

## Enhance production of recombinant lumbrokinase by optimizing gene codon usage for expression in *Pichia pastoris* and its properties

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Received: September 7, 2015; accepted: October 30, 2015.

Lumbrokinase (LK), as a group of potential fibrinolytic enzymes from earthworm having molecular weights of 25 to 40 kDa that removal of these fibrinopeptides leads to production of soluble fibrin clot and prevents the ability of blood clots. In this study, the gene encoding for a LK from the earthworm *Eisenia fetida* (GenBank Accession No. AF304199) was optimized codon for expression in *P. pastoris* under the control of an AOX1 promoter. The recombinant lines were screened based on the ability to express LK. The molecular weight of recombinant LK was determined by SDS-PAGE, was 47 kDa, purified and renatured using nickel-chelating resin with a recovery rate of 12.96%. The concentration of recombinant lumbrokinase was 215 mg/l and fibrinolytic activity of rLK was 3.58 U/mg calculated by comparison with the activity of a plasmin standard. The main physiochemical features of the lumbrokinase including temperature stability and pH resistance. Metal ions, detergents, and organic solvents tested indicated a significantly influence on rLK activity. These results suggested that the lumbrokinase expressed in *P. pastoris* could potentially be used as additive for the treatment of diseases associated with thrombosis.

**Keywords:** Fibrinolytic activity; lumbrokinase; *Pichia pastoris*; thrombosis; fibrin; earthworm.

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### Introduction

Lumbrokinase is alkaline serine proteases that are present in the body cavity and digestive organs of the earthworm [1]. Their isoelectric points (pI) are 3-5 [2, 3]. These enzymes were considerably stable at pH 2-11 and a temperature up to 60°C [4]. They are highly stable in solution *in vitro* for long periods, having a strong tolerance to organic solution [5]. Most importantly, it can be absorbed through the intestine [6, 7]. So, lumbrokinase now been widely used for the treatment of thrombosis [8-11].

The traditional process of producing lumbrokinase is to extract and purify it from the earthworm, a procedure that can yield

preparations contaminated with multiple components [12-16]. To elucidate the mechanism of the fibrinolytic property of lumbrokinase, high yields of recombinant LK are needed. Therefore, scientists have been attempting to produce recombinant LK by gene engineering. Several of them have been expressed in different expressing system such as *Escherichia coli*, insect cell, yeast, goat mammary glands, and plants [17-20]. Among these studies, the majority of expressed proteins were either inactive or partially active compared to the native enzymes. Yeast expression systems utilizing *P. pastoris* may be a good option for producing LK proteins [21-23].

In this study, we have purified the active recombinant LK, and then characterized its

main physiochemical features in a purified form. Our results showed that the fibrinolytic activity of secreted recombinant LK produced by *P. pastoris*. Blood clot lysis was observed when rLK was tested *in vitro*. The results may lead to the development of a therapeutic thrombotic agent for the treatment of diseases associated with thrombosis.

## Materials and method

### Chemicals and reagents

Bovine thrombin, plasmin was purchased from Biochemica (Sigma Aldrich Co., St. Louis, MO, USA). Bovine fibrinogen was obtained from the Laboratory of Enzyme (IBT, VAST). Yeast extract, peptone, tritonX-100, casamino acids, and sorbitol were purchased from Bio Basic Inc. (Ontario, Canada), and protein MW standard were from Fermentas Corp. (USA). Kit ProBond™ Nickel-Chaleting Resin, zeocin (100 µg/ml) were from Invitrogen Corp. (Carlsbad, CA, USA). Some other chemicals such as potassium phosphate, tris-HCl, sodium acetate were from Merck (Germany).

### Vectors, strains and culture conditions

Gene sequence encoding lumbrokinase optimized codon for expression in *Pichia pastoris* based on the protein sequence of earthworm *Eisenia fetida* (GenBank Accession No. AF304199) was obtained from Genscript (USA). Expression vector pPICZαA contained I<sub>k</sub> gene was obtained from previous studies (pPLK). *Pichia pastoris* host strain X33 (Invitrogen Corp., Carlsbad, CA, USA) were used for expression of the lumbrokinase. Yeast extract-peptone (YP) containing 1% (w/v) yeast extract and 2% (w/v) peptone was used for the cultivation of *P. pastoris*. YP agar contained additionally 2% (w/v) agar and 100 µg/ml zeocin.

### Optimization of codon for expression in *Pichia pastoris*

Codons of lumbrokinase gene were optimized for expression in *P. pastoris* by Genscript (USA)

based on the protein sequence obtained from earthworm *Eisenia fetida* (GenBank Accession No. AF304199) to produce the single gene that can reach the highest possible level of expression. The codon usage bias in *P. pastoris* was increased by upgrading the Codon Adaptation Index (CAI). GC content and unfavorable peaks have been optimized to prolong the half-life of the mRNA. The Stem-Loop structures, which impact ribosomal binding and stability of mRNA, were broken. In addition, their optimization process has screened and successfully modified those negative cis-acting sites such as cryptic splicing sites and premature Poly A sites.

