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High-level secretory production of recombinant bovine enterokinase light chain by *Pichia pastoris*

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Abstract

Enterokinase (EC 3.4.21.9) is a serine proteinase with a specific digest sequence $(Asp)_4$-Lys in the duodenum. Its high specificity for the recognition site makes enterokinase (EK) a useful tool for an in vitro cleavage of fusion proteins. In this work, an active bovine enterokinase light chain (EK_L) was produced in secretory form by a recombinant strain of the methylotrophic yeast *Pichia pastoris*. The influences of methanol utilization phenotype of the host strain, induction pH, and carbon source on the recombinant production were studied. The production of recombinant EK_L by Mut^s strain was much higher than that by Mut^+ strain. When inducted at pH 6.0, on a glycerol/methanol medium, the concentration of recombinant EK_L (rEK_L) reached 350 mg l^{-1}, which was 20-fold higher than that reported previously. The recombinant EK_L was purified in a simple procedure on the anion exchange chromatography and 15 mg pure active EK_L were obtained from 100 ml culture broth supernatant. The specific activity of purified rEK_L was approximately 9000 u mg^{-1}. To facilitate purification and removal of rEK_L after cleavage of fusion protein, the C-terminal His-tagged EK_L (EK_L/His) was also expressed in *P. pastoris*, and this His-tagged EK_L exhibited a similar enzymatic activity to the untagged EK_L.

Keywords: *Pichia pastoris*; Enterokinase; Heterologous protein expression

1. Introduction

Enterokinase (EC 3.4.21.9) is a serine proteinase in the duodenum that plays a critical role in mammalian digestion. It is the physiological activator of pancreatic trypsinogen. It converts trypsinogen into its active form trypsin, by cleaving its aminoterminal hexapeptide $Val(Asp)_4$-Lys (Kunitz, 1939; Yamashina, 1956). More recently, the enterokinase has been shown to have a broad utility in cleaving fusion proteins produced in *Escherichia coli* (LaVallie et al., 1993). The enzyme is particularly suitable for this role because of its high degree of specificity, its tolerance to a wide range of reaction conditions, and the fact that its recognition sequence lies entirely on the aminoterminal side of the scissile bond. This enzymatic activity allows release of carboxyl-terminal fusion partners from fusion proteins without leaving unwanted amino acid

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residues on their amino termini (Collins-Racie et al., 1995; Hosfield and Lu, 1999).

Partial purifications of enterokinase from porcine, bovine, and human have been reported (Maroux et al., 1971; Liepnieks and Light, 1979; Grant and Hermon-Taylor, 1976). All these purified enzymes have been shown to be serine proteases by their shared susceptibility to inhibitors such as diisopropyl fluorophosphates and tosyl-L-lysine chloromethyl ketone. Bovine enterokinase is a disulfide-linked heterodimer comprised of a 35 kDa light chain and a 115 kDa heavy chain derived from a single-chain precursor (Kitamoto et al., 1994). The heavy chain plays a role in trypsinogen recognition and membrane association (Fonseca and Light, 1983), and the light chain is released by partial reduction of the heterodimer, and exhibits the activity and specificity (Light and Fonseca, 1984). The cDNA encoding bovine enterokinase light chain has been isolated and expressed in various hosts: mammalian COS cells (LaVallie et al., 1993), *E. coli* (Collins-Racie et al., 1995; Yuan and Hua, 2002), filamentous fungus (Svetina et al., 2000), and methylotrophic yeast *Pichia pastoris* (Vozza et al., 1996). However, the applications of rEK_L have not been fully exploited because of its low expression level and high cost.

In this paper we describe a successful production of large amounts of EK_L and EK_L/His in *P. pastoris* by using fed-batch culture. The influence of methanol utilization phenotype of the host strain, induction pH, and carbon source is studied subsequently. A simple purification procedure is also reported.

2. Materials and methods

2.1. Materials

All restriction enzymes were purchased from New England Biolabs. Chelating Sepharose, and Q Sepharose were purchased from Pharmacia-LKB. Plasmid pPICZαA and *P. pastoris* GS115 were purchased from Invitrogen.

