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Expression and Purification of Tobacco PR-1a Protein for Function Analysis

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Pathogenesis-related proteins are assigned an important role in plant defense and in general adaptation to stressful environment. Although tobacco PR-1a is the first pathogenesis-related protein to be purified and characterized, its function is the least known of all PR-families. In the present study, coding sequence for tobacco mature PR-1a protein was amplified and subcloned in pGEX-5X-3 expression vector to overexpress soluble GST-PR1a fusion protein (42 kDa) in *Escherichia coli*. The cleaved PR-1a protein (15.5 kDa) was isolated after removal of GST-tag by Factor Xa. Purified recombinant GST-PR1a protein was used to prepare antiserum which can be used to detect the native tobacco PR-1 in the acidic extract of the TMV-infected leaves. Antifungal assay *in vitro* showed that both GST-PR1a and cleaved PR-1a proteins had antifungal activity against *Phytophthora infestans*, suggesting that the free N-terminus is not necessary for PR-1a in its antifungal activity. These results provide some clues for investigating the action mechanism of PR-1a.

Key Words: PR-1a, Pathogenesis-related protein, Antifungal activity, *Escherichia coli* expression, Antiserum.

INTRODUCTION

A group of plant-coded proteins induced by different stress stimuli, termed as pathogenesis-related proteins (PRs) is assigned an important role in plant defense against pathogenic constraints and in general adaptation to stressful environment[1]. The recognized PR proteins have been extensively reviewed[2-6] and recently classified into 17 families based on their primary structure, serological relationships and biological activities[7]. Several types of proteins are common, while others have so far been found to occur more specifically in some plant species.

The PR-1 proteins almost represented in every plant species researched up to the present[7,8]. The acidic tobacco PR-1a was the first pathogenesis-related protein to be purified and characterized in 1978[9] and has been regarded as the type member of the PR-1 proteins[10]. In tobacco and tomato, PR-1 proteins belong to small multigene families[8]. PR-1 proteins are extractable and stable at low pH (< 3), thermostable and highly resistant to protease. Comparative analysis find that the PR-1

family shows a high level of sequence conservation throughout different plant families and contains 6 conserved cysteines forming three disulphide bridges[8]. Fernández *et al.*[11] solved the structure of tomato PR-1b (P14a) by nuclear magnetic resonance and found that the protein contained four α-helices and four β-strands arranged antiparallel between helices. Edreva[1] suggested that the high stability and the resistance to several proteases of PR-1 proteins were probably determined by that unique compact molecular structure.

Many researchers have tried to assess the function of PR-1 proteins in plants. Earlier studies found that transgenic plants expressing the PR-1 cDNA had no resistance against tobacco mosaic virus, alfalfa mosaic virus and potato virus Y, respectively[12-14]. Subsequently, some data suggested that PR-1 members had antifungal activity. It was reported that transgenic tobacco plants constitutively expressing the PR-1a gene exhibited significantly increased tolerance to two oomycete pathogens, *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae*[14]. Direct antifungal activity of tomato PR-1 was demonstrated both *in vitro* as an inhibition of germination of *P. infestans* zoospores and *in vivo* as a reduction in the surface area of leaf discs infected with this fungus. Results showed that the basic tomato PR-1c and tobacco PR-1g proteins had the highest antifungal activities, while the acidic tobacco PR-1a showed lower but significant antifungal activity[15]. Overexpression of a TMV-inducible basic PR-1 gene from pepper enhanced tolerance to *P. parasitica* var. *nicotianae*, *Ralstonia solanacearum* and *P. syringae* pv. *tabaci*[16].

Surprisingly, the prominent PR-1 proteins are often used as markers of the enhanced defensive state conferred by pathogenesis-induced systemic acquired resistance (SAR), but its function is the least known of all PR-families. The mechanism of its action as well as the cellular and molecular targets of PR-1 protein is still not known[1,7]. In order to investigate whether the N-terminus of PR-1a play an important role in its antifungal mechanism, we expressed, purified recombinant PR-1a from *E. coli* and carried out antifungal assay *in vitro*.

