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Proteomic analysis of pupal gut serine protease of Silkworm, *Bombyx mori*: Partial purification and biochemical characterization

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ABSTRACT

In the present study, one-dimensional gel electrophoresis (1-DE) and two-dimensional gel electrophoresis (2-DE) coupled zymography were performed for pupal gut proteins which revealed a single prominent proteolytic band/spot at molecular weight of 37 kDa and an isoelectric point (pI) at 5–6 approximately. The proteolytic spot was identified as 37 k-protease of *B. mori* using mass spectrometry. This 37k-Pupal gut serine protease (PGSP) was purified by anionic exchange and gel filtration chromatography. The biochemical characteristics of PGSP were evaluated using quantitative protease assay. PGSP has substrate specificity with gelatin and cannot be inhibited by various protease inhibitors. However, it was sensitive to reducing agents, dithiothreitol and β-mercaptoethanol. The proteolytic activity was not affected by metal ions (10 mM) except Cu^{2+} and Hg^{2+}. The optimum pH and temperature of PGSP was determined as pH 9.0 and 60 °C. The purified protease revealed significant stability and compatibility towards surfactants, oxidizing agent and commercial detergents at 25% concentration. The PGSP (90 μg) with 1% detergent (Rin) showed complete destaining of blood stained cloth. It has significant clot lysis (> 40%) activity within 3 h. In addition, the PGSP shows stable activity in presence of 25% concentration of organic solvents. The overall results suggest that PGSP of *B. mori* could be utilized as an additive agent in detergent industry.

1. Introduction

The enzymes have been employed for a diverse array of applications in industries and scientific research, ranging from food processing, detergent and textile industry, in order to manipulate DNA/RNA in biotechnological research (Li et al., 2012). Proteases represent a major group of enzymes, and account for 70% of worldwide sale of the total industrial enzyme. The global enzyme market is estimated to rise about 7% at a healthy pace to $ 8.0 billion in 2015 (Dewan, 2011). Enzyme demand in the United States is estimated to increase 3% annually to $ 2.2 billion in 2019. At present, there are almost 4000 known enzymes. Of these, about 200 with microbial origin are used commercially. The large scale production of microbial proteases has a drawback due to their high cost requirement for intensive filtration to obtain microbe-free preparations (Phadtare et al., 1997). In addition, usage of microbial proteases in modern detergent formulation has some limitations such as low activity and stability towards sodium dodecyl sulfate, bleach powder and hydrogen peroxide (H_2O_2). Moreover, the production cost of microbial enzymes is not affordable. Henceforth, we are in urge to search alternative and reliable sources for proteases with high activity and stability in various conditions.

In this context, insect gut can act as a promising option for isolating proteases that hold industrially important characteristics. The insect gut contains several proteases such as trypsins, chymotrypsins, cathepsin-b like proteases, aminopeptidases, elastases, carboxypeptidases and serine proteases, which are responsible for the hydrolysis of leaf organic matter, and synthesis of small peptides and amino acids for its growth and development (Applebaum, 1985; Patankar et al., 2001; Srinivasan

Abbreviations: B. mori, *Bombyx mori*; 1-DE, one-dimensional gel electrophoresis; 2-DE, two-dimensional gel electrophoresis; pI, isoelectric point; PGSP, Pupal gut serine protease; PBS, Phosphate-buffered saline; PTU, Phenylthiourea; EDTA, Ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; H_2O_2, hydrogen peroxide; IEF, Isoelectric focusing; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-Time of Flight Mass Spectrometry; DEAE-C, Diethylaminoethyl cellulose; PMSF, phenylmethylsulfonyl fluoride; DMSO, Dimethyl sulfoxide; ANOVA, Analysis of variance; DTT, Dithiothreitol; β-ME, Mercaptoethanol; DFP, Diisopropyl fluorophosphates

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et al., 2006; Chougule et al., 2008; Tabatabaei et al., 2011). The insect gut proteases are considered as a target for insect pest management and industrial application because of their unusual potential to function in the alkaline microenvironment of the gut (pH 10.0–12.0) (Christeller et al., 1992).

The alkaline protease from gut of insect, *Spilosoma obliqua* shows compatibility with various commercial detergents, degrades protein substrate and removes stains (Anwar and Saleemuddin, 2000). Alkaline protease is used in various industries such as waste management, medical applications, leather processing and food industry (Dalev, 1994; Kudrya and Simonenko, 1994; Varela et al., 1997; Neklyudov et al., 2000). The use of insect trypsin like serine proteases has increased remarkably because of its unique features. These include the stability and solid activity of insect trypsin under harsh conditions, such as temperatures of 35–70 °C (Hivrale et al., 2005; Sanatan et al., 2013) and high pH values of 8.0–11.0 (Saboia-Vahia et al., 2013) against detergents, oxidizing, reducing and bleaching agents (Sanatan et al., 2013). In addition, solvent stable proteases from insect gut and its opportunity in pharmaceutical industry for synthetic reaction and peptide synthesis were also studied (Hivrale et al., 2005; Sanatan et al., 2013). Therefore, proteases are a promising tool in industries such as brewing, leather and textile, detergent, dairy and food processing industries (Hamed and Attias, 1987; Ahmad et al., 1980; Anwar and Saleemuddin, 2000).

For industrial prospects, identification of such an enzyme from the insect gut with varying essential properties may be a good alternative approach for existing protease. Thus, in the present study, the pupal gut serine protease was characterized using proteomics analysis, purified and evaluated by quantitative protease assay for biochemical characterization.