2.2. Gene construction and plasmids construction

A synthetic gene encoding wild-type bovine enterokinase light chain was designed on the basis of its amino acid sequence and preferred codons of *P. pastoris* (Sreekrishna et al., 1997) and cloned into the *P. pastoris* expression vector pPICZαA (Fig. 1). The constructed vector pPICZαA-EK_L contains complete EK_L structural gene downstream α-factor sequence and the vector pPICZαA-EK_L/His contains His-tagged sequence downstream EK_L sequence.

2.3. Transformation of P. pastoris and selection of transformants

P. pastoris GS115 strain was transformed with *Sal*I-linearized pPICZαA-EK_L and pPICZαA-EK_L/His by electroporation following the manufacturer's instructions (Invitrogen). All Zeocin-resistant colonies were replica-plated onto MMH plates (1.34% YNB, 0.5% methanol, 1.61 uM biotin, 0.004% histidine, 1.5% agar) and MDH plates (same composition as

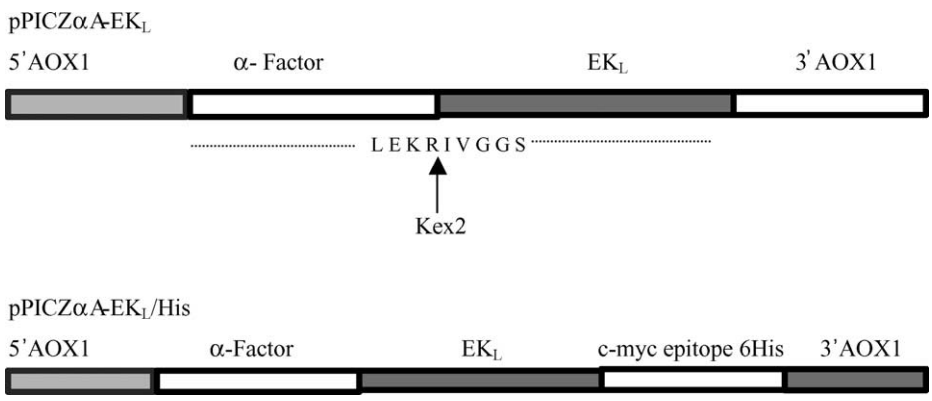

Fig. 1. Schematic representation of the EK_L expression vectors used.

MMH but with 2% glucose instead of methanol) to determine the methanol-utilizing phenotypes. After 3–4 days of incubation, the Mut^+ phenotypes grew normally on both MMH and MDH, whereas the Mut^s phenotypes grew very slowly on MMH plates. Both Mut^s and Mut^+ strains were used for shake-flask cultivation.

2.4. Shake-flask cultivation of P. pastoris

A single colony of transformants was grown with 100 ml of BMGYH (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 1.61 uM biotin, 0.004% histidine, 1% glycerol) in 500 ml shaking flask until O.D.600 = 5 at 30 °C. The growth culture was centrifuged and the pellet was suspended with 20 ml of BMMYH (identical to BMGYH except for 0.5% methanol instead of 1% glycerol). The resuspended culture was grown for 48 h by addition of 0.5% methanol every 24 h. Samples were taken for the detection of the recombinant expression by Western blot using the rabbit-anti-enterokinase serum.

2.5. Fed-batch culture studies

All fed-batch cultures were carried out in a 7-l bioreactor. Seed culture for the bioreactor was started from the fresh glycerol stock and inoculated directly into 500 ml baffled flasks (50 ml working volume) containing minimum glycerol medium (1.34% YNB, 1% glycerol, 1.61 uM biotin, and 0.004% histidine). After 24 h of growth at 30 °C, seed culture was inoculated with 1% inoculum, then grown for 16–20 h and finally used to inoculate the bioreactor. Ten percent inoculum was used for inoculation of a 7-l bioreactor containing 3 l of medium ($(NH_4)_2SO_4$ 20 g l^{-1}; glycerol 40 g l^{-1}; KH_2PO_4 12 g l^{-1}; $CaSO_4{\cdot}2H_2O$ 1 g l^{-1}; histidine 0.4 g l^{-1}; PTM1 ($CuSO_4{\cdot}5H_2O$ 6.0 g l^{-1}, NaI 0.08 g l^{-1}, $MgSO_4{\cdot}H_2O$ 3.0 g l^{-1}, $ZnCl_2$ 20.0 g l^{-1}, HBO_4 0.02 g l^{-1}, $FeSO_4{\cdot}7H_2O$ 65 g l^{-1}, $CoCl_2$ 0.5 g l^{-1}, Biotin 0.2 g l^{-1}, $NaMnO_4{\cdot}2H_2O$ 0.2 g l^{-1}, H_2SO_4 4 ml l^{-1}) 4.35 ml l^{-1}, buffered to pH 5.5 using 2 M NH_4OH). Dissolved oxygen was kept at over 30% air saturation, temperature was kept at 30 °C and aeration was maintained at 2 v.v.m. Fermentation was carried out in two phases as follows.

