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Isfahan University of Medical Sciences

Original Article

A novel approach to enhance the performance of kallikrein 6 enzyme using *Pichia pastoris* GS115 as a host

Fatemeh Mahmoodi[1,2], Hamid Bakherad[2], Navid Mogharrab[1,*], and Mohammad Rabbani[3,*]

[1]Biophysics and Computational Biology Laboratory (BCBL), Department of Biology, College of Sciences, Shiraz University, Shiraz, Iran.
[2]Department of Pharmaceutical Biotechnology and Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.
[3]Department of Pharmacology and Toxicology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

Abstract

Background and purpose: Enzyme engineering is the process of raising enzyme efficiency and activity by altering amino acid sequences. Kallikrein 6 (KLK6) enzyme is a secreted serine protease involved in a variety of physiological and pathological activities. The increased expression of KLK6 plays a key role in various diseases. Instability and spontaneous activation and deactivation are major challenges in the study of this enzyme. This study aimed to create a stable pro-KLK6 enzyme by enzyme engineering, designing a specific cleavage site for enterokinase, and using *Pichia pastoris* GS115 as a host cell. Then, recombinant pro-KLK6 was used to introduce a novel inhibitor for it.
Experimental approach: An engineered pro-KLK6 gene was cloned into the pPICZα A expression vector. Then, it was expressed in *P. pastoris* GS115 and purified by Ni-NTA chromatography. An inactive engineered pro-KLK6 gene was cleaved by enterokinase and converted to an active KLK6. The KLK6 enzyme activity and its kinetic parameters were measured using N-benzoyl-L-arginine ethyl ester (BAEE) substrates.
Findings/Results: The secretory form of the pro-KLK6 was expressed at about 11 mg/L in *P. pastoris* (GS115). Before activation with enterokinase, pro-KLK6 was inactive and did not activate spontaneously. The kinetic parameters, including K_m and V_{max}, were estimated at 113.59 μM and 0.432 μM/s, respectively.
Conclusion and implications: A stable pro-KLK6 enzyme was produced using *P. pastoris* (GS115) as the host cell and a specific cleavage site for enterokinase. Additionally, this study assessed the kinetic parameters of the KLK6 enzyme using the BAEE substrate for the first time.

Keywords: Enterokinase; Kallikrein6; Kinetic; Nα-Benzoyl-L-arginine ethyl ester; *P. pastoris* (GS115).

INTRODUCTION

Enzymes naturally evolve in living organisms, so they enable the organism to function efficiently. For industrial or laboratory production of an enzyme, it is necessary to alter its structure to optimize its function and characteristics. During the process of protein or enzyme engineering, changes are made in the amino acid sequence with the help of mutations in the DNA encoding the enzyme (1). Enzyme engineering is typically used to improve enzyme properties, such as catalytic activity, pH, and stability (1). A major issue, after protein engineering, is the production of the engineered enzyme in a suitable host. A host cell is selected based on the enzyme's structure, which is crucial for the successful production of an engineered enzyme (2).

Enzymes play an important role in various diseases. To find out how they work, it is necessary to produce a stable form of them in a laboratory (3).

*Corresponding authors:
M. Rabbani; Tel: +98-3137927085, Fax: +98-3136680011
Email: rabbani@pharm.mui.ac.ir
N. Mogharrab; Tel: +98-9177018316, Fax: +98-7132280926
Email: mogharrab@shirazu.ac.ir

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The kallikrein enzyme family is one of the enzyme families that plays an important role in a variety of diseases and has been currently studied a lot. Kallikrein-related peptidases are a family of secretory serine proteases with trypsin and chymotrypsin-like activity (4). Kallikrein family members are used as diagnostic or prognostic markers for various diseases (5). Kallikrein 3 or prostate-specific antigen, is used as a useful biomarker in the diagnosis of prostate and breast cancers (6), and kallikreins 9-11 are involved in the diagnosis of ovarian cancer (7). An important member of the kallikrein family is kallikrein 6 (KLK6), also called protease M, neurosin, or zyme (8). KLK6, like other members of the kallikrein family, is synthesized as an inactive pre-proenzyme (244 amino acids). After the removal of the signal peptide, the enzyme turns into an immature pro-enzyme. Finally, the KLK6 is activated (223 amino acids) by digesting a short piece in the N-terminal (9). KLK6, like other kallikrein-related proteases, is involved in physiological and pathological activities (10). This enzyme is highly expressed in ovarian (8), colon (11), stomach (12), and early stages of breast (8) cancers. It is also involved in neurological disorders such as epilepsy, degradation of amyloid beta-protein, and demyelination of nerve cells due to inflammation (13). Previous studies have demonstrated that the increased expression of this enzyme plays a role in inflammatory skin diseases, such as psoriasis (14), and inflammatory diseases of the central nervous system, such as multiple sclerosis and Alzheimer's disease (13).

