การโคลนและผลิตเอ็นไซม์เอนเทอโรไคเนสสายสั้นจากวัว

นางสาวชนิดา กุประดิษฐ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต สาขาวิชาเทคโนโลชีวภาพ
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ปีการศึกษา 2549

BOVINE ENTEROKINASE LIGHT CHAIN

CLONING AND PRODUCTION

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A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Biotechnology

Suranaree University of Technology

Academic Year 2006

BOVINE ENTEROKINASE LIGHT CHAIN CLONING AND PRODUCTION

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ชนิดา กุประดิษฐ์ : การโคลนและผลิตเอ็นไซม์เอนเทอโรไคเนสสายสั้นจากวัว
(BOVINE ENTEROKINASE LIGHT CHAIN CLONING AND PRODUCTION)
อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.มารินา เกตุทัต-คาร์นส์, 156 หน้า.

เอนเทอโรไคเนสเป็นเอ็นไซม์จำพวกซีรีนโปรติเอสซึ่งจะตัดพันธะเปปไทด์ทางด้านปลายคาร์บอกซี่ของตำแหน่งตัดจำเพาะ (Asp_4Lys) และเนื่องจากเอ็นไซม์เอนเทอโรไคเนสสามารถทำงานได้ในสภาวะที่หลากหลาย จึงทำให้เอนเทอโรไคเนสมีความเหมาะสมอย่างยิ่งในการนำมาใช้ตัดฟิวชันโปรตีนที่ตำแหน่งจำเพาะ ในงานวิจัยนี้ ผู้วิจัยได้ทำการโคลนและผลิตเอ็นไซม์เอนเทอโรไคเนสสายสั้นในระบบรีคอมบิแนนท์ (rEK_L) จากลำไส้วัว

จากการเพิ่มจำนวนดีเอนเอของยีนเอนเทอโรไคเนสสายสั้น (EK_L) จากลำไส้วัวและควายในประเทศไทยโดยเทคนิค RT-PCR และ nested PCR พบว่า ผลิตภัณฑ์ดีเอนเอที่เพิ่มจำนวนได้มีขนาด 708 เบส และทำนายลำดับกรดอะมิโนได้ 235 กรดอะมิโน ซึ่งลำดับกรดอะมิโนของโปรตีน EK_L จากควายที่ได้จากงานวิจัยนี้มีความใกล้เคียงกับลำดับกรดอะมิโนของโปรตีน EK_L จากวัวที่มีการรายงานจากงานวิจัยที่ผ่านมา และพบว่ากรดอะมิโนของยีน EK_L จากวัวในประเทศไทยมีความแตกต่างเพียง 1 ตำแหน่งเท่านั้น ในขั้นตอนการชักนำยีน EK_L จากวัวที่ได้นี้ให้เกิดการแสดงออกและทำเอ็นไซม์ให้บริสุทธิพบว่า rEK_L ที่ผลิตในระบบโปรคาริโอตไม่สามารถทำงานได้ แต่ rEK_L ที่ได้จากการผลิตในระบบยูคาริโอตโดยใช้ *Pichia pastoris* สายพันธุ์ Y11430 สามารถทำงานได้และสามารถตรวจพบกิจกรรมของเอ็นไซม์นี้ในอาหารที่ได้จากการเลี้ยง *Pichia* ในถังหมักในช่วงที่มีการชักนำให้เกิดการแสดงออกของเอ็นไซม์นี้ในระบบรีคอมบิแนนท์ด้วยเมธานอล ในระหว่างกระบวนการเหนี่ยวนำให้เกิดการแสดงออกนั้นพบว่า การผลิตเอ็นไซม์ที่อุณหภูมิต่ำนั้นไม่สามารถปรับปรุงคุณภาพของเอ็นไซม์ที่ผลิตให้มีคุณภาพที่ดีขึ้น แต่สามารถเพิ่มผลผลิตของโปรตีนในระบบรีคอมบิแนนท์ได้ หลังจากสิ้นสุดกระบวนการทำเอ็นไซม์ให้บริสุทธิโดยเทคนิคการแลกเปลี่ยนประจุพบว่า ความเข้มข้นของ rEK_L บริสุทธิที่ได้คือ 433 มิลลิกรัมต่อลิตรจากการเหนี่ยวนำให้เกิดการแสดงออกของ rEK_L ที่อุณหภูมิต่ำ สำหรับการทดสอบคุณสมบัติต่าง ๆ ของ rEK_L ที่บริสุทธิพบว่า rEK_L ที่ได้จากงานวิจัยนี้มีกิจกรรมเอ็นไซม์ลดลงอย่างชัดเจนเมื่ออยู่ในสภาวะที่มีค่า pH ต่ำ สำหรับความสามารถในการตัดฟิวชันโปรตีนที่มีตำแหน่งตัดของเอ็นไซม์ EK (Asp_4Lys) พบว่าเพียง 0.09 ไมโครกรัม ของ rEK_L ที่บริสุทธิซึ่งได้จากงานวิจัยนี้สามารถตัด 6 ไมโครกรัม สับเสรตที่เป็นฟิวชันโปรตีนของ rice BGlu1-thioredoxin ได้มากกว่า 95 % เมื่อบ่มที่ 30 องศาเซลเซียสเป็นเวลา 21 ชั่วโมง ซึ่งผลิตภัณฑ์ที่ได้จากการตัดฟิวชันโปรตีนด้วย rEK_L ที่ได้จากงานวิจัยนี้และ

rEK_L ที่ผลิตขายตามท้องตลาดนั้นมีรูปแบบของแถบโปรตีนที่ปรากฏบนเจล SDS-PAGE คล้ายคลึงกัน

สาขาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

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CHANIDA KUPRADIT : BOVINE ENTEROKINASE LIGHT CHAIN CLONING AND PRODUCTION. THESIS ADVISOR : ASST. PROF. MARIENA KETUDAT-CAIRNS, Ph.D. 156 PP.

BOVINE ENTEROKINASE LIGHT CHAIN/CLONING/FUSION PROTEIN

Enterokinase is a serine protease which catalyzes the hydrolysis of peptide bonds at the C-terminal end of the specific cleavage site $(Asp)_4Lys$. It retains full activity in various reaction conditions, which makes it suitable for site-specific cleavage of fusion proteins. In this research, cloning and production of recombinant bovine enterokinase light chain (rEK_L) were achieved.

Thai bovine and buffalo EK_L gene amplification by RT-PCR and nested PCR produced 708 bp PCR products, which encoded 235 predicted amino acids. Only one amino acid mutation was found in the Thai bovine EK_L. The obtained protein sequence of Thai buffalo EK_L in this research was closely related to that previously reported for bovine EK_L. In the step of bovine rEK_L expression and purification, rEK_L active could be obtained from eukaryotic expression system using *Pichia pastoris* Y11430, but not in the prokaryotic system. The enzymatic activity was detected in the recombinant *Pichia* fermentor culture supernatant during the methanol production phase. Low temperature production did not improve the quality of rEK_L, but did increase the yield of recombinant protein. After ion exchange purification, 433 mg/L of purified rEK_L was obtained from fermentation under the low induction temperature condition. The properties of the obtained purified rEK_L were also observed. The enzymatic activity of rEK_L was reduced significantly at low pH. The ability of rEK_L to cleave a specific $(Asp)_4Lys$ site of rice BGlu1-thioredoxin fusion proteins showed that

more than 95% of 6 µg fusion protein was cleaved by 0.09 µg of the purified rEK_L in 21 h at 30°C. The products of cleavage of the fusion protein with commercial rEK_L and rEK_L from this research showed similar patterns on SDS-PAGE.

School of Biotechnology Student's Signature_____

Academic Year 2006 Advisor's Signature_____

Co-advisor's Signature _____

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LIST OF ABBREVIATIONS

AOX	alcohol oxidase enzyme
DOT	dissolved oxygen tension (%)
DsbA	disulfide oxidoreductase
EK_L	enterokinase light chain enzyme
GBS	glycerol basal salt medium
GF	glycerol feed medium
MF	methanol feed medium
MLFB	methanol-limited fed-batch
Ni-IDA	nickel- iminodiacetic acid column
Ni-NTA	nickel-nitrilotriacetic acid
OLFB	oxygen-limited fed-batch
OTR	oxygen transfer rate (mol/h)
OUR	oxygen uptake rate (mol/h)
PCR	polymerase chain reaction
pI	isoelectric point
rEK_L	recombinant enterokinase light chain
RT	reversed transcription
SP column	sulfopropyl column
STI–Sepharose	soy bean trypsin inhibitor-sepharose column
Trx	thioredoxin fusion partner
$Y_{p/x}$	specific production yield on methanol (unit/g cell)

LIST OF ABBREVIATIONS (Continued)

$Y_{x/s}$	biomass yield on methanol (g cell/ g methanol)
μ	specific growth rate (1/h)

CHAPTER I

INTRODUCTION

1.1 Introduction

Recently, with advances in genetic engineering, the use of fusion protein technologies as a tool for recombinant protein production is well known in the biopharmaceutical and biotechnology industry. Fusing the coding sequence for a desired recombinant protein to a well expressed gene has several advantages. Most fusion protein strategies position the protein of interest at the C-terminal of the highly expressed fusion partner, which allows translation initiation to occur on a "*proven*" gene sequence that is known to be well translated, thus high expression levels of the desired protein will occurred (Huang et al., 2004). To obtain the interested protein in the purification step, the fusion tagged is removed by chemical reagents or enzymes. Compare with the chemical reaction of using cyanogens bromide (CNBr), enzymatic cleavage has more advantages such as higher specificity and milder reaction conditions (Suh et al., 2004). An important requirement for any fusion protein expression technology is the availability of site-specific proteases to cleave the interested protein from the fusion partner (Song et al., 2002). Several highly specific proteases have been used for this purpose, including factor Xa, thrombin and enterokinase. Cleavage can occur within the recognition sequence, thus any amino acid downstream of the cleavage point are retained by the target protein. The

complete removal of tag sequence requires that the cleavage point reside on the C-terminal side of recognition sequence. Only factor Xa and enterokinase cleave target on the C-terminal side of the recognition sequence allowing complete removal of affinity tag sequence (McCormick and Berg, n.d.). Enterokinase displays a high degree of specificity thus it is suitable for preparing target protein by cleavage at the fusion junction (Song et al., 2002).

More recently, the enterokinase has been shown to have a broad utility in cleaving fusion protein. The enzyme is particularly suitable for this role because of its high degree of specificity, and its tolerance to a wide range of reaction conditions. Currently most commercial enterokinases are purified as holoenzymes from bovine or porcine intestines. They are expensive and the purified preparations are prone to be contaminated by trace of other gut proteases (Yuan and Hua, 2002). It can be obtained only in small quantities from bovine duodenal mucosa, which makes industrial scale production very expensive. Recombinant cell containing bovine enterokinase gene have been used to solve this problem (Kim et al., 2005). However, the application of recombinant enterokinase light chain has not been fully succeeded due to the low expression level and still high production cost (Suh et al., 2004).

In Thailand, the imported recombinant bovine enterokinase light chain which used for cleavage the fusion proteins in laboratory is very expensive, 84 USD for 0.063 µg and 336 USD for 0.32 µg from New England Biolabs (http://www.neb.com). To reduce the amount of imported enterokinase, it is important and necessary to produce this recombinant enzyme in Thailand. The expression of recombinant bovine enterokinase light chain in the suitable host and appropriate technology to obtain high quality and large amount of this enzyme to supply for research use will be good for

the recombinant protein production laboratory. The cost of recombinant protein production research in Thailand will be reduced,if this research is successful.

In this work, recombinant bovine enterokinase light chain was produced. Cloning the enterokinase light chain gene from bovine and buffalo duodenum tissues were done (chapter II). Shake flask scales expression of recombinant bovine enterokinase light chain was performed in *Escherichia coli* and *Pichia pastoris*. To obtained large amount of recombinant enzyme, the recombinant bovine enterokinase light chain was produced in a 2 L fermentor under the optimal production conditions (chapter III). The activity and enzymatic properties of recombinant enzyme were tested to confirm that high quality of recombinant enterokinase light chain was obtained (chapter III).

1.2 Research Objectives

- To clone Thai bovine and buffalo enterokinase light chain from duodenum tissue.

- To express the recombinant bovine enterokinase light chain in *E. coli* and *P. pastoris*.

- To produce the recombinant bovine enterokinase light chain under the optimum production condition in a 2 L fermentor.

- To purify and test the properties of recombinant bovine enterokinase light chain which produced in this work.

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CHAPTER II

BOVINE AND BUFFALO ENTEROKINASE LIGHT CHAIN CLONING

2.1 Introduction

2.1.1 The functions of enterokinase

All animal need to digest exogenous macromolecules without destroying similar endogenous constituents. The regulation of digestive enzyme is required. Vertebrate have solved this problem by a two-step enzymatic cascade to convert pancreatic zymogens (inactive enzyme) to active enzyme in lumen of the gut (Kitamoto et al., 1994). As early as 1899, Schepowalnickow in Pavlov's labolatory discovered that a proteolytic activity was generated in pancreatic juice upon addition of duodenal juice. The agent responsible for this effect, enterokinase (enteropeptidase, EC 3.4.4.8.), was first considered by Waldschmidt-Leitz to be a sort of cofactor adding to component of pancreatic juice to form active trypsin (Maroux et al., 1971).

Enterokinase is a glycoprotein and is now designated enteropeptidase (Mann and Mann, 1994). In all animal species studied, enterokinase is expressed highest in duodenum and rapidly decreases, becoming undetectable by the distal jejunum (Yuan et al., 1998). It is produced by the mucosa of small intestine (Bett,

1979). This enzyme is associated with the brush border of enterocytes and appears to be absent in other cell types, although enterokinase antigen was reported in some goblet cell. Substantial quantities of free enterokinase also occur in mucinous secretions of bovine and porcine small intestine, suggesting that enterokinase could be secreted by other cells and localized secondarily on enterocytes (Yuan et al., 1998). It controls a primordial enzymatic cascade that is conserved among vertebrates and is essential for normal intestinal digestion. When pancreatic secretions enter the duodenum, enterokinase recognized the acidic activation peptide of trypsinogen and cleaves it. The trypsin product then cleaves and activates the other zymogens in pancreatic fluid, enabling the digestion of food (Lu et al., 1999).

In human, congenital deficiency of enteropeptidase causes severe intestinal malabsorption with diarrhea, vomiting, and growth failure that can be treated successfully by supplementation with pancreatic extract. Mutations in the proenteropeptidase gene were the molecular cause of congenital enteropeptidase deficiency (Gasparian et al., 2003).

2.1.2 Structure of enterokinase

The enterokinase enzymes have molecular weight range from 150,000 to 300,000 Dalton depending on the species (Matsushima et al., 1994). Mammalian enterokinases contain 30-50% carbohydrate, which may contribute to the apparent differences in polypeptide masses (Kitamoto et al., 1994). Bovine enterokinase is a disulfide linked two-chain polypeptide a light chain and a heavy chain that is derived from a single chain precursor (Song *et al*., 2002)

The enzyme was reported to be composed of three chain, light chain, mini chain and heavy chain, in human and pig (Matsushima et al., 1994). The N-termial hydrophobic domain of a single-chain serine protease zymogen could mediate membrane association (Yuan et al., 1998). A transmembrane segment in the heavy chain anchors enterokinase in the brush border of duodenal enterocytes. The removal of heavy chain domain by reduction proteolysis or mutagenesis reduces the rate of trypsinogen activation ~ 500-fold, demonstrating that the heavy chain is necessary for optimal cleavage of trypsinogen (Lu et al., 1999). The characteristic of serine protease active site, serine and histidine residues, have been localized to the smaller subunits, thus for these enzymes the smaller (light) chain are the catalytic domains (La Vallie et al., 1993). Available data indicated that in all cases the smaller polypeptide chain, called the light chain, is the catalytic chain (Matsushima et al., 1994). The N-terminal isoleucine of enterokinase light chain is preceded by Ser-Pro-Lys (bovine) or Thr-Pro-Lys (human), suggesting that enterokinase is activated by a trypsin-like enzyme. The identification and location of the proenterokinase activator may indicate another level in the control of digestion (Kitamoto et al., 1994). Duodenase is assumed to be responsible for the activation of proenterokinase (Choi et al., 2001).

The enterokinase serine protease domain contains a basic tetrapeptide segment consisting of Arg96-Arg-Arg-Lys99 for porcine, mouse and human enterokinase; or Lys96-Arg-Arg-Lys99 for bovine and rat enterokinase. This segment is not conserved in other serine proteases, and computer modeling suggested that it is located on the protein surface where it might bind the acidic activation peptide residue of trypsinogen. Thus, enterokinase appears to have an extended binding site or "*exosite*", distinct from the catalytic center, which recognizes substrate amino acid residues on

the N-terminal side of the cleaved bond (Lu et al., 1999).

Enterokinase activated bovine trypsinogen by cleaving after the sequence Val$(Asp)_4$Lys, releasing an amino-terminal activation peptide (Kitamoto et al., 1994). The conversion of trypsinogen to trypsin is achieved by hydrolysis of the Lys(6)-Ile(7) bond, with the subsequent release of an activation peptide, containing an unusual tetra-aspartic acid sequence, which has been shown to play an important role in the recognition and activation of trypsinogen by enteropeptidase (Jeno et al., 1987). The acidic -$(Asp)_4$-Lys sequence of the trypsinogen activation peptide is conserved among vertebrate. The similar sequence of trypsinogens from lungfish (Ile-$(Glu)_2$-Asp-Lys or Ile-Glu-$(Asp)_2$-Lys) and African clawed frog (Phe-$(Asp)_4$-Lys) have been observed (Kitamoto et al., 1994).

In 1991, Light and Janska determined the amino-terminal 27 residues of S-vinylpyridyl derivative of the light chain by gas-phase Edman degradation. The results showed that the sequence has homologies with other serine proteases containing one or two chain. The homology confirms that the catalytic subunit has the same three-dimensional structure and, therefore, the same mechanism of enzymatic action as pancreatic chymotrypsin, trypsin, and elastase. The nature of the N-terminal amino acid of enterokinase light chain is very important for the catalytic activity. The three dimensional structure shows that the N-terminal Ile residue is buried inside the protein and interacts with other amino acid to maintain active conformation.

2.1.3 Application of enterokinase

Recently, quite few recombinant proteins of pharmaceutical interest have been expressed as fusion proteins for various reasons (Suh et al., 2004) include stability, high yield, improve conformational folding and secretion of the expressed product as well as purification and detection (Liew et al., 2005). Fusion tags are usually placed at the N- or C-termini of the target protein, with a specifically design protease cleavage site located between the tag and the target protein for convenient removal of the tag (Hosfield and Lu, 1998). In recent years, enterokinase light chain (EK_L) has been widely used to cleave the fusion protein in vitro with a high specificity for $(Asp)_4Lys$ (Kim et al., 2005). This enzyme keep its activity under a variety of reaction conditions for cleaving fusion protein at wide pH values, ranging from 4.5 to 9.5 at temperatures ranging from 4 to 45^{o}C, and in the presence of various detergents and denaturants (Yuan et al., 2002). An important characteristic of this enzyme is that its recognition sequence lies completely on the N-terminal side of the scissile bond, allowing any downstream fusion partner to retain its native N-terminus (Collins-Racies et al., 1995). For these reasons, enterokinase is one of the most popular proteases used for cleaving the fusion protein.

In 1996, Vozza et al. observed that the activity of the holoenzyme was found to be three-fold less than the recombinant bovine enterokinase light chain (rEK_L) based on equimolar amounts of protein when fluorescence substrate was used. When the ability to hydrolyze the Lys-Ile bond of trypsinogen was assayed, the activity of the rEK_L was lower by 30-folds in comparison to the holoenzyme. The released EK_L by partial reduction of disulfide-linked heterodimer exhibit almost the same enzymatic activity and specific activity as the holoenzyme for both synthetic substrate and fusion

protein carrying the $(Asp)_4Lys$ target sequence (Song et al., 2002).

Although the cleavage of enterokinase is highly specific, it has been reported that after cleavage of fusion protein, removal of enzyme is highly recommended to prevent the further degradation of the target protein (Choi et al., 2001).

The light chain alone exhibits the proteolytic activity (Kim et al., 2005). Therefore, in this report, only the EK_L was cloned and recombinant enzyme produced with N- or C-terminal His tag to later on easily remove the rEK_L

2.1.4 Molecular cloning of enterokinase light chain

cDNA encoding enterokinase gene from various mammalian species have been cloned and sequenced. In 1994, Kitamoto et al. determined the full-length cDNA sequence for bovine enterokinase and partial cDNA sequence for human enterokinase. The deduced amino acid sequence indicated that the active two-chain enterokinase is derived from a single-chain precursor. Membrane association may be mediated by a potential signal-anchor sequence near the amino terminus

In 1995, Kitamoto et al. isolated overlapping cDNA clones that encode the complete human enterokinase amino acid sequence from a human intestine cDNA library. Starting from the first ATG codon, the composite 3,966 bp. cDNA sequence contains an open reading frame of 3,057 bp. that encodes a 784 amino acid heavy chain followed by a 235 amino acid light chain. The heavy chain contains a potential N-terminal myristylation site, a potential signal anchor sequence near the N-terminus. The researcher reported that the EK_L is homologous to the trypsin-like serine proteinases. The active serine protease domain is positioned at the C- terminus with

the active site serine residue at amino acid 971. By northern blot analysis, a 4.4 kb enterokinase mRNA was detected only in the small intestine.

In 1993, LaVallie et al. cloned a cDNA encoding the catalytic domain (light chain) of bovine enterokinase. The nucleotide sequence of the cloned cDNA contains a 235 amino acid polypeptide that shares a high degree of homology with variety of mammalian serine proteases involved in digestion, coagulation, and fibinolysis.

In 2002, Yuan and Hua cloned a cDNA encoding EK_L from Chinese bovine duodenum tissue. The research finding found that the obtained Chinese bovine EK_L cDNA is 708 bp encodes 235 amino acid. This sequence is almost identical to the previous report by La Vallie et al. (1993) except for the two one-base mutations at the third position of triplet codons which do not change the encode amino acid.

In 2004, Huang et al. reported the results of Chinese northern yellow bovine EK_L gene cloning. The result showed that all bases matched exactly with the sequence of known EK_L deposited in GenBank. This result did not support Yuan result. They found three different codons in Chinese bovine EK_L when compare to La Vallie (L19663) sequence. The researcher discussed that the mutation detected by Yuan and Hua (2002) may have been caused by the process of RT-PCR or PCR.

In this work, bovine EK_L gene were cloned and sequenced for further expression work in chapter III. In this part, the buffalo EK_L gene was also cloned and sequenced. Because of the close relations between cow and buffalo, thus the properties of EK_L gene from buffalo and cow should be interesting to compare for enzymes relationship. The EK_L gene from buffalo have never been cloned and sequenced before. Thus the buffalo EK_L gene was cloned and sequenced in this research part. The similarity of sequence information of EK_L gene from buffalo and

cow were compared to other EK_L gene from GenBank databases at NCBI (http://www.ncbi.nlm.nih.gov/) using BLAST program (Altschul et al., 1997).

2.2 Objectives

-To clone Thai bovine enterokinase light chain for expression in chapter III

- To clone Thai buffalo enterokinase light chain

- To compare sequence information of enterokinase light chain from Thai cow and buffalo to other mammalian enterokinase in GenBank

2.3 Research Methodologies

Bovine and Buffalo enterokinase light chain cloning

2.3.1 Design oligonucleotide primers

The bovine EK_L gene was amplified with gene specific primers. These gene specific primers were designed base on the nucleotide sequence of bovine EK_L gene in GenBank, accession number L19663. Due to the close relation between cow and buffalo, the buffalo enterokinase light chain gene was also cloned using the bovine EK_L primers.

2.3.2 Total RNA extraction

Fresh Thai bovine or buffalo duodenum tissue from slaughterhouse approximately 30 mg was grinded in liquid nitrogen. Then, the total RNA was extracted from this tissue using Nucleospin RNA extraction kit. Aliquot of total RNA was kept at -70^{o}C.

2.3.3 Cloning of cDNA encoding Thai bovine enterokinase light chain

First strand cDNA was synthesized using oligodT (Q_T) primer (Table 2.1), and superscriptIII reverse transcriptase. The reaction was carried out at 55^{o}C for 1 h. PCR amplification was performed using Taq DNA polymerase with primer EK_L_1 and Qo (Table 2.1). Primer EK_L_1 corresponding to the part of heavy chain of bovine enterokinase. Primer Qo was used to anneal the complementary sequence of 5' end of Q_T primer. PCR cycles were carried out as follow: denaturation at 94^{o}C for 30 seconds, annealing at 53^{o}C for 30 second, and extension at 72^{o}C for 1 minute for 35 cycles. Nested PCR was performed with primers EK_L_2 and EK_L_3 (Table 2.1), which anneal to the N-terminal and C-terminal of bovine EK_L, respectively. Nested PCR condition was described follow the PCR condition except the annealing temperature which depends on each primer pair. All steps of EK_L gene amplification are summarized and shown in Fig. 2.1b. The expected PCR product was excised and purified from agarose gel then ligated into pGEM-T easy vector (Fig. 2.1a) (Promega) by T4 DNA ligase and transformed into *E. coli* DH5α. The clones which contain the plasmid with bovine or buffalo EK_L gene inserted were screened using colony PCR technique. The positive clones were selected and plasmid extracted. Sanger dideoxy technique was done to determine the nucleotide sequence of the buffalo and the EK_L genes. The similarity of the sequence obtained in this step was compared to GenBank databases at NCBI (http://www.ncbi.nlm.nih.gov/) using BLAST program (Altschul et al., 1997).

Table 2.1 Primers sequence information

Primer name	Sequence
Q_T	5’ CCAgTgAgCAgAgTgACgAggACTCgAgCTCAAgC$(T)_{17}$ 3’
Q_o	5’CCAgTgAgCAgAgTgACg 3’
EK_L_1	5’gATgTgTgTCAgCTgCTggg 3’
EK_L_2	5’ gggAATTCAgATTgTCggAggAAgTgACTC 3’
EK_L_3	5’ ggCCgCggATgTAgAAAACTTTgTATCCAC 3’
EK_L_4	5’ ggCCATggCTATTgTCggAggAAgTgACTCC 3’
EK_L_5	5’ ggCTCgAgATgTAgAAAACTTTgTATCCACTCT G 3’
EK_L_6	5’ gAAgATTgTCggAggAAgTg

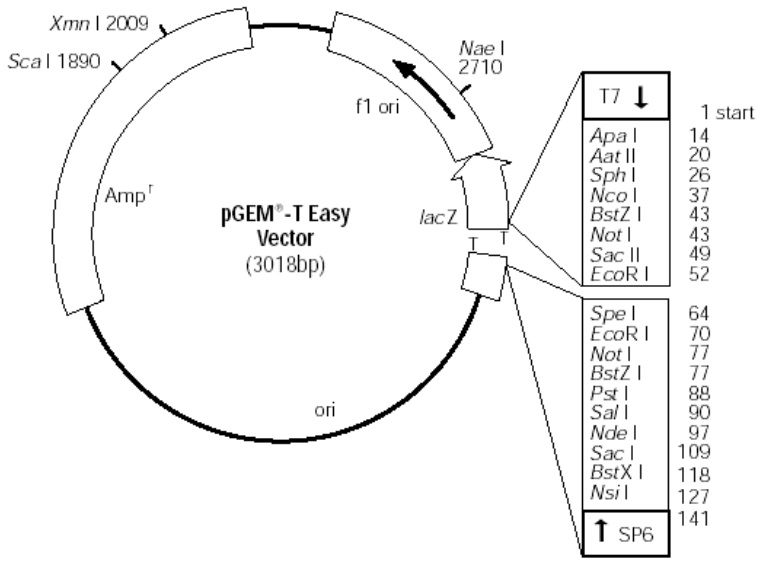

Fig. 2.1a pGEM-T Easy Vector Circle map (Promega Technical manual No.042)

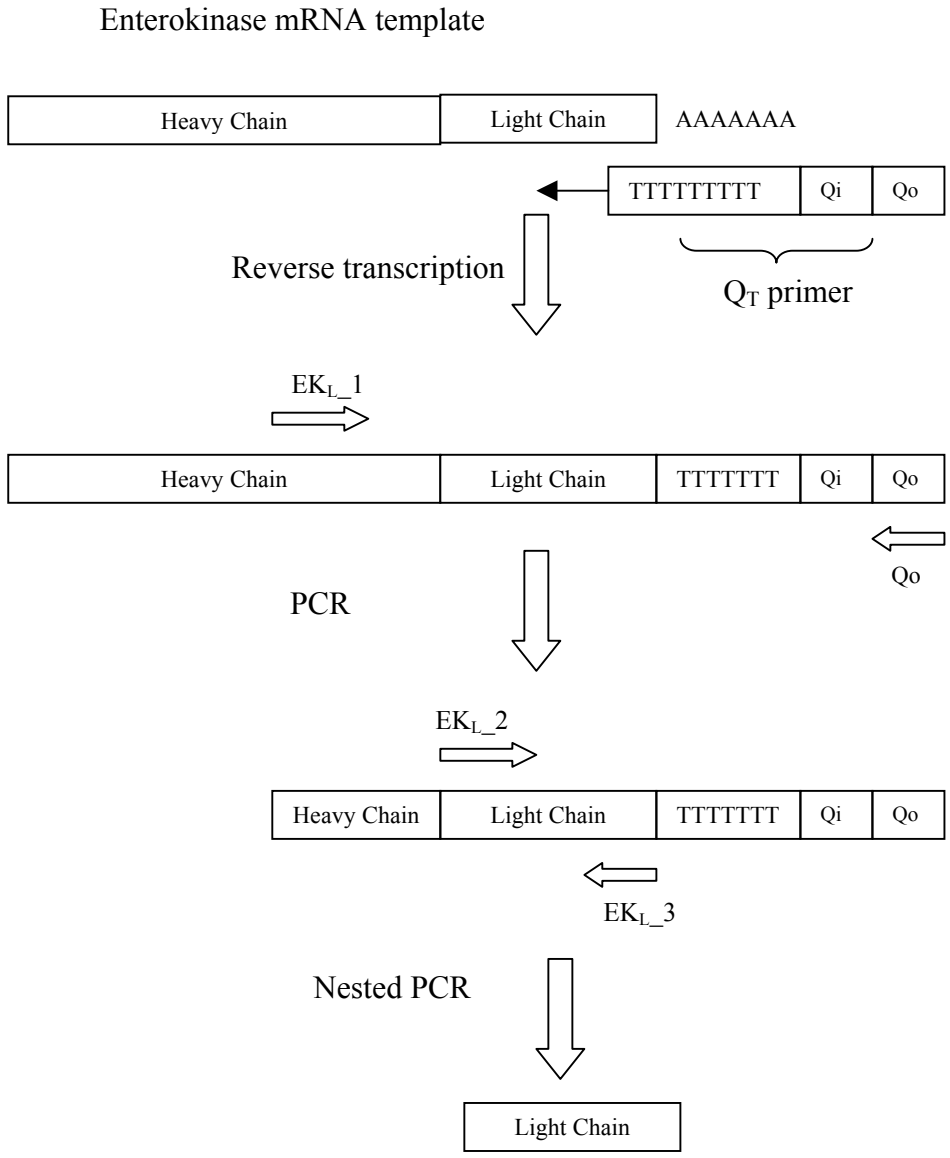

Fig. 2.1b Summary of enterokinase gene amplification steps. First strand cDNA of enterokinase gene is synthesized by reverse transcription technique with Q_T primer. Double stand DNA of enterokinase gene is amplified by PCR technique using EK_L_1 and Qo primers. Only the part of EK_L is obtained by nested-PCR technique in the final step with EK_L_2 and EK_L_3 primers.

