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Functional recombinant human Legumain protein expression in *Pichia pastoris* to enable screening for Legumain small molecule inhibitors

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ABSTRACT

Legumain (LGMN) is a lysosomal protease that can specifically hydrolyze proteins after carboxyl-terminal asparagine residues. It has been reported that Legumain is highly expressed in many human tumors and promotes the migratory and invasive activity of cancer cells. Due to the limitation of an abundant and affordable source of endogenous active Legumain for further function studies, we produced the recombinant protein in *Pichia pastoris*. The pPICZα-LGMN expression plasmid was constructed and transformed into *Pichia pastoris* strain and positive recombinants were identified. Fermentation conditions were optimized and it was found that Legumain was most highly expressed under pH 6 culture conditions. In addition, the enzyme activity of the purified Legumain was tested using a fluorogenic substrate (Z-Ala-Ala-Asn-AMC) assay and the optimum pH for the autocatalytic activation of recombinant Legumain was very acidic at a pH value of 3. The recombinant protein was then used to screen a library of compounds and small molecule 1773 (Terramycin) was shown to effectively inhibit Legumain enzyme activity. These results indicate that the *Pichia pastoris* expression system can produce highly active recombinant Legumain protein allowing it to be used for High-throughput screening (HTS) applications.

1. Introduction

Legumain, an asparaginyl endopeptidase, is a member of the C13 family of cysteine proteases and has specific activity for hydrolysis of asparagine peptide bonds [1]. It is composed of 433 amino acids, including signal peptide, propeptide and the Legumain catalytic mature domain [2]. Legumain is inactive in its precursor form and is activated by self-cleaving at N^{323} site under acidic conditions.

Legumain is highly conserved amongst biological species and is located in intracellular vesicles, such as lysosomes and endosomes, as well as on the cell surface and extracellular matrix. Legumain is highly expressed in most solid tumors [3,4]. Furthermore, it has been reported that overexpressing Legumain in cancer cells increases their migratory and invasive activity [5–8]. Legumain therefore plays an important role in tumor invasion and metastasis.

As a protease, Legumain can specifically hydrolyze the carboxyl-terminal peptide bonds of asparagine residues. In addition to its protein-degrading function, Legumain can also hydrolyse certain precursor proteins such as pro-enzymes and pro-hormones, or activate other proteolytic enzymes and then participate in a variety of physiological processes [9–11]. Moreover, it has been used as a prognostic indicator in breast cancer [3,12]. These findings were compared with the clinical outcome and other established cancer specific properties to reveal its prognostic significance. A study shows cytoplasmic immunoreactivity of Legumain was over-expressed in gastric cancer compared with paired normal gastric mucosa. Patients with Legumain-positive localized tumors had lower 5-year overall survival (OS) than those with Legumain-negative tumors. Multivariate survival analysis showed that Legumain was an independent prognostic marker for OS [13,14]. Legumain is also associated with amyloid precursor protein (APP) cleavage and tau protein cleavage in Alzheimer's disease (AD). It has also been reported that Legumain has some effects on cognitive function in both the 5 × FAD mouse and P301S mouse [10,11,15]. Due to the limitation of a source of endogenous active Legumain, it was important to explore the production of functional Legumain using a recombinant protein expression system. The eukaryotic expression system, *Pichia pastoris* has more advantages than other eukaryotic expression systems, such as protein processing, protein folding and posttranslational modification.

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In this study, we successfully expressed Legumain in *Pichia pastoris*, and the purified active Legumain was used to screen a compound library for small molecule inhibitors.

2. Materials and methods

2.1. Plasmid construction

The plasmid pPICZα was obtained from Invitrogen. mRNA was extracted from the breast cancer cell line MCF-7 and the amplification of cDNA fragments encoding Legumain (1251bp) was conducted by PCR. The following primers was used for PCR: (Legumain-F, 5′- GGAATTC GTTCCTATAGATGATCCTG-3; Legumain-R, 5′-GCTCTAGAAAGTAGTG ACCAAGGCACACG -3′), The PCR products were digested with *EcoRI* and *Xba I* and cloned into pPICZα by utilizing the same restriction enzymes to produce the expression vector pPICZα-Legumain. The plasmid was transformed into *E. coli* TOP10 cells. The plasmid DNA was amplified, purified, linearized with the restriction enzyme *ScaI I*, and integrated into the chromosomal DNA of *Pichia pastoris* X33 strain through electroporation (Bio-Rad, Hercules, USA).

The transformed cells were grown on YPD agar plates containing 100 μg/ml, 300 μg/ml, 600 μg/ml, 1000 μg/ml Zeocin respectively at 30 °C for 3–5 days incubation. Two transformants grew on YPD plates with the highest level of 1000 μg/ml Zeocin. Twenty-seven transformants grew on YPD plates with 600 μg/ml Zeocin. Afterwards, eight transformants were picked out from the YPD plate with 1000 μg/ml and 600 μg/ml Zeocin. The AOX1 locus is the site for the gene of interest integrated into the *Pichia pastoris* genome. The genomic DNA of these transformants were extracted and the positive recombinants were identified by PCR using the upstream/downstream Alcohol Oxidase 1 (AOX1) gene primers (AOX1-F, 5′-GACTGGTTCCAATTGACAAGC-3; AOX1-R, 5′- GCAAATGGCATTCTGACATCC -3′).

