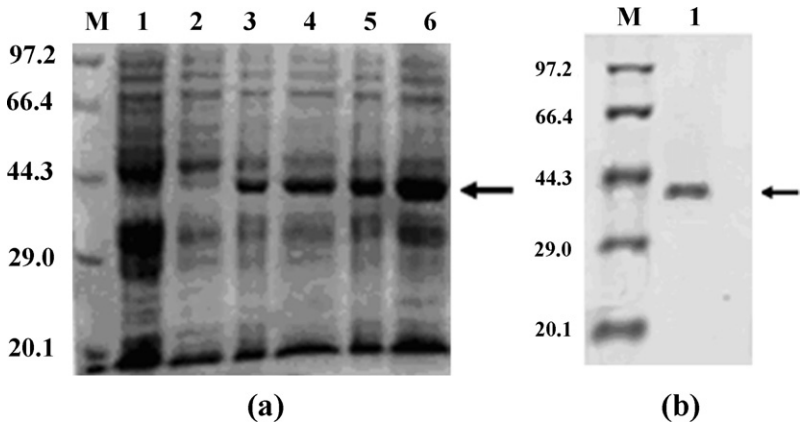

Fig. 2. (a) The SDS-PAGE analysis of the expression of the heparinase in *E. coli* BL21. M: protein molecular weight marker; lane 1: the control strain transformed with the primitive plasmid pET-28a (+); lane 2–6: the recombinant strains transformed with the expression plasmid pET-Hep. Samples were taken after the induction with 0.5 mM IPTG for 0 (lane 2), 3(lane 3), 5 (lane 4), 7 (lane 5) and 9 h (lane 6). The expressed heparinase is indicated by an arrow. (b) The purification result of the recombinant heparinase by a Hi-Trap metal chelating affinity column loaded with 100 mM nickel sulfate.

3. Results and discussion

3.1. Construction of the expression vector pET-Hep

A PCR product was amplified with the genomic DNA from *F. heparinum* as the template. The PCR product was purified, digested with *Eco*RI and *Hind* III, and ligated into the *Eco*RI–*Hind* III digested pET28a (+) vector to construct the recombinant plasmid pET-Hep. The correct clone was confirmed by PCR amplification. The recombinant plasmid was also confirmed by the restrict digestion with *Eco*RI and *Hind* III and DNA sequencing. All these results indicate that the expression vector pET-Hep is constructed successfully (Fig. 1). In order to enhance the soluble expression of the heparinase in the periplasmic space of *E. coli* and purify this enzyme more

