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Recombinant expression, refolding, purification and characterization of *Pseudomonas aeruginosa* protease IV in *Escherichia coli*

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ABSTRACT

Several protease IV enzymes are widely used in proteomic research. Specifically, protease IV from *Pseudomonas aeruginosa* has lysyl endopeptidase activity. Here, we report the recombinant expression, refolding, activation, and purification of this protease in *Escherichia coli*. Proteolytic instability of the activated intermediate, a major obstacle for efficient production, is controlled through ammonium sulfate precipitation. The purified protease IV exhibits superior lysyl endopeptidase activity compared to a commercial product.

1. Introduction

Protease IV, also named lysyl endopeptidase and Lys-C (EC3.4.21.50), has been found in several microorganisms, such as *Achromobacter lyticus* (Achromobacter protease I, API) [1] *Lysobacter enzymogenes* (lysyl endopeptidase, Lys-C) [2,3] and *Pseudomonas aeruginosa* (prpL) [4]. These proteases robustly and specifically recognize and cleave C-terminal to the basic amino acid lysine, even following with proline or glutamic acid, making them useful enzymes for various applications, including proteomic sample preparation and human insulin production [2,5,6]. Current proteomic grade protease IV products of high purity and activity supplied by vendors such as Wako, and others, are extracted from the native bacteria *L. enzymogenes* using an inefficient process [2,7].

P. aeruginosa is a major pathogen associated with burns, cancer, cystic fibrosis and immunodeficiency diseases. Protease IV of *P. aeruginosa* was previously purified from the PA103-29 strain [8,9]. The activity of this protease can be inhibited by 1 mM TLCK, 1 mM DTT, or 150 mM 2-mercaptoethanol [9]. These enzymes were expressed as a “pre-proenzyme” and were cleaved into the active form *in vivo*. The prpL gene encodes a signal sequence of 24 amino acids, a pro-sequence of 187 amino acids and a mature protease IV of 251 amino acids [10]. Gene mutation studies have shown that the active site residues, His-72, Asp-122 and Ser-198, could form a catalytic triad; however, there is no detailed structural information [11]. This gene has been cloned and expressed in *Pseudomonas*

Abbreviations: TRX, thioredoxin; LB medium, Luria-Bertani medium; ORF, open reading frame; DO, dissolved oxygen; FCM, fermentation complex medium; IPTG, isopropyl thio-β-D-galactoside; IB, inclusion bodies; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LC-MS, Liquid chromatography-mass spectrometry; HPLC, High performance liquid chromatography; TCL, total cell lysate; FASP, filter-aided sample preparation; UPLC, ultra performance liquid chromatography; PSMs, peptide spectrum matches; FDR, false discovery rate; MW, molecular weight; TLCK, *N*-tosyl-L-lysyl chloromethyl-ketone; CD, circular dichroism.

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putida successfully, but the expression level is very low [12].

In this paper, we designed a prokaryotic expression vector containing the pro-protease IV of *P. aeruginosa* with a thioredoxin (TRX) tag (TRX-pro-protease IV). After high-density fermentation of the *Escherichia coli* strain containing the expression vector, protease IV of high purity was generated using a newly developed process including refolding, activation and purification. The expression level of TRX-pro-protease IV was 1.7 g/L and the final yield of mature protease IV was 34.4 mg/L (based on the fermentation volume). This recombinant protease IV showed superior enzymatic activity and comparable specificity to current commercial products extracted from the native source, making it a potential replacement for proteomic research.

2. Materials and methods

All experiments were performed at room temperature (RT, 20 °C) unless otherwise noted.

2.1. Bacterial strains, plasmids and genetic manipulation

The *E. coli* BL21 (DE3) strain and the pET-32a vector were purchased from Novagen (Germany). The strain was maintained on LB medium (1.0% tryptone, 0.5% yeast extract and 1.0% NaCl, pH 7.5) containing 100 μg/mL ampicillin (Amp) at 37 °C. The open reading frame (ORF) of the pro-protease IV gene (GenBank accession: AY062882.1) was codon-optimized according to *E. coli* biases. This pro-protease IV gene was synthesized and inserted into the *EcoR* I/*Xho* I -digested pET-32a plasmid to yield a pET-32a-TRX-pro-protease IV vector. The vector was transformed into the *E. coli* BL21 (DE3) cells.

2.2. TRX-pro-protease IV expression in a bioreactor

The *E. coli* BL21 (DE3) strain containing the pET-32a-TRX-pro-protease IV vector was grown on a solid LB plate containing 100 μg/mL ampicillin (Amp) at 37 °C. A single colony was inoculated into a 250-mL flask containing 50 mL of liquid LB/Amp medium and cultured in a shaker at 37 °C to an optical density of 2.0 (OD_{600}) and was saved as the seed culture.