Growth phase: this phase consisted of a glycerol batch phase until glycerol in the medium was utilized. To achieve a high cell density, the glycerol (50% glycerol, 4.3 ml PTM1, feeding rate: 18 ml h^{-1} l^{-1}) fed-batch phase was initiated and lasted for 4 h.

Production phase (induction phase): this phase consisted of a methanol fed-batch phase where cells were induced by methanol. The feed-medium used for this phase contained: methanol (100% methanol, 12 ml l^{-1} PTM1, feeding rate: 3 ml h^{-1} l^{-1}) or methanol + glycerol (50% methanol, 50% glycerol, 12 ml l^{-1} PTM1, feeding rate: 6 ml h^{-1} l^{-1}) or methanol + sorbitol (50% methanol, 50% sorbitol, 12 ml l^{-1} PTM1, feeding rate: 6 ml h^{-1} l^{-1}). During this phase the pH of cell batch of cultured medium was kept at a fixed value. Batches at pH 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5 were tested.

2.6. Purification of the secreted rEK_L

One hundred milliliter of cultured medium supernatant was dialyzed overnight in TEB (10 mM Tris–Cl, pH 8.0, 1 mM EDTA, 10 mM β-mercaptoethanol). The dialysate was loaded onto 1.6 × 10 cm Q Sepharose fast flow column equilibrated with TEB. Protein was eluted with a gradient of 0.0–0.5 M NaCl. The EK_L fractions were pooled and dialyzed overnight against a solution containing 50 mM potassium phosphate, 50 mM NaCl and 50% glycerol, pH 8.0.

2.7. Purification of the secreted rEK_L/His

One hundred milliliter of cultured medium supernatant was dialyzed overnight in 20 mM sodium phosphate buffer (pH 7.5) containing 300 mM NaCl (buffer A) and applied to a nickel chelating sepharose column (1.6 × 10 cm) pre-equilibrated with buffer A. Protein was eluted with a gradient of 0.0–0.5 M imidazole. The rEK_L/His fractions were pooled and dialyzed overnight against a solution containing 50 mM potassium phosphate, 50 mM NaCl and 50% glycerol, pH 8.0.

2.8. Analysis of proteins

The purity of the protein sample was checked by 12% SDS-PAGE. The protein concentration of the enzyme was determined using the Bio-Rad dye agent with BSA as a standard and the N-terminal sequence of rEK_L was determined by Edman

degradation method on Applied Biosystems Procise sequencer. Enterokinase activity was determined by using the fluorogenic enterokinase substrate Gly-Asp-Asp-Asp-Asp-Lys-β-naphthylamide (Sigma; Grant and Herman-Taylor, 1979). Fluorescence was measured by excitation at 337 nm and emission at 420 nm. The activity of rEK_L was also detected by cleavage of the fusion protein TRX/PTH harbouring the EK recognition sequence DDDDK in the linker. EK_L from Invitrogen (EKMax^{TM}) was used as a standard to which the measured values were aligned.

3. Results and discussion

3.1. Influence of methanol utilization phenotype of the host on EK_L production

The expression level of recombinant protein by Mut^s and Mut$^+$ strains may be different (Sreekrishna et al., 1997). So at first we investigated the expression of recombinant EK_L by both Mut^s and Mut$^+$ strains in shake flasks. Western blot revealed that the EK_L level expressed by Mut^s strain was much higher than that of Mut$^+$ (Fig. 2B), so Mut^s was chosen for fed-batch culture. In addition, Mut^s phenotype was selected over the Mut$^+$ phenotype because of the latter's higher oxygen requirement that can result in oxygen-deficient conditions within the bioreactor.

3.2. Fed-batch culture studies

3.2.1. Effect of pH

P. pastoris is known to grow over a wide pH range, from 3 to 7, with minimal effect of pH on the growth rate. However, pH has been shown to significantly affect secreted recombinant proteins due to protease activity in the fermentation broth (Cregg et al., 1993). In order to find out the optimal pH for the expression of recombinant EK_L, we conducted experiments under pH between 4 and 6.5 during the fed-batch production phase.

