ISSN 0582-9879 Acta Biochimica et Biophysica Sinica 2004, **36**(7): 513–517 **CN** 31-1300/Q

Short Communication

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 (EK_L) 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-EK_L-His_6 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 EK_L-His_6 was attained by methanol induction and its molecular weight is 43 kD. Because of the existence of His_6-tag, EK_L-His_6 was easily purified from *P. pastoris* fermentation supernatant by using Ni^{2+} affinity chromatography and the yield is 5.4 mg per liter of fermentation culture. This purified EK_L-His_6 demonstrates excellent cleavage activity towards fusion protein containing EK cleavage site.

Key words recombinant enterokinase; secreted expression; Ni^{2+} affinity chromatography; fusion protein cleavage

EK (enterokinase) is a serine proteinase which consists of a heavy chain and a light chain linked by a disulfide bond. The light chain of EK contains a chymotrypsin-like serine proteinase domain sufficient for the normal recognition and cleavage of EK substrate [1,2]. Under physiological condition, enterokinase recognizes amino acid sequence DDDDK↓X of trypsinogen and turns it into active trypsin after cleavage [3]. Enterokinase allows any downstream fusion target protein to retain its native N-terminus, without leaving any unwanted amino acid residues on their amino termini. Enterokinase retains its activity under a variety of reaction conditions and is capable of cleaving fusion proteins at wide pH values, ranging from 4.5 to 9.5, at temperatures ranging from 4 °C to 45 °C, and in the presence of various detergents and denaturants. Therefore EK is widely utilized as a tool protease in the research and production of gene engineering [4].

During the past 15 years, the methylotrophic yeast *Pichia pastoris* has developed into a highly successful system for the production of a variety of heterologous proteins [5]. The increasing popularity of this particular expression system can be attributed to several factors, most importantly: (1) the simplicity of techniques needed for the molecular genetic manipulation of *P. pastoris* and their similarity to those of *Saccharomyces cerevisiae*, one of the most well-characterized experimental systems in modern biology; (2) the ability of *P. pastoris* to produce foreign proteins at high levels, either intracellularly or extracellularly; (3) the capability of performing many eukaryotic posttranslational modifications, such as glycosylation; and (4) the availability of the expression system as a commercially available kit.

In the present paper, EK_L gene was fused to the 3′ end of α-MF secretion signal gene to get α-MF signal-EK_L-His_6 coding sequence, and the whole coding sequence was cloned into the integrative plasmid pAO815 and transformed into GS115 methylotrophic strain of *Pichia pastoris*. Secreted expression of recombinant EK_L-His_6 was attained by methanol induction. The secreted EK_L was

Received: April 19, 2004 Accepted: May 29, 2004

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

The study was supported by a grant from the Teaching and Research Award Program for the Outstanding Young Teachers in Higher Education Institutions of Ministry of Education of China

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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™ 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 α-mating factor prepro secretion signal peptide from *Saccharomyces cerevisiae* was amplified from plasmid pPIC9K (Invitrogen) using the upstream primer 1: 5′-c cgg tct cga att caa acg atg aga ttt cct tca att ttt act gc-3′ and downstream primer 2: 5′-c tct gga gtc act tcc tcc gac aat tct ttt ctc gag aga tac ccc ttc ttc-3′. The 705 bp fragment encoding the 235 amino acid residues of recombinant EK was PCR-amplified from previously reported EK_L expression plasmid pET32a-EK_L using the upstream primer 3: 5′-ctc gag aaa aga att gtc gga gga agt gac tcc aga g-3′ and downstream primer 4: 5′-c cgg tct cga att cta atg atg atg atg atg tag aaa act ttg tat cc-3′. The downstream primer 4 contains a sequence encoding the His_6-tag. Primer 2 and 3 were designed to be overlapped and paired so that EK_L could be integrated and fused to the 3′ end of α-MF secretion signal gene to get α-MF signal-EK_L-His_6 by PCR. PCR products of EK_L and α-MF signal were mixed, and PCR was performed as follows: denaturation at 94 °C for 1 min, annealing and extension at 68 °C for 5 min for 8 cycles. The resulting product α-MF signal-EK_L-His_6 was used as template, primer 1 and primer 4 were added into the mixture to amplify the α-MF signal-EK_L-His_6 gene. PCR cycles were as following: denaturation at 94 °C for 1 min, annealing at 62 °C for 1 min and extension at 72 °C for 1 min for 28 cycles.

The obtained DNA fragment was digested with *Eco*RI and cloned into the same site of pAO815 to get pAO815-EK_L-His_6. The orientation of the fragment was validated by restriction enzyme digestion by using *Bam*HI, located on the vector, and *Eco*RV, located inside the cloned fragment, and confirmed by DNA sequencing.

Transformation and screening

To generate recombinants, GS115 cells were transformed with *Sal*I-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^s 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 μl of culture was added into a 20 μl PCR reaction system, PCR were performed as recommended by Invitrogen's instruction.

Expression and purification of EK_L-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 EK_L-His_6 was collected.