KLK6 is a trypsin-like enzyme that can cleave peptide bonds after amino acids, such as lysine or arginine (15). This enzyme activates and inactivates itself by digesting peptide bonds after Lys21 and Arg80, respectively (16). To study the properties of the KLK6 enzyme, a stable form is needed, and the enzyme's ability to self-activation is a major challenge. The native form of pro-KLK6 is produced in commercial and converted into active form with Lys C because Lys C is non-specific and cuts the peptide bond after all Lys amino acids, the enzyme becomes completely inactive after 20-30 min. For this reason, the commercial form of pro-KLK6 is also not suitable for studying enzyme properties. In these cases, enzyme or protein engineering is used to improve enzyme performance. In this study, it was attempted to produce a stable engineered pro-KLK6 enzyme that is activated only in the presence of enterokinase. Consequently, a specific cleavage site was designed for the enterokinase enzyme at the N-terminus of KLK6. According to the characteristics of the KLK6 enzyme, from *P. pastoris* GS115 was used as the host cell. The most common expression host used in *P. pastoris* yeast, strain GS115, has a Mut^+ phenotype. The strains containing this type of phenotype have both genes encoding alcohol oxidase 1 and 2 promoters. In this group, methanol is used in a high amount and quickly, and with the presence of methanol as a carbon source, protein expression will be done at a high level (17). The recombinantly engineered pro-KLK6 was then expressed in *P. pastoris* GS115. Next, the KLK6 kinetic parameters were measured using N-benzoyl-L-arginine ethyl ester (BAEE) substrates. Considering the importance of KLK6 in various diseases, the stable pro-enzyme produced in this study can be used to identify the function, structure, and substrate characteristics, and also to design an effective and specific inhibitor for this enzyme.

MATERIALS AND METHODS

Materials

BAEE and reagents were provided by Sigma-Aldrich (USA). *P. pastoris* GS115, *E. coli* TOP10, and pPICZα A were obtained from Pasteur Institute (Iran). All restriction enzymes and T4 DNA ligase were obtained from Thermo Fisher (USA). The Pfu master mix was obtained from DNA Biotech (Iran). Bradford kit and mouse anti-His tag specific antibody were provided from Sinagen (Iran) and Abcam (ab18184) (USA), respectively.

Preparation of engineered pro-KLK6 and cloning of recombinant KLK6 in expression vector pPICZα A

Briefly, cDNA encoding the KLK6 enzyme (MCF10A cell line) was amplified by two polymerase chain reaction (PCR) steps. The first step of PCR was performed

using primer pairs, forward: 5'-AAGTTGGTGCATGGCGGACCCT-3' and reverse: 5'-ACTTGGCCTGAATGGTTTTTT GGATCCAGTT-3'. During the first step of PCR, the gene encoding the KLK6 was amplified (Lys 21 - Lys 224). In the second step of PCR, specific restriction sites for the enterokinase, *Eco*RI, and *Xba*I were included at the 5′ and 3′ ends, respectively, by forward: 5'-GAATTCGACGATGATGACAAGTTGGTG CAT-3' and reverse: 5'-ACTCTAGAA-CCTTGGCCTGAAT-3'. In this step, the gene encoding the engineered pro-KLK6 enzyme was amplified (Fig. 1). PCR conditions included an initial denaturation step at 94 °C for 10 min, 94 °C for 35 s, annealing at 60 °C (65 °C, the second step of PCR) for 35 s, extension at 72 °C for 10 min, 35 cycles and finally an extension for 10 min.

Then, the purified vector and PCR products were digested with the mentioned enzymes and after purification from the gel, they were used for ligation. The PCR fragment was cloned into the expression vector pPICZα A. pPICZα A/pro-KLK6 was transformed into *E. coli* TOP10 by using the heat shock method. The cells were then cultured on a Luria-Bertani agar plate containing 100 µg/mL zeocin. The resulting colonies were confirmed by colony PCR and enzymatic digestion. Plasmid purification was then performed from one of the confirmed colonies using a DNA plasmid purification kit (DNA Biotech, Iran).

