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ORIGINAL PAPER

Isolation and identification of a plant lysozyme from *Momordica charantia L*

Shaoyun Wang · Biao Shao · Jingli Chang · Pingfan Rao

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Abstract A plant lysozyme exhibiting antifungal activity and with a molecular mass of 20.1 kDa in SDS–polyacrylamide gel electrophoresis was isolated from *Momordica charantia L* using a procedure that involved aqueous extraction, vacuum freeze-drying of extraction, and ion exchange chromatography on high-performance liquid chromatography on Source Q and POROS 50 HS. Its N-terminal sequence was determined to be Q-L-C-E-L-A-A-C-M-K-R-H-G-L-D, showing relatively high homology (about 80%) with that of hen egg white lysozyme. Its pI was estimated to be 9.9. The lysozyme exhibited a pH optimum at pH 5.5 and a temperature optimum at 50 °C. Moreover, the lysozyme exerted an antifungal action toward *Mucorracem osus* and *Rhizoctonia solani*, in addition to an antibacterial action against *Escherichia coli* and *Staphylococcus aureus*.

Keywords *Momordica charantia L* · lysozyme · Antibacterial · Antifungal

Introduction

Lysozyme, as a self-defense enzyme, was serendipitously discovered by Alexander Fleming in 1922 [1]. Lysozyme, because of its muramidase activity, has long been known to exert its antimicrobial action by specifically hydrolyzing the 1,4 β-D-linkage between N-acetylmuramic acid and N-acetylglucosamine of cell wall peptidoglycan [2]. To date, 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) [3]. Thus far, the chicken type of lysozyme has been the most extensively studied, and several molecules have already been purified and cloned [4]. 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 [3–10].

There are extensive reports on lysozyme's antibacterial activity against Gram-positive and Gram-negative bacteria. For example, lysozyme is reported to be able to kill bacteria in the chick egg white [5], milk [8], eastern oyster [11, 12], white shrimp [13], and kuruma shrimp [14]. It has been widely developed in agricultural and industrial applications [2, 4, 5]. The catalytic mechanism of its bactericidal activity is hypothesized to reside in its muramidase activity, leading to degradation of the murein layer and reduction in the mechanical strength of the bacterial cell wall. These actions eventually result in the killing of the bacteria by lysis [15]. There are several literatures regarding its lytic activity against fungal pathogens, such as, *Phytophthora nicotianae* and *Fusarium oxysporum* [15], *Candida albicans* [16], and *Pythium aphanidermatum* and *Botrytis cinerea* [3]. However, reports on the antifungal activity of lysozyme are relatively few in number.

Momordica charantia L is a member of *Cucurbitaceae* family and has been used as a traditional plant medicine for many years in China and at the same time, it serves as dietary component, well known as a source of nutrients and proteins. It also serves as healthy diet to human well-being because of its extensive function of antidiabetic, antiviral,

Shaoyun Wang and Biao Shao are the co-first authors.

S. Wang (✉) · B. Shao · J. Chang · P. Rao
Institute of Biotechnology,
Fuzhou University, Fuzhou 350002,
People's Republic of China
e-mail: shywang@fzu.edu.cn

antibacterial, antioxidant, and anti-inflammatory activities [17–19]. We report herein a protein in *Momordica charantia L*, which demonstrates lysozyme activity, antibacterial activity, and antifungal activity against a variety of bacterial and fungal species. It has not previously been reported that lysozyme with the antibacterial and antifungal activity has been isolated from *Momordica charantia L.*

Materials and methods

Materials

The fresh fruits of *Momordica charantia L* were purchased from a local market. The fungi *Mucorracem osus* and *Rhizoctonia solani* were kindly provided by the Department of Plant Pathology, Fujian Agricultural University, Fujian, China. *Micrococcus lysodeikticus* was purchased from Sigma Co (Shanghai, China). Source Q and POROS-50 HS were purchased from TOSOH Biosciences (Japan) and PerSeptive Biosystem (USA), respectively. All chemicals were of the highest purity available.

Sample preparation

Exactly 100 g of *Momordica charantia L* with seeds removed was soaked in distilled water for several hours and homogenized in 0.2 M phosphate-buffered saline (PBS) (pH 6.0). The homogenate was centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was designated as the crude extract and dialyzed against distilled water for several times, and then the supernatant was centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was lyophilized in the freeze dryer. The frozen powder was preserved for further investigations.