EXPERIMENTAL

Bacterial strains, plasmid, fungus and reagents: *Escherichia coli* strain JM109 and BL21 (DE3) preserved in this laboratory were used as host. The expression vector pGEX-5X-3 containing *Schistosoma japonicum* 26 kDa glutathione transferase was purchased from Amersham Bioscience (USA). *Phytophthora infestans* used for antifungal assay was kindly supplied by Shui-ying Yang (College of Plant Protection, Southwest University, Chongqing, China). Tobacco PR-1a clone was gift from Leslie Friedrich. *Taq* DNA polymerase and T4 DNA ligase were purchased from Stratagene. The Gel Extraction Kit, the Plasmid Mini Kit, restriction enzymes and DNA Markers were purchased from TaKaRa (Dalian, China). Factor Xa Cleavage Capture Kit was obtained from Novagen (Germany). Isopropyl β-D-thiogalactoside (IPTG), ampicillin and reduced glutathione were from Amresco. Leupeptin, antipain, DTT and PMSF were from Sigma (St. Louis, USA). Glutathione Sepharose 4 Fast

Flow affinity column was supplied by Amersham Biosciences (USA). Primers synthesizing and DNA sequencing were performed by Invitrogen Biotechnology Co., Ltd. (Shanghai, China). All other reagents were of research grade or better and were obtained from commeraial sources.

Construction of expression vector: pBluescript SK vector containing the cDNA sequence of tobacco PR-1a gene (Gen Bank accession number X06930) was used as the template to amplify the coding sequence of mature tobacco PR-1a corresponding with 136 amino acids (signal sequence not included). The engineering was achieved by PCR amplification using forward primer 5'-CGCGGATCCCCCAAAATTCTCAACAAG-3' and reverse primer 5'-CCGCTCGAGTTAGTATGGACTTTCGCCTCT-3'. The forward and reverse primers included the sites of *Bam*H I and *Xho*I (underlined), respectively. The conditions for PCR were 94 °C, 1 min; 61 °C, 1 min; 72 °C, 1 min for 35 cycles. The amplification products of PCR were purified and digested with *Bam*H I and *Xho*I and then ligated into GST fusion expression vector pGEX-5X-3 which had been double digested with the same restriction enzymes. The constructs were transformed into *E. coli* JM109 competent cells to construct recombinant plasmid pGEX-PR1a. Transformants were selected on LB plates containing ampicillin (100 μg/mL). Desired clones were confirmed by restriction and DNA sequence analyses. All of DNA manipulations above were performed as described by Sambrook *et al.*[17].

Expression and purification of GST-tagged proteins: The recombinant plasmids pGEX-PR1a were transformed into *E. coli* strain BL21 (DE3) and transformed cells were grown on LB agar plate containing ampicillin (100 μg/mL) overnight at 37 °C. To choose the more efficient colonies, a small-scale expression was carried out and the most efficient colony was screened for the fusion protein expression henceforward. The efficient single colony was incubated overnight at 37 °C in fresh liquid LB medium containing 100 μg/mL ampicillin with vigorous agitation. One mL of overnight culture was used to inoculate 100 mL of LB containing 100 μg/mL ampicillin and the culture was grown at 37 °C with vigorous agitation. When the optical density of cells at 600 nm reached 0.6, IPTG was added to the culture at a final concentration of 1.0 mM. The bacterial cells were incubated for 4 h at 29 °C under stirring (200 rpm) and were harvested afterwards by centrifugation at 8000 rpm for 10 min at 4 °C. The cell pellets were resuspended in ice-cold lysis buffer (140 mM NaCl, 2.7 mM KCl, 5 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 10 μg/mL leupeptin, 10 μg/mL antipain, 1 mM PMSF, 1 mM DTT, 5 mM EDTA, pH 7.2) and then sonicated on ice in short burst. Triton-X-100 was added to 1 % final concentration (v/v) and the solution was gently stirred in ice-water for 1 h to facilitate solubilization of proteins. The supernatant was then collected by centrifugation at 8000 rpm for 15 min at 4 °C and was applied to a Glutathione Sepharose 4 Fast Flow affinity column equilibrated in advance. After the target protein was bound to the matrix, the column was washed with PBS containing 0.5 M NaCl to remove non-specifically bound protein. The target protein was eluted with 10 mM reduced glutathione. The

purified protein was supplemented with 10 % glycerol, frozen in liquid nitrogen and then transferred to -80 °C for following experiments.