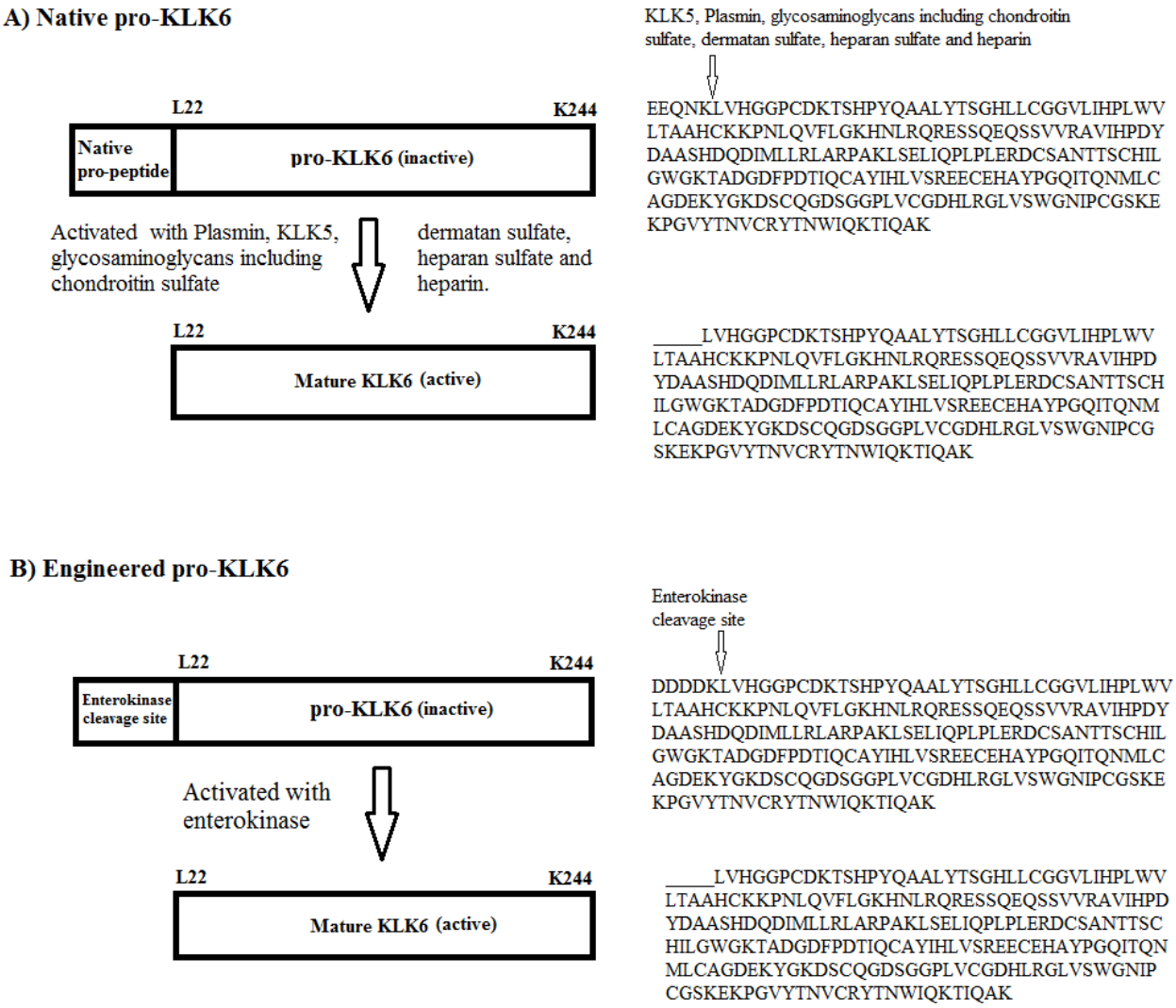

Fig. 1. Native pro-KLK6 sequence and pro-KLK6 sequence after protein engineering. (A) Schematic figure of native pro-KLK6 activation and its conversion to mature form by KLK5, plasmin, glycosaminoglycans including heparin, heparan sulfate, dermatan sulfate, and chondroitin sulfate (16); (B) schematic figure of engineered pro-KLK6 activation and its conversion to mature form by enterokinase. KLK, Kallikrein.