Isolation and purification

The frozen powder was dissolved and dialyzed against 0.02 M borate sodium buffer (pH 8.0) with several changes of buffer and was chromatographed on a Source Q column. Chromatography was carried out on a BioCAD700E perfusion chromatography workstation from PerSeptive Biosystem (PB, USA) at room temperature, with a flow rate of 1.0 mL/min. The column was eluted with a linear gradient of NaCl (0–700 mM) in the same buffer. Unadsorbed proteins and adsorbed proteins were collected, which was monitored at 280 nm. Lysozyme activity was determined for all the fractions. The fraction with lysozyme activity was condensed, dialyzed against 0.02 M sodium citrate buffer (pH 3.4) for 24 h, and subsequently chromatographed on a POROS-50 HS column equilibrated with 0.02 M sodium citrate buffer (pH 3.4). The absorbances of all fractions were monitored at 280 nm. After elution of nonabsorbed materials, the column was eluted with several concentrations of NaCl (0.08 M, 0.15 M, 0.21 M, 0.3–0.7 M) in the same buffer to get further isolation.

Characterization of the purified lysozyme

Enzyme assays

Lysozyme activity was determined by using a turbidimetric method [3, 20]. It was followed by the clearance of a suspension of *Mtcrococcus luteus* dried cells (300 μg/mL, 100 mM PBS buffer, pH 7.0). Lyophilized cells of *M. lysodeikticus* were suspended in 100 mM PBS (pH 7.0, OD 450 = 1.0–1.3). 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.

Protein determination

Protein concentrations were determined by using the method of Folin-Phenol [20], using bovine serum albumin as a standard.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE (12.5% T, 4% C) was performed according to the method of Laemmli and Favre [21]. 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.

Isoelectric focusing electrophoresis

The isoelectric focusing (IEF)-PAGE was performed using a 2-Dimensional Electrophoresis and Data Analysis System (Investigator™ 5000, Tokyo, Japan). The PhastGel IEF for standard proteins was bought from BIO-RAD Company, USA, covering the pH range 3–10.

Determination of optimum pH and temperature

The effects of pH and temperature on the enzymatic activity of the purified lysozyme were investigated within a pH range of 4.0–8.0 using 0.02 M sodium acetate buffer (pH 4.0, 5.0 and 5.4), 0.02 M sodium citrate buffer (pH 6.0 and 6.6) and 0.02 M Tris–HCl buffer (pH 7.2 and 8.0), and a temperature range from 25 to 80 °C. The samples were incubated for 5 min at each given pH or temperature as mentioned earlier.

N-terminal amino acid sequence analysis

The N-terminal amino acid sequence of the purified lysozyme was performed by Edman degradation using a protein sequencer (Applied Biosystems Model 476A, Perkin Elmer Co. MA, USA). Phenylthiohydantoin derivatives were separated and identified by capillary reversed phase HPLC in a C18 column with an analyzer.

Assay for antibacterial activity

The assay for antibacterial activity toward *Staphylococcus aureus* and *Escherichia coli* was conducted using sterile Petri dishes (90 × 15 mm) containing 10 mL Luria–Bertani (LB) medium (1.5% LB agar). Three milliliters of warm nutrient agar (0.7%) containing 95 cfu/mL of bacteria inoculum 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 (75 and 380 μg) in 20 mM PBS buffer (pH 7.0) was put on the disk. The plates were incubated at 37 °C for 15–20 h. A transparent ring around the paper disk signifies antibacterial activity [3].

To further evaluate the antibacterial effect of the enzyme, the minimal concentration of inhibition was observed through the change of absorbance in bacteria cultivation process. Five milliliters of warm nutrient medium containing 95 cfu/mL of *Escherichia coli* inoculum was poured into every tested tube. Then, a solution of lysozyme (0.8, 0.6, 0.4, 0.2, 0.1 and 0.05 mg/L) in 20 mM PBS buffer (pH 7.0) was added into each tube, which formed a series of concentrations. After incubation at 37 °C for 4 h, incubating liquid was monitored at 450–650 nm to determine the content of *Escherichia coli* and the minimal concentration of inhibition (MIC) was calculated. No visible bacterial growth was observed at minimal concentration of inhibition.