Fed-batch cultures of *P. pastoris* were maintained at pH 5.5 for the growth phase. This phase lasted for approximately 24 h, followed by a 5 h glycerol fed-batch phase. During this phase the pH was adjusted to the desired value and then maintained for the remainder of the experiment. As shown in Fig. 2A, the highest yield of recombinant EK_L was observed at pH 6.0.

3.2.2. Effect of carbon source and induction time

The influence of the carbon source on EK_L production was studied by monitoring concentrations and yields. After a batch phase on glycerol, the fed-batch phase on either pure methanol, methanol + glycerol or methanol + sorbitol was studied. The kinetics of biomass and concentration of rEK_L over 144 h cultivation was followed by analyzing samples removed from the cultured broth. As shown in Fig. 3, when induced

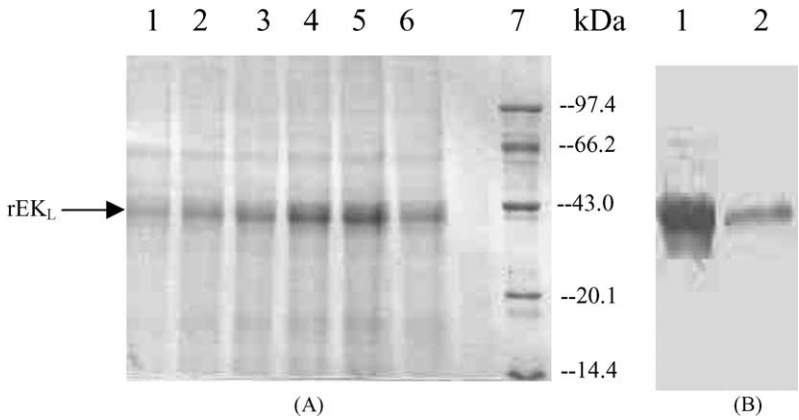

Fig. 2. (A) SDS-PAGE of crude cultured broth supernatant proteins induced at different pHs. Lanes 1, 2, 3, 4, 5, 6 correspond to experiments done with pHs of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, respectively; Lane 7: protein standard. (B) Western blot analysis of secreted rEK_L: 1 shows GS115 carrying pPICZαA-EK_L (Mut^s); 2 shows GS115 carrying pPICZαA-EK_L (Mut$^+$).

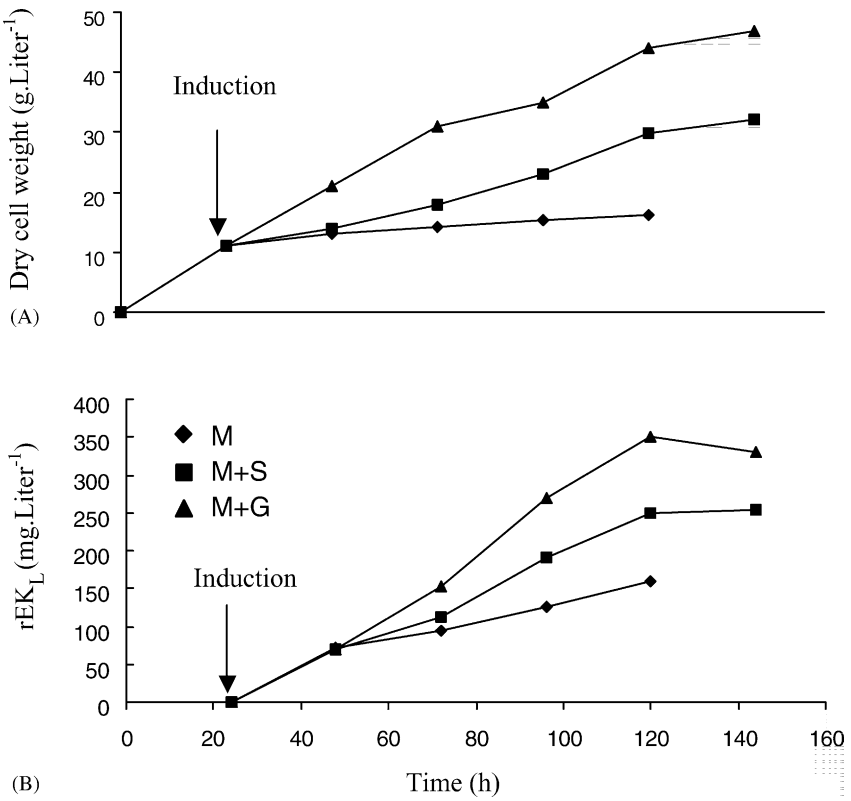

Fig. 3. Effects of different induction conditions on cell and rEK_L concentrations of *P. pastoris* during fed-batch culture. (A) Cell concentrations; (B) rEK_L concentrations; G, glycerol; M, methanol; S, sorbitol.