2.4 Results and discussion

2.4.1 Bovine enterokinase light chain

The enterokinase light chain (EK_L) gene was amplified from Thai bovine duodenum tissue by RT-PCR and nested PCR techniques. The double band of PCR product approximately 700 and 800 bp were found (data not shown). One of them, 700 bp, matched with the bovine EK_L size in GenBank. Both of them were excised and purified from agarose gel. Because the concentration of the 700 bp PCR product was much higher than the other, only the single band of 700 bp was found in the purification step. The purified DNA fragment was ligated into pGEM-T easy vector (Fig. 2.1a). The recombinant plasmid was transformed into *E. coli* DH5α. Colony PCR technique with primer EK_L _2 and EK_L _3 was used to screen the clones containing recombinant plasmid. The positive clones were selected and plasmid extracted for sequencing. The results of sequencing and predicted amino acid sequence are shown in Fig. 2.2. Bovine EK_L cDNA comprises 708 bp and 235 translated amino acid. The result of nucleotide sequencing showed 99 % identical to bovine EK_L in GenBank. The nucleotide sequence of EK_L in this research is almost identical to the previous sequence accession number L19663. There are five bases mutations but four of them are silent mutations which do not change the encoded amino acid. Only one amino acid is changed because of one mutation was at the second position of the triplet codon. The other four positions of single-base changes are at the third position of the triplet codon are the Ala-147 (GCT ----> GCC), Thr-167 (ACG ----> ACA), Tyr-175 (TAT ----> TAC) and Thr- 205 (ACA ---->ACG). The one position of one-base changes at the second position of triplet codon

(CAA ----> CGA) caused amino acid changes from Gln-58 to Arg-158. These results indicated that the 4 silence mutations in Thai bovine EK_L may be caused by mutation of the gene. However, the nucleotide missence (Gln 158 ---->Arg 158) mutation may occurred by the process of RT-PCR and PCR error. In the future, repeat EK_L cloning and sequencing should be required to confirm the corrected nucleotide sequence of Thai bovine EK_L.

The result of bovine EK_L amino acid sequence compare with other EK_L from various origins in GenBank is shown in Fig. 2.3. To compare with EK_L amino acid from research of Kitamoto et al. in 1994, LaVallie et al. in 1993 and Northern Chinese cow (accession number AAT84164) the similarity of Thai cow showed 99%, 99% and 98% identity, respectively. There are two amino acid residues in Northern Chinese cow (at Arg-82 and Asp-76) and one amino acid residues in Thai cow (Arg-158) different from the result of Kitamto et al. in 1994 and LaVallie et al. in 1993. The EK_L amino acid sequence alignment of cow indicated that it share the conservation of serine protease sequence motifs among various origin, in particular, the active site residues can be identified as His 41, Asp 92 and Ser 187. The bovine EK_L amino acid sequence from various origins also has the same position of three potential N-glycosylation and nine cysteine residues (Fig. 2.3). This result indicated that EK_L gene is highly conserved between cows of different origin.

In conclusion, the Thai bovine EK_L obtained has one amino acid mutation which is different from researches of Kitamoto et al. in 1994 and LaVallie et al. in 1993. Because of the changed amino acid in Thai bovine EK_L was not found in catalytic active site, the obtained clone Thai bovine EK_L was used for the expression work in chapter III.

```
DNA: ATTGTCGGAGGAAGTGACTCCAGAGAAGGAGCCTGGCCTTGGGTCGTTGCT  51
 +1: I  V  G  G  S  D  S  R  E  G  A  W  P  W  V  V  A   17

DNA: CTGTATTTCGACGATCAACAGGTCTGCGGAGCTTCTCTGGTGAGCAGGGAT  102
 +1: L  Y  F  D  D  Q  Q  V  C  G  A  S  L  V  S  R  D    34

DNA: TGGCTGGTGTCGGCCGCCCACTGCGTGTACGGGAGAAATATGGAGCCGTCT  153
 +1: W  L  V  S  A  A  H  C  V  Y  G  R  N  M  E  P  S    51

DNA: AAGTGGAAAGCAGTGCTAGGCCTGCATATGGCATCAAATCTGACTTCTCCT  204
 +1: K  W  K  A  V  L  G  L  H  M  A  S  N  L  T  S  P    68

DNA: CAGATAGAAACTAGGTTGATTGACCAAATTGTCATAAACCCACACTACAAT  255
 +1: Q  I  E  T  R  L  I  D  Q  I  V  I  N  P  H  Y  N    85

DNA: AAACGGAGAAAGAACAATGACATTGCCATGATGCATCTTGAAATGAAAGTG  306
 +1: K  R  R  K  N  N  D  I  A  M  M  H  L  E  M  K  V   102

DNA: AACTACACAGATTATATACAGCCTATTTGTTTACCAGAAGAAAATCAAGTT  357
 +1: N  Y  T  D  Y  I  Q  P  I  C  L  P  E  E  N  Q  V   119

DNA: TTTCCCCCAGGAAGAATTTGTTCTATTGCTGGCTGGGGGGCACTTATATAT  408
 +1: F  P  P  G  R  I  C  S  I  A  G  W  G  A  L  I  Y   136

DNA: CAAGGTTCTACTGCAGACGTACTGCAAGAAGCCGACGTTCCCCTTCTATCA  459
 +1: Q  G  S  T  A  D  V  L  Q  E  A  D  V  P  L  L  S   153

DNA: AATGAGAAATGTCGACAACAGATGCCAGAATATAACATTACAGAAAATATG  510
 +1: N  E  K  C  R  Q  Q  M  P  E  Y  N  I  T  E  N  M   170

DNA: GTGTGTGCAGGCTACGAAGCAGGAGGGGTAGATTCTTGTCAGGGGGATTCA  561
 +1: V  C  A  G  Y  E  A  G  G  V  D  S  C  Q  G  D  S   187

DNA: GGCGGACCACTCATGTGCCAAGAAAACAACAGATGGCTCCTGGCTGGCGTG  612
 +1: G  G  P  L  M  C  Q  E  N  N  R  W  L  L  A  G  V   204

DNA: ACGTCATTTGGATATCAATGTGCACTGCCTAATCGCCCAGGGGTGTATGCC  663
 +1: T  S  F  G  Y  Q  C  A  L  P  N  R  P  G  V  Y  A   221

DNA: CGGGTGCCAAGGTTCACAGAGTGGATACAAAGTTTTCTACATTAG        708
 +1: R  V  P  R  F  T  E  W  I  Q  S  F  L  H  *         235
```

Fig. 2.2 Coding sequence of Thai bovine EK_L is shown with the translated aminoacid sequence below it. Compare with bovine EK_L accession number L19663, the intramolecular disulfide bridge in Thai bovine EK_L are predicted at nine cysteine residues which are boxed. The three potential N-glycosylation sites are underlined. Three catalytic amino acids are highlight.

```
Thai_cow                    IVGGSDSREGAWPWVVALYFDDQQVCGASLVSRDWLVSAAHCVYGRNMEP 50
AAB40026_Kitamoto_          IVGGSDSREGAWPWVVALYFDDQQVCGASLVSRDWLVSAAHCVYGRNMEP 50
AAA16035_LaVallie_E.R._     IVGGSDSREGAWPWVVALYFDDQQVCGASLVSRDWLVSAAHCVYGRNMEP 50
AAT84164_Tan_H.D._          IVGGSDSREGAWPWVVALYFDDQQVCGASLVSRDWLVSAAHCVYGRNMEP 50
                            *********************************************

Thai_cow                    SKWKAVLGLHMASNLTSPQIETRLIDQIVINPHYNKRRKNNDIAMMHLEM 100
AAB40026_Kitamoto_          SKWKAVLGLHMASNLTSPQIETRLIDQIVINPHYNKRRKNNDIAMMHLEM 100
AAA16035_LaVallie_E.R._     SKWKAVLGLHMASNLTSPQIETRLIDQIVINPHYNKRRKNNDIAMMHLEM 100
AAT84164_Tan_H.D._          SKWKAVLGLHMASNLTSPQIETRLIDQIVINRHYNKRRKNNDIAMMHLEM 100
                            ****************************** *****************

Thai_cow                    KVNYTDYIQPICLPEENQVFPPGRICSIAGWGALIYQGSTADVLQEADVP 150
AAB40026_Kitamoto_          KVNYTDYIQPICLPEENQVFPPGRICSIAGWGALIYQGSTADVLQEADVP 150
AAA16035_LaVallie_E.R._     KVNYTDYIQPICLPEENQVFPPGRICSIAGWGALIYQGSTADVLQEADVP 150
AAT84164_Tan_H.D._          KVNYTDYIQPICLPEENQVFPPGRICSIAGWGALIYQGSTADVLQEADVP 150
                            ************************************************

Thai_cow                    LLSNEKCRQQMPEYNITENMVCAGYEAGGVDSCQGDSGGPLMCQENNRWL 200
AAB40026_Kitamoto_          LLSNEKCQQQMPEYNITENMVCAGYEAGGVDSCQGDSGGPLMCQENNRWL 200
AAA16035_LaVallie_E.R._     LLSNEKCQQQMPEYNITENMVCAGYEAGGVDSCQGDSGGPLMCQENNRWL 200
AAT84164_Tan_H.D._          LLSNEKCQQQMPEYNITENMVCAGYDAGGVDSCQGDSGGPLMCQENNRWL 200
                            *******:*****************:**********************

Thai_cow                    LAGVTSFGYQCALPNRPGVYARVPRFTEWIQSFLH 235
AAB40026_Kitamoto_          LAGVTSFGYQCALPNRPGVYARVPRFTEWIQSFLH 235
AAA16035_LaVallie_E.R._     LAGVTSFGYQCALPNRPGVYARVPRFTEWIQSFLH 235
AAT84164_Tan_H.D._          LAGVTSFGYQCALPNRPGVYARVPRFTEWIQSFLH 235

                            **********************************
```

Fig. 2.3 Multiple amino acid sequence alignment of bovine EK_L gene from various origins. Thai bovine EK_L are compared with bovine EK_L accession number AAB40026, AAA16035 and AAT84164 in GenBank which was cloned and sequenced by Kitamoto et al., LaVallie et al. and Tan et al., respectively. The consensus amino acid residues shown below the alignment indicates in the stars. The catalytic amino acid, histidine, aspartic acid, and serine residues at the active-site are shown in the bold letters.

2.4.2 Buffalo enterokinase light chain cloning

After buffalo enterokinase light chain (EK_L) gene was amplified by RT-PCR and nested PCR techniques using bovine primer (Table 2.1), a 708 bp. PCR product similar size to the bovine EK_L gene was found. The result in Fig. 2.4 shows the expected size of DNA fragment which was excised and purified from agarose gel. The purified fragment was ligated into pGEM-T easy vector (Fig. 2.1a). The clones which contained recombinant plasmid were selected. The nucleotide sequence of buffalo EK_L was analyzed according to Sanger's dideoxy method. The results of sequencing analysis and the translated product are shown in Fig. 2.5.

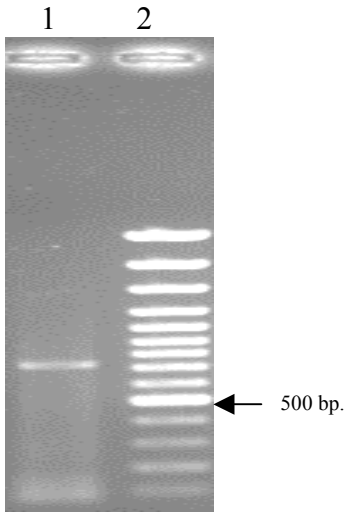

Fig. 2.4 The amplification of buffalo EK_L gene using RT-PCR and nested PCR techniques. The expected nested PCR product is shown in the single band of 708 bp. Lane 1: nested PCR product of buffalo EK_L gene, Lane 2: 100 bp. DNA Marker (Fermentas).

```
DNA: ATTGTCGGAGGAAGTGACTCCAAAGAAGGAGCCTGGCCTTGGGTCGTTGCT 51
 +1: I  V  G  G  S  D  S  K  E  G  A  W  P  W  V  V  A   17

DNA: CTGTATTTCGACGATCAACAGGTCTGCGGAGCTTCTCTGGTGATCAGGGAT 102
 +1: L  Y  F  D  D  Q  Q  V  C  G  A  S  L  V  I  R  D    34

DNA: TGGCTGGTGTCGGCCGCCCACTGCGTGTACGGGAGAAATATGGAGCCGTCT 153
 +1: W  L  V  S  A  A  H  C  V  Y  G  R  N  M  E  P  S    51

DNA: AAGTGGAAAGCAGTGCTAGGCCTGCATATGGCATCAAATCTGACTTCTCCT 204
 +1: K  W  K  A  V  L  G  L  H  M  A  S  N  L  T  S  P    68

DNA: CAGATAGAAACTAGGTTGATTGACCAAATTGTCATAAACCCACACTACAAT 255
 +1: Q  I  E  T  R  L  I  D  Q  I  V  I  N  P  H  Y  N    85

DNA: AAACGGAGAAAGGACAATGACATCGCCATGATGCATCTTGAAATGAAAGTG 306
 +1: K  R  R  K  D  N  D  I  A  M  M  H  L  E  M  K  V   102

DNA: AACTACACAGATTATATACAGCCTATTTGTTTACCAGAAGAAAATCAAGTT 357
 +1: N  Y  T  D  Y  I  Q  P  I  C  L  P  E  E  N  Q  V   119

DNA: TTTTCCCCAGGAAGAATTTGTTCTATTGCTGGCTGGGGGACACTTATATAT 408
 +1: F  S  P  G  R  I  C  S  I  A  G  W  G  T  L  I  Y   136

DNA: CAAGGTTCTACTGCAGACGTACTGCAAGAAGCTGACGTTCCCCTTCTATCA 459
 +1: Q  G  S  T  A  D  V  L  Q  E  A  D  V  P  L  L  S   153

DNA: AATGAGAAATGTCAACAACAGATGCCAGAATATAACATTACGGAAAATATG 510
 +1: N  E  K  C  Q  Q  Q  M  P  E  Y  N  I  T  E  N  M   170

DNA: GTGTGTGCAGGCTACGAAGCAGGAGGGGTAGATTCTTGTCAGGGGGATTCA 561
 +1: V  C  A  G  Y  E  A  G  G  V  D  S  C  Q  G  D  S   187

DNA: GGCGGACCACTCATGTGCCAAGAAAACAACAGATGGCTCCTGGCTGGCGTG 602
 +1: G  G  P  L  M  C  Q  E  N  N  R  W  L  L  A  G  V   204

DNA: ACGTCATTTGGATATAAGTGTGCACTGCCTAATCGCCCAGGGGTGTATGCC 663
 +1: T  S  F  G  Y  K  C  A  L  P  N  R  P  G  V  Y  A   221

DNA: CGGGTCCCAAGGTTCACAGAGTGGATACAAAGTTTTCTACATTAG       708
 +1: R  V  P  R  F  T  E  W  I  Q  S  F  L  H  *        235
```

Fig. 2.5 The predict amino acid sequence of buffalo EK_L. The cDNA encoding buffalo EK_L (GenBank accession DQ518426) is shown with the translated amino acid sequence below. The same position of nine cysteine residues and three potential N-linked glycosylation were found in buffalo and bovine EK_L. The 9 cysteine residues are boxed. The 3 potential N-linked glycosylation sites are underlined. Three catalytic amino acids are highlight.

A homology search for buffalo EK_L nucleotides sequence by BLAST program (Altschul et al., 1997) revealed that buffalo EK_L has the most identity with bovine EK_L (98%). The comparison of EK_L nucleotide from the various mammalian species in GenBank showed that the buffalo EK_L gene was 98 %, 89 %, 85 %, 85% and 81% identical with cow, pig, chimpanzee, human and rat, respectively. The cDNA of buffalo EK_L was translated to amino acid sequence. The open reading frame encodes a polypeptide of 235 amino acids residues. The calculated mass and pI (http://www.expasy.org/) are 26.28 kDa and 5.21, respectively. Compare with cow, pig, chimpanzee, human and rat, the buffalo EK_L had 97%, 88%, 85%, 85% and 77% identical amino acid sequence residues, respectively (Fig. 2.7). This result can be concluded that amino acid sequence of EK_L is highly conserved among various mammalian species. The buffalo EK_L amino acid sequences is almost identical to the previously reported bovine EK_L (Accession number L19663) and Thai cow excepted that only 6 amino acid in buffalo includes Lys 8, Ile 32, Asp 90, Ser 121, Thr 133 and Lys 210 are different (Fig. 2.6)**.** The comparison of amino acid sequence between buffalo and bovine EK_L in GenBank was found that they have the same position of three potential N-glycosylation and nine cysteine residues (Fig. 2.5). By analogy with bovine EK_L, it is predicted that intramolecular disulfide bonding in buffalo EK_L occurs between cysteine pairs 26/42, 126/193, 157/172, 183/211. The remaining cysteine at position 112 is probably involved in single disulfide bridge with the noncatalytic chain (La Vallie et al., 1993).

In conclusion, this research is the first buffalo EK_L gene cloned and sequenced. Therefore, the buffalo EK_L sequence was submit to GenBank and obtained the

accession number DQ518426. The nucleotides and amino acids sequence of buffalo EK_L showed highest similarity score when compared with bovine EK_L. This result indicated that buffalo EK_L gene is closely related to bovine EK_L. However, this data information is only the basic knowledge. The works of expression and enzymatic properties studied should be done in the future**.**

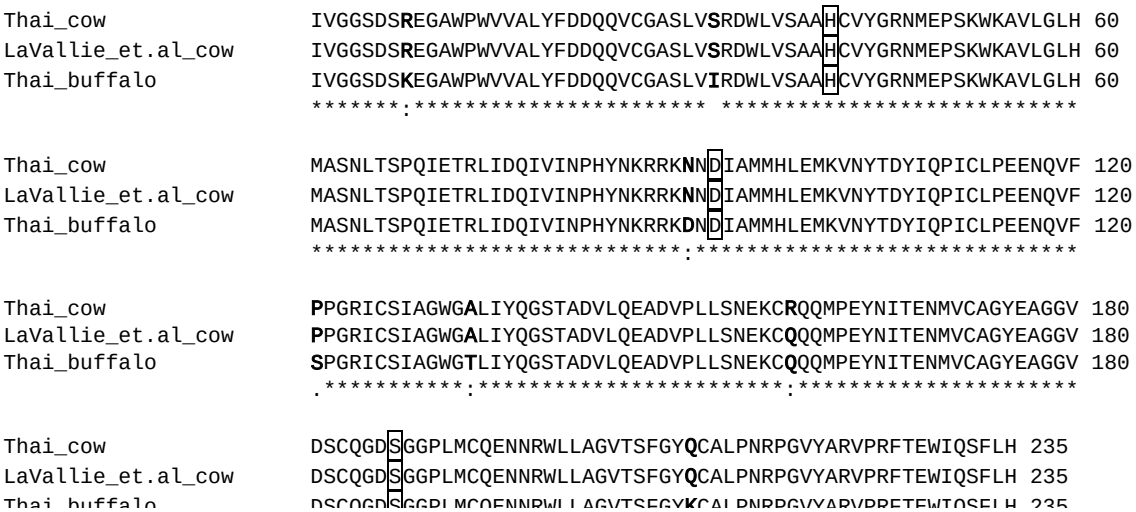

Fig. 2.6 The amino acid sequence alignment of EK_L from Thai buffalo, Thai cow and cow in NCBI database accession number L19663. The same amino acid residues of EK_L which found in all cow and buffalo origin are shown in the star below the letters. The different amino acid residues are shown in the bold letters. The active-site histidine, aspartic acid, and serine residues are boxed.

```
Human           IVGGSNAKEGAWPWVVGLYYGGR----LLCGASLVSSDWLVSAAHCVYGRNLEPSKWTAI 56
Chimpanzee      IVGGSNAKEGAWPWVVGLYYGGR----LLCGASLVSSDWLVSAAHCVYGRNLEPSKWTAI 56
Cow             IVGGSDSREGAWPWVVALYFDDQ----QVCGASLVSRDWLVSAAHCVYGRNMEPSKWKAV 56
Thai_cow        IVGGSDSREGAWPWVVALYFDDQ----QVCGASLVSRDWLVSAAHCVYGRNMEPSKWKAV 56
Thai_Buffalo    IVGGSDSKEGAWPWVVALYFDDQ----QVCGASLVIRDWLVSAAHCVYGRNMEPSKWKAV 56
Pig             IVGGNDSREGAWPWVVALYYNGQ----LLCGASLVSRDWLVSAAHCVYGRNLEPSKWKAI 56
Rat             IVGGSDTQAGAWPWVVALYYRDRSGDRLLCGASLVSSDWLVSAAHCVYRRNLDPTRWTAV 60
                ****.::: *******.**: .:     :******  ********** **::*::*.*:

Human           LGLHMKSNLTSPQTVPRLIDEIVINPHYNRRRKDNDIAMMHLEFKVNYTDYIQPICLPEE 116
Chimpanzee      LGLHMKSNLTSPQTVPRLIDEIVINPHYNRRRKDNDIAMMHLEFKVNYTDYIQPICLPEE 116
Cow             LGLHMASNLTSPQIETRLIDQIVINRHYNKRRKNNDIAMMHLEMKVNYTDYIQPICLPEE 116
Thai_cow        LGLHMASNLTSPQIETRLIDQIVINPHYNKRRKNNDIAMMHLEMKVNYTDYIQPICLPEE 116
Thai_Buffalo    LGLHMASNLTSPQIETRLIDQIVINPHYNKRRKDNDIAMMHLEMKVNYTDYIQPICLPEE 116
Pig             LGLHMTSNLTSPQIVTRLIDEIVINPHYNRRRKDSDIAMMHLEFKVNYTDYIQPICLPEE 116
Rat             LGLHMQSNLTSPQVVRRVVDRIVINPHYDKRRKVNDIAMIHLEFKVNYTDYIQPICLPEE 120
                ***** *******   *::*.**** **::*** .***:***:**************

Human           NQVFPPGRNCSIAGWGTVVYQGTTANILQEADVPLLSNERCQQQMPEYNITENMICAGYE 176
Chimpanzee      NQVFPPGRNCSIAGWGTVVYQGTTANILQEADVPLLSNEKCQQQMPEYNITENMICAGYE 176
Cow             NQVFPPGRICSIAGWGALIYQGSTADVLQEADVPLLSNEKCQQQMPEYNITENMVCAGYD 176
Thai_cow        NQVFPPGRICSIAGWGALIYQGSTADVLQEADVPLLSNEKCRQQMPEYNITENMVCAGYE 176
Thai_Buffalo    NQVFSPGRICSIAGWGTLIYQGSTADVLQEADVPLLSNEKCQQQMPEYNITENMVCAGYE 176
Pig             NQVFPPGRICSIAGWGKVIYQGSPADILQEADVPLLSNEKCQQQMPEYNITENMMCAGYE 176
Rat             NQTFTPGRMCSIAGWGYNKINGSTVDVLKEADVPLVSNEKCQQQLPEYDITESMLCAGYE 180
                **.*.*** *******    :*:..::*:******:***:*:**:***:***.*:****:

Human           EGGIDSCQGDSGGPLMCQENNRWFLAGVTSFGYKCALPNRPGVYARVSRFTEWIQSFLH 235
Chimpanzee      EGGIDSCQGDSGGPLMCQENNRWFLAGVTSFGYKCALPNRPGVYARVSRFTEWIQSFLH 235
Cow             AGGVDSCQGDSGGPLMCQENNRWLLAGVTSFGYQCALPNRPGVYARVPRFTEWIQSFLH 235
Thai_cow        AGGVDSCQGDSGGPLMCQENNRWLLAGVTSFGYQCALPNRPGVYARVPRFTEWIQSFLH 235
Thai_Buffalo    AGGVDSCQGDSGGPLMCQENNRWLLAGVTSFGYKCALPNRPGVYARVPRFTEWIQSFLH 235
Pig             EGGIDSCQGDSGGPLMCLENNRWLLAGVTSFGYQCALPNRPGVYARVPKFTEWIQSFLH 235
Rat             EGGTDSCQGDSGGPLMCQENNRWFLVGVTSFGVQCALPNHPGVYARVSQFIEWIHSFLH 239
                 ** ***********  *****:*.****** :*****:*******.:* ***:****
```

Fig. 2.7 Multiple amino acid sequence alignment of EK_L protein between buffalo and the other mammalian species. The translated composite cDNA sequence of buffalo EK_L were aligned with sequence of cow, pig, human, chimpanzee and rat. The active-site histidine, aspartic acid, and serine residues are shown in the bold letters.

2.5 Conclusions

The results of Thai Bovine and buffalo EK_L gene amplification showed 708 bp of PCR product and 235 predict amino acid. The comparison of Thai bovine EK_L amino sequence showed 99% and 97 % identical to bovine EK_L in GenBank accession number L19663 and buffalo EK_L accession number DQ518426 (Fig. 2.6), respectively. These results can be concluded that bovine EK_L gene is closely related to buffalo EK_L.

From the alignment in Fig. 2.7 we can somewhat concluded that Arg 158 in the Thai bovine is a definite PCR error since at the position 158 of EK_L from other species are all Gln 158. The result of EK_L amino acid sequence alignment indicated that it shares the conservation of the active site residues, His 41, Asp 92 and Ser 187. There are nine cysteine residues in Thai bovine and buffalo EK_L (Fig. 2.2 and 2.5) which were predicted to be involved in intramolecular disulfide bonding in EK_L. When bovine EK_L was aligned with other serine protease, particularly pancreatic elastase whose tertiary structure is known and which has 8 cysteines and four intrachain disulfide bridges, the 8 positions of the 9 cysteine residues in EK_L are highly conserved (La Vallie et al., 1993). The segment of Lys86-Arg-Arg-Lys89

which was found in cow and buffalo (Fig. 2.7) has been reported that it is located on the protein surface where might bind the acidic activation peptide residue of trypsinogen (Lu et al., 1999).

The Ile-Val-Glu-Glu sequence of enterokinase N-terminal is conserved in various mammalian species (Fig. 2.7). In cow, the presence of the conserved amino-terminal activation peptide sequence, Ile-Val-Glu-Glu, shows that enterokinase must have a zymogen precursor and that the two-chain enzyme arises from limited proteolysis during posttranslational processing (Light and Janska, 1991). Previous researchs have reported that the N-terminal amino acid of enteropeptidase is buried into hydrophobic pocket according to the crystal structure. This interaction is believed to play an important role for maintaining the conformation for the catalytic activity. The N-terminal amino acid, Ile, is highly conserved among enteropeptidase light chain in various sources (Song et al., 2002). The N-terminal Ile of EK_L is involved in a critical salt bridge within the structure. The three-dimensional structure of EK_L shows that the N-terminal Ile residue is positioned inside the protein for salt bridge where as the C-terminal portion is exposed at outer surface (Choi et al., 2001).

Although one amino acid mutation, Arg 158, was found in Thai bovine EK_L but this amino acid residues is not at the active site of the enzyme, so the enzymatic activity of this recombinant EK_L should not be change. The obtained clone of Thai bovine enterokinase light chain in this chapter was used for expression work in chapter III.

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CHAPTER III

EXPRESSION OF BOVINE ENTEROKINASE LIGHT CHAIN

3.1 Introduction

3.1.1 Review of enterokinase production

Enterokinase enzyme has been purified from duodenal tissue, however small amount of native enterokinase can be obtained. The method of purification has been described by Waldschmidt-Leitz (1924-25). The method essentially dry swine duodenal mucosa with acetone and ether. Then extracted the dried mucosae with dilute ammonium hydroxide, removing foreign protein from the aqueous extract by acetic acid and further purify by precipitation with alcohol, tannic acid and finally by fractional adsorbtion on $Al(OH)_3$ and kaolin. By this method, the researcher obtained a preparation which was 100 times more active per unit dry weight than the acetone dried mucosa. For the purification method of Kunitz in 1938, the principle of purification is pH adjustment and fractional precipitation using ammonium sulfate, the preparation of enterokinase was 5,000-50,000 times as active as the acetone dried mucosa (Kunitz, 1938). In 1984, Light and Fonseca separated the heavy chain and light chain subunit of bovine enterokinase. A reduction of the disulfide bonds of bovine enterokinase was performed in 50 mM dithioerythritol (DTT) at pH 9 and 4^oC.

The researcher examined the properties of catalytic subunit (light chain) toward synthetic substrates and protein. The results were found that the specificity was restricted to lysine and arginine residues and the catalytic efficiency toward ester substrates was the same or greater than the intact enzyme. The catalytic subunit retained the specificity of intact enterokinase, but the rate of activation of trypsinogen was much slower. These results were discussed that the absence of the heavy subunit would decrease the number of subsite interactions and alter the specificity of binding or the loss of heavy subunit causes a small conformational change in the catalytic subunit.

Until recently purified enterokinase from bovine and porcine intestine was available, and even highly purified enzyme preparations were prone to be contamination by other gut proteases (Collins-Racie et al., 1995). Thus, using enterokinase has been limited in the past because of proteases contaminant from intestine preparation, small quantity and low specific activity. These proteases often degrade the product of fusion protein cleavages extensively (Lavallie et al., 1993; Vozza et al., 1996). The recombinant DNA technologies have been used to solve these problems. The cDNA encoding enterokinase light chain has been cloned and inserted into different expression vectors. Various host cells have been used to express the recombinant enterokinase light chain.

- Prokaryote expression host

The gram-negative bacterium *Escherichia coli* is one of the most attractive systems for heterologous protein expression, because of its ability to grow rapidly, its well characterized genetics, and the availability of an increasing large number of cloning vectors and mutant host strains (Austin., 2003). However, there are many

proteins which are very difficult to express in an active form. *E. coli* frequently generates inclusion bodies instead of producing soluble and active protein (Turner et al., 2005) especially for mammalian proteins production which comprise the multi-domains and or disulphide-bonds (Austin, 2003). Fusion protein technologies have been used to improve the solubility of recombinant protein. Maltose-binding protein (MBP), glutathione-S-transferase (GST) and thioredoxin (Trx) are the most commonly used for the fusion system in terms of their abilities to produce properly folded and biological active protein (Collins-Racie et al., 1995). From several reports, *E. coli* have been used as the expression host for rEK_L production as describe below.

In 1995, Collins-Racies et al. produced the bovine rEK_L in *E. coli* by a secretory expression system that utilized *E. coli* DsbA protein (Disulfide oxidoreductase) as an N-terminal fusion partner. The DsbA-EK_L fusion was design with an inter-domain linker containing a hexa-histidine sequence and an enterokinase cleavage site (-Asp_4Lys-). The EK_L cDNA was fused in-frame to the 3'-end of the coding sequence for DsbA. Expression of the DsbA-EK_L fusion is transcriptionally regulated by the pL promoter (bacteriophage lambda). Promoter was induced by adding tryptophan to the culture medium. The DsbA-EK_L was purified using Ni-IDA (iminodiacetic acid) resin and STI–Sepharose column. The autocatalytic cleavage of fusion protein occurred when incubated elution fraction from Ni-IDA at 4^oC for 16 h. The active rEK_L generated by autocatalytic cleavage was purified using the affinity chromatography resin STI-sepharose. The yield of finally purification step, active recombinant EK_L, was 1 mg from 125 g of starting cell paste. The enzymatic properties of rEK_L from mammalian and bacterial cells were similar. Both products exhibited decreased ability to activate trypsinogen when compared to the native

heterodimeric enzyme. The rEK_L derived from *E. coli* were capable of cleaving fusion protein with much higher activity than that in the native heterodimer enterokinase.

In 2002, Yuan et al. inserted the cDNA encode bovine enterokinase catalytic subunit into pET32a. The EK_L fragment was fused downstream to the fusion partner thioredoxin (Trx) and following enterokinase recognition sequence. *E. coli* BL21 was chosen for the expression system. The rEK_L was expressed under IPTG induction condition. The active rEK_L was released from the fusion protein after autocatalytic of Trx-rEK_L then purified by DEAE chromatography and gel filtration. The result was found that the expected protein band, approximately 26 kDa, was found by SDS-PAGE technique. SDS-PAGE data showed that there were no intensity-increased of protein bands around 40 kDa which was related to the theoretical molecular weight of Trx-rEK_L fusion protein. It indicated that most products were produced in soluble form and was able to autocleave the Trx_rEK_L. The specific activity of rEK_L was 720 AUs/mg protein. The yield of purified active rEK_L was 4.3 mg from 100 ml shake flask culture.

In 2004, Ile et al. cloned cDNA encoding Chinese bovine EK_L gene. The corrected EK_L DNA fragment was inserted into a pET39b then expressed in *E. coli* BL21 (DE3). The data from 12% SDS-PAGE showed that under IPTG induction condition, pET39b-EK_L protein had an expected protein band of 65 kDa and absent in un-induced cell. The rEK_L was purified by Ni-IDA resin then desalt and buffer changed. A high autocatalytic cleavage activity was found after incubated the elution fraction at 21^oC for 72 h. The post-cleavage products without additional enterokinase showed 28 and 37 kDa band of DsbA and rEK_L, respectively.

From several researches indicated that *E. coli* is a suitable host for rEK_L expression in prokaryotic systems.

- Eukaryote expression host

Various eukaryotic hosts have been used as the expression host for rEK_L expression.