Assay for antifungal activity

The assay for antifungal activity was executed using Petri plates (90 × 15 mm) 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. A volume of 20 μL solution of lysozyme (75 and 380 μg) in 20 mM PBS buffer (pH 7.0) was put on the disk. The plates were incubated at 28 °C for 24 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 *Mucorracem osus* and *Rhizoctonia solani*.

For a quantitative assay for antifungal activity, *Rhizoctonia solani* was taken as an example; three doses (containing 5, 15, and 30 μM) of the sample in 20 mM PBS buffer pH 7.0 were added separately to three aliquots each containing 4 mL potato dextrose agar at 45 °C, mixed rapidly and poured into 3 separate small Petri dishes. After the agar cooled down, the same small amount of mycelia was inoculated onto 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 was expressed as IC_{50}, which represents the protein concentration required for 50% growth inhibition.

Statistical analyses

All experiments were conducted in triplicate. All data were presented as means (standard deviations, SDs) of three independent experiments. Statistical analysis was done using Student's *t* test. A value of $p < 0.05$ was considered statistically significant.

Results and discussion

Purification of *Momordica charantia L* lysozyme

The solution of the frozen powder was applied to chromatography on a Source Q column. It was carried out at room temperature, with a flow rate of 1.0 mL/min. The column was eluted with a linear gradient of NaCl (0–700 mM) in the same buffer. Following a linear NaCl concentration gradient, two main adsorbed fractions (P1, P2) were desorbed from the column (Fig. 1). Lysozyme activity was determined for all of the fractions. The P2 fraction demonstrated lysozyme activity. It was condensed, dialyzed against 0.02 M sodium citrate buffer (pH 3.4) for 24 h, and chromatography on a POROS-50HS column was carried out. The first eluted peak (HS-1) represents lysozyme activity, a purified *Momordica charantia L* antifungal and antibacterial protein (Fig. 2). Its SDS–PAGE pattern showed a single band (Fig. 3).

The protein yield and enzymatic activity at each purification step are presented in Table 1. There was an almost 55-fold increase in the specific activity of the purified lysozyme when compared with that of the crude extract. The specific activity of the lysozyme was 640 U mg^{-1} at pH 5.0 and 40 °C.

N-terminal amino acid sequence

The 15 N-terminal amino acid sequence of the purified *Momordica charantia L* lysozyme was determined to be Q-L-C-E-L-A-A-C-M-K-R-H-G-L-D. The first 15 N-terminal residues present 80% homology with the amino acid sequence 22–36 of hen egg white lysozyme (Table 2).

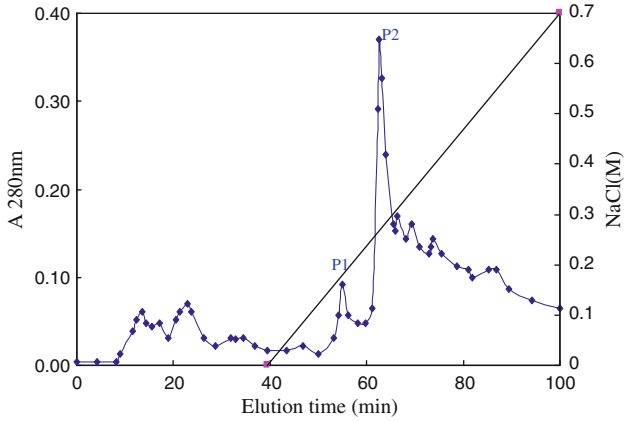

Fig. 1 Fractionation of a solution of the frozen powder on a Source Q column equilibrated with the binding buffer (0.02 M borate sodium buffer, pH 8.0). The gel was washed with the binding buffer and eluted with a linear gradient from 0 to 0.7 M NaCl in the same buffer

