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First report of a novel plant lysozyme with both antifungal and antibacterial activities

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Abstract

A novel lysozyme exhibiting antifungal activity and with a molecular mass of 14.4 kDa in SDS–polyacrylamide gel electrophoresis was isolated from mung bean (*Phaseolus mungo*) seeds using a procedure that involved aqueous extraction, ammonium sulfate precipitation, ion exchange chromatography on CM-Sephadex, and high-performance liquid chromatography on POROS HS-20. Its N-terminal sequence was very different from that of hen egg white lysozyme. Its p*I* was estimated to be above 9.7. The specific activity of the lysozyme was 355 U/mg at pH 5.5 and 30 °C. The lysozyme exhibited a pH optimum at pH 5.5 and a temperature optimum at 55 °C. It is reported herein, for the first time, that a novel plant lysozyme exerted an antifungal action toward *Fusarium oxysporum*, *Fusarium solani*, *Pythium aphanidermatum*, *Sclerotium rolfsii*, and *Botrytis cinerea*, in addition to an antibacterial action against *Staphylococcus aureus*.
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Keywords: Mung bean; Lysozyme; Antifungal protein

Lysozyme (muramidase, EC.3.2.1.17) is widely distributed among eukaryotes and prokaryotes. It catalyzes the hydrolysis of bacterial cell walls and acts as a nonspecific innate immunity molecule against the invasion of bacterial pathogens [12]. Lysozymes are classified into six types: chicken-type lysozyme (c-type) which includes stomach lysozyme and calcium-binding lysozyme, goose-type lysozyme (g-type), plant lysozyme, bacterial lysozyme, T4 phage lysozyme (phage-type), and invertebrate lysozyme (i-type) [1,5,8,9,11,17,24–26]. Thus far, the chicken type of lysozyme has been the most extensively studied, and several molecules have already been purified and cloned [9]. It has also been reported that the chicken type of lysozyme, such as hen egg white lysozyme (HEWL), is a naturally occurring protein found in many organisms such as viruses, bacteria, plants, insects, reptiles, birds, and mammals [21].

Lysozyme is well known as an antibacterial protein with activity against Gram-positive and Gram-negative bacteria. Its bactericidal activity is hypothesized to reside in its muramidase activity, leading to degradation of the murein layer and reduction of the mechanical strength of the bacterial cell wall. These actions eventually result in the killing of the bacteria by lysis [4]. There are also some reports concerning its lytic activity against fungal pathogens, such as *Phytophthora nicotianaae* and *Fusarium oxysporum* [4], *P. aphanidermatum* and *Botrytis cinerea* [32], and *Candida albicans* [27]. However, reports about the antifungal activity of lysozyme are relatively few in number, and none has been none isolated from plant sources.

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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 protease inhibitors [30], amylase inhibitors [39], lectins [14], antifungal polypeptides [2,33], and ribosome inactivating proteins [16]. All of these polypeptides may contribute to a common function of defence against predators such as insects [20] and pathogens such as fungi [40]. To date, a large number of antifungal polypeptides have been reported. Structurally they can be divided into different types; for example, thaumatin-like proteins [41], chitinases [2,36], glucanases [33], embryo-abundant proteins [34], miraculin-like proteins [40], cyclophilin-like proteins [42], allergen-like proteins [43], and antifungal proteins [44]. Sometimes a combination of antifungal proteins is found in a single species of bean [45].

The mung bean is popular in the Orient for use in cakes and soups, and as bean sprouts. A cyclophilin-like protein [42], a trypsin inhibitor [22], a lectin [3], a malate dehydrogenase [35], a chitinase [36], and a non-specific lipid transfer protein [37] have been isolated from the mung bean. We report herein a protein in mung bean, which exerts both lysozyme activity and antifungal activity against a variety of fungal species. It has not previously been reported from the mung bean or other plants.

Materials and methods

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

CM-Sephadex C-50 and POROS-HS were purchased from Amersham Biosciences (Sweden) and PerSeptive Biosystem (USA), 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.

