

Capsule Treatment by Soluble Expressed Recombinant Heparinase II Enhance the Foreign Gene Transformation to *Escherichia coli* K5

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In the present study, a recombinant *E. coli* BL21 strain containing heparinase II gene was utilized to express and purify soluble heparinase II. When incubated at a low culture temperature of 22°C and in an auto-induction medium, the recombinant *E. coli* BL21 could successfully enhance the express of heparinase II in soluble and active forms. The purified recombinant heparinase II could eliminate heparosan *in vitro* completely and de-polymerized *in vivo* the capsular heparosan of *E. coli* K5. Heparinase II treatment on *E. coli* K5 capsular polysaccharide enhanced the transformation efficiency of foreign genes into K5. It will improve the metabolic engineering operation in this strain. To our knowledge, it was first report to utilize heparinase II to eliminate K5 capsule.

Key words: Heparinase II, heparosan, Depolymerization, Capsule, *E. coli* K5, Gene transformation.

Heparin is a uniquely important polysaccharide as it is one of the oldest drugs and is currently in widespread clinical prevention of blood coagulation¹. Isolated by extraction from porcine intestines², heparin's use was associated in 2008 with a life-threatening rapid onset, acute side effect³. These adverse events were caused by an oversulfated chondroitin sulfate (OSCS) contaminant found in certain lots of heparin^{4,5}. Laboratory-scale studies have shown that heparosan (average molecular weight (MW_{avg}) > 10,000, obtained from *E. coli* K5 strain⁶, can be enzymatically converted to an anticoagulant polysaccharide similar to heparin^{7, 8}. The

preparation of a bioengineered heparin, from microbially produced heparosan, offers a potentially safer alternative for animal-sourced heparin⁹.

Heparosan, the capsular polysaccharide of *E. coli* K5, also named N-acetylheparosan, the structure of which is [-GlcUA-1, 4-GlcAc-1,4-]_n (GlcUA, glucuronic acid; GlcNAc, N-acetylglucosamine). Heparosan can be used as precursor for the biosynthesis of heparin/heparin sulfate¹⁰. Heparin is a very important glycosaminoglycan, having the function of anticoagulated blood, antithrombotic, hypolipemic, antiinflammatory, antitumor, and inhibition of bacterial adhesion. The production of heparosan in large quantity is necessary for realizing the industrial production of bioengineering heparin. Therefore, the research on metabolic pathway of heparosan synthesis in *E. coli* K5 and over-

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expression of the key enzyme genes in native or heterogeneous host cells are very interesting.

The synthesis of heparosan in *E. coli* K5 is regulated by a group of enzymes, named KfiA, KfiB, KfiC and KfiD, located in Region II of genomic DNA. KfiA is a α -UDP-GlcNAc glycosyltransferase. KfiC is a β -UDP-GlcA glycosyltransferase and has polymerization activities. KfiD is a UDP-Glc dehydrogenase and can catalyze the linkage of monosaccharide to UDP-GlcUA. KfiB is thought to be a binding protein and may be the initiator for K5 synthesis at the cytoplasmic membrane^{11,12}. Single gene or gene combination of kfiA, kfiB, kfiC, and kfiD can be cloned and expressed in suitable host bacteria to investigate the effect of the four genes on heparosan synthesis. The genetic modified strains might produce heparosan in higher quantity than the wild strain.

However, in our previous study, it was found that the transformation ratio of the foreign genes into *E. coli* K5 was very low. It was possible that the native capsule of *E. coli* K5 might prevent the foreign gene transformation and result in the decrease of transformation ratio of the foreign genes into *E. coli* K5. In the present study, a recombinant *E. coli* BL21 strain containing heparinase II gene was utilized to express and purify soluble heparinase II (Hep II). The effect of Hep II on the elimination of *E. coli* K5 capsule and consequential effect on the transformation ratio of foreign genes into *E. coli* K5 was then evaluated. To our knowledge, it was first report to utilize Hep II to eliminate K5 capsule.

MATERIALS AND METHODS

Bacteria and media

Escherichia coli strains grown in Luria-Bertani (LB) broth or on LB agar, in which 50 μ g/ml kanamycin sulfate (BBI, Canada) was added for *E. coli* with plasmid pET28b(+), pET28b-kfiA and pET-28b-kfiA-C, and 50 μ g/ml carbenicillin (BBI, Canada) was added for *E. coli* with plasmid expressing Hep II.

