Homologous Expression and Enzymatic Properties of Kex2 Protease from *Pichia pastoris* (Postprint)

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Date: 2018-10-15T00:00:00+00:00

Abstract

Kex2 protease is a proprotein-processing protease derived from yeast. This study employed Pichia pastoris for the homologous expression of Kex2 protease from Pichia pastoris (PPKex2) to investigate its expression characteristics and enzymatic properties, and compared it with Saccharomyces cerevisiae Kex2 protease (SCKex2) expressed in Pichia pastoris. First, the Kex2 genes were obtained from the genomes of Pichia pastoris and Saccharomyces cerevisiae, respectively, inserted into the expression vector pPIC9K, and transformed into Pichia pastoris strain GS115. Following methanol induction of the recombinant strains, the results demonstrated that the specific activity of PPKex2 in the fermentation supernatant was 7-fold that of SCKex2. The Kex2 protease was purified using a Q-FF strong anion exchange column for enzymatic characterization. Enzymatic characterization revealed that the optimal reaction pH for PPKex2 was 8.0–9.0, and the optimal reaction temperature was 37 °C, which are similar to those of SCKex2. Regarding stability, PPKex2 was most stable at pH 7.0, exhibited higher stability than SCKex2 under alkaline conditions, lower stability than SCKex2 under acidic conditions, and its thermal stability was slightly lower than that of SCKex2. Enzyme kinetic studies showed that the kcat and kcat/Km values of PPKex2 were 4.8 and 3.3 times those of SCKex2, respectively. This study reports for the first time the expression characteristics and enzymatic properties of homologously expressed Pichia pastoris Kex2 protease, establishing a foundation for its future research and applications.

Full Text

Homologous Expression and Enzymatic Characterization of Kex2 Protease from *Pichia pastoris*

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Abstract

Kex2 protease is a precursor-processing protease derived from yeast. In this study, Kex2 protease from *Pichia pastoris* (PPKex2) was homologously expressed in *P. pastoris* to investigate its expression characteristics and enzymatic properties, which were further compared with those of Kex2 protease from *Saccharomyces cerevisiae* (SCKex2) expressed in the same host system.

First, Kex2 genes were cloned from the genomes of *P. pastoris* and *S. cerevisiae*, inserted into the expression vector pPIC9K, and transformed into *P. pastoris* strain GS115. Following methanol induction, the specific activity of PPKex2 in the fermentation supernatant was seven times higher than that of SCKex2. The recombinant proteases were purified using Q-FF strong anion exchange chromatography for subsequent enzymatic characterization.

The enzymatic characterization revealed that PPKex2 exhibited an optimal pH of 8.0–9.0 and optimal temperature of 37 °C, similar to SCKex2. In terms of stability, PPKex2 was most stable at pH 7.0, showing higher stability than SCKex2 under alkaline conditions but lower stability under acidic conditions. Additionally, the thermostability of PPKex2 was slightly lower than that of SCKex2. Kinetic analysis demonstrated that the kcat and kcat/Km values of PPKex2 were 4.8-fold and 3.3-fold higher than those of SCKex2, respectively. This study reports for the first time the expression characteristics and enzymatic properties of homologously expressed PPKex2, establishing a foundation for its future research and application.

Keywords: Kex2, *Pichia pastoris*, homologous expression, enzymatic characterization

Introduction

Kex2 protease (EC 3.4.21.61) is a precursor-processing protease derived from yeast, belonging to the subtilisin family of calcium-dependent neutral serine proteases. It specifically cleaves peptide bonds at the carboxyl terminus of dibasic amino acid residues such as Lys-Arg, Arg-Arg, and Pro-Arg [1-3]. In yeast, Kex2 protease plays a critical role in the secretory pathway by cleaving signal peptides from precursor proteins to release mature secreted proteins [4, 5].

Since the initial discovery of Kex2 protease from *Saccharomyces cerevisiae* (SCKex2) by Thorner' s laboratory in 1984 [1], extensive research has been conducted on its enzymatic properties and industrial applications [6, 7]. Studies have shown that SCKex2 can process precursor proteins not only in *S. cerevisiae* but also when heterologously expressed in *P. pastoris* and mammalian cells, where it recognizes and cleaves target sites to produce active proteins [8, 9]. Additionally, the structural properties and catalytic mechanism of SCKex2 have been reported [10-13]. Currently, SCKex2 expressed in *P. pastoris* is commercially available as a tool enzyme from companies such as PeproTech and Shanghai Yaxin. While SCKex2 has been thoroughly studied and applied, Kex2 proteases from other yeast species remain less characterized. For instance, Bader et al. reported Kex2 protease from *Candida glabrata* [14, 15] and subsequently compared the substrate specificity of four yeast-derived Kex2 proteases, including *P. pastoris* Kex2 (PPKex2), though detailed expression and enzymatic properties of PPKex2 were not elaborated. Sreenivas et al. [4] enhanced the processing efficiency of recombinant insulin glargine precursor by overexpressing PPKex2 in *P. pastoris*.