In 1993, La Vallie et al. cloned the cDNA encoding the catalytic subunit of bovine enterokinase and expressed in mammalian cell. The expression of COS-1 cell showed low level of secreted protein and the degradation of small amount of the secreted protein occurred. They concluded that the majority of the expressed protein was not folded properly.

In 1996, Vozza et al. produced bovine EK_L in the methylotrophic yeast *P. pastoris*. The EK_L gene was inserted in fame with the alpha mating factor, the secretion signal, into the expression plasmid pAO815-S. *Pichia pastoris* GS115 was chosen as the expression host. The molecular weight of the secreted protein detected on the immunoblot was 46 kDa. Large amount of secreted rEK_L was produced in 2 L fermentor containing 1 L of fermentor culture by fed-batch cultivation technique. The supernatant was harvest after a total methanol induction of 72 h which the highest yield of rEK_L obtained. The secreted rEK_L was purified using ion exchange (Q Sepharose Fast Flow column) follow by the affinity (soybean trypsin inhibitor) chromatography. The researcher reported that the yield 6.3 mg of purified rEK_L was obtained from 1 L fermentation culture broth which was higher than the results from previous reports that produced in *E. coli* and COS-1 cell. The results of fusion protein cleavage showed that the high specific activity of rEK_L requires 100-fold less enzyme than the holoenzyme to cleavage fusion proteins.

In 2004, Peng et al. produced the recombinant bovine EK_L in secretory form by *P. pastoris* GS115 and used His-tagged purification system to purify the enzyme. The chosen expression vector was pPICZαA. The researcher reported that the production of rEK_L from mut^s strains was much higher than that by mut^+ strains. Large scale productions of rEK_L were performed in 7 L fermentor containing 3 L of medium using fed-batch cultivation technique. The estimated molecular weight of rEK_L containing His-tagged was 44 kDa. The rEK_L was purified by Nickel chelating chromatography. The highest concentration of rEK_L reached 350 mg/L at the optimum production condition and specific activity of purified rEK_L-His_6 was about 8000 unit /mg which was just a little less than un-tagged rEK_L. The researchers also indicated that the different glycosylation pattern was important to the specific activity of rEK_L.

In 2004, Fang et al. amplified the Chinese bovine EK_L then fused these DNA fragment to 3'end of secretion signal of α-factor and 5' end His_6. The fragment of α-factor-EK_L-His_6 was obtained by PCR technique. This fragment was inserted into pAO815 under Alcohol Oxidase promoter (AOX) which can be induced by methanol. *P. pastoris* GS115 was chosen as the expression host. The 43 kDa of protein band was found on polyacrylamide gel. The rEK_L containing His_6 was purified using Ni^{2+} affinity chromatography. The yield of purified rEK_L was 5.4 mg/L of fermentation culture. The activity of purified rEK_L was tested using GST-VAS fusion protein containing an EK cleavage site as substrate. The reaction was done at 16^oC for 16 h in 50 mM Tris-Cl pH 8. The protein product was detected using SDS-PAGE and the quantity or intensity of the product was performed by software Grab-it 2.5 and Gelwork (UVP) then calculated the cleavage efficiency towards substrate. The

research finding indicated that the fusion protein was completely cleaved by 16 ng of rEK_L in 16 h.

Strains of *Saccharomyces cerevisiae* have also been used as host for production of rEK_L. In 2005, Kim et al. produced C-terminal His-tagged rEK_L in *S. cerevisiae*. The recombinant plasmid of pIL20XEK_L-Ca-His encoding the C-terminal His-tagged EK_L was transformed into *S. cerevisiae* 2805. The media composition and cultivation condition were determined to find the optimum condition for efficient rEK_L production. The results showed that when the ratio of yeast extract to bacto peptone was 5.5, the concentration of 3.8 mg/L rEK_L and final ethanol concentration of 0.4 g/L were obtained. The researcher reported the optimum agitation speed and aeration feed rate in 5 L jar was 700 rpm and 2 vvm, respectively. They also suggested that DOT should be maintained at high level, more than 60%, in order to suppress ethanol accumulation. When excessive amount of ethanol accumulates in the culture broth, the fraction of plasmid-containing cells is reduced, leading to low product yield.

In 2001, Choi et al. cloned bovine EK_L gene and expressed in *S. cerevisiae*. The researcher incorporated His tag at either the N- or C-terminus of rEK_L to produce the N-terminal His-tagged EK_L and C-terminal His-tagged EK_L. The His-N-rEK_L was not purified as efficiently as the rEK_L-C-His under the same condition. The results of enzymatic activity assay using fluorometric substrate showed that the activity of rEK_L-C-His is similar to that of rEK_L, whereas no activity was detected with His-N-rEK_L. They concluded that the nature of N-terminus residue of EK_L is critical for its enzymatic activity whereas that the C-terminus is not.

In 2000, Svetina et al. expressed bovine rEK_L containing sequence of Kex2 protease cleavage site in the protease deficient filamentous fungus *Aspergillus niger*. The plasmid pAN56-2M was used as the expression vector. The fusion protein of glucoamylase-EK_L was expressed in soya milk medium. The secreted rEK_L was purified by ion exchange and affinity chromatography. The highest quality of rEK_L was up to 5 mg/L in the medium. Then, in the purification step, 1.9 mg/L of highly active rEK_L was recovered which showed a specific activity of 19,880 unit /mg protein.

From several reports, the expression levels and specific activity of purified rEK_L were difference in various hosts as summarized in table 3.1

Table 3.1 The comparison of rEK_L concentration and specific activity of this purified enzyme in the various hosts

Host cell	**Final concentration of rEK_L (mg / L of media culture)**	**Specific activity of purified rEK_L (unit / mg of purified protein)**
E. coli (In 2002 by Yuan et al.)	268	720
S. cerevisiae (In 2004 by Kim et al.)	3.8	-
P. pastoris (In 2004 by Peng et al.)	350	9,000
A. niger (In 2000 by Svetina et al.)	5	19,880

From these results, the methylotrophic yeast *P. pastoris* showed that it is the best system for rEK_L expression, thus it was chosen as the host for rEK_L production in this research. Although the specific activity of rEK_L which was produced in

P. pastoris was less than that in *A. niger* but the final concentration of this enzyme which was produced in *P. pastoris* was higher. The cultivation of *P. pastoris* is also easier than that of *A. niger*. Even through, the rEK_L expression in eukaryote system is better than in prokaryote system, but the bacteria system is simple, rapid, inexpensive, highly effective, fast high density cultivation and easily scaleable, thus in this work the rEK_L was expressed in both prokaryotic (*E. coli* systems) and eukaryotic (*P. pastoris* system). The enzymatic properties of rEK_L from both systems were compared to obtain the system which cheaper and higher quality rEK_L production.

3.1.2 *Pichia pastoris*

Pichia pastoris is an ascomycetous budding yeast that most common exists in a vegetative haploid state. On nitrogen limitation, mating occurs and diploid cells are formed. Since cells of the same strain can rapidly mate with each other, *P. pastoris* is by definition homotallic (Cregg et al., 1998). It is an eukaryotic host and the expressed of recombinant protein can undergo the necessary post-translational processing and secretion, result in a product that is either identical or more similar to the native protein (Lin Cereghino and Cregg, 2000). Compared to other eukaryotic expression systems, *Pichia* have many advantages, because it does not have the endotoxin problem associated with bacteria nor the contamination problem of proteins produced in animal cell culture (Cino, 1999). Compare to *S. cerevisiae*, the glycoproteins which are produced by *S. cerevisiae* are often over-glycosylated and terminal mannose residues in N-linked glycan are added by an α1,3 bond that is suspected to be allergenic (Terentiev et al., 2004). In high cell density for *S. cerevisiae* cultivation, ethanols (the product of *S. cerevisiae* fermentation) rapidly

build up to toxic levels which limit further growth and foreign protein production (Lin Cereghino and Cregg, 2000).

Heterologous proteins that are expressed by *P. pastoris* cells can be glycosylated on the Asn component of the Asn-Xaa-Thr/Ser consensus sequence and on Ser or Thr hydroxyl groups to provide N-linked and O-linked saccharides, respectively (Bretthauer and Castellino, 1999). Some researchers observed that the *S. cerevisiae* invertase secreted from *P. pastoris* is not hyperglycosylated but contains outer chains of only 8-14 mannose residues (Man8-14), compared to an average length of > 50 when the same enzyme is secreted from *S. cerevisiae* (Higgin and Cregg, 1998). The little differences in the size of the oligosaccharides could be correlated to whether the recombinant protein was prokaryotic or eukaryotic and the composition of the growth medium of culturing in shake flask or fermentor (Bretthauer and Castellino, 1999). For bovine rEK_L from *P. pastoris* expression, it is predicted that it has three potential N-linked glycosylation sites. Vazza et al. (1996) reported that the product of rEK_L which was treated with endoglycosidase H, which cleaves high mannose glycans, showed a shift in size from 46 kDa to 33 kDa.

Pichia pastoris is a methylotrophic yeast. It can grow on methanol as a sole carbon and energy source. It has a highly inducible methanol utilization pathway. Methanol oxidase, the first enzyme of the pathway, accounts for up to 35% of the total protein in cells grown on limited amounts of methanol (Sreekrishna et al., 1997). The first step in metabolism is the oxidation of methanol to formaldehyde, generating hydrogen peroxide in the process, by the enzyme alcohol oxidase (AOX). To avoid the hydrogen peroxide toxicity, this first step methanol metabolism take place within a specialized organelle, called the peroxisome that sequesters toxic hydrogen peroxide

away the rest of cell (Higgins and Cregg, 1998). A portion of the formaldehyde generated by AOX leaves the peroxisome and is further oxidized to formate and carbon dioxide by two cytoplasmic dehydrogenases, reactions that are a source of energy for cells growing on methanol (Fig. 3.1). There are two genes that encode alcohol oxidase in *P. pastoris*: AOX1 and AOX2; AOX1 is responsible for a vast majority of alcohol oxidase activity in the cell (Lin Cereghino and Cregg, 2000). A characteristic of AOX1 promoter is that it is strongly repressed in cells grown on glycerol, but is induced over 1000-fold when cells are shifted to a medium containing methanol as a sole carbon source (Damasceno, et al., 2004). The strains that are deleted at AOX1 causes to force them to reply on the transcriptionally weak AOX2 gene. AOX2 encodes a protein that is 97% identical to and has approximately the same specific as that of AOX1. AOX is undetected in cells cultured on carbon sources such as, glycerol, glucose or ethanol, but constitutes up to 30% of the total soluble protein in methanol grown cell (Cregg et al., 1993). In all methylotrophic yeast, they show the diauxic growth pattern when grow on a mixture of glucose and methanol. Glucose is utilized first and repressed C1 utilization enzyme. *P. Pastoris* is fully repressed in ethanol and unlimited glucose. Under the non-limiting glycerol and carbon starvation, some degree of the derepression of the promoter occurs (Tschopp et al., 1987).

The most common used approach for heterologous protein expression has been to express the gene of interest under the control of the AOX1 promoter. Genes of interest are integrated into *P. pastoris* genome via homologous recombination (Damasceno, et al., 2004). AOX synthesis is regulated at the transcription level and the promoter of this gene would be most useful for controlling the expression of

foreign genes (Cregg et al, 1993). The regulation of the AOX1 gene involves two mechanisms: repression/derepression and induction (Egli et al., 1980). The repression occurs when the cell is grown at moderate levels of repressing substrate. Gene is derepressed when the repressing carbon source is limited (Jahic et al., 2002; Egli et al., 1980).

Linear vector DNAs can be generated and stable transformants of *P. pastoris* can be obtained via homologous recombination between sequences shared by the vector and host genome. All *P. pastoris* expression vector carry at least one *P. pastoris* DNA segment (the AOX1 or GAP promoter fragment) with unique restriction sites that can be cleaved and used to direct the vector to integrate into the host genome by a single crossover type insertion event. Vector containing the *P. pastoris* His4 gene can also be directed to integrate into *P. pastoris* genomic at *his4* locus (Fig. 3.2a). Three methanol-utilizing phenotypes (mut^+, mut^s and mut^-) of *P. pastoris* have been used for heterologous proteins production (Higgins and Cregg, 1998). Insertion of the expression cassette into *his4* locus generates a mut^+ strain (methanol utilization plus), a phenotype indistinguishable from wild type *P. pastoris* (Lin Cereghino and Cregg, 2000). The crossover at both the AOX1 promoter and 3' AOX1 regions of the vector and the genome cause AOX1 coding region to be deleted. Transformants resulting from an AOX1 replacement event are phenotypically His^+ and mut^s (Fig. 3.2b) (Higgins and Cregg, 1998). The mut^s phenotype was obtained from the disruption of AOX1 gene by gene insertion (Inan and Meagher, 2001). This strain must rely on the much weaker AOX2 gene for AOX and grows on methanol at a slow rate (Higgins and Cregg, 1998.). This strain sometimes expresses higher level of foreign protein than wild-type host, especially in shake flask culture (Cregg et al.,

1993). The mut$^-$ (methanol utilization negative) strain is the strain which both AOX1 and AOX2 genes are disrupted (Inan and Meagher, 2001). All of these strains, even the mut$^-$ strain, retain the ability to induce expression at high levels from the AOX1 promoter (Lin Cereghino and Cregg, 2000).

With *P. pastoris*, heterologous proteins can either be expressed intracellularly or secreted into the medium. Because *P. pastoris* secretes only low levels of endogenous proteins and its culture medium contains no added proteins, a secreted heterologous protein comprises vast majority of the total protein in the medium. Thus, secretion serves as a major first step in purification and separating the foreign protein from the bulk of cellular proteins (Higgins and Cregg, 1998). Fermentation of *P. pastoris* is especially important for secreted proteins because the concentration of product in the medium is proportional to the concentration of cell in culture. The level of transcription initiated from the AOX1 promoter can be 3-5 times greater in cells fed methanol at growth-limiting rate compared to cells grown in excess methanol (Higgins and Cregg, 1998). The low levels of foreign proteins expression have been observed from shake flask culture of *P. pastoris*. Potential reasons for this include: a lack of pH control in typically shake flask cultures, inadequate aeration of cultures, or inability to control feeding of carbon sources at optimal (growth-limiting) rate. To solve these problems, the medium buffering that is adequate for the culture density, adjust to a pH that stabilized and minimized proteolytic degradation of product and addition of a modest amount of peptone or casamino acid which may protect the product from proteolysis, have been used (Cregg et al, 1993). Although some foreign protein have expressed well in shake-flask cultures but the expression level are still low when compare to fermentor cultures (Lin Cereghino and Cregg, 2000). Because

of these reasons, in this research, large scale production of rEK_L was performed in 2 L fermentor to obtain large amount of the secreted recombinant protein. The rEK_L protein was expressed in *P. pastoris* using the α-factor secretion signal to secrete the rEK_L into the medium for easily separation and purification.

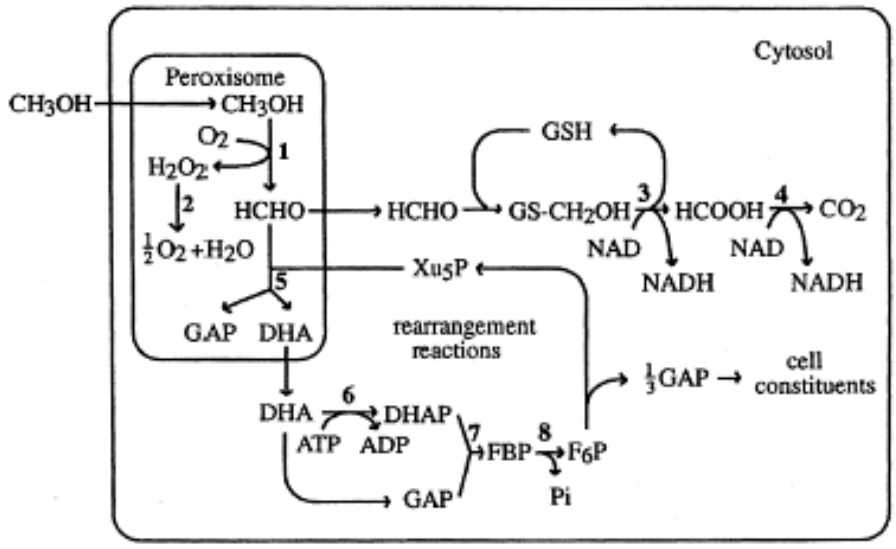


Fig. 3.1 The methanol pathway in *P. pastoris*. 1, alcohol oxidase; 2, catalase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase, 5, dihydroxyacetone synthase; 6, dihydroxyacetone kinase; 7, fructose 1,6-bi-phosphate aldolase; 8, fructose 1,6-biphosphate (Lin Cereghino and Cregg, 2000).

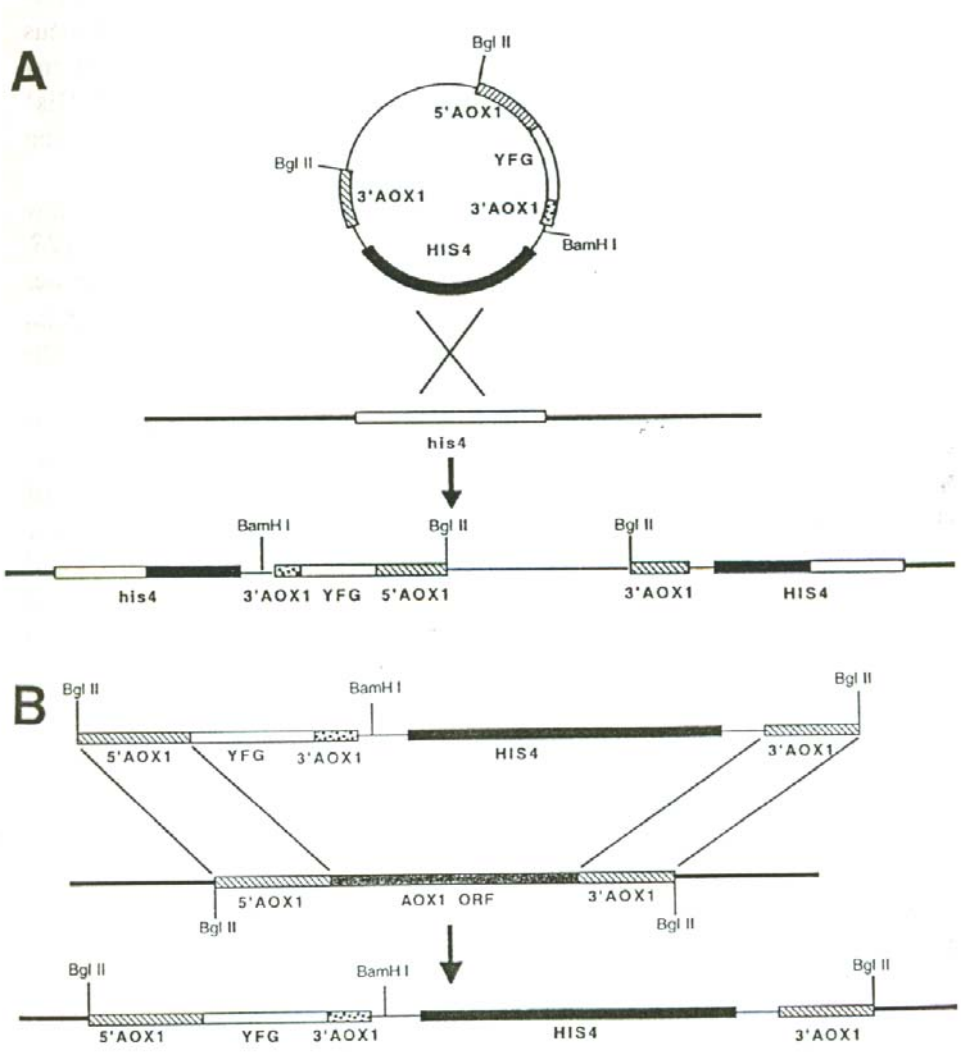


Fig. 3.2 Integration of expression vectors into the *P. pastoris* genome. (A) Single crossover integration into the *his4* locus. (B) Integration of vector fragment by replacement of AOX1 gene (Higgins and Cregg, 1998).

3.1.3 Fermentor cultivation of *P. pastoris*

The growth conditions for *P. pastoris* are ideal for large scale production of heterologous protein, because the medium components are inexpensive and defined, consisting of pure carbon sources (glycerol and methanol), biotin, salts, trace elements, and water. Since *P. pastoris* is cultured in media with relatively low pH and methanol, it is less likely to become contaminated by most other microorganisms (Lin Cereghino and Cregg, 2000). The necessary of fermentor culture is that the environment control in fermentor can be improved resulting in high cell density of *Pichia* cells (100 g/L dry cell weight or 500 OD_{600}) (Higgins and Cregg, 1998). Production of recombinant proteins by *P. pastoris* fermentation is typically carried out in a fed-batch mode, resulting in high cell densities and high levels of product (Zhang et al., 2005). Four stages fermentation protocol involve glycerol batch phase, glycerol fed-batch phase, methanol induction and methanol production phase has been suggested for recombinant protein expression in *P. pastoris* (Jahic et al., 2002).

The first step is glycerol batch phase; the culture is grown in a salt medium containing glycerol which was used as a sole carbon source. During this time, biomass accumulates but heterologous gene expression is fully repressed (Lin Cereghino and Cregg, 2000). Upon glycerol depletion, the second phase is initiated by adding glycerol at a growth-limiting rate (Inan and Meagher, 2001). The glycerol is fed to the bioreactor. Limiting glycerol concentration during this phase gives a short period of derepression to the AOX1 promoter which allows the cells to transition smoothly change from glycerol phase to methanol phase (Higgins and Cregg, 1998; Chiruvolu et al., 1998). The third step is the methanol induction phase, which is

initiated by adding limited amount of methanol. The methanol concentration at this phase must be carefully monitored by gas chromatography or dissolved oxygen tension (DOT) because in the presence of excess oxygen and methanol, formaldehyde, the first product of methanol methabolism, rises to toxic level for the cells (Higgins and Cregg, 1998). It is very important to feed methanol slowly in the first 2-3 h of the methanol induction phase to adapt the culture to grow on methanol (Invitrogen Corporation, n.d.). Then the methanol feed rate is adjusted to match the increasing of the AOX activity (Jahic et al., 2003). When the culture is adapted to methanol, the methanol production phase is initiated. During the main production phase, the methanol feed rate is adjusted to maintain the DOT reaches about 25% air sat to advoid oxygen limitation. Under this conditions, the concentration of methanol is very low and growth rate was limited (Charoenrat et al., 2005). The dissolve oxygen tension or DOT, the value related to the oxygen concentration in the medium can be used to monitor the substrate concentration in the culture medium. *Pichia* will consume oxygen as it grows causes the DOT value to reduce. Because oxygen is required for the first step of methanol catabolism and also in the energy metabolism, thus it is important to maintain the DOT at a high enough level to ensure growth of *Pichia* on methanol (Invitrogen Corporation, n.d.).

The increase of the oxygen transfer rate (OTR) in the *Pichia* system has been report to enhance the oxygen uptake rate (OUR) due to the increase of the methanol (substrate) consumption rate, cell growth and productivity of many recombinant proteins. Two fermentation techniques have been designed base on the general OTR model [$OTR = K_L a(C^*-C)$]. OTR is affected by three parameters include the oxygen transfer rate coefficient (K_L), the specific air bubble area per volume and driving

force(C*-C). The first technique was designed to run the process under oxygen limited conditions due to decreasing the oxygen concentration in liquid outside the film (C~0), thereby resulting in an increase in the driving force. The second technique was designed to increase the oxygen solubility in equilibrium with the gas phase (C*) by increasing the total air pressure in the bioreactor (Charoenrat , 2005).

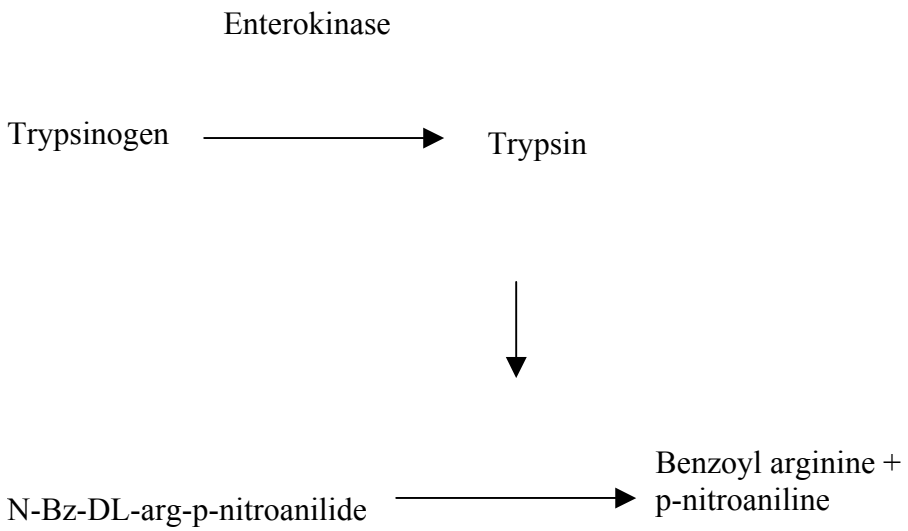
To achieve high cell density, the control environment of bioreactor is very important. Several researchers have shown that optimum protein production in *P. pastoris* occurred at 30^{o}C, and that all protein expression ceases at 32^{o}C (Cino, 1999). However, the secretion of the protease to the medium occurred by dead cell causes the decrease of secreted recombinant proteins. The proteolysis degradation is a significant problem in many high cell density cultures. To solve this problem, several researches have adjusted pH, temperature and in the other strategies have added casamino acid and peptone or using protease deficient strains to reduce the secreted proteases (Jahic et al., 2003). In 2003, Jahic et al. reported that decreasing pH and temperature during methanol production phase from 5 to 4 and 30 to 22^{o}C, respectively can increase the yield of full-length fusion protein. The secreted full-length fusion protein from this condition, 1.5 g/L, was higher when compared to the original cultivation. For rEK_L, Peng et al. (2004) reported that the optimal pH for rEK_L production was 5.5-6.

In this work, rEK_L was expressed in 2 L fermentor using four stages fermentation protocol involve glycerol batch phase, glycerol fed-batch phase, methanol induction and methanol production phase. The optimal pH was adjusted to 5.5 which reported by Peng et al. (2004) as an optimum pH to obtain the maximum yield of secreted rEK_L. The temperature of recombinant protein production was

compared between 30 and 20°C to obtain the best condition for high quality of rEK_L production.

3.1.4 Enzymatic activity assay

The native enterokinase enzymatic activity assays have been described base on two step procedures by Kunitz since 1939 (Jeno et al., 1987). Assay of enterokinase activity using trypsinogen as substrate was performed based on the following reactions.



Trypsinogen was activated to trypsin by pre-incubation with enterokinase at pH 6 and trypsin formed was assayed by adding N-Bz-DL-arg-p-nitroanilide. After stop reaction by adding 30% acetic acid, the color of generated p-nitroaniline was measured at 410 nm. One unit of enterokinase estimated is defined as that amount which produced one amidolytic unit of trypsin under the specified assay conditions. One amidolytic unit of trypsin is that amount which liberated 1 μmol of p-nitroaniline in 1 min (Bhat et al., 1981). The main difficulty lies in the prevention of trypsinogen

auto activation (Jeno et al., 1987). In 1976, Hesford et al. reported the synthesis of an artificial substrate tert-butyl-oxylcarbonyl-tetra-L-aspartyl-L-lysyl-2-naphthylamide; the 2-naphthylamide released was assayed colorimetrically with diazo dye. This substrate exploits the tetra-aspartyl sequence characteristic of the activation peptides of mammalian trypsinogens. The artificial substrate has a lysine residue on the carbonyl side of the target bond it should, like trypsinogen, be cleaved by trypsin (Grant and Taylor, 1979). In 1979, Grant and Taylor reported that the artificial substrate for enterokinase activity assay using glycine-tetra-L-aspartyl-L-lysyl-2-naphylamide (Gly-$(Asp)_4$-Lys-Nap) as a substrate. The researcher reported that the pH optimum for human enterokinase was 8.4 in 25 mM Tris-HCl buffer and did not hydrolyze this substrate at pH below 7. The research finding showed that in the presence of 10 mM Ca^{2+}, the hydrolysis of Gly-$(Asp)_4$-Lys-Nap by human enterokinase was three times faster than that in 0.1 mM Ca^{2+}. An increasing due to a decrease in k_m but also the rise in k_{cat}. Ca^{2+} would seem to increase the affinity of enterokinase for the tetra-aspartyl and promoting dissociation of the product. The researcher also reported that Gly-$(Asp)_4$-Lys-Nap was also hydrolyzed by trypsin but it was not possible to determine any values of k_m; their data indicated that the mechanism of hydrolysis was complex and did not obey Michaelis-Menten kinetics. The Lys-Nap bond of this fluorogenic substrate is hydrolyzed by enteropeptidase generated 2-Napthylamide which can be measured fluorometrically (Jeno et al., 1987). Recently, Gly-$(Asp)_4$-Lys-Nap have been used as the fluorogenic substrate for enterokinase activity assay in several researches. The bovine rEK_L activity was measured by an increase of fluorescence (excitation at 337 nm, emission 420 nm)

caused by the release of β-napthylamide. One unit (AU) of rEK_L was defined as one absorbance increase of fluorescence (excitation at 337 nm, emission 420 nm) in a half minute caused by enterokinase (Yuan and Hua, 2002). There have been several researches that determined the activity of rEK_L using the ability of cleaving the fusion protein containing the enterokinase recognition site and detected the expect product by SDS-PAGE technique. This technique is cheaper than the fluorometric method and can be used to confirm that the hydrolysis site of active rEK_L is correct. In 2004, Fang et al. determined the cleavage activity of rEK_L using GST-VAS fusion protein containing enterokinase recognition site as a substrate. The researcher determined the quantities or intensity of the product on SDS-PAGE by software Grab-it 2.5 and Gelwork (UVP) to calculate the cleavage efficiency of rEK_L.

In this works, the activity of rEK_L was assayed using fluorescence (Gly-$(Asp)_4$-Lys-Nap) and fusion protein containing enterokinase recognition site as a substrates. The activity of rEK_L was calculated as the change in fluorescence over time. The product from fusion protein cleavage was detected by SDS-PAGE technique. The results of activity assay were compared to the activity of commercial rEK_L from NEB.

3.1.5 Protein purification

There are a range of chromatographic techniques available for purification of biomolecules include salt precipitation, ion exchange, hydrophobic interaction, affinity, size exclusion, chiral, thiophilic. Ion exchange, hydrophobic interaction, affinity chromatographies are adsorptive techniques enabling selective adsorption/ desorption of one or more components of the feed stock (Levison, 2001). Separation

by chromatography depends on the differential partition of proteins between a stationary phase (the chromatographic medium or the adsorbent) and a mobile phase (the buffer solution). Normally the stationary phase is packed into a vertical column of plastic, grass or stainless steel, where as the buffer is pumped through this column. Highly specific methods, such as bioaffinity, e.g., antibody-antigen interaction, do in some cases give a highly pure protein in a single step (Jason and Ryde'n, 1989). In this research, the protein purification techniques focused on the basic purification method using ion exchange chromatography and the engineered polyHis-tagged to improve purification process.

3.1.5.1 Ion exchange process

Ion exchange chromatography is a widely used protein separation technique in both small laboratory scale and large-scale purification. It is cheap compare with other chromatographic methods (Hedhammar et al., 2004). The basis for the ion exchange process is the reversible interaction between a charged protein and an oppositely charged chromatographic medium (Protein Purification, 2001). Proteins bind to ion exchangers by electrostatic forces between the protein's surface charges (mainly) and the dense cluster of charge groups on the exchanger. The charges are balanced by counterions such as metal ions, chloride ions and sometimes buffer ions (Scopes, 1994). When above its pI a protein will bind to an anion exchanger, when below its pI a protein will bind to a cation exchanger (Protein Purification, 2001). The interaction between the proteins and the ion exchanger depends on several factors include net charge and surface charge distribution of the protein, the ion strength and the nature of the particular ion in the solvent, pH, other additives to the solvent. The

binding of protein to charged group on the stationary phase competes with the binding of other ions in the solvent. The pH is one of the most important parameters which determine protein binding as it determines the effective charge on both the protein and the ion exchanger. At pH far away from the pI, proteins bind strongly and do not desorb at all low ionic strength. Near to its pI, the net charge of a protein is less and consequently it binds less strongly (Janson and Rydén, 1989).

