Biochemical Engineering Journal 114 (2016) 237–243

Contents lists available at ScienceDirect

Biochemical Engineering Journal

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

Regular article

# Improving the activity of heparinase I by the directed evolution, its enzymatic properties and optimal conditions for heparin degrading by recombinant cells

Ping Yu*, Tianmei Jia, Yanxia Chen, Yunting Wu, Yishu Zhang

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

ARTICLE INFO

*Article history:*
Received 1 February 2016
Received in revised form 29 June 2016
Accepted 18 July 2016
Available online 19 July 2016

*Keywords:*
Directed evolution
Error-prone PCR
Heparinase I
Enzymatic properties
Heparin degradation
Recombinant cells

ABSTRACT

Heparinase I can degrade heparin and heparin sulfate to produce low molecular weight heparins (LMWHs) that are widely used as an anticoagulant and antithrombotic drug. In this study, directed evolution was used to improve the activity of heparinase I. The clone *E. coli*-heparinase-I133/P316 with two amino acid substitutions was screened and identified. This clone produced 366 U/L of heparinase I, which was 57.8% higher than that from the control clone (232 U/L). The optimal temperature of heparinase-I133/P316 was lower than that of the original heparinase I. The mutated enzyme and original one presented almost the same varying trend as an function of pH, and their optimal pH was near 7.5. $Ca^{2+}$ at 1 mM could significantly increase the activity of heparinase-I133/P316. $Cu^{2+}$, $Ni^{2+}$, $Mg^{2+}$, $Mn^{2+}$ and $Zn^{2+}$ at 1 mM were found to inhibit its activity, and $Ba^{2+}$ and $Fe^{2+}$ at 1 mM almost had no obvious effect on its activity. The optimal substrate concentration, temperature, pH, shaker speed and cell concentration for heparin degradation by *E. coli*-heparinase-I133/P316 were 125 μg/mL, 40 °C, 7.5, 400 rpm and 40 mg/mL, respectively. After reaction for 6 h, degradable products are basically concentrated in a small molecular weight region. This study provides a new insight into the high production of heparinase I and the preparation of LMWHs by *E. coli*-heparinase-I133/P316 cells.



## 1. Introduction

Heparinase I can degrade heparin and heparin sulfate to produce low molecular weight heparins (LMWHs) (Fig. 1), an anticoagulant and antithrombotic drug widely used in clinical practice [1]. Since heparinase I was firstly discovered from *Flavobacterium heparinum*, a lot of studies, including the cloning and expression of its encoding genes, its bioactivities, and its purification and characterization, have been made [2–5]. Heparinase I not only functions to detect and remove heparin [6,7], but also has other bioactivities, such as the inhibition of the invasion and metastasis of tumor cells [8,9], and the regional heparinization of blood [10–12]. It is also involved in inhibiting the tumor angiogenesis [13–15], which makes it become a very promising and useful target of the anti-tumor therapy in future [16,17]. Heparinase I has currently been prepared from several microorganisms, such as *F. heparinum*, *Bacillus circulans* and *Bacteroides stercoris* [18–20]. *F. heparinum* is a major commercial producer of heparinase I [21,22]. To date, however, it has been expensive due to its low yield from natural microorganisms.

Heparin is a highly sulphated glycosaminoglycan which comprises of β-1-4-linked hexuronic acid and glycosamine residues [23]. Although heparin has been widely used as an anticoagulant and antithrombotic drug in clinical practice for more than 70 years, many side effects, such as the hemorrhage and the reduction of hematoblasts, etc., cannot be avoided owning to its large molecular weight [24]. Compared to the unfractionated heparin, LMWHs not only retain the antithrombotic and other bioactivities, but also reduce or avoid above side effects [25]. Moreover, LMWHs have longer half-life, better bioavailability, higher safety and more convenience compared to heparin. These characters make them become more popular in the clinical application [26]. LMWHs can be prepared by the physical separation from natural organisms and the controlled lysis. Porcine intestinal mucosa is a good source for the physical separation of LMWHs [27,28], but the yield of LMWHs is so low that the large-scale use is limited. The controlled lysis includes the chemical lysis and the heparinase I degradation [29]. The former method is widely used in China [30]. Although the chemical lysis is relatively simple, it has many disadvantages,

* Corresponding author.
*E-mail address:* yup9202@hotmail.com (P. Yu).

http://dx.doi.org/10.1016/j.bej.2016.07.011
1369-703X/

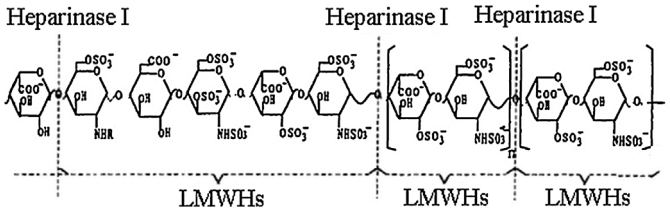


**Fig. 1.** Schematic diagram of the enzymatic degradation of heparin to LMWHs by heparinase I.

including the heavy environmental pollution and the low pharmaceutical activity of prepared LMWHs owning to the use of nitrite during the lysis. Moreover, sulfate groups of heparin, the important ones for keeping heparin bioactivities, are damaged owning to reactions of hypochlorous acid groups and strong oxidants used during the chemical lysis. Therefore, the heparinase degradation has been deserved more attention in recent ten years because it has temperate reaction conditions, high selectivity and environmental friendship, and is easy to achieve continuous production. Thus, LMWHs preparation by the heparinase degradation has become an ideal alternative for replacing the traditional chemical lysis for future use.