by pure methanol for 96 h, the concentration of rEK_L had reached 150 mg l^{-1}, and biomass was 16.3 g l^{-1} DCW. While adding glycerol or sorbitol, the concentration of rEK_L reached 350 and 250 mg l^{-1}, respectively. Adding glycerol resulted in a 2.3-fold concentration increase of rEK_L compared to pure methanol. We found that glycerol demonstrated beneficial effect in supporting cell growth and improving final EK_L concentration over sorbitol. We also investigated the influence of adding casamino acids during production phase, but we found that casmino acids had little effect on the expression level of rEK_L. Compared to the shake flask procedure, the culture of *P. pastoris* using high cell density fed-batch cultivation generated a significantly high level expression of rEK_L. A 20-fold increase in rEK_L concentration was observed in culture medium compared to the report (Vozza et al., 1996).

The induction time also has effect on the production of EK_L (Fig. 3). After 96 h of induction, the concentration of EK_L reached peak value. A longer induction period more than 120 h led to a decrease of recombinant protein.

3.3. Purification of rEK_L and rEK_L/His

The recombinant EK_L was purified to homogeneity from cultured broth supernatants by a simple procedure on Q Sepharose Fast Flow chromatography (Fig. 4A). This procedure is highly efficient in providing large amounts of pure enzyme, and 15 mg of pure recombinant EK_L were obtained from 100 ml of crude extract (Table 1). The N-terminal sequence of the recombinant enzyme was determined to be IVGGS which is the same as native bovine EK_L. Using nickel

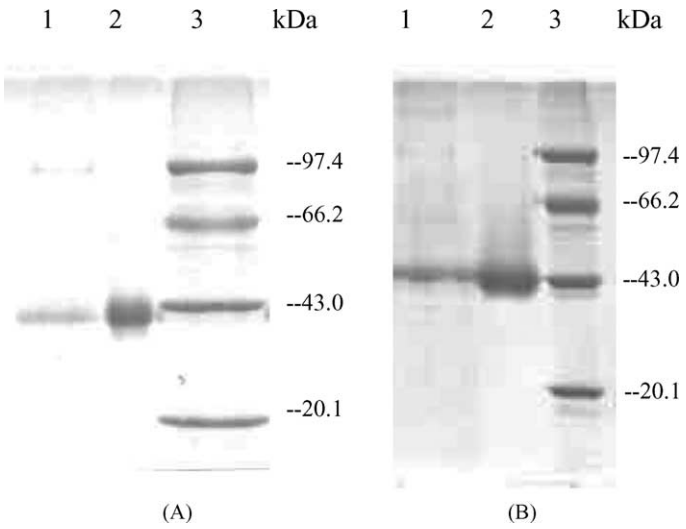

Fig. 4. SDS-PAGE analysis of purification of rEK_L and rEK_L/His: (A) 1, crude extract of rEK_L; 2, rEK_L eluted from Q Sepharose Fast Flow; 3, protein standard; (B) 1, crude extract of rEK_L/His; 2, rEK_L/His eluted from Ni^{2+} chelating Sepharose Fast Flow; 3, protein standard.

chelating sepharose chromatography, rEK_L/His could be easily purified to homogeneity (Fig. 4B).

3.4. Enzymatic assays of rEK_L and rEK_L/His

The estimated molecular weight of rEK_L on SDS-PAGE was approximately 41 kDa. The activity of rEK_L was compared to the activity of the standard (Invitrogen EKMAX™). Fluorometric assay showed that the specific activity of our rEK_L was approximately 9000 u mg^{-1}, while rEK_L/His had a molecular weight of 44 kDa and 8000 u mg^{-1} specific activity. Previously we have expressed rEK_L in *E. coli*, but the activity of rEK_L from *E. coli* was very low. Other reports of rEK_L expressed by *E. coli* also showed the low activity (Collins-Racie et al., 1995; Yuan and Hua, 2002). Svetina et al. (2000) reported that the specific activity of rEK_L expressed by filamentous fungus *A. niger* was 19,880 u mg^{-1} which was approximately twice the specific activity of rEK_L expressed by *P. pastoris*. Considering the different glycosylation pattern among these enzymes, we concluded that glycosylation was important to the activity of EK_L.