In this study, we cloned the Kex2 protease gene from the *P. pastoris* genome, achieved homologous expression of PPKex2 using the *P. pastoris* expression system, and purified it to electrophoretic homogeneity to characterize its enzymatic properties. Simultaneously, we expressed SCKex2 in *P. pastoris* and compared the expression levels and enzymatic properties of both proteases, providing a foundation for further research and application of PPKex2.

Materials and Methods

1.1 Plasmids and Strains

Pichia pastoris GS115 strain, *Saccharomyces cerevisiae* S288c strain, *Escherichia coli* JM109 strain, and the pPIC9K expression vector were maintained in our laboratory.

1.2 Reagents and Media

Fast-digest restriction enzymes SnaB I, Avr II, Sal I, Not I, and T4 DNA Ligase were purchased from Takara. G418 was obtained from Shanghai Sangon Biotech. The protease substrate Boc-Gln-Arg-Arg-pNA was purchased from Hefei Bomei Biotechnology. Media compositions were as follows: LB medium (0.5% w/v yeast extract, 1% w/v peptone, 1% w/v NaCl), YPD medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose), fermentation medium BMGY (1% w/v yeast extract, 2% w/v peptone, 1.34% w/v YNB, 100 mM potassium phosphate buffer pH 6.0, 4×10 % w/v biotin, 1% v/v glycerol), and BMMY (1% w/v yeast extract, 2% w/v peptone, 1.34% w/v YNB, 100 mM potassium phosphate buffer pH 6.0, 4×10 % w/v biotin, 1% v/v methanol).

1.3 Construction of Expression Strains

Genomic DNA from *P. pastoris* and *S. cerevisiae* was extracted using a bead-beating method. Primers were designed based on sequences obtained from NCBI to amplify the Kex2 genes, which were then ligated into pMD19T vector and sequenced. The pMD19T-PPKex2 and pPIC9K plasmids were double-digested with SnaB I and Avr II at 37 °C for 20 min, while pMD19T-SCKex2 and pPIC9K were digested with SnaB I and Not I under the same conditions. After digestion, fragments were ligated at 16 °C for 16 h and transformed into *E. coli* JM109 competent cells. Positive clones were selected and verified by sequencing. Correct plasmids were linearized with Sal I and electroporated into *P. pastoris* GS115 competent cells. Following electroporation, 1 mL of sorbitol was added, and cells were recovered at 30 °C for 1 h before plating on MD plates and incubating at 30 °C for 3 days. Positive clones were selected, inoculated into YPD tubes, and verified by genomic PCR.

1.4 Expression of Kex2 Protease Strains

Positive clones were streaked on YPD+G418 plates and incubated at 30 °C for 3 days. Single colonies were inoculated into BMGY medium and cultured for 18 h until OD reached 2–6. Cells were harvested by centrifugation, resuspended in BMMY medium, and induced with 1% (v/v) methanol. Methanol (1% v/v) was added every 24 h, and cell density and protein concentration were monitored at each time point. After 96 h of induction, the supernatant was collected by centrifugation for subsequent purification.

1.5 Purification of Recombinant Kex2 Protease

The fermentation supernatant was ultrafiltered and buffer-exchanged into 10 mM NaAc-HAc buffer (pH 5.0). The sample was then loaded onto a Q-FF strong anion exchange column (3 cm × 30 cm) pre-equilibrated with the same buffer. Bound proteins were eluted with a continuous gradient of 0–500 mM NaCl. Fractions were collected and analyzed for A and protease activity. The purity of eluted proteins was assessed by SDS-PAGE.