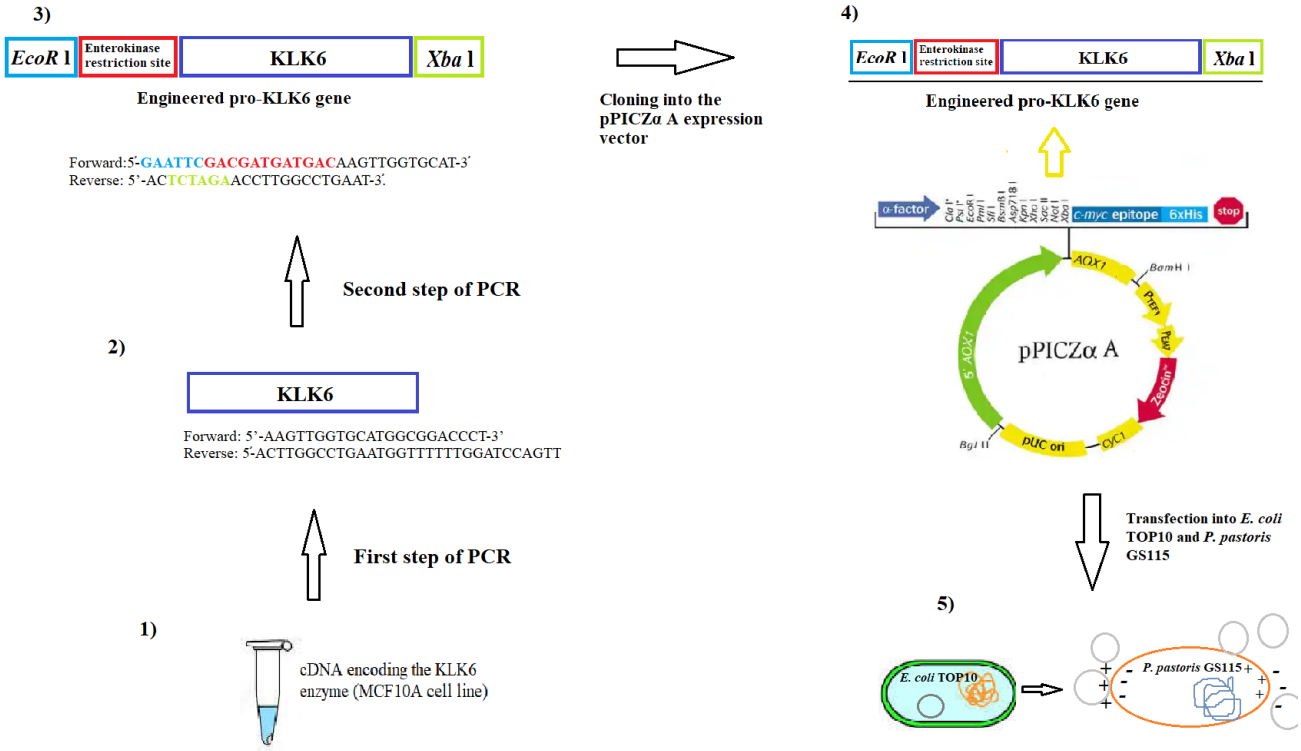

Fig. 2. Schematic figure of production and cloning of recombinant pro-*KLK6* in *the* expression vector pPICZα A. KLK, Kallikrein; PCR, polymerase chain reaction.

The resulting plasmid was linearized by *SacI* enzyme and transferred to *P. pastoris* GS115 competent cells by electroporation method (18) at 1,500 V, 5 ms (Bio-Rad, USA), and immediately cultured on YPDS-agar culture medium containing 100 µg/mL zeocin for 72 h at 30 °C (Fig. 2).

Expression and purification of recombinant pro-KLK6 by affinity chromatography

A selected clone containing pPICZα A / pro-KLK6 was cultured in 20 mL of YPD medium containing 100 µg/mL of zeocin, and incubated at 28 °C and 200 rpm for two days. After reaching OD_{600} = 2-4, 1% methanol was added to the yeast culture medium to induce expression. Every 24 h, 1% methanol was added to the culture medium. The supernatant was collected 48 h after induction, and sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to evaluate the expression of the pro-KLK6 enzyme. In the next step, all the above steps were performed in the volume of one liter of yeast culture. Due to the presence of His-tag at the C-terminal of pro-KLK6, the Ni-NTA chromatography and the native method were used to purify the enzyme. The column was washed with different concentrations of imidazole buffer (20-60 mM). Then, it was eluted with a 500 mM imidazole elution buffer. The purity of the purified protein was evaluated by SDS-PAGE gel.

Evaluation of protein concentration using the Bradford method

The concentration of the purified pro-KLK6 was measured using the Bradford method and bovine serum albumin as a standard.

Western blot

The western blotting technique was used to confirm pro-KLK6 expression. Briefly, after the transfer of protein bands from 12% SDS-PAGE gel to a nitrocellulose membrane, the pro-KLK6 enzyme was identified by mouse anti-His tag specific antibody (1: 1000), and the diamino-benzidine peroxidase substrate was used to detect the pro-KLK6 band on the nitrocellulose membrane.

Activation of human KLK6 by enterokinase

Enterokinase was used to activate pro-KLK6. The enterokinase cleaves the N-terminal propeptide fragment of the pro-KLK6 enzyme turning to a mature and active

form. Enterokinase was expressed and purified in the same laboratory. The concentration used was 25 IU: 1 IU, pro-KLK 6: enterokinase.

Kinetic parameters of KLK6

The synthetic substrate BAEE is a specific substrate for trypsin-like enzymes, such as kallikrein. Different concentrations of the substrate (10-1000 µM) and a constant concentration of KLK6 (12 nM) were prepared to a volume of 1000 µL using a 67 mM Na_2HPO_4 buffer (pH = 7.6). Enzymatic activity was measured at 254 nm by spectrophotometer (JASCO, V-670, Japan). The kinetic parameters of the enzyme, such as K_m and V_{max}, were calculated by the Michaelis-Menten model and Lineweaver-Burk equation (1).

$$1/V = K_m/V_{max} \times 1/[S] + 1/V_{max} \quad (1)$$

RESULTS

Production and cloning of recombinant pro-KLK6 in expression vector pPICZα A

The PCR products electrophoresed on an agarose gel showed two bands of 673 bp and 700 bp, respectively (Fig. 3A). The second step PCR product was extracted from agarose gel (Fig. 3B). Then, the cleanup product from the second round of PCR and the cloning vector was digested by *Eco*RI and *Xba*I (Fig. 3C).

