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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

solution, and 1 ml of 1% (w/v) colloidal chitin (pH 5.4) was incubated at 50 °C for 60 min. The reaction was terminated by adding 2 ml dinitrosalicylic acid reagent and heating in boiling water for 5 min, then cooled to room temperature, and centrifuged at 6000 rpm for 10 min. The supernatant was subjected to spectrophotometric measurement at 530 nm. One unit of chitinase activity was defined as the amount of enzyme that liberates 1 μg *N*-acetamino-glucose per minute at pH 5.4 and 50 °C.

Isoelectric focusing electrophoresis

The isoelectric point (p*I*) was determined using a Two-Dimensional Electrophoresis and Data Analysis System (Investigator 5000). The standard proteins used covered the pH range 3–10.

Determination of optimum pH and temperature

The effects of pH and temperature on the enzymatic activity of the purified chitinase preparation were investigated within a pH range between pH 4.0 and 8.0 using 0.2 M sodium acetate buffer (pH 4.0, 5.0, and 5.4), sodium citrate buffer (0.2 M, pH 6.0 and 6.6), and 0.2 M Tris–HCl buffer (pH 7.2 and 8.0), and a temperature range from 30 to 80 °C.

Thermal stability

According to the method proposed by Whitaker [24], the thermal stability of the purified chitinase was estimated by determining the residual activity of the enzyme solution after incubation at various temperatures from 37 to 80 °C.

SDS–PAGE

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (15% T, 4% C) was performed according to the method of Laemmli and Favre [25]. Gels were stained in 0.1% (w/v) Coomassie blue–30% (v/v) methanol–10% (v/v) acetic acid in water. The destaining solution was 30% (v/v) methanol–10% (v/v) acetic acid in water.

Assay of chitinase for antifungal activity

The zone of inhibition assay [11] for antifungal activity was executed using 100 × 15 mm petri plates containing 10 ml of potato dextrose agar. After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed around and at a distance of 1 cm away from the rim of the mycelial colony. An aliquot (8 μl containing 60 or 300 μg) of chitinase in 20 mM PBS buffer (pH 6.0) was introduced to a disk. The plates were incubated at 23 °C for 72 h until mycelial growth had enveloped peripheral disks containing the control (buffer) and had produced crescents of inhibition around disks containing samples with antifungal activity. The fungal species included *S. rolfsii*, *F. solani*, *P. aphanidermatum*, *M. arachidicola*, *F. oxysporum*, and *B. cinerea*.

