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Co-overexpression of *Pp*PDI Enhances Secretion of Ancrod in *Pichia pastoris*

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Abstract Ancrod, a serine protease purified from the venom of *Agkistrodon rhodostoma*, is highly specific for fibrinogen. It causes anticoagulation by defibrinogenation and has been used as a therapeutic anticoagulant for the treatment of moderate to severe forms of peripheral arterial circulatory disorders in a variety of countries. The DNA of ancrod was amplified by recursive PCR with a yeast bias codon and cloned into the pGEM-T Easy vector. In order to achieve a high level secretion and a full activity expression of ancrod in *Pichia pastoris* (*P. pastoris*), the *P. pastoris* protein disulfide bond isomerase (*Pp*PDI) was co-overexpressed in the strain. The secretion characteristics of ancrod with and without *Pp*PDI were examined. With co-overexpression of *Pp*PDI, the production of recombinant ancrod (rAncrod) was increased to 315 mg/L in the culture medium, which is twofold higher than the control strain carrying only the ancrod gene. Through purified by Ni^{2+} affinity chromatography and phenyl Sepharose column, the purity of rAncrod was found to be as high as 95.2%. The fibrinogenolytic and zymographic activities of the rAncrod were determined and found to be similar to that of the native protein. This improved expression system can facilitate further studies and the industrial production of ancrod.

Keywords Co-overexpression · *Pichia pastoris* · *Pp*PDI · Purification · rAncrod

Introduction

Ancrod is a snake venom serine protease purified from the venom of *Agkistrodon rhodostoma*. It is used for the treatment of patients with a variety of arterial occlusive

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diseases, including stroke [1, 2]. Ancrod has a fibrinolytic activity similar to thrombin, but it specifically cleaves only the alpha chain of fibrinogen, producing the characteristic fibrinopeptides α-, β-, and γ-chains [3]. Ancrod acts at different stages of coagulation and blood circulation by depleting the substrate needed for thrombus formation; the depletion of fibrinogen reduces blood viscosity, which improves blood circulation and enhances thrombolysis by stimulation of endogenous plasminogen activators [4, 5]. Because ancrod has been used in patients with heparin-induced thrombocytopenia, it is important to ensure that it does not directly affect the platelets and potentially increase bleeding [6].

Ancrod has been used in Europe and Canada for several decades for treatment of central retinal vein thrombosis, deep venous thrombosis (DVT), myocardial infarction, and peripheral vascular disease [6–12]. Anticoagulant therapy using ancrod has been shown to be at least as effective as conventional anticoagulants and may even be safer in terms of reduced bleeding complications. It appears to induce the dissolution of preformed thrombi in a variety of clinical syndromes, such as hypercoagulable states, glomerular nephritis, and thrombotic-related stroke [7, 11, 13, 14]. The results have indicated that ancrod has considerable potential value in the treatment of various thrombo-embolic states and in the prevention of thrombosis. The clinical effect of ancord is based on the combination of an anticoagulant effect caused by the inhibition of fibrin polymerization and the fibrinolysis of thrombin-induced thrombi by the degradation of fibrin(ogen).

In previous reports, Burkhart et al. attempted to express ancrod in *Escherichia coli*, but the recombinant protein was retained in the bacteria as inactive inclusion bodies [15]. Subsequently, recombinant ancrod (rAncrod) was produced in a mouse epithelial cell [16]. However, using the mouse epithelial cell system is more costly and more time-consuming. Finally, Yu et al. were able to express ancrod in *P. pastoris*, and about 132 mg/L of the recombinant protein was obtained [17].

The goals of this study were to increase the amount of recombinant ancrod in *P. pastoris* and to evaluate the effects of co-overexpression of *Pp*PDI on the recombinant production.

Material and Methods

Vectors, Host Strains, and Materials

The pGAPZA, pPIC9k plasmid, and *P. pastoris* KM71 (*his4*, *aox1*) strains were from Invitrogen (San Diego, CA). Media components were from Difco (Detroit, MI). MD, MDG, YPD, BMGY, and BMMY were all prepared following the Invitrogen expression manuals. pGEM-T Easy Kit, DNA restriction enzymes, and T_4 DNA ligase were purchased from Promega. Oligonucleotide fragments were synthesized by Bioasia (Shanghai, China). Protein molecular weight standards and Pfu DNA polymerases were from Sangon (Shanghai, China). Bovine fibrinogen and bovine thrombin were purchased from Sigma (St. Louis, MO).