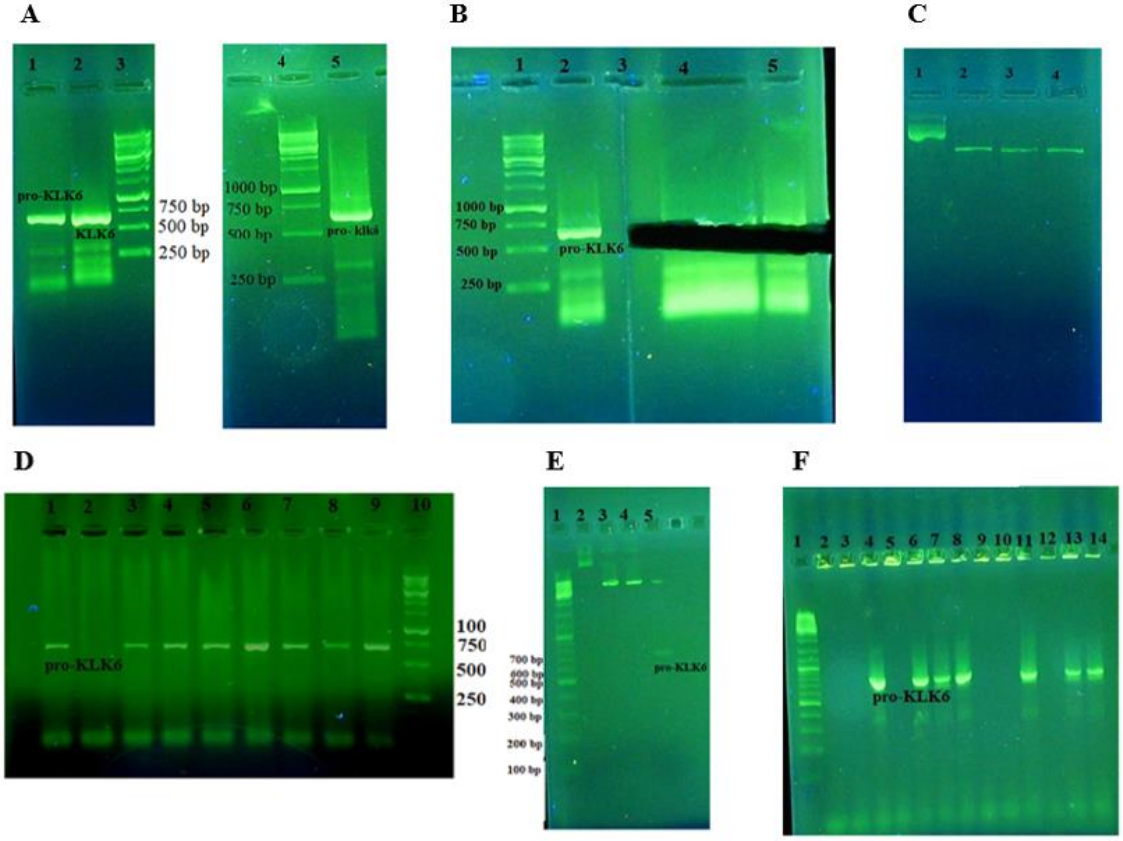

Fig. 3. Production and cloning of recombinant pro-KLK6 in expression vector pPICZα A. (A) Agarose gel analysis of the PCR products of the KLK6 gene. Lane 1: 700 bp band as a result of the second stage of PCR of KLK6 gene, lane 2: 673 bp band as a result of the first stage of PCR of KLK6 gene, lane 3: 1-kb DNA ladder (Smobio, Taiwan), lane 4: 1-kb DNA ladder, lane 5: 700 bp band as a result of the second stage of PCR of KLK6 gene; (B) PCR product clean up from agarose gel; lane 1: 1-kb DNA ladder (Smobio, Taiwan), lane 2: the second stage of PCR product, lanes 4 and 5: cut and separated parts of the agarose gel containing the PCR product; (C) double enzymatic digestion of expression vector pPICZα A without gene; lane 1: expression vector pPICZα A, lane2: enzymatic digestion of expression vector pPICZα A with *Eco*RI, lane 3: enzymatic digestion of expression vector pPICZα A with *Xba*I, lane 4: double enzymatic digestion of expression vector pPICZα A with *Xba*I and *Eco*RI; (D) colony PCR results of different colonies of TOP10 transformed with pPICZα A / pro-KLK6; (E) analysis of the pPICZα A / pro-KLK6 with restriction enzymes; lane 1: ladder, lane 2: expression vector pPICZα A / pro-KLK6, lane 3: enzymatic digestion of expression vector pPICZα A / pro-KLK6 with *Eco*RI, lane 4: enzymatic digestion of expression vector pPICZα A / pro-KLK6 with *Xba*I, lane 5: double enzymatic digestion of expression vector pPICZα A / pro-KLK6 with *Eco*RI and *Xba*I; (F) colony PCR results of different colonies of *Pichia pastoris* GS115 transformed with pPICZα A / pro-KLK6 with a specific primer for KLK6. KLK, Kallikrein; PCR, polymerase chain reaction.

Pro-KLK6 DNA was cloned into the pPICZα A vector in the α-MF frame. It was transferred to *E. coli* TOP10 by the heat shock method and verified by colony PCR and enzymatic digestion. Colony PCR results confirmed that the TOP10 strain contains the pro-KLK6 gene (Fig. 3D). Furthermore, enzymatic digestion and electrophoresis confirmed the presence of a band at 700 bp (Fig. 3E). Also, the sequencing results confirmed the presence of the desired fragment. The pPICZα A / pro-KLK6 was transferred to *P. pastoris* by electroporation method and confirmed by colony PCR (Fig. 3F).

Evaluation of pro-KLK6 expression by SDS-PAGE and Western blotting

Initial clones containing pPICZα A / pro-KLK6 were cultured in 20 mL of YPD medium to evaluate the expression of the pro-KLK6 enzyme. The SDS-PAGE results of the samples collected after 48 h of induction indicated a very weak band in the 34-kDa region. Due to the presence of c-Myc and His-tag in the C-terminal of pro-KLK6, the observed 34-kDa band confirmed the expression of pro-KLK6. Next, pro-KLK6 was expressed in liter. To concentrate the sample and facilitate its purification, the isolated supernatant was freeze-dried, and then, dissolved in 50 mL of base buffer (300 mM Na2HPO4 and 50 mM NaCl). Finally, the pro-KLK6 enzyme was purified by Ni-NTA chromatography (Fig. 4A) and confirmed by Western blotting using a specific antibody against His-tag (Fig. 4B).