1.6 Kex2 Protease Activity Assay

Protease activity was determined spectrophotometrically using Boc-Gln-Arg-Arg-pNA (Boc-QRR-pNA) as substrate [16]. Kex2 protease cleaves this substrate to release p-nitroaniline. The reaction mixture contained 100 mol/L Boc-QRR-pNA, 50 mM Tris-HCl (pH 8.0), and 2 mM Ca^2 . At 25 °C, 200 L of substrate solution was added to each well of a 96-well microplate. Kex2 protease was diluted to 100 g/mL, and 20 L was added to each well. Absorbance at A was recorded every 20 s for 3 min. One unit (U) of enzyme activity was defined as the amount of enzyme required to catalyze the conversion of 1 mol of Boc-QRR-pNA per minute at 25 °C and pH 8.0. Activity was calculated using the formula: U(mol/L) = ΔA/min × F, where F = TV/(×L×SV) × 10 .

TV represents the total reaction volume (0.220 mL), SV is the sample volume (0.020 mL), L is the light path length (0.220 mL system in microplate), and is the molar extinction coefficient of the detected substance (7840.58 L/(mol · cm)). A blank control containing substrate but no enzyme was used for baseline correction.

1.7 Determination of Enzymatic Properties

1.7.1 Optimal pH and Temperature For optimal pH determination, purified Kex2 protease was assayed at 25 °C using Boc-QRR-pNA prepared in 50 mM NaAc-HAc buffer (pH 3.0–6.0), Tris-HCl buffer (pH 7.0–8.0), or Gly-NaOH buffer (pH 9.0–11.0). Relative activity was calculated with the highest activity set as 100%. Optimal temperature was determined using two methods. Method 1 measured initial reaction rates at different temperatures (10–90 °C) in 50 mM Gly-NaOH buffer (pH 9.0). Method 2 involved incubating the enzyme at various temperatures (25–45 °C) for 3 h in the same buffer, terminating the reaction by boiling for 10 min, and then measuring product formation spectrophotometrically.

1.7.2 pH and Thermal Stability For pH stability, purified enzyme was incubated at 25 °C for 1 h in 20 mM NaAc-HAc (pH 3.0–6.0), Tris-HCl (pH 7.0–8.0), or Gly-NaOH (pH 9.0–10.0) buffers. Residual activity was then measured under optimal pH and temperature conditions, with the highest activity set as 100%. For thermal stability, enzyme solutions were incubated at 25 °C, 35 °C, 45 °C, and 55 °C for 1, 2, 4, 8, and 12 h, followed by residual activity measurement under optimal conditions. Activity was expressed relative to untreated enzyme.

1.7.3 Kinetic Parameters Kinetic parameters were determined using Boc-QRR-pNA at concentrations ranging from 5×10 to 2×10 mol/L under optimal conditions. Data were fitted using GraphPad Prism 7.0 to calculate Km and kcat values.

Results

2.1 Sequence Analysis of PPKex2 and SCKex2

The amino acid sequences of PPKex2 and SCKex2 were aligned using DNAMAN and ESpript 3.0, and the secondary structure of PPKex2 was predicted [Figure 1: see original paper]. The two Kex2 proteases shared 43.43% amino acid sequence identity.

2.2 Construction and Expression of Recombinant Strains

The expression plasmids pPIC9K-PPKex2 and pPIC9K-SCKex2 were constructed [Figure 2: see original paper], in which the native signal peptide of

Kex2 was replaced by the -factor signal peptide from pPIC9K, and the Kex2 gene was placed downstream of the AOX1 promoter for methanol-induced expression. During the methanol induction phase, cell density and protein concentration were monitored every 24 h, and samples were analyzed by SDS-PAGE. As shown in [Figure 3: see original paper], the concentration of Kex2 protease in the fermentation supernatant increased gradually with induction time. After 96 h of methanol induction, the protein concentration in the supernatant reached 0.42 mg/mL for PPKex2 with a specific activity of 70 U/g, while SCKex2 reached 0.85 mg/mL with a specific activity of 10 U/g.

2.3 Purification of Kex2 Proteases

After 96 h of induction, fermentation broth was harvested, centrifuged, ultrafiltered, and subjected to ion exchange chromatography. PPKex2 eluted as a major peak at 250–400 mM NaCl, with the fraction collected at 260–300 mM NaCl. SCKex2 eluted at 350–500 mM NaCl, with the fraction collected at 380–450 mM NaCl. Both proteases were purified to electrophoretic homogeneity, showing single bands on SDS-PAGE [Figure 4: see original paper] with apparent molecular masses of approximately 88 kDa, consistent with theoretical predictions.

2.4 Enzymatic Characterization

2.4.1 Optimal pH and Temperature The optimal pH profiles are shown in Figure 5: see original paper. Both proteases exhibited similar pH-dependent activity patterns, with PPKex2 and SCKex2 showing optimal activity at pH 8.0–9.0 and pH 9.0, respectively. Activity was essentially abolished below pH 5.0, while both maintained relatively high activity between pH 7.0 and 10.0. Notably, SCKex2 showed higher activity than PPKex2 between pH 6.0 and 7.5, whereas PPKex2 was more active between pH 7.5 and 9.0; above pH 9.0, both showed similar activity.