### Comparison and analysis of expression gene in *P. pastoris* with Genbank records

Graphical Codon Usage Analyser (Gcua) (<http://gcua.schoedl.de/>), BLAST, ClustalOmega EMBL, and DNASTAR software were used to analyze and compare the frequency of using codon of the expression gene sequence in *P. pastoris* with Genbank records.

### Yeast transformation and screening

The plasmid pPLK linearized with *SacI* was transformed into *P. pastoris* X33 according to the instruction of EasySelect™ *Pichia* Expression Kit (Invitrogen Corp., Carlsbad, USA). Transformants were screened on YPDS plates containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 1 M sorbitol, and 2% (w/v) agar with zeocin™ at a final concentration of 0.1 mg/ml. The presence of the I<sub>k</sub> gene in the transformants was confirmed by PCR using yeast genomic DNA as template and I<sub>k</sub> specific primers. Clones showing the right size of the PCR product and zeocin resistance were chosen for expression.

### Gene expression

*P. pastoris* X33 strains contained vector pPLK transformants were grown in 5 ml of YP medium [1% (w/v) yeast extract, 2% (w/v) peptone] supplemented with 1% (w/v) glycerol at 28°C with agitation at 220 rpm until an OD<sub>600</sub> nm absorbance of 5-6 was reached. The cell

**Table 1.** Recombinant LK activity in various expression medium

Medium	OD <sub>600</sub> nm	rLK activity (U/ml)	Protein concentration (mg/ml)	Specific rLK activity (U/mg)
BMMY	9.755	0.112 ± 0.004	0.267 ± 0.007	0.419 ± 0.121
MMY	9.575	0.112 ± 0.121	0.211 ± 0.019	0.53 ± 0.155
MM	6.87	0.1006 ± 0.061	0.178 ± 0.006	0.565 ± 0.062
YPM	8.725	0.3105 ± 0.045	0.259 ± 0.002	1.198 ± 0.090
YPTM	9.325	0.435 ± 0.016	0.271 ± 0.025	1.605 ± 0.049
YPTCM	9.64	0.767 ± 0.053	0.389 ± 0.004	2.654 ± 0.071

pellet was harvested by centrifugation at 4,000 rpm for 5 min. For AOX1 promoter-controlled expression of LK, the cell pellet was resuspended in 25 ml of YP medium supplemented with 0.5% (v/v) methanol and was induced every 24 h to maintain induction. Cultivation was performed at 28°C with 220 rpm agitation. The culture supernatant was collected periodically to detect LK activity.

#### Optimal medium condition

The expression strain *P. pastoris* X33/pPLK was grown in various medium that contained different ingredients (Table 1). Mediums supplemented with 0.5% (v/v) methanol and changed every 24 h to maintain induction. Cultivation was performed at 28°C and 220 rpm agitation. The culture supernatant was collected periodically to detect LK activity.

#### Optimal concentration of Methanol

Various concentrations of methanol ranging from 0.5 to 2% (v/v) was added to YP medium and cultured with the expression strain *P. pastoris* X33/pPLK every 24 h to maintain induction. Cultivation was performed at 28°C and 220 rpm agitation. The culture supernatant was collected periodically to detect LK activity.

#### Optimal culture time

The expression strain *P. pastoris* X33/pPLK was grown in YP with 0.5 % (w/v) methanol and every 24 h to maintain induction. Cultivation was performed at 28°C and 220 rpm agitation. The culture supernatant after every 24 h was collected periodically to detect LK activity.

#### Purification of recombinant LK

One liter of cell-free supernatant of YPTCM medium (2% peptone, 1% yeast extract, 0.01% tritonX-100, 1% casamino acids, 0.5% methanol) stored at 4°C was thawed and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to 70% saturation with stirring. The mixture was kept at 4°C overnight, and then was centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was discarded. The precipitate was dissolved in 20 mM potassium phosphate buffer (pH 7.4). The sample was loaded onto His-Bind Column (10 cm glass column with 2 ml of Ni-agarose) pre-equilibrated with binding buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.5 M NaCl, 10 mM imidazole, pH 8.0) at the rate of 1 ml/5 min for twice. The column was washed with washing buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.5 M NaCl, 20 mM imidazole, pH 8.0) until the baseline was stable. Protein was eluted with 5 column volumes of elution buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.5 M NaCl, 250 mM imidazole, pH 8.0) at the flow rate of 0.25 ml/min and collected in fractions. All chromatography procedures were carried out at room temperature. The eluted protein was analyzed by SDS-PAGE and visualized by silver staining. Imidazole was removed from this protein sample by dialysis before the following assays.