Ammonium sulfate precipitation. The crude lysozyme sample was first fractionated by ammonium sulfate precipitation, in which the crude lysozyme solution was treated with ammonium sulfate to 20% saturation. Ammonium sulfate was added to the resulting supernatant to 80% saturation. 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 lysozyme 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 of buffer, 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 flow rate was 0.5 ml/min, i.e., 10 min/tube. The absorbance of the eluate was monitored at 280 nm. Lysozyme activity was determined for all of the fractions. The second fraction (P2) demonstrating lysozyme activity was pooled, incubated at 70 °C for 10 min, and centrifuged at 10,000 rpm for 10 min to remove denatured protein. The supernatant was condensed, dialyzed against 0.02 M phosphate-buffered saline (PBS), (pH 6.0) 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). Chromatography was carried out on a BioCAD700E perfusion chromatography workstation from PerSeptive Biosystem (PB, USA) at room temperature, with a flow rate of 1 ml/min. The absorbances of all fractions were monitored at 280 nm. After elution of a sizeable quantity of unadsorbed materials, the column was eluted with a linear gradient of NaCl (0–0.5 M) in the same buffer to yield two peaks. Lysozyme activity was determined for all of the collected fractions. The first eluted peak represents lysozyme, a purified mung bean antifungal protein.

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

Enzyme assays. Lysozyme activity was determined by using a turbidimetric method [19,38]. Lyophilized cells of *M. lysodeikticus* were suspended in 100 mM sodium acetate buffer (pH 5.5, $OD_{450} = 0.6$–1.0). The initial decrease in OD_{450} of the suspensions caused by the lysis of *M. lysodeikticus* cells was measured at 30 °C for 1 min. A decrease of 0.001 in OD was defined as 1 unit of lysozyme activity.

SDS–PAGE. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (12.5%T, 4%C) was performed according to the method of Laemmli and Favre [15]. 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.

Protein determination. Protein concentrations were determined by using the method of Lowry et al. [18] using bovine serum albumin as a standard.

N-terminal amino acid sequence analysis. The N-terminal amino acid sequence of the purified enzyme was performed by Edman degradation using a protein sequencer (Applied Biosystems Model 476A, Perkin–Elmer, MA, USA). Phenylthiohydantoin derivatives were separated and identified by capillary reverse-phase high-performance liquid chromatography on a C18 column with an analyzer (Applied Biosystems Model ABI 140D, Perkin–Elmer, MA, USA).

Determination of optimum pH and temperature. The effects of pH and temperature on the enzymatic activity of the purified lysozyme preparation were investigated within a pH range between pH 3.0 and pH 8.0 using 100 mM sodium citrate buffer (pH 3.0), sodium acetate buffer (pH 4.6, 5.0, and 5.5), phosphate (pH 6.0), and 100 mM Tris–HCl buffer (pH 7.2 and 8.0), and a temperature range from 20 to 100 °C.

Thermal stability. According to the method proposed by Whitaker [8], the thermal stability of the purified lysozyme was estimated by determining the residual activity of the enzyme solution after incubation at various temperatures from 30 to 100 °C, which was performed at pH 3.0, 5.5, and 7.2, respectively, in order to investigate the effect of pH on its thermal stability.

Assay of lysozyme for antibacterial activity. The assay for antibacterial activity toward *Staphylococcus aureus* and *Salmonella* sp. was conducted using sterile petri dishes (100 × 15 mm) containing 10 ml LB agar (1.5% agar). Three milliliters of warm nutrient agar (0.7%) containing the bacteria was poured into the plates. A sterile blank paper disk (0.625 cm in diameter) was placed on the agar. Then a solution of lysozyme (containing a $2^0 \sim 2^{-9}$ gradient of 17 μM) in 20 mM PBS buffer (pH 6.0) was introduced to a disk. The plates were incubated at

37 °C for 12–20 h. A transparent ring around the paper disk signifies antibacterial activity.

Assay of lysozyme for antifungal activity. The assay for antifungal activity was executed using 100 × 15 mm petri plates containing 10 ml potato dextrose agar. Around and at a distance of 1 cm away from the central disk (0.625 cm in diameter) were placed sterile blank paper disks of the same size. An aliquot (8 μl containing 60 or 300 μg) of lysozyme 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 from the central disk had enveloped peripheral disks containing the control (buffer) and had produced crescents of inhibition around disks containing samples with antifungal activity. The fungal species tested included *S. rolfsii*, *F. solani*, *P. aphanidermatum*, *F. oxysporum*, *B. cinerea*, and *M. arachidicola*.

