Cloning and expression of recombinant thrombin in *Escherichia coli* JM109 (DE3)

**Research article**

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Prothrombin, a protein involved in blood coagulation, is a plasma glycoprotein composed of the Gla domain, two adjacent kringle domains, and a serine protease domain. Prothrombin is a thrombin precursor playing the important role in the coagulation physiological as well as pathological condition. Thrombin is the key to convert the fibrinogen into fibrin by switching activation of XIII factor, pushed plasminogen into plasmin, the development of the fibroblast and helps the stabilization of thrombolysis. In this study, the prothrombin gene was 936 bp in length and encoded 312 amino acids from bovine lung was optimized codon, was cloned in pET21a vector and expression in *E. coli*, in order to replace traditional bandages having slow effect, reduce the cost of products, cater the community health. The results showed that initially the successful cloning and expression of recombinant prothrombin in *E. coli* JM109(DE3).

Prothrombin, 1 glycoprotein huyết tương liên quan tới quá trình đông máu gồm 2 vùng Gla, 2 vùng Kringle và 1 vùng serine protease. Prothrombin là tiền chất của thrombin có vai trò quan trọng trong sinh lý đông máu cũng như tình trạng bệnh lý. Thrombin được sản sinh từ chất hoà lý fibrinogen thành fibrin bằng cách hoà hóa các yếu tố đông máu như XIII, thúc đẩy chuyển plasminogen thành plasmin và kích thích tăng sinh các tế bào tổ (fibroblast), giúp ổn định quá trình lâm tan huyết khối. Trong nghiên cứu, các gen prothrombin được tách dòng từ phối bộ có kích thước 936 bp, mã hóa cho 312 axit amin được tói ưu hóa, đông dòng vào vector pET21a và biểu hiện trong *E. coli*. Mục đích của nghiên cứu nhằm tạo ra băng gạc cầm máu nhanh, gia thành thức, phục vụ sức khỏe cộng đồng và thấy thể bằng gạc truyền thống. Kết quả nghiên cứu bước đầu cho thấy đã nhận dòng và biểu hiện thành công prothrombin tái tổ hợp ở chúng *E. coli* JM109(DE3).

**Keywords:** fibrin, fibrinogen, prothrombin, thrombin

1. Introduction

Thrombin plays a crucial role in the conversion of fibrinogen into fibrin which are plasma protein engaged in the final stage of coagulation. In 1940, the purified thrombin was applied in surgery (Walter 1940) and used widely multiple. In USA, it was used for more than 1 million patients at a cost of 250 million dollars (Lawson 2006). It may be useful for controlling the bleeding from capillaries and small veins, but not efficient and is not indicated for arterial bleeding quickly and massively (Greenhalgh, Gamelli et al. 2009, Singla, Ballard et al. 2009). In USA, thrombin has been applied to the nano gauze to apply on wounds to stop the bleeding faster than conventional gauze. In the biochemistry, thrombin is a valuable biochemical tool because of high proteolysis. The cutting site (Leu-Val-Pro-Arg-Gly-Ser) includes linked domains in the recombinant protein’s structure. After the purification of protein, thrombin is used for cutting on Arg-Gly linked sites to remove the His tags.

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http://dx.doi.org/10.13141/JVE
ISSN 2193-6471
Thrombin was isolated from bovine and buffalo appears have studied and applied in hemostasis process from XIX century to present with many labeled patents in USA (Glover and Shaw 1971, Silbering, Blythe et al. 1990, OVERHOLSER and Woodell-May 2015). Also, thrombin was extracted from human plasma and snake venoms (Markland and Damus 1971, Fenton, Fasco et al. 1977, Naski, Lorand et al. 1991, Aizawa, Winge et al. 2008, D.Zaqueo, Kayano et al. 2014). In the present study, we cloned a thrombin cDNA from bovine liver. The new prethrombin gene was expressed in E. coli, recovered, further, purified with a nickel-chelating resin column and assayed the initial conversion of fibrinogen into fibrin. Results showed that recombinant prethrombin with molecular weight about 37 kDa have been obtained and has activity of conversation. The results may lead to the development of new approach for the treatment of hemostasis to replace the traditional methods.