#### SDS-polyacrylamide gel electrophoresis

The eluted protein was analyzed by polyacrylamide gel electrophoresis (SDS-PAGE). The fractions containing LK were pooled and

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AF304199.1-Genbank      ATGTTACTTCTCGCCCTTGCATCGTTGGTAGCGGTGGGTTTTGCGCAACCACCAGTCTGG
LK-optP.                ATGCTTCTTTTGGCACTTGCTTCTTTGGTTCGCAGTCGGTTTTCGCCAGCCTCCAGTTTGG
*** * *** * ** ***** ** ***** ** * ** ***** ** * ** ***** **
AF304199.1-Genbank      TACCCCGGTGGTCAATGCAGTGTGAGCCAGTACTCAGATGCTGGTGACATGGAACCTCCT
LK-optP.                TATCCAGGTGGTCAATGTTCTGTCTCCAATACAGTGATGCAGGTGACATGGAATTGCCA
** ** ***** ***** ** * ** ***** ***** ***** * **
AF304199.1-Genbank      CCCGGAACAAAAATTGTCGGAGGAATTGAAGCCAGACCATACGAGTTCCCATGGCAGGTG
LK-optP.                CCTGGAAC TAAGATCGTTGGTGAATTGAAGCTAGACCATACGAATTTCTTGGCAGGTT
** ***** ** * ** * ** ***** ***** ***** ** * ** *****
AF304199.1-Genbank      TCCGTCCGAAGGAAGTCTTCCGATTCCCATTTCTGCGGAGGTAGCATCATCAACGATCGT
LK-optP.                TCAGTCAGAAGAAAATCTTCCGATAGTCATTTCTGTGGTGGATCTATTATCAACGACAGA
** *** ** * ** ***** ***** ***** ** * ** ***** *
AF304199.1-Genbank      TGGGTTGTCTGCGCTGCTCACTGCATGCAGGGAGAGCCCTGCCCTGGTTTCATTGGTC
LK-optP.                TGGGTTGTCTGTGCTGCCACTGTATGCAAGGAGAATCACCAGCTTTGGTCAGTCTTGT
***** ***** ***** ***** ***** ** * ** ***** * **
AF304199.1-Genbank      GTCGGTGAGCAGATAGCAGCGCTGCGAGTACAGTACGTCAGACTCATGACGTTGACAGC
LK-optP.                GTCGGAGAGCATGATTCAAGTGCAGCTTCCACTGTTAGACAGACACATGATGTTGATTCA
***** ***** ** * ** * ** * ** * ** ***** ***** *****
AF304199.1-Genbank      ATCTTCGTCCACGAGGACTACAACGGAAATACCTTTGAGAACGACGTTTCTGTCAATCAAG
LK-optP.                ATCTTCGTTACGAAGATTACAACGGTAATACCTTCGAGAACGACGTTTCCGTCATCAAG
***** ***** ** ***** ***** ***** ***** ***** *****
AF304199.1-Genbank      ACAGTTAACGCCATCGCCATCGACATCAACGATGGGCCAATCTGCGCTCCAGATCCAGCC
LK-optP.                ACTGTTAACGCCATTGCAATCGATATTAATGACGGTCTTATTTGTGCTCCAGATCCTGCC
** ***** ** * ** * ** * ** * ** ***** ***** *****
AF304199.1-Genbank      AACGATTACGCTTACCCTAAGAGCCAGTGTCTCCGGATGGGGAATCAACTCAGGTGGA
LK-optP.                AATGACTACGTTTATAGAAAATCTCAATGCTCCGGTTGGGGAACAATTAAGTCTGGTGG
** ** ***** ** * ** * ** ***** ***** ***** ***** *****
AF304199.1-Genbank      GTCTGCTGCCCCAACGTTCTGCGATATGTGACACTGAACGTCACAACCAACGCCTTCTGC
LK-optP.                GTTTGTTGCCCAAATGTTTTGAGATATGTCACTCTTAACGTTACTACAAATGCCTTTTGT
** ** ***** ** * ** * ** ***** ** * ** ***** ** * ** ***** **
AF304199.1-Genbank      GATGATATCTACAGCCATTATATACAATTACCAGCGACATGATCTGCGCCACGGACAAC
LK-optP.                GATGACATCTACAGTCTTTGTATACCATCACTTCTGATATGATTTGTGCTACAGACAAC
***** ***** ** * ** ***** ** * ** * ** ***** ** * ** *****
AF304199.1-Genbank      ACCGGACAGAACGAGAGAGACTCTTGCCAGGGTGACTCTGGCGGCCCTCTGAGCGTCAAG
LK-optP.                ACCGGTCAAAATGAGAGAGATTCTTGCCAGGGAGACTCCGGTGGACCATTGTCAAGTCAAG
***** ** * ** ***** ***** ***** ** * ** * ** *****
AF304199.1-Genbank      GATGGCAACGGAATCTTCCAGCCTCATTGGTATTGTGCTTGGGGAATCGGTTGCGCATCT
LK-optP.                GATGGTAATGGAATCTTCTCCCTTATCGGTATTGTTTATGCGGGTATTGGATGTGCTTCT
***** ** ***** ***** ** * ** ***** ** * ** ***** ** * ** *****
AF304199.1-Genbank      GGCTATCCAGGAGTCTACGCCCGCTCGGATCCCAAAC TGGATGGATCACAGACATTATT
LK-optP.                GGATACCTGGAGTTTACGCAAGAGTCCGGAAGT CAGACAGGTTGGATTACAGACATTATT
** ** * ** ***** ***** * ***** ** * ** * ** ***** *****
AF304199.1-Genbank      ACCAACAAC TAG
LK-optP.                ACTAACAAT ---
** *****
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Figure 1. Comparison lk gene sequence for expression in *P. pastoris* with natural gene in Genbank by using the ClustalOmega EMBL comparison tool.

concentrated by ultrafiltration. SDS–PAGE was performed in a 12% gel on a Mini-II apparatus (Bio-Rad Laboratories, Hercules, CA, USA) according to the method of Sambrook *et al* [24]. After electrophoresis, the gel was stained and visualized by silver staining [25]. Protein concentration was measured by using the Bradford assay with crystalline bovine serum albumin as the standard [26].