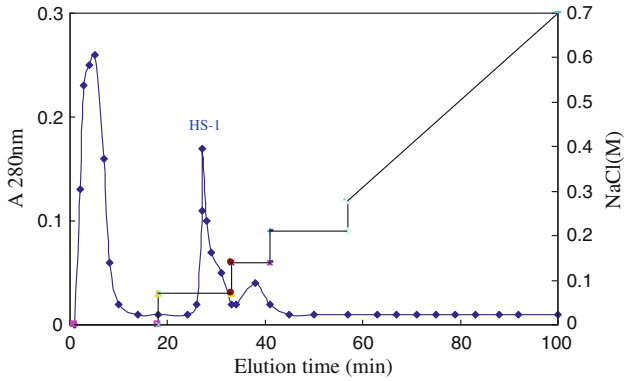

Fig. 2 Elution profile of fraction P2 from the POROS 50 HS column. The fraction P2 from the Source Q column was dialyzed and then applied to a POROS 50 HS column in 0.02 M citrate-buffered saline (pH 3.4). Adsorbed proteins HS-1 were eluted with 0.08 M in the same buffer

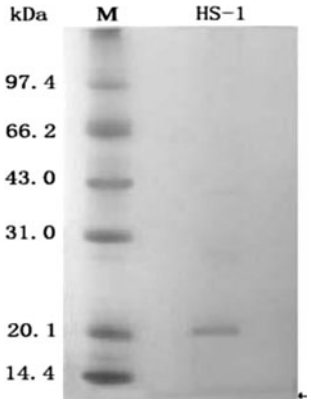

Fig. 3 SDS–PAGE of *Momordica charantia L* lysozyme. *Lane M* was molecular mass standards; *lane HS*-1 was the sample loaded

Properties of the lysozyme

Molecular weight

The purified enzyme exhibited a single band on SDS–PAGE as shown in Fig. 3. The molecular weight of the lysozyme obtained was estimated by SDS–PAGE to be 20.1 kDa. There is actually a range of molecular weight reported for lysozymes. Lysozyme from hemolymph of *Heliothis virescens larvae* displays a molecular weight of 17 kDa [9]. T4 phage lysozyme has a molecular weight of 18.7 kDa [22]. Lysozyme from pawpaw displays a molecular weight of 28 kDa [6], while goose egg white lysozyme reported by Schoentgen et al.[23] has a molecular weight of 21 kDa. The mentioned molecular weights are all obtained by SDS–PAGE [6, 9, 22, 23].

Isoelectric point

Based on the results of isoelectric focusing electrophoresis (Fig. 4a), the isoelectric point (*p*I) of the newly reported lysozyme was determined to be 9.9, as standard proteins of IEF covered the pH range from 3 to 10 shown in Fig. 4b.

Lysozymes include acidic and basic in nature. The *p*I of *Momordica charantia L* lysozyme was very similar to its counterparts from other c-type sources [3, 9]. Most of the c-type lysozymes reported are basic proteins [10]. However, the digestive lysozymes isolated from the midgut of *Musca domesta* [24] and *Drosophila melanogaster* [25] were shown to have neutral or even acidic isoelectric points, respectively. The *p*I may further indicate that the newly purified lysozyme is a c-type-like one. The individual lysozymes are often different in their molecular mass and pI represents the diversity of lysozymes in the nature.

pH and temperature optima

The pH and temperature optima of lysozyme are shown in Fig. 5. The optimum pH was 5.5 (Fig. 5a), and the optimum temperature was 50 °C (Fig. 5b). The optimum pH of the purified enzyme was similar to that of plant lysozyme. For example, mung bean lysozyme has optimum pH of 5.5 [3], isolysozymes of radish seeds have optimum pH of 5.8 and 5.5 [26]. The similarity of optimum pH suggests homology among plants. The optimum temperature of the purified lysozyme was similar to its counterparts from kuruma shrimp [14] and radish [26]; the former was at a temperature of 50 °C and the latter was at a temperature of 55 °C. However, thermostability of the purified enzyme was somewhat similar to that of lysozyme from the gut of the soft tick reported by Kopacek et al. [10].