To further evaluate the antifungal effect of the enzyme, the inhibition of hyphal growth of *F. solani* by the purified protein was observed under a light microscope. Cells and spore suspensions (10^5 or 10^6 in 1 ml) of the fungus were prepared. Then 50 μl of cells and spore suspension treated with the purified protein (300 μg) was added into a tube with 1 ml medium. After incubation at 27 °C for 48 h, cells were separated from the growth medium by centrifugation and plated for observation under an optical microscope at 100× magnification.

For a quantitative assay to determine the IC_{50} of antifungal activity, three doses of the antifungal protein were added separately to three aliquots each containing 4 ml potato dextrose agar at 45 °C, mixed rapidly, and poured into three separate small petri dishes. After the agar had cooled down, the same amount of mycelia was inoculated to each plate. Buffer only without antifungal protein served as a negative control. After incubation at 27 °C for 72 h, the area of the mycelial colony was measured and the inhibition of fungal growth and hence the IC_{50} value were determined. The IC_{50} value is the concentration required to reduce the area of the mycelial colony to 50%.

Results

Purification of mung bean lysozyme

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 (PO), the second adsorbed fraction (P2) exhibiting lysozyme activity was desorbed from the column with a linear NaCl concentration gradient (Fig. 1A). The active peak was pooled, incubated at 70 °C for 10 min, and then centrifuged at 10,000 rpm for 10 min to remove denatured protein. The supernatant was concentrated, the buffer was changed to 0.02 M PBS (pH 6.0), and chromatography on a POROS-HS column was carried out. The first adsorbed peak (hsl) from the column possessed both lysozyme and antifungal activities (Fig. 1B). Its SDS–PAGE pattern is shown in Fig. 2. The molecular mass of the lysozyme obtained was estimated by SDS–PAGE to be 14,400. The purified lysozyme was shown by capillary reverse-phase high-performance liquid chromatography (Fig. 3) to be of high purity. It possessed a p*I* exceeding 9.7.

The protein yield and enzymatic activity at each purification step are presented in Table 1. There was an almost 479-fold increase in specific activity of the purified lysozyme compared with that of the crude extract.