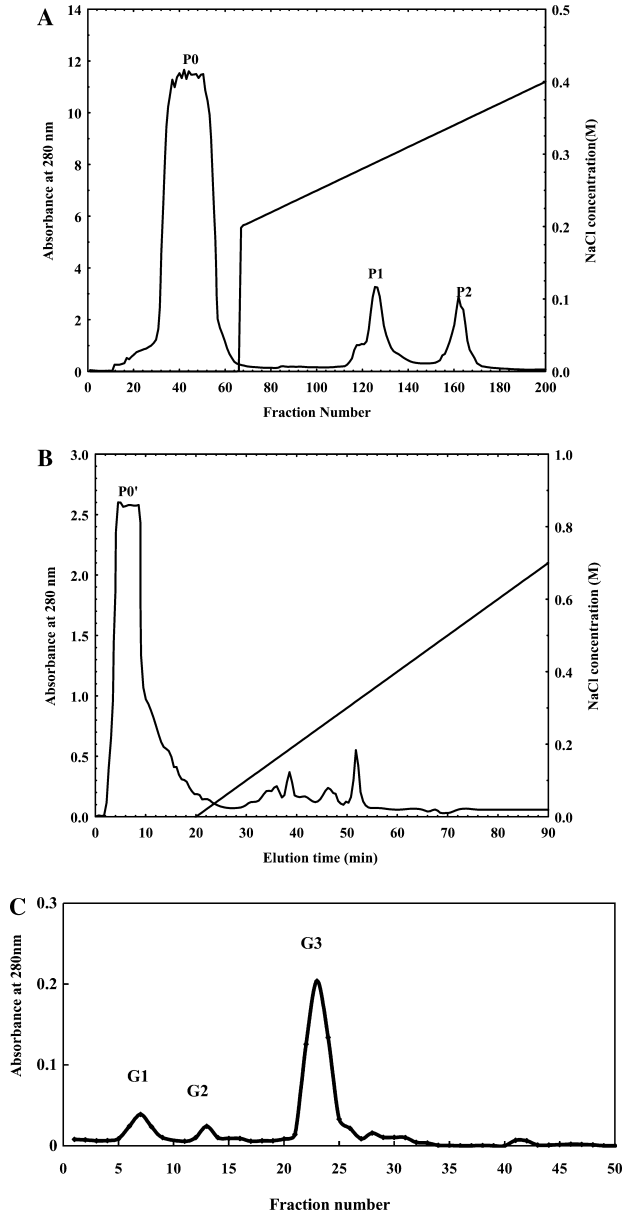

Fig. 1. (A) Fractionation of a solution of the $(NH_4)_2SO_4$ precipitate of mung bean extract on a CM-Sepharose column equilibrated with the binding buffer (0.02 M sodium acetate buffer, pH 5.4). The gel was washed with the binding buffer and eluted with a linear gradient of 0.2–0.4 M NaCl in the same buffer. (B) Elution profile of fraction P1 from the POROS-HS column. The adsorbed fraction P1 from the CM-Sepharose column was dialyzed and then applied to a POROS-HS column in 0.02 M PBS buffer (pH 6.0). The column was then washed with the binding buffer. Adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 1 M in the same buffer. (C) The unadsorbed fraction with chitinase activity from POROS-HS column chromatography was pooled and applied to gel filtration chromatography on a Sephadex G-75 column. Protein elution was carried out with 0.02 M PBS (pH 6.0) containing 0.1 M NaCl.

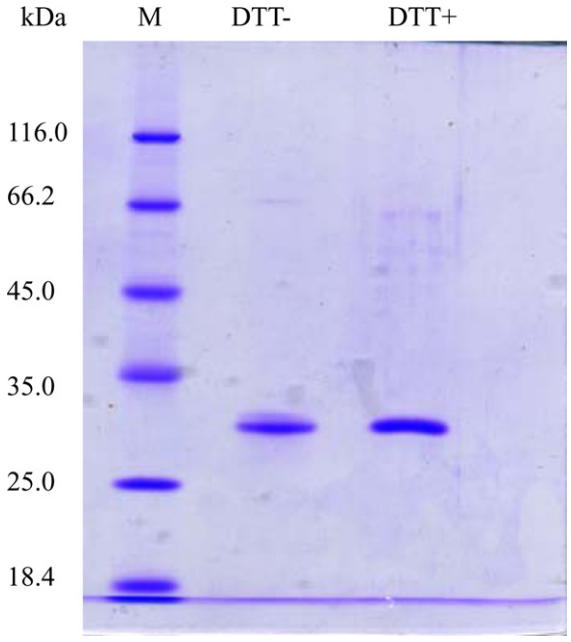

Fig. 2. SDS–polyacrylamide gel electrophoresis of mung bean chitinase. From left to right: lane M was molecular mass standards; lane dithiothreitol− (DTT−) was loaded with 15 μg chitinase under non-reducing conditions (without addition of DTT);. Lane DTT+ was loaded with 15 μg chitinase under reducing conditions (with DTT added).

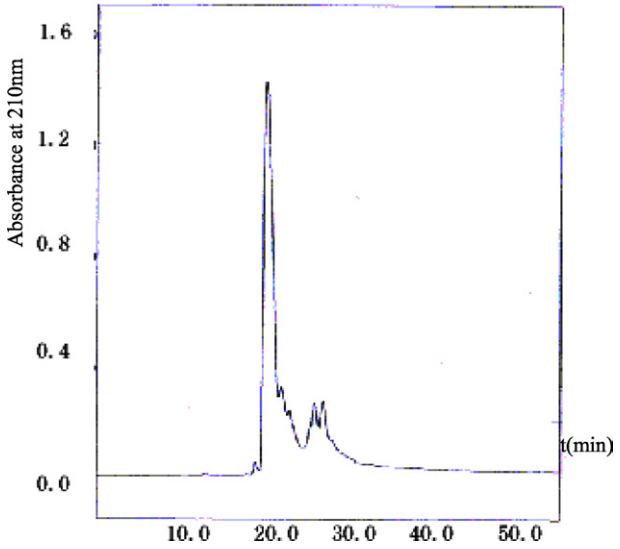

Fig. 3. Capillary reverse-phase high-performance liquid chromatography of the purified chitinase using a C18 column.

