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Purification and characterization of a novel chitinase from *Trichosanthes dioica* seed with antifungal activity

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ABSTRACT

Chitinases are a group of enzymes that show differences in their molecular structure, substrate specificity, and catalytic mechanism and widely found in organisms like bacteria, yeasts, fungi, arthropods actinomycetes, plants and humans. A novel chitinase enzyme (designated as TDSC) was purified from *Trichosanthes dioica* seed with a molecular mass of 39 ± 1 kDa in the presence and absence of β-mercaptoethanol. The enzyme was a glycoprotein in nature containing 8% neutral sugar. The N-terminal sequence was determined to be EINGGGA which did not match with other proteins. Amino acid analysis performed by LC-MS revealed that the protein was rich in leucine. The enzyme was stable at a wide range of pH (5.0–11.0) and temperature (30–90 °C). Chitinase activity was little bit inhibited in the presence of chelating agent EDTA (ethylenediaminetetraaceticacid), urea and Ca^{2+}. A strong fluorescence quenching effect was found when dithiothreitol and sodium dodecyl sulfate were added to the enzyme. TDSC showed antifungal activity against *Aspergillus niger* and *Trichoderma* sp. as tested by MTT assay and disc diffusion method.

1. Introduction

Chitinases are glycosyl hydrolases with sizes ranging from 20 kDa to 90 kDa [1], widely found in organisms like bacteria, yeasts, fungi, arthropods actinomycetes, plants and humans. These enzymes are considered to be a plant defense pathogenesis-related proteins (PRPs) to protect plants against pathogens by degrading chitin, a linear polymer of β-1,4-*N*-acetylglucosamine into lower molecular weight chitooligomers. Chitinase expression is induced or expressed by defensive and non-defensive mechanisms in nature. As a defense mechanism, chitinases expression is induced by pathogens or exogenous chemicals, and the chitinases induce the production of phytoalexins by release of oligosaccharide elicitors [2,3]. In addition to defensive role some plant chitinases play others physiological roles such as in early plant somatic embryo development [4–7].

Chitinases are a huge and diverse group of enzymes that show differences in their molecular structure, substrate specificity, and catalytic mechanism [8]. Chitinases have been divided into 2 main groups: Endochitinases and exochitinases. According to their primary structures, plant chitinases have been categorized into three members, class I, II, and III [9]. More recently, they are further divided into seven classes, I–VII [10]. In general, classes of I, II and IV enzymes contain globular GH-19 catalytic domain and α + β structure while the class III and V plant chitinases have GH-18 catalytic domain with (β/α)8 barrel folding. [11]. Possibly, these structural differences are related to the biological roles of chitinases in vivo. Although chitin is the primary component of the exoskeleton in a large number of organisms, including the cell walls of fungi and of some algae and the shells or cuticle of arthropods, normal mammalian cells contain no chitin, nor has it been reported in cancer cells [11]. A significant cell surface damage was observed, when chitinase was added into the culture medium

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of MCF-7 breast cancer cells [11]. Due to their significant functions in human health care and medical uses as insecticidal, fungicidal activities, and anti tumor activities, chitinases have become an interesting research target to a number of researchers [11–14].

In plants, chitinases are mainly present in seeds, stems, tubers and flowers. Specific chitinases are present in seeds of many plant species to perform their physiological functions such as protecting from bacterial diseases and chitinous polysaccharide degradation. They also play the role of a storage protein [15]. All known isoforms of seed chitinases belong to classes I, II, IV and VII, which are encoded by *Chia* genes, as well as to the more divergent class III encoded by *Chib* genes [16]. Belonging to the Cucurbitaceae family, *Trichosanthes dioica* is known as pointed gourd or 'Potol' in Bengali. It is a common vegetable in Asia and widely cultivated in the eastern and some part of northern India and Bangladesh. It is a good source of carbohydrates, vitamin A, and vitamin C. It also contains major nutrients and trace elements (magnesium, potassium, copper, sulfur, and chlorine) which are needed in small quantities, for playing essential roles in human physiology. As a lectin was already purified from *T. dioica* seeds [17] and large amount of peptide content in the range of 2–8 kD was reported [18], it is more likely to find any chitinase or chitinase-like protein in these seeds giving protection from the microbial attack. In the present study, we are reporting purification of a novel toxic chitinase with antifungal activity from *Trichosanthesdioica* seeds first-time, which can contribute significantly to the current knowledge of seed-specific chitinase protein family.