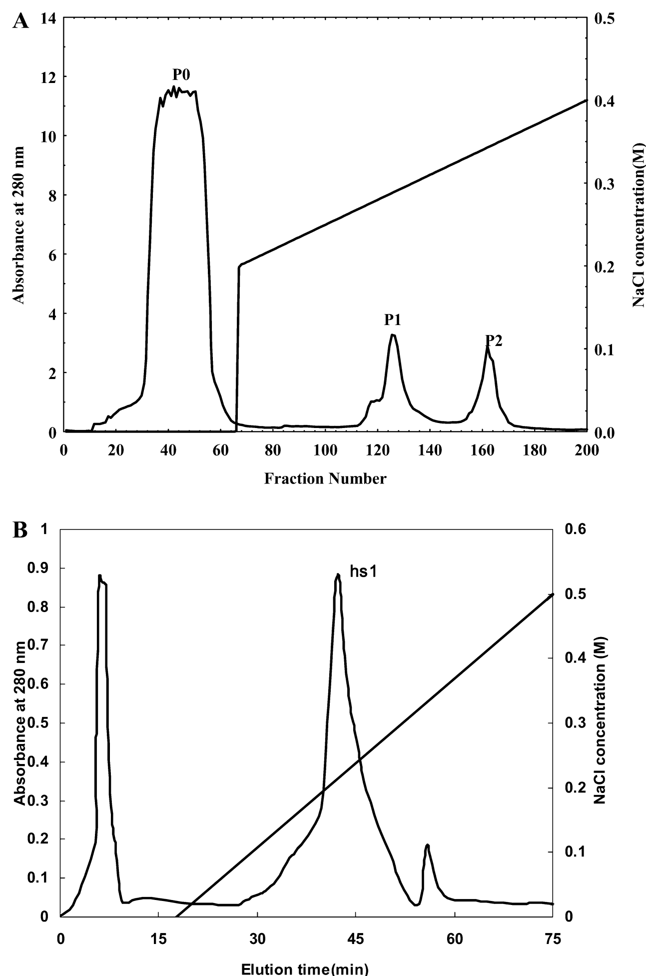

Fig. 1. (A) Fractionation of a solution of the $(NH_4)_2SO_4$ precipitate derived from mung bean extract on a CM-Sephadex column equilibrated with the binding buffer (0.02 M sodium acetate buffer, pH 5.4). The gel was washed with the binding buffer and eluted with a linear gradient of 0.2–0.4 M NaCl in the same buffer. (B) Elution profile of fraction P2 from the POROS-HS column. The adsorbed fraction P2 from the CM-Sephadex column (P2) was pooled, incubated at 70 °C for 10 min, and centrifuged at 10,000 rpm for 10 min to remove denatured protein. The supernatant was dialyzed and then applied to a POROS-HS column in 0.02 M phosphate-buffered saline (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 0.5 M in the same buffer.

N-terminal amino acid sequence

The 13 N-terminal amino acid sequence of the purified mung bean lysozyme was determined to be D-M-P-G-K-V-A-L-T-A-Q-S-F. It has low homology (about 23%) with hen egg while lysozyme (Table 2).

Other properties of the enzyme

pH and temperature optima

Lysozyme activity, as a function of pH and temperature, is shown in Fig. 3. The optimum pH was 5.5

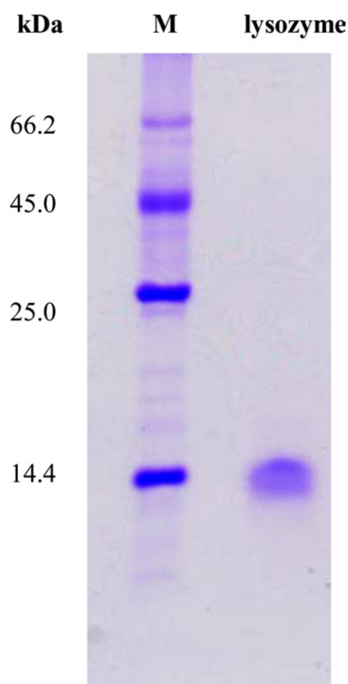

Fig. 2. SDS–polyacrylamide gel electrophoresis of mung bean lysozyme. From left to right: lane M was molecular mass standards; sample lane on the right was loaded with 15 μg mung bean lysozyme.

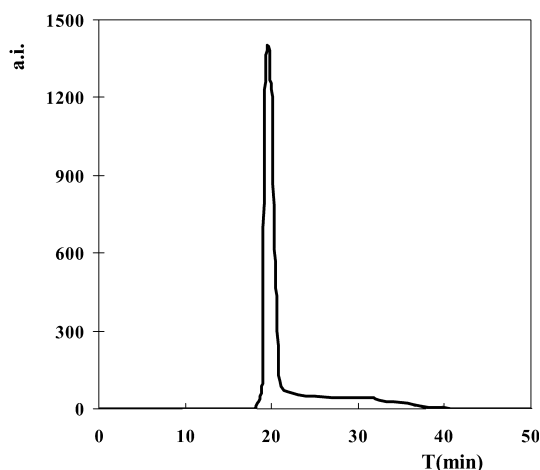

Fig. 3. Capillary reverse-phase high-performance liquid chromatography of the purified lysozyme using a C18 column.

(Fig. 4A), and the optimum temperature was 55 °C (Fig. 4B). In fact, it showed relative stability between 50 and 60 °C. When it was incubated at 70 °C, the purified enzyme lost only 11% of its activity. However, it was significantly inactivated when the ambient temperature rose above 80 °C, and it was almost completely inactivated at a pH above 8.0 or at a temperature of 100 °C.

Thermostability

The thermal stability of the purified lysozyme, as estimated by determining the residual activity of the enzyme solution, was performed at pH 3.0, 5.5, and 7.2, in order to investigate the effect of pH on its thermal stability. The results were interesting in that the stability to heat at an acidic pH of 3.0 was the highest in spite of its intermediate enzyme activity, with the bulk of enzyme activity remaining even at 90 °C. When at pH 5.5, it was stable below 60 °C; incubation at 70 °C for 30 min resulted in 10% loss of activity, and it was rapidly inactivated when it was incubated at a temperature above 80 °C. However, the stability to heat above the neutral pH (pH 7.2) was very poor with a lower enzyme activity. It was almost completely inactivated after incubation at a temperature above 40 °C for 30 min (Fig. 5).