A large variety of types of ion exchangers have been used for protein purification. Ion exchangers consist of matrix substituted with either basic or acidic groups. The basic ion exchangers containing positive groups are called anion exchangers, while the acidic ones containing negative groups are called cation exchangers. The charge of the ion exchanger depends on the pH (Janson and Ryde´n, 1989). The carboxylmethyl group of a cation exchanger and diethylaminoethyl groups of an anion exchanger have been attached to the suitable material such as cellulose, agarose, dextran, silica and synthetic polymer. The more recent trend is to attach charged group that remain charged at extreme of pH, such as quaternary amino groups and sulfopropyl (SP-). At low pH operation, the “stronger” acidic group of sulfonate is needed. Sulfo groups remain fully charged right down to pH 1 (Scopes, 1994).

For mobile phase, normally the concentration of the buffer salt during protein adsorption is low around 0.01 M – 0.05 M. To avoid pH disturbances in the initial part of the chromatogram, the maximum buffer capacity is needed. A buffer has a maximal buffering capacity at its p*Ka*. One pH unit away from the pK, the buffering capacity has decreased five-fold since 90% of the proteolytic stem is in either the base or the acid form. To avoid disturbances in the pH causes by the buffering species itself participating in ion exchange, the buffering ion should not interact with or bind

the ion exchanger. For anion exchanger a positive buffering ion, such as Tris, p*Ka* 8.2, is preferred and has Cl^- as counterion. For cation exchanger, the buffering ion should be negative charged, for example phosphate, carbonate, acetate, and the counterion are K^+ or Na^+ (Janson and Ryde'n, 1989).

Before using ion exchanger column, the ion exchanger must be equilibrated. The purpose of the equilibration procedure is to ensure that the ion exchanger is in equilibrium with the counter-ions which will be used during elution. It is important that the ion exchanger is equilibrated to the pH and ionic composition of the starting buffer changing in the composition of the mobile phase during the chromatography are to be avoided (Janson and Ryde'n, 1989). The protein is now in an appropriate buffer for applying to the ion-exchange column. Ion exchange involves buffer ions, and a rapid displacement of counterions by protein molecules should result in sharp local changes in pH and salt concentrations. Usually, the condition for adsorption, the protein will stick to the top of the column and stay there until buffer conditions are altered for the elution procedure. At any point on the column reach by the protein, 10% remain in solution and pass down to the next point. On average, after 10 column volumes of the starting buffer have been applied, the protein will begin to emerge from the bottom (Scope, 1984).

For the elution procedure, two general methods for eluting proteins are available. First is to change the buffer pH to a value where binding is weakened, lower pH for an anion exchanger (to make the adsorbed proteins less negative), or higher pH for a cation exchanger (to make the proteins less positively charge). Second is to increase the ionic strength, thereby weakening the electrostatic interaction between protein and adsorbent. The change of pH buffer method for elution procedure

is not very successful. That because, unless there is a very high buffering capacity, sudden large pH changes happen as protein become eluted, so there is little separation of individual components. Salt gradients have been used to elute protein from ion exchanger. Usually either potassium or sodium chloride is used to generate the gradient. The action of the salts can be considered that the salt can directly displace the protein; the ions (e.g., Cl^- on the DEAE-adsorbent) occupy the positively charged sites and block reattachment by protein (Scope, 1984).

3.1.5.2 Poly His tag for protein purification

Affinity tags are often very selective and thereby minimize the number of unit operation necessary for a pure product (Hedhammar et al., 2004). Although, several protein purification systems have been used such as using maltose-binding protein, glutathione-s-transferase protein fusion but the problem is their large size may interfere the corrected folding and function of the fusion protein. To solve the problems, the small tag, poly histidine tag has been widely used (Schäfer et al., 2002).

The His-tagged protein purification system exploits the affinity of histidine residues for metal cations. A polyhistidine fusion has high affinity and with optimization of the process, the fusion protein can be purified to a very high level in a single step. The immobilized metal affinity chromatography (IMAC) is the chromatography which support with a chelating agent and charged with cation such as Zn^{2+}, Ni^{2+} (Brewer et al., 2001). Using hexahistidine tag (6xHis tag) and IMAC purification technique has many advantages. The 6xHis tag is the smallest tag available (6 amino acids). It is non charged at physiological pH and therefore usually shows the least interference with protein structure and function. The high affinity of

6xHis tag for Ni-NTA (nickel-nitrilotriacetic acid) , the high capacity matrices, has been applied in commercial His-tagged protein purification system (Schäfer et al., 2002).

The 6xHis tag can be incorporated into any expression vector using PCR, or fragment ligation. The gene or a cDNA encoding the interested protein is inserted into the expression vector in the appropriate site and in frame of 6 histidine residues (at C- or N-terminal). Under the production condition the protein is expressed as a fusion protein containing 6xHis tag (The Qiaexpressionist, 2001).

Nickel ions (Ni^{2+}) can form six electron coordination bonds. NTA binds to four of these bonds. Two ligand binding sites are then available to readily coordinate with a His-tagged protein. The picture of Ni^{2+} and His-tagged interaction is shown in Fig. 3.3. In 2001, Quigen developed the method of 6xHis-tagged protein purification system for recombinant protein purification. The system is base on the selective and affinity of Ni-NTA metal-affinity chromatography matrices for biomolecules which have been tagged with 6 consecutive histidine residues. The imidazole rings which is the part of histidine residue bind to the nickel ions and disrupt the binding of dispersed histidine residues in the nontagged background proteins. The untagged proteins that have many histidine residues on their surface will also bind to Ni-NTA but the interaction will be much weaker than the binding of 6xHis-tagged protein.

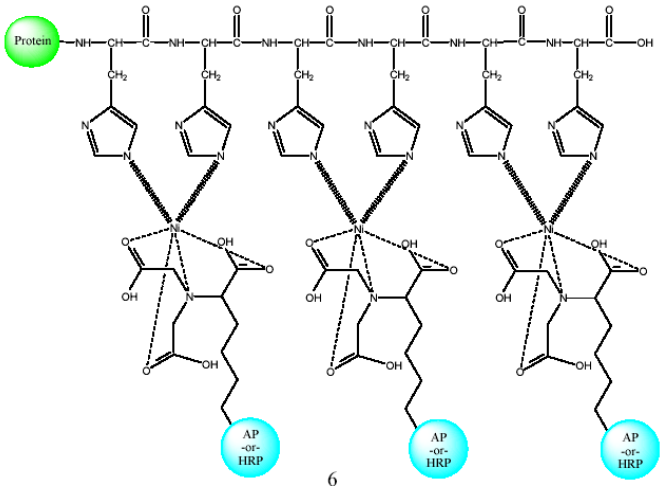


Fig. 3.3 His-tagged protein interaction with Ni-NTA conjugated to HRP/AP) (KPL Inc., www, 2007)

Since the binding of tagged protein to Ni-NTA resin is not conformation-dependent and is not affected by most detergents and denaturants thus protein can be purified either under native or denaturing conditions. The condition for purification (denature or native) depends on protein location, protein stability and the accessibility of the 6xHis-tagged to retain biological activity. The purification under native condition, the 6xHis-tagged protein must be soluble. In the case of 6xHis-tagged is hidden by the tertiary structure of the native protein, the soluble protein should be denatured before purification on the Ni-NTA. The purification under denaturing condition, strong detergent such as 6 M GdnHCl or 8 M urea completely solubilize 6xHis-tagged protein, the 6xHis-tagged on a protein will be fully exposed and bind to Ni-NTA (The Qiaexpressionist, 2001). GdnHCl is a salt and, therefore, at low concentrations, Gdn^+ and Cl^- ions are presume to mask the positive charge and negative charged amino acid side chains, thereby, the reducing or destabilizing electrostatic interaction can occur. The binding of Gdn^+ ions to the proteins is

presumed to predominate and to push the equilibrium toward the unfold state. The denaturing action of urea was presumed to mainly base on its ability to bind to the protein. Because the urea molecule is uncharged, it is not effect to have any significant effect on the intermolecular and intramolecular electrostatic interactions in the protein. Thus, free energy of unfolding from urea denaturations indicated the stabilizing effect of hydrophobic interactions and the destabilizing effect of electrostatic repulsions (Monera et al., 1994). Using this condition, the binding of 6xHis-tagged protein to Ni-NTA can be improved.

Endogenous proteins with histidine residues that interact with the Ni-NTA groups can be washed out of the matrix by lowering the pH value or by adding 10-50 mM imidazole. The elution procedure can be done by increasing the concentration of imidazole or decrease the pH values. The indole ring of histidine has p*Ka* of 6.5, therefore at a pH below 6.5 the His tag will have a net positive charge (Mcalister, 2007). To elute 6xHis-tagged protein by decreasing pH values, at pH below 6, the histidine side chain is protonated and the binding of the Ni^{2+} and His-tagged is disrupted. To avoid the protein damaged by reduction in pH, increase the concentration of imidazole for 6xHis-tagged protein elution is mildest and is recommended under native conditions. When imidazole concentration is increased to 100-250 mM, the 6xHis-tagged protein will dissociate because they can no longer compete for binding sites on the Ni-NTA resin (The Qiaexpressionist, 2001).

From several researches, the rEK_L has been purified by ion exchange and affinity chromatography. The fusion protein cleavage by rEK_L has been suggested that after fusion protein are cleaved, removal of enzyme is highly recommended to prevent the further degradation of the target protein (Choi et al., 2001). Because of

this reason, in this research, the bovine EK_L was inserted inframe into the expression plasmid containing His tag at 5' or 3' end allows selective adsorption of rEK_L containing His-tagged on to the Ni^{2+} chelating column. Using His-tagged purification technique, the purification and separation of rEK_L are very easy. In this research part, to obtain high quality of rEK_L, the recombinant enzyme was purified by ion exchange and His-tagged purification system.

3.2 Objectives

- To express the rEK_L in *E. coli* and *P. pastoris* Y11430 and compared the enzymatic properties of rEK_L from both systems

- To produce large amount of rEK_L in a 2 L fermentor by *P. pastoris* under optimal production condition.

- To purified and test enzymatic properties of rEK_L then compared the enzymatic activity of the obtained rEK_L to the commercial EK_L

3.3 Research methodologies

3.3.1 Materials

The methylotrophic yeast *P. pastoris* strain Y11430 is a wild-type mut^+ and His^+. The expression vectors used for *Pichia* expression system in this work are pPICZαB thrombin and pPICZαB NH8 which were modified from pPICZαB from Invitrogen. Both of them are expression vectors used for expression of secreted recombinant proteins in *P. pastoris*. They contain AOX1 promoter which can be induced by methanol. Selection of these vectors is base on the selectable marker, Zeocin resistance, which is bifunction in both *P. pastoris* and *E. coli*. The pPICZαB

thrombin and pPICZαB NH8 are different in the position of His tag. The pPICZαB thrombin contains C-terminal 6xHis-tagged and the pPICZαB NH8 contains N-terminal 8xHis-tagged.

The plasmid vector, pET32a, and *E. coli* strain Origami, BL21 pLysS and Rosetta gami were purchased from Novagen. The pET32a is designed for high expression of recombinant protein of interested fused to the C-terminus of the 109 amino acid of Trx-Tag or thioredoxin protein. Cloning sites are available for producing fusion proteins containing His-tagged and S-tag at the C-terminus for detection and purification (Fig. 3.5). The expression of the fusion protein is transcribed by T7 RNA polymerase (Novagen, www, 2007). Origami host strains are the strain that have mutations in both the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes resulting in enhances disulfide bond formation in the cytoplasm (Prinz et al., 1997). Rosetta strains carry an additional feature, Novagen has added the *leu W* and *proL* tRNA genes to create pRARE plasmid, which encodes tRNAs for all of the rarely used codons encoding Arg, Ile, Gly, Leu and Pro. These host strain are well suited to enhance the expression levels of proteins from target gene containing rare codons that would otherwise impede translation (Novy et al., 2001). Rosetta gami strains are derived from Origami strains, and carry the pRARE plasmid for rare tRNAs in a K-12 background with the *trxB*/*gor* mutations to enhanced disulfide bond formation in the cytoplasm (Novagen, www, 2007). The BL21 (DE3) pLysS strain is a derivative of *E. coli* B. It is deficient in both *lon* and *omp T* proteases, resulting in superior isolation of intact recombination proteins. This host carries a chromosomal copy of the T7 RNA polymerase gene that is controlled by the *lacUV5* promoter and utilizes the T7 RNA promoter to control protein expression. The strain carries a low-

level expression plasmid that expresses the T7 lysozyme. T7 lysozyme binds to T7 RNA polymerase, thus the transcription is inhibited. On IPTG induction, overproduction of the T7 RNA polymerase shuts down any low-level inhibition by T7 lysozyme, thus the recombinant protein can be transcribed. This is ideal for extremely toxic proteins expression because expression does not occur until the induction condition will be initiated (Nextgensciences, www, 2007).

All restriction enzymes were purchased from NEB. Antifoam was purchased from Fluka. The fluorogenic enterokinase substrate (Gly-$(Asp)_4$-Lys-β-naphthylamide) was obtained from Sigma.

3.3.2 Constructions of expression vectors

For *E. coli* expression system, the fragment of EK_L was amplified with EK_L_4 and EK_L_5 primers, contains *Nco*I and *Xho*I restriction site, respectively (Table 2.1). After this PCR product and pET32a expression plasmid were cleaved with *Nco*I and *Xho*I restriction enzyme, they were purified. The digested PCR product was then ligated between the *Nco*I and the *Xho*I sites of the cut vector resulting in EK_L fused with thioredoxin (Trx) fusion partner at N-terminal followed by the enterokinase cleavage site and 6xHis-tagged at C-terminal (Fig. 3.4).

For *Pichia* expression system, the entire cDNA encoding bovine EK_L gene in the pGEM -T easy vector was amplified with primers EK_L_2 and EK_L_3 which was flanked by *Eco*RI and *Sac*II restriction site, respectively (Table 2.1). This PCR product was digested with *Eco*RI and *Sac*II restriction enzymes then inserted inframe with the prepro α-mating factor, secretion signal, between *Eco*RI and *Sac*II restriction

site of pPICZαB thrombin and pPICZαB NH8 expression vectors resulting in rEK_L containing His tag at C- or N-terminal, respectively (Fig. 3.5).

All of plasmids constructed were transformed into *E. coli* DH5α by heat shock method. The clones containing the recombinant plasmid were sequenced to ensure preservation of the original DNA sequence and to ensure the correct in frame translation.

3.3.3 Expressions of recombinant bovine EK_L in shake flask scale

3.3.3.1 Prokaryotic expression system

The recombinant plasmid, pET32a_EK_L, and the control of plasmid no inserted, pET32a, were transformed into *E. coli* strain Origami, BL21 pLysS and Rosetta gami by heat shock method. The colonies containing plasmid were selected on LB-ampicillin/kanamycin/tetracycline (Appendix I) for Origami strain and LB-ampicillin/chloramphenicol (Appendix I) for BL21 pLysS and Rosetta gami strains. In the step of expression screening, the transformant colonies were selected and transferred to 125 ml shake flask containing 20 ml LB-ampicillin/kanamycin/tetracycline medium for Origami strain. For the expression of BL21 pLysS and Rosetta gami strain, the single colony was transferred to 100 ml of LB-ampicillin/chloramphenicol (induced and un-induced condition).

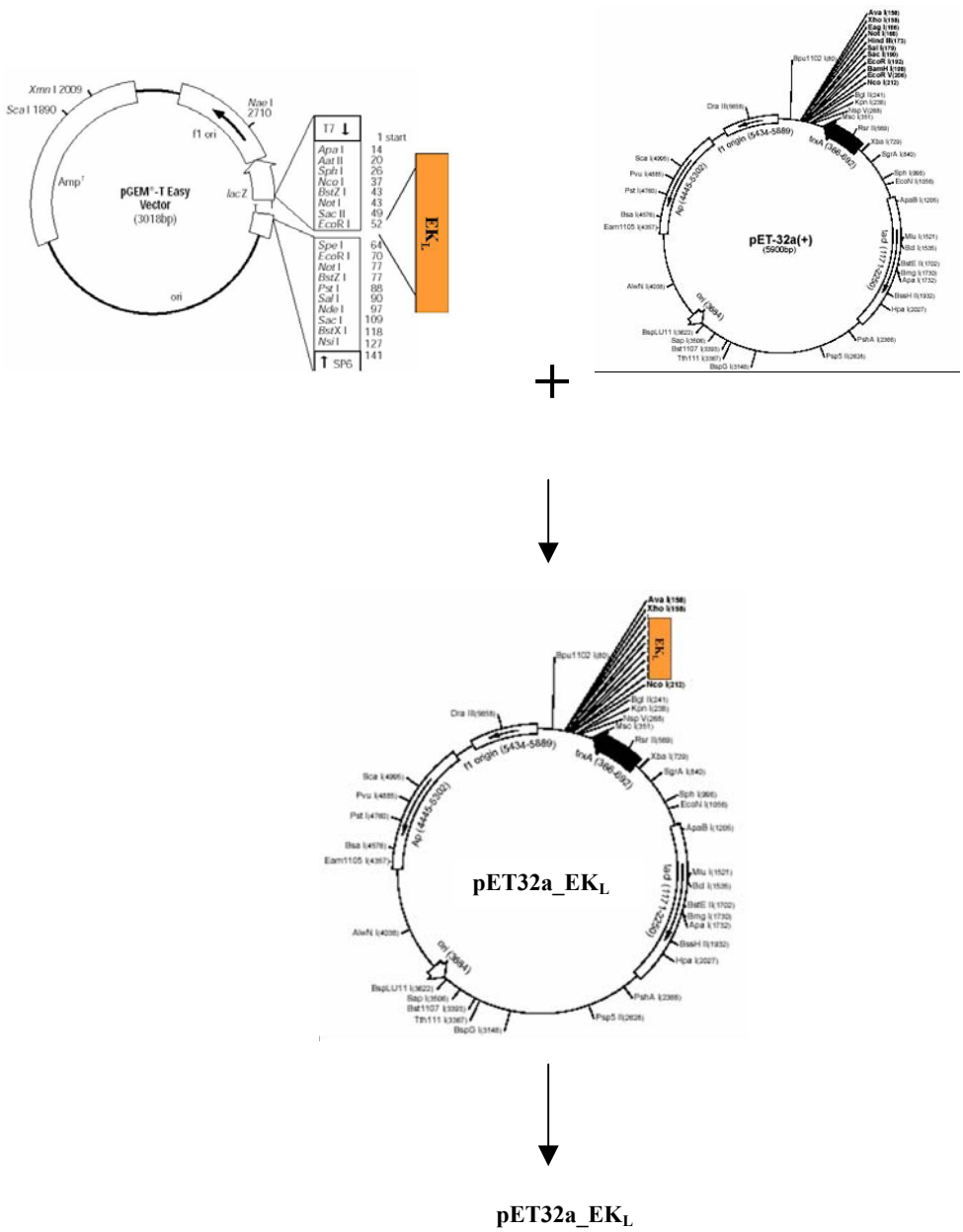


Fig. 3.4 Map of the pGEM-T EK_L, and pET32a for rEK_L expression in *E. coli*. A cDNA of EK_L was fused in-frame to 3' coding sequence of thioredoxin follow by sequence encoding 6xhistidine purification tag, enterokinase recognition site and 6xHis- tagged C-terminal of rEK_L.

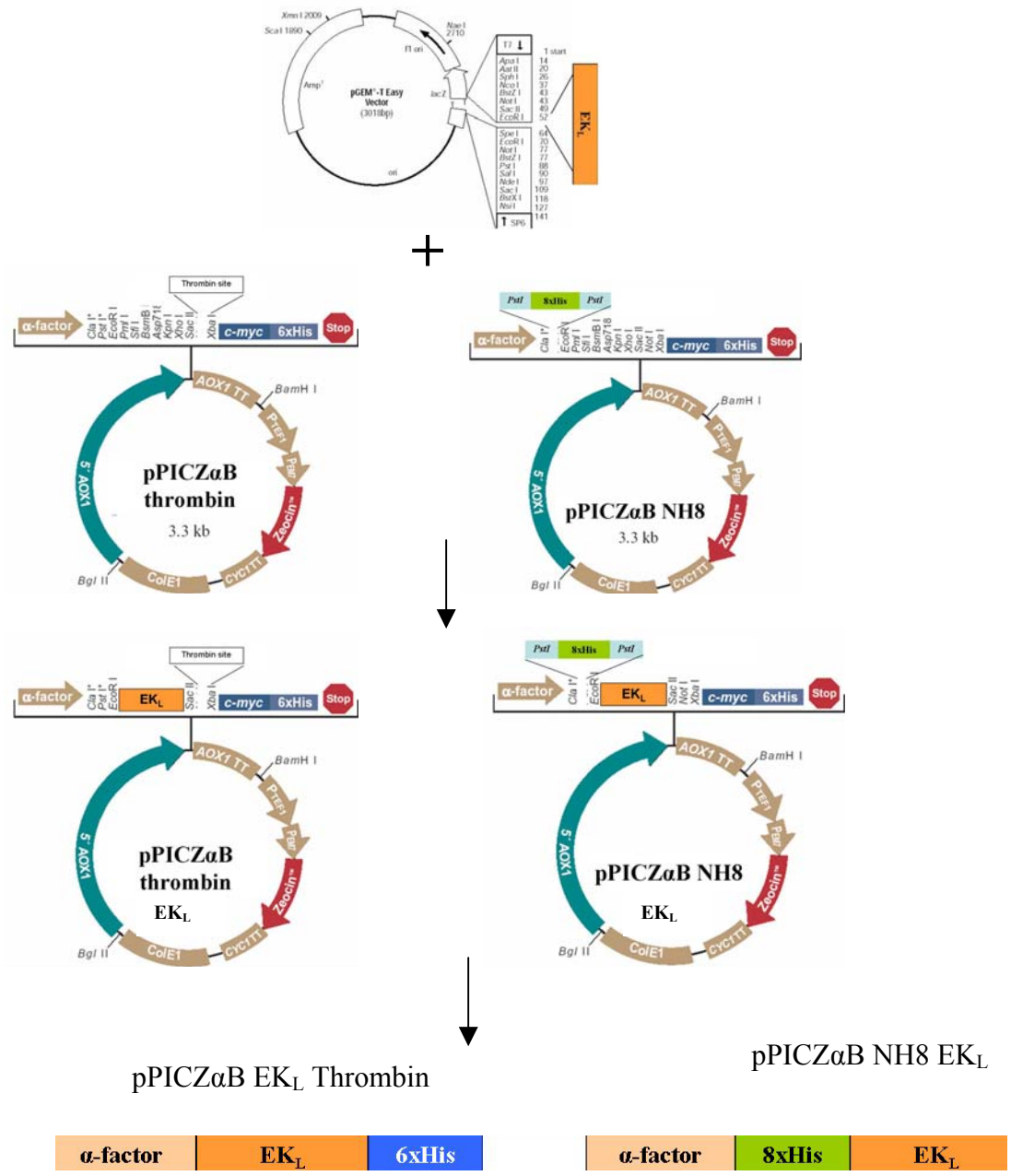


Fig. 3.5 Map of the pGEM-T EK$_L$, pPICZαB thrombin and pPICZαB NH8 for rEK$_L$ expression in *P. pastoris*. A cDNA of EK$_L$ was fused in-frame to 3' coding α-factor sequence of pPICZαB thrombin and pPICZαB NH8 resulting in rEK$_L$ containing 6xHis-tagged at C-terminal and 8xHis-tagged at N-terminal, respectively.

The cultures were incubated at 37^{o}C, 200 rpm for 3-4 h (or until OD_{600} ~ 0.6-0.8) then the induction were initiated by adding 0.8 mM IPTG into 10 ml Origami culture broth and 0.4 mM IPTG into 100 ml BL21 pLysS and Rosetta gami culture broth of the induced condition flask. Both induced and un-induced cultures of pET32a_EK_L and the control, pET32a, from each strain, were incubated at 20^{o}C, 200 rpm for 16 h. At the end of the cultivation, the cell pellet were harvested by centrifugation at 4,000 rpm, 4^{o}C for 30 min then resuspend in 50 mM Tris-Cl pH 8 and freeze at -70^{o}C for 30 min then rapidly thaw at room temperature. To lyses the cell, lysozyme was added into the thaw suspension to a final concentration of 200 μg/ml and incubated on ice for 30 min then transferred to sonicator. The fractions of insoluble and soluble were separated by centrifugation at 14,000 rpm for 15 min. Both fractions of each culture were checked for the protein band by SDS-PAGE technique. The enzymatic activities of crude soluble fraction were tested using fluorogenic substrate.

3.3.3.2 Eukaryotic expression system

The pPICZαB thrombin_EK_L and pPICZαB NH8_EK_L were digested with *Sac*I to generate linearized plasmid and introduce into *P. pastoris* Y11430 by electroporation method (Invitrogen, n.d.). The colonies containing recombinant plasmid were selected on YPD-zeocin (Appendix I) and screened by colony PCR technique with gene specific primers, EK_L_2 and EK_L_3 primers, to verify the integration of plasmid into *Pichia* genome.

The transformants were tested the expression level of rEK_L in shake flask scale. A single colony of transformants were inoculated in 1,000 ml shake flask containing 100 ml BMGY medium (Appendix I) and grown at 28^{o}C in incubator

shaker for 48 h. The induction was initiated by adding methanol to a final concentration of 1-3 % (v/v) every 12 h to induce AOX1 promoter. After 72 h of induction, supernatant and cell pellet were separated by centrifugation at 4,000 rpm for 30 min. Cell pellet was tested for transcription of EK_L mRNA by RT-PCR technique. Total RNA of *P. pastoris* was extracted using Trizol reagent (Invitrogen) then expression of EK_L mRNA was detected by two step RT-PCR and nested PCR techniques with EK_L_2 and EK_L_3 primers for pPICZαB thrombin_EK_L and EK_L_6 and EK_L_3 primers (Table 2.1) for pPICZαB NH8_EK_L. The transformant containing pPICZαB thrombin or pPICZαB NH8 without the EK_L gene insert were used as negative control. The rEK_L which was secreted in supernatant was concentrated by centricon (10 kDa molecular weight cut off) (Pall Life Sciences) then the protein band was detected using SDS-PAGE technique. And the enzymatic activity was tested using fluorogenic substrate. The transformants that showed EK_L mRNA expression was chosen and transferred to 2 L fermentor for large scale production.

3.3.4 Large scale production in 2 L fermentor

Starter culture preparation was started from transferred the chosen transformant colony into 250 ml shake flask containing 70 ml BMGY medium (Appendix I) containing 100 μg/ml zeocin. Cultivation was performed at 30^{o}C, 200 rpm for 24 h. After 24 h of growth, 5% starter culture (50 ml) was inoculated into 2 L fermentor containing 950 ml Glycerol Basal Salt medium (GBS) (Appendix I).

Fed-batch fermentation was carried out in 2 L fermentor containing 950 ml GBS medium. Before sterilization of the fermentor, DOT electrode was checked and pH probe was calibrated (Appendix II). The PTM1 trace salts element (Appendix I)

was added to the fermentor to final concentration of 4.35 ml/l after sterilization. Ammonia solution 25% was used as base to control and adjust pH. Antifoam was added manually when high level of foam occurred. Standard protocol for recombinant protein production by *P. pastoris* in fermentor has been suggested by Charoenrat (2005). Four stages recombinant protein production in fermentor include glycerol batch, glycerol fed-batch, methanol induction and methanol production phase were used.

First step was glycerol batch phase. This phase was used to increase cell density. Before starter was inoculated, the fermentor was set up at cultivation condition, 30^{o}C, pH 5.5, aeration rate 1 vvm, agitation speed rate 1,00 rpm, then DOT electrode was calibrated (Appendix II). The DOT was close to 100% before inoculation. Glycerol batch phase was initiated by inoculation of 5% starter culture (50 ml) into 2 L fermentor containing 950 ml sterile GBS medium. During this phase, DOT was decreased continuously because *P. pastoris* consumed oxygen for their energy metabolism. The process was run until the sharp increasing in DOT can be detected which presumable mean that the glycerol was completely consumed. This phase take varying from 18 to 24 h depended on cell density of starter (Invitrogen, n.d.). At the end of the phase, 20 ml of sample was taken for cell growth analyses include OD_{600} measurement and dry cell weight analysis. For dry cell weight analysis, the cell pellets were dried at 105^{o}C for 6 h then let it cool down in desiccators. The enzymatic activity in crude culture supernatant was tested using fluorogenic substrate. The protein bands were detected on 15% SDS-PAGE gel.

The second phase is glycerol fed-batch phase which applied to increase the cell density and derepression of AOX1 promoter (Chiruvolu et al., 1998). After

glycerol was completely consumed in the first phase, the manual feed of glycerol feed medium (GF) (Appendix I) was applied. To obtain similar cell concentration as reported by Charoenrat (2005) at the end of the second phase (~ 40 g/L), the amount of GF medium feeding into fermentor have to calculate base on the data obtained from Charenrat (2005).

- Cell concentration at the beginning of this phase (X_o) equal to 20 g/L
- Cell concentration at the end of this phase (X_t) equal to 40 g/L
- Culture volume at the beginning of this phase (V_0) equal to 1 L
- Biomass yield ($Y_{x/s)}$ was approximately 0.7 g cell/g sub.

Thus, total cell concentration increasing in this phase was 20 g/L and total amount of glycerol needed to get this value can be calculated as

Glycerol (g) = (20 x 1)/ 0.7

= 28.517 g.

However, GF medium contains only 50% glycerol and its density is 1.109 g/ml. Thus, the total amount of GF medium needed is

GF medium (g) = (28.571 x 1109) 500

= 63.37 g.

The glycerol feed rate can be calculated based on specific growth rate (μ) which defined as

$$(1/X)\ (dX/dt) = \mu$$

This differential equation contains only two variables, biomass concentration (X; g/L) and time (t; h); and μ is constant. The variables are easy to separate by multiplying both sides by dt:

$$(1/X)\, dX = \mu\, dt$$

The equation is now ready to integrate:

$$\int_{X_0}^{X_t} (1/X)\, dX = \mu \int_0^t dt$$

Thus,

$$\ln (X_t/X_0) = \mu t$$

Where; X_t = 40 g/L

X_0 = 20 g/L

μ = 0.18 h^{-1} (Jahic et al., 2002)

Substitution these values into the equation;

$$t = (\ln Xt/X0)/ \mu$$

$$= (\ln 40/20) / 0.18$$

$$= 3.85 \text{ h.}$$

So, the GF medium 63.37 g have to feed into fermentor in 3.85 h or feed rate in 63.37 / 3.85 = 16.46 g/h. When the GF feed rate is slower than 16.46 g/h, feeding time will longer than 3.85 h and the substrate will be limited. However, if the GF feed rate is faster than 16.46 g/h, the accumulation of glycerol will occur and the AOX1 promoter can be repressed. During this stage, a little of recombinant proteins is produced by derepressed of AOX1 promoter under low repressor (glycerol) concentration (Jahic et al., 2002). The sample was taken at the end of this phase. This sample was analyzed for cell growth, enzymatic activity and quality of protein.

The third stage is methanol induction phase, the methanol feed medium (MF) (Appendix I) was added to the fermentor at very low final concentration. During this

phase, the *P. pastoris* was started to produce the enzyme in methanol metabolism pathway. For 1 L of initial fermentation volume, 0.633 ml of MF medium was injected into fermentor. During the first methanol injection, while the *P. pastoris* adapted to grow on methanol, DOT decreased slowly and erratic because of the methanol accumulation. After the methanol was completely consumed which take about 1 h, DOT was rapidly increase up to about 50%, the second methanol induction was performed by again adding 0.633 ml of MF medium into the fermentor. During this time, DOT rapidly decreased after MF injection because the increasing of AOX activity thus the rate of methanol utilization of the culture was faster than the first time induction. The DOT rapidly increases up to 50% only in 30 min. The third methanol induction was performed by adding 0.633 ml of MF medium to the fermentor when the increase of DOT was observed. These methanol inductions were repeated three times. At the end of this phase, the *P. pastoris* should be fully adapted to use methanol as substrate, then the methanol production phase was started.

