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Study of antimicrobial activities of chitinases from a potato prototype cultivated in Bangladesh

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ABSTRACT

Chitinases (designated as SPCs) were isolated from 'Shilbilati' potatoes, a potato prototype cultivated in Bangladesh by affinity chromatography on a chitin column. SPCs agglutinated rat erythrocytes at the minimum concentration of 7 µg/mL and showed toxicity against brine shrimp nauplii with the LC_{50} value of 20 µg/mL. The chitinases also agglutinated seven bacterial strains among the twelve as studied. *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Salmonella typhi* were the most sensitive towards the SPCs and were agglutinated at 1.2, 2.5 and 5.0 µg/mL protein concentrations respectively. Antibacterial tests demonstrated that SPCs showed inhibitory activity against the pathogenic bacteria *Staphylococcus aureus*, *Bacillus subtilis and Salmonella typhi*. Antifungal activity was investigated by the disc diffusion method. Five fungal species (*Candida albicans*, *Aspergillus niger*, *Fusarium vasinfectum, Aspergillus fumigatus* and *Aspergillus flavus*) and two fungal genus (*Penicillium* and *Mucor* sp.) were examined in the assay. SPCs showed antifungal activity against *Candida albicans*, *Fusarium vasinfectum* and *Penicillium* sp.

Keywords: lectins, antibacterial, antifungal, toxicity, hemagglutination

INTRODUCTION

Microorganisms are frequently a cause of prevailing diseases in developing countries. The economic crisis, inefficient public access to medical and pharmaceutical care, emergence of resistant strains, drug toxicity and the side-effects caused by synthetic drugs are some of the main factors influencing the search for the natural products having antimicrobial and antifungal activities.

For the past twenty years there has been an increased interest in the investigation of natural materials. Some of those have been approved as new antimicrobial drugs, but there is still an urgent need to identify novel substances that are active towards pathogens with high resistance (Recio, 1989; Cragg *et al.,*1997). Potato (*Solanum tuberosum*, Solanaceae), is the only major tuber crop that is grown in temperate regions. It is also the most important tuber crop in terms of production, accounting for about 45% of the total world production of all tuber crops (Shewry, 2003). Varieties of potatoes are cultivated worldwide and a number of chitin-binding proteins have been identified in potatoes (*Solanum tuberosum* L.) on the basis of their affinity, enzymatic properties or amino acid sequence (Allen and Neuberger, 1973; Desai and Allen, 1979; Kilpatrick, 1980; Matsumoto *et al.*, 1980; Owens and Northcote, 1980; McCurrach and Kilpatrick, 1986; Millar *et al.*, 1992; Pramod and Venkatesh, 2006). A major chitin binding component of potato is the lectin, which is a glycoprotein with an unusual amino acid composition characterized by a very high content (up to 30%) of hydroxyproline (Van Damme *et al.*, 2004).

Recent publications suggest that plant extracts may be used as microbicidal agents *in vitro*. A novel antimicrobial protein (AP1) was purified from leaves of the potato (*Solanum tuberosum*, variety MS-42.3) with a procedure involving ammonium sulphate fractionation, molecular sieve chromatography with Sephacryl S-200 and hydrophobic chromatography with Butyl-Sepharose using a FPLC system. The inhibition spectrum investigation showed that AP1 had good inhibition activities against five different strains of *Ralstonia solanacearum* from potato or other crops, and two fungal pathogens, *Rhizoctonia solani* and *Alternaria solani* from potato (Feng *et al.,* 2003). The present study was undertaken to investigate the potential antimicrobial activity of the chitinases on a panel of microorganisms obtained from the potato prototype cultivated in Bangladesh.

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MATERIALS AND METHODS

Isolation of lectins

The edible portion of the potato prototype named 'Shilbilati' cultivated in the Rangpur region of Bangladesh was homogenized in 50 mM Tris-HCl buffer (pH 8.0) containing 1% NaCl and 0.02% sodium metabisulfite. The homogenate was centrifuged at 17,000 rpm for 15 min and the supernatant was collected and subjected to affinity chromatography on a chitin column (2 × 25 cm), previously equilibrated with 10 mM Tris-HCl buffer, pH 8.0. The column was first washed by 10-15 volume of 10 mM Tris-HCl buffer, pH 8.0 and then followed by 2 volume of distilled water. Finally the proteins were eluted by 0.5 M acetic acid. The eluted fraction was dialyzed against distilled water for overnight. Protein was estimated by the Lowry method using lipid-free BSA as the standard.

