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Purification, biochemical characterization and structural modeling of a potential htrA-like serine protease from *Bacillus subtilis* DR8806

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ABSTRACT

The production of an extracellular htrA-like serine protease by *Bacillus subtilis* DR8806 was studied in this study. The enzyme was purified using ammonium sulphate precipitation and Sephacryl S-200 size exclusion chromatography. The analysis by SDS-PAGE and zymogram of enzyme showed a molecular weight of 37 kDa. Isoelectric focusing revealed a p*I* value of 6.6 for the enzyme. By the use of casein as substrate, the enzyme was active and stable at the wide range of temperatures with maximum activity at 45 °C and pH 8. The enzyme activity was increased by Ca^{2+}, K^{+}, Mg^{2+}, Fe^{2+}, dimethylsulfoxide (DMSO), whereas its activity was decreased by Hg^{2+}, Ba^{2+}, Cu^{2+}, Zn^{2+}, H_2O_2, CTAB (cetyltrimethylammonium bromide) and SDS (sodium dodecyl sulphate). In addition, Mn^{2+}, Na^{+}, Triton X-100, β-mercaptoethanol, EDTA (ethylenediaminetetraacetic acid) had no significant effect on the enzyme activity. Among organic solvents, ethanol and methanol enhanced the activity. The gene of the protease showed a 1200 bp open reading frame with 97% similarity to other htrA-like proteases. The computational modeling of the protease showed two distinct domains: a PDZ domain and protease core domain. The catalytic triad also demonstrated a degree of discrepancy in comparison to other serine proteases. It is composed of a serine residue as a nucleophile and a proline as a base center, while the acidic center was not fully identified. The obtained results suggested a new type of htrA-like protease with no previous records in bacillaceae family.

1. Introduction

Nearly two third of commercial proteases are produced by fungal species, yeasts and bacteria [1]. Among them, proteases are one of the most important enzymes, which contribute about 60% of the total world enzyme market [2]. They have numerous applications in different industries such as food processing, pharmaceuticals, detergents, leather, brewing, photographic, textile, silk, bakery, dairy, bioremediation, biosynthesis, and biotransformation. The main application of proteases is usage in laundry and detergents [3,4].

Bacillus genus, especially *Bacillus subtilis* is a well-known source for protease production due to the high growth rate, ability to secrete proteins into medium and safety as well [5]. It has more than six types of extracellular serine proteases, which have different properties. Due to growing demands for these enzymes, researchers are interested in finding new source of bacteria to produce proteases with appropriate properties such as activity and stability in a wide range of pH and temperature and also withstanding under harsh conditions [6]. HtrA-like proteases are a family of enzymes acting as trypsin-like serine proteases [7–10]. The structure of this type of proteases is slightly unusual in compare with the other industrial and well-characterized proteases. Their structures usually contained at least one PDZ domain, which had a role in binding to a carboxyl terminus of other proteins. The catalytic triad in this family of proteases was comprised of His-Asp and a Ser residue. However, in htrA-like proteases, only Ser residue is definitely conserved [9,11].

B. subtilis DR8806 has been isolated from Dig Rostam hot mineral spring in Iran, and some thermostable enzymes purified from this strain such as glucoamylo-pullulanase and lipase [12,13]. In current study, we have characterized a new type of protease in the medium of this strain. This enzyme was active in a wide range of temperature and pH. It was stable in the presence of many organic solvents and active toward many substrates. We also identified the potential gene of the enzyme as well as its protein sequence. Based on this sequence, the computational model of the enzyme was resolved by using Accelrys Discovery Studio 2.5.5 (Dassault Systemes, France) and Schrodinger suite 2013-1 (Schrödinger LLC, USA).

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2. Materials and methods

Chemicals used in the cultivation of microorganism were purchased from Merck (Darmstadt, Germany). Sephacryl S-200 was supplied by Pharmacia (GE Healthcare Life Sciences, Uppsala, Sweden). All other chemical were of analytical grade.

2.1. Optimizing culture conditions

The enzyme was produced by of *B. subtilis* DR8806 using a basic medium composed of sucrose (1%, w/v), yeast extracts (1%, w/v), peptone (0.5%, w/v), NaCl (0.5%, w//v), $MgSO_4{\cdot}7H_2O$ (0.2%, w/v) and KH_2PO_4 (0.1, w/v). The inoculated medium was incubated at 37 °C in a shaker at 120 rpm for 32 h. To optimize culture conditions and maximum enzyme production, the medium was also supplemented with 1% (w/v) of different carbon (glucose, sucrose, maltose, fructose, starch, and lactose) and nitrogen sources (peptone, ammonium acetate, ammonium nitrate, ammonium chloride).

2.2. Protease purification

All purification steps were carried out at 4 °C except otherwise stated. After cultivation of *B. subtilis* DR8806 at 37 °C for 32 h, the cells were removed by centrifuging the medium at 8000 rpm, for 20 min. Total proteins in the cell-free supernatant were precipitated by adding ammonium sulphate to 85% saturation and the resulting suspension was kept for an overnight. Subsequently, the precipitate was then collected using centrifuge at 8000 rpm for 30 min. The precipitate was dissolved in a minimum volume of 20 mM phosphate buffer pH 6.5 and dialyzed against the same buffer at 4 °C and then loaded onto gel filtration column (Sephacryl S-200, 90 cm × 1 cm) already equilibrated with 20 mM phosphate buffer pH 6.5 and 0.1 M NaCl. The sample was eluted off at flow rate of 0.4 ml/min. The active fractions were dialyzed against 20 mM Tris–HCl buffer pH 8. Thereafter, the fractions were freeze dried and stored at −20 °C for further characterization.