The last stage is methanol production phase. This stage the methanol feed rate was adjusted to increase the expression of the AOX1 promoter. The control of methanol feed rate in this phase is very important. The methanol feed rates must be controlled to provide methanol concentration at the proper rate to allow for just enough for protein synthesis but not excess methanol addition which can causes toxicity (Cino, 1999). The MF medium feed rate was controlled manually by setting up the starting feed rate at the very low speed. In the first 2-3 h of this phase the DOT value may be erratic and finally it was stable and constant. After DOT reading was constant, for the recombinant protein production at low temperature, the temperature was slowly decreased to 20^{o}C and kept the production stage under this condition until

the end of process. During this phase, the methanol feed rate depend on methanol utilization rate of the culture which monitored by DOT. The methanol feed rate was adjusted to increase the recombinant protein expression every 24 h until 90 h of the total methanol production time. When very low DOT was shown, the MF was shut off until the increasing of DOT was observed. Samplings were performed one time a day for analysis of cell growth, enzymatic activity and quality of the protein. At the end of this phase, culture broth was collected and centrifuged at 4,000 rpm for 30 min. After the cell pellet was separated, supernatant was centrifuged again at 12,000 rpm for 15 min and filtered through 0.45 μm filter paper then kept at 4^{o}C.

All fermentation processes of both construct at the two induction temperature have summarized in table 3.2

Table 3.2 The fermentation processes of pPICZαB thrombin_EK_L (EK_L_thrombin) and pPICZαB NH8_EK_L (NH8_EK_L) constructs at two induction temperatures

fermentation	construct	Initial fermentation volume (ml)	Induction temperature (^{o}C)
I	EK_L_thrombin	700	30
II	EK_L_thrombin	1000	30
III	NH8_EK_L	1000	30
IV	EK_L_thrombin	1000	20
V	NH8_EK_L	1000	20

3.3.5 Purifications of rEK_L

3.3.5.1 Purification of rEK_L by nickel column

The filtrated supernatant sample was dialyzed and concentrated in 50 mM Tris-Cl pH 8 using Vivaflow membrane (10 kDa molecular weight cut-off) (Sartorius) then applied to nickel column (His Trap^{TM} FF 1 ml). Before the purification process was started, the column was equilibrated with 10 column volume (CV) of 50 mM Tris-Cl pH 8 then the dialyzed sample was load on the column using syringe injection. The flow-through fractions were collected. After all of sample was loaded then unbound protein was eliminated using 10 CV of washing buffer (50 mM Tris-Cl pH 8, 300 mM NaCl, 20 mM imidazole) and collected wash fractions. The recombinant protein containing His-tagged was eluted with 5 CV of elution buffer (50 mM Tris-Cl pH 8, 300 mM NaCl, 250 mM imidazole). The elution fractions were collected. The fractions of flow-through, wash and elution were detected the protein band using SDS-PAGE and tested for enzymatic activity.

To purify the rEK_L under denaturing condition, the dialyzed sample was treated with 6 M urea or 50 mM EDTA and codenaturing condition using 6 M urea and 50 mM EDTA before purification. The samples were incubated with each reagent and shaken on ice for 6 h then kept at 4^oC overnight before applied to nickel column. For EDTA treated sample, EDTA was eliminated before the purification processes was started. The rEK_L solution was changed into 50 mM Tris-Cl pH 8 by centricon (10 kDa molecular weigh cut-off). For urea denaturing condition, the denaturing buffer components were similar to the native purification buffer except for urea was

dissolved in equilibration, wash and elution buffer. Protein in each fraction was detected using SDS-PAGE technique.

3.3.5.2 Purification of rEK_L by ion exchange chromatography

The culture supernatant was dialyzed and concentrated in 50 mM sodium acetate buffer pH 5 using Vivaflow and centrifuged at 12,000 rpm for 15 min before loaded on the SP column (SP_FF column 5 ml). The purification process was carried out using FPLC machines (ÄKTA purifier, Amersham Pharcacia Biotech). The column was equilibrated with 50 mM sodium acetate buffer pH 5 then the concentrate protein sample was injected into the sampling loop of FPLC. To remove the unbound fraction, the column was washed with 5 CV of 50 mM sodium acetate buffer pH 5. The recombinant protein was eluted with a gradient of 0-1 M NaCl. The protein concentration was detected by spectrophotometer at 280 nm. The flow-through, wash and elution fractions were collected by automatic fraction collecting of the FPLC machine. Each fraction was tested for enzymatic activity and checked the protein band by SDS-PAGE. The elution fractions which showed high activity were pooled and concentrated. The contaminated protein in the purify fraction was checked by SDS-PAGE gel and stained in silver staining solution (e-Zi silver stain kit)**.** To determine the total protein concentration, 50 µl samples were mixed with 1.5 ml of Coomassie Plus Protein assay reagent kit (Merk) and incubated at room temperature for 10 min. The total protein concentration was detected at 595 nm and using the standard concentration curve of BSA to calculate the total protein concentration.

3.3.6 Enzymatic activity assay and properties analysis of purified rEK_L

The enterokinase activity was determined using the fluorogenic substrate Gly-$(Asp)_4$-Lys-β-naphthylamide (GD_4K β-naphthylamide). One hundred μl of the crude culture broth was added to 2 ml substrate solution (50 μM GD_4K-β-naphthylamide in 70 mM Tris-Cl pH 8 and 10% DMSO) then immediately mixed and placed cuvette containing the reaction in the cuvette holder of fluorospectrophotometer. Enzymatic activity was measured by an increasing of fluorescence (excitation at 337, emission at 420 nm) caused by the release of β-naphthylamide over one minute interval. The activity of rEK_L was also detected by cleavage of fusion protein, Trx_β-glucosidase (Chenchor et al., 2006), which has the enterokinase recognition sequence in the linker. The cleavage products were checked on 15% SDS-PAGE gel. The commercial rEK_L from NEB was used as a positive control.

The pH stability of purified rEK_L was tested by incubate 10 μl of purified enzyme in 300 μl of 50 mM sodium acetate buffer pH 3, 4, 5 or potassium phosphate buffer pH 6, 7, 8 or Tris-Cl pH 7, 8, 9 and 10 at 30^{o}C for 17 h then checked the quality of purified protein by SDS-PAGE and tested for the enzymatic activity using fluorogenic substrate. From these results, the pooled elution fraction was kept in the suitable buffer containing 50% glycerol at -20^{o}C.

The effect of induction temperature on the quality of recombinant protein production by *P. pastoris* was also considered. The specific enzyme activity, fluorogenic substrate assay and ability of fusion protein cleavage, of purified rEK_L which was produced at 30 and 20^{o}C were compared to obtain the best system for rEK_L production.

3.4 Results

3.4.1 Constructions and expression of rEK$_L$ in shake flask

3.4.1.1 Prokaryotic expression system

After the EK$_L$ gene in pGEM-T easy vector was amplified and ligated into pET32a between the *Nco*I and *Xho*I cloning site yielding the Trx_rEK$_L$ containing enterokinase cleavage site at the inter-domain linker. The ligation reaction was transformed into *E. coli* DH5α then colonies containing recombinant plasmid were screened using colony PCR technique with gene specific primers. Screening fourteen ampicillin resistant clones, only six clones showed positive band. Two of them were chosen for plasmid extraction and sequencing to verify the junction sequence. The analysis of nucleotide obtained from the C-terminal junction of pET32a_EK$_L$ showed that the nucleotide sequence was correct and the translated product was in-frame and identical to the design of plasmid construction (Fig. 3.6).

In the step of expression screening, the verified pET32a_EK$_L$ was transformed into *E. coli* Origami strain. One ampicillin/tetracycline/kanamycin resistant colony was selected and transferred to 125 ml shake-flask containing 20 ml LB-ampicillin/tetracycline/kanamycin broth and incubated at 37^{o}C, 200 rpm for 4 h. After 4 h of cultivation, the culture was poured into two 50 ml sterile centrifuged tube (10 ml each) for induced and un-induced condition.

```
EKL_clone       PPGRICSIAGWGALIYQGSTADVLQEADVPLLSNEKCRQQMPEYNITENMVCAGYEAGGV 180
pET32a_EKL      PPGRICSIAGWGALIYQGSTADVLQEADVPLLSNEKCRQQMPEYNITENMVCAGYEAGGV 94
                ************************************************************

EKL_clone       DSCQGDSGGPLMCQENNRWLLAGVTSFGYQCALPNRPGVYARVPRFTEWIQSFLH----- 235
pET32a_EKL      DSCQGDSGGPLMCQENNRWLLAGVTSFGYQCALPNRPGVYARVPRFTEWIQSFLHLEHHH 154
                ********************************************************

EKL_clone       ---
pET32a_EKL      HHH 157
```

Fig. 3.6 Amino acid sequence alignment of C-terminal junction pET32a_EK_L and EK_L clone from chapter II. The C-terminal junction region is shown in the bold letters. By this constructed, the rEK_L containing 6xHis-tagged at C-terminal can be produced.

The induction was initiated by adding IPTG into the culture broth to a final concentration of 0.8 mM. Then the induce and un-induce culture were incubated at 20^{o}C for 16 h. After16 h, the cells were harvested and lysed by sonication in 50 mM Tris-Cl pH 8 then the soluble and insoluble fraction were separated by centrifugation at 14,000 rpm for 15 min.

The fusion protein of Trx_rEK_L was designed with an inter-domain linker containing an EK cleavage site. It has been demonstrated that if the active rEK_L is expressed, it should be produced as soluble form and will be able to autocleave the Trx from Trx_rEK_L (Yuan and Hua, 2002). The SDS-PAGE data (Fig. 3.7) from this experiment showed that only 47 kDa of the fusion protein band was found in the fraction of insoluble induced culture. And small amount in un-induced culture of colony containing pET32a_EK_L but this protein band was not found in the control culture (colony containing pET32a). This result indicated that the rEK_L was inactive resulting in no protein band around 26 kDa which is the size of rEK_L.

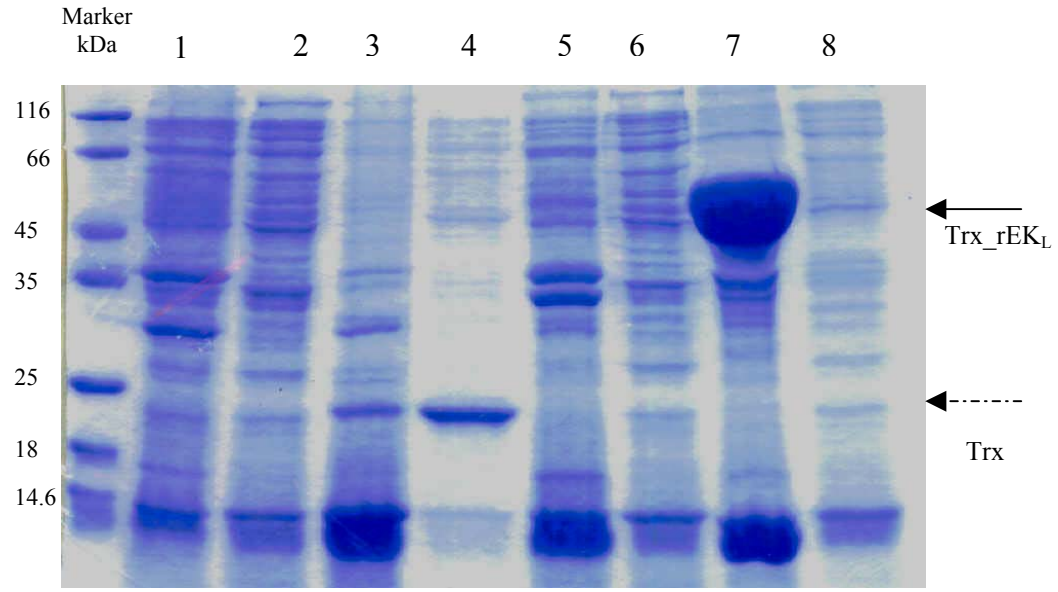


Fig. 3.7 SDS-PAGE analysis of Trx_rEK$_L$ expression in *E. coli* Origami strain. Lane 1-2: the insoluble and soluble fraction of un-induced cell containing pET32a, respectively; Lane 3-4: the insoluble and soluble fraction of induced cell containing pET32a, respectively; Lane 5-6: the insoluble and soluble fraction of un-induced cell containing pET32a_EK$_L$, respectively; Lane 7-8: the insoluble and soluble fraction of induced cell containing pET32a_EK$_L$, respectively. Arrow demonstrated the Trx_rEK$_L$ in the insoluble fraction, dash arrow (◄----) demonstated Trx (fusion partner) in the soluble fraction.

In attempts to express active rEK$_L$ in *E. coli* with this construct plasmid, other host strains were tried. The strain of Rosetta gami and BL21 pLysS were chosen as the new expression host. The culture volume was increased up to 100 ml. After IPTG induction, the cell pellets were harvested and detected for the protein band in soluble and insoluble fraction. SDS-PAGE data showed that no autocleavage products from

Trx_rEK$_L$ fusion protein were detected in soluble fraction of Rosetta gami (Fig. 3.8) and BL21 pLysS (data not shown). Almost all of the rEK$_L$ produced as inactive form and accumulated in the insoluble fraction. The EK$_L$ activity was also determined in soluble fraction of the Rosetta gaimi and BL21 pLysS using fluorogenic substrate, no activity can be detected.

From the results, it can be concluded that the expression of rEK$_L$ in *E. coli* was not successful because the product of rEK$_L$ was inactive and no enzymatic activity was detected in soluble fraction of Rosetta gami and BL21 pLysS. Therefore, production of active rEK$_L$ was tried to express in eukaryotic system.

3.4.1.2 Eukaryotic expression system

*EcoR*I and *Sac*II restriction site was added to the cDNA encoding 235 amino acid of EK$_L$ by PCR technique. After digested this PCR product with *EcoR*I and *Sac*II, the digested product was inserted in frame with α-factor secretion of the *P. pastoris* expression plasmid pPICZαB thrombin and pPICZαB NH8 resulting in pPICZαB thrombin_EK$_L$ and pPICZαB NH8_EK$_L$. The recombinant plasmids were sequenced to verify that the junction sequence of the recombinant plasmids were correct. The obtained nucleotide sequenced was translated into amino acid then amino acid sequence of pPICZαB thrombin_EK$_L$ and pPICZαB NH8_EK$_L$ were aligned (Fig. 3.9).

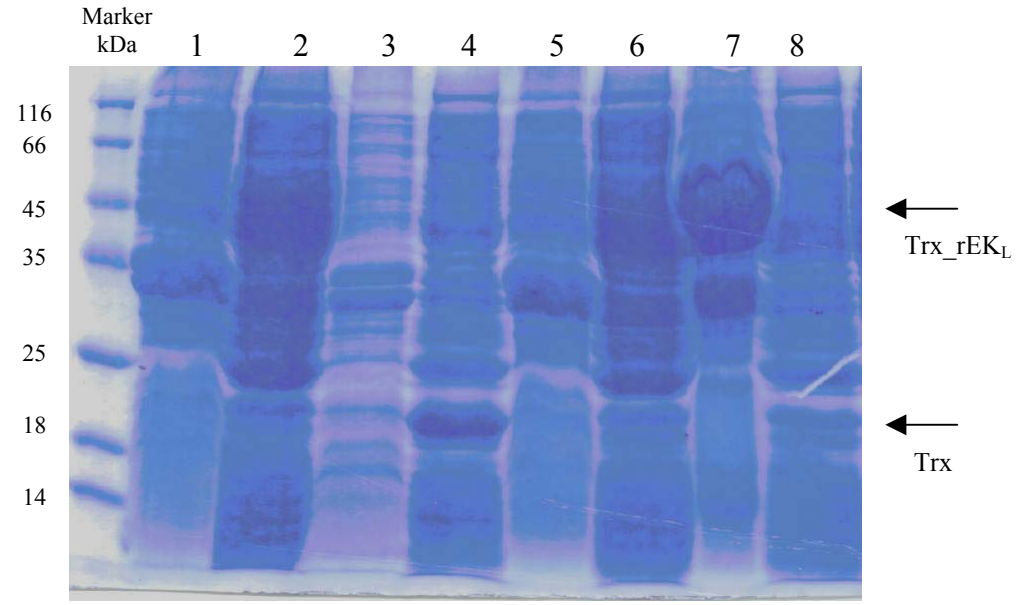


Fig. 3.8 SDS-PAGE analysis of Trx_rEK$_L$ expression in *E. coli* strain Rosetta gami. Lane 1-2: the insoluble and soluble fraction of un-induced cell containing pET32a, respectively; Lane 3-4: the insoluble and soluble fraction of induced cell containing pET32a, respectively; Lane 5-6: the insoluble and soluble fraction of un-induced cell containing pET32a_EK$_L$, respectively; Lane 7-8: the insoluble and soluble fraction of induced cell containing pET32a_EK$_L$, respectively. Arrow demonstrated the Trx_rEK$_L$ in the insoluble fraction, dash arrow (⟵) demonstated Trx in the soluble fraction.

```
NH8_EKL          LFETMRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFS 60
Thrombin_EKL     ------------------------------------------------------------
                 ::  : :  ::  :::     :   :         :    ::    :   : : :: :

NH8_EKL          NSTNNGLLFINTTIASIAAKEEGVSLEKREAEAAHHHHHHHHAAVRRIQIVGGSDSREGA 120
Thrombin_EKL     ----------------------QRTLRRELSCG------------IQIVGGSDSREGA 24
                       ::::   :  :          :** ...          :  ************

NH8_EKL          WPWVVALYFDDQQVCGASLVSRDWLVSAAHCVYGRNMEPSKWKAVLGLHMASNLTSPQIE 180
Thrombin_EKL     WPWVVALYFDDQQVCGASLVSRDWLVSAAHCVYGRNMEPSKWKAVLGLHMASNLTSPQIE 84
                 ************************************************************

NH8_EKL          TRLIDQIVINPHYNKRRKNNDIAMMHLEMKVNYTDYIQPICLPEENQVFPPGRICSIAGW 240
Thrombin_EKL     TRLIDQIVINPHYNKRRKNNDIAMMHLEMKVNYTDYIQPICLPEENQVFPPGRICSIAGW 144
                 ************************************************************

NH8_EKL          GALIYQGSTADVLQEADVPLLSNEKCRQQMPEYNITENMVCAGYEAGGVDSCQGDSGGPL 300
Thrombin_EKL     GALIYQGSTADVLQEADVPLLSNEKCRQQMPEYNITENMVCAGYEAGGVDSCQGDSGGPL 204
                 ************************************************************



NH8_EKL          MCQENNRWLLAGVTSFGYQCALPNRPGVYARVPRFTEWIQSFLHPRRPPAF--------- 351
Thrombin_EKL     MCQENNRWLLAGVTSFGYQCALPNRPGVYARVPRFTEWIQSFLHPRLVPRGSILEQKLIS 264
                 ******************************************  *

NH8_EKL          ---------------
Thrombin_EKL     EEDLNSAVDHHHHHH 279
```

Fig. 3.9 Amino acid sequence alignment of pPICZαB NH8_EK$_L$ and pPICZαB thrombin_EK$_L$. The C-terminal 6xHis-tagged of pPICZαB thrombin_EK$_L$ and N-terminal 8xHis-tagged of pPICZαB NH8_EK$_L$ are boxed. The junction between inserted EK$_L$ and plasmid at N- and C-terminal of each construct are shown in bold letters. NH8_EKL and Thrombin_EKL refer to amino acid sequence of pPICZαB NH8_EK$_L$ and pPICZαB thrombin_EK$_L$, respectively.

Amino acid sequence at the junction of N- and C-terminal of pPICZαB thrombin_EK$_L$ and pPICZαB NH8_EK$_L$ (Fig. 3.9) are in-frame with polyHis-tagged resulting in rEK$_L$ containing C-terminal 6xHis-tagged for pPICZαB thrombin_EK$_L$ and N-terminal 8xHis-tagged for pPICZαB NH8_EK$_L$. The expression was performed using the construct of pPICZαB thrombin_EK$_L$ or pPICZαB NH8_EK$_L$. Both recombinant

expression vectors were linearized by digestion with *Sac*I to facilitate the homologous recombination of plasmid and host genome at AOX1 promoter. This linearized plasmid was transformed into *P. pastoris* Y11430 by electroporation method. The zeocin resistant colonies were screened using colony PCR technique with 5' and 3'AOX or EK_L_2 and EK_L_3 primers (Table 2.1) to confirm the integration of pPICZαB thrombin_EK_L or pPICZαB NH8_EK_L into the *Pichia* genome, respectively. The positive clone of pPICZαB thrombin_EK_L showed the predicted 1200 bp PCR product that included portions of the AOX1 and the integrated EK_L. The positive clone of pPICZαB NH8_EK_L showed 700 bp PCR product of the inserted EK_L in *Pichia* genome.

To identify clones which were able to express rEK_L, the EK_L mRNA were detected in shake-flask expression experiment. Colonies containing the integrated of EK_L were grown in 100 ml BMGY medium until glycerol was completely consumed which take about 48 h. Then methanol was added to the culture in a final concentration of 1-3% (v/v) every 24 h to induce AOX1 promoter until 72 h of total induction times. After 72 h of induction, the cell pellet and supernatant were separated. The mRNA of EK_L was detected from the induced pellet culture carrying pPICZαB thrombin_EK_L by two steps RT-PCR and nested PCR with EK_L_2 and EK_L_3 primer (Fig. 3.10). For the construct of pPICZαB NH8_EK_L, the EK_L mRNA was detected by RT-PCR with gene specific primer. The results showed that the expected PCR and nested PCR product of 700 bp was observed in both transformant carrying pPICZαB NH8_EK_L or pPICZαB thrombin_EK_L, respectively. It is clear that the positive results were found in the colony which was able to express EK_L mRNA only.

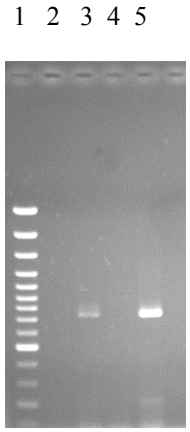


Fig. 3.10 The screening of EK_L expression in mRNA level from *P. pastoris* induced cell pellet using RT-PCR and nested PCR techniques. Lane 1: DNA Marker (Fermentas); Lane 2 is the nested PCR product of the transformants containing pPICZαB thrombin (control); Lanes 3-5 are the nested PCR product of the transformants containing pPICZαB thrombin_EK_L. The expected nested PCR product is shown in the major band of 708 bp found in colony 1 and 3 which was represent in lane 3 and 5, respectively.

The supernatant from shake-flask culture of the transformant carrying pPICZαB thrombin_EK_L was concentrated then analyzed for the secreted rEK_L on 15% SDS-PAGE gel. The result of SDS-PAGE showed that the expected protein band of 43 kDa (Fig. 3.11 lane 6 and 7) was observed in induced culture supernatant of transformants containing pPICZαB thrombin_EK_L which was able to express the EK_L mRNA. However, no enzymatic activity was detected in this crude culture when used the fluorogenic substrate. The problem of activity assay in the shake-flask culture

broth was the interfering of color of the medium broth. To confirm the expression of rEK_L in culture broth, the pH of concentrated rEK_L containing 6xHis-tagged at C-terminal was adjusted to pH 8 then purified by Ni-NTA column (Quigen Kit). The elution fractions were analyzed for the enzymatic activity using fluorogenic substrate. The results of rEK_L purification indicated that no increasing of fluorescence unit in the elution fractions was found. This result showed that the expression of rEK_L in shake-flask scale was very low level. To solve this problem, large scale production was carried out in a 2 L fermentor to increase the amount of rEK_L.

3.4.2 Large scale production in 2 L fermentor

3.4.2.1 pPICZ*α*B thrombin_EK_L fermentation

Large scale production was performed using the transformants containing the construct of pPICZαB thrombin_EK_L which was able to express the EK_L mRNA. For the first fermentation, which will be called "fermentation *I*", only 700 ml was achieved. Thirty five ml of an overnight starter culture was used to inoculate into 665 ml fermentation medium. Four stages recombinant protein productions in fermentor using fed-batch culture technique were performed as described earlier in the material and method section. The cultivation condition was controlled at 30^{o}C, pH 5.5, aeration rate 1 vvm and agitation speed 1,000 rpm. The samples were taken at the end of each fermentation stage and one time a day for the methanol production phase.

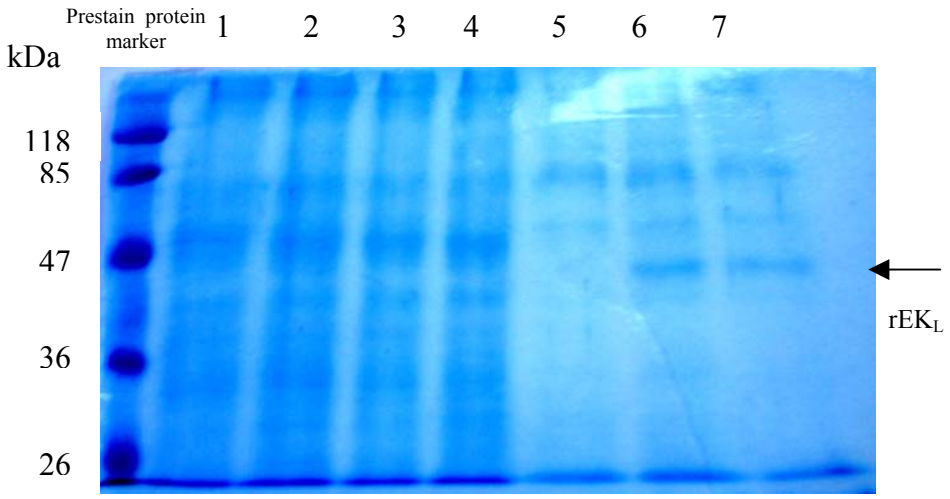


Fig. 3.11 SDS-PAGE of total cellular protein (soluble fractions) and the concentrated culture broth supernatant from the induce culture. Lanes 1-4 are total cellular protein (soluble fraction) of the induced culture of pPICZαB thrombin and pPICZαB thrombin_EK$_L$ clone 1, 2, and 3, respectively; Lane 5-7 are the concentrated culture broth supernatants of the induced culture of pPICZαB thrombin, and the colony containing pPICZαB thrombin_EK$_L$ number 1 and 3 which show mRNA expression, respectively. Arrow demonstrated the secreted rEK$_L$ in the culture broth supernatant in lane 6 and 7.

Duplicated of 5 ml culture broth was centrifuged to separate cell pellet and supernatant. The cell pellet was washed with distilled water then centrifuged and dry to determine the cell dry weight. For supernatant analysis, the visible protein band in the supernatant was detected on SDS-PAGE gel and tested for the enzymatic activity of the crude enzyme by fluorogenic substrate. SDS-PAGE profile showed that the intensity of the 63 kDa protein band increase when the times passed especially in the

methanol production phase. However the expected rEK_L band of 43 kDa which was found in shake-flask culture was not detected in the fermentor culture broth (Fig. 3.12). The rEK_L enzymatic activity were detected in the crude culture broth of the methanol production samples. But the sample of glycerol batch or fed-batch phase, no enzymatic activity was detected. After 90 h of methanol production phase, fermentor culture was harvested by centrifugation twice at 8,000 rpm for 30 min. The supernatant was dialyzed and concentrated in 20 mM sodium phosphate buffer pH 8, 300 mM NaCl and kept at 4^oC then purified by Ni-NTA column (Quigen kit).

However, some problem happened with the condenser during the fermentation, so a high portion of the fermentation broth was evaporated. Therefore, the concentration of the measure value might not be as accurate as in further fermentation experiments.

Because of the problem in fermentation *I* and the purification of rEK_L using nickel column was not successful, the second fermentation was performed. The second fermentation of *P. pastoris* Y11430 containing pPICZαB thrombin_EK_L was performed with 1 L initial fermentation volume. The cultivation condition was controlled at 30^oC, pH 5.5, aeration rate 1 vvm and agitation speed 1,000 rpm. During the production of rEK_L, the samples were taken at the end of each fermentation stage and one time a day for the methanol production phase. The pattern of protein bands in each sample were analyzed by SDS-PAGE technique.

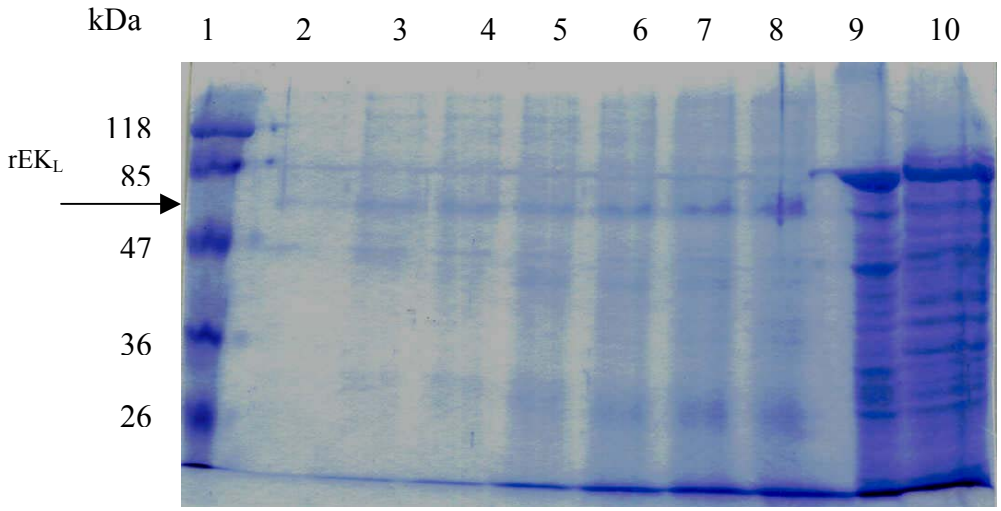


Fig. 3.12 Analysis of SDS-PAGE of crude culture broth supernatant from fermentation *I.* [12 µl supernatant sample and 10 µl of soluble or insoluble fraction was loaded on 15% SDS-PAGE gel]. Lane 1: Prestained protein molecular marker (Fermentas); Lane 2: sample from the end of glycerol batch phase; Lane 3: sample from the end of glycerol fed-batch phase; Lane 4: sample from the end of methanol induction phase; Lane 5-8: samples during methanol production phase at 18, 43, 66 and 90 h of induction, respectively; Lane 9-10: soluble and insoluble fraction of *P. pastoris* at the end of fermentation process, respectively. Arrow demonstrated the secreted rEK$_L$ in the crude culture supernatant.

The SDS-PAGE data indicated that the major visible band of 63 kDa was found in the fermentor culture broth. The intensity of this protein band increased especially in the methanol production phase (Fig. 3.13). The results of enzymatic activity analysis in

the crude culture broth were observed that the activity was found in the samples of the methanol production phase after 12 h of methanol induction. This result indicated that the increasing of activity depended on the ability of methanol utilization of the culture. After 84 h of the induction time, the culture was harvested by centrifugation twice at 8,000 rpm for 30 min. Then, 350 ml of culture supernatant was filtered through 0.45 μm filter then concentrated to the final volume of 30 ml. The concentrated supernatant was dialyzed with 50 mM sodium acetate buffer pH 5 then purified by ion exchange chromatography.

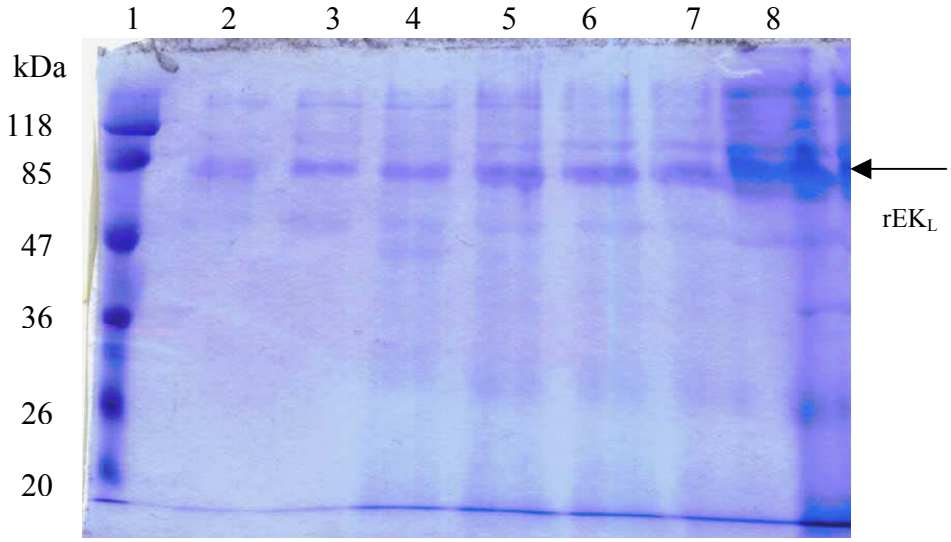


Fig. 3.13 Analysis of SDS-PAGE of crude culture broth supernatant from fermentation *II*. Lane 1: Prestained protein molecular marker (Fermentas); Lane 2-3: sample from the end of glycerol batch and fed-batch phase, respectively; Lane 4-7: samples during the methanol production phase at 12, 40, 66 and 84 h of induction, respectively; Lane 8: the dialyzed and concentrated supernatant. Arrow demonstrated the rEK$_L$ in the crude culture supernatant.