Results

Purification of the chitinase

The solution of the ammonium sulfate precipitate was applied to an open column of CM-Sephadex. Following removal of a large amount of unadsorbed proteins, the first adsorbed fraction (P1) exhibiting chitinase activity was desorbed from the column with a linear NaCl concentration gradient (Fig. 1A). The active peak was pooled. The buffer was changed to 0.02 M PBS (pH 6.0) and chromatography on a POROS-HS column was carried out. The unadsorbed material (P0′) demonstrated chitinase activity and antifungal activity (Fig. 1B). The active fraction was further fractionated by gel filtration on Sephadex G-75 column (Fig. 1C). The third peak, which is designated as G3, displayed chitinase activity. Its SDS–PAGE pattern is shown in Fig. 2. The purified chitinase was shown by CLC chromatography (Fig. 3) to be of high purity. When it was subjected to sequence analysis, no sequence information could be obtained, indicating that its N-terminal was blocked.

The protein yield and enzymatic activity at each purification step are presented in Table 1. There was a 3.9-fold increase in specific activity of the purified chitinase compared with that of the crude extract.

Properties of the enzyme

Molecular mass

The molecular mass of the chitinase obtained was estimated by SDS–PAGE to be 30,800 under both non-

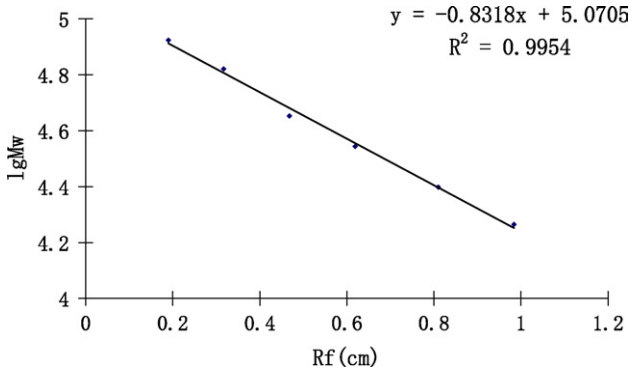

Fig. 4. A plot of logarithm of protein molecular mass vs migration rate.

Table 1
Summary of purification of a chitinase from *Phaseolus mungo*

Purification step	Total protein (mg)	Total PLU (U)	PLU/mg	Purification (fold)	Yield (%)
Ammonium sulfate precipitation (20–80% saturation)	903.5	876.7	0.97	1.0	100
CM-Sephadex C-50 (first I peak)	246.0	360.1	1.46	1.50	41.1
POROS HS (unadsorbed peak)	24.3	46.2	1.9	2.0	5.3
Sephadex G-75 (third peak)	4.3	16.4	3.81	3.9	1.9

Note. Protein obtained from 100 g *Phaseolus mungo* seeds.

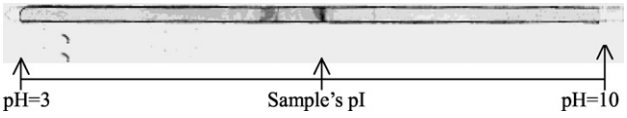

Fig. 5. Isoelectric focusing results.

reducing and reducing conditions, i.e., in the absence or presence of dithiothreitol, as shown in Fig. 4, indicating that the purified chitinase is a monomeric protein. The standard curve for molecular mass determination is shown in Fig. 4.

Isoelectric point

The isoelectric point (p*I*) was determined as 6.33 (Fig. 5).

pH and temperature optima

Chitinase activity as a function of pH and temperature is shown in Fig. 6. The optimum pH was 5.4 and the optimum temperature was 40–50 °C. The purified enzyme lost 43% of its activity when it was incubated at 60 °C for 60 min, and it was completely inactivated at pH above 8.0 and also at temperatures above 85 °C.

Thermostability

Mung bean chitinase was stable below 50 °C, but was rapidly inactivated when incubated at temperatures above 50 °C. Incubation at 60 and 80 °C for 60 min resulted in 43 and 57% loss of activity, respectively. The chitinase was completely inactivated after incubation at a temperature above 85 °C for 5 min.

Antifungal activity of chitinase

Figs. 7a–e present the antifungal activity of mung bean chitinase on five fungal species. It can be seen that the antifungal protein showed strong antifungal activity toward *F. solani* (Fig. 7a). Antifungal activity toward *F. oxysporum* (Fig. 7b), *M. arachidicola* (Fig. 7c), *P. aphanidermatum* (Fig. 7d), and *S. rolfsii* (Fig. 7e) was discernible. However, it had no antifungal effect on *Botrytis cinera* (data not shown).