#### Measurement of recombinant LK activity

Fibrinolytic activity was measured by using plasminogen-rich fibrin plates according to the method of Astrupt *et al* (1952) with plasmin as a standard. Samples (10  $\mu$ l of purified protein) were spotted on the artificial fibrin plate and then incubated at 37°C for 5 h. The lytic area diameter of each standard was measured from different directions at least four times to get the average. Then a standard curve was established with the logarithm of different activity as the x-axis and the logarithm of the average of each lytic area diameter as the y-axis. The activity of each sample can be obtained according to the diameter of its lytic area shown on the fibrin plate [27].

#### Temperature and pH optimum

The recombinant LK was (1) spotted on the artificial fibrin plate, incubated for 5 h at 20–60°C, and assayed the relative residual activity (%); (2) dissolved in 20 mM sodium acetate buffer (pH 3.0– 5.5), 20 mM potassium phosphate buffer (pH 5.5– 8.0), and 20 mM tris-HCl buffer (pH 8.5– 11). Mixture was incubated for 30 min at room temperature. The relative residual activity (%) was determined in comparison to the buffer that contained no enzyme at the corresponding pH values.

#### Temperature and pH stability

To observe the thermal stability of the enzyme, the enzyme activity was measured after incubation of the enzyme at various temperatures (30, 37, 40, and 50°C) in 20 mM potassium phosphate buffer (pH 7.4) with various time periods (10, 20, 30, 40, 50, and 60 min).

The pH stability of the enzyme was estimated by measuring the remaining fibrinolytic activity of enzyme after incubation for 10–60 min in 20 mM potassium phosphate at various pHs (pH 6–8) at room temperature.

#### Effect of metal ions, detergents, and organic solvents

The purified rLK was incubated with 5–15 mM of various metal ions ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Zn}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Al}^{3+}$ ) and EDTA, in 0.5–2% (w/v) of different detergents (SDS, tween 20, tween 80, and triton X-100), and 10–30% (v/v) of different solvents (methanol, ethanol, isopropanol, n-butanol, and acetone) at room temperature for 2 h. The residual activity was then determined.

## Results

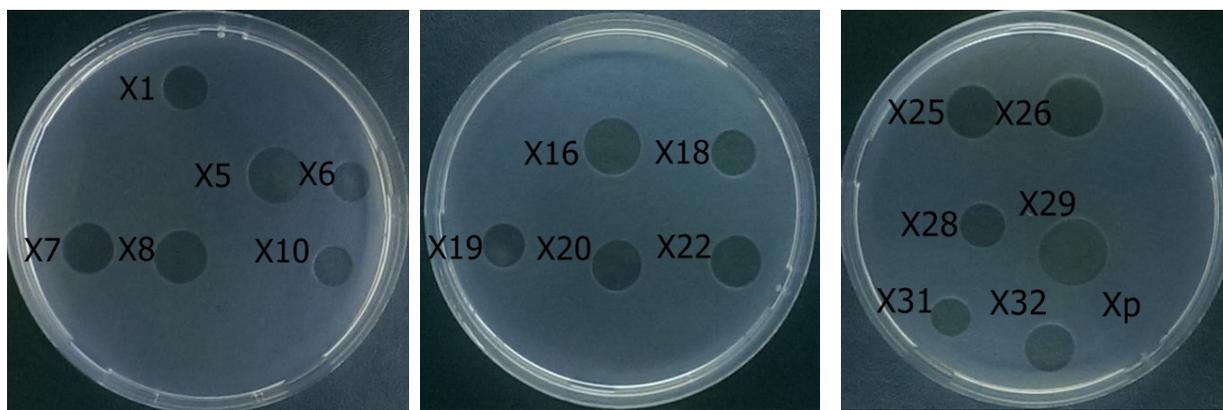
#### Comparison and analysis of expression gene encoding lumbrokinase in *P. pastoris*

Sequence of lk gene for expression in *P. pastoris* was compared to gene sequence (AF304199) in Genbank. Figure 1 shows that more than 50% of the codons were changed comparing to the natural gene. The BLAST comparison result demonstrated that the degree of genetic similarity of lk gene for expression in *P. pastoris* was 76% to that of the natural gene. In addition, the average GC content was decreased from 53.74% to 44.2%, and the GC distribution was more even than it before modification. The high GC content and uneven distribution of secondary structure in natural gene will make it difficult for the interaction between mRNA and rRNA, and therefore, affecting the efficiency of foreign protein expression.