Cleavage of recombinant protein: The GST-tag of the fusion protein was removed by digesting the fusion protein with Factor Xa according to instructions provided by Novagen. Each 500 μg portion of fusion protein was incubated with Factor Xa (10 units) for 12 h at 21 °C in 1×Factor Xa cleavage/capture buffer containing 50 mM *Tris*-HCl, pH 8.0, 100 mM NaCl and 5 mM $CaCl_2$. After the cleavage reaction, Factor Xa could be quantitatively removed with agarose Xarrest™. All protein samples were dialyzed with 50 mM *Tris*-HCl, pH 8.0 overnight at 4 °C and concentrated to about 1 mg/mL protein. The protein concentration and the purity were assessed by the method of Bradford and SDS-PAGE, respectively.

Production and identification of antiserum against GST-PR1a: Antibodies against GST-PR1a were raised in rabbits. The purified GST-PR1a was emulsified in Freund's complete adjuvant for the first injection and for the subsequent three booster injections in incomplete adjuvant. 400 μg of pure GST-PR1a antigen were used for the first injection and 200 μg were used for the others. After 5 weeks, the last booster immunization, the serum was collected. It was placed without any motion for 1 h at room temperature and then at 4 °C overnight. After clot removal, serum was clarified by centrifugation and stored in small batches at -80 °C. The verification of antibodies activity and specificity were characterized by double immunodiffusion and western blotting.

Extraction of tobacco acidic pathogenesis-related proteins: Glasshouse-grown tobacco plants (8 weeks old) were inoculated with TMV and control plants were mock-inoculated with10 mM sodium phosphate. After 10 d, the lower leaves were inoculated with TMV or mocked-inoculated with 10 mM sodium phosphate, leaves were removed and extracted with buffer (71 mM citric acid, 32 mM K_3PO_4, 33 mM 2-mercaptoethanol, 2 mM ascorbic acid, pH 2.8) for acidic PR-proteins[18]. After centrifugation at 12000 g for 20 min, the supernatant was dialyzed to 10 mM *Tris*-HCl, pH 8.3[19].

Electrophoresis and western blotting: Proteins were separated on 12 % SDS-PAGE gel electrophoresis. Protein samples were denatured in sample buffer containing 5 % β-mercaptoethanol for 5 min at 100 °C and then centrifuged at 10,000 g for 5 min before loading the gel. After SDS-PAGE, proteins in gel were transferred to PVDF membrane using Mini Transfer-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories) at 80 mA for 180 min at 4 °C[20]. The transfer buffer was 25 mM *Tris*, 190 mM glycine and 20 % methanol. The blot was incubated 4 h at room temperature in TBST (20 mM *Tris*-HCl, 100 mM NaCl, 0.1% Tween 20, pH 7.6) containing 5 % non-fat powdered milk. Rabbit antiserum against GST-PR1a was diluted 1:1000 in TBST to detect PR-1a in the extract from tobacco leaves. After incubating the blot for 1 h at room temperature, it was washed three times with TBST. The blot was incubated with a 1:10000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Invitrogen) for 1.5 h at room temperature. The blot was washed 3 times

with TBST and the secondary antibody was detected using ECL Chemiluminescent Substrate (Amersham Bioscence, USA) according to manufacturer's instructions.