Construction of Expression Plasmids

A DNA sequence encoding for ancrod was designed by selecting high-usage codons in yeast according to the amino acid sequence of GenBank number EF210486. The gene was divided into 18 oligonucleotide fragments with 60 bases each and similar melting temperatures (45–50 °C) in their overlapping regions, simultaneously creating an *Eco*R I

site, a His-tag in the 5′-end, and a *Not* I site in the 3′-end to facilitate cloning and purification. According to the idea of recursive PCR [18], the full length DNA was assembled using overlapping oligonucleotides that matched regions of the sense and antisense strands of the DNA sequence. The PCR product was cloned into the pGEM-T Easy vector. Correct sequences were confirmed by sequencing and releasing from *Eco*RI and *Not*I sites and were cloned into pPIC9k at the corresponding site. The fusion gene was made following the α factor signal sequence for secreted expression and under the control of AOX1, the highly methanol-inducible and tightly regulated promoter. Colony PCR and restriction digestion by *Eco*RI and *Not*I were carried out to confirm the recombinant transformants, and the positive clones achieved were verified by sequencing.

The *Pp*PDI fragment, lacking a signal peptide, was amplified from the *P. pastoris* genomic DNA using forward primer 5′-CATGGAATTCATGCAATTCAACTGGGATATT and reverse primer 5′-CGCCTCGAGATTAAAGCTCGTCGTGAGCG based on a published sequence [19]. The PCR products of *Pp*PDI was digested with *Eco*RI and *Xho*I and inserted into the vector pGAPZA.

Transformation and Selection in *P. pastoris* KM71 Expression Host

The pPIC-his6-ancrod plasmids, ligated with in-frame DNA sequence, were digested with *Sac*I in the AOX1 promoter region to linearize the vector and then transformed into *P. pastoris* KM71 with MicroPulser electroporator (Bio-Rad, USA) under the conditions described in the report [20]. His+ transformants were firstly isolated from MD medium and then replica-plated onto MDG medium containing increasing concentrations of G418 (0.5, 1.0, 1.5, 2.0, and 3.0 mg/mL). After the genomic DNA of the His+ G418-transformants were extracted by the modified "boiling–freezing–boiling method" [21], correct integration of pPIC9K-his6-ancrod was confirmed by PCR. Clones with putative multicopy strains of pPIC9K-his6-ancrod were retained for further expression studies. The one with the highest expression level was used to incept pGAP-*Pp*PDI.

The ancrod expressing transformant KM71/pPIC-his6-ancrod was transformed with pGAP-*Pp*PDI, a plasmid with a sequence encoding for *Pp*PDI under the control of the *GAP* promoter. The integration of *Pp*PDI expression cassettes in the double transformants was confirmed by PCR amplification.

Selection of High Producers and Recombinant Ancrod Expression

Several putative high level expression transformants of each *P. pastoris* strain were evaluated in the pre-expression process. The promising transformants obtained in the pre-expression process were grown at 28 °C in 150 mL baffled shake flasks containing 25 mL BMGY liquid medium until the A600 value reached 2–3. The cultures were centrifuged at 5,000 r/min for 5 min, and the cell pellets were resuspended in 4× the original volume in BMMY and followed by methanol induction for 72 h. Then, 1.0 mL of the cell-free culture supernatants was collected daily and concentrated with 10% saturated trichloroacetic acid (TCA). The concentration of the total protein was obtained by A280 analysis with the Biophotometer (Eppendorf). The relative abundance of rAncrod (%) as compared to the total protein present in the samples was evaluated by SDS-PAGE analysis with a GeneSnap scanning software, and determination of rAncrod concentration was calculated by multiplying the two corresponding values obtained above. Western blotting detected rAncrod specificity in the total protein of the optimal supernatants, and the highest expressing clone was selected for further study.

For routine recombinant ancrod production, the selected *P. pastoris* expression clones were cultured at 28 °C with vigorous shaking, wherein biomass accumulation was carried out in 300 mL of YPD for 36 h using 2,000 mL baffle shake flasks and was followed by methanol induction for 72 h in 60 mL of BMMH using 500 mL baffle shake flasks. Each culture was also supplemented with methanol to a final concentration of 1% at 24 h of methanol induction.

Western Blotting of Culture Supernatants

One milliliter of culture supernatant was mixed with 1/9 volume of 100 % TCA and incubated on ice for 30 min. After centrifugation (15,000×*g*, 15 min, 4 °C), the pellet was washed once with cold acetone, air-dried, and then resuspended in 20 μL non-reducing loading buffer. Samples were separated on 12% polyacrylamide gels.