0.2 L of EK_L-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, 10 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 EK_L-His_6

Using recombinant GST-VAS (GST-vasostatin) fusion protein containing an EK cleavage site as substrate, the cleavage activity of EK_L-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 EK_L-His_6 was added to 20 μg GST-VAS respectively and incubated at 16 °C for 16 h. Reaction buffer was 50 mM Tris-HCl, pH 8.0.

Results

Construction of expression vector and transformation into *P. pastoris*

EK_L-His_6 coding sequence was fused to the 3′ end of α-MF secretion signal gene to get α-MF signal-EK_L-His_6. Then the whole coding sequence was cloned into an integrative plasmid pAO815 (Fig. 1).

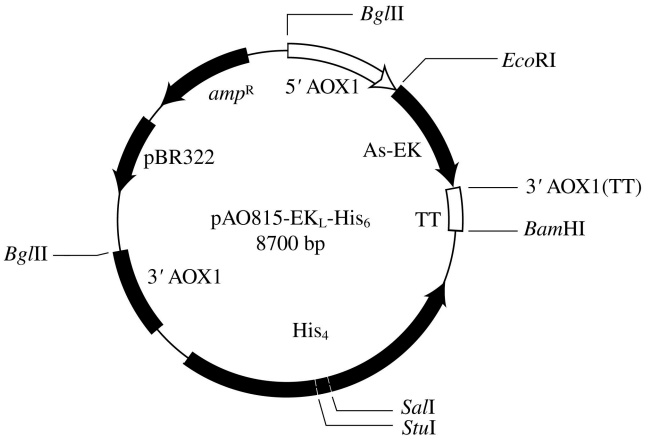

Fig. 1 Diagram of the recombinant expression plasmid pAO815-EK_L-His_6

Gene encoding α-MF signal-EK_L-His_6 was inserted into the *Eco*RI site of inte-grative plasmid pAO815 to get pAO815-EK_L-His_6.

pAO815-EK_L-His_6 was linearized by *Sal*I and transformed into GS115 *Pichia pastoris*. Positive recombinant transformants were selected by PCR (Fig. 2) and utilized for secreted expression of recombinant EK_L-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).

Expression and purification of recombinant EK_L-His_6

The secreted expression of EK_L-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 EK_L-His_6 was easily purified since there are very few proteins in the *P. pastoris* fermentation supernatant. The SDS-PAGE of purified EK_L-His_6 was stained with Coomassie Brilliant Blue and exhibited homogenous with the molecular weight around 43 kD (Fig. 3).

Biological activity analysis of EK_L-His_6

Cleavage activity of EK_L-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

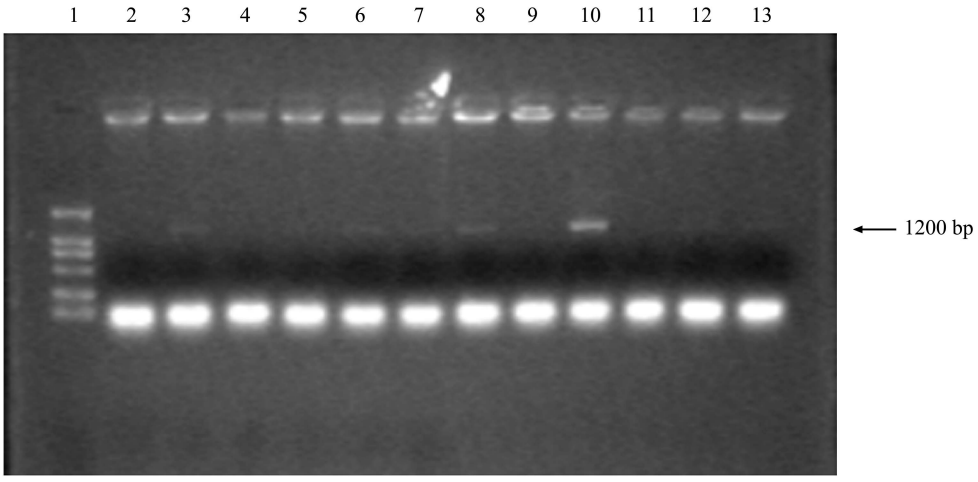

Fig. 2 PCR screening for pAO815-EK_L-His_6/GS115 positive clones

1, DL2000 DNA marker; 2–13, PCR screening results of 12 pAO815-EK_L-His_6/GS115 clones. The arrow at 1200 bp indicates the PCR product of α-MF signal-EK_L-His_6 expression cassette in positive transformants using 5′ and 3′ AOX1 primers.