Gcua and DNASTAR tools were applied to compare the frequency of codon usage of natural lk gene and expression gene in *P. pastoris*. For amino acid Ala, the frequency of the usage of the natural gene codon GCG was higher than its usage in *P. pastoris* while the frequency of the usages of GCA and GCT codons were reversed. To balance the frequency of

**Table 2.** Purification procedure of rLK from the culture supernatant of *P. pastoris* X33/pPLK

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Culture supernatant	6.35	2.16	2.94	100	1
Ni <sup>2+</sup> -ProBond™ resin column	0.82	0.23	3.58	12.96	1.22



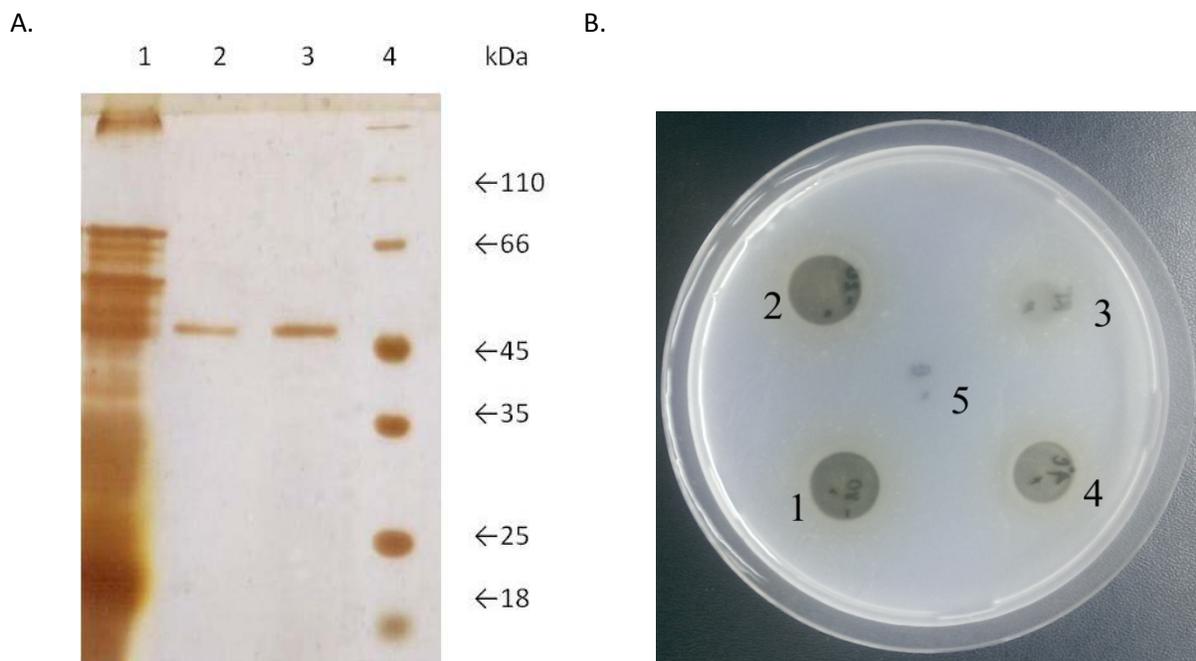
**Figure 2.** Screening the LK expression strains. Fibrinolytic activity was measured by using plasminogen-rich fibrin plates by the method of Astrupt *et al* (1952) with plasmin as a standard. The culture supernatants (10  $\mu$ l purified protein) were spotted on the artificial fibrin plate and then incubated at 37°C for 5 h. The lytic area diameter of each standard was measured from different directions at least four times to get the average.

codon usage in expression and to reduce the GC content, gene lk1 was optimized to increase the usage frequency of GCT and GCA codons while reduce the frequency of GCC and GCG codons. For Arg, CGG and CGC codons were rarely found in *P. pastoris*, while the frequencies of these two codons in natural lk1 gene are relatively higher than the others. For optimization of codon usage and reduction of GC content, the expression gene primary used AGA codon instead of other five codons AGG, CGA, CGC, CGG, and CGT. For Asn, the frequency of using AAC codon in natural gene was different to that of AAT codon, while the frequency of using both codons in *P. pastoris* was relatively balanced. In optimized gene for expression, the condon usage frequencies for amino acids Cys, Gln, Glu, His, Ile, and Lys were similar. For Gly, reduced GC content and codon usage frequency balance were applied in the host cell. Expression gene focused on the usages of two codons (GGT and GGA) only without using GGC and GGG codons.

