Expression of Recombinant Chinese Bovine Enterokinase Catalytic Subunit in P. pastoris and Its Purification and Characterization

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Abstract

Enterokinase is a tool protease widely utilized in the cleavage of recombinant fusion proteins. cDNA encoding the catalytic subunit of Chinese bovine enterokinase (EKL) was amplified by PCR and then fused to the 3′ end of prepro secretion signal peptide gene of α-mating factor from Saccharomyces cerevisiae to get the α-MF signal-EKL-His6 encoding gene by PCR. Then the whole coding sequence was cloned into the integrative plasmid pAO815 under the control of a methanol-inducible promoter and transformed GS115 methylotrophic strain of Pichia pastoris. Secreted expression of recombinant EKL-His6 was attained by methanol induction and its molecular weight is 43 kD. Because of the existence of His6-tag, EKL-His6 was easily purified from P. pastoris fermentation supernatant by using Ni2+ affinity chromatography and the yield is 5.4 mg per liter of fermentation culture. This purified EKL-His6 demonstrates excellent cleavage activity towards fusion protein containing EKL cleavage site.

Key words recombinant enterokinase; secreted expression; Ni2+ affinity chromatography; fusion protein cleavage

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Abbreviations: EK, enterokinase; EKL, catalytic subunit of Chinese bovine enterokinase; GST-VAS, GST-vasostatin

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easily purified from the few native proteins found in the *P. pastoris* fermentation supernatant, using a single step purification of Ni\(^{2+}\) affinity chromatography.

### Materials and Methods

#### Materials

A *Pichia* expression kit and plasmid pAO815 were purchased from Invitrogen (Houston, USA). Primers were synthesized by Shanghai Bio-Color Company, Shanghai, China. Restriction enzymes, T4 DNA ligase and high fidelity thermostable polymerase Pyrobest were from TaKaRa (Dalian, China). His-Select\(^{TM}\) HC nickel affinity gel was purchased from Sigma (St. Louis, USA). GST-vasostatin expressing plasmid was constructed previously in our lab.

#### Construction of expression plasmid pAO815-"EK\(_L\)-His\(_6\)"

The sequence encoding the N-terminal 85 amino acids of \(\alpha\)-mating factor prepro secretion signal peptide from *Saccharomyces cerevisiae* was amplified from plasmid pPIC9K (Invitrogen) using the upstream primer 1: 5'ctc cga att caa acg atg aga ttt cct tca att ttt act gc-3' and downstream primer 2: 5'ttt ctc gag aga tac ccc ttc ttc-3'. The 705 bp fragment encoding the 235 amino acid residues of recombinant EKL was PCR-amplified from previously reported EKL encoding the 235 amino acid residues of recombinant EKL-His\(_6\). The orientation of the fragment was validated and cloned into the same site of pAO815 to get pAO815-"EK\(_L\)-His\(_6\)".

#### Transformation and screening

To generate recombinants, GS115 cells were transformed with *Sall*-linearized pAO815-"EK\(_L\)-His\(_6\)" according to the manual to the *Pichia* expression kit. Cells were spread on RD plates and grew at 30 °C for 3 d. Fifty transformants were replica plated on MM and MD medium for Mut\(^{+}\) and Mut\(^{-}\)screening. The selected integrants were confirmed by PCR using 5' and 3'AOX1 primers. An easy screening method was improved in which the culture medium was used as PCR template directly without extracting the total genomic DNAs of integrants. 2 \(\mu\)l of culture was added into a 20 \(\mu\)l PCR reaction system, PCR were performed as recommended by Invitrogen’s instruction.

#### Expression and purification of EKL-"His\(_6\)"

A single colony was inoculated into 25 ml BMGY (made in our lab) and grew at 200 r/min at 30 °C until the culture reached an \(A_{600}\) of 2.0–6.0 (approximately 24 h). Cells were harvested by centrifugation, resuspended in 200 ml BMMY (made in our lab) to an \(A_{600}\) of 1.0, and induced to express at 30 °C with methanol to be added to a final concentration of 0.5% every 24 h to maintain induction. After 4 days, the culture was centrifuged at 10,000 r/min for 5 min and the supernatant containing the secreted recombinant EKL-"His\(_6\)" was collected.

0.2 L of EKL-"His\(_6\)" expression supernatant was dialyzed overnight against 4 L of 20 mM Tris-HCl, pH 8.0. The dialysate was loaded onto Ni\(^{2+}\) affinity column (1 cm ×5 cm) equilibrated with equilibration buffer (50 mM Tris-HCl, pH 8.0, 250 mM imidazole). The column was washed with wash buffer (50 mM Tris-HCl, pH 8.0, 10 mM imidazole) and then eluted with the elution buffer (50 mM Tris-HCl, pH 8.0, 250 mM imidazole). The elution fraction was dialyzed overnight against 20 mM Tris-HCl, pH 8.0, and stored at −70 °C.

#### Protease activity analysis of EKL-"His\(_6\)"

Using recombinant GST-VAS (GST-vasostatin) fusion protein containing an EK cleavage site as substrate, the cleavage activity of EKL-"His\(_6\)" was determined. Quantitative analysis of SDS-PAGE was performed by software Grab-it 2.5 and Gelwork (UVP) and the cleavage efficiency towards substrate was calculated. For the quantitative cleavage efficiency assay, 0, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 or 16.0 ng EKL-"His\(_6\)" was added to 20 \(\mu\)g GST-VAS respectively and incubated at 16 °C for 16 h. Reaction buffer was 50 mM Tris-HCl, pH 8.0.