Antifungal assays: *Phytophthora infestans* was grown on potato tuber slices at 18 °C in the dark. Mycelium was collected after 7 d and sporangia were washed out from the mycelium with cold water. The washing fluid was then incubated at 18 °C in the dark to allow zoospore release. The sporangia were separated from swimming zoospores by gentle centrifugation (30 g). The protein solutions to be assayed for antifungal activity were mixed with 2×10^4 zoospores/mL in a final volume of 20 µL on depression glass slides and incubated overnight at 18 °C and 100 % humidity in the dark. Germination inhibition was observed under a light microscope and inhibitory activities of proteins were compared to control samples[15]. Buffer was used as negative control.

RESULTS AND DISCUSSION

The N-terminal region of the primary PR-1a translation product in tobacco contains a signal sequence which would be cleaved upon maturation. In this assay, the PR-1a fragment PCR cloned did not contain this signal sequence. The coding sequence of mature tobacco PR-1a (0.43 kb) was successfully amplified from the cDNA clone of tobacco using the forward and reverse primers containing a *Bam*H I restriction site at the 5'-end and an *Xho*I site at the 3'-end of their sequences. The amplified production was subcloned in pGEX-5X-3 vector, inserted into *E. coli* strain JM109 first. As shown in Fig. 1, the exact cDNA fragments were observed when the recombinant plasmids were digested with corresponding restriction enzymes. Furthermore, DNA sequencing of both strands showing that the desired insert was successfully cloned in the pGEX-5X-3 expression vector and in the correct reading frame. Then the correct constructs were transformed into *E. coli* strain BL21 (DE3) to overexpress the recombinant GST-tag proteins.

The overexpression of recombinant proteins in transformed cells was analyzed by SDS-PAGE. After IPTG induction, there was an obvious extra band around the molecular weight of 42 kDa (Fig. 2, Lane 3), which was consistent with the expected molecular weight of GST-PR1a. When expressed at 29 °C, many GST- PR1a fusion proteins existed in the soluble expression fraction (Fig. 2, Lane 4). Then the fusion proteins could be purified by one-step Glutathione-Sepharose affinity chromatography and a pure preparation of 42 kDa was obtained (Fig. 2, Lane 5). The yield was *ca.* 6 mg of recombinant proteins/1 L of bacterial culture.

Using a specific protease, Factor Xa whose cleavage site located between the GST moiety and the PR-1a protein, the recombinant protein was divided into two portions (Fig. 3, Lane 2). Then Factor Xa was captured by agarose and the GST moiety was removed by rechromatographing the sample on the glutathione Sepharose column. Finally, the protein preparation (15.5 kDa), named cleaved PR-1a was obtained after the N-terminal GST moiety was removed from the protein with the apparent homogeneity, as shown in Fig. 3, Lane 4.

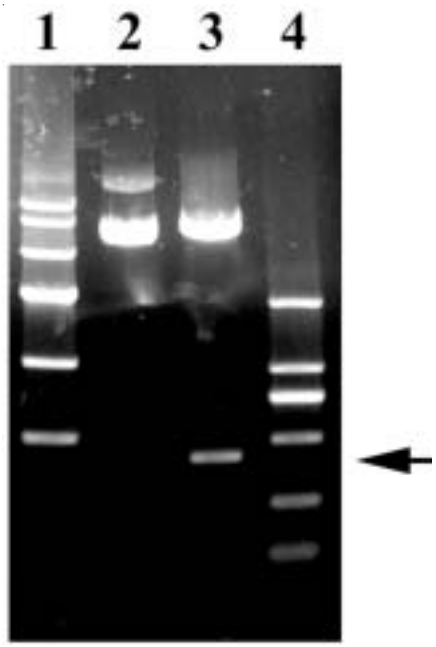


Fig. 1. Verification of positive clone with pGEX-PR1a by double enzymes digestion; **Lane 1**, Marker IV; **Lane 2**, recombination plasmid pGEX-PR1a; **Lane 3**, product of pGEX-PR1a digested with *Bam*H I and *Xho*I; **Lane 4**, DL 2 000 DNA marker