Table 1 Summary of purification of a lysozyme from *Momordica charantia L*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Crude extract	1356.9	1.56×10^4	11.5	1.0	100
Vacuum freeze-drying	420.1	8.70×10^3	20.7	1.8	55.8
Source Q	72.4	6.89×10^3	95.1	8.3	44.2
POROS 50HS	10.1	6.43×10^3	637.2	55.4	41.2

Protein obtained from 100 g of fresh *Momordica charantia L*

Table 2 Comparison of the amino acid sequence of *Momordica charantia L* lysozyme with that of hen egg white

Lysozyme source	Amino acid sequence	Identity (%)
Momordica charantia L	1QLCELAACMKRHGLD15	100
Hen egg white	22GYCELAAAMKRHGLD36	80

Number 1 and number 15 of the amino acid sequence refer to Q and D being the 1st and 15th amino acid residue of *Momordica charantia L* lysozyme; number 22 and number 36 of the amino acid sequence refer to G and D being the 22nd and 36th amino acid residue of hen egg white lysozyme, respectively. Underlined characters, identical amino acids with the purified lysozyme from *Momordica charantia L*

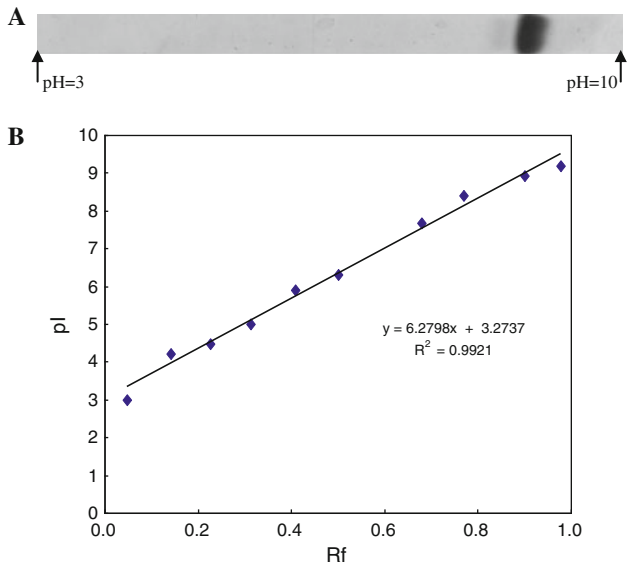

Fig. 4 Isoelectric focusing electrophoresis result of *Momordica charantia L* lysozyme (**a**) and the standard curve of standard proteins covering the pH range 3–10 (**b**)

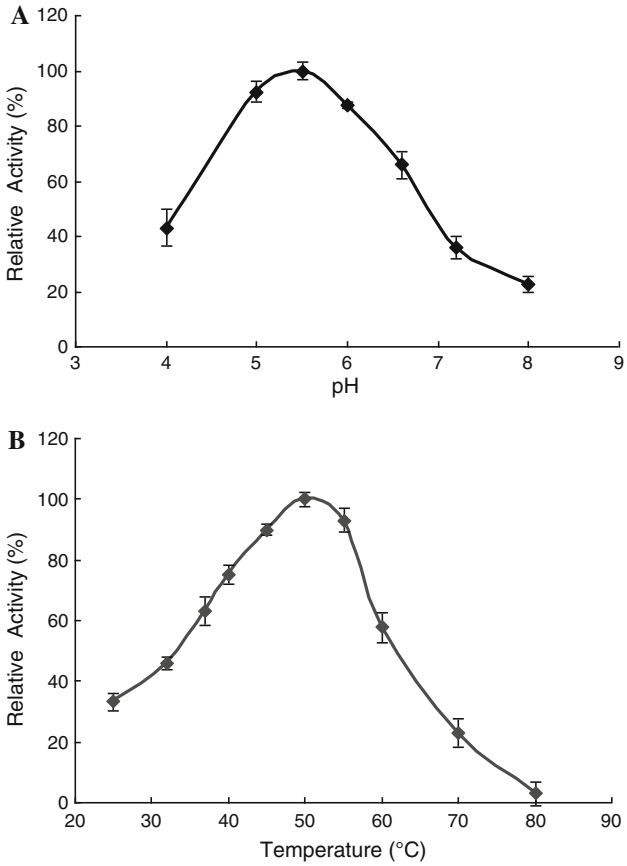

Fig. 5 Effect of **a** pH and **b** temperature on activity of the purified lysozyme. Activity at pH 5.5 and 40 °C was used as standard for comparison; 40 °C and pH 5.5 was used to determine the optimal pH, and the optimal temperature, respectively

Antibacterial activity

The purified lysozyme exerted antibacterial activity on *Escherichia coli* (Fig. 6a) and *Staphylococcus aureus* (Fig. 6b). Moreover, the antibacterial activity showed was concentration dependent, and the crescents of inhibition on disks containing samples of 380 μg were more significant than those with samples of 75 µg as shown in Fig. 6. The minimal concentration of inhibition (MIC) on *Escherichia coli* was calculated to be 0.2 g lysozyme/L, which shows comparable inhibition activity with the reported chicken egg lysozyme (E. coli as a target) [3, 5].