2. Materials and methods

2.1. Purification of chitinase

Mature seeds of *T. dioica* were dried, crushed and the fat content was removed by treating with *n*-hexane. Then fat free powder was mixed with 10 mM Tris–HCl buffer pH 8.0 and centrifuged at 10,000 × *g* for 25 min at 4 °C. The supernatant was dialyzed against 10 mM Tris–HCl buffer pH 8.0 and centrifuged again at 10,000 × *g* for 15 min at 4 °C. The supernatant that was marked as crude protein was applied on a QA-cellulose column, previously equilibrated with the said buffer. The unbound fraction was collected and the bound fraction was eluted with the linear gradient of NaCl (0.0–1.0 M) and monitored at 280 nm. The homogeneity was checked by using SDS–PAGE in 15% polyacrylamide gel (Atto, Japan) in the presence and absence of β-mercaptoethanol as described by Laemmli [19]. Phosphorylase b (97.2 kDa), bovine serum albumin (66.4 kDa), ovalbumin (44.3 kDa), carbonic anhydrase (29.0 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa) were used as standard marker proteins. The glycoprotein was detected by PAS (Periodic Acid-Schiff) stain of the SDS–PAGE gel [20].

2.2. Chitin binding assay

Purified chitinase in 10 mM Tris–HCl was applied on a chitin column (1 cm × 7 cm) previously equilibrated with the same buffer and washed by 30 ml of 10 mM Tris–HCl buffer and then eluted by 20 mM Sodium acetate buffer, pH 4.0. Finally absorbance was measured at 280 nm.

2.3. N-terminal sequence determination and amino acid analysis

N-terminal sequence of TDSC was determined by Edman degradation using a protein sequencer (Shimadzu, Japan). Homologous sequences were searched by the BLAST program and FASTA program accessed by NCBI. Amino acid analysis was carried out with a Shimadzu LCMS-2020 including a quadrouple mass spectrometer equipped with an electrospray ionization interface (ESI) source. Samples were prepared using Phenomenex EZ:faast amino acid analysis kit following manufacturer's instruction. Liquid chromatographic separation were achieved by using phenomenex EZ:faast AAA-MS column (250 × 3.0 mm). The column and autosampler tray temperature were kept constant at 35 °C and 10 °C, respectively. Mobile phase conditions comprised of (A) 10 mM ammonium formate in water, and (B) 10 mM ammonium formate in methanol. A gradient starting at 68 to 83% (B) over 13 min, and then ramping to 68% by 13.01 min was used for analyses The column was then allowed to re-equilibrate at 68% (B) for 4 min. The mobile phase was delivered at a flow rate of 0.25 ml/min and the sample injection volume was 1 μl. Samples were ionized by positive ion electrospray ionization mode under the following source conditions: Nebulizing gas flow 1.5 l/min, detector voltage was fixed as tuning, DL temperature 250 °C and heat block temperature 200 °C. Mass spectra were obtained at selected ion monitoring (SIM) mode. Analysis was carried out by using SIM for specific *m*/*z* for different amino acid derivatives. Peak areas for all components were automatically integrated by using Shimadzu LC-MS lab solution software.

2.4. Preparation of the substrate (colloidal chitin) and enzyme assay

The substrate colloidal chitin 1% (w/v) was prepared according to the method described by Zhang et al. [12] with minor modification. One g of chitin (Carl Roth, Germany) was dissolved in cold concentrated HCl (20 ml) and placed at 4 °C for 1 h. Then 240 ml of DW was added and kept 18 h at 4 °C. The mixture was centrifuged at 4 °C for 6000 rpm for 15 min. The precipitate was washed several times with water until the pH reached to neutral and finally precipitate was dissolved in 10 mM of phosphate buffer at pH 7.0. The chitinase activity was assayed using DNS (3,5-dinitrosalicylic acid) method, measuring the reducing end group of *N*-acetylglucosamine (GlcNAc) using 1% colloidal chitin (w/v) as substrate. The standard reaction mixture consisting of 0.5 ml of suitably diluted enzyme and 0.5 ml of 1% (w/v) colloidal chitin (pH 7.0) was incubated at 45 °C for 30 min. Then 1 ml DNS was added to terminate the reaction and heated for 10 min in boiling water. The samples were rapidly cooled to room temperature and centrifuged at 6000 rpm for 5 min. The absorbance of the supernatant was measured at 540 nm. One unit of chitinase activity was defined as the amount of enzyme liberating 1 μmol of *N*-acetylglucosamine per minute under described conditions.

2.5. Determination of kinetic parameters

Michaelis-menten constants were determined using the optimal reaction conditions in experiments designed to calculate the reaction velocity at each substrate concentrations. Then K_m and V_{max} of the chitinase were determined using the Lineweaver–Burk plot.

2.6. Estimation of protein and sugar content

Protein was estimated by the Lowry method [21] using lipid-free BSA as the standard. The sugar content of TDSC was determined according to the phenol-sulfuric acid method [22] using D-glucose as standard.

2.7. Determination of optimal pH and pH stability

The optimal activity of the chitinase was investigated by checking the enzyme activities of 0.5 ml of chitinase (1 mg/ml) in 100 mM of different buffers with pH values ranging from 4 to 10. The buffers used were sodium acetate buffer (pH 4.0–6.0), phosphate buffer (pH 7.0), Tris–HCl (pH 8.0), Glycine–NaOH buffer (pH 9.0–10.0). To