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Purification and biochemical characterization of stable alkaline protease Prot-2 from *Botrytis cinerea*

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ABSTRACT

An extracellular alkaline protease (Prot-2) selectively secreted by *Botrytis cinerea* growing in medium containing *Spirulina algae* as inducer was purified to homogeneity by a combination of ammonium sulfate precipitation, gel filtration and ion-exchange chromatography, followed by size-exclusion chromatography. Prot-2 presented a single 30-kDa band on SDS-PAGE, which showed proteolytic activity following renaturation. Prot-2 has a monomeric structure, is active in the pH range 5.0–9.0 and shows an optimal temperature of activity at 50 °C. Prot-2 is thermostable, and activated by Ca^{2+}. The inhibitory action of reducing agents (dithiothreitol or β-mercaptoethanol) was suppressed by dithiobis-nitrobenzoic acid (DTNB) addition, indicating the role played by disulfide in enzyme activity and/or stability. Prot-2 showed extreme stability towards non-ionic surfactants (5% Tween 20, 5% Triton X-100 and Nonidet P-40). It was relatively stable in 25% aqueous/organic solvent mixtures and was activated by oxidizing agents (H_2O_2 and sodium perborate). A broad specificity of Prot-2 was tested using a range of natural and synthetic oligopeptide substrates. It was further supported by the hydrolysis profile of the insulin B chain by Prot-2.

1. Introduction

Recent years have witnessed a significant increase in the use of enzymes as industrial catalysts. The global market for industrial enzymes is estimated at $3.3 billion in 2010. This market is expected to reach $4.4 billion by 2015, a compound annual growth rate of 6% over the 5-year forecast period [1]. Proteases in particular, represent an important group of enzymes produced industrially and they account for 60% of the worldwide sales value of the total industrial enzymes [2]. Alkaline proteases enjoy a big share of the enzyme market primarily as detergent additives. Microbial alkaline proteases are also very useful in other industrial sectors, such as leather, food, feed, textile, organic synthesis, pharmaceutical and silk, for recovery of silver from used X-ray films [3] and for waste water treatment [3,4]. Proteases have also clinical and medical applications such as reduction of tissue inflammation [5]. However, microbial [6], marine yeast [7] and fungal [8] proteases are also used in hydrolysis to produce bioactive peptides.

Proteases from microbial origin have long been used in industry. They are mostly of fungal origins because of their biochemical diversity and by the fact that the ease of their production may be still increased by environmental and/or genetic manipulations [9]. They are easily extracted and separated from mycelium.

The amount of produced proteases varies greatly with strains and media used. Distinct proteases from different strains, which catalyze the same reactions, allow flexibility in choice of fermentation conditions since they may have different stabilities and different pH and temperature optima [4].

Filamentous fungi are used in many industrial processes for the production of enzymes and metabolites [10]. On an industrial scale, exo-proteases such as alkaline proteases are produced in complex media containing glucose and other costly substrates. Cultivation conditions are essential in successful enzyme production and optimization of parameters such as pH, temperature and media composition is important in developing the production process. Despite all the work dedicated to production of proteolytic enzymes, little information is available on keratinases [11]. For potential industrial applications, hyper-productive organisms growing on economical substrates are required. To this end, during a screening program on protease-producing strains, *Botrytis cinerea* producing a large amount of alkaline proteases was studied and optimal conditions for its high yield production and its potential industrial applications were investigated. To the best of our knowledge, studies on proteases from *Botrytis* are rare. Only a thiol-dependent serine alkaline protease from *B. cinerea* was purified and characterized [12].

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In this study, we describe the purification and biochemical characterization of a novel extracellular alkaline protease produced by *B. cinerea*.

2. Materials and methods

2.1. Fungal strains

Filamentous fungi used in this study were obtained from the collection of the Laboratory of Plant Protection, National Institute of the Agronomic Research of Tunisia. Fungi were isolated from infected plants, identified and maintained on potato dextrose agar (PDA) plates at 20 °C. *B. cinerea* strain was successively cultivated on PDA plates in the presence of 0.5% of yeast extract.

2.2. Growth conditions for proteases production

For production of proteases the fungal culture was carried out on complete medium (Potato Dextrose Broth, PDB). Mycelia plugs (4 mm Ø) from a 3 days-old PDA culture were transferred to 15 ml of PDB and the culture was grown for 3 days at 28 °C, with shacking at 150 rpm and used to inoculate liquid basal medium used initially for screening fungi hyper-producer of extracellular protease.