Hemagglutination assay

The hemagglutination assay was performed in 96-well microtiter U-bottomed plates in a final volume of 100 µL containing 50 µL of 2% suspension of albino rat erythrocytes previously washed with 0.15 M NaCl and 50 µL of two-fold serially diluted lectin solutions. After gentle shaking, the plate was kept at room temperature for 30 min; the agglutination titer of the maximum dilution giving positive agglutination was recorded.

Brine shrimp nauplii lethality assay

Toxicity of the proteins was studied using brine shrimp nauplii (*Artemia salina* L). Ten shrimps in artificial sea water (pH 7.0) were transferred to 50 vials and Shilbilati potato chitinases (SPCs) was added at the concentration of 0.005-0.5 mg/mL. Finally the volume was adjusted to 3 mL by the addition of artificial sea water. All tests were performed at room temperature (around 30 °C), under a continuous light regime. Three replicates were used for each treatment and control. From these data, the percentage of mortality of the nauplii was calculated for each concentration and the LC_{50} values were determined using Probit analysis as described by Finney (1971).

Bacterial agglutination activity

The bacterial agglutinating activity was performed by *Bacillus cereus, Staphylococcus aureus*, *Bacillus megaterium*, *Sarcina lutea*, *Pseudomonas aeruginosa*, *Shigella shiga*, *Shigella dysenteriae*, *Shigella sonnei*, *Salmonella typhi*, and *Klebsiella* sp. Bacteria were grown in 37 °C overnight in nutrient broths (liquid nutrient medium) and collected by centrifugation at 4,000 rpm for 3 min, washed with 10 mM Tris-HCl and re-suspended in the same buffer. 50 µL of each bacterial suspension (2%) was mixed with a serial dilution of partially purified protein solutions to a final volume of 100 µL in 96-well microtiter plate. The plate was agitated for 2 min and the mixture was kept at room temperature for 2 h. Finally the bacterial agglutinating activity was monitored by a light microscope.

Antibacterial assay

Antibacterial activity of the chitin binding proteins was investigated by the disc diffusion method (Cole, 1994), performed using sterile-petri dishes (100 × 15 mm) containing 30 mL nutrient agar. Twelve species of pathogenic bacteria were seeded separately onto the surface of nutrient agar plates, followed by the placement of a sterile double filter paper disc (5 mm diameter) on the agar surface of each plate. An aliquot (10 µL) of each sample was then added to each disc and the bacterial cells were allowed to grow at 30 ^{0}C for 12 h. A transparent ring around the paper disc revealed the antimicrobial activity. Eleven bacterial species (*Bacillus cereus, Staphylococcus aureus, Bacillus subtilis, Bacillus megaterium, Sarcina lutea, Pseudomonas aeruginosa, E. coli, Shigella shiga, Shigella dysenteriae, Shigella sonnei, Salmonella typhi*) and one bacterial genus *Klebsiella* sp. were examined in the assay.

Antifungal assay

Antifungal activities of the chitin binding lectins were performed using sterile-petri dishes (100 × 15 mm) containing 30 mL potato dextrose agar. Fungal mycelia were placed over the solid potato dextrose agar and the sterile filter paper discs (5 mm in diameter) were distributed over the plate. Protein solutions at different concentration in Tris-HCl buffer (pH 7.8) containing 0.15 M NaCl (TBS) and the TBS as the control, were applied on each paper disc of Petri dishes. Incubation of the Petri dishes were carried out at 31°C until mycelial growth had enveloped discs containing the control and formed crescents of inhibition around the discs with antifungal samples. Five fungal species (*Candida albicans*, *Aspergillus niger*, *Fusarium vasinfectum*, *Aspergillus fumigatus* and *Aspergillus flavus*) and two fungal genus (*Penicillium* and *Mucor* sp.) were examined in the assay.

RESULTS AND DISCUSSION

Hemagglutination assay

SPCs were isolated from a variety of potatoes named Shilbilati, by using single step affinity chromatography on chitin column. The unbound fractions did not show any agglutination activity whereas the bound fraction (designated as SPCs) showed high agglutination activity against the albino rat erythrocytes with the minimum concentration of 7.0 µg/mL. The proteins might be included in the category of lectin as they agglutinated rat erythrocytes.

Brine shrimp nauplii lethality assay

Some lectins are toxic and showed toxicity against brine shrimp nauplii. Our present data showed the mortality rate of brine shrimp nauplii raised with the increase of the

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concentration of the lectin. In the present study 50% mortality of the nauplii occurs (LC_{50}) at 20 µg/mL concentration of SPCs (Figure 1), where as LC_{50} of MSL (mannose specific lectin) was 21.87 µg/mL (Absar *et al*., 2005). This result revealed that SPCs toxicity is similar to the MSL.