The seed culture was inoculated into a 5-L fermenter (Applikon Biotechnology, the Netherlands) with a working volume of 2.5 L of fermentation complex medium (FCM) [13]. The starting inoculation OD_{600} was approximately 0.01, and the air-flow rate was 6.0 L/min. The temperature was set to 37 °C, and the pH value was maintained at 7.0 by acid or base addition through automatic feedback controls. Dissolved oxygen (DO) was maintained during the batch period at 30% air saturation. When the DO was lower than 15%, the stirring speed was increased in a cascade mode depending on the DO. The maximum stir speed was 1200 rpm. The feed medium (275.0 mL/L glycerol, 225.0 g/L yeast extract) was added at 18.0 mL/h into the fermenter when the OD_{600} reached 25.0. Ten hours after inoculation (OD_{600} had reached 31.0), the temperature was decreased to 32 °C, and protein expression was induced by addition of 1 mM isopropyl thio-β-D-galactoside (IPTG). After 10 h of induction (OD_{600} had reached 100), the cells were recovered by centrifugation using a J6-MI centrifuge (Beckman, USA) at 4500*g* for 30 min at 4 °C. The cell density measurement was performed using an Ultraspec 10 at 600 nm (GE Healthcare, USA).

2.3. Cell disruption and inclusion body recovery

The *E. coli* cell pellet containing overexpressed TRX-pro-protease IV was re-suspended in PBS and lysed by high-pressure homogenization on an APV 1000 homogenizer (SPX, USA) two times at 800 bar. The inclusion bodies (IB) were recovered by centrifugation at 4500*g* for 30 min at 4 °C using a J6-MI centrifuge (Beckman, USA). The pellet was washed with PBS containing 2 M urea for 1 h with stirring. The suspension was then centrifuged again to obtain the TRX-pro-protease IV IB.

2.4. TRX-pro-protease IV refolding, activation and purification

The TRX-pro-protease IV IB was solubilized in denaturing solution (20 mM Tris, 8 M urea, 20 mM DTT, pH 8.5) at a ratio of 1:40 (gram wet weight of IB:mL volume) and the suspension was shaken gently for 4 h at RT. After centrifugation at 27,000*g* for 30 min using a J-26XP centrifuge (Beckman, USA), the clarified and denatured IB supernatant was recovered. The supernatant was diluted directly into refolding buffer (20 mM Tris, 1 mM cystine, 3 mM cysteine, 2 M urea, pH 10.0) at a volume ratio of 1:20. After refolding at RT overnight, the sample pH was adjusted to 7.5 by addition of 1 M HCl and incubated at room temperature for 6 h with stirring. Solid ammonium sulfate was added to 60.0% saturation [14,15]. The suspension was stirred for 1 h at 4 °C followed by incubation without stirring for 4 h on ice. Precipitates containing mature protease IV were recovered by centrifugation at 4500*g* for 40 min using a J6-MI centrifuge. After removal of the supernatant, the pellet was solubilized with resolubilization buffer (20 mM Tris, 150 mM NaCl, pH 7.5) and clarified by centrifugation at 27,000*g* for 30 min at 4 °C using a J-26XP centrifuge.

Solubilized protein samples were loaded onto a pre-equilibrated HiTrap Chelating HP (5 mL, GE Healthcare, USA) at a flow rate of 10 mL/min in buffer A (20 mM Tris, 2 M NaCl, pH 7.5). The column was then washed with five column volumes (CV) of buffer A followed by a linear gradient to 2% buffer B (20 mM Tris, 2 M NaCl, 0.5 M imidazole, pH 7.5) in 5 CV. The active fraction was eluted with a linear gradient of 2–100% buffer B in 10 CV. Based on SDS-PAGE, the fractions containing mature protease IV were pooled together.

The pooled sample was concentrated in an Amicon Ultra-15 mL concentrator (10 kDa, Merck-Millipore, German) at 4000*g* for 30 min at 4 °C using a J-26XP centrifuge. The buffer exchange was repeated three times with buffer C (20 mM Tris, 8 M urea, pH 8.5). Concentrated sample was loaded onto a Sephacryl S-100 column (XK 16 × 40 with a 33 cm column height, GE Healthcare, USA). The column was pre-equilibrated with buffer D (50 mM acetic acid, 6.7 mg/mL trehalose). The flow rate was 2 mL/min. Fractions containing purified protease IV were also pooled based on SDS-PAGE.

2.5. Evaluation of TRX-pro-protease IV and protease IV by SDS-PAGE and LC-MS

For SDS-PAGE sample preparation, aliquots of solubilized IB, refolded, and activated samples were mixed with 5 μL of 5× reduced loading buffer (20% glycerol, 10% SDS, 50 mM Tris, 0.1% bromophenol blue, 5% β-mercaptoethanol, pH 6.8) and heated immediately at 100 °C for 10 min. Samples were analyzed on 15% SDS-PAGE according to standard methods [16]. Briefly, electrophoresis was performed on a Mini-Protean Electrophoresis Cell system (Bio-Rad, USA) and stained with Coomassie Brilliant Blue G-250. The purity and concentrations of the samples were calculated by ScanImage (4.0.3.2) software (National Institutes of Health, USA).

The molecular weights (MWs) of refolded TRX-pro-protease IV and purified mature protease IV were measured using LC-MS (Agilent, ESI-TOF 6210, USA). The HPLC is connected with a C_{18} column (Kinetex 2.6 μm C_{18}, 50 × 4.60 mm, Phenomenex). The m/z detection range for ESI-TOF is 1000–10,000.