Western blot detection of the optimal supernatants was done with the His· tag monoclonal antibody (Ab_1; Novagen) against the 6× histidine tag of rAncrod, with the Rb anti-mo IgG/HRP (Ab_2; Beijing Biosynthesis Biotechnology) against Ab_1 and with the Strengthen HRP-DAB Western Blotting Detection kit (Tiangen).

Purification of rAncrod

Purification of the rAncrod was performed by a combination of Ni^{2+} affinity chromatography and hydrophobic interaction chromatography. The cultures were harvested by centrifugation at the optimal time points, filtration through 0.22 μm filters, and then application to the His·bind purification chromatography columns, which were prepared and used according to the kit manual. The column was washed with the washing buffer, and recombinant protein was eluted with the elution buffer containing 1 M imidazole. An equal volume of 3.0 M sodium sulfate was added to the NTA-Ni resin eluate, and this material was loaded onto a phenyl Sepharose 6 FF (low sub) column equilibrated in 50 mM Tris (pH 7.5) and 1.5 M ammonium sulfate. After washing with the same buffer, ancrod was eluted by a linear gradient of 1.5–0 M ammonium sulfate (in 50 mM Tris, pH 7.5), and the active fractions were pooled together. The purity of rAncrod was adjusted to a final concentration of 5 mg/mL and analyzed with SDS-PAGE on 12% polyacrylamide gel.

Fibrinogenolytic Activity Assay

Fibrinogenolytic activity [22] was assayed by incubating 0.1 mL of a 0.2% bovine fibrinogen solution with 0.05 mL recombinant ancrod solution (containing 1–100 ng ancrod) in 50 mM Tris–HCl buffer (pH 7.5) at 37 °C. Samples were subsequently analyzed by SDS-PAGE.

Fibrinogen Clotting Assay

The fibrinogen clotting activity of ancrod was examined according to the technique used for thrombin but with a slight modification. Briefly, 30 μL of recombinant ancrod (about 150 ng) was added into 450 μL of bovine fibrinogen (in 10 mM Tris–HCl, 0.15 M NaCl, pH 7.5), and the mixture was incubated at room temperature. Then, 0.1 NIH units human thrombin in 50 μL buffer were used as a positive control, and the 50 μL buffer was used as a negative control. The absorbance at 405 nm, which reflects the formation of clotted fibrin, was recorded on a DU-70 Spectrophotometer (Beckman, USA) at different time points.