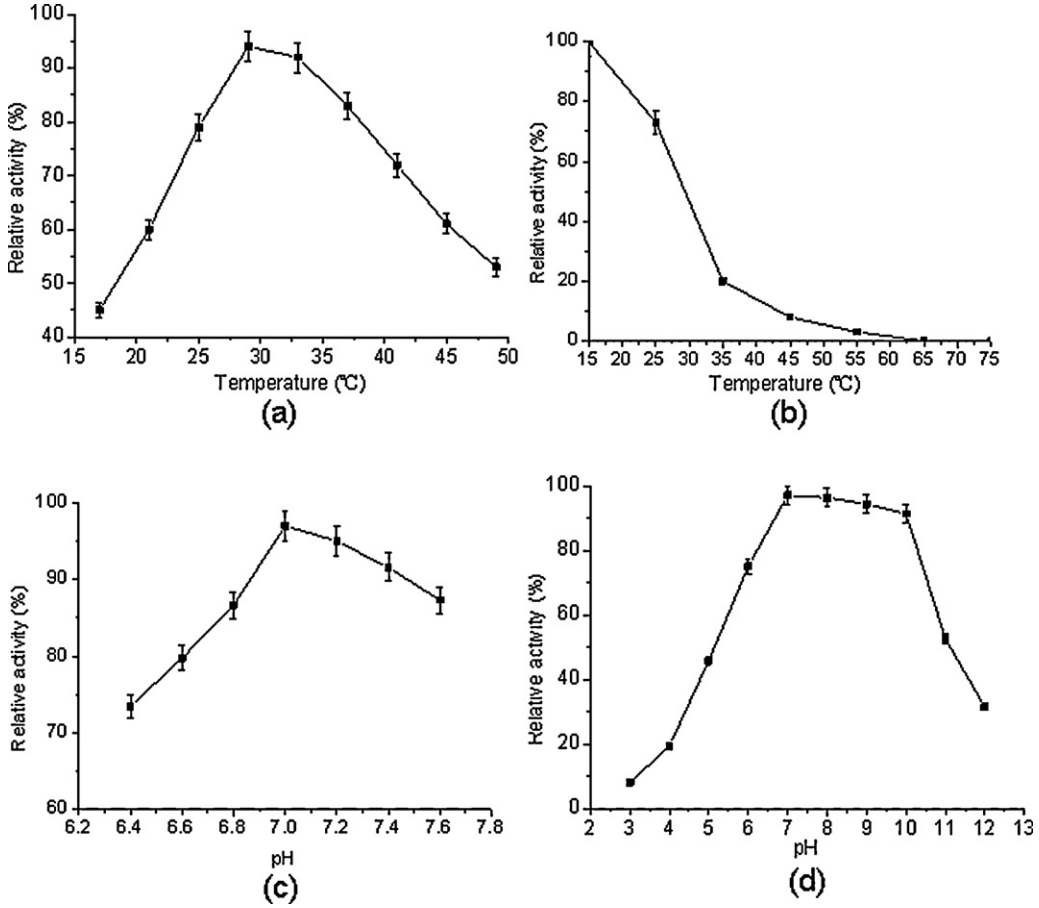

Fig. 3. The enzymatic properties of the recombinant heparinase. (a) The impact of temperature on the activity of the heparinase. (b) The impact of temperature on the stability of the heparinase. (c) The impact of pH on the activity of the heparinase. (d) The impact of pH on the stability of the heparinase.

Table 1
The effect of metal ions on the activity of the heparinase.

Metal ions	Relative activity (%)
Control	100
Ba^{2+}	98
Ca^{2+}	161
Zn^{2+}	93
Mg^{2+}	104
Mn^{2+}	39
Pb^{2+}	36
Cu^{2+}	127
Fe^{2+}	94

easily, its DNA sequence was designed to fuse to the downstream of 6× His-tag of N-terminal of the pET28a (+) vector when the plasmid pET-Hep was constructed.

3.2. Expression and purification of the heparinase

The expression analysis of the heparinase in the recombinant cells is presented in Fig. 2(a). A significant band with an estimated molecular weight of 43 kDa was presented after SDS-PAGE compared to the control sample. It was also found that with the increase of the induction time, the activity of the expressed heparinase increased and a maximal heparinase activity of 1061 IU/L toward the substrate heparin was achieved after the recombinant strain was induced at 28 °C for 9 h. This activity was about 1.7-fold higher than that from the control train *F. heparinum* (Lohse & Linhardt, 1992). The heparinase was purified by a metal chelating affinity column and the result is shown in Fig. 2(b). Only a single protein band was shown in the SDS-PAGE gel. This indicates that the heparinase obtained is electrophoretically pure and may be used for the analysis of its enzymatic properties.

3.3. Enzymatic properties of the heparinase

The activity of the heparinase was determined at different temperatures ranging from 15 to 50 °C and the result is presented in Fig. 3(a). The variation of the activity of the purified heparinase at different temperatures presented an inverted U-shape with the increase of temperatures. The optimal temperature of it was found to be 30 °C, which was similar to those of some other ones (Kuang et al., 2006). The thermal stability of the purified enzyme was determined and the result is presented in Fig. 3(b). It was found that the recombinant heparinase was heat-unstable. The enzymatic activity decreased dramatically with the increase of temperatures (from 15 to 75 °C). Only about 20% of the original enzymatic activity was kept at 35 °C. The enzymatic activity lost completely by the treatment at 65 °C for 30 min. The effect of pH on the enzymatic activity is presented in Fig. 3(c). The optimal pH of this enzyme was found to be 7.0. The heparinase had a relatively higher activity in alkali solutions than acidic solutions. The pH stability of heparinase is presented in Fig. 3(d). It was found that the heparinase had a higher stability at pHs from 7.0 to 10.0 than other pHs. Metal ions can bind strongly with the sulfhydryl group of cysteine in the molecule of enzyme and this can change structures and activities of enzymes (Coolbear, Whittaker, & Daniel, 1992). The effect of metal ions on the activity of heparinase is presented in Table 1. Observed activities of the recombinant heparinase were the highest in the presence of Ca^{2+} and Cu^{2+} and the lowest in the presence of Mn^{2+} and Pb^{2+}. The other metal ions used had little effect (within 10%) on the activity of the heparinase. This implies that the addition of Ca^{2+} and Cu^{2+} may decrease the dosage of the heparinase during its biocatalysis.

4. Conclusions

The results of the present research demonstrate that *E. coli* is a more convenient and excellent expression system for an efficient production of the heparinase. The expression vector bearing a heparinase gene from *F. heparinum* was constructed and transformed into the *E. coli* BL21 strain, and the recombinant strain which could express the heparinase effectively was screened successfully by the SDS-PAGE and the quantitative analysis of the heparinase activity. The activity of heparinase was up to 1061 IU/L after 9 h induction with 0.5 mM IPTG. The analysis of enzymatic properties indicated that the optimal temperature and pH were 30 °C and 7.0, respectively. The heparinase was heat-unstable and had a relatively higher stability at pHs ranging from 7.0 to 10.0. Ca^{2+} and Cu^{2+} were found to have a significant activating function to the activity of the heparinase. All these results lay a good foundation for the preparation of LMWHs by the heparin degradation using the heparinase. Continuous efforts should be given to further enhance the thermal stability of the heparinase by the enzymatic modification and prepare LMWHs with the recombinant heparinase.

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