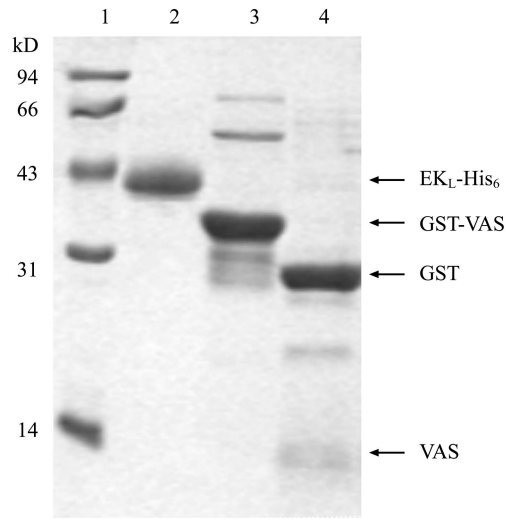

Fig. 3 SDS-PAGE analysis of purity and activity of recombinant EK$_L$-His$_6$

1, protein molecular weight markers are 94, 66, 43, 31, and 14 kD, respectively, from top to bottom; 2, the purified EK$_L$-His$_6$; 3, GST-VAS fusion protein containing EK cleavage site; 4, cleavage product of GST-VAS by EK$_L$-His$_6$.

was effectively and almost completely cleaved by 16 ng recombinant EK$_L$-His$_6$.

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 EK$_L$ via recombinant DNA route, and EK$_L$ 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 EK$_L$, EK$_L$ was fused to DsbA to obtain secreted expression in *E. coli*. But most products were inactive and the yield of recombinant active EK$_L$ was low, only 1 mg from 125 g of starting cell paste [4]. When expressed in *P. pastoris*, 6.3 mg of EK$_L$ was purified from one liter of fermentation culture [10].

In the present paper, secreted expression of EK$_L$-His$_6$ 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

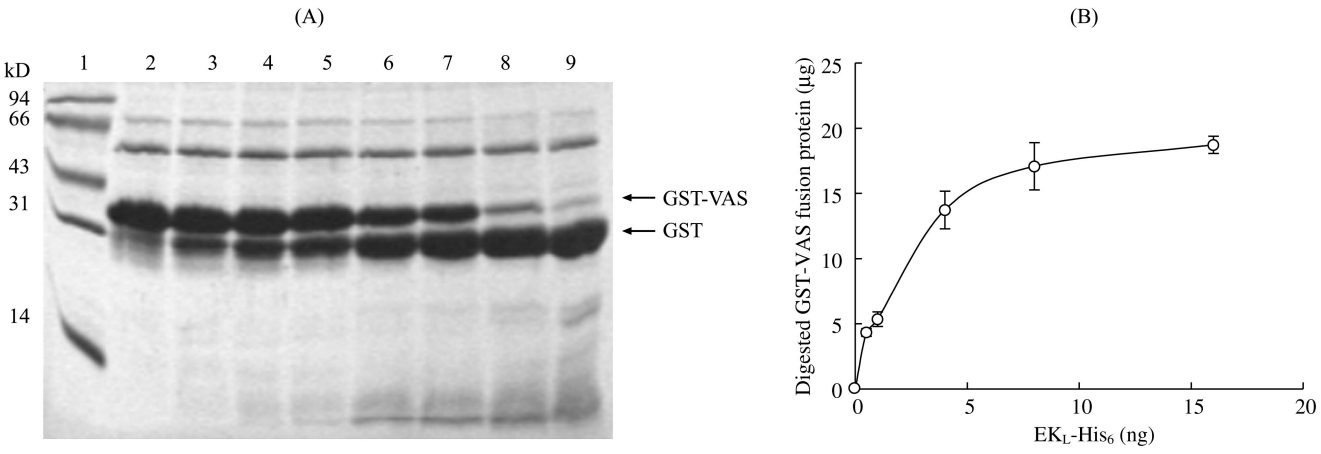

Fig. 4 SDS-PAGE and quantitative analysis of GST-VAS fusion protein digested by EK$_L$-His$_6$

(A) SDS-PAGE analysis. 20 μg GST-VAS was incubated at 16 °C for 16 h in 50 mM Tris-HCl, pH 8.0, with different doses of EK$_L$-His$_6$ (0, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, or 16.0 ng, respectively). All samples were analyzed by 12% SDS-PAGE. Cleavage of the 36 kD GST-VAS protein produced a 28 kD GST and an 8 kD VAS products. (B) Quantitative analysis. The enzyme/substrate correlation curve of EK$_L$-His$_6$ digestion was plotted according to the quantitative analysis of SDS-PAGE performed by software.

expression in *P. pastoris* and should be considered. The sequence of EK_L 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 EK_L-His_6 was not affected by the glycosylation. The molecular weight of the secreted EK_L-His_6 is approximately 43 kD on SDS-PAGE, not the predicted 26.3 kD, due to glycosylation.

The yield of EK_L-His_6 from one liter of *P. pastoris* fer-mentation (5.4 mg) was low compared to other proteins expressed in this system. The obtained EK_L-His_6 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_6-tag, the purification process of EK_L-His_6 is very simple and convenient by using a single step puri-fication of Ni^{2+} affinity chromatography. The addition of His_6-tag does not impact the activity of EK_L. Furthermore, protease EK_L-His_6 can be easily removed or excluded from the cleft target protein products by affinity chromatography. Considering the high price of available commercial EK, the establishment of recombinant Chinese bovine EK_L expression strain and the corresponding purification method has extensive practical foreground.

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Edited by
Xiang-Fu WU

Correction

2004, 36(6): 385–389

Synthesis and Characteristics of an Aspartame Analogue, *L*-Asparaginyl *L*-3-Phenyllactic Acid Methyl Ester

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