2. Materials and Methods

2.1. Chemicals and reagents

Bovine liver tissue, bovine fibrinogen was obtained from the Laboratory of Enzyme (IBT, VAST). Yeast extract, peptone, dNTPs, EcoRI, Xhol, Tl, ligase, dNTP set, Taq polymerase, RNase, GeneRuler™ 1kb DNA Ladder, bacto tryptone, agar, ampicillin, IPTG, lysozym, protein MW were purchased from Bio Basic Inc. (Ontario, Canada) and Fermentas Corp. (USA). Kit ProBond™ Nickel-Chaleting Resin and Kit SuperScript™ First - StrDNA Synthesis System for RT-PCR, IPTG were from Invitrogen Corp. (Carlsbad, CA, USA) and some other chemical such as potassium phosphate, tris-HCl, sodium acetate from Merck (Germany). Primers was designed based on cDNA sequence of thrombin publishing on Genbank and was synthesized by Invitrogen Corp. (USA).

2.2. Vectors, strains and culture conditions

Expression vector pET21a+ contained prethrombin gene. E. coli host strain DH10B and JM109(DE3) (Invitrogen Corp., Carlsbad, CA, USA) were used for cloning and expression of the prethrombin, respectively. Luria-Bertani (LB) containing 0.5% (w/v) yeast extract, 1% (w/v) peptone, 1% NaCl, pH 7-7.5 was used for the cultivation of E. coli; LB agar contained additionally 1.5% (w/v) agar and 100 µg/ml ampicillin.

2.3. RT-PCR amplification and gene cloning

The total RNA was isolated from bovine liver tissue by using the extract Kit according to the manufacturer’s instructions. The reaction of first-strand cDNA synthesis was catalyzed by reverse transcriptase using random primers. A reaction product of 3 µl was added in the 25 µl PCR amplification system. The primers were as follows forward primer and reverse primer. Prethrom-F: 5'-ggaagtctaggggaagcg-3' and Prethrom-R: 5'-gcgctgaactactg-3'. PCR reaction were catalyzed by Taq DNA polymerase (5 U/µl) and the product was digested with Xhol and EcoRI, and the DNA fragment including the ORF was ligated into vector pET21a+ to get pEThr vector.

2.4. Expression of the recombinant pEThr

Overnight culture of cells bearing the construct for expression was dilute 1% into fresh LB broth in the presence of 100 µg/ml ampicillin. When the cell suspension reached an absorbance of 0.6 - 0.8 at 600 nm, the expression was induced by the addition of 1mM IPTG for 6 h. Cells were harvested by centrifugation at 4000 rpm for 10 min and re-suspended in lysis buffer. The cells were sonicated on ice bath and the lysate was centrifuged at 12500 rpm for 10 min. Cells was dissolved in 50mM Tris-HCl buffer containing 8 M Urea. Expression of the fusion protein was analyzed by SDS-PAGE.

2.5. Purification of the recombinant pEThr

The inclusion body was dialysed in 50mM Tris HCl, pH 8.0 to remove urea and dialysis sample was replaced the buffer for 1 time every 2 hours. After 3 times, samples were centrifuged 12500 rpm for 30 min to receive the supernatant. The sample was loaded onto His-Bind Column (10 cm glass column with 2 ml of Ni-agarose) pre-equilibrated with denatured buffer (50 mM NaH2PO4, 0.5 M NaCl, 10 mM imidazole, pH 8.0) at a rate of 1 ml/ 5 min for twice. The column was washed with 8ml washing denatured buffer for 3 times. Recombinant prethrombin was pushed out of the column with 8 ml eluted denatured buffer. The results showed on SDS-PAGE and dying with the Coomassie blue were performed according to Sambrook and Russell.