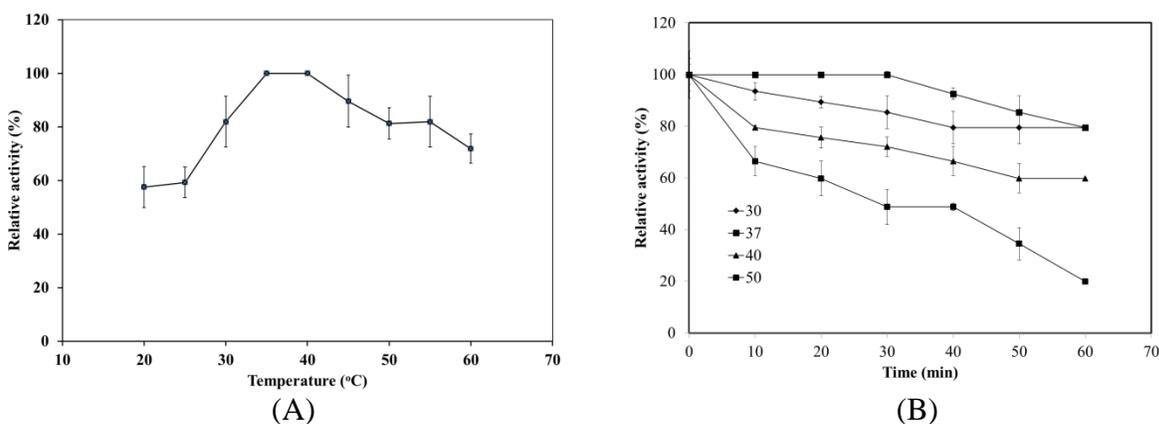
The frequency of codon usage was appropriate balanced in *P. pastoris* host cells. It was similar to the amino acids of Leu, Pro, and Ser. LK1 expression gene codon usage frequency in *P. pastoris* host cells was optimized.

#### Expression and purification of rLK

*P. pastoris* X33 strains were transformed with the yeast expression plasmid pPLK. Recombinant yeasts were screened on YPDS zeocin plates (100  $\mu$ g/ml). 31 positive clones were obtained and grown in YP medium supplemented with 0.5% (v/v) methanol. The medium was changed every 24 h to maintain induction. Cultivation was performed at 28°C and 220 rpm agitation. The culture supernatant was collected periodically to detect LK activity after 96 hours period. In fibrinolytic clones, X29 clone showed the highest amount of fibrinolytic activity (2.47 U/mg) and was selected for the lumbrokinase production (Figure 2).



**Figure 3.** Determination of the molecular weight and activity of the fibrinolytic enzyme. (A) SDS- PAGE analysis of 96 h fermentation supernatant of pPICZαLK/X33 strain (lane 1), fractions of purified rLK (lane 2, 3), and molecular weight marker (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA) (lane 4). (B) Fractions of purified rLK (1, 2, 3); plasmin (4) and supernatant of pPICZαA/X33 strain (5).

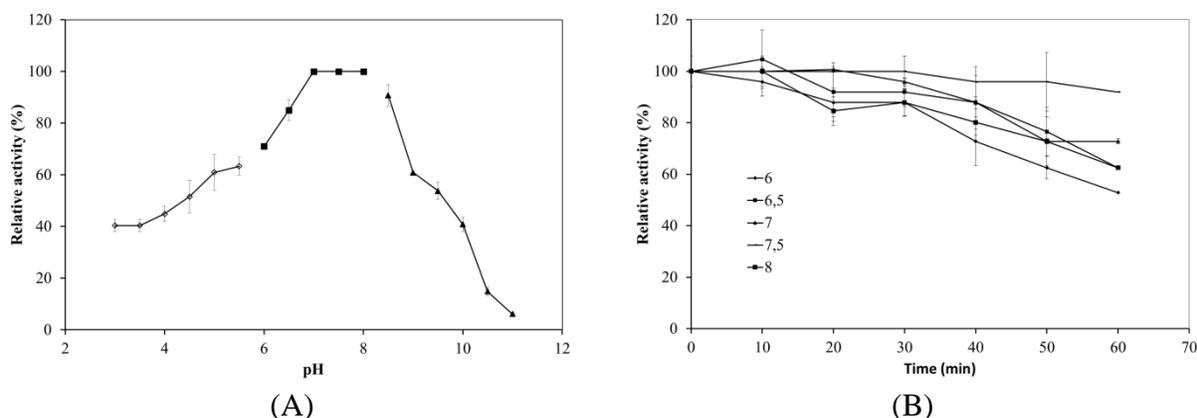


**Figure 4.** Enzyme activity (A) and stability of the recombinant LK (B) in 20 mM potassium phosphate (pH 7.4) at different temperature for different periods.

After induction of the gene by 0.5% (v/v) methanol, the culture supernatant was checked by SDS-PAGE analysis. The results indicated that the *P. pastoris* X33/pPLK strain produced maximum LK (2.94 U/mg) after induction for 96 h in YPTCM media (data not shown). Also, a relative increase from 2.548 to 2.962 U/mg occurred in specific activity of rLK between 0.5% and 1% (v/v) methanol when concentration of

methanol was optimized ranging from 0.5%- 2% (v/v) in YPM media.

His-tagged rLK was purified and re-natured by using nickel-chelating resin column (table 2). The peak fractions were collected and subjected to SDS-PAGE analysis. Figure 3 showed a protein band with molecular weight approximately 47 kDa. These data also indicated that the increase



**Figure 5.** Enzyme activity (A) and stability of the recombinant LK (B) in various buffers with pH values ranging from 3 to 11 for different periods. The remaining fibrinolytic activities were assayed by a fibrin plate as described in the text.

in molecular weight of rLK caused by glycosylation was a form of post-translational modification. A site (193 Asp) of N-glycosylation in peptide sequence rLK (NVTT sequence) were identified by using NetNGlyc 1.0 server.