Two kinds of media were used to express Hep II. Luria-Bertani (LB) medium contains 10 g/L peptone, 10 g/L NaCl, and 5 g/L yeast extract with original pH 7.0. Auto-induction medium (AIM)¹³ contains 8.95 g/L Na₂HPO₄, 3.40 g/L KH₂PO₄, 2.68

g/L NH₄Cl, 0.710 g/L Na₂SO₄, 0.241 g/L MgSO₄, 0.0273 g/L FeCl₃, 5.00 g/L peptone, 2.50 g/L yeast extract, 2.00 g/L glucose, 1.00 g/L lactose, and 20.0 g/L glycerol with original pH 7.0.

Expression and purification of heparinase II

E. coli BL21 cells (carrying Hep B) was inoculated in LB containing 50 μ g/ml carbenicillin and incubated at 37°C and 200 rpm overnight. Then 2 ml of overnight culture was inoculated to 100 mL LB and AIM, respectively, and then incubated at 37°C and 200 rpm for 3-4 hours. When the OD_{600nm} reached 0.6-0.8, the culture temperature was lowered to 22°C. After the subsequent culture for at least 30 min, IPTG was added into the LB broth (No IPTG was added to AIM) to the final concentration of 0.1 mM for additional culture at 22°C overnight (usually 18 hours of incubation after induction gives the optimum yield).

The cells were harvested by spinning at 6000 \times g for 15 min (Sorvall Biofuge Stratos, Thermo Scientific, USA), and then was suspended in 25 ml Buffer A containing 25 mM Tris (pH 7.5), 500 mM NaCl, and 10 mM imidazole (single cell suspension is essential for obtaining the maximum yield). To disrupt the cells, the cells suspension was treated (lysed) by an ultrasonic processor, VC505A, Sonics & Materials, Inc, USA, under the sonication conditions as the following: 3 \times 30 seconds, 250 watt of output power, and 50% amplitude. The bacterial suspension must keep on ice during the process of sonication. The supernatant of the cells lyase was harvested by centrifuge at 12,000 g for 30 min (Sorvall Biofuge Stratos, Thermo Scientific, USA). Cell debris was suspended in the same volume of Buffer A as mentioned above and homogenized using a magnetic stirrer. Hep II in the supernatant and cell debris was detected by enzyme activity analysis and denaturing polyacrylamide gel electrophoresis (PAGE). Then the soluble heparinase II in supernatant was harvested as the crude enzyme of Hep II.

The supernatant of lyase was filtered through a 0.45 μ m filter and then was loaded on to a Ni-NTA agarose column (Ni-NTA Sefinose 6 Fast Flow, BBI, Canada) which is equilibrated with Buffer A at a flow rate of 2-3 ml/min. The column was washed with 5 ml of buffer A, and eluted with a linear gradient of imidazole from 10 mM to 250 mM in Buffer A in 60 min. The enzyme activity of the eluent was measured via a method described in

the following section. The purity of the protein was measured by SDS-PAGE.

Protein determination and SDS-PAGE electrophoresis

Bradford assay was applied to determine the content of protein with bovine serum albumin as a standard. SDS-PAGE electrophoresis was performed using a discontinuous polyacrylamide gel (12% separating gel, 5% stacking gel, 1 mm thickness) under denaturing conditions via a mini electrophoresis system (MinRun GE-100, Hangzhou Bioer Technology Co. LTD, China). 100 μ l samples of total cell protein (cell pellets, obtained with harvesting 1 ml cell cultures, then suspended with buffer A), soluble fraction (supernatant), and insoluble fraction (cell debris) were mixed with 100 μ l 2 \times sample buffer (pH 6.8, containing 100 mmol L⁻¹ DTT, 2% SDS, 100 mmol L⁻¹ Tris-HCl, 0.02% bromophenol blue, and 20% glycerol) respectively. These samples were heated for 5 min at 100°C to denature proteins, and then 7.5 μ l of each sample was loaded for SDS-PAGE analysis. As the tracking dye reaches the anodic end of the electrophoresis gel, the electrophoresis was terminated and the gel was stained with Coomassie brilliant blue R250. The molecular weights of the purified enzymes were estimated by comparison with standard molecular weight markers (MBI, Fermentas, Canada).

Enzyme activity determination

According to the method of the references¹⁴⁻¹⁵ which was further modified by Sigma (File # SSHEPA02, revised: 08/13/1997), the enzyme activity of Hep II was calculated as the increase of optical density at 235 nm due to the production of unsaturated uronic acid when heparin (Sangon, Shanghai) was eliminated by Hep II. The unit definition of Hep II was: One unit will form 0.1 μ mole of unsaturated uronic acid per hour at pH 7.0 at 25°C.