The results show the purified lysozyme has more activity against the Gram-negative bacteria such as *E. coli*. In contrast, egg white lysozyme shows antibacterial properties especially against Gram-positive bacteria. Antibacterial activity of lysozyme is connected with the structure of the cell wall. This enzyme catalyzes hydrolysis of β-glycoside

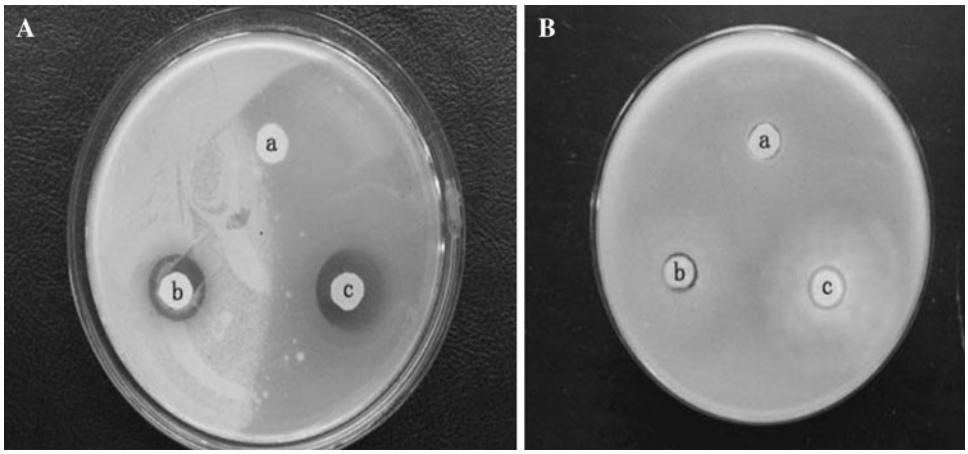

Fig. 6 Inhibitory activity of *Momordica charantia L* lysozyme toward *Escherichia coli* (Fig. 6a) and *Staphylococcus aureus* (Fig. 6b). **a** 0.02 M phosphate-buffered buffer, pH 7.0, **b**75 μg lysozyme, and **c** 380 μg lysozyme

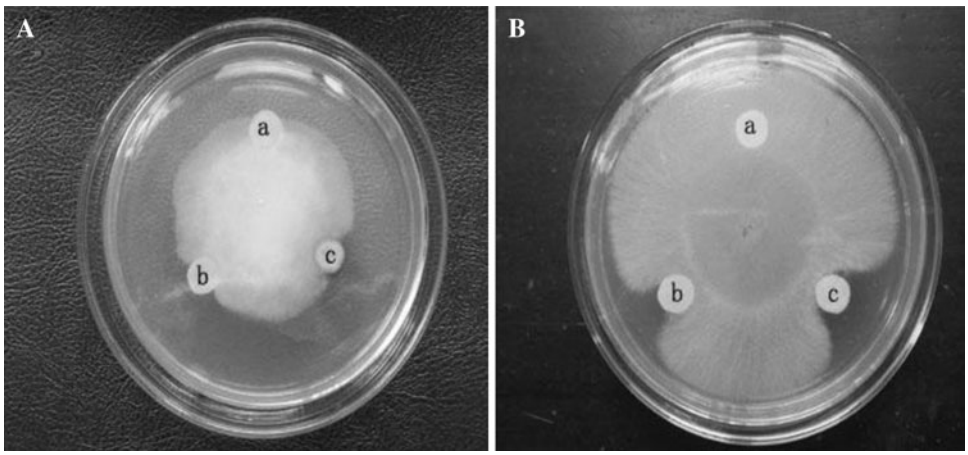

Fig. 7 Inhibitory activity of *Momordica charantia L* lysozyme toward *Mucor racem osus* (Fig. 7a)and *Rhizoctonia solani* (Fig. 7b). **a** 0.02 M phosphate-buffered saline, pH 7.0, **b** 75 μg lysozyme, and **c** 380 μg lysozyme