3.4.2.2 pPICZαB NH8_EK_L fermentation

For the third fermentation, the colony containing pPICZαB NH8_EK_L which was able to express EK_L mRNA was chosen and transferred to 2 L fermentor for large scale production. Four stages of fed-batch culture for recombinant protein production by *P. pastoris* were used to produce rEK_L. The cultivation condition was controlled at 30^oC, pH 5.5, aeration rate 1 vvm and agitation speed 1,000 rpm. During fermentation, the samples were taken at the end of each fermentation stages and one time a day for the methanol production phase. After 92.6 h of methanol production phase the maximum activity was obtained but a small decreased in the amount of total protein in the crude culture broth was detected after 250 g of MF medium was fed into the culture. The culture broth was centrifuged to separate the cell pellet and supernatant. Three hundred and fifty ml of supernatant was concentrated to 30 ml then dialyzed with 50 mM Tris-Cl pH 8 and kept at 4^oC. All culture supernatant samples were analyzed on SDS-PAGE gel and the results showed the major band of 63 kDa and the intensity of this band increase with time (and methanol feed) (Fig. 3.14). These results indicated that the pattern protein bands which were detected from the fermentor culture broth of both construct were the same. The visible major protein band of 63 kDa should be the secreted rEK_L. The result of enzymatic activity showed that it can be detected in the methanol production samples after 21 hours of induction time.

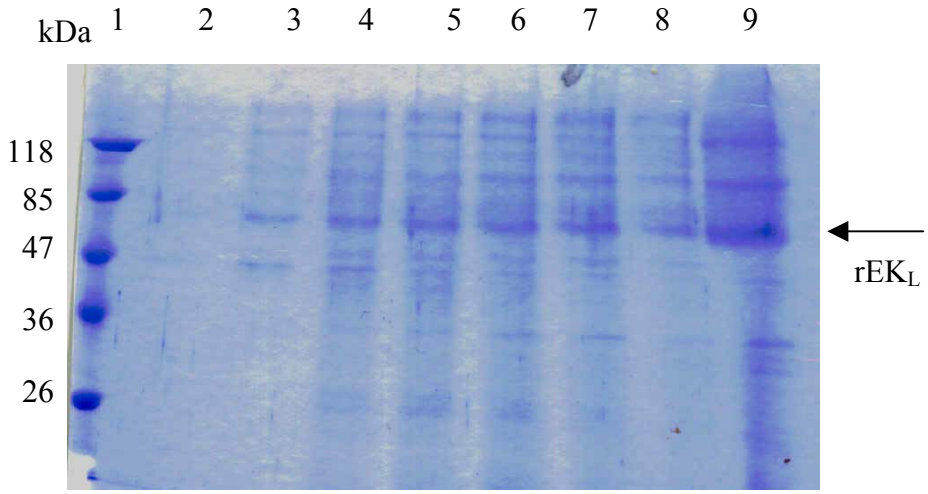


Fig. 3.14 Analysis of SDS-PAGE of crude culture broth supernatant from fermentation *III*. Lane 1: Prestained protein molecular marker (Fermentas); Lane 2-3: sample from the end of glycerol batch and fed-batch phase, respectively; Lane 4-7: samples during the methanol production phase at 21, 45, 69 and 92 h of induction time, respectively; Lane 8: the culture broth supernatant at the end of fermentation process; Lane 9: the dialyzed and concentrated supernatant. [The visible protein band was obtained from 12 µl of the sample for lane 2-8 and 8 µl of dialyzed sample for lane 9.] Arrow demonstrated the rEK$_L$ in the culture broth supernatant.

3.4.2.3 The rEK$_L$ production at low temperature

For the forth (EK$_L$_thrombin) and fifth (EK$_L$_NH8) fermentation, both construct of rEK$_L$, Thrombin_EK$_L$ and NH8_EK$_L$, were used to produce the secreted rEK$_L$ at low induction temperature. The fermentation process initiated by inoculation

50 ml of an overnight starter culture into 2 L fermentor containing 950 ml initial fermentation volume. Before the methanol production phase was initiated, the cultivation condition was controlled at 30^{o}C, aeration rate 1 vvm and agitation speed rate 1,000 rpm. And after 4 h of the glycerol fed-batch phase, approximately 40 g/L of cell dry weight was observed from low induction temperature in both construct. The result of cell dry weigh obtained at the end of glycerol fed-batch phase was not too different in all fermentation. In the first 2-3 h of the methanol production phase, MF medium was fed into the culture at very slow flow rate. After 3 h of methanol production phase, the culture fully adapted to grow on methanol which showed constant DOT in the range of 20-28%. At this time, the temperature was adjusted with slowly decrease from 30^{o}C to 20^{o}C and the culture was kept at this temperature until the end of the fermentation process. At the end of the fermentation process, 130.17 g/L and 98.26 g/L of cell dry weight was observed from fermentation *IV* and *V*, respectively (Table 3.3). However, very high enzymatic activity of 9,730 unit/g cell, were obtained after 91.5 h of methanol induction from fermentation *V*. Compare to the production of rEK$_{L}$ at 30^{o}C, all methanol consumption in fermentations *III* and *V* (pPICZαB NH8_EK$_{L}$) were not much different but the cell dry weigh from the low temperature production was higher than that of the 30^{o}C production. Data analyses of rEK$_{L}$ production are summarized in table 3.3.

Table 3.3 Comparison of cell dry weight, total methanol consumption and enzymatic activity from crude culture broth at the end of fermentation process of transformant carrying pPICZαB thrombin_EK_L or pPICZαB NH8_EK_L. In parentheses [ ] and ( ) indicate each construct and time in hour (h) for each stage, respectively. Time with * indicate time only in the induction period.

Fermentation no. and construct	Initial volume (ml)	Induction temperature (^oC)	Cell dry weight at the end of glycerol batch phase (g/L)	Cell dry weight at the end of glycerol fed-batch phase (g/L)	Total methanol consumption (g)	Cell dry weight at the end of fermentation (g/L)	Specific product yield (unit/g cell)
Fermentation *I* [EK_L_thrombin]	700	30	28.66 (18.33 h)	43.75 (3.5 h)	188.2 (90.3 h*)	113.33	1,152
Fermentation *II* [EK_L_thrombin]	1,000	30	32.14 (24.5 h)	39.11 (4 h)	233.9 (84 h*)	63.74	1,272
Fermentation *III* [_NH8_EK_L]	1,000	30	17.06 (22.7 h)	40.83 (3.5 h)	375.9 (92.6 h*)	89.33	6,475
Fermentation *IV* [EK_L_thrombin]	1,000	20	29.24 (20.75 h)	43.55 (4 h)	467.4 (95.2 h*)	130.17	1,407
Fermentation *V* [_NH8_EK_L]	1,000	20	12.5 (21.5 h)	38.98 (4 h)	371.2 (91.5 h*)	98.26	9,730

From the table 3.3, the time of glycerol batch phase was difference (18-24 h) depend on the physical properties of each cell culture and the initial concentration of starter culture. The cell dry weight of 12-32 g/L was observed at the end of glycerol batch phase. In fermentation *II*, slow growth of cell culture was due to the starter culture which was prepared from a colony on YPD agar plate of over a week old resulting in slower glycerol consumption in the glycerol batch phase than the other fermentation (Table 3.3). This indicated that for a good starter preparation, the *P.*

pastoris on YPD agar must be not older than a week. At the end of glycerol fed-batch phase, approximately 40 g/L cell dry weight can be obtained which was as high as expected.

The results of cell dry weight and the methanol consumption during the methanol production phase of fermentations *II* to *V* are shown in Fig. 3.15A and 3.15B, respectively. The increasing of cell dry weight depended on the methanol consumption of each culture and also depended on the induction temperature. The cell dry weight of both culture constructs obtained from low induction temperature was higher than that of the high induction temperature. Compare to the biomass yield of each culture, have similar biomass yield ($Y_{x/s)}$ which was approximately 0.2 g cell/ g methanol consumption. When compare between the same construct, high specific production yield (unit /g cell) (Fig. 3.15C) was found when high amount of methanol was consumed. This result indicated that increase of methanol feed can increase the amount of rEK_L (detected by the increase of enzymatic activity). However, the adjustment of methanol feed rate in the methanol production phase must depend on the ability of methanol consumption of the culture. The investigation in this experiment found that MF medium feed rate should be carefully controlled not higher than 2 g/L/h in the first 24 h of methanol production phase. During the methanol production phase, the MF medium was stopped when very low DOT occurred to ensure that the methanol was not accumulated and presented at toxic concentration. The methanol pump was turned on when the methanol was completely consumed which indicated by the increasing of DOT. The increasing of MF medium feed rate was introduced when cell density increase. The observation during the methanol-

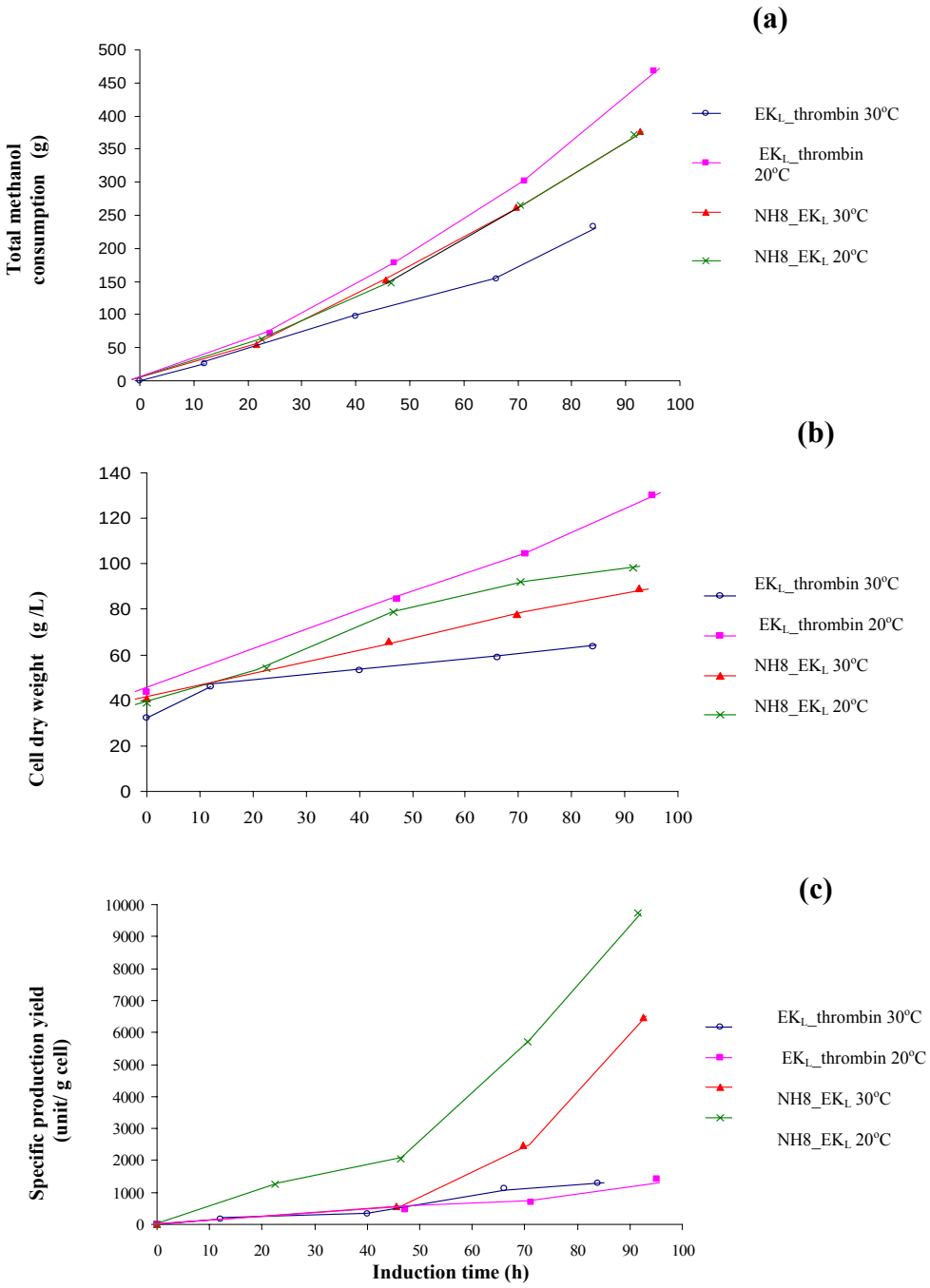


Fig. 3.15 The relationship between (a) total methanol consumption, (b) cell dry weight, (c) specific production yield of rEK_L obtained from fermentation of transformant carrying pPICZαB thrombin_EK_L (EK_L_thrombin) and pPICZαB NH8_EK_L (NH8_EK_L) at 30 and 20°C induction temperature and induction time.

production phase found that dissolved oxygen levels were close to 0.1-3% when increasing the MF medium feed rate to 4 g/h for 1 L initial fermentation volume. During fermentation process, the metabolism of the cell culture was monitored by DOT and pH value. The decrease in pH was indicated that the culture was properly grown and produced some acid metabolite. The decreased in DOT mean that the oxygen was used for catabolism and metabolism. At the end of the fermentation, 63-130 g/L of cell dry weigh was obtained.

The specific production yield of rEK_L, $Y_{p/x}$, (unit/g cell) from different construct, the C- and N-terminal His-tagged were compared (Fig. 3.15C). High specific production yield of rEK_L was found in the fermentation of rEK_L containing N-terminal His-tagged at both high and low induction temperature. However rEK_L from both construct were not able to be purified by nickel column (see results of His-tagged protein purification) thus the difference of activity can not be concluded that it is because the influence of His-tagged. The different of activity from both constructs may be caused by the different of physical of each transformant or clonal variation. The ability of methanol consumption for cell generation in both constructs was not different but the ability of rEK_L production of each construct was different. High amount of the methanol fed into the culture carrying pPICZαB NH8_EK_L resulted in high amount of secreted rEK_L which indicated by increasing of enzymatic activity. Because of this reason the transformant of rEK_L containing N-terminal His-tagged was chosen for investigation of the effect of low temperature production on the production of rEK_L.

The effect of low temperature production on the production of rEK_L was considered in the construct of pPICαB NH8_EK_L. The total protein concentration obtained from both temperature induction systems was not much different as shown in Fig. 3.16. The slight decrease of total protein concentration occurred at high induction temperature but not at low temperature. At low temperature production, the heat generation from methanol consumption can be reduced at low temperature thus the cell death should be decreased. The comparison of specific production yield (unit/g cell dry weight) at the end of fermentation process (fermentation *III* and *V*) from 20°C production, 9,730 unit/g cell, was higher than that from 30°C production, 6,475 unit/g cell. This comparison indicated that because large amount of survival cell occurred at low temperature production thus almost all of the culture was able to utilize the methanol to induce AOX1 promoter. Because of this reason, the enzymatic activity at 20°C production was higher than that at 30°C production when equal amount of g cell was compared.

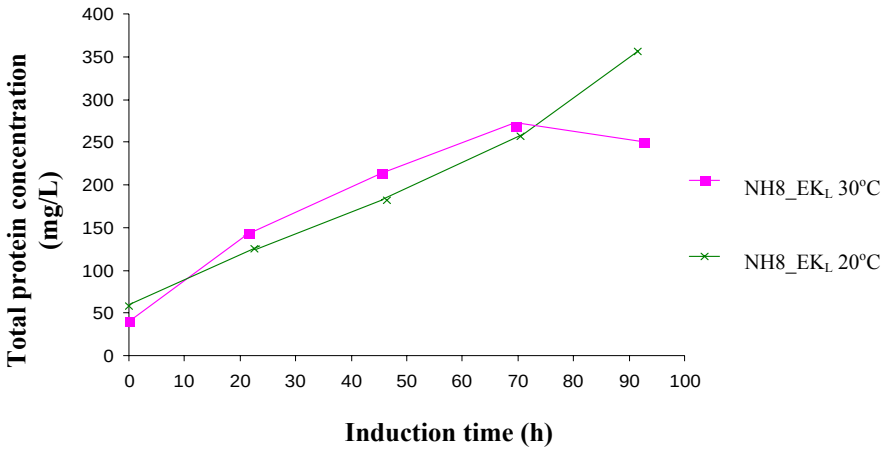


Fig. 3.16 The relationship between total protein concentration obtained from fermentation of transformant carrying pPICZαB NH8_EK_L at 30 and 20°C induction temperature and induction time.

However, the comparison of rEK_L unit / mg of total protein indicated that rEK_L production at low temperature was much higher than that at high temperature production (Fig. 3.17a). Total protein concentration obtained from both conditions was not much different but the amount of rEK_L obtained from low induction temperature was higher than that of high temperature. Because of this reason, enzymatic activity from low induction temperature (20^oC) was higher than that at high induction temperature when equal amount of total protein (mg) was compared. The comparison of product yield of rEK_L (unit /g methanol) during induction phase obtained from 30 and 20^oC induction temperature are shown in Fig. 3.17b. Although the enzymatic activity (unit) from both induction temperatures, 30 and 20^oC, increased over the induction times but product yield (unit/ g methanol) from low induction temperature was higher than that of high temperature. These results indicated that ability of methanol consumption for product formation in the low induction temperature culture was better than that in high temperature. Due to large amount of survival cell occurred at low temperature production thus almost all of the culture was able to utilize the methanol and produced the large amount of secreted rEK_L. However, the quality of rEK_L of both temperature inductions was considered after purification.

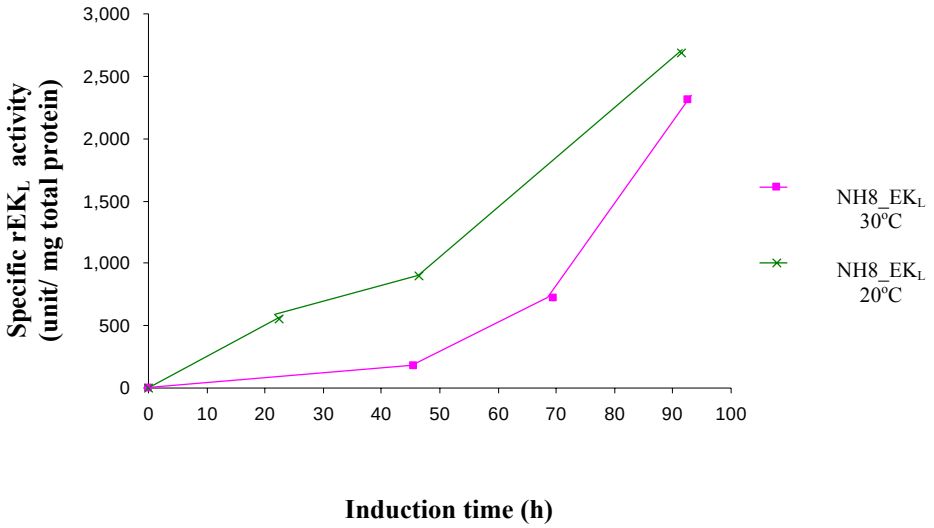


Fig. 3.17a The relationship between specific rEK$_L$ activity (unit/ total protein concentration) obtained from fermentation of transformant carrying pPICZαB NH8_EK$_L$ at 30 and 20°C induction temperature and induction time

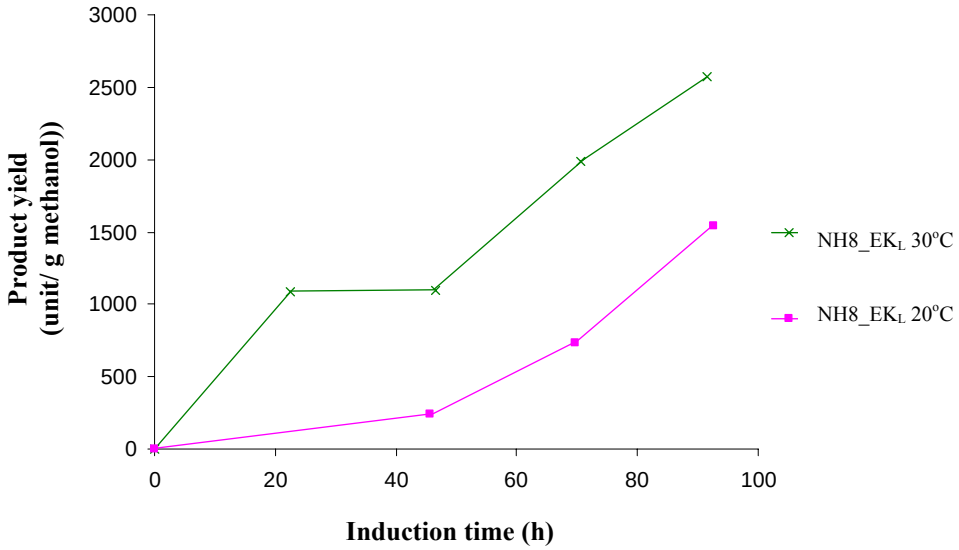


Fig. 3.17b The rEK$_L$ product yield obtained from fermentation of pPICZαB NH8_EK$_L$ construct at 30 and 20°C induction temperature

3.4.3 Purification of rEK_L

3.4.3.1 Purification of rEK_L by nickel column

For the first fermentation of the transformants containing the pPICZαB thrombin_EK_L with 700 ml initial fermentation volume, after the cell pellet was removed, the supernatant was concentrated and dialyzed in 20 mM sodium phosphase buffer pH 8, 300 mM NaCl and then purified by Ni-NTA column (Quigen kit). Before the sample was loaded into the Ni-NTA column, the column was equilibrated with 50 mM sodium phosphase buffer pH 8, 300 mM NaCl. After 6 ml of dialyzed sample was loaded into the column, the column was washed twice with 600 µl of 50 mM sodium phosphase buffer pH 8, 300 mM NaCl, 20 mM imidazole and eluted three time with 100 µl of 50 mM sodium phosphase buffer pH 8, 300 mM NaCl, 250 mM imidazole. All fractions, flow-through, wash and elution, were collected and analyzed for enzymatic activity. The result of enzymatic activity analysis of the purification fractions indicated that the binding of the 6xHis-tagged rEK_L was not found because almost all of the enzymatic activity was detected in the flow-through fraction but no activity was found in elution fractions. This result indicated that 6xHis-tagged at C-terminal of rEK_L dose not work and it can not bind Ni^{2+} thus new construct was design to put the His-tagged at the N-terminal of rEK_L.

For the N-terminal His-tagged protein purification, at the end of the fermentation process, the culture was harvested and the cell was removed. The crude supernatant was filtered pass 0.2 µm filter then concentrated and dialyzed in 50 mM Tris-Cl pH 8. Twenty ml of the dialyzed sample was loaded into pre-equilibrated nickel column (His Trap^{TM} FF 1 ml) with 50 mM Tris-Cl pH 8 and collected the flow-

through fractions. The column was washed with 10 ml (10 CV) of washing buffer containing 50 mM Tris-Cl pH 8, 300 mM NaCl pH 8, 20 mM imidazole then eluted with 7 ml elution buffer (50 mM Tris-Cl pH 8, 300 mM NaCl pH 8, 250 mM imidazole). During the step of elution, every 500 µl of elution fractions were collected. Each fractions were analyzed on SDS-PAGE (Fig. 3.18) and enzymatic activity tested. The results of SDS-PAGE analysis showed that the pattern protein bands of the dialyzed sample before load into nickel column and the unbound fractions were not different. Because almost all of the rEK_L did not bind to the column thus almost all of the expected band of 63 kDa was detected in the flow-through fraction and only small amount in the wash fraction. These results were supported by the results of the enzymatic activity assay which almost all of the enzymatic activity were found in the flow-through fraction but no activity were detected in the elution fractions. Both the results from the purification of the construct of rEK_L containing C- and N-terminal His-tagged indicated that no binding of rEK_L to the nickel column was found.

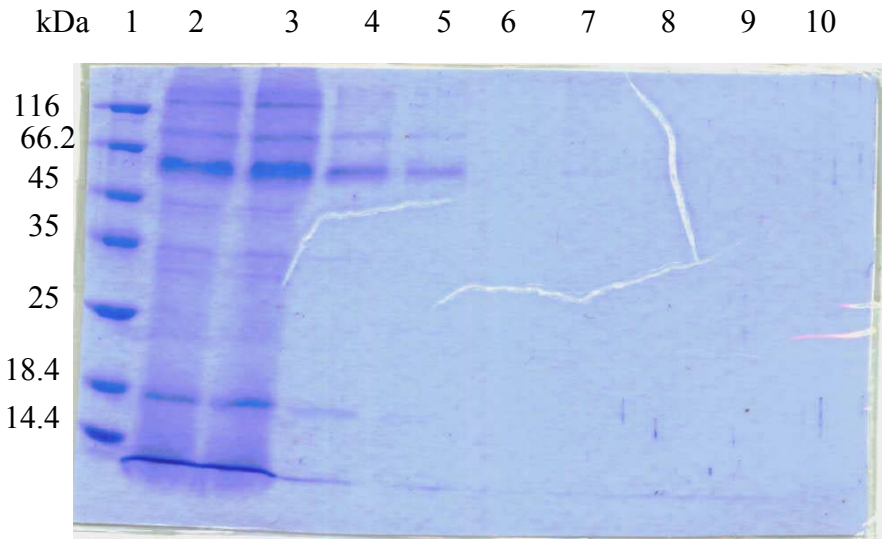


Fig. 3.18 The SDS-PAGE analysis of rEK_L purification fractions from nickel column. The dialyzed fermentor supernatant of the transformant containing pPICZαB NH8_EK_L at 30°C production was purified using nickel column. Lane1: Protein molecular marker (Fermentas); Lane 2: 10 µl of dialyzed sample; Lane 3: 10 µl of flow-through fraction; Lane 4-5: 12 µl of wash fraction; Lane 6-10: 12 µl of elution fraction number 1-5.

To obtain rEK_L from His-tagged purification, the affinity metal ion for His-tagged purification was changed from Ni^{2+} to Co^{2+}. Ten ml of dialyzed sample of N-terminal His-tagged construct in 50 mM Tris-Cl pH 8 was incubated on ice with 1ml Co^{2+} beads for 30 min. The unbound fraction was collected by centrifugation at 900 rpm for 5 min. The Co^{2+} beads were washed with 5 ml washing buffer (50 mM Tris-Cl pH 8, 150 mM NaCl, 10 mM imidazole). Then eluted with 5 ml of elution buffer (50 mM Tris-Cl pH 8, 150 mM NaCl, 250 mM imidazole). In the step of elution, the cobalt beads were incubated with 1 ml elution buffer for 1 min then centrifuged at 900

rpm for 3 min to spin the cobalt beads down. One ml of each elution fraction was collected. The elution procedures were performed for 5 times. Each fraction was collected then analyzed for protein bands on SDS-PAGE gel and also tested for enzymatic activity using fluorogenic substrate. The results of SDS-PAGE (Fig. 3.19) and enzymatic activity analysis indicated that no binding of rEK_L N-terminal His tagged to Co^{2+} ion occurred. Thus major protein bands and almost all of the activity were detected in the unbound fraction and no protein band or enzymatic activity was found in the elution fractions.

The problem of no binding of the rEK_L to the Ni^{2+}column nor Co^{2+} beads was assumed that some component in the crude concentrated sample may block the binding of rEK_L containing His-tagged and the metal ions. To solve this problem, the rEK_L was purified by ion exchange chromatography first to remove it away from inhibitor in the crude fermentor culture broth then the purified enzyme was pH adjusted and loaded into nickel column again. The purified rEK_L of the C- or N-terminal His-tagged construct which was obtained from ion exchange purification was adjusted pH to 8 by 50 mM sodium phosphate buffer or 50 mM Tris-Cl pH 8. The rEK_L at pH 8 was purified using nickel column again. No enzymatic activity was detected in the elution fractions but almost all of the activity was found in the flow-through fraction again.

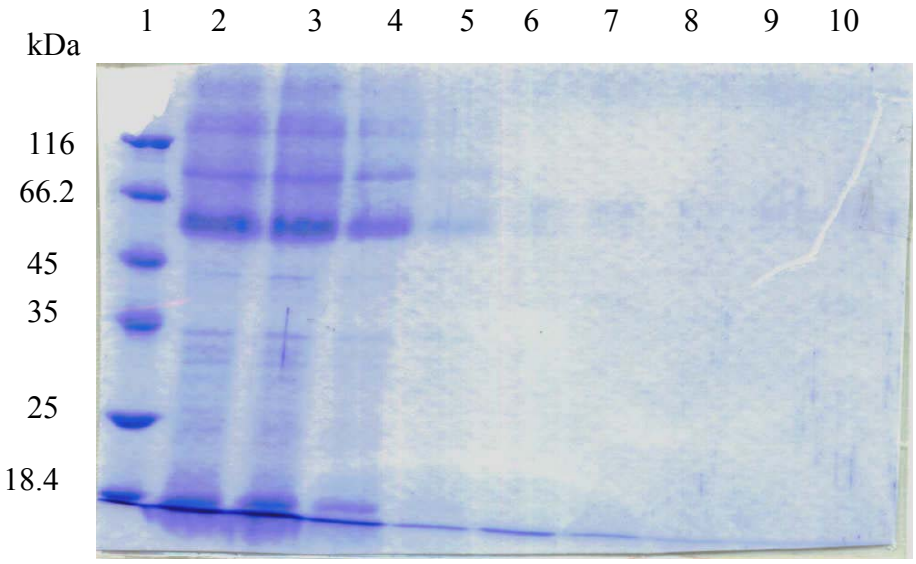


Fig. 3.19 The SDS-PAGE analysis of rEK_L fractions from purification using cobalt bead. The dialyzed fermentor supernatant of the transformant containing pPICZαB NH8 at 30°C production was purified using cobalt bead. Lane1: Protein molecular marker (Fermentas); Lane 2: 10 µl of dialyzed sample; Lane 3: 10 µl of flow-through fraction; Lane 4-5: 12 µl of wash fraction; Lane 6-10: 12 µl of elution fraction number 1-5.

Another attempt to purify the rEK_L containing N-terminal His-tagged was to purify the recombinant protein under denaturing condition or treated the sample with EDTA. This is under the hypothesis that the His-tagged was not expose to the medium because of the protein folding, the purification was then performed under denaturing condition with 6 M urea. And under the hypothesis that metal ion of the fermentor medium were bound to the His-tagged of the rEK_L, the sample was treated with 50 mM EDTA to eliminate the binding of other metal ion. Three ml of dialyzed sample in 50 mM Tris-Cl pH 8 was incubated with 6 M urea and shaken on ice for 6 h

then kept at 4^{o}C overnight. Three ml of the denature sample was loaded into the pre-equilibrated nickel column (His Trap^{TM} FF 1 ml) then flow-through fraction was collected. The column was washed with 5 ml washing and eluted with 5 ml elution buffer containing urea at 6 M final concentration. Every 1 ml of wash fractions, 100 µl of elution fractions 1 and 2 and 250 µl of elution fractions 3 to 10 were collected. The fractions were analyzed for protein bands on SDS-PAGE gel. The SDS-PAGE data showed that almost all of the major protein bands were found in the flow-through fraction and small amount was detected in the wash fraction (Fig. 3.20). No single protein band was detected in the elution fractions.

For the EDTA sample treatment, 1.8 ml of the dialyzed rEK$_L$ N- terminal His-tagged was incubated with 50 mM EDTA pH 8. The reaction was shaken on ice for 6 hours then kept at 4^{o}C overnight. Before loading into the pre-equilibrated nickel column, the buffer of rEK$_L$ was changed with 50 mM Tris-Cl pH 8 to eliminate EDTA. Two ml of EDTA treated rEK$_L$ was loaded into the nickel column (His Trap^{TM} FF 1 ml) then wash with 5 ml washing buffer and eluted with 5 ml of elution buffer. After elution, each fraction was analyzed on SDS-PAGE gel. The result was not difference from the purification under neither native condition nor urea denaturing condition. No protein band was detected in elution fractions but almost all of the major protein bands were found in the flow-through fraction (Fig. 3.21). All of these result indicated that His-tagged at N-terminal could not bind to nickel ion column neither denature nor native conditions. This may be due to His-tagged of rEK$_L$ molecule was cleaved off.