The lysozyme from mung bean was stable to heat at acidic pH. The purified protein was obtained using incubation at 70 °C and pH 5.4, in order to inactivate some extraneous protein. It was found that the thermal stability at acidic pH 3.0 was the highest, while the enzyme activity at pH 3.0 was less than that of pH 5.5.

Antibacterial activity of mung bean lysozyme

The mung bean lysozyme exerted antibacterial activity toward the Gram-positive bacterium *S. aureus*. However, it had no effect on Gram-negative bacteria such as *Salmonella* sp. (data not shown). The antibacterial gradient to *S. aureus* was determined (Table 2). Antibacterial activity was demonstrated in the lysozyme concentration gradient of $2^0 \times 17$ to $2^{-5} \times 17$ μM. The MIC to *S. aureus* was 0.53 μM (see Table 3).

Antifungal activity of mung bean lysozyme

The antifungal activity of mung bean lysozyme on five fungal species is illustrated in Figs. 6A–E. It can

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

Purification step	Total protein (mg)	Total activity ($U \times 10^3$)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	1.21×10^4	8.97	0.741	1.0	100
Ammonium sulfate precipitation (20–80% saturation)	2.37×10^3	5.98	2.52	3.4	66.7
CM-Sephadex (second peak)	334	3.08	9.22	12.4	34.3
CM-Sephadex (second peak) incubation (70 °C, 10 min)	40.6	2.87	70.7	95.4	32.0
POROS HS (first peak)	5.6	1.99	355	479	22.2

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

Table 2
Comparison of N-terminal sequence of mung bean lysozyme with that of hen egg white lysozyme

Lysozyme	Residue number	N-terminal sequence	Residue number	% identity in N-terminal sequence
Mung bean	1	DMPGKVALTAQSF	13	100
Hen egg white	1	KVFGRCELAAAMK	13	23

Residue number 1 and residue number 13 for mung bean lysozyme refer to D and F being the first and 13th amino acid residue in mung bean lysozyme, respectively.

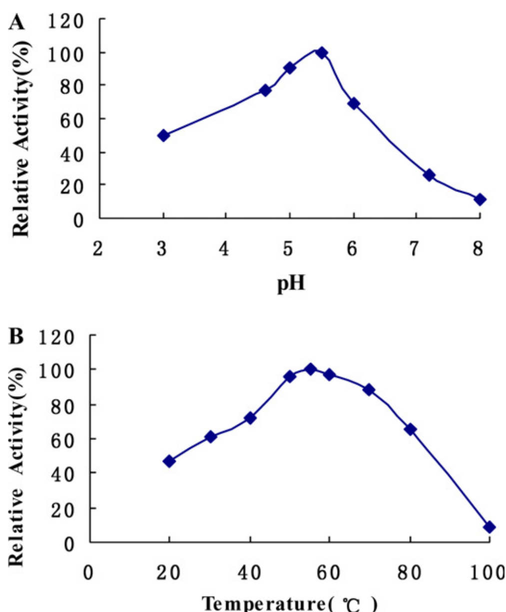

Fig. 4. Effect of (A) pH and (B) temperature on activity of the purified mung bean lysozyme. Activity at pH 5.5 and 30 °C was used as standard for comparison.

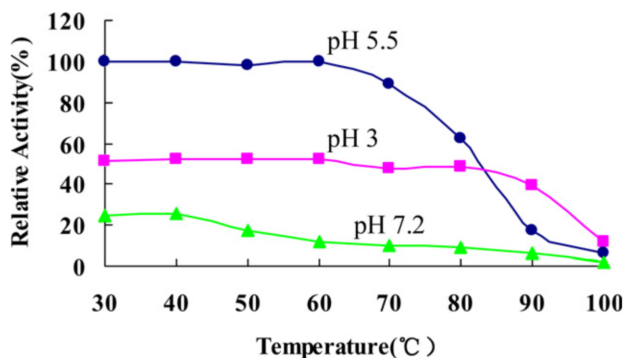

Fig. 5. Thermal stability of the purified lysozyme. The results were estimated by determining the residual activity of the enzyme solution after incubation at various temperatures from 30 to 100 °C. The enzyme assay was performed under pH 3.0 (■), 5.5 (●), and 7.2 (△), respectively.