Table 1
Purification of recombinant EK_L

Sample	Volume (ml)	rEK_L% of total protein	Total protein (mg)	Yield (%)
Fermentation broth	100	27	130	100
Dialyzation	124	29	114	94
Q Sepharose	6	95	15	41

The activity of rEK_L was also determined by cleavage of the fusion protein TRX/PTH (TRX, thioredoxin; PTH, parathyroid hormone). Under optimal conditions the PTH product with expected size of 9 kDa was well detected on SDS-PAGE (Fig. 5). The N-terminal sequence analysis of the product confirmed that correct cleavage occurred at the expected DDDDK recognition sequence of the fusion protein. But when the reaction time prolonged, the target protein (PTH) was degraded obviously. Further degradation of the protein in cleavage reaction was probably due to an intrinsic broad specificity of the enzyme (Choi et al., 2001). Thus removal of the enzyme is highly recommended to prevent further degradation of the target protein after cleavage of fusion protein using benzamidine or soybean trypsin inhibitor chromatography. For efficient purification and post-cleavage removal of the enzyme, we also expressed the rEK_L with C-terminal His-tag by *P. pastoris* that exhibited activity equivalent to untagged EK_L. The His-tagged EK_L could be easily removed by a single passage of the reaction mixture on nickel chelating column. The nickel chelating chromatography has advantages over benzamidine or soybean

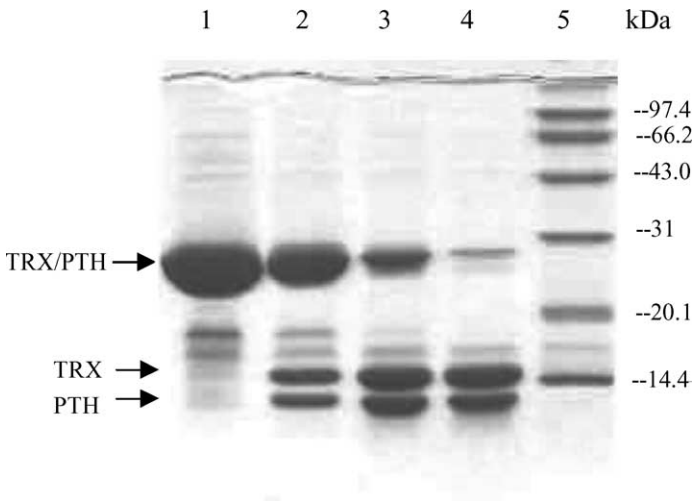

Fig. 5. Cleavage of fusion protein with purified rEK_L. The fusion protein was cleaved in 50 mM Tris–HCl (pH 8.0) with purified rEK_L for 2, 4, 8 h at 37 °C. The cleaved products were applied on 15% SDS-PAGE and stained with Coomassie Blue. Lane 1: fusion protein only; Lane 2: 2 h; Lane 3: 4 h; Lane 4: 8 h; Lane 5: protein standard.

trypsin inhibitor chromatography for removal of the enzyme, since the resin is stable, inexpensive, and highly specific (Porath et al., 1975). Furthermore, the His-tag tightly binds to metal chelating matrix under broad conditions such as high salt, detergent, and denaturant. Therefore, the cleavage reaction could be conducted on a wide range of conditions tailor-made to the protein of interest.

4. Conclusion

Recombinant bovine EK_L was expressed in *P. pastoris* using a fed-batch culture system. The influences of pH, carbon source and induction time were studied. By optimizing the fermentation conditions, the expression level of rEK_L reached 350 mg l^{-1}, and the secreted rEK_L could be purified in a simple procedure. To facilitate purification and removal of rEK_L after cleavage of fusion protein, EK_L with C-terminal His-tag was also expressed in *P. pastoris*. The results of the present study demonstrate that *P. pastoris* represents a viable host for the heterologous expression of functional EK_L.

Acknowledgements

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