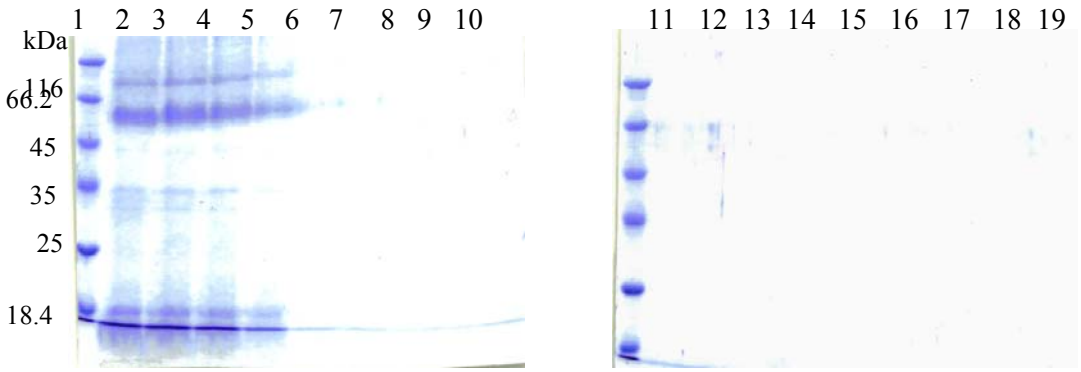


Fig. 3.20 The SDS-PAGE analysis of rEK_L purification fractions from nickel column under denaturing condition. The denaturing dialyzed and concentrated fermentor supernatant of the transformant containing pPICZαB NH8 at 30^{o}C production was purified using nickel column. Lane1: Protein molecular marker (Fermentas); Lane 2: 10 µl of dialyzed sample; Lane 3: 10 µl of denature dialyzed sample; Lane 4: 10 µl of flow-through fraction; Lane 5-9: 12 µl of wash fraction 2-5; Lane 10-19: 12 µl of elution fraction number 1-10.

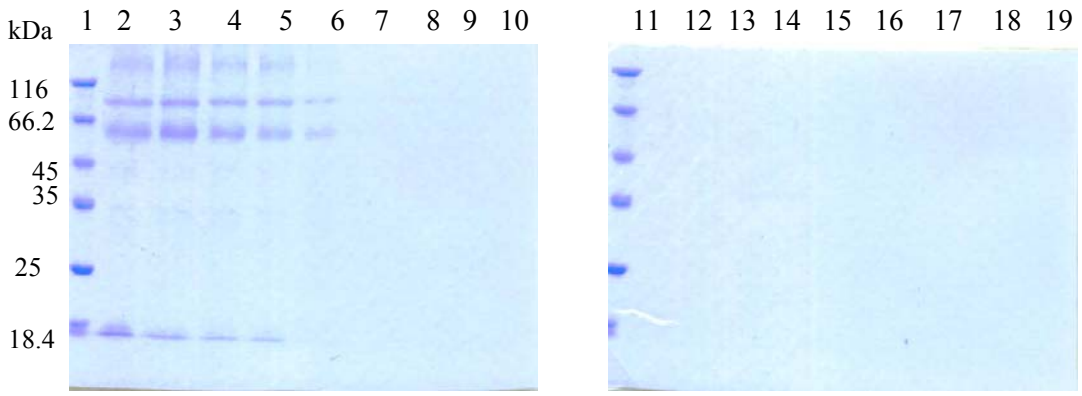


Fig 3.21 The SDS-PAGE analysis of rEK_L purification fractions using nickel column. The dialyzed and concentrated fermentor supernatant of the transformant containing pPICZαB NH8 at 30°C production was treated with 50 mM EDTA before loaded into nickel column. Lane1: Protein molecular marker (Fermentas); Lane 2: 10 µl of dialyzed sample; Lane 3: 10 µl of dialyzed sample which was treated with EDTA before buffer changed; Lane 4: 10 µl of dialyzed sample which was treated with EDTA after buffer changed; Lane 5: 10 µl of flow-through fraction; Lane 6-9: 12 µl of wash fraction 1-4; Lane 10-19: 12 µl of elution fraction number 1-10.

Low temperature production of rEK_L was tried, in the hope that the rEK_L would be able to purify by nickel column. The purpose of this experiment was that the cleavage of His-tagged by protease in the fermentor culture medium may be reduced due to low temperature production which can decrease the cell death and protease from death cell. One hundred ml of fermentor culture broth (from 20°C) was concentrated then dialyzed in 50 mM phosphate buffer pH 8. The dialyzed sample was purified using Ni-NTA column (Quigen kit). The result of enzymatic activity assay from the purification fractions showed that almost all of the enzymatic activity

was found in the flow-through fraction and no activity can be detected in the elution fraction.

To prove that His-tagged was removed from the rEK_L molecule; western bolt analysis was performed using His tag antibody (Invitrogen) as the probe. The result showed that very small smear band occurred in the sample of dialyzed and concentrated fermentor supernatant from the production of transformant containing pPICZαB NH8_EK_L at 20^oC (data not shown). This result can be concluded that 8xHis-tagged at N-terminal of rEK_L was removed from the rEK_L protein which indicated by the appeared of the very small band of 8xHis-tagged in the supernatant.

3.4.3.2 Purification of rEK_L by ion exchange chromatography

The culture supernatant from the end of process was dialyzed and concentrated in 50 mM sodium acetate buffer pH 5 then centrifuged the concentrated sample of both constructs at 12,000 rpm for 20 min, this sample was ready to load into SP column (cation exchanger). The rEK_L was eluted by 0-1 M gradient NaCl then the elution fractions were collected for enzymatic activity assay. The profiled of rEK_L purification from ion exchange showed that almost all of the rEK_L was eluted at 40 – 60 % conductivity in all rEK_L production (data not shown). The results of activity assay from each fraction indicated that almost all of the activity were found in the elution fractions but no activity could be detected in the fraction of flow-through or wash. Similar single protein band of elution fraction, 63 kDa, were found from the purification of fermentor supernatant from both constructed of pPICZαB NH8_EK_L and pPICZαB thrombin_EK_L. The result of SDS-PAGE analysis of each fraction from the fermentor supernatant purification of transformant carrying pPICZαB NH8_EK_L

from 30°C production is shown as an example in Fig. 3.22a. The elution fractions that showed high activity were pooled then concentrated and analyzed on SDS-PAGE gel (Fig. 3.22b). The results of the pPICZαB NH8_rEK$_L$ purification are summarized in Table 3.4. After the 30°C production rEK$_L$ was purified by cation exchanger column the result showed that only 224 mg/L was obtained from 5,118 mg/L of total protein concentration. For the low temperature production, 433 mg/L was obtained from 4,972 mg/L of total protein. The quality of obtained rEK$_L$ was considered from the specific activity from both systems temperature. The specific activity of purified rEK$_L$ comparison showed that the rEK$_L$ from 30°C production was higher than that at 20°C production. This result can be indicated that low induction temperature condition can not improve the quality of rEK$_L$ but only the yield of recombinant protein can be improved.

Because large amount of the purified rEK$_L$ can be obtained at the low temperature production thus the purified rEK$_L$ from 20°C production was chosen for the properties analysis of the purified rEK$_L$.

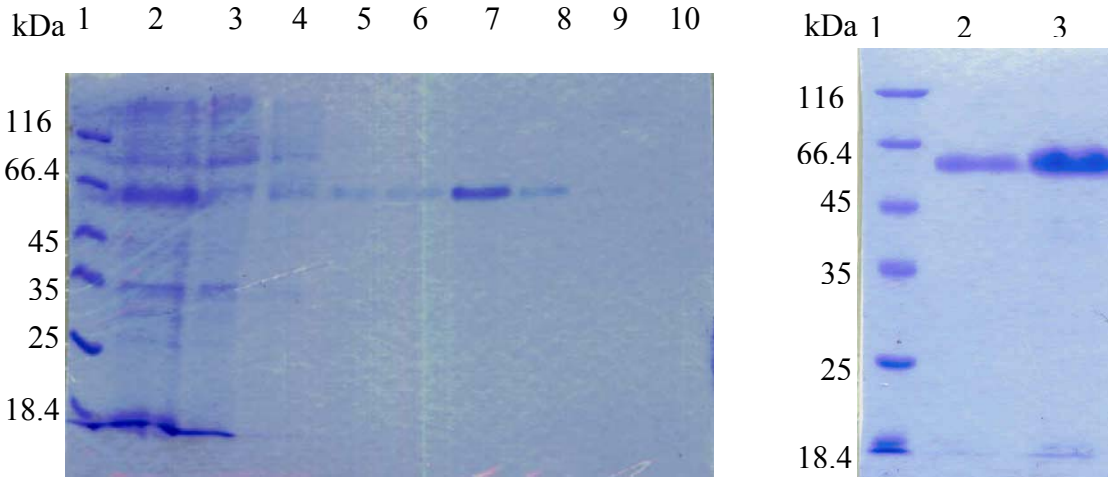


Fig. 3.22 (a) Represent the result of SDS-PAGE analysis of rEK_L purification from SP column (Cation exchanger). Three ml of the dialyzed and concentrated fermentor supernatant of the pPICZαB NH8_EK_L was load into ion exchange column. Lane1: Protein molecular marker (Fermentas); Lane 2: 10 µl of dialyzed sample; Lane 3: 10 µl of flow-through fraction; Lane 4: 12 µl of wash fraction; Lane 5-10 : 12 µl of elution fraction number 43, 44, 51, 53, 55, 57, respectively. **(b)** Represent the result of pooled elution fraction (43-51) and concentrated. Lane 1: protein molecular marker (Fermentas); Lane 2: 12 µl pooled fraction; Lane 3: 12 µl of concentrated pooled fraction (11 ml of initial volume to 2.3 ml of final volume).

Table 3.4 Recovery of rEK_L from fermenter culture broth of pPICZαB NH8_rEK_L construct which was induction at 30°C and 20°C.

Induction temperature	After dialysis		Pooled fraction from elution step of SP column	
	Total protein (mg/L)	Specific activity (U /mg total protein)	Total protein (mg/L)	Specific activity (unit /mg total protein)
20°C	4,973	26,068	433	170,926
30°C	5,118	18,301	224	213,016

3.4.4 Analysis of the purified rEK_L

3.4.4.1 pH stability

The pH stability was initiated by incubating 10 µl of the purified enzyme in 300 µl of 50 mM sodium acetate buffer pH 3, 4, 5 or potassium phosphate buffer pH 6, 7, 8 or Tris-Cl pH 7, 8, 9, 10 at 30°C for 17 h. Then the effects of pH on the quality of purified protein were tested by SDS-PAGE and enzymatic activity assay. During enzymatic activity assay, no changing of pH after the incubation reaction was performed. Ten µl of the rEK_L in the pH stability test buffer were added directly to 2 ml of the substrate solution. The results of activity analysis indicated that very low activity was observed in the rEK_L which was incubated at the pH lower than 5. Highest activity was found in the rEK_L incubation with 50 mM Tris-Cl pH 7 (Fig. 3.23). For SDS-PAGE result of this experiment showed very thin band because 30 fold of rEK_L concentration was diluted. The second pH stability tested for SDS-PAGE analysis was initiated by incubated 5 µl of the purified enzyme in 140 µl of 100 mM sodium acetate buffer pH 3, 4, 5 or potassium phosphate buffer pH 6, 7, 8 or Tris-Cl pH 7, 8, 9, 10 at 30°C for 17 h then checked the quality of purified protein by SDS-

PAGE. The results of SDS-PAGE showed that no degradation of the purified band protein in all of pH buffer (Fig. 3.24). Although no degradation of the rEK_L was observed in low pH buffer but low enzymatic activity of rEK_L was found. Therefore, the obtained purified rEK_L in the step of ion exchange purification was buffer changed with 50 mM Tris-Cl pH 7 containing 50% glycerol and kept at -20°C.

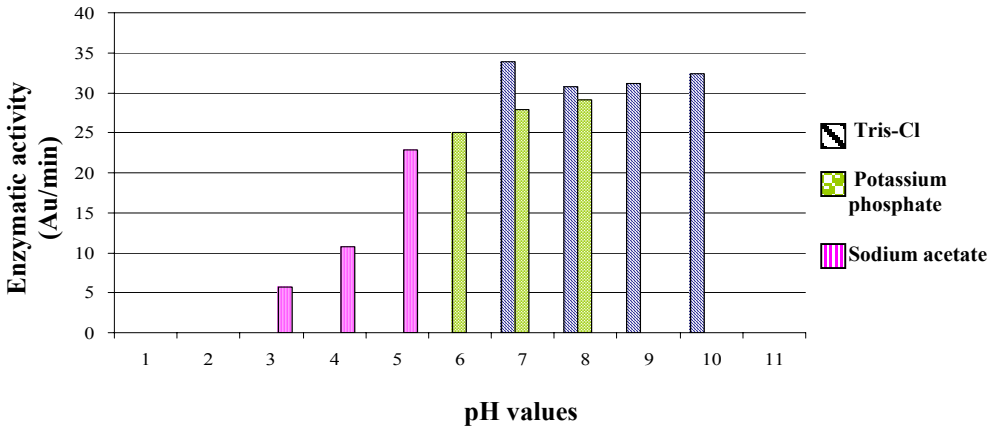


Fig. 3.23 Effect of pH on the enzymatic activity of the purified rEK_L. Ten µl of the purified enzyme was incubated with 300 µl of the various pH buffers at 30°C for 17 h. After 17 h of incubation, 10 µl of the each incubation reaction was added into 2 ml of enterokinase fluorogenic substrated solution and detected the increasing of fluorescence unit for 5 min.

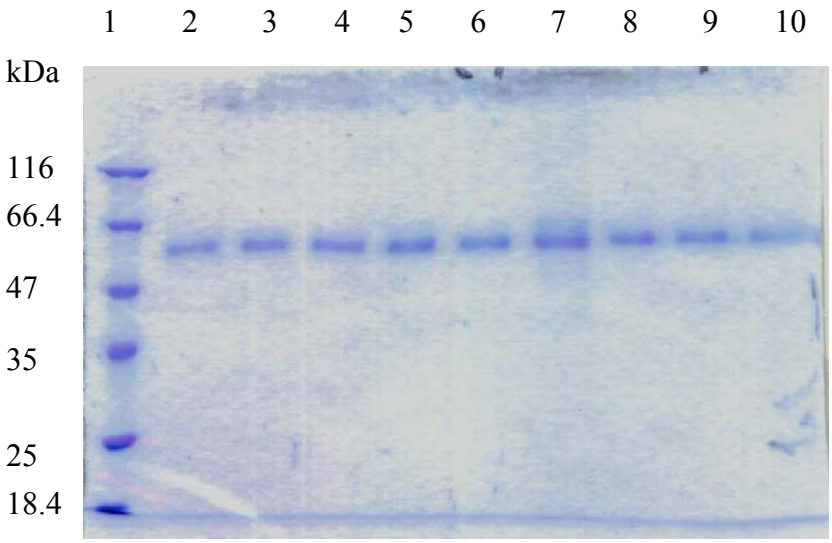


Fig. 3.24 SDS-PAGE analysis of the purified rEK_L which was incubated in the various pH buffers. Five ul of purified rEK_L was incubated with 140 µl of the various pH buffers at 30°C for 17 h. After 17 h of incubation, 12 µl of each incubation reaction was mixed with 3 µl loading dye then loaded on 15% SDS-PAGE gel. Lane 1: Protein molecular marker (Fermentas); Lane 2-4: The incubation reaction of rEK_L with 100 mM sodium acetate buffer pH 3, 4 and 5, respectively; Lane 5-7: The incubation reaction of rEK_L with 100 mM potassium phosphate buffer pH 6, 7 and 8, respectively; Lane 8-10: The incubation reaction of rEK_L with This-Cl buffer pH 7, 8, 9, respectively.

3.4.4.2 Silver staining

The protein contamination of purified rEK_L was analyzed by SDS-PAGE then stained with silver staining solution (e-Zi silver stain kit). The silver staining is the technique which used for detected the small amount of protein because this technique is high sensitivity than the coomassie staining. The purified rEK_L fraction from ion exchange purification was separated by 15 % SDS-PAGE gel then stained with silver staining solution. The big band of 63 kDa and the smaller band were detected on SDS-PAGE gel after stained with silver staining solution (Fig. 3.25).

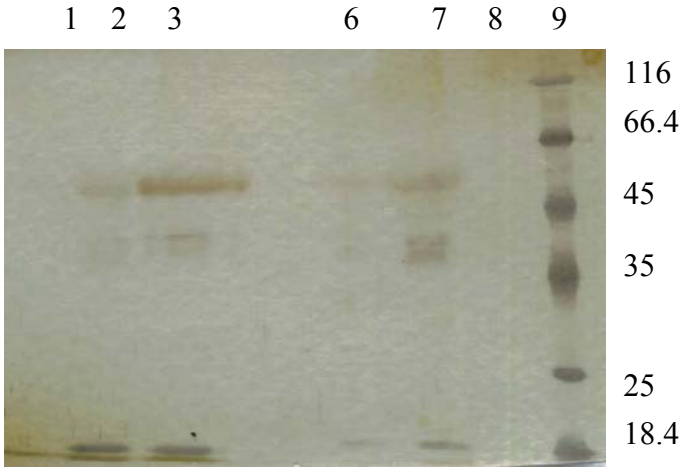


Fig. 3.25 The SDS-PAGE analysis of rEK_L purification fraction from SP column after stained with silver staining solution. Each fraction was loaded on 15% SDS-PAGE gel. Lane 1-3: 12 µl of wash fraction, pooled elution fraction and 6 µl of concentrated pooled fraction from 20°C production, respectively; Lane 6- 7: 12 µl of pooled elution fractions and concentrated pooled fraction from 30°C production, respectively.

This results indicated that the contamination of the other protein in the purify fraction of rEK_L was very small amount. However, to ensure that the larger band of 63 kDa was rEK_L thus this larger band was cut off from SDS-PAGE gel and identified by LC/MS/MS technique.

3.4.4.3 Protein identification

To verify that the larger band, 63 kDa, of elution fraction from ion exchange purification of culture supernatant from fermentation of transfromant containing pPICZαB thrombin_EK_L is rEK_L, the band was cut out of the SDS-PAGE gel and identified by LC/MS/MS method. The principle of LC/MS/MS method is to compare the digested peptide from experiment with the theoretical fragmentation spectrum. Tryptic peptides are separated in HPLC first then the desorbed peptides from the column are eluted directly into the mass spectrometer. In the ion trap, the mass of intact peptides are measured. Then each peptide is isolated in the trap, and the collision energy is increased, fragmenting the peptide to result in an MS/MS spectrum that represents the sequence of peptide. These MS/MS spectra are subjected to search against a database (protein, DNA or EST) to find a peptide with the corresponding intact mass and the fragment mass. The result of the purify band identification indicated that only a short peptide fragment is similar to the bovine enterokinase and a very low similarity amino acid score can be obtained (data not shown). This problem may be due to the interfering of hyper glycosylation in *Pichia* which caused the mass of peptide obtained shift from the intact peptide fragment. However, even with the only very low match, it does confirm that the 63 kDa band is rEK_L.

3.4.4.4 Ability of fusion protein cleavage by the purified rEK_L

The activity of rEK_L was also detected by cleavage of fusion protein, rice BGlu1-Trx (Chenchor et al., 2006) which has the enterokinase recognition sequence in the linker. The purified rEK_L of the transformant containing pPICZαB NH8_EK_L at 20 and 30°C production was incubated with 6.22 μg of rice BGlu1-Trx in 50 mM Tris-Cl pH 8 at 30°C for 4 h. The cleavage products were checked on 15% SDS-PAGE gel. The commercial rEK_L from NEB was used as positive control. The result of SDS-PAGE analysis is shown in Fig. 3.26. The major cleavage products of 50 kDa were found in the cleavage reaction which was cleaved by the rEK_L. The pattern of band product which generated by the cleavage of the rEK_L in this experiment and the commercial rEK_L from NEB were similar. This result indicated that the active rEK_L from 20 and 30°C production was able to cleave the fusion protein of rice BGlu1-Trx containing enterokinae cleavage site. However, in the total reaction of 22 μl, almost all of the 6.22 μg of rice BGlu1-Trx was completely cleaved by 0.002 μg of commercial rEK_L and approximately 90% of this fusion protein was cleaved by 0.093 – 0.651 μg of rEK_L obtained from 30°C production or 0.193 – 1.351 μg of rEK_L which obtained from 20°C production. To improve the %cleavage, the incubation time was extended to 21 h. The cleavage products are shown in Fig. 3.27. The intensity of the major product of 50 kDa from the cleavage of rEK_L in this experiment was not different when compare to the intensity of the major cleavage product which was cleaved by commercial rEK_L after 4 h (Fig. 3.27a). However, the degradation of the major product of 50 kDa occurred by cleavage of commercial rEK_L after 21 h of incubation at 30°C but not for rEK_L obtained from this research. From SDS-PAGE analysis in Fig. 3.27b indicated that the un-complete fusion protein can be reduced by

increasing the incubation time when using rEK_L obtained from the 30°C production.

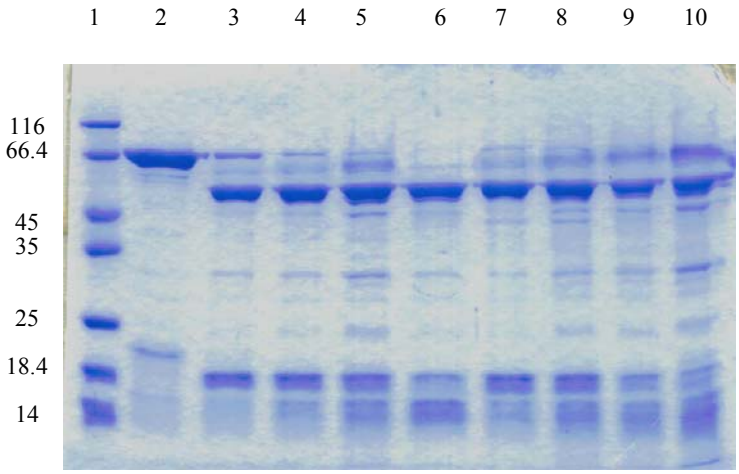


Fig. 3.26 Cleavage of fusion protein, rice BGlu1-Trx, with purified rEK_L of *P. pastoris* Y11430 carrying pPICZαB NH8_EK_L which was produced at 20 and 30°C. The fusion protein was cleaved with purified rEK_L which was produced at 20 or 30°C and the commercial rEK_L from NEB in 50 mM Tris-Cl pH 8 in total reaction of 22 µl for 4 h at 30°C. The cleavage products were analyzed on 15% SDS-PAGE gel and stained with Coomassie Blue R250. Lane 1: protein molecular marker (Fermentas); Lane 2: fusion protein only in the reaction buffer, Lane 3-5: the incubation of fusion protein with 0.093, 0.279 and 0.651 µg of 30°C production of purified rEK_L, respectively; Lane 6: the incubation of fusion protein with 0.002 µg of commercial rEK_L from NEB; Lane 7-10: the incubation of fusion protein with 0.193, 0.579, 0.965 and 1.351 µg of 20°C production of purified rEK_L, respectively.

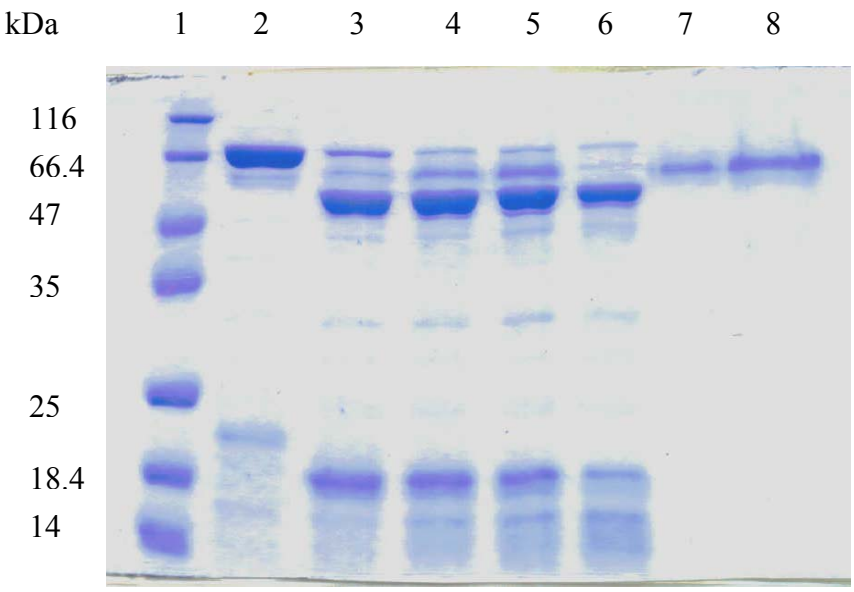


Fig. 3.27(a) Cleavage of fusion protein, rice BGlu1-Trx, with purified rEK_L from 30°C production of *P. pastoris* Y11430 carrying pPICZαB NH8_EK_L. The cleavage reaction (22 µl) was incubated for 4 h at 30°C. Lane 1: Protein molecular marker (Fermentas); Lane 2: fusion protein only in the reaction buffer; Lane 3-5: the incubation of fusion protein with 0.093, 0.279 and 0.651 µg of purified rEK_L, respectively; Lane 6: the incubation of fusion protein with 0.002 µg of commercial rEK_L from NEB; Lane 7: 0.651 µg of the stock purified rEK_L in 22 µl of 50 mM Tris-Cl pH 8; Lane 8: 0.651 µg of the stock purified rEK_L.

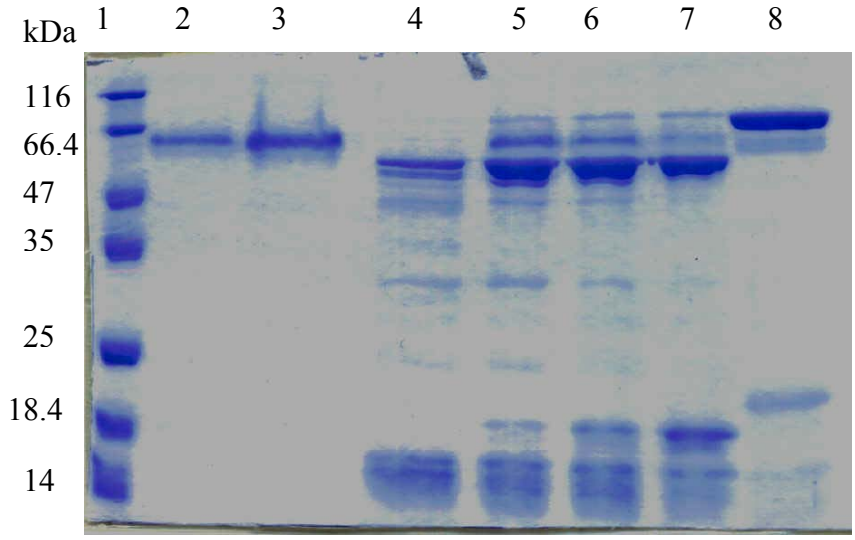


Fig. 3.27(b) Cleavage of fusion protein, rice BGlu1-Trx, with purified rEK_L from 30°C production of *P. pastoris* Y11430 carrying pPICZαB NH8_EK_L. The cleavage reaction (22 µl) was incubated for 21 h at 30°C. Lane 1: Protein molecular marker (Fermentas); Lane 2: 0.651 µg of the stock purified rEK_L in 22 µl of 50 mM Tris-Cl pH 8; Lane 3: 0.651 µg of the stock purified rEK_L; Lane 4: the incubation of fusion protein with 0.002 µg of commercial rEK_L from NEB; lane 5-7: the incubation of fusion protein with 0.651, 0.279 and 0.093 µg of the purified rEK_L, respectively; Lane 8: fusion protein only in the reaction buffer.

3.5 Discussion

Enterokinase is a serine protease which catalyzes the hydrolysis of peptide bonds in polypeptides and protein. Enterokinase is synthesized by the enterocytes of the proximal small intestine and incorporated into the brush border membrane (Jeno et al., 1987). It play a key role in initiating proteolytic digestion by converting trypsinogen into trypsin, which in turn activates other pancreatic zymogens, via cleavage at highly specific sequence of $(Asp)_4Lys$ (Hosfield and Lu, 1999: Ile et al., 2004). Enterokinase allows any downstream fusion target protein to retain its native amino-terminus, without leaving any unwanted amino acid residues on their amino termini. Because of its specific cleavage and it also retains full activity in various reaction conditions thus this make it suitable for fusion protein cleavage (Yuan and Hua, 2002).

Various researchers reported the expression of rEK_L in several hosts. In this research, the expressions of rEK_L were investigated in both prokaryotic and eukaryotic systems. In prokaryotic system, the rEK_L was designed to be expressed as the Trx_rEK_L fusion protein contains the enterokinase cleavage site at an inter-domain linker. If the expressed rEK_L is soluble and active, it will be able to autocleave and remove Trx from rEK_L molecule. This fusion protein of Trx_rEK_L was expressed in cytoplasm of *E. coli* strain Origami, Rosetta gami and BL21 pLysS. The results showed that almost all of the expressed rEK_L was inactive and insoluble thus it was not able to autocleave and remove Trx from its molecule. Only the major band of fusion protein was found in insoluble fraction. Formation of inclusion bodies in recombinant expression systems is the results of an unbalanced equilibrium between in vivo protein aggregation and solubilization. There are a set of structurally complex

aggregates often perceived to occur as a stress response when recombinant protein is expressed at high rates (SØrensen and Mortensen, 2005). This research finding was similar to the investigation of Collin-Racie et al. in 1995 who found that the production of rEK_L was inactive when expressed in cytoplasm of *E. coli*.

In 1995, Collin-Racie et al. reported that the other cytoplasmic fusion partner, glutathione-s-transferase and mature maltose binding protein (MBP), have been chosen to fuse with enterokinase. After expression of fusion protein, the expressed product was found to be inactive and much of rEK_L in the cell lysate was insoluble. The same results have been reports by Gasparian et al. (2003) who express the fusion of recombinant human Trx_EK_L using pET32a as the expression vector. The researchers found that 90% of 44 kDa fusion protein was insoluble and formed inclusion body (Gasparian et al., 2003). These results may be due to the folding environment in cytoplasm of *E. coli* was not suitable for the folding of rEK_L. The reducing environment within the cytoplasm prevents folding and/or reduces the stability of rEK_L (Collin-Racie et al., 1995). Macromoleculular crowding of proteins at concentrations of 200-300 mg/ml in the cytoplasm of *E. coli* suggest a highly unfavorable protein-folding environment, especially during recombinant high-level expression (SØrensen and Mortensen, 2005). However, contradictable results by Yuan and Hua in 2002 showed that the active and soluble rEK_L can be successfully expressed in cytoplasm of *E. coli*. It was able to autoclave Trx from the rEK_L molecule. But their later publication reported that the obtained rEK_L can not completely cleaved 5 fusion protein with EK cleavage site (Fang et al., 2003).

In prokaryotic expression system, the expression of soluble and active rEK_L in *E. coli* was fulfilled via fused the rEK_L to Dsb fusion partner. By this system, the

rEK_L was allowed proper folding and disulfide bond formation in periplasmic space. However, the total protein obtained by this strategy was soluble only 40% (Vozza et al., 1996). Because of this reason, the rEK_L was produced in eukaryotic system.

In eukaryotic expression system, the secreted rEK_L was expressed in *P. pastoris* Y11430. The rEK_L was designed to contain C- or N-terminal His-tagged to rapidly and easily separate the rEK_L from the cleavage product to prevent further degradation of the cleavage products. The expression of rEK_L was controlled by AOX1 promoter and secreted by secretory pathway using α-MF of *S. cerevisiae*. The processing of *S. cerevisiae* α-MF involves three steps. The first is the removal of the pre signal pepide, the first 19 amino acid signal, by signal peptidase in the endoplasmic reticulum. Second, Kex2 endopeptidase cleaves between Arg-Lys of the pro leader sequence. Then it is rapidly followed by cleavage of Glu-Ala repeated by the Ste 13 protein (Lin Cereghino and Cregg, 2000) and secreted the rEK_L out of the cell into the medium.

In shake-flask cultivation, the 43 kDa expected band was found in supernatant culture broth but no enzymatic activity was detected. However, the main problem of enzymatic activity assay in shake-flask crude culture broth was the medium's color interfering when using fluorogenic substrate. For transformant expression screening step, independently isolated *P. pastoris* strains transformed with the same expression vector routinely display a range of product level. The clonal variation is observed even within collections of transformants harboring the same number of expression cassette. Thus, to find the best producer, it is necessary to screen a significant number of transformants (Cregg et al., 1993). In this research, because the problem of color interfering in enzymatic activity assay and low expression level in shake-flask

cultivation thus the step of transformants screening was done by checking the EK_L mRNA expression. For large scale production, only 1 clone from each construct which was able to express high level of EK_L mRNA was chosen for large scale production in fermentor.