bonds, releasing N-acetylglucosamine and N-acetylmuramic acid [2–6]. The spectrum of antibacterial activity of lysozyme may be extended thanks to modifications of the enzyme. It was found that modified egg white lysozyme exhibit effective action against Gram-negative bacteria *Pseudomonas fluorescens,* and more effective action against bacteria *Staphylococcus epidermidis* was observed [27, 28]. Lysozyme with antibacterial activity shows potential in food technology as antibacterial agent in milk products and in wine production processes, and in pharmacological technology as a drug for treatment of ulcers and infections [4, 5].

Antifungal activity

The antifungal activity of *Momordica charantia L* lysozyme on *Mucorracem osus* (Fig. 7a) and *Rhizoctonia solani* (Fig. 7b) is illustrated. It can be seen that the protein showed obvious antifungal activity toward *Mucorracem osus* (Fig. 7a) and strong antifungal activity toward *Rhizoctonia solani* (Fig. 7b). The IC_{50} value of the antifungal activity toward *Rhizoctonia solani* was calculated to be 12.3 μM, as shown in Fig. 8.

Antibacterial activity of lysozyme against Gram-positive and Gram-negative bacteria has been well studied, and it has been widely developed in agricultural and industrial applications [2, 4, 5]. However, relatively limited information is available on its antifungal activity. There are several literatures regarding its lytic activity against fungal pathogens, such as *P.nicotianae*, *F. oxysporum*, *P.aphanidermatum*, *B. cinerea*, and *C. albicans* [3, 15, 16, 26]. After we reported the mung bean lysozyme with antifungal activity 5 years ago [3], we now further report a novel plant lysozyme with N-terminal amino acid sequencing. It demonstrates antifungal activity against *Mucorracem osus* and *Rhizoctonia solani,* and antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. The present investigation suggested the new purified lysozyme exhibits its specific but some limited antifungal spectrum; the spectrum of antifungal activity may be extended through modifications of the enzyme in the further study.

In conclusion, a plant lysozyme has been isolated from *Momordica charantia L*. The purified lysozyme was monomer protein with a molecular mass of 20.1 kDa, and its pI was estimated to be 9.9. The lysozyme exhibited a pH optimum at

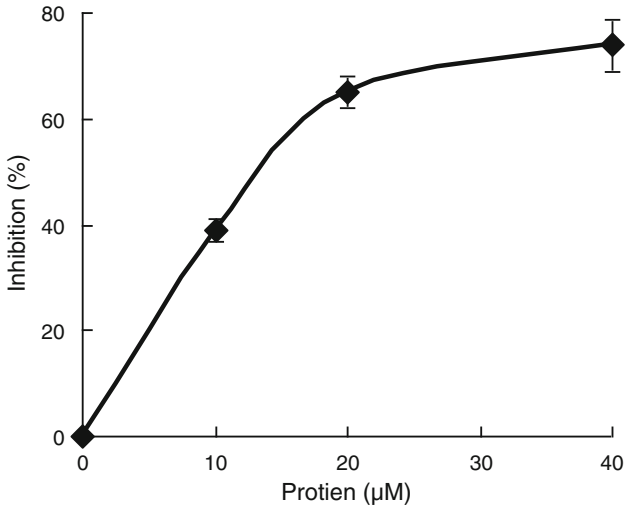

Fig. 8 Determination of the IC_{50} value of the antifungal activity of *Momordica charantia L* lysozyme toward *Rhizoctonia solani*. Each data point represents mean ± SD of triplicate inhibition radius of mycelia. The IC_{50} was calculated to be 12.3 μM

pH 5.5 and a temperature optimum at 50 °C. It first revealed that the lysozyme of *Momordica charantia L* demonstrated antifungal activity and antibacterial activity. This report may provide more evidence to understand the complicated characteristics of lysozyme family.

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