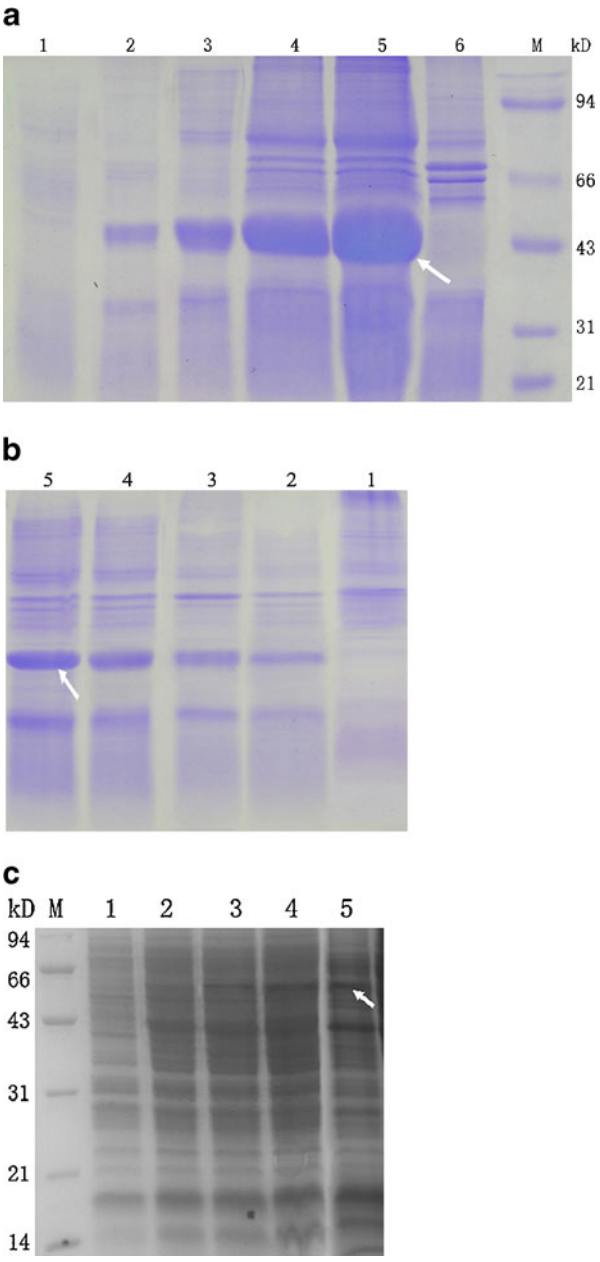

Fig. 1 SDS-PAGE of the ancrod's expression. **a** Supernatants of ancrod co-expression with *Pp*PDI. *M* protein marker, *1* supernatants of KM71, *2*, *3*, *4*, *5* the 1d, 2d, 3d, and 4d supernatants of the double transformant, respectively. **b** Supernatants of ancrod expression alone. *1* supernatants of KM71, *2*, *3*, *4*, *5* the 1d, 2d, 3d, and 4d supernatants of the double transformant, respectively. **c** Precipitates of the double transformant. *M* protein marker, *1* supernatants of KM71, *2*, *3*, *4*, *5* the 1d, 2d, 3d, and 4d precipitates of the double transformant, respectively

Zymographic Assay

Fibrin zymography was carried out as described by Granelli-Piperno and Reich [23]. In brief, samples were separated by gel electrophoresis in the presence of SDS under non-reducing conditions. Subsequently, the gel was washed in a Triton X-100 solution to remove SDS and renature the proteins. The gel was overlaid on a fibrinogen (0.7%, *w*/*v*)–agarose (0.8%, *w*/*v*) plate and incubated at 37 °C. Ancrod activation resulted in the lysis of fibrin and revealed itself as dark bands on an opaque background.

Results and Analysis

Expression Vector Construction

The ancrod DNA sequence was manipulated through a five-step PCR and ligated into pGEM-T. The ancrod expression vector pPIC-his6-ancrod was constructed by placing the ancrod DNA downstream of the AOX1 promoter and was fused with the signal peptide α factor. After transformation and selection, the recombinant transformants were identified firstly by colony PCR and then by restriction digestion, in which fragments of about 0.7 and 9.3 kb were screened as expected (data not shown). Plasmids with correct insertion and right open reading frames of his6-ancrod in pPIC9k were also confirmed by DNA sequencing.

Transformation and Selection

By high efficiency transformation of the *Sac*I-linearized pPIC-his6-ancrod into the *P. pastoris* KM71 strains, about 900 His^+ transformants were isolated. Thirty His^+ transformants were chosen for inoculation on MDG medium containing G418 (2.0 mg/mL or higher), and 23 of them had the ability to grow well, indicating that the *Sac*I-linearized pPIC-his6-ancrod was integrated into the AOX1 sites of *P. pastoris* genome. Genomic DNA PCR further confirmed the correct integration of pPIC9K-his6-alf into the corresponding chromosomal locus of *P. pastoris* (data not shown).

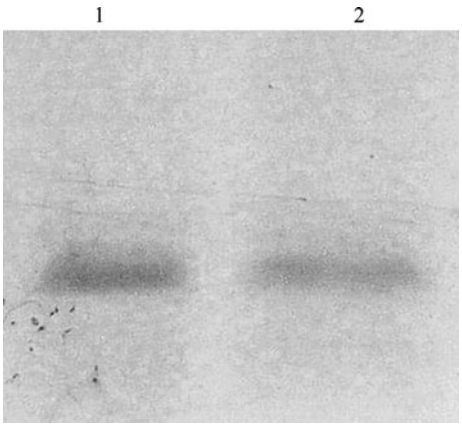

Fig. 2 Western blotting analysis of rAncrod 1, 2: rAncrod from double transformant and ancrod expression alone, respectively

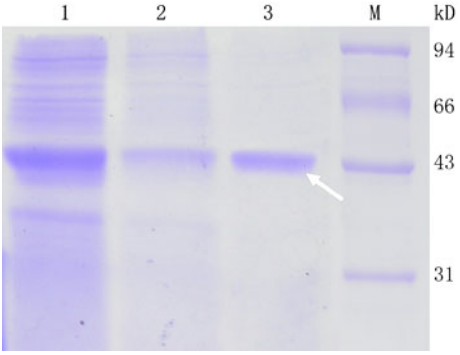

Fig. 3 SDS-PAGE analysis of the purified rAncrod. The *arrows* showed the target protein induced by methanol. *M* protein marker, *1* unpurified supernatants of double transformant, *2* purified rAncrod from Ni^{2+} affinity chromatography, *3* purified rAncrod from HiTrap 1 mL Q-Sepharose 6 FF