Recombinant LK concentration in expression medium was 215 mg/l, which were 1.21 and 1.32 folds higher than it in rLK based on previous reports [22, 28]. According to the diameter of lytic area on the artificial fibrin plate and the protein concentration determined by Bradford assay, the specific fibrinolytic activity of the purified rLK was about 3.58 U/mg, with a recovery rate of 12.96 % and purification fold is 1.22.

#### Effects of temperature and pH on enzyme activity

The optimum temperature of the enzyme was determined by varying the reaction temperatures between 20-60°C for 5 h. As shown in figure 4A, the activity of enzyme was increased at temperatures ranging between 20-40°C with the maximal activity at 37-40°C. Their activities gradually decreased from 90% to 72% at the temperature above 40°C. In 60°C, the enzyme activities were down to 72%. These results indicated that the optimal temperature for rLK enzyme appeared to be ranging between 37-40°C. The enzyme was also very stable over 30-40°C. Stability of the enzyme was decreased

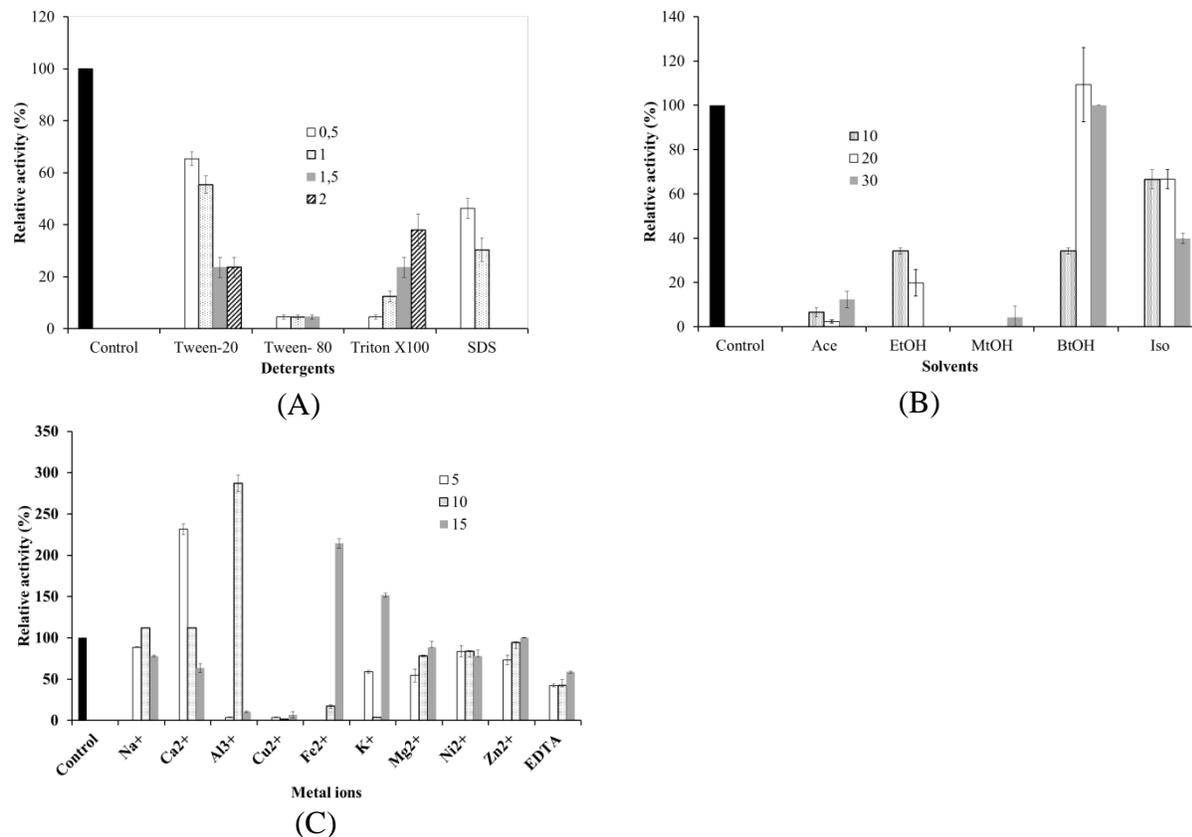
rapidly at 50°C after incubated for 60 min (20%) (Figure 4B).

The effect of pH on the activity of the purified enzyme was determined by using various buffers with pH values ranging from 3 to 11. The enzyme was most active in a range of 7.0-8.0. At pH range from 8.5 to 11, the enzyme activities were decreased rapidly from 90% to 6% (Figure 5A). The enzyme was stable over a pH range of 6.0- 8.0 at 37°C for 60 min (Figure 5B).