Investigation of pro-KLK6 enzyme activity after purification and activation by enterokinase

Using the Bradford method, 11 mg/L of protein was obtained. The enzyme's activity was evaluated and confirmed with the BAEE substrate at 254 nm. Following purification and incubation at 37 °C for 24 h, the pro-enzyme did not show any activity. In the next step, enterokinase converted the pro-KLK6 to its active form (Fig. 5A and B). Then, KLK6 enzyme activity was investigated in the presence of BAEE substrate at10, 50, and 100 µM and the constant concentration of the enzyme (12 nM). The results are reported in Fig. 6.

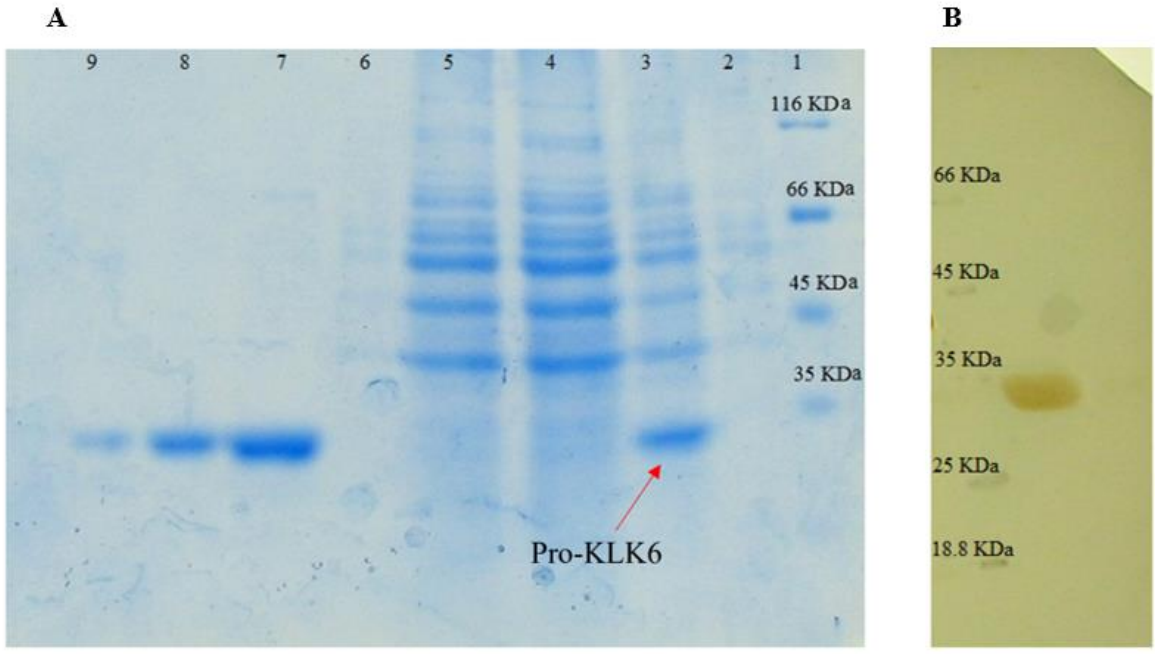

Fig. 4. (A) SDS-PAGE gel and western blot analysis. Lane 1: molecular weight marker (Cytomatingen, Iran), lane 2: supernatant GS115 / pPICZα A / KLK6 before induction with methanol; lane 3: the expressed of KLK6, 48 h after induction with 1% methanol, lanes 4-6: the fractions after washing with different concentrations of imidazole buffer (20-60 mM), lanes 7-9: the KLK6 eluted by 500 mM imidazole buffer; (B) the western blotting technique was used to confirm pro-KLK6 expression. KLK, Kallikrein.