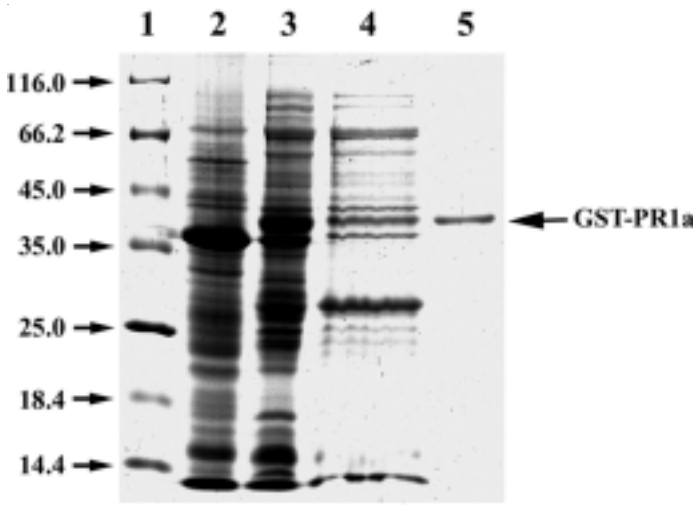


Fig. 2. SDS-PAGE analysis of recombinant GST-PR1a protein; **Lane 1** is protein molecular weight markers: 116.0, 66.2, 45.0, 35.0, 25.0, 18.4 and 14.4 kDa, from top to bottom; **Lanes 2 and 3** were total bacterial protein from BL21 (DE3) containing plasmid pGEX-PR1a without and with IPTG induction respectively; **Lane 4**, the soluble fraction from centrifugation of induced sonicated cells; **Lane 5** is the purified GST-PR1a fusion protein

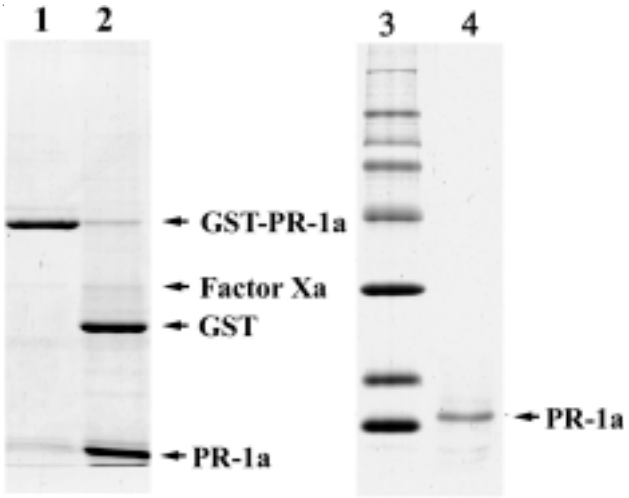


Fig. 3. SDS-PAGE analysis of GST- PR1a fusion protein digested by Factor Xa; **Lane 1**, purified GST-PR1a fusion protein; **Lane 2**, cleavage of the 42 kDa GST-PR1a produced a 26.5 kDa GST and a 15.5 kDa PR-1a product; **Lane 3**, low molecular mass protein marker; **Lane 4**, the purified PR1a protein

Antibodies against GST-PR1a were raised in rabbits. The titer of antiserum characterized by double immunodiffusion was 1:32. When the antiserum reacted with the extract of Samsun NN tobacco leaves inoculated by TMV, two bands around 17 kDa were detected as Fig. 4, Lane 4, while no band was shown in negative control of the extract of mock-inoculated tobacco leaves (Fig. 4, Lane 3). The extract of TMV-infected Samsun NN tobacco leaves contains acidic pathogenesis-related proteins (Fig. 4, Lane 2). According to Van Loon *et al.*[21], molecular mass of Samsun NN tobacco PR-1a and -1b were both 17 kDa while that of PR-1c was 16.5 kDa. Because tobacco acidic PRs of group 1 (PR-1a, -1b and -1c) were shown to belong to a serological group in tobacco and to be serologically related to PRs from other plant species[22], the antiserum against GST-PR-1a can react with all of them. So, the upper band immunodetected was PR-1a and -1b, the lower was PR-1c of Samsun NN tobacco (Fig. 4, Lane 4).