Expression of Recombinant Ancrod

The clone with the best activity was chosen for upscaled protein production. After cultivation, ancrod was expressed in both the double transformants and the pPIC-his6-*ancrod/KM71*. As shown in Fig. 1a and b, the recombinant ancrod was successfully expressed in *P. pastoris* strains as a secreted protein. The secretion of recombinant ancrod was detectable on day 1 and reached a maximal level after 4 days. The expressed recombinant ancrod was about 45 kDa (Fig. 1a and b), which is larger than the theoretical size of the unglycosylated (24 kDa) protein. This indicated that the recombinant ancrod was glycosylated after the posttranslational modification. Under the optimized culture parameters of pH value, methanol daily addition concentration, and induction time length, the production of rAncrod reached up to 160 mg/L for KM71 transformants. The production of rAncrod with co-overexpression of *Pp*PDI was increased to 315 mg/L in the culture medium. This was twofold higher when compared with the control strain carrying only the ancrod gene (compare Fig. 1a and b). A band at approximately 55 kDa, indicating the presence of PDI in intra cellular fractions, is shown in Fig. 1c.

Western Blot

For detection of the recombinant ancrod, Western blot analysis was used under non-reducing conditions. As shown in Fig. 2, the Western blot resulted in only one band on the membrane, revealing that Ab_1 bound specifically to the 6× histidine tag of rAncrod.

Table 1 Purification of recombinant ancrod from 1 L of cell culture supernatants of the recombinant *P. pastoris* strains

	Total protein (mg)	Purity quotient (%)	Recovery efficiency (%)
Culture supernatant	315.0	34.6	100
rAncrod from Ni^{2+} column	267.1	85.5	84.8
rAncrod from Q-Sepharose 6 FF	199.3	95.2	63.3

Protein concentration was estimated by Bradford assay

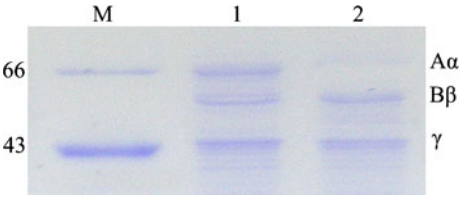

Fig. 4 SDS-PAGE analysis of fibrinogen after an 18-h incubation alone with rAncrod. *M* protein marker, *1* fibrinogen, *2* sample incubated with fibrinogen for 1 h

Purification of rAncrod

Due to the 6× histidine tag of rAncrod, purification of the secreted rAncrod was easily achieved in a small scale by using the His·bind purification chromatography kit. The culture supernatant was loaded on a Ni^{2+} column for initial purification. The ancrod protein eluted from the Ni^{2+} column was >80% pure (Fig. 3, lane 2). Impurities either flowed through the phenyl Sepharose 6 FF column when purified ancrod was being eluted or were removed by washing after purified it was eluted.

SDS-PAGE of the purified rAncrod showed that there was only one band on 15% polyacrylamide gel (Fig. 3, lane 3). This, along with the Western blot result (Fig. 2), demonstrated that the protein with a molecular weight of about 45 kDa was probably a native protein of the KM71 transformant and was likely the glycosylated form of rAncrod. As summarized in Table 1, the recovery efficiency of rAncrod was 74.6% of double transformants. And the GeneSnap scanning analysis demonstrated that the purity of rAncrod reached up to 95.2%. This might be attributed to the fewer native proteins secreted by the host strain.

Fibrinolytic Activity Assay

The fibrinogenolytic activity of these purified proteins was tested by analyzing the digestion of fibrinogen. The enzyme prepared was active in a fibrinogen solution (Fig. 4). Ancrod could cleave the fibrinogen α chain, but neither β chain nor γ chain was degraded. The activity of the recombinant enzyme showed fibrinolytic activity similar to what was previously described [9].