2.2. Optimization of protein expression conditions

Positive clones were inoculated into 100 ml BMGY medium(10 g/l Yeast extract, 20 g/l Peptone, 100 ml 1 M Potassium phosphate buffer, 100 ml 10 × YNB, 2 ml 500 × Biotin, 100 ml 10 × glycerin) at 30 °C in a shaking incubator until the OD_{600} reached 4 to 6. The culture broth was chilled after centrifugation at 8000 rpm for 5 min at 30 °C, the supernatant was removed and the cells were then transferred to 100 ml BMMY medium (10 g/l Yeast extract, 20 g/l Peptone, 100 ml 1 M Potassium phosphate buffer, 100 ml 10 × YNB, 2 ml 500 × Biotin, 100 ml 10 × Methanol) at different pHs (3, 4, 5, 6, 7, adjusted by phosphoric acid and ammonia) at 30 °C in a shaking incubator. Methanol to a final concentration of 0.5% was added every 12 h and samples were collected every 24 h up to a 5 day period. Samples were centrifuged at 8000 × g for 15 min at 4 °C to remove the cells, and the supernatant containing expressed proteins were subsequently frozen at −20 °C for further purification.

2.3. Purification of recombinant Legumain by ammonium sulfate precipitation

Different amounts of solid $(NH_4)_2SO_4$ were added to the culture supernatant to a final concentration of 80%–90% saturation and incubated for 30 min, then centrifugated at 8000 rpm for 5 min. The pellets were dissolved in a buffer containing 50 mM Na_3PO4 (pH 7.0), 100 mM NaCl and then dialyzed overnight. Purified recombinant Legumain was then analyzed by SDS-PAGE.

2.4. Protein concentration measurement

BSA protein solution containing 0, 0.5, 1, 2, 4, 6, 8, 10 μg protein was pipetted into a 96-well plate, and the volume was adjusted to 20 μl with a buffer containing 50 mM Na_3PO4 (pH 7.0), 100 mM NaCl. 5, 10, 15 μl of recombinant Legumain was added to the 96-well plate respectively and the volume was adjusted to 20 μl with the same buffer. 200 μl Coomassie Brilliant Blue G-250 was added into the protein solution, the absorbance at 595 nm was measured after 2 min and no more than 10 min after mixing. The quantity of protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein concentration of the samples.

2.5. Legumain activity assay

The activity of recombinant Legumain was measured by cleaving a synthetic fluorescent substrate Z-Ala-Ala-Asn-AMC (R and D). Briefly, 10ul aliquots of Legumain (0.6 mg/ml) were mixed with 50 μl of different activation buffers (containing 50 mM Sodium Acetate and 100 mM NaCl, pH 4) and pre-incubated at 37 °C for 2 h respectively. Samples were then diluted to 1 ng/μl in assay buffer (50 mM MES, 250 mM NaCl, pH 5). 30 μl of 150 μM Z-Ala-Ala-Asn-AMC was added and the enzymatic activity measured using a microplate reader (Biotek) at 380 and 430 nm. Readings were taken every 2 min for 20 cycles respectively. One unit of enzyme activity was defined as the activity required to release 1 μM of AMC per minute at 37 °C.

To determine the optimum pH for autocatalytic activation, 20 μl aliquots of purified recombinant Legumain (0.6 mg/ml) were mixed with 50 μl of Activation Buffer (containing 50 mM Sodium Acetate, 100 mM NaCl with a pH gradient between 2 and 6). Acetic acid and NaOH were used to adjust the pH. The samples were incubated at 37 °C for 2 h, and then analysed by SDS-PAGE.

2.6. Screening of Legumain small molecule inhibitors

To screen for small molecule inhibitors of Legumain, 30 μl of 1 ng/μl activated Legumain and 30 μl of 10 μM compounds (FDA-Approved-Drug-Library, Selleck, USA) were combined and added into a black well plate then incubated for 30 min. Afterwards, 30 μl of 150 μM Substrate Z-Ala-Ala-Asn-AMC (R and D) was added, including a control containing 60 μl Assay Buffer and 30 μl of 150 μM Substrate. Enzymatic activity was measured using a microplate reader (Bio-tek) at 380 and 430 nm respectively, read every 2 min for 20 cycles.

3. Results

3.1. Vector construction and analyzing recombinant Legumain expression

To express recombinant human Legumain in *Pichia pastoris* X33, the full-length *LGMN* cDNA was cloned into expression vector pPICZα. DNA sequencing confirmed the accuracy of the cloning. The expression vector was linearized using the restriction enzyme SacI and transformed into *Pichia pastoris* X33 by electroporation. The transformants were screened on YPD plates with 600 and 1000 μg/ml zeocin. Eight Zeocin-resistant transformants were further analyzed by PCR, and six were positive recombinants, which produced a band of 1839 bp (*LGMN* gene 1251 bp plus the 588 bp *AOXl* gene sequence from the expression vector) (Fig. 1). The weak 2200 bp PCR band presented the amplification of the *AOXI* gene in the Pichia pastoris X33 genome. Three positive recombinants, named 1, 2 and 6, were used for the expression of recombinant Legumain. As shown in Fig. 2, the SDS-PAGE analysis of the culture supernatant of samples demonstrated that the recombinant 6 produced a high level Legumain protein at 120 h fermentation, with a single predominant protein band of approximately 56 kDa. The expected molecular weight of the recombinant Legumain is 50 kDa, though it is slightly smaller than the size of expressed protein, this might be duo to the protein modification in the yeast cells. It has been reported that the molecular weight of the mammalian cell expressed precursor form of Legumain is 56 kDa [1].