be seen that the protein showed strong antifungal activity toward *F. oxysporum* (Fig. 6A), and some antifungal activity toward *F. solani* (Fig. 6B), *S. rolfsii* (Fig. 6C), *P. aphanidermatum* (Fig. 6D), and *Botrytis cinera* (Fig. 6E). However, it had hardly any antifungal effect on

Table 3
The antibacterial gradient of mung bean lysozyme to *Staphylococcus aureus*

Sample concentration gradient (×17 μM)	Antibacterial effect
2^{0}	+
2^{-1}	+
2^{-2}	+
2^{-3}	+
2^{-4}	+
2^{-5}	+
2^{-6}	−
2^{-7}	−
2^{-8}	−
2^{-9}	−

M. arachidicola (data not shown. The IC50 of its antifungal activity toward *F. solani* was 11 μM (Fig. 7)).

The IC_{50} value of the antifungal activity toward *F. solani* was calculated to be 11 μM. Light microscopic examination disclosed lysozyme-induced distortions of the fungus. Photographs revealed hyphal morphological alterations and a reduced number of hyphae for fungus growing in the presence of lysozyme (Fig. 8B) as compared with normal hyphal growth on control medium (Fig. 8A).

Transmission electron microscopic observation of a hypha treated with the purified lysozyme showed detachment of cell membrane and cell wall, and appearance of a wide space filled with amorphous material (data not shown). It is surmised that the antipathogenic activity of purified protein was due to corrosion of the pathogen wall and subsequent leaking out of its cytoplasm.

Discussion

There are a range of molecular masses reported for lysozymes. Lysozyme from chicken egg-white or belonging to c-type has a molecular mass between 14.0 and 15.0 kDa. Others have somewhat different molecular masses. Lysozyme from hemolymph of *Heliothis virescens* larvae displays a molecular mass of 16 kDa [31]. T4 phage lysozyme has a molecular mass of 18.7 kDa [10], while goose egg-white lysozyme reported by Jollés [28] has a molecular mass of 21 kDa. Thus, the lysozyme from mung bean exhibits a molecular mass within the range of c-type molecular masses. The p*I* of mung bean

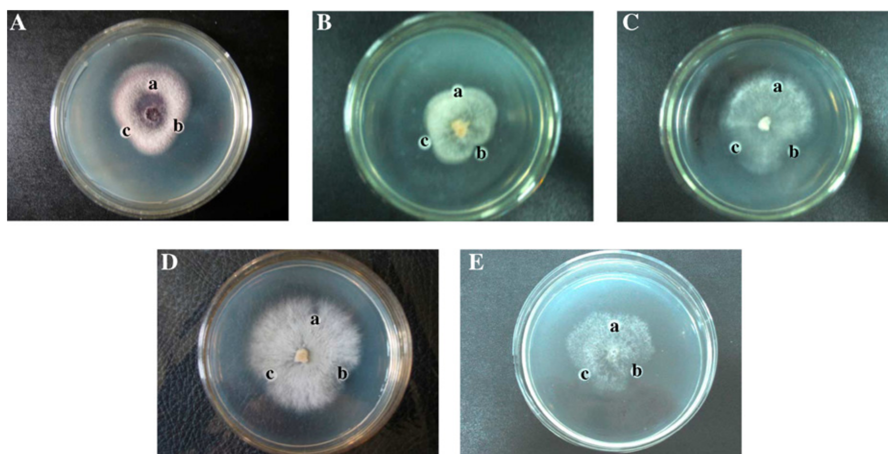

Fig. 6. (A–E) Inhibitory activity of mung bean lysozyme toward *Fusarium oxysporum*, *Fusarium solani*, *Pythium aphanidermatum*, *Sclerotium rolfsii*, and *Botrytis cinerea.* (a) 0.02 M phosphate-buffered saline, pH 6.0, (b) 300 μg mung bean lysozyme, and (c) 60 μg mung bean lysozyme.

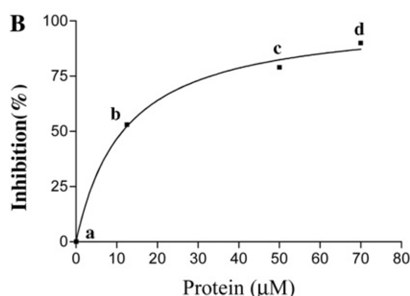

Fig. 7. (A,B) Determination of the IC_{50} value of the antifungal activity of mung bean lysozyme toward *Fusarium solani*. Plate a represents the control and plates b, c, and d represent treatment with 12.5, 50, and 70 μM mung bean lysozyme, respectively. The IC_{50} was calculated to be 11 μM.