An efficient method for improving the enzymatic production is the use of the directed evolution technology. Error-prone PCR is an effective technology for the directed evolution. In the past ten years, a large number of enzyme-encoding genes have been cloned, and have been subject to the directed evolution for improving their activity and production [31–35]. Thus, it is interesting to investigate if the activity of heparinase I from *F. heparinum* can be improved by the directed evolution using the error-prone PCR, and if LMWHs can be effectively prepared by the heparinase degradation for avoiding the drawbacks of the chemical lysis.

In this study, the heparinase I from *F. heparinum* was subject to the directed evolution for improving its activity by the error-prone PCR, and enzymatic properties of the original heparinase and the mutated one were compared. Optimal conditions of the heparin degrading by the mutated heparinase I were also investigated. To the best of our knowledge, this is the first report regarding the directed evolution of heparinase I by the error-prone PCR.

## 2. Materials and methods

### 2.1. Bacterial strains and plasmids

*Escherichia coli* DH5α and BL21 (DE3) were used as the host for the amplification of vectors and the expression of heterologous genes, respectively. The clone *E. coli* BL21-pET-Hep containing the original heparinase I encoding gene (*hepI*) was constructed before in our laboratory, and was used as the control strain for comparing the heparinase I activity [32]. The plasmid pET-28a (+) under the control of $T_7$ promoter was purchased from the Novagen Co. Ltd, USA, and was used as the primitive expression vector. The plasmid pET-Hep from the clone *E. coli* BL21-pET-Hep was used as the template of the error-prone PCR for the directed evolution of heparinase I. PCR mutagenesis Kit containing TaqDNA polymerase, restriction endonucleases and $T_4$ DNA ligase were purchased from the TaKaRa, Co. Ltd, Japan. Isopropyl-β-D- thiogalactopyranoside (IPTG), peptone, yeast extract and beef extract were purchased from the BBI Co. Ltd, USA. The other reagents were of analytical grade, and were used as the routine method.

### 2.2. Construction of the error-prone PCR library

The plasmid pET-Hep was extracted from the clone *E. coli* BL21-pET-Hep constructed before [33], and was used as the template of the error-prone PCR. A primer set, $F_1$: 5′-TAGAATTC CAGCAAAAAAAATCCG-3′ and $R_1$: 5′-GGCAAGCTTGTCTGGCAGTTTCGCTGTA-3′, was designed for amplifying the gene encoding the heparinse I (*hpaI*) by the error-prone PCR. Underlined letters represent *Eco*R I and *Hin*d III-digested sites for the cloning purpose. The error-prone PCR was carried out by changing concentrations of dATP, dGTP (from 0 to 1.0 mM) and $MnCl_2$ (from 0 to 1.0 mM) according to the specification of the PCR mutagenesis Kit provided by manufacture. The error-prone PCR procedure 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. Resultant products were purified and digested with restriction endonucleases *Eco*RI and *Hin*dIII, and were then ligated into the plasmid pET-28a (+) digested with the same restriction endonucleases, giving the plasmid pET-HepI, which was then transformed into *E. coli* BL21 (DE3) supercompetent cells by the heat-shock method [34]. Cells were streaked onto LB plates containing 100 μg/mL of ampicillin, and were incubated at 37 °C until the single colony appeared, giving the mutant library of heparinase I.

### 2.3. Screening of the mutants

The single colony from the mutant library of heparinase I was separately picked up with a sterile toothpick, and was resuspended in each well of 96-well microtiter plates pre-filled with 150 μL of LB medium. Plates were incubated at 37 °C with vigorous shaking on a shaker until $OD_{600}$ reached 0.6, and then 0.5 mM IPTG was added to induce the production of heparinase I at 20 °C for 12 h. Cells were harvested by centrifugation at 3000 rpm and 4 °C for 30 min. Pellets were resuspended in a 150 μL of the nondenaturing lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Trixon X-100, 1% NP-40, 2 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM $Na_3VO_4$, 0.5 μg/mL leupeptin) at 37 °C for 2 h. The cell debris was removed by centrifugation at 16,000 rpm and 4 °C for 20 min for obtaining the supernatant. The activity of heparinase I was determined as described by Galliher et al. [21] with minor modification. The sample was centrifuged at 3000 × *g* for 10 min to remove cell pellets. 200 μL of the supernatant was mixed with 100 μL of the heparin solution (25 mg/mL in 0.25 M sodium acetate-0.0025 M calcium acetate at pH 7.0). The mixture was incubated at 30 °C in a water bath. At different time intervals, 10 μL of the mixture was taken out, and was added to 10 mL of 0.02 mg/mL Azure A dye solution. The dye showed a metachromatic shift from blue to red in the presence of heparin. The change in the optical density at 620 nm was measured within 1 h. The degrading amount of heparin can be obtained by the difference in the optical density at 620 nm. The activity of heparinase I was calculated as given below:

$$X = A \times 10^{-3} \times 0.25 \times 10^{6} \times \frac{1}{T}$$

where X is the activity of heparinase I (U/L), A is the degrading amount of heparin (mg) and T is the catalytic time (h).

One unit of the heparinase I activity is defined as the amount of enzyme that can degrade 1 mg heparin per hour at 30 °C and pH 7.0. The activity of heparinase I from the control clone *E. coli* BL21-pET-Hep was also determined according to the above procedure for comparison to those from the mutated clones. The transformant with the highest activity of heparinase I was screened, and was designated as *E. coli*-heparinase-I133/P316.