Discussion

There is a range of molecular masses reported for antifungal chitinases. Some chitinases have a molecular mass near 30 kDa, while others are well over 30 kDa in molecular mass [22]. Delandin, the chitinase-like antifungal protein from rice bean, has a molecular mass of 28 kDa [26], similar to its counterparts from field beans and pinto beans [27,28]. However, two chitinases from chickpea and an endochitinase from bean leaves have a molecular mass over 30 kDa [4,5]. So, the chitinase from mung bean has a molecular mass (30.8 kDa) within the range of molecular masses (between 25 and 35 kDa) reported for chitinases [26]. The results of the dithiothreitol experiment indicate that it is a monomeric protein as are the previously reported chitinases [26–28].

The p*I* values of chitinases from leguminous plants have not been reported previously. The p*I* value of mung bean chitinase was found to be 6.33. This constitutes an addition to the existing knowledge about chitinases.

The optimum pH of the purified chitinase was somewhat different from chitinases obtained by either Boller et al. [22] or Yang and Luo [30]. The former was at pH 6.5 and the latter was at pH 6.0, while mung bean chitinase underwent 40% loss of activity at pH 6.0. However, its optimum temperature was similar to those of the above-mentioned chitinases.

The thermostability of the mung bean chitinase is similar to that of the chitinase reported by Boller et al. [22], but higher than that of the chitinase reported by Yang and Luo [30]. Based on a comparison of the zone/crescent of inhibition of mycelial growth, the inhibitory action of mung bean chitinase on fungal growth was much more potent than that from the bean seeds [30], similar to that of the French bean thaumatin-like protein [22], but weaker than that of the rice bean chitinase-like protein [26].

According to Schlumbaum et al. [31], chitinases from plants are potent inhibitors of fungal growth, but there are different classes. We select as examples, chitinases from chickpea (*Cicer arietinum*) cell-suspension cultures [5] and ethylene-treated bean leaves [4]. In the first case only one of the chitinases, the basic chitinase with a blocked N-terminal, possessed antifungal activity [5]. The acidic class III chitinase was devoid of activity on fungi and showed N-terminal sequence identity with class III acidic chitinases from *Cucumis sativis*, *Parthenocissus quinquifolia*, *H. brasiliensis*, and *Arabidopsis thaliana*. In the latter case, the enzyme has been evaluated in detail for its antifungal effect. Light microscopic examination disclosed chitinase-induced swelling of hyphal tips and hyphal distortions in the fungus. Wall disruption, release of chitin oligosaccharides from cell walls, and cytoplasm leakage were observed in ultrastructural and cytochemical studies [4]. In the present investigation, a chitinase was isolated from mung bean seeds and its antifungal activity was displayed in several fungal species including *F. solani*, *F. oxysporum*, *M. arachidicola*, *S. rolfsii*, and *P. aphanidermatum*. So it could be deduced that the purified antifungal protein belongs to the aforementioned latter case, due to its degradation of chitin which is a component of the fungal wall.

In sum, a chitinase with antifungal activity against a variety of fungal species was isolated from mung bean seeds. This is noteworthy since not all chitinases have been shown to possess antifungal activity. The purified chitinase was somewhat different from other reported chitinases from bean leaves and bean seeds in molecular

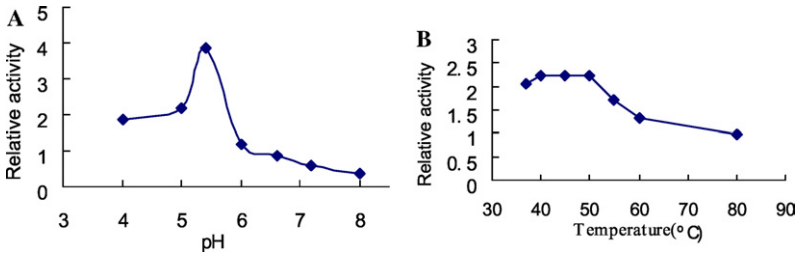

Fig. 6. Effects of pH and temperature on activity of the purified mung bean chitinase. Activity at pH 5.4 and 50 °C was used as reference.