To test antifungal activities of recombinant GST-PR1a proteins before and after the remove of GST-tag, we monitored the degree of inhibition of *Phytophthora infestans* zoospore germination *in vitro*. As a control, most of zoospores germinated in the PBS buffer pH 7.2 (Fig. 5A). However, it was observed that the germination of the zoospores and germ-tube length were reduced in the presence of GST-PR1a at a concentration of 600 μg/mL (Fig. 5B). Furthermore, in the presence of 250 μg/mL cleaved PR-1a, the germination of the zoospores was almost stopped completely (Fig. 5C). The effect on growth inhibition of *P. infestans* with increasing concentrations of GST-PR1a and cleaved PR-1a protein were shown in the Fig. 6. IC_{90} value of cleaved PR-1a protein was found to be about 250 μg/mL (Fig. 6B).

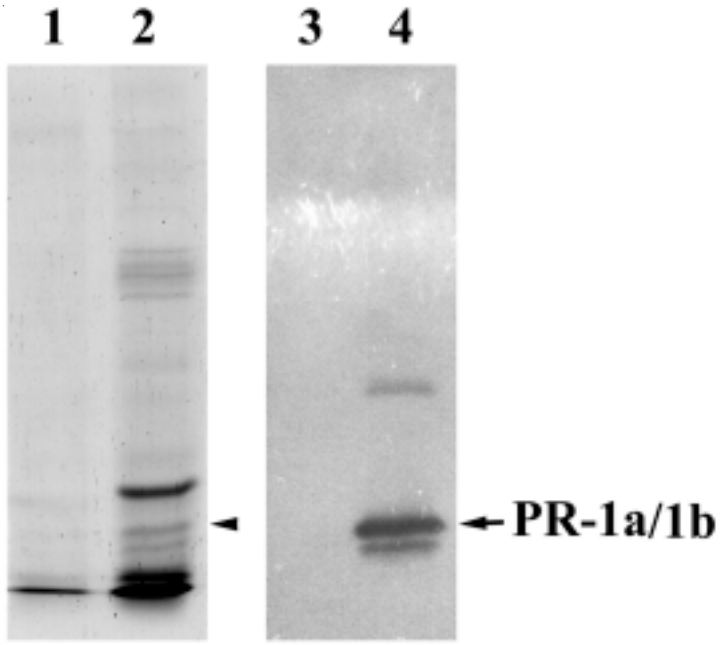
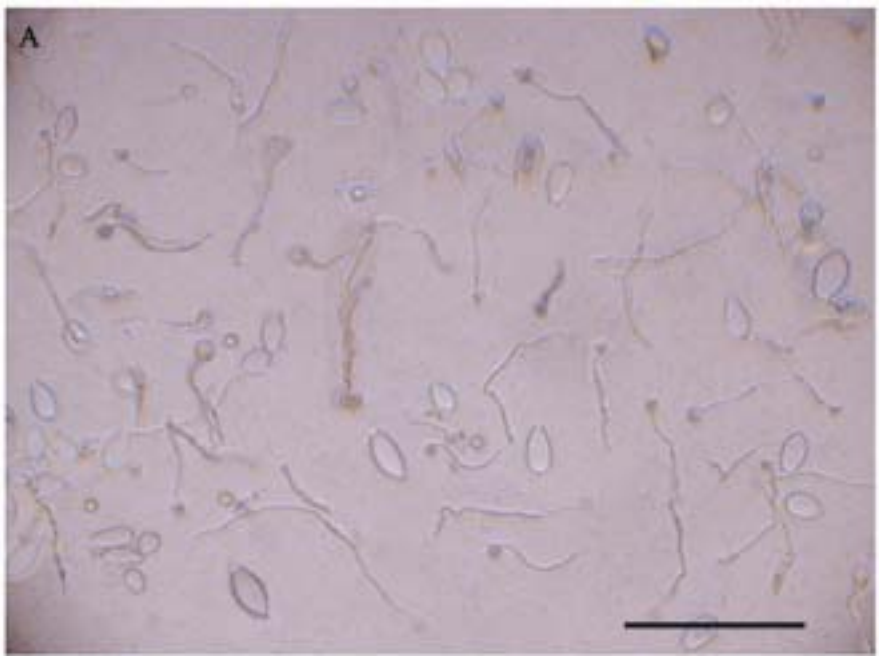