The culture medium for fungal growth and protease hyper production was that described by Bennett and Lasure [13] with some modifications. *B. cinerea* strain was grown in liquid basal medium containing 1.0% (w/v) yeast extract, 0.5% (w/v) glucose, 0.03% (w/v) $MgSO_4$, 0.1% (w/v) KH_2PO_4, along with 2% sheet *Spirulina algae*, or gelatin or soya proteins as inducer [14]. After adjusting its pH at 6.0 by adding 20% (w/v) Na_2CO_3, the medium was sterilized by autoclaving at 120 °C for 20 min. The culture was grown in 250 ml Erlenmeyer flasks containing 50 ml medium.

Culture medium and mycelia were harvested by vacuum filtration through Whatman No. 1 filter paper on Büchner funnel, and centrifugation at 5500 × *g* and 4 °C for 15 min. The obtained supernatant was used as crude enzyme preparation.

2.3. Protease assay

Azocasein hydrolysis was measured by the method described by Segers et al. [15] and Philips et al. [16] with the following modifications: commercial azocasein (Sigma Chemical Co., St. Louis, MO, USA) or azoalbumin (Sigma) was dissolved at 5% (w/v) in 0.1 M Tris–HCl buffer, pH 8.0.

Briefly, 50 μl of diluted enzyme was added to 200 μl reaction buffer (100 mM Tris–HCl, pH 8.0) containing 5% azocasein (w/v) and the mixture was incubated at 50 °C for 30 min. The reaction was stopped by adding 600 μl of 10% (w/v) trichloroacetic acid and left for 15 min on ice, followed by centrifugation at 15,000 × *g* for 10 min to remove the precipitated protein. Six hundred microliters of the supernatant was neutralized by adding 700 μl of 1 N NaOH and absorbance at 440 nm was recorded with an UV/Visible spectrophotometer (Shimadzu model 1240, Tokyo, Japan). One unit of enzyme activity was defined as the amount, which yielded an increase A_{440} of 0.01 in 30 min at 60 °C under the assay conditions, mentioned above [17].

2.4. Protein determination

Protein concentration of samples was estimated by the method of Bradford [18] using Bio-Rad protein dye (Bio-Rad, Hercules, CA, USA) as reagent concentrate and bovine serum albumin (BSA) to prepare the standard solution range. Protein concentration in various steps of purification was estimated by absorbance at 280 nm.

2.5. Zymography

Zymography was performed on SDS-PAGE according to the method of Schmidt et al. [19] with slight modification. Polyacrylamide gels (10%) were copolymerized with 0.05% gelatin. Samples were mixed with SDS sample buffer without β-mercaptoethanol (β-ME), without heat denaturation and run at 100 V. After electrophoresis, the gels were immersed in 100 mM Tris–HCl buffer (pH 8.5) containing 2.5% Triton X-100 at 4 °C, with shaking for 30 min to remove SDS. Triton X-100 was removed by washing the gels three times with 100 mM Tris–HCl buffer (pH 8.5). The gels were then incubated in 100 mM Tris–HCl buffer (pH 8.5) at 50 °C for 30 min. Finally, the gels were stained with a 0.2% (w/v) solution of Coomassie blue (10% acetic acid and 30% methanol) for 30 min and then destained (10% acetic acid and 30% methanol) for 30 min. Proteolytic activity was observed as clear lysis bands of degraded protein on a uniformly blue background.

2.6. Enzyme purification

The culture broth was filtered and centrifuged at 5500 × *g* for 15 min at 4 °C, and the supernatant was used for further purification.

2.6.1. Ammonium sulfate precipitation

Ammonium sulfate was added to the supernatant to bring the saturation to 0–80% in an ice-bath. The precipitate was collected by centrifugation at 20,000 × *g*, 4 °C, for 60 min. The enzyme was recovered by re-suspending the protein pellet in 50 mM Tris–HCl buffer at pH 8.0. Then, the suspension was dialyzed thoroughly against the same buffer for desalting.

2.6.2. Superdex G-75 gel filtration

The dialyzed fraction was subjected to gel filtration on a Superdex G-75 column (60 × 1.6 cm), which has been equilibrated previously with 25 mM Tris–HCl buffer, pH 8.0. Fractions of 3 ml were collected at a flow rate of 1 ml/min with the same buffer. Protein content (absorbance at 280 nm) and protease activity were determined. Fractions showing protease activities were pooled. Two peaks of protease activity were eluted from the column. The fractions of a major peak were polled and dialyzed against 25 mM phosphate buffer (pH 6.0) before further purification by anion-exchange chromatography onto a Sulfopropyl (SP)-Sepharose column. The FPLC apparatus used was a Sprint Biocad model (PerSeptive Biosystems, Applied Biosystems, Life Technologies, Carlsbad, CA, USA).