Optimal temperature was determined using two methods. Method 1 measured initial reaction rates at various temperatures [FIGURE:5(b)], revealing a linear increase in reaction rate with temperature up to 90 °C under the assay conditions. Blank controls containing only substrate showed no change in absorbance, confirming substrate stability. However, since Method 1 measures initial rates over only 3 min, and practical applications require 1-3 h processing times during which enzymes may denature at high temperatures, Method 2 was employed to determine the optimal temperature for practical use. In Method 2, reactions were carried out at various temperatures for 3 h before measuring product yield. As shown in [FIGURE:5(c)], the optimal temperature for PPKex2 was 37 °C, while SCKex2 exhibited optimal activity at 40 °C. PPKex2 also displayed a broader temperature range for activity.

2.4.2 pH and Thermal Stability pH stability results are presented in Figure 6: see original paper. Both proteases were essentially inactive after treatment

at pH 3.0 or pH 10.0. PPKex2 was relatively stable between pH 5.0 and 8.0, with maximum stability at pH 7.0. In contrast, SCKex2 was most stable between pH 4.0 and 6.0, with optimal stability at pH 6.0. Below pH 6.0, SCKex2 demonstrated higher stability than PPKex2, whereas above pH 6.0, PPKex2 was more stable.

Thermal stability is shown in Figure 6: see original paper-(c). Both proteases lost activity more rapidly at higher temperatures. PPKex2 retained 88% relative activity after 2 h at 25 °C, but only 45% remained after 12 h. At 55 °C, PPKex2 was completely inactivated within 1 h. SCKex2 was more stable at 25 °C, retaining 75% activity after 12 h, and survived up to 4 h at 55 °C before complete inactivation. Overall, PPKex2 exhibited lower thermostability than SCKex2 under identical conditions.

2.4.3 Kinetic Parameters Kinetic analysis results are summarized in . While the Km value of PPKex2 was higher than that of SCKex2, its kcat and kcat/Km values were 4.8-fold and 3.3-fold higher, respectively.

Discussion

Kex2 protease plays a crucial role in the yeast secretory pathway and has been widely applied in mammalian cell studies. SCKex2 is currently commercially available as a tool enzyme. Although the *P. pastoris* expression system is commonly used for Kex2 production, only Sreenivas [4] and Bader [15] have reported studies on PPKex2, and detailed research on its heterologous expression and enzymatic properties remains scarce.

Through comparative analysis of amino acid sequences and secondary structures, we found that both PPKex2 and SCKex2 comprise a signal peptide, propeptide, catalytic domain, P-domain, Thr/Ser-rich region, and transmembrane domain, but share only 43.43% sequence identity. This low sequence similarity suggested potentially significant differences in enzymatic properties. In this study, we amplified the Kex2 protease gene from the *P. pastoris* genome, achieved homologous expression of PPKex2, and purified it to electrophoretic homogeneity for comprehensive characterization and comparison with the well-studied SCKex2.

At the shake-flask level, the specific activity of PPKex2 in fermentation supernatant was seven times higher than that of SCKex2. After purification, enzymatic characterization revealed that PPKex2 exhibited significantly higher catalytic activity than SCKex2, with a kcat value 4.8 times greater. PPKex2 showed optimal activity at pH 8.0–9.0 and 37 °C, with highest stability at pH 7.0. SCKex2 exhibited optimal activity at pH 9.0 and 40 °C, with highest stability at pH 6.0, consistent with previous reports [12, 13]. Overall, PPKex2 demonstrated superior stability under alkaline conditions but lower stability under acidic conditions compared to SCKex2. Conversely, PPKex2 showed lower thermostability and was more prone to inactivation at elevated temperatures.

These findings demonstrate that PPKex2 possesses high catalytic activity and significant potential for industrial applications. Genetic engineering strategies for achieving high-level homologous expression represent an effective approach to reduce production costs. Established methods for enhancing heterologous protein expression in *P. pastoris* include promoter engineering [17], selection of high-copy strains [18], and fermentation optimization [19]. Future work will focus on improving PPKex2 expression levels to facilitate its commercialization.

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Foundation item: National Natural Science Foundation of China (31671799); Six Talent Peaks Project in Jiangsu Province (NY-010)

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