Heparosan elimination by Hep II

Heparosan, obtained by fermentation in our laboratory, was used to test the elimination effect of Hep II on heparosan. According to the method for enzyme activity determination, 5% heparin solution was instead by 5% heparosan solution¹⁶. All other situation was according to enzyme activity determination. Sampling interval was 0.5 hour. Then the effect of heparinase II on the elimination of heparosan was determined by PAGE. The heparosan samples were collected from

the supernatant of heparosan elimination test. After electrophoresis, Gels were stained with Alcian blue (Biosharp, Hefei, China) for visualization. In PAGE analysis, 5% spacer gel and 10% separation gel was applied under constant voltage with 400 volt.

TEM analysis of *E. coli* K5 capsule elimination

To evaluate the effect of heparinase II on *E. coli* K5 capsule elimination, the in vivo degradation of capsule heparosan was tested. After culture at 37°C and 200 rpm for 10 h, the cells in one milliliter of the broth were harvested by centrifuge at 4000 \times g and 4°C for 5 min. Then the cells were incubated with Hep II in a reaction mixture containing Regent A, C and E (described in enzyme activity analysis), at 25°C for 2 hours. The cells from another one milliliter of the broth without Hep II treatment were used as the control. As described by the previous reports¹⁷⁻¹⁹, the effect of Hep II on K5 capsule elimination was detected by transmission electron microscope (TEM, H-7650, HITACHI, Japan). Before TEM analysis, the cells were fixed by 0.5 mL 2.5% glutaraldehyde at 4°C overnight.

Transformation of foreign genes into *E. coli* K5

Two groups of strains were chosen for transformation. Group 1 was *E. coli* K5 strains after capsule eliminated by Hep II for 1 hour in enzyme reaction system. Group 2 was wild *E. coli* K5. Plasmids pET-28b-kfiA and pET-28b-kfiA-C were chosen as the foreign genes. Both plasmids contain kanamycin resistant genes. Strains containing pET-28b-kfiA or pET-28b-kfiA-C can grow in LB media with kanamycin. CaCl₂ method was applied for transformation. After transformation, the mixture was smeared onto agar plate and incubated at 37°C overnight. Then the number of colony on the plate was counted.

RESULTS

Expression and purification of heparinase II

After incubation in LB media with IPTG induction and incubation in auto-induction media, Hep II was successfully expressed as detected by SDS-PAGE. After ultrasonication treatment of cells, Hep II was detected mainly in cell debris when incubation in LB media, suggesting most of the Hep II was insoluble inside cells. However, Hep II could be detected both in supernatant and cell debris when incubation in auto-induction media

and increasing amount of Hep II was soluble and excreted. So auto-induction media was applied for Hep II preparation in this study.

After incubation in auto-induction media, a large amount of Hep II can be found in supernatant after ultrasonic treatment of cells (Fig. 1). Because the vector contains *hepB* which was the Hep II expression gene, and the fragment His-tag, Ni-NTA affinity chromatography was applied for purification of Hep II from the supernatant. After one-step purification by Ni-NTA affinity chromatography, Hep II had 2.6 purification fold. After analysis via Un-Scan-it gel software based on Fig. 2, the final concentration of heparinase II reached 96.29% and the molecular weight of purified heparinase II was 84.82 kDa (Fig. 2). This result coincided with the result of references²⁰⁻²¹.

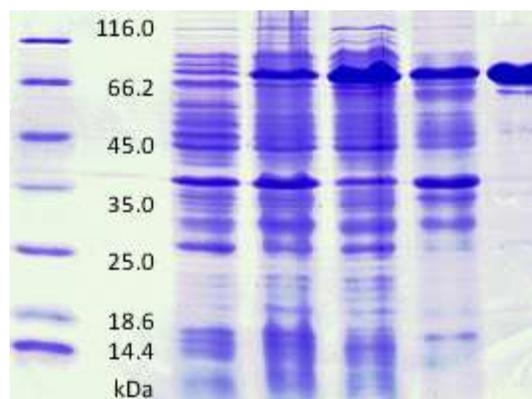


Fig. 1. Purification of heparinase II by Ni-NTA affinity chromatography, lane 1, Protein marker; lane 2 and 3, Total protein of the LB broth without IPTG inducing and the AIM broth inducing by lactose; lane 4 and 5, Supernatant and cell debris of the lysate after ultrasonic treatment of cells induced by lactose in AIM; lane 6 Heparinase II purified by Ni-NTA