2. Material and methods

2.1. Insect rearing

Eggs of domesticated silkworm, *Bombyx mori* (crossbreed race, Tamilnadu White X NB4D2) were obtained from the Government Grainage Centre, Tiruchirapalli, India. The eggs were incubated at 27 ± 2 °C and 75 ± 5% relative humidity for a successful hatch. Newly hatched larvae were fed with chopped, tender mulberry leaves (MR2 variety) until third instar and with coarse leaves up to the last instar (Nirmala et al., 1999). After the animals had ecdysed to the last larval stage (fifth instar), they were staged and synchronized according to Franzetti et al. (2012).

2.2. Gut isolation and protein extraction

The whole gut was collected from fifth instar larvae (4th day) to the second day of the pupa (L5D4 to P2) of silkworm *B.mori*. The gut was washed twice in 1X Phosphate-buffered saline (PBS) buffer (pH 7.2). The gut was lysed by grinding at 4 °C using Phenylthiourea (PTU) buffer containing 50 mM Tris, 10 mM Ethylenediaminetetraacetic acid (EDTA), 15% glycerol and 0.005% PTU. The extracts were then centrifuged at 13,000 rpm at 4 °C for 10 min to remove insoluble material. Total protein was quantified using Bradford method (Bradford, 1976). The supernatant was stored at −85 °C until further use.

2.3. Zymography assay

To analyze the proteolytic profile of midgut proteases, approximately 30 µg of proteins were subjected to electrophoresis (80 V at 4 °C) on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels copolymerized with 0.1% gelatin. After electrophoresis, the gel was washed twice in zymogram buffer for 30 min at 4 °C (50 mM Tris base, pH 7.4, 200 mM $CaCl_2$ and 16 mM sodium azide) containing 2.5% Triton X-100. Then, the gel was incubated overnight in the reaction buffer without Triton X-100 at 37 °C. The proteolytic bands were visualized by staining the gel with 0.25% Coomassie blue R-250 and subsequent destaining with 10% glacial acetic acid. The molecular mass of the proteases was calculated by comparing with the mobility of protein molecular weight marker. All results were derived from triplicates.

2.4. 2DE-zymography

The 2DE-zymography was performed as described by Grudkowska et al. (2013) with slight modification. The pupal gut extract containing soluble proteins were precipitated with 100% ice-cold acetone and then the precipitate was collected by centrifugation at 13, 000 rpm for 10 min at 4 °C. The pellet was washed twice in 80% acetone and air-dried at room temperature. It was dissolved in non-denaturing non-reducing Isoelectric focusing (IEF) sample buffer containing 1% NP-40% and 5% glycerol. The protein was quantified using Bradford assay.

Approximately, 100 µg of protein was re-suspended in 125 µL of rehydration buffer containing 1% NP-40% and 5% glycerol, 0.001% bromophenol blue and 0.25% carrier ampholytes (40% BioLyte® 3/10 Ampholyte, Bio-Rad, USA). The Immobilized pH gradient strips (IPG strips :7 cm, Bio-Rad) having the pH range from 3 to 10 was rehydrated overnight and subjected to IEF. It was performed at a constant temperature of 20 °C and 50 µA/strip. The IEF program was as follows: 250 V for 30 min (Step) 4000 V for 2 h (Grad), 4000 V for 4 h (Step) and finally the volt hrs achieved in 9, 966. The strips were frozen at −85 °C. Next day, the strips were equilibrated with equilibration buffer (0.375 M Tris-HCl, 2% SDS and 20% glycerol) for 15 min. After equilibration, the strips were sealed on the top of 12% SDS-PAGE gels using 0.5% agarose in 1X SDS-PAGE tank buffer (pH 6.8) containing 0.001% bromophenol blue. For 2D-zymography, the resolving gel was co-polymerized with 0.1% of gelatin and the run was performed until the dye front reached the bottom of the gel.

After electrophoresis, the gel was washed twice for 30 min at room temperature in wash buffer (50 mM Tris-base pH 7.4, 200 mM $CaCl_2$ and 1 mM $ZnCl_2$ and 2.5% Triton X-100). Then, the gel was incubated at 37 °C in reaction buffer (50 mM Tris-base (pH 7.4), 200 mM $CaCl_2$ and 1 mM $ZnCl_2$) overnight. Spots of gelatin degradation were visualized by staining the gel with 0.25% Coomassie blue R-250 in methanol: glacial acetic acid (30:10) for 30 min and subsequent destaining with methanol: glacial acetic acid (40:10) for 5 min. The normal 2-DE gel was subjected to Coomassie staining (0.25% Coomassie Brilliant Blue R-250, 30% methanol and 10% acetic acid) for 2 h and destained (40% methanol and 10% acetic acid) until clear spots were visualized beyond blue background. 2-DE spot at 37 kDa stained by Coomassie blue corresponding to the proteolytic spot was analyzed by matrix-assisted laser desorption/ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS) analysis.

2.5. In-gel trypsin digestion and MALDI-TOF-MS analysis

Gel spots were subjected to in-gel reduction and alkylation, followed by trypsin digestion, as previously described by Shevchenko et al. (2006). Peptides were extracted using 100 µL of extraction buffer (1:2 [v/v] of 5% formic acid/acetonitrile). Extracted peptides were dried in a SpeedVac to remove organic solvent. Dried samples were dissolved in 10 µL of molecular biology grade water before mass spectrometry analysis by MALDI-TOF/TOF (UltrafleXtreme, Bruker Daltonics, Germany) and the Peptide mass fingerprint (PMF) data were analyzed using Moscot server as described by Kannan et al. (2016).

2.6. Purification of PGSP

Anion exchange chromatography was performed with minor modification for the purification of PGSP (37 kDa) from the gut of *B.mori* as described by Singh et al. (2013). Approximately 2 g of Diethylaminoethyl