2.3. Determination of serine protease activity

Serine protease activity was determined according to the method described by Moradian et al. with some modification [14]. One hundred microliters of enzyme was incubated with 0.5 ml casein (5 mg/ml dissolved in 20 mM Tris–HCl buffer, pH 8) at 37 °C for 30 min. The reaction was stopped by adding 0.75 ml trichloroacetic acid (TCA) 10% (w/v) and kept at 4 °C for 1 h. Afterwards, the precipitate was removed by centrifugation at 12,000 rpm for 10 min and the absorbance of the supernatant was measured at 280 nm. One unit (U) of protease activity was defined as the amount of enzyme that hydrolyzes casein to release 1 μg tyrosine in 1 min under assay condition.

2.4. SDS-PAGE, zymography and isoelectric focusing

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the Laemmli method using (5%, w/v) stacking gel and (12%, w/v) resolving gel [15]. To determine the molecular weight and purity of protease, the gel was stained with Coomassie Brilliant Blue R-250 prepared with methanol, acetic acid, water (4:1:5, v/v/v). The stained gel was decolorized with methanol, acetic acid, and water (4:1:5, v/v/v). The relative molecular weight of the purified protein was estimated by comparing its mobility to standard protein size marker.

For zymography analysis, 0.1% (w/v) gelatin was co-polymerized with separating gel. Samples were mixed with β-mercaptoethanol-free gel loading buffer and were loaded to the gel without heating. Electrophoresis was performed at 4 °C. Subsequently, the gel was soaked in renaturing buffer (2.5% (v/v) Triton X-100) for 1 h and then transferred to the developing buffer (Tris 50 mM, NaCl 0.2 M and $CaCl_2$ 5 mM at pH 8), and incubated for an overnight at 37 °C. Finally, the gel was stained with 0.2% (w/v) Coomassie Brilliant Blue R-250 for 30 min. A clear zone on the distained gel was considered as protease activity.

Isoelectric focusing of the purified protease was carried out on gel strips with immobilized pH gradients (IPG) from 3 to 9 (Amersham Bioscience, UK) according to the manufacture instructor. Briefly, the IPG strip was rehydrated in a solution containing 8 M urea, 1% (w/v) CHAPS, 0.2 (w/v) DTT, and 0.25% (v/v) IPG buffers overnight. The sample was applied at the anodal end and run at 2500 V for 90 min at 10 °C. Finally the band was fixed by TCA treatment and stained with Coomassie Blue. Subsequently, the p*I* of the sample was measured based on its instruction manual.

2.5. Effect of pH on activity and stability of the protease

The effect of pH on the enzyme activity was measured at different pH between 3 and 12 with the interval of 0.5 units using the following buffers; 0.1 M of sodium acetate buffer (pH 3–5), phosphate buffer (5.5–7.5), Tris–HCl buffer (8–10) and glycine-NaOH (10.5–12). To assay relative activity, the enzyme was dissolved in each pH and the mixture was incubated for 30 min. The pH stability of the enzyme was also determined by pre-incubation of purified enzyme in aforementioned buffers for 1 h at 37 °C. Afterward, the residual activity was measured under assay condition. The activity at beginning of the experiment was considered as control (100%).

2.6. Effect of temperature on activity and stability of the protease

Thermal activity of the purified protease was studied by incubating the enzyme reaction at temperatures between 20 °C and 90 °C for 30 min using 20 mM Tris–HCl buffer pH 8. Subsequently, the reaction was stopped using trichloroacetic acid (TCA), and the relative activity was measured as described before. To determine the enzyme stability, the purified protease was incubated at different temperature (20–90 °C) at pH 8 for 1 h in the presence of casein 0.5% (w/v). Afterwards, the residual activity was measured under the assay condition. The non-heated enzyme was considered as control (100%).

2.7. Effects of various metal ions on the protease activity

Effect of metal ions on activity of the purified protease was examined. Different ions such as Na^+, K^+, Mg^{2+}, Ba^{2+}, Ca^{2+}, Hg^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+} and Cr^{2+} at 1, 5 and 10 mM final concentration was dissolved in 5 mg/ml casein solution in 20 mM Tris–HCl buffer pH 8.0. Subsequently, the enzyme was incubated with each of these solutions for 15 min at 37 °C then the enzyme activity was measured as described earlier. The activity of the enzyme in the absence of any metal ions was considered as 100%.

2.8. Effect of inhibitors on the protease activity

The effect of two well-known serine protease inhibitors, phenylmethylsulfonyl fluoride (PMSF) and ethylenediaminetetraacetic acid (EDTA), on activity of the purified enzyme was determined using at 5 mM and 10 mM final concentration in 20 mM Tris–HCl buffer pH 8.0. The enzyme was pre-incubated with inhibitors at 37 °C for 1 h to evaluate the influence of inhibitors on protease activity. Casein as a substrate was then added and the residual activity was measured under standard assay condition. The activity