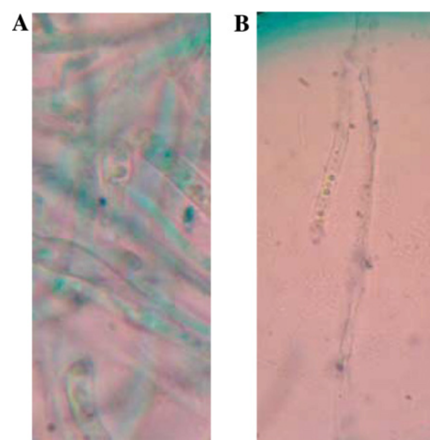

Fig. 8. Light microscopic observation on inhibition of hyphal growth in *Fusarium solani* by purified mung bean lysozyme. (A) Mycelia of fungus after 48 h of growth in the absence of mung bean lysozyme; (B) mycelia of fungus after 48 h of growth, in the presence of 300 μg mung bean lysozyme.

lysozyme was calculated to be above 9.7 (data not shown), which was very similar to its counterparts from other sources [31,13]. Most of the c-type lysozymes reported are basic proteins [13]. On the other hand, the digestive lysozymes isolated from the midgut of *Musca domesta* [17] and *Drosophila melanogaster* [26] were shown to have neutral or even acidic isoelectric points, respectively. The p*I* may further indicate that the lysozyme from mung bean is a c-type-like one.

The optimum temperature of the purified lysozyme was similar to its counterparts from both kuruma shrimp [7] and radish [29]; the former was at a temperature of 50 °C and the latter was at a temperature of 55 °C. As regards the optimum pH, except for lysozymes from plant sources, such as radish seed lysozyme with a similar optimum pH of 5.8 [29], its optimum pH was somewhat different from others. For example, the pH optimum of the tick gut lysozyme was in the range of pH 5–7 [13], with a wide pH scope. The optimum pH in kuruma shrimp and chicken egg lysozymes were about pH 7.5 and 8.0, respectively [7,24]. However, this newly isolated mung bean lysozyme was almost inactivated (by 74% and 90%), respectively, at pH 7.2 and 8.0.

The results show that thermostability of the purified enzyme was somewhat similar to that of lysozyme from the gut of the soft tick reported by Kopacek et al. [13]. Lysozyme from *Heliothis* [31] was very stable at high temperatures and low pH. Its activity was stable at 100 °C at pH 3.0 but was labile at 100 °C at pH 6.8. Podboronov [23] reported that heating at 100 °C for 3 min reduced the activity of tick lysozymes by 10% and 45% when performed in an acidic (pH 5–6) and alkaline (pH 8–9) medium, respectively. Gao et al. [6] reported that chick egg-white lysozyme showed almost full activity at 100 °C.

Although lysozyme is well known as an antibacterial protein, with activity against Gram-positive and Gram-negative bacteria, reports about its antifungal activity are scanty. There are some reports about the lytic activity of animal lysozymes against some fungal pathogens, such as *P. nicotianaae* and *F. oxysporum* [4], *P. aphanidermatum* and *B. cinerea* [32], and *C. albicans* [27]. However, no reports about antifungal activity of lysozymes from plant sources are available. In the present investigation, a lysozyme was isolated from mung bean seeds and its antifungal activity was displayed in broad fungal species including *F. oxysporum*, *F. solani*, *S. rolfsii*, *P. aphanidermatum*, and *B. cinerea.*

In conclusion, a lysozyme has been isolated from mung bean seeds. It is the first time that it has been revealed that the lysozyme of mung bean seeds demonstrated potent antifungal activity. The purified lysozyme was somewhat different from other reported lysozymes in terms of molecular mass, and pH and temperature optima. The observation that the purified protein showed antifungal activity toward a variety of fungal species suggested an important role of this purified protein in constitutive host defense mechanisms against microbial pathogens. This may contribute to the development of a strategy of biological control of fungal pathogens typical of the crop.

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