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A chitinase with antifungal activity from the mung bean

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Abstract

A chitinase with antifungal activity was isolated from mung bean (*Phaseolus mungo*) seeds. The procedure entailed aqueous extraction, $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography on CM-Sepharose, high-performance liquid chromatography (HPLC) on Poros HS-20, and gel filtration on Sephadex G-75. The protein exhibited a molecular mass of 30.8 kDa in SDS–polyacrylamide gel electrophoresis. Its p*I* was 6.3 as determined by isoelectric focusing. The specific activity of the chitinase was estimated to be 3.81 U/mg. The enzyme expressed its optimum activity at pH 5.4 and was stable from 40 to 50 °C. It exerted antifungal action toward *Fusarium solani*, *Fusarium oxysporum*, *Mycosphaerella arachidicola*, *Pythium aphanidermatum*, and *Sclerotium rolfsii*.

Keywords: Mung bean; Chitinase; Antifungal protein

Leguminous plants have formed a popular subject of research owing to the abundance of proteins and polypeptides with important biological activities that they elaborate. Examples of these proteins are amylase inhibitors [1], lectins [2], and antifungal polypeptides [3,4]. These polypeptides play a role of defense against predators such as insects [5] and pathogens such as fungi [8]. To date, a large number of antifungal polypeptides have been reported. Structurally they can be divided into many types that comprise protease inhibitors [7], ribosome inactivating proteins [8], thaumatin-like proteins [9], chitinases [4,5,9], glucanases [5], embryo-abundant proteins [10], miraculin-like proteins [8], cyclophilin-like proteins [11], allergen-like proteins [12], and novel proteins [13]. Sometimes a combination of antifungal proteins is found in a single species [14].

Chitinases are an integral part of the reaction of plants to infections and stress, responding in concert with other defense related proteins [15]. They can be divided into three classes. Class I chitinases are basic and possess a cys-rich chitin-binding N-terminal domain. Class II chitinases are acidic lacking in the N-terminal domain but highly homologous to class I chitinases within the catalytic domain. Class III chitinases are serologically unrelated to class I or II chitinases but highly homologous to *Hevea brasiliensis* lysozyme in sequence [16]. Class IV chitinases comprise several chitinases with structural similarities but sequence dissimilarities to class I chitinases [17]. Chitin is a main component of fungal cell wall chitinases, together with β-1,3-glucanases, breaks down fungal cell wall, and inhibits fungal growth [18].

The mung bean is popular in the Orient for use in cakes and soups and as bean sprouts. A cyclophilin-like protein [11], a trypsin inhibitor [19], and a lectin [20] have been isolated from the mung bean. We report herein a protein in mung bean, which exerts both chitinase activity and antifungal activity against a variety of fungal species. It has not been previously reported from the mung bean. The use of $(NH_4)_2SO_4$ precipitation,

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cation-exchange chromatography, and gel filtration for the isolation is described below.

Materials and methods

Materials

Mung bean (*Phaseolus mungo*) seeds were purchased from a local market. The fungi *Fusarium oxysporum*, *Mycosphaerella arachidicola*, and *Botrytis cinerea* were kindly provided by Department of Biochemistry, The Chinese University of Hong Kong, Hong Kong, China. The fungi *Fusarium solani*, *Pythium aphanidermatum*, and *Sclerotium rolfsii* were obtained from Department of Plant Pathology, Fujian Agricultural University, Fujian, China.

CM-Sephadex C-50, POROS-HS, and Sephadex G-75 were purchased from Amersham Biosciences (Sweden), PerSeptive Biosystem (PB, USA), and TOSOH (Japan), respectively. Standard proteins for molecular mass determination were purchased from Gibco-BRL (Life Tech., USA). All chemicals were of the highest purity available.

Sample preparation

Exactly 100 g of mung bean seeds was soaked in distilled water for several hours and homogenized in 0.2 M sodium acetate buffer (pH 5.4). The homogenate was centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was designated as the crude extract for further investigations.

Isolation and purification

Ammonium sulfate precipitation

The crude chitinase sample was first fractionated by ammonium sulfate precipitation, in which the crude chitinase solution was treated with ammonium sulfate to 20% saturation. The resulting supernatant was then adjusted to 80% saturated ammonium sulfate. After centrifugation at 12,000 rpm for 20 min, the supernatant was discarded while the precipitate was collected and dissolved in 100 ml of 0.02 M sodium acetate buffer (pH 5.4). The chitinase activity of the solution was determined as described below.

Cation-exchange chromatography

The solution of ammonium sulfate precipitate was dialyzed against 0.02 M sodium acetate buffer (pH 5.4) with several changes and then applied to an open column of CM-Sephadex C-50 column (2.5 × 55 cm) previously equilibrated with the starting buffer (0.02 M sodium acetate buffer, pH 5.4). Following removal of a large amount of unadsorbed proteins, the column was eluted with a linear gradient of NaCl (200–400 mM) in the same buffer. The absorbance of the eluate was monitored at 280 nm. Chitinase activity was determined for all the fractions. The first fraction (P1) demonstrating chitinase activity was pooled, dialyzed against 0.02 M phosphate-buffered saline (PBS) (pH 6.0) at 4 °C for 24 h, and subsequently chromatographed on a column of POROS-HS (0.75 × 7.5 cm), which had been equilibrated with 0.02 M PBS (pH 6.0). After elution of a sizeable quantity of unadsorbed materials, the column was eluted with a gradient of NaCl (0–1.0 M) in the same buffer to yield four peaks. Chitinase activity was determined for all the fractions collected. The unadsorbed fraction (P0′) demonstrating chitinase activity was pooled for further purification. Chromatography was carried out on a Bio-CAD700E perfusion chromatography workstation from PerSeptive Biosystem (PB, USA) at room temperature, and the absorbances of all fractions were monitored at 280 nm.

Gel filtration

The unadsorbed fraction with chitinase activity from POROS-HS chromatography was pooled and subjected to gel filtration on a Sephadex G-75 column. Protein elution was carried out with 0.02 M PBS (pH 6.0) containing 0.1 M NaCl [26–29]. The flow rate was 0.3 ml/min and the eluate was monitored at 280 nm. The chitinase activity of each fraction was determined.

CLC chromatography

The purified chitinase was chromatographed on a C18 capillary reverse-phase high-performance liquid chromatography column using an analyzer (Applied Biosystems Model ABI 140D, Perkin–Elmer, MA).

Characterization of the purified chitinase

Analysis of N-terminal sequence

Sequencing was conducted using a Hewlett–Packard HPG1000A Edman degradation unit and an HP 1000 HPLC column [6].

Protein determination

It was conducted as described by Lowry et al. [21].

Enzyme assays

Chitinase activity was determined by measuring the reducing end group *N*-acetamino-glucose produced from colloidal chitin according to the method of Boller et al. [22], with some modifications. Preparation of colloidal chitin was performed by the method of Li et al. [23]. In a typical reaction, the reaction mixture consisting of 0.1 ml of 3.3 mM sodium azide, 1 ml enzyme