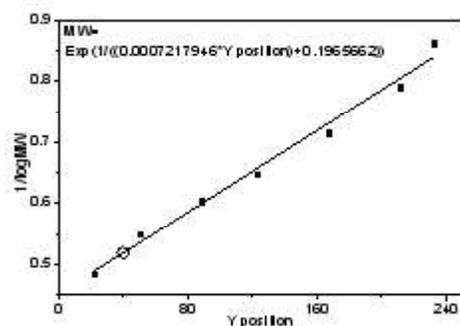


Fig. 2. Molecular weight analysis of Heparinase

Elimination of heparosan and capsule of *E. coli* K5 via heparinase II

Heparosan could be eliminated by Hep II. The longer the elimination time was, the less high-molecular-weight heparosan detected. After 4 hours, all of the heparosan was eliminated into very low molecular weight molecule.

The effect of Hep II on capsular elimination of *E. coli* K5 was detected by TEM. Fig 4A and B showed the TEM results for wild strains grown on LB agar plate. The outside surface of the strain looks not smooth and flocculent, coinciding with the structure characteristics of capsule, although it's not thick enough. Fig 4C and D showed the TEM results for wild strains grown in flask with LB media. The outside surroundings of the strain looks much thicker than that in Fig 4A and B. Fig 4E and F showed the TEM results for strains grown in flask with LB media

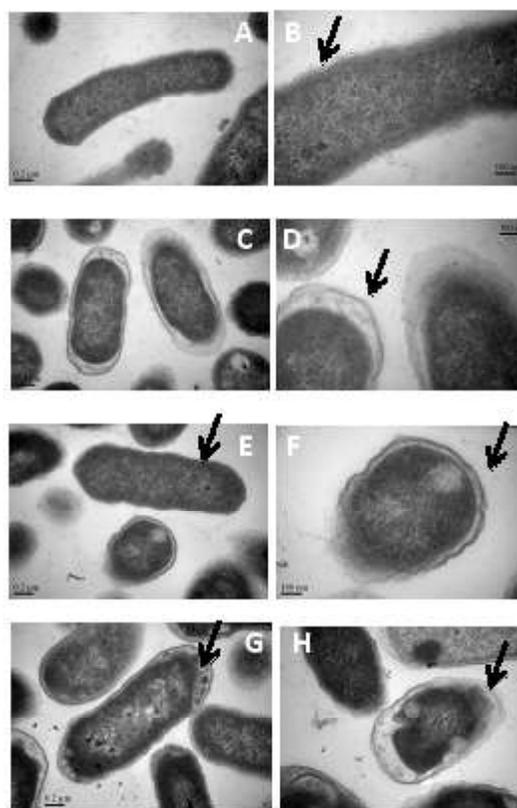


Fig. 4. TEM graphs of *E. coli* K5 cultured on agar plate (A, 70000 \times , B, 150000 \times) and cultured in shake flask (C, 70000 \times , D, 150000 \times) and *E. coli* K5 cultured in shake flask eliminated by Heparinase II (E, 70000 \times , F, 150000 \times , G, 70000 \times , H, 80000 \times), respectively

and than treated by Hep II. The outside surface of the strains in Fig 4E and F is smoother than that in Fig 4A and B, and the outside surface layer in Fig 4E and F is much thinner than that in Fig 4C and D. These results suggested that the capsule polysaccharide layer become thinner and smoother after the treatment of Hep II and part of heparosan at the outside surface of *E. coli* K5 was eliminated by Hep II.

Another phenomenon found after treatment by Hep II is that the appearance in and outside the cells has changed a bit (Fig. 4 E, F, G, H). The inside of the cells become not homogeneous and the outside surface become irregular compared with cells in Fig 4 A, B, C, D. This change might result from the response of cells to the detrimental environmental of the enzyme reaction systems which was necessary for the Hep II activity.

Effect of heparinase II on transformation ratio of foreign genes into *E. coli* K5

To detect the effect of Hep II treatment on transformation ratio of foreign genes into *E. coli* K5 via CaCl₂ method, two groups of strains, i.e., wild and treated *E. coli* K5, were simultaneously chosen as the host cell for foreign genes transformation (Table 1). Plasmid, pET-28b-kfiA and pET-28b-kfiA-C, carrying kanamycin-resistant gene, were chosen as the vectors for the foreign genes transformation. Their transformant could grow in media containing kanamycin. The results (Table 1) indicated that the cells after treated with Hep II exhibited higher transformation ratio than wild cells. It is obviously that Hep II can improve plasmid transformation of *E. coli* K5.