#### Effects of detergents, organic solvents and metal ions on enzyme activity

The effects of various detergents on enzyme activity were determined by measuring residual enzyme activity after incubation of rLK with 0.5-2% (v/v) detergents for 30 min at room temperature. The enzyme activity was inhibited by Tween 20 and SDS, and was significantly inhibited by Tween 80. However, in contrast, the relative activity of enzyme was increased gradually when the concentration of Triton-X100 was increased from 0.5-2% (v/v) (Figure 6A).

Most of organic solvents with the different concentrations had affected the enzyme activities. The relative activity of enzyme was increased quickly to 109.4% when rLK was incubated with 20% (v/v) n-butanol. However, the enzyme activities were inhibited by



**Figure 6.** Effects of detergents (A), organic solvents (B) and metal ions (C) on rLK activity.

acetone, ethanol, methanol, and isopropanol, especially, 10-20% (v/v) methanol could fully inactivate the enzyme (Figure 6B).

The levels of the effects of metal ions with different concentrations indicated the differences in activities of enzyme. A drastic increase of enzyme relative activity was recorded with the appearance of 5 mM Na<sup>+</sup> and Ca<sup>2+</sup>, which reached the highest at 113% and 191%, respectively. However, this enzyme was inhibited by Al<sup>3+</sup>, Cu<sup>2+</sup>, Fe<sup>2+</sup>, K<sup>+</sup>, and EDTA, but not Mg<sup>2+</sup> and Zn<sup>2+</sup> after incubation of the enzyme with 5-15 mM of each metal ion for 30 min at room temperature. Ion Ni<sup>2+</sup> did not affect the enzyme activity (Figure 6C).

### Discussion

In this study, we expressed recombinant LK in *P. pastoris*. The purified enzyme was highly active.

Currently, there are many studies about enzymes which extracted from earthworm. However, there have been only a few studies about expressing recombinant LKs. In addition, capsules of fibrinolytic enzyme used in clinics come from the extracts of earthworms and are mixtures of different components including different proteins. The successful purification of the recombinant lumbrokinase provides a single component with fibrinolytic activity. In this study, we were successful to optimize codon encoding gene for expressions in *P. pastoris*. The resulted rLK concentration was higher than it in previous reports [22, 28]. Other studies also indicated that the increase in molecular weight of rLK caused by glycosylation, was a form of post-translational modification. In that case, protein was attached to a hydroxyl or other functional group of another molecule (a glycosyl acceptor) [5]. However, the glycosylated pattern of the recombinant LK protein has not been investigated. Our study has shown the

same results as that of the study by Wu and colleagues using *E. fetida* [29], in which, Glycan measurement showed that all eight proteases were glycoprotein with different carbohydrate contents. The glycosylation of LKs might play important role in LKs' fibrinolytic activity, stability, and proteolysis resistance [30].

The results of this study demonstrated that the effects of temperature on enzyme activity and stability were dissimilar to that of previous study [17]. Besides, the result of Cho and colleagues indicated that six isozymes which were purified from *Lumbricus rubellus* had the highest caseinolytic activity at 50°C [31]. However, the optimal temperature of rLK in our study was lower than that in other studies. This difference can be explained as the difference in amino acid sequences. When the temperature increase is limited, enzyme activity will reduce because the high temperature breaks some bonds in the protein molecule, and therefore, changing the molecular structure, especially, the structure in the active site of the enzyme, which will affect enzyme's catalytic activity.

Thermal stability is very important for practical applications of enzyme. The results also showed that the enzyme rLK was less stable with heat than that in Hu's study [17] and the isozymes extracted from earthworm. The unstable ability of the enzyme can be related to the level of low glycosylation or expression in various expression systems. For example, N-glycosylation of cycloinulo-oligosaccharide fructanotransferase enzyme altered the thermal stability of the enzyme that was expressed in *S. cerevisiae* [32]. Other studies have also demonstrated the similar role of glycosylation in thermal stability of protein [33, 34].

pHs of buffers affect the ionized state of the hydrocarbons of the amino acids in the enzyme. The ionization of functional groups in the active site and/or substrate will affect the activity of enzyme. When we studied the effects of pH on enzyme activity, the optimal pH values were the same as the values of the fibrinolytic enzyme

that was extracted from *L. rubellus*. However, the values of pH stability of rLK were dissimilar to the values of the recombinant enzyme PM246 (pH 2-11, 55°C) [17] and the six isozymes purified from *L. rubellus* (pH 4-12, 50°C and pH 2-11, 60°C) [31, 35].

We found that the activity of enzyme was inhibited by some detergents, organic solvents, and metal ions. These results were the same as the results of Hu's studies [17]. However, the figures were quite different from other results [31, 36]. The isozymes extracted from *L. rubellus* and *Perionyx excavatus* showed strong tolerance to detergents and organic solvents for a long time and were not affected by EDTA. Overall, further studies should be done before the practical applications, especially, in treatment of thrombotic-associated diseases.

### Acknowledgments

This work was supported by the program "Research Development and Application of Biotechnology, 2012- 2014", Ministry of Science and Technology (Project: Research technological process to produce recombinant lumbrokinase makes the drug for prevention of vascular congestion).

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