2.6.3. Anion-exchange chromatography on FPLC with SP-Sepharose

The active fractions of the major peak pooled from Superdex G-75 gel filtration, dialyzed against 25 mM phosphate buffer (pH 6.0) were applied to a SP-Sepharose column (20 × 2.6 cm, GE Healthcare, Amersham, Uppsala, Sweden) using FPLC system (Sprint Biocad model).

Column was first equilibrated with 25 mM phosphate buffer (pH 6.0). Active fractions were applied to the SP-Sepharose column, and then bound proteins were eluted with a linear salt gradient of NaCl in the range of 0–0.5 M in the equilibration buffer. Fractions were collected at a flow-rate of 1.0 ml/min and monitored by the absorbance at 280 nm. The active fractions obtained from SP-Sepharose purification were pooled. The pool was dialyzed against 25 mM Tris–HCl buffer at pH 8.0, concentrated by filtration on a membrane with a 10-kDa cut off (Millipore, cassette: 5.0-SQ.FT) and then subjected to HPLC gel filtration chromatography.

2.6.4. Gel filtration on TSK-G2000 SWXL HPLC column

The active protease fraction obtained by anion-exchange chromatography (2 ml) was further purified by gel filtration chromatography onto TSK-G2000 SWXL column (300 × 7.8 mm; Tosohaas, Montgomeryville, PA) using a Waters (Milford, MA, USA) HPLC Alliance 2695 system. The column was previously equilibrated with 25 mM Tris–HCl buffer, pH 8.0. Samples were injected 10 times in 200 μl boucle. Elution with the same buffer was performed at a flow rate of 0.8 ml/min, and detection was performed with a Waters 996 photodiode array detector at 220 nm. The active fraction was re-injected in the same conditions in HPLC system. The column was previously calibrated with thyroglobulin (670 kDa), bovine-γ-globulin (158 kDa), ovalbumin (43 kDa), equine myoglobulin (17 kDa) and vitamin B12 (1.35 kDa). Protease activity was tested in the chromatographic fractions by azocasein assay.

2.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE was carried out for the control of the purity and the determination of enzyme molecular weight as described by Laemmli [20] in a discontinuous system made of 5% stacking gel and 10% running gel by using a Mini-Protean II gel system (Bio-Rad). Samples were heated at 100 °C for 5 min in Laemmli buffer before electrophoresis. The molecular weight of the enzyme was estimated using a low molecular weight calibration kit (Bio-Rad) containing pre stained SDS-PAGE standards: phosphorylase B (107 kDa), bovine serum albumin (81 kDa), ovalbumin (48.7 kDa), carbonic anhydrase (33 kDa), soybean trypsin inhibitor (27 kDa), and lysozyme (20.7 kDa). For the final steps of purification we used another molecular weight markers (Sigma) containing: β-galactosidase (116 kDa) phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (34 kDa), soybean trypsin inhibitor (24 kDa). The proteins were visualized by the Coomassie blue staining method previously described.

2.8. Biochemical properties of the purified protease

2.8.1. Effect of pH on the enzyme activity and stability

To determine the effect of pH on protease activity, the rate of azocasein degradation with purified enzyme was evaluated at various pH values. The optimum pH of activity of the purified enzyme was studied over a pH range of 4.0–11.0. For the study of pH stability, the enzyme was incubated overnight at room temperature in different buffers and the residual proteolytic activity was determined under standard assay conditions. The following buffer systems were used at 100 mM: sodium acetate buffer, pH 4.0–6.0; potassium phosphate buffer, pH 7.0; Tris–HCl buffer, pH 8.0–9.0; glycine–NaOH buffer, pH 10.0–11.0. The experiment was repeated twice and average values were taken.

2.8.2. Effect of temperature on the protease activity and stability

To investigate the effect of temperature, the protease activity was tested at different temperatures ranging from 30 to 80 °C for 30 min at pH 8.0. Heat stability and half-life times of the purified protease were determined after incubation at 40, 50, 55 and 60 °C for 120 min. Aliquots were withdrawn at regular time intervals to test the remaining protease activity with azocasein assay at pH 8.0 and 50 °C. The non-heated enzyme was considered as 100% control. The experiment was repeated twice and average values were taken.