Table 1. Comparison of plasmid transformation into *E. coli* K5 with and without heparinase II treatment

Media/inoculum	HepII-treated K5, cfu	K5, cfu wild
K5+plasmid+Kan*	Tens of	Several
K5+Kan**	None	None
K5***	Full of plate	Full of plate

*Plasmid was transformed into *E. coli* K5 and the mixture was smeared onto LB agar plate containing kanamycin; **Wild and treated *E. coli* K5 were smeared onto LB agar plate containing kanamycin; ***Wild and treated *E. coli* K5 were smeared onto LB agar plate without kanamycin.

DISCUSSION

Most reported and applied heparinase (I, II, III) was mainly from *F. heparinum*. Upon induction with heparin, *F. heparinum* synthesizes and secretes into its periplasmic space heparinase I, II, and III. When these enzymes were expressed in IPTG-induced *E. coli* HB101 in LB at 37°C, most of each recombinant enzyme was insoluble, and most of the heparinase III protein was degraded. However, when they were expressed at 25°C, heparinase I and II were both present predominantly in a soluble, active form²². When heparinase I was expressed with an N-terminal histidine tag, the enzyme was insoluble and inactive, but could be refolded, and was purified to homogeneity by nickel-chelate chromatography with 43% of cumulative yield and 14.4 mg/l of recovery of purified enzyme to culture²³. Recombinant heparinase III was also expressed in *Escherichia coli* using the T7 polymerase pET expression system²⁴.

Heparinase I specifically cleaves heparin and heparan sulfate in a site-dependent manner, showing great promise for producing low molecular weight heparin (LMWH). However, the commercial use of heparinase I (Hep I) has been largely hampered by its low productivity and extremely poor thermostability²⁵. Heparinase II cleaves endolytically heparin-like glycosaminoglycans in a nonrandom manner via two distinct active sites, one of which is heparinase I-like, cleaving at hexosamine-sulfated iduronate linkages, whereas the other is presumably heparinase III-like, cleaving at hexosamine-glucuronate linkages. However, little about the soluble expression of heparinase II in *E. coli* BL21 was found in the previous literature. Thus, this study focused on heparinase II expression and application.

The *hep B*-expressing strain has been constructed by Liu via the pET System with *E. coli* BL21 (DE3) as the host cell. However, the disadvantage of the pET System is that large amounts of target protein were produced and frequently resulted in accumulation as inclusion bodies. A key strategy is to decrease the culturing temperature to enhance the solubility of the target protein as reported by Su *et al.*,²². Another strategy is using auto induction medium (AIM). AIM improved the soluble and active express of

recombinant protein in *E. coli* BL21 harboring pET plasmid. Additionally, *E. coli* cultures induced by galactose can saturate at higher density than cultures induced by IPTG²⁶.

In this study, a strategy of lowering the culture temperature of *E. coli* BL21 to 22°C and in auto-induction media, was applied for Hep II preparation and resulted in a successful enhanced express of soluble and active Hep II. We also discovered that cells grown in auto-induction media exhibited much higher protein yield than cells grown in LB media induced with IPTG. Cells in auto-induction media slow down transcription and translation rates thus increasing the proportion of soluble fraction. Although co-expression of molecular chaperones of recombinant proteins may aid the protein folding to reduce the formation of inclusion body, the auto-induction method seems to more suitable. It shows several advantages. First, it does not require monitoring the culture or adding inducer during cell growth. Second, it avoids the use of IPTG which is high cost and shows toxicant to humans. This strategy should be applicable to other recombinant protein (especially active enzyme) production in *E. coli* BL21 (DE3) driven by T7 promoter, in which protein easily expressed in inclusion bodies as an insoluble form.

Heparinase is commonly used as a tool to degrade heparin and heparan sulfate. As one kind of heparin-like GAGs, heparosan is also possible to be de-polymerized by heparinase, during which Hep II might be more suitable than heparinase I and III because Hep II could cleave at two kind of linkage in heparin, and possible in heparosan. However, little experiment result was reported in the past decades. This study is the first to report the heparosan de-polymerization by Hep II. This result suggested a potential pathway to prepare LMWHs, i.e., de-polymerize heparosan before it was modified to be LMWH.

In this study, we discovered that Hep II treatment on *E. coli* K5 capsular polysaccharide enhanced the transformation efficiency of foreign genes into K5. It will improve the metabolic engineering operation in this strain.

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