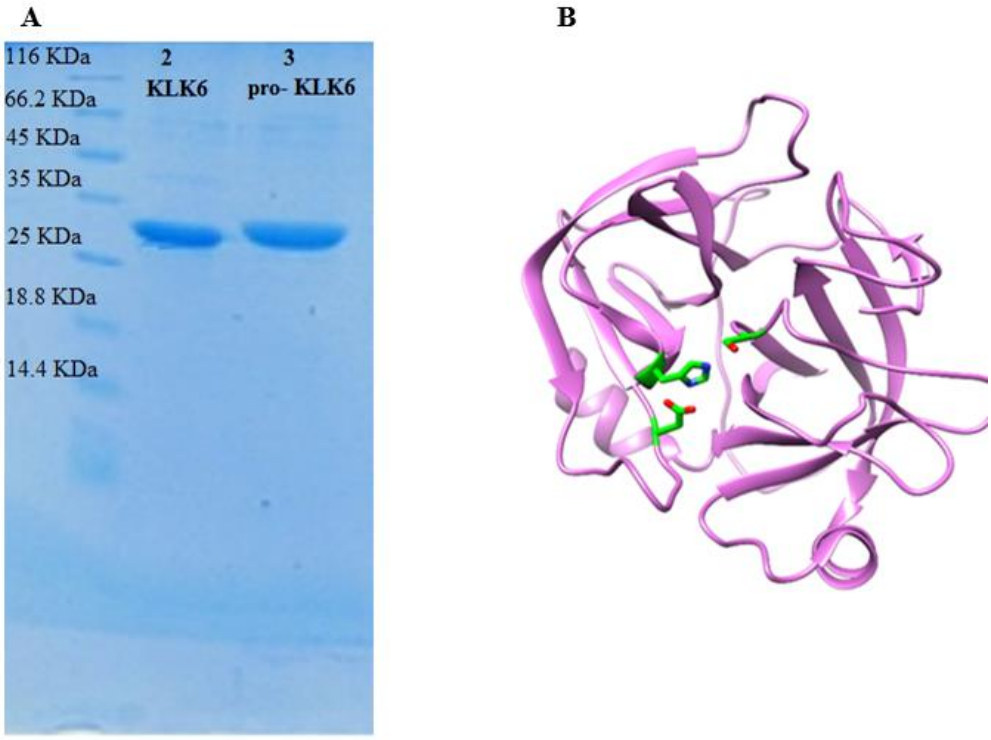

Fig. 5. SDS-PAGE gel analysis after activation of pro-KLK6 and its conversion to its active form (KLK6) by enterokinase. (A) SDS-PAGE gel analysis; lane 1: molecular weight marker (Cytomatingen, Iran), lane 2: KLK6 after activation with enterokinase, lane 3: pro- KLK6 (inactive); (B) a schematic structure of active KLK6 (pdb:1L2E). UCSF Chimera 1.11 was used for the visualization. KLK, Kallikrein.

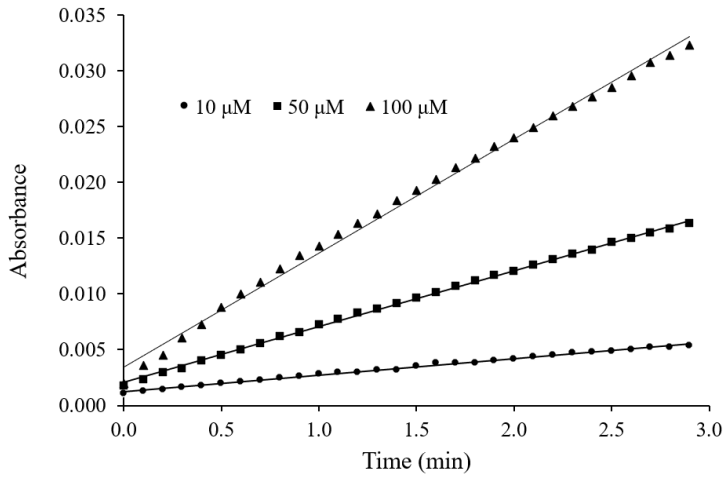

Fig. 6. KLK6 activity assay. The activity of KLK6 enzyme after activation by enterokinase, in the presence of different concentrations of N-benzoyl-L-arginine ethyl ester substrate (10-100 µM) and constant concentration of the enzyme (12 nM) was investigated. KLK, Kallikrein.

Determination of KLK6 kinetic properties based on the Michaelis-Menten model

The kinetic properties were also determined based on the Michaelis-Menten model. Using the Lineweaver-Burk plot, Michaelis constants (K_m) and maximal velocity (V_{max}) were calculated. To calculate the K_m and V_{max} parameters, the enzyme concentration was kept unchanged at 12 nM and the BAEE concentration ranged from 10-1000 µM (Fig. 7A). The resulting values for K_m and V_{max} were 113.59 µM and 0.432 µM/s, respectively (Fig. 7B).

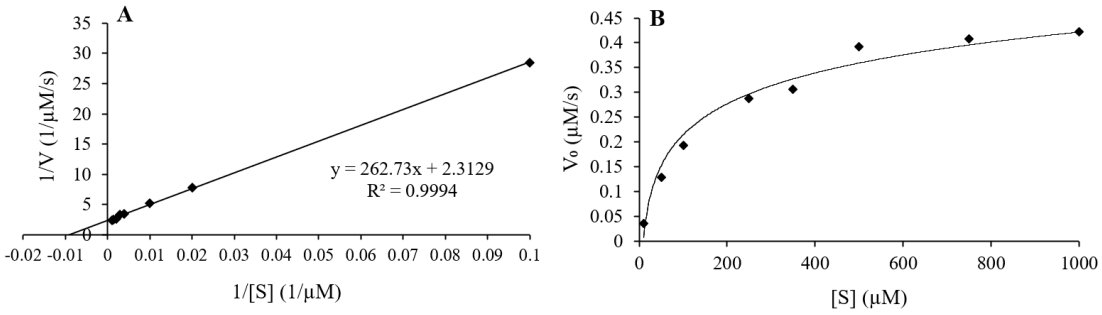

Fig. 7. Investigation of kinetic parameters. (A) Lineweaver-Burk plot of KLK6 kinetics in 67 mM Na_2HPO_4 buffer at pH 7.6, and 12 nM enzyme concentration, in the presence of different concentrations of BAEE. A linear regression analysis was performed using the reciprocals of experimentally determined reaction velocity (1/v) versus substrate concentration (1/[S]). A slope of "a" is equal to K_m divided by V_{max}, while the y-intercept "b" represents the reciprocal of V_{max}; (B) Michaelis-Menten plot of KLK6 kinetics in the presence of different concentrations of BAEE. KLK, Kallikrein; BAEE, N-benzoyl-L-arginine ethyl ester.