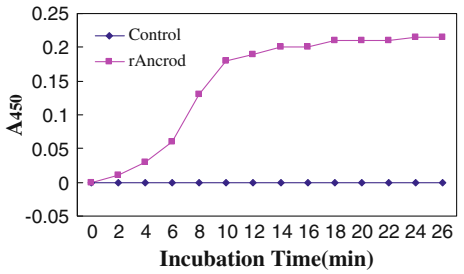

Fig. 5 Time-dependent clotting of bovine fibrinogen by the recombinant ancrod. *Diamond* represents recombinant ancrod, *square* represents human thrombin, and *triangle* represents buffer

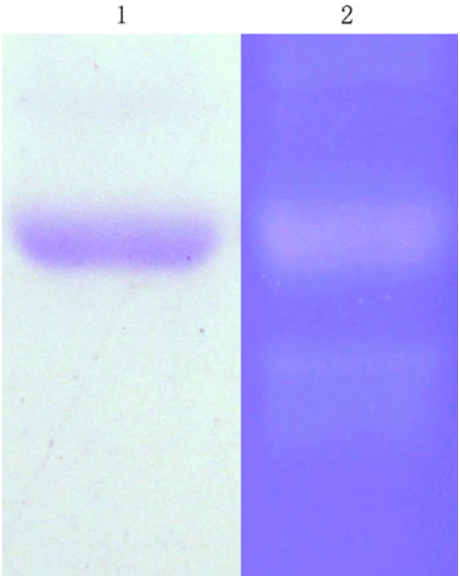

Fig. 6 Zymographic assay of rAncrod. *1* recombinant ancrod on 15% polyacrylamide gel under non-reducing condition, *2* recombinant ancrod of converted fibrinogen into fibrin, which results in the formation of turbid band at the corresponding site on the bovine fibrinogen–agar plate

Fibrinogen Clotting Assay

The interaction between the recombinant ancrod and fibrinogen were primarily studied. Like the human thrombin, the recombinant ancrod increased the turbidity of a fibrinogen solution in a time-dependent manner (Fig. 5).

Zymographic Assay

The activity of ancrod using the method of fibrin zymography was examined. The reverse fibrin autography showed broad bands clearly visualizing fibrinolysis against a blue background of undigested fibrin substrate on the fibrin/agar indicator gel (Fig. 6, lane 2). The bands had apparent molecular weights of 45 kDa. These results indicated that the proteolytic activity of ancrod was relatively stable.

Discussion

Since different kinds of thrombotic diseases, such as thrombosis, DVT, and stroke, occur much more often than in the past decades, the development of new thrombolytics for the antithrombotic therapies has become indispensible.

Ancrod is a rapid fibrinolytic agent that is immunologically distinct from heparin and does not cause immune thrombocytopenia. It has a fibrinolytic activity similar to thrombin but specifically cleaves only the α chain of fibrinogen. Ancrod has been shown to be a safe alternative for patients with HITT/HATT who need anticoagulation for CPB. The risk of bleeding, coagulopathy, and thromboembolism is minimal [10–12, 24]. Ancrod is a foreign protein to humans, but allergic reactions are rarely found [25].

Previously, expressions of ancrod in *E. coli*, mouse epithelial cell, and *P. pastoris* were attempted [15–17]. Yet the recombinant ancrod obtained presented a number of challenges: the inability to produce active molecules at an acceptable production level, inferior amount of recombinant protein, and the more costly and time-consuming process.

In the present report, we chose the *P. pastoris* system to express our target gene his6-ancrod under the control of the high level transcription of AOX1 promoter. When comparing the levels of ancrod expression with the co-overexpression *Pp*PDI and without it, a significant difference was found. A high level of the recombinant ancrod was expressed and secreted into the broth by α-factor preprosequence during shaking cultivation. Purification of the secreted rAncrod was easily achieved due to the relatively specific 6× histidine tag. Analysis of the SDS-PAGE and A_{280} results showed that rAncrod had been efficiently secreted with the highest production of 315 mg/L for double transformants. As such, we propose that the disulfide bond formation and isomerization are rate-limiting in ancrod folding and that the correct formation of disulfide bonds is the crucial factor responsible for solubility and activity of rAncrod. A twofold increase in ancrod secretion in fermentor cultures shows the importance of this study for large-scale production of rAncrod.

In addition, the fibrinolytic activity identification results indicated that rAncrod had been functionally expressed and that the 6× histidine tag at the N terminus of rAncrod had no clear negative influence to its activity. Thrombolysis of rAncrod was demonstrated to be dose-dependent and time-relative.

In conclusion, we designed the gene for ancrod based on its primary amino acid sequence. A functional ancrod was expressed in *P. pastoris*. It was successfully purified by Ni-NTA affinity chromatography and hydrophobic chromatography. Such a process may allow for the development of effective thrombolytic agents involving this enzyme.

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