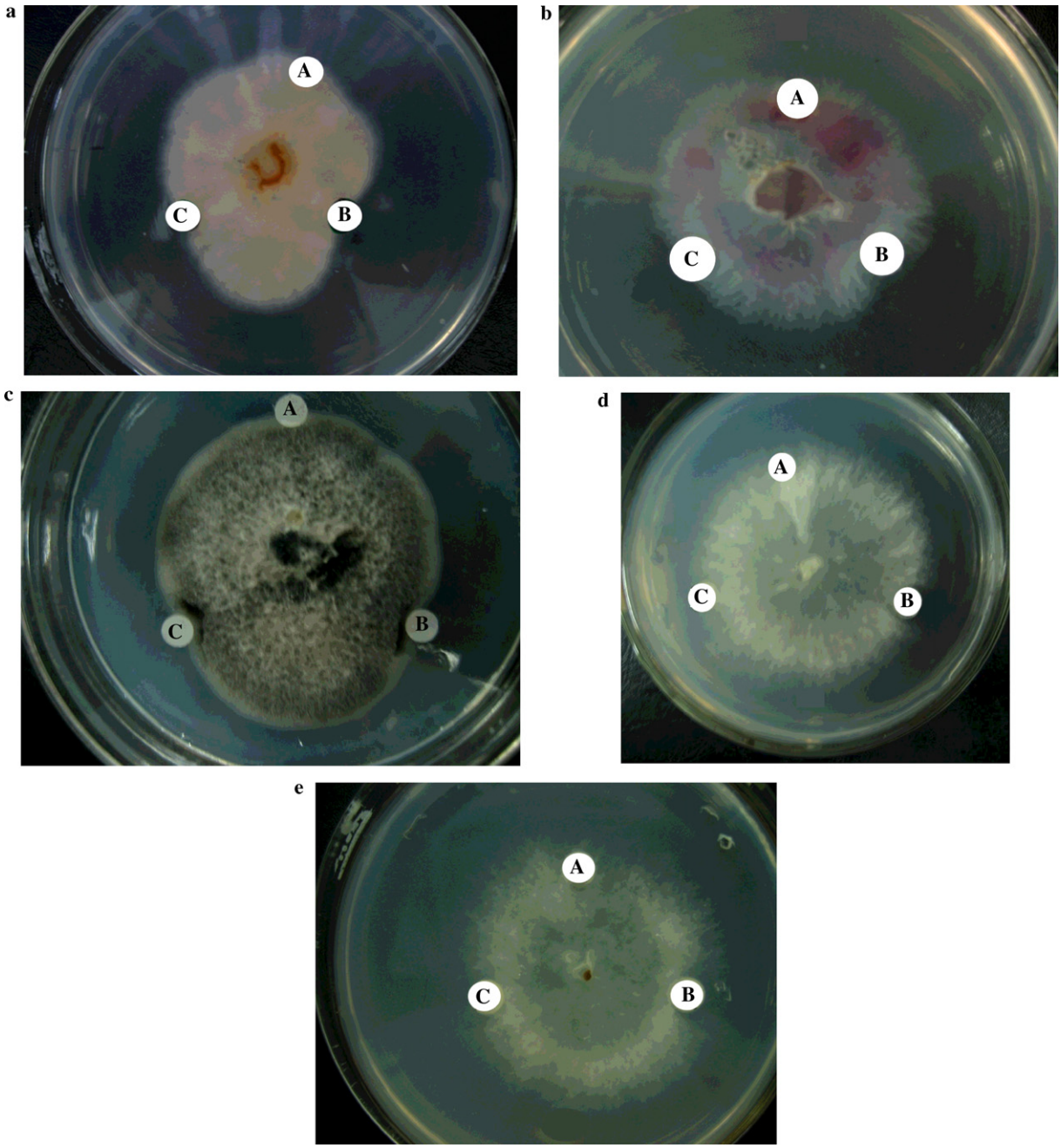

Fig. 7. Inhibitory activity of mung bean chitinase toward *Fusarium solani*, *Fusarium oxysporum*, *Mycosphaerella arachidicola*, *Pythium aphanidermatum*, and *Sclerotium rolfsii*, respectively. (A) 0.02 M PBS buffer, pH 6.0, (B) 300 μg mung bean chitinase, and (C) 60 μg mung bean chitinase.

mass, and pH and temperature optima. The observation that the purified protein showed antifungal activity toward different fungal species may have important implications in agriculture.

Acknowledgments

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