DISCUSSION

The pro-KLK6 enzyme is activated spontaneously during purification by hydrolyzing the peptide bond after Lys21 and then, it is inactivated by cleaving the Arg80-Glu81 peptide bond (16). This intrinsic self-cleavage property of KLK6, which leads to enzyme instability, makes it difficult to study the structure and substrate properties of the enzyme. Different solutions have been used to overcome this problem in previous studies (16,19,20). Here, a specific cleavage site was designed for enterokinase at the N-terminal of KLK6 to prevent spontaneous activation of the enzyme. As a result, the enzyme would only be activated in the presence of enterokinase. Since KLK6 contains multiple disulfide bonds and a glycosylation site (21), it needs to be expressed in a eukaryotic host. *P. pastoris* is an expression host known to produce a variety of proteins and enzymes (22). On the other hand, *P. pastoris* GS115 was used as the host cell. The GS115 strain was the first strain used for the expression of recombinant proteins (23). It includes both genes encoding alcohol oxidase-1 and alcohol oxidase-2, so it can use a lot of methanol as a carbon source. It can tolerate higher concentrations of methanol and it is not toxic to them (24). Other strains, such as the KM71, lack the alcohol oxidase 1 promoter. Methanol consumption is lower in this strain and they are sensitive to methanol concentration (25,26). Additionally, this strain produces protein after 150 to 200 h of methanol consumption (27-29), compared to about 50 h for GS115. In this study, we were able to produce 11 mg/L of the enzyme. Bayen *et al.* expressed pro-KLK6 enzyme at KM71 and purified 15 mg/L (16). Their results showed that the enzyme is activated spontaneously after expression in the host and the mature enzyme is unstable (16). The enzyme produced in GS115 in the present study showed no activity until being activated by enterokinase and it was stable. This may be due to the shorter expression time in GS115. Several studies have also used the baculovirus/insect cell line system (9,19,30) and the human embryonic kidney 293 cells as a host (20), which is more expensive than the yeast expression system. KLK6 is a trypsin-like serine protease that accepts the amino acids arginine and lysine at its active site. Several studies have reported that KLK6 prefers arginine over lysine, with a several-fold higher affinity in its P_1-position (20,31,32). Using the BAEE substrate, which contains an arginine at P1, the K_m and V_{max} kinetic parameters were measured for the KLK6 enzyme. Michaelis-Menten is a classic model of enzyme kinetics. According to Michaelis-Menten kinetics, the rate of an enzyme-catalyzed reaction depends on the concentrations of the enzyme and substrate (33). An alternative representation of Michaelis-Menten kinetics is the Lineweaver-Burk plot, which displays the inverse of the reaction rate (1/V) against the inverse of the substrate concentration (1/[S]) (34). In this study, K_m and V_{max} were calculated as 113.59 μM and 0.432 μM/s for the enzyme using Michaelis-Menten and Lineweaver-Burk equations, respectively. Kinetic parameters of the KLK6 enzyme have been measured using different substrates. Several researchers have

reported the K_m of KLK6 enzyme for Tosyl-Gly-Pro-Arg-AMC substrate as 1562 μM (35,36). Another study reported a K_m value of 777 μM for the substrate Tosyl-Gly-Pro-Lys-AMC (35,36). Bernett *et al.* obtained K_m equal to 0.41 mM for Phe-Ser-Arg-AMC substrate hydrolysis by KLK6 enzyme and also reported 0.335 mM value for Asp-Pro-Arg-AMC substrate (35,36). K_m shows the affinity of the enzyme for the substrate. The lower this value is, the greater the affinity of the enzyme for the substrate. The results of the previous studies and the present study indicated the affinity of KLK6 enzyme to BAEE substrate.

CONCLUSION

The increased expression of KLK6 plays a role in developing various diseases, including multiple cancers, neurological disorders, and inflammatory diseases. Instability and spontaneous activation and deactivation are major challenges in the study of this enzyme. In this research, with the help of the targeted design of a recombinant enzyme by adding a specific cleavage site to enterokinase to prevent autoactivation of the enzyme and selection of *P. pastoris* (GS115) as a suitable host cell, a stable pro-enzyme was successfully produced.

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Conflict of interest statement

The authors declared no conflicts of interest in this study.

Authors' contribution

M. Rabbani, N. Mogharrab, and H. Bakherad designed the study. M. Rabbani and N. Mogharrab supervised the study and gave the final approval of the manuscript to be submitted for publication. F. Mahmoodi and H. Bakherad performed the experiments, collected the data, and drafted the manuscript. The final version of the manuscript was read and approved by all authors.

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