Fig. 4. SDS-PAGE analysis and western blotting; **Lanes** 1and **3**: acidic extract of the mocked tobacco leaves; **Lanes 2** and **4**: acidic extract of the TMV infected tobacco leaves; **Lanes 3** and **4**: western blotting analysis with the antiserum against GST-PR1a



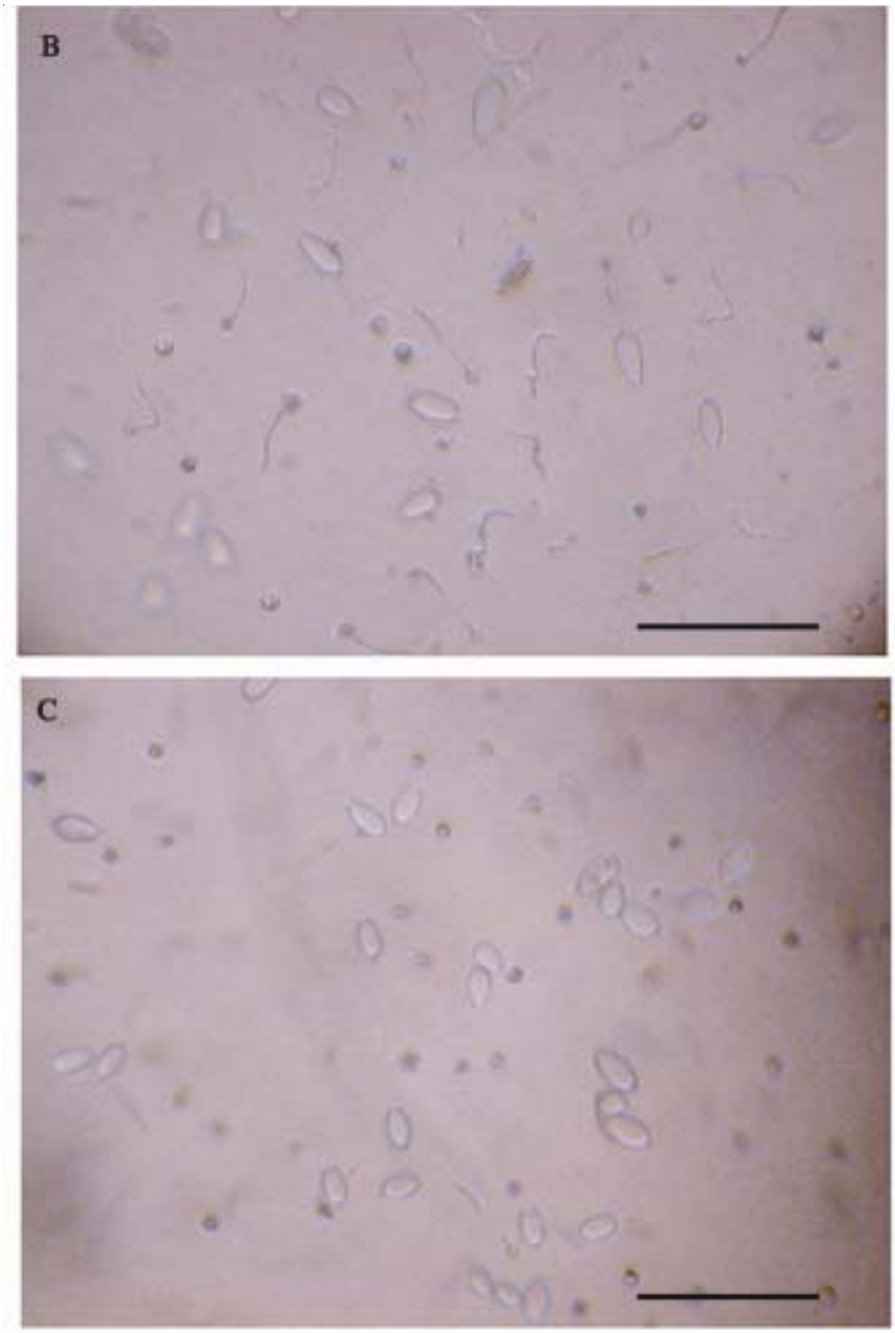


Fig. 5. *In vitro* inhibitory activity on the release and germination of zoospores of *P. infestans*. Bars, 80 μm; (A) PBS buffer (pH 7.2); (B) GST-PR1a (600 μg/mL); (C) cleaved PR-1a (250 μg/mL) were added to 4×10^4 sporangia per mL.

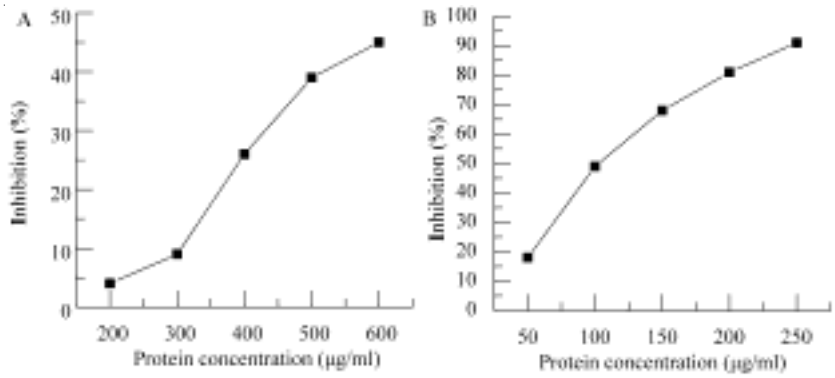


Fig. 6. Dose-response curve of inhibition of *P. infestans* zoospore germination (A) GST-PR1a, (B) cleaved PR-1a proteins

In present expression, due to the cloning procedure, cleaved PR-1a contained glycine, isoleucine and proline residue additionally at the N-terminal region, compared with the native tobacco PR-1a protein. Antifungal analysis demonstrated that cleaved PR-1a protein could stopped the germination of *P. infestans* zoospore. Niderman *et al.*[15] reported that the purified PR-1a protein from tobacco leaves infected with TMV was able to inhibit the germination of *P. infestans* zoospore almost completely *in vitro* at a concentration of 200 μg/mL. It exhibited that the cleaved PR-1a protein had antifungal property similar to that of the purified native PR-1a. The effectiveness of PR-1a with three N-terminal amino acid residues in inhibiting *P. infestans* suggests that the free N-terminus is not necessary for PR-1a in the antifungal activity. However, Caruso *et al.*[23] found that recombinant wheat PR4 proteins which contain an additional N-terminal methionine residue due to the cloning procedure lose the activity in inhibiting hyphal growth. After removal of the N-terminal methionine, they were able to inhibit the fungus growth with the same efficiency with respect to the native proteins, which suggests that the N-terminal region may play an important role in the antifungal mechanism of wheat PR4 proteins.

It was noted that GST-PR1a protein had less inhibition against *P. infestans* than the cleaved PR-1a protein, since 600 μg/mL GST-PR1a protein, equal to containing about 200 μg/mL PR-1a protein, only caused 45 % growth inhibition. However, the cleaved PR-1a protein at a concentration of 200 μg/mL inhibited the germination of zoospores by 81 %. These results suggest that the GST-tag in the recombinant protein probably interfere the folding of PR-1a giving raise to the decrease of antifungal activity.

Present finding suggests that the recombinant PR-1a with three additional N-terminal amino acid residues had antifungal property against *P. infestans* similar to that of the native PR-1a provides some clues for investigating the action mechanism of PR-1a. Furthermore, antiserum against GST-PR1a was obtained. It can be used further for GST pull-down assay to detect the targets of PR-1a.

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