Large scale production in fermentor was performed to increase amount of rEK_L. The protein analysis of culture broth by SDS-PAGE showed that very few protein from *P. pastoris* were secreted into the medium and the secreted rEK_L was shown to be the major band in fermentor culture broth. This result indicated that only small amount of the *Pichia* own protein was secreted to culture medium. This is the advantage of selecting recombinant protein secretion in *P. pastoris* since this organism secretes only very low levels of native protein. Thus secretion of foreign protein become an effective purification step and easily separated product from most other cellular components (Cregg et al., 1993). However, the expected protein band in shake-flask and fermentor culture supernatant was different. Because the rEK_L was produced by secretory pathway using the α-MF of *S. cerevisiae*, thus this make the secreted product permitted post-translational events such as proteolytic maturation, glycosylation and disulfide formation (Cregg et al., 1993). Some foreign protein secreted in *P. pastoris* appeared to be hyperglycosylated (Lin Cereghino and Cregg, 2000). N-linked oligosaccharides originate from an oligosaccharide of $Glc_3Man_9GlcNAc_2$ (Glc= glucose; GlcNAc= N-acetylglucosamine) that is assempled on dolicol (pyrophosphate) in endoplasmic reticulum (ER), and that is transferred to the appropriate Asn of nascent protein in a co-translational event. In most yeast that have been studies, the three glucose residues and one specific α-1,2-linked mannose residue are removed by specific glycosidases in the ER, giving the protein N-linked

$Man_8GlcNAc_2$ core structure that is further process in the Golgi complex. In *S. cevisiae*, N-linked core units are elongated in the Golgi through the addition of mannose outer chain (Lin Cereghino and Cregg, 2000). Little differences in the size of oligosaccharides could be correlated to composition of the growth medium or cultivating in shake-flask or fermentor (Bretthayer and Castellino, 1999). In this research, the difference protein size in shake-flask and fermentor cultivation possible dues to the difference glycosylation process between the cultivation.

Large amount of secreted rEK_L could be obtained from fermentor cultivation and enzymatic activity could be detected in crude culture supernatant. This activity was not found in the fermentor culture supernatant of the other recombinant protein production nor from the control fermentation. These results confirm that the detection of enzymatic activity was specific for EK enzyme and had no background effect of the other serine protease in *Pichia* culture broth. Compare to shake flask cultivation, the rEK_L production in fermentor cultivation has more advantages includes high-cell density, AOX1 promoter can be continually induced, reduced the oxygen limitation and cultivation environments can be controlled effectively thus large amount of the secreted rEK_L can be obtained by fermentor cultivation.

During the first fermentation with 700 ml initial fermentation volume, some of the culture medium evaporated because the temperature of condenser was not low enough resulting in high cell concentration. Because of this problem, dry cell weight which obtained from 5 ml of the taken sample was higher than that from the other fermentations. The activity comparison of both constructs indicated that higher activity obtained by the cultivation of transformant carrying pPICαB NH8_EK_L. Due to the unsuccessful purification of rEK_L using nickel column in both constructs, C-

and N-terminal His-tagged, thus the difference of enzymatic activity of both constructs may be due to the effect of clonal variation rather than the N- or C-terminal His-tagged. In the construct of C-terminal His-tagged, almost all of the methanol in the culture was use as the carbon source for cell growth and low amount for induce AOX1 promoter. This indicated that the variation properties of each clone or clonal variation are the important factor for recombinant protein production. Clonal variation was first observed by K. Sreekrishna, who found large differences in the level of TNF produced by different transplacement transformants (from 1-30% of total protein) (Clare et al., 1998). Viader-Salvado et al. (2006) reported that a clonal variation was obtained after the transformation process due to genetic differences generated during the transformation event of the host strain. The transformants with lesser genetic differences with respect to the *P. pastoris* host strain are the recombinant strains with the highest level of recombinant protein production.

At the low temperature production, cell dry weigh in crude culture broth of both constructs was higher than that of 30^{o}C production. These results indicated that the organism growth rate can increase at low induction temperature (20^{o}C) (Fig. 3.15B). For total protein investigation, the slight decrease of total protein in fermentor culture broth occurred after 250 g of MF medium was fed into the culture at 30^{o}C production temperature but not at 20^{o}C production (Fig. 3.16). However, the quality of rEK_L was not improved even this condition can improve the rEK_L yield. Low temperature can improve the yield of heterologous protein expression in *P. pastoris* by enhancing protein folding in endoplasmic reticulum and/or by reducing the medium protease activity. A low induction temperature was associated with a low and constant level of dead cells during induction (<2%) and reduce protease activity (Woo

et al., 2004). In this research, 433 mg/L of purified rEK_L was recovered from 330 ml fermentation broth of 20^oC production condition which was as high as the previous reports by Peng et al (2004). From all results of rEK_L production by *P. pastoris* using fed-batch culture indicated that the increasing of enzymatic activity was proportional to ability of methanol consumption of each culture to induce AOX1 promoter (compare to the same transformant). During methanol production phase, the methanol feed rate was manually controlled depended on cell density and DOT. Increasing of methanol feed rate can increase amount of the secreted recombinant protein. However, high methanol concentration can not be kept in high-cell density cultures without oxygen limitation or temperature limitation (Charoenrat et al., 2005; Jahic et al., 2002). In this research DOT was closely to 0.1-3% for several hours during methanol production phase but the organism still survived and the rEK_L was still produced. This can be concluded that the oxygen limitation did not inhibit rEK_L accumulation which is similar to the previous reported by Charoenrat et al. (2005). However, one of the disadvantage of the process control in this research was the adjustment of methanol feed rate which was controlled manually. In this research, the adjustment of methanol feed rate depended on the health of the culture which was indicated by a reading of DOT and pH. But the variable of methanol concentration in the culture during the production phase can not be monitored. Thus, only small amount of methanol concentration was introduced into fermentor culture. Monitoring and controlling this variable are important because high levels of this inducer substrate can be toxic to cell and low levels of methanol may not be enough to initiate the AOX transcription (Cos et al., 2006). Because of this reason the adjustment of methanol feed rate should be controlled by the feed-back regulation using the methanol concentration as the

parameter to control the speed of methanol feed to ensure that the methanol concentration in the culture not present as high as toxic. By this technique, the methanol consumption can be increased resulting in increased recombinant protein production. However, This work was not able to do this technique due to the limitation of the controller at SUT. In 2005, Charoenrat et al. have reported the oxygen-limited fed-batch technique (OLFB) for the production of recombinant protein Thai Rosewood β-glucosidase with *P. pastoris* can improve the oxygen transfer rate and methanol consumption. In the OLFB, the methanol concentration was automatically controlled at 350 mg/L, and it resulted in a rapid drop of DOT below the detection limit. The researches found that the total methanol consumption was about 40% higher in this technique thus the increasing oxygen transfer rate obtained by the use of the OLFB technique. Thus, the higher oxygen uptake rate, higher methanol consumption rate and also higher β-glucosidase production obtained when compared to the conventional methanol limited fed-batch technique. However, the low oxygen concentration in the OLFB might cause an accumulation of intracellular methanol if it limits the oxidation rate of methanol but the analysis of cell viability indicated that no difference in viability was observed between the OLFB and the MLFB process control.

In the step of rEK_L purification of both constructs using nickel column, no rEK_L binding to nickel resin were detected. Almost all of the enzymatic activity and expected band was detected in the initial flow-through fraction. To ensure that the problem was not caused by the nickel column, cobalt bead was used to purify this rEK_L. The results were not different when compare to the using of nickel column. Almost all of the rEK_L was found in the flow-through fraction. This problems may be

due to the protein folding resulting in His-tagged not expose to the medium, or a alternatively, the exposed His-tagged of rEK_L was bound to some other metal ion from the fermentation medium thus this exposed His-tagged can not bind to metal ion resin. To solve these two problems, the rEK_L were purified under denaturing condition or with 50 mM EDTA treatment. The result of protein analysis by SDS-PAGE showed that no protein band can be detected in elution fraction in both cases. All results of rEK_L N- and C-terminal His-tagged purification by nickel column or cobalt resin can be concluded that the purification of rEK_L was unsuccessful under neither native nor denature conditions.

The effect of N-terminal His-tagged on enzymatic activity of rEK_L has been reported by Choi et al. (2001) who found that no enzymatic activity can be detected in rEK_L containing N-terminal His-tagged. The researcher reported that because of N-terminal extension of EK_L can blocks its active site. The importance of enterokinase N-terminal has been reported by Choi and Seong in 1999. The researchers showed that the N-terminal amino acid of rEK_L plays a crucial role for the enzyme activity. Ile at N-terminal of EK_L is highly conserved among EK_L from various sources and it is buried into hydrophobic pocket according to the crystal structure. The modifications at N-terminal end, such as substitution, addition, or deletion, can delete its enzyme activity (Choi and Song, 2001). The construct of N-terminal His-tagged of rEK_L in this research showed high enzymatic activity and the very small smear band of 8xHis-tagged from N-terminal of rEK_L was detected in the concentrate fermentor culture supernatant by western blot analysis using His tag antibody. Because of this reasons, unsuccessful rEK_L purification using nickel column was hypothesized that the His-tagged at N-terminal of rEK_L was removed. However, N-terminal sequence of

obtained rEK_L should be performed in the future to prove the hypothesis. The problem of weak binding of 6xHis-tagged to IMAC column has been reported by Hellwig et al. in 1999 who produced the carcinoembryonic antigen by *P. pastoris*. The researcher discussed that this problem seems to be a problem specific to each protein.

The ion exchange purification using a strong cation exchange resin has proven to be an effective starting point for purification of a variety of secreted products. The sample in 50 mM sodium acetate buffer pH 5 was loaded through the SP (cation exchanger) column and eluted with a linear gradient of 0 to 1 M NaCl in sodium acetate buffer. This eluted protein will be typically 90% pure or grater (Cregg et al., 1993). The purification of rEK_L in this research was fulfilled by ion exchange purification as described by Cregg et al. (1993). Almost all of the other native host proteins were eliminated by this technique. Only the secreted rEK_L tightly bind to the column and it can be eluted in the final step of purification. However, the size of this purified rEK_L was larger than that from the other previous reports which was produced in *P. pastoris* GS115 strain. But the ability of cleavage of fusion protein with rEK_L in this research and commercial EK was not different. This is the first reported of rEK_L production in the wild type strain of *P. pastoris* Y11430 thus the difference of rEK_L size may be due to the difference in glycosylation patterns of each *Pichia* strain.

The decreasing of enzymatic activity occurred when incubated the rEK_L with the buffer pH lower than 5. This indicated that rEK_L was not stable in low pH environment. In 2004, Song et al. reported that the optimal pH for rEK_L production by *P. pastoris* was 6 and decreasing of the recombinant yield at pH was lowers than 4.

The pH optimum for human enterokinase was found to be 8.4 in 25 mM Tris-HCl buffer. Enterokinase did not hydrolyze $Gly(Asp)_4Lys$-Nap at pH below 7. However, hydrolysis of trypsinogen by enterokinase is carried out at pH 5.6 since activation of trpsinogen by trypsin become increasingly significant at higher pH. Nevertheless, the ratio k_{cat}/k_m for enterokinase hydrolysis of trypsinogen at pH 5.6 was of the same order as the k_{cat}/k_m ratio for $Gly(Asp)_4Lys$-Nap at pH 8.4. This indicated that the tetra-aspartyl sequence of trypsinogen is the high affinity binding site for enterokinase (Grant and Hermon-Taylor, 1979). A reduced of activity was observed in phosphate buffer when compared to Tris-Cl this may be due to phosphate buffer inhibit the enzymatic activity. The information of commercial enterokinase enzyme from Roche Applied Science (http://www.roche-applied-science.com/pack-insert/1334115a.pdf) has reported that the optimal pH for the reaction is between 7.0 -8.0, but the enzyme can be used within pH 6.0-8.5. In phosphate buffer the activity is significantly reduces.

The ability of cleavage of fusion protein with the obtained rEK_L was not different from the commercial enzyme. However, a litter slower cleavage rate occurred when cleaved with rEK_L obtained from this research. Because the commercial enzyme is supplied in Tris-Cl buffer containing Ca^{2+} which has been reported that it is a cofactor and can increase the reaction rate of human enterokinase light chain. Thus the rate of fusion protein cleavage with commercial rEK_L was a little faster than that with rEK_L obtained in this research. Uncompleted cleavage product which was cleaved with rEK_L obtained by this research can be reduced by increasing the incubation time. However the long time incubation can reduce the uncompleted cleavage product when cleaved with rEK_L obtained in this research but the further degradation product was found when it was cleaved with commercial rEK_L. The result

of further degradation of cleavage product by rEK_L has been reported by Choi et al. (2001). In 2000, Choi et al. found that the further degradation was due to enterokinase itself, not any other contaminating protease. The cleavage product generated by both rEK_L showed non-target cleavage products of Trx, 15 and 18 kDa, on SDS-PAGE gel. The same results has been reported by Gaspariean et al. who also used the pET-32a vector and have also observed similar non-target cleavage of the Trx moiety when thioredoxin-fused human interleukin-13 was digested with human enterokinase light chain (Liew et al., 2005). This due to non-specific cleavage site of EK_L occurred in Trx.

In conclusions, the cheaper, active and high purity rEK_L could be obtained after the purification process in this research.

3.6 Conclusions

Active rEK_L could be obtained from eukaryotic expression system using *P. pastoris* Y11430 as the expression host. Large scale production of rEK_L was carried out using fed-batch cultivation technique in 2 L fermentor. Enzymatic activities of the 63 kDa secreted rEK_L from fermentor culture supernatant during the methanol production phase were detected. The comparison of induction temperature showed that low temperature can not improve the quality of rEK_L but increase only the yield of recombinant protein. The purification of rEK_L by nickel column was unsuccessful which was assumed that C- and N-terminal His–tagged of rEK_L was removed. However, the purification of rEK_L was fulfilled by ion exchange purification technique. The effect of pH on enzymatic activity of rEK_L indicated that the enzymatic activity of rEK_L reduced significantly in the low pH environment. The

ability of rEK_L to cleave the fusion protein showed that more than 95% of 6 μg fusion protein was cleaved by 0.09 μg of obtained purified rEK_L in 21 h at 30°C. All results demonstrated that the main research objective, the production of cheaper, highly active and pure $rEK_{L,}$ were fulfilled.

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CHAPTER IV

CONCLUSIONS

The results of Thai Bovine and buffalo EK_L gene amplification showed 708 bp of PCR product and 235 predict amino acid. The comparison of Thai bovine EK_L amino sequence results can be concluded that bovine EK_L gene is closely related to buffalo EK_L. Although one amino acid mutation was found in Thai bovine EK_L but that amino acid residues was not in the active site, so the enzymatic activity of recombinant EK_L should not be change. Because of these reasons, the obtained clone of Thai bovine enterokinase light chain in this research was used for the expression work.

In the step of rEK_L expression and purification, active rEK_L could be obtained from eukaryotic expression system using *P. pastoris* Y11430 as the expression host. The enzymatic activities from the 63 kDa secreted rEK_L were detected from fermentor culture supernatant during the methanol production phase. In the fermentation process, the effects of induction temperature on the rEK_L production were investigated. The comparison of induction temperature showed that low temperature can not improve the quality of rEK_L but increase only the yield of recombinant protein. At the end of fermentation process, the purification of rEK_L was fulfilled by ion exchange purification technique. The effect of pH on enzymatic activity of rEK_L indicated that the enzymatic activity of rEK_L reduced significantly in the low pH environment. The ability of rEK_L to cleave the fusion protein showed that

more than 95% of 6 µg fusion protein was cleaved by 0.09 µg of obtained purified rEK_L in 21 h at 30^oC. All results demonstrated that the research objective, cloning of rEK_L and the production of cheaper, highly active and pure $rEK_{L,}$ were fulfilled.

APPENDICES

APPENDIX I

MEDIA PREPARATION

1. LB-ampicillin/kanamycin/tetracycline broth

Yeast extract	5	g/L
Tryptone	10	g/L
Sodium chloride	10	g/L

Autoclave at 121^{o}C, for 15 min. Kanamycin, Tetracycline, and Ampicillin are added in the warm medium at 15 µg/ml, 12.5 µg/ml and 50 µg/ml final concentrations, respectively after autoclaving. If the agar medium is desired, 15 g/L of agar is added into the medium before autoclaving.

2. LB-ampicillin/chloramphenicol broth

Yeast extract	5	g/L
Tryptone	10	g/L
Sodium chloride	10	g/L

Autoclave at 121^{o}C for 15 min. Chloramphenicol and Ampicillin are added in the warm medium at 34 µg/ml and 50 µg/ml final concentrations, respectively after

autoclaving. If the agar medium is desired, 15 g/L of agar is added into the medium before autoclaving.

3. Yeast extract peptone dextrose (YPD) Zeocin broth

Yeast extract	10	g/L
Peptone	20	g/L
Dextrose	20	g/L

Autoclave at 121^{o}C for 20 minutes. then cooling down to 55^{o}C and add zeocin at 100 µg/ml final concentration. If the agar medium is desired, 15 g/L of agar is added into the medium before autoclaving.

4. Buffered glycerol complex (BMGY) medium

Yeast extract	10	g
Peptone	20	g
Glycerol	10	g

Dissolved in 100 mM potassium phosphate buffer pH 6 and the final volume is adjusted to 1 L. The media is sterilized for 15 min at 121^{o}C. Zeocin is added into the sterilized medium at 100 µg/ml final concentration.

5. Glycerol Basal Salt medium (GBS)

H_3PO_4 85%	26.7	ml
$CaSO_4$	0.93	g
K_2SO_4	18.2	g
$MgSO_4.7H_2O$	14.9	g
KOH	4.13	g
Glycerol	40.0	g

Bring the volume up to 1 L with distillate water. Fermentor containing 950 ml GBS medium is autoclaved at 121^{o}C for 35 min. The sterile fermentor is cooling down to the cultivation temperature and adds 4.35 ml of PTM1 trace salt to 1 L of this sterile medium then adjust pH of the medium to 5.5 with ammonium solution 25 %.

6. Glycerol feed medium (GF)

Dissolved 500 g of 99.5 % glycerol with distillate water and adjust the volume to 1 L. The GF medium is autoclaved at 121^{o}C for 35 minutes. After autoclaving, 12 ml of PTM1 trace salt is added into 1 L of the sterile medium.

Note. Density of glycerol 50 % (w/v) is equal to 1.109 g/ml

7. Methanol feed medium (MF)

Add 12 ml of PTM1 trace salts into 1 L of 99.9 % methanol.

8. PTM1 trace salts

$CuSO_4$. $5H_2O$	6.0	g
KI	0.08	g
$MnSO_4.H_2O$	3.0	g
$Na_2MoO_4.2H_2O$	0.2	g
H_3BO_3	0.02	g
$ZnCl_2$	20.0	g
$FeCl_3$	13.7	g
$CoCl_2.6H_2O$	0.9	g
H_2SO_4	5.0	g
Biotin	0.2	g

Dissolve in distillated water and adjust the volume to 1 L. Filter sterilization with a 0.45 µm sterile and store at room temperature.

APPENDIX II

FERMENTOR OPERATION AND ANALYTICAL METHOD

1. Fermentor operations (Biostat B, B.Braun, Germany)

The 950 ml of GBS medium is added in the clean 2 L fermentor. The temperature is controlled at 30^{o}C, impeller speed rate at 1,000 rpm and aeration rate 1 vvm. For pH probe calibration, the power line of pH probe must be connected to the fermentor controller. When the fermentor temperature is 30^{o}C then pH probe will be calibrated out side the fermentor jar. Before pH probe calibration, the monitor of fermentor controller is moved to the pH calibration mode and then pressed the arrow key at the controller to move the cursor in front of the position of pH 4 calibration. The pH probe is rinsed with DI water then now the probe can be immersed into room temperature standard solution buffer pH 4 and then presses the "Enter" key at the controller. The correct value is accepted, the cursor will be skipped to the position of pH 7 calibrations. For pH 7 calibration, the pH probe is rinsed with DI water then immersed into room temperature standard solution buffer pH 7 and then presses "Enter" key at the controller. After the pH probe is calibrated for both pH values, the slope (54 to 60 mV/ pH) and zero (-30 to + 30 mV) of pH calibration will be checked to confirm that the calibration value is in the acceptable range. To ensure the reading

of pH value is accurate, the pH calibrations were repeated 3 times before sterilization of the fermentor.

For DOT electrode calibration, the DOT electrode calibration is performed after fermentor sterilization. Before calibration, the fermentor condition is operated at the cultivation conditions and the power line of DOT electrode must be connected to the fermentor controller. The temperature is controlled at 30^{o}C, impeller speed rate at 1,000 rpm and 1 vvm of aeration rate. For 1 L GBS medium, 4.35 ml of PTM1 is added in to the fermentor containing 950 ml GBS medium. Then pH is adjusted to 5.5 by adding ammonia solution 25%. When pH and temperature is reached to 5.5 and 30^{o}C, respectively, the DOT calibration is initiated. Move the cursor of the fermentor controller monitor to the calibration mode and then presses the arrow key at the controller to move the cursor to the position of DOT = 0% calibration then disconnect the DOT electrode and presses the “Enter” key at the controller. After the corrected reading value of 0 % DOT is accepted, the cursor will be skipped to the position of 100 % DOT. At 100 % DOT calibration, power line of DOT electrode is reconnected to the controller again and the increasing of the reading of DOT is observed if the DOT electrode has no problem then presses the “Enter” key at the controller. When the 100 % DOT is accepted the cursor will pass to the pO_2 slope and zero values. To ensure that the calibration value is in the acceptable range, the values of pO2 slope (25 to 200 nA) and zero (0 to +15 nA) must be in the range as recommended in the fermentor manual control book. The DOT calibration is recalibrated for 3 times before starter inoculation to ensure that the reading of DOT value is accurate.

Before starter inoculation, all tubing, ammonium solution 25%, antifoam, GF

and MF medium, which connected to the fermentor is filled with each solution. During the glycerol fed-batch and methanol production phase, the GF and MF medium reservoirs is placed on a balance. Thus weigh of glycerol and methanol (g) which is fed into the culture is calculated by the weigh lose from the beginning (after the tubing is filled with GF or MF medium).

2. Enzymatic activity calculation

Enzymatic activity is calculated by the increasing of fluorescence unit in one minute because the released of β-naphthylamide by rEK_L.

1 unit is defined as 1 Au (Absorbance fluorescence unit) changes over 1 min interval which is detected at 337 excitation and 420 emissions at room temperature for 10 min.

Example

One hundred μl of fermentor crude culture supernatant is added into 2 ml substrate solution (50 μM GD_4K-β-naphthylamide in 70 mM Tris-Cl pH 8 and 10% DMSO). The reaction is then immediately mixed and placed cuvette containing the reaction in the cuvette holder of fluorospectrophotometer. Enzymatic activity is measured by an increasing of fluorescence (excitation at 337, emission at 420 nm) for 10 minutes at room temperature. The increasing of fluorescence unit in one minute interval which is detected at 337 excitation and 420 emissions is shown in Fig. A1.1. from Fig. A1.1, unit (Au/minute) of rEK_L is 95.606.

Table A2.1 The increasing of fluorescence unit during the enzymatic activity assay

time (min)	Abs
0	446.783
1	539.179
2	630.162
3	734.176
4	831.314
5	933.642
6	1009.075

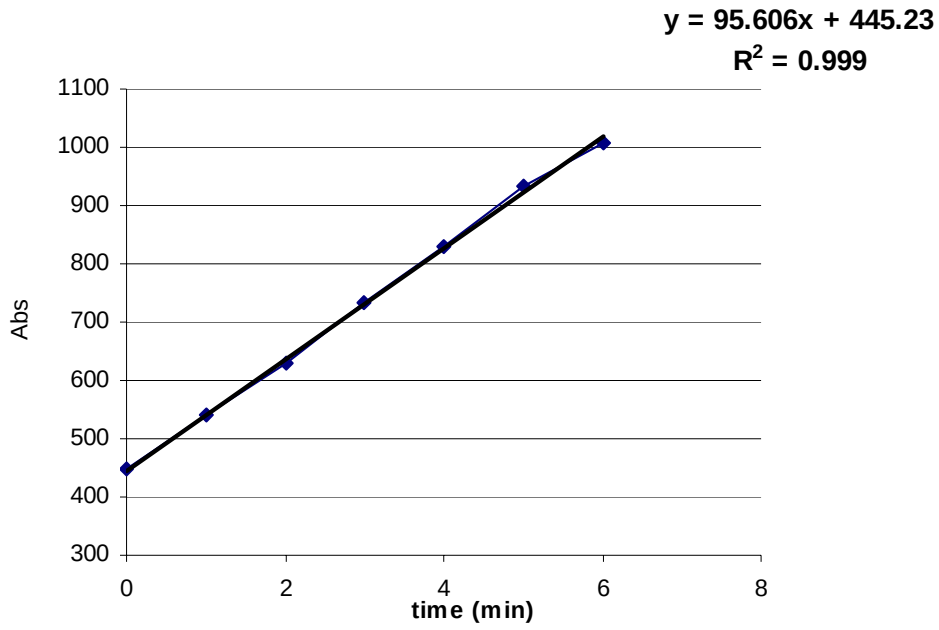

Fig. A2.1 The increasing of fluorescence unit over time

3. Analysis of total protein concentration

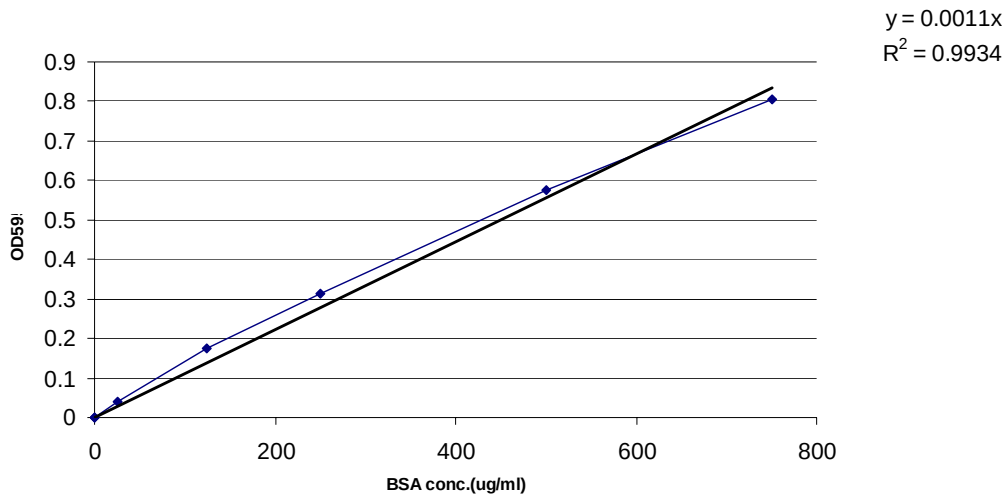

Fig. A2.2 standard curve of OD_{595} versus BSA concentration

The reaction is initiated with adding 50 µl of sample or water into 1.5 ml Coomassie plus protein assay reagent kit (Merk). Then the reactions are mixed well and incubated at room temperature for 10 min. The protein concentration is detected by spectrophotometer at 595 nm. The reaction mixture contains water is used as the blank. The protein concentration is calculated as follow

Protein concentration (µg/ml) = (1/slope) x (Abs_{595nm}) x (1/dilution factor)

APPENDIX III

TABLE OF RAW DATA FROM FERMENTATION

Table A3.1 Raw data from fermentation of transformant carrying pPICZαB thrombin_EK_L at 30^{o}C induction temperature

sample no.	phase	DOT (%)	Cultivation time (h.)	Induction time (h.)	cell dry weight (g/L)	Methanol feed medium (g)	rEK_L (unit)	Unit / g cell
1	glycerol batch	< 26.2	24.5	0	32.14	0	0	0
2	glycerol fed-batch	< 30.8	28.7	0	39.11	0	0	0
3	methanol production	40.2	43.5	12	45.80	26.5	0.71	155
4	methanol production	24	71.5	40	53.21	98.1	1.71	321
5	methanol production	0.3	97.5	66	58.85	154.2	6.56	1114
6	methanol production	0.1	115.5	84	63.74	233.9	8.11	1272

$Y_{x/s}$ (g cell/ methanol consumption) = 0.27

Table A3.2 Raw data from fermentation of transformant carrying pPICZαB thrombin _EK_L at 20^oC induction temperature

sample no.	phase	DOT (%)	cultivation time	Induction time	cell dry weight (g/L)	Methanol feed medium (g)	rEK_L (unit)	unit /g cell
1	glycerol batch	42.8	20.75	0	29.24	0	0	0
2	glycerol fed- batch	50.7	24.5	0	43.55	0	0	0
3	Methanol production	12-13.4	74	24.2	60.63	71.4	0	0
4	Methanol production	19.4	97	47.2	84.42	178.1	4.03	478
5	Methanol production	15-17	121	71.2	104.52	301.9	7.18	687
6	Methanol production	12.8	145	95.2	130.17	467.4	18.31	1407

$Y_{x/s}$ (g cell/ methanol consumption) = 0.28

Table A3.3 Raw data from fermentation of transformant carrying pPICZαB NH8_EK_L at 30^oC induction temperature

sample no.	phase	DOT (%)	cultivation time (h)	Induction time (h)	cell dry weight (g/L)	Methanol feed medium (g)	rEK_L (unit)	unit / g cell
1	glycerol batch	63.6	22.7	0	17.06	0	0	0
2	glycerol fed-batch	42.2	27	0	40.83	0	0	0
3	methanol production	4	47.4	21.6	54.17	54.2	0	0
4	methanol production	22-30	71.4	45.6	66.2	152.5	3.72	562
5	methanol production	1	95.4	69.6	78.03	261.9	19.26	2468
6	methanol production	0.2	119.4	92.6	89.33	375.9	57.84	6475

$Y_{x/s}$(g cell/ methanol consumption) = 0.24

Table A3.4 Raw data from fermentation of transformant carrying pPICZαB NH8_EK_L at 20^{o}C induction temperature

sample no.	phase	DOT (%)	Cultivation time (h)	Induction time (h)	cell dry weight (g/L)	Methanol feed medium (g)	rEK_L (unit)	unit / g cell
1	glycerol batch	72.5	21.5	0	12.5	0	0	0
2	glycerol fed-batch	47.4	25.5	0	38.98	0	0	0
3	methanol production	1.5	50	22.5	54.15	63	6.86	1267
4	methanol production	6	74	46.5	79.01	148	16.26	2058
5	methanol production	0.3	98	70.5	92.25	265.5	52.66	5708
6	methanol production	0.5	119	91.5	98.26	371.2	95.61	9730

$Y_{x/s}$(g cell/ methanol consumption) = 0.26

Table A3.5 Enzymatic activity unit / g methanol of transformant carrying pPICZαB NH8_EK_L from 30^oC induction temperature

sample no.	phase	Cultivation time (h)	Induction time (h)	protein concentration (mg/L)	Methanol feed medium (g)	rEK_L (unit)	unit / mg total protein	unit / g methanol
1	glycerol batch	22.7	0	20.45	0	0	0	0
2	glycerol fed-batch	27	0	39.55	0	0	0	0
3	methanol production	47.4	21.6	143.64	54.2	0	0	0
4	methanol production	71.4	45.6	212.73	152.5	3.72	174.78	243.8
5	methanol production	95.4	69.6	268.64	261.9	19.23	716.83	735.3
6	methanol production	119.4	92.6	250.00	375.9	57.84	2313.60	1538.7

Table A3.6 Enzymatic activity unit / g methanol of transformant carrying pPICZαB NH8_EK_L from 20^{o}C induction temperature

sample no.	phase	Cultivation time (h)	Induction time (h)	protein concentration (mg/L)	methanol feed medium (g)	rEK_L (unit)	unit / mg total protein	unit / g methanol
1	glycerol batch	21.5	0	25	0	0	0	0
2	glycerol fed-batch	25.5	0	57.73	0	0	0	0
3	methanolproduction	50	22.5	125.00	63	6.86	548.75	1088.8
4	methanol production	74	46.5	181.82	148	16.26	894.25	1098.6
5	methanol production	98	70.5	256.36	265.5	52.66	2053.99	1983.3
6	methanol production	119	91.5	355.91	371.2	95.61	2686.25	2575.6

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