2.6. Analysis the conversion of fibrinogen into fibrin

The activity of purified thrombin was analyzed by the conversion of fibrinogen into fibrin in the reaction included 20 ml fibrinogen, 200 µl NaCl 0.9% and 50 µl thrombin. Control sample has no thrombin. Time of transformation of fibrinogen solution into fibrin strands was observed.

3. Results and discussion

3.1. Amplification of prethrombin gene from liver bovine tissue

The total RNA extracting from bovine liver was extracted by RNA purification Kit. Content of total RNA reached 328 µg/ml and the A260/280 value reached 2.02. The results showed that total RNA was purified successful and was used in other studies.

RT-PCR reaction was performed as described in the method section. Figure 1 (lane 3) shows a specific DNA fragment about 1000 bp in size, in correspondence with the theoretical size of prethrombin is 936 bp.

The sequence of DNA fragment was analyzed by BLAST data. The data indicated the DNA fragment are cloned is gene coding for prethrombin is composed 936 nucleotides to encode a polypeptide of 312 amino acid residues (Fig. 2).
In addition, BLAST analysis revealed that the DNA fragment encoding prethrombin has high homology reached 98-99% compared with some published sequences.

Figure 1. The agarose gel electrophoresis of total RNA from bovine liver (1, 2) and PCR products of prethrombin gene (3)

3.2. Construction of recombinant expression vector pEThr

The resulting PCR product and pET21a+ were digested with EcoRI/XhoI, inserted into the pEThr plasmid and transformed into E. coli JM109 (DE3). Overnight incubation of this strain in LB agar contained 100 µg/ml ampicillin. Plasmid was extracted from some selected clones. The results show the recombinant plasmids were higher than pET21a+ plasmid in size, they may be due to contain the inserted prethrombin gene. These plasmids were digested again with EcoRI/XhoI to check exactly insertion of prethrombin gene. The figures showed a band with size was approximately 1000 bp of prethrombin gene and 5500 bp of pET21a+ plasmid (Figure 3). So, prethrombin gene was inserted into pET21a+.

3.3. Expression of recombinant prothrombin

The recombinant pEThr plasmid was transformed into E.coli JM109 (DE3) by chemical method to get the recombinant strains (JMThr). The pET21a+ was also transformed into E.coli JM109 (DE3) to get the control sample (pET21a+/JM109). These strains contained prothrombin gene was cultured in LB medium added to 100 µg/ml ampicillin and induced with 1 mM IPTG. In the SDS-PAGE gel, a protein band about 37 kDa appeared after 6 h induction and the molecular mass agrees with the theoretical estimate based on the size of pETThr (Figure 4).

Figure 2. Sequence of the cDNA encoding prethrombin

Figure 3. The agarose gel electrophoresis of recombinant plasmid pEThr (lane 1-6) and the digested products with EcoRI/XhoI (lane 6-10); pET21a+ plasmid (lane C)
3.4. Purification of recombinant prethrombin

The cells of E. coli JMThr strain were sonicated to get the inclusion bodies containing prethrombin. The pellets were dissolved in 8 M Urea solution and dialysed in 50 mM Tris-HCl, pH 8.0. After dialysis, the lysate was centrifuged to remove the pellets. The supernatant was used for purification using affinity chromatography. The results showed getting the soluble recombinant prethrombin (Figure 6A).

4. Conclusions

Here, the gene encoding thrombin were cloned from bovine liver and created the E. coli JM109(DE3) strain which expressed intracellular recombinant prethrombin to get 12% in yield of total cellular protein based on the analysis of expressed protein by Dolphin 1D software. The recombinant protein was purified using ion exchange chromatography columns Ni²⁺. Prethrombin had initial activity to convert the fibrinogen into fibrin after the activation with CaCl₂.

5. Acknowledgement

The study was supported by the Project of Institute of Biotechnology, Vietnam Academy of Science and Technology, (Project: CS16-04 “Study on expression conditions and purification of recombinant thrombin for application create rapid hemostatic dressings”), 2016-2017.

6. References


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