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Results

Construction of expression vector and transformation into \( P. \) pastoris

\( \text{EK}^\_\text{His}_6 \) coding sequence was fused to the 3’ end of \( \alpha\)-MF secretion signal gene to get \( \alpha\)-MF signal-\( \text{EK}^\_\text{His}_6 \). Then the whole coding sequence was cloned into an integrative plasmid pAO815 (Fig. 1).

Expression and purification of recombinant \( \text{EK}^\_\text{His}_6 \)

The secreted expression of \( \text{EK}^\_\text{His}_6 \) was approximately 10 mg/L culture. 200 ml fermentation supernatant was dialyzed overnight against 20 mM Tris-HCl, pH 8.0 and loaded onto Ni\( ^2+ \) affinity column. The secreted \( \text{EK}^\_\text{His}_6 \) was easily purified since there are very few proteins in the \( P. \) pastoris fermentation supernatant. The SDS-PAGE of purified \( \text{EK}^\_\text{His}_6 \) was stained with Coomassie Brilliant Blue and exhibited homogenous with the molecular weight around 43 kD (Fig. 3).

Biological activity analysis of \( \text{EK}^\_\text{His}_6 \)

Cleavage activity of \( \text{EK}^\_\text{His}_6 \) was determined by utilizing GST-VAS fusion protein as substrate [Fig. 4(A)]. Quantitative cleavage efficiency curve [Fig. 4(B)] and time course curve were plotted (data not shown). The relationship between cleavage efficiency and enzyme/substrate ratio was investigated in detail and the results showed that after incubation for 16 h at 16 °C, 20 µg GST-VAS

pAO815-\( \text{EK}^\_\text{His}_6 \) was linearized by SalI and transformed into GS115 Pichia pastoris. Positive recombinant transformants were selected by PCR (Fig. 2) and utilized for secreted expression of recombinant \( \text{EK}^\_\text{His}_6 \). The DNA sequencing result is consistent with that of Chinese bovine enterokinase catalytic subunit we reported before [6] and it has two bases difference from the sequence reported by foreign scientists [2]. The 141th and 177th triplet codons for Ala (GCA) were substituted by Thr (ACA).
was effectively and almost completely cleaved by 16 ng recombinant EK₁-His₆.

Discussion

Fusion expression of heterologous protein is a popular strategy widely used in gene engineering to improve the yield and solubility of target protein, and made target protein easily purified [7–9].

Currently most commercial EKs are purified holoenzymes from bovine or porcine intestines, and are expensive. Even highly purified preparations are prone to be contaminated by traces of other gut proteases [6]. Researchers tried to produce EK₁ via recombinant DNA route, and EK₁ has been expressed in *E. coli* and methylotrophic yeast *Pichia pastoris* [6,10]. To avoid the formation of inclusion bodies and to get the biologically active recombinant EK₁, EK₁ was fused to DshA to obtain secreted expression in *E. coli*. But most products were inactive and the yield of recombinant active EK₁ was low, only 1 mg from 125 g of starting cell paste [4]. When expressed in *P. pastoris*, 6.3 mg of EK₁ was purified from one liter of fermentation culture [10].

In the present paper, secreted expression of EK₁-His₆ was fulfilled with the help of α-mating factor prepro secretion signal gene from *Saccharomyces cerevisiae*. The total length of α-MF signal peptide is 89 amino acids, but the auto-cleavage site is the 85th amino acid near the C-terminal of it and therefore there are four residual amino acids at the N-terminal of target protein. The sequence encoding these four amino acids were removed when primers were devised for amplifying α-MF signal gene and the 85 amino acids also exhibited the ability to lead secretion and auto-cleavage.

Glycosylation also impacts the heterologous protein...
expression in *P. pastoris* and should be considered. The sequence of EKL predicts three potential N-linked glycosylation sites and glycosylation in *P. pastoris* is more similar to that in mammalian cells [10]. Although excessive glycosylation can occur in *P. pastoris*, and the general pattern of glycosylation remains to be characterized, the activity of EKL-His<sub>6</sub> was not affected by the glycosylation. The molecular weight of the secreted EKL-His<sub>6</sub> is approximately 43 kD on SDS-PAGE, not the predicted 26.3 kD, due to glycosylation.

The yield of EKL-His<sub>6</sub> from one liter of *P. pastoris* fermentation (5.4 mg) was low compared to other proteins expressed in this system. The obtained EKL-His<sub>6</sub> displays excellent cleavage activity towards protein substrate and is capable of cleaving substrate thoroughly at a enzyme/substrate ratio of 1/1000 (*W*/*W*). Owing to the introduction of His<sub>6</sub>-tag, the purification process of EKL-His<sub>6</sub> is very simple and convenient by using a single step purification of Ni<sup>2+</sup> affinity chromatography. The addition of His<sub>6</sub>-tag does not impact the activity of EKL. Furthermore, protease EKL-His<sub>6</sub> can be easily removed or excluded from the clef target protein products by affinity chromatography. Considering the high price of available commercial EK, the establishment of recombinant Chinese bovine EK expression strain and the corresponding purification method has extensive practical foreground.

## References


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**Correction**


Synthesis and Characteristics of an Aspartame Analogue, *L*-Asparaginyl

*L*-3-Phenyllactic Acid Methyl Ester

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