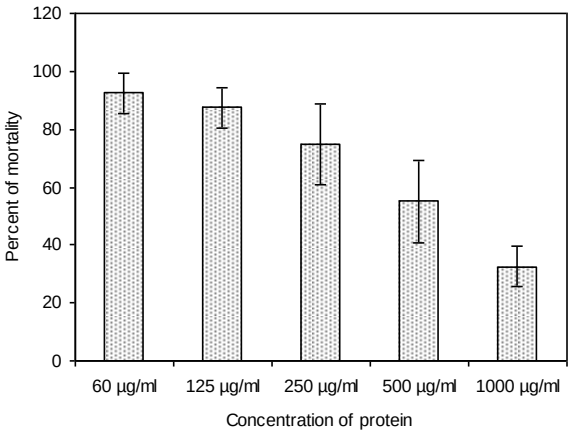

Figure 1: Percent of mortality of brine shrimp nauplii treated with SPCs solution after exposure for 24 h.

Bacterial agglutination activity

SPCs agglutinated both gram positive and gram negative bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella* sp., *Shigella shiga*, *Shigella dysenteriae*, *Shigella sonnei* and *Salmonella typhi*) as presented in Table 1.

It was reported that EuniS lectin agglutinated *Bacillus cereus*, *Staphylococcus* sp., *Bacillus subtilis*, *Pseudomonas aeruginosa* and *E. coli* (Oliveira *et al.*, 2008). The bacterial agglutination might be due to the presence of cognate glycan antigen on the cell surface of induced bacteria. The inhibition study suggested that the bacterial agglutination occurred as a result of the interaction of lectins with bacterial surface carbohydrates (Ghanekar and Pérombelon, 1980). The result showed that *Pseudomonas aeruginosa*, *Shigella dysenteriae, Salmonella typhi* and *Klebsiella* sp. were most sensitive towards SPCs and the minimum agglutination concentration were 1.2, 2.5, 5.0 and 10.0 µg/mL respectively. Agglutination of several bacterial strains confirms the interaction between the lectins and the strains.

Antibacterial assay

SPCs exhibited antibacterial activity against the tested pathogenic bacteria *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Salmonella typhi*. The antibacterial activity of the SPCs against *Salmonella typhi* is shown in Figure 2. A zone of inhibition is formed at a concentration of 4.0 mg/mL that gives higher inhibition than the zones formed at concentrations of 2.0 and 0.5 mg/mL of SPCs. So it is clear that the inhibitory zone became larger with the increase of concentrations of SPCs. Figure 2 also represents the antibacterial activity of SPCs against *Bacillus subtilis.* SPCs showed visible inhibitory zone at the concentration of 1.5 mg/mL. When the applied concentration was twofold of the previous one then the inhibitory zone increased remarkably. The antibacterial activity of SPCs against *Staphylococcus aureus* represented a different result from those of the above. It showed higher activity at a low concentration (1.5 mg/mL) as compared to that as observed at 3.0 mg/mL.

Table 1: Minimum concentrations of Shilbilati potato chitinases (SPCs) needed for visible agglutination of some pathogenic bacteria.

Bacterium	SPCs (µg/mL)
Bacillus cereus	-
Staphylococcus aureus	86
Bacillus subtilis	-
Bacillus megaterium	-
Sarcina lutea	-
Pseudomonas aeruginosa	1.2
Klebsiella species	10
Escherichia coli	-
Shigella shiga	86
Shigella dysenteriae	2.5
Shigella sonnei	43
Salmonella typhi	5

In recent years, it was reported that various plant extracts showed high antimicrobial activity (Sağdıç *et al.*, 2002). Lectins in higher plants defend against pathogenic bacteria and fungi by recognizing the infecting microorganisms via binding; thereby preventing their subsequent growth and multiplication (Etzler, 1986). It was reported that heat-stable serine protease inhibitor from the potato tuber inhibited the growth of a variety of bacterial strains, *Straphylococcus aureus* and *E. coli* (Kim, 2006); and EuniS lectin showed inhibitory activity against *Staphylococcus* sp., *Bacillus subtilis*, *E. coli* and *Pseudomonas aeruginosa* (Oliveira *et al.*, 2008).

Antifungal assay

SPCs showed antifungal activity against three fungi among the seven as studied. The inhibitory activity of the protein solution against *Candida albicans* on PDA was presented in Figure 3. A zone of inhibition is formed at a concentration of 2.5 mg/mL whereas in case of *Fusarium vasinfectum* and *Penicillium* sp. antifungal activities were found at a lower concentration of 1.5 mg/mL.

Chitin-binding lectins have been isolated from different sources; including bacteria, insects, plants and mammals (Campos-Olivas *et al.*, 2001; Rebers and Willis, 2001; Suzuki *et al.*, 2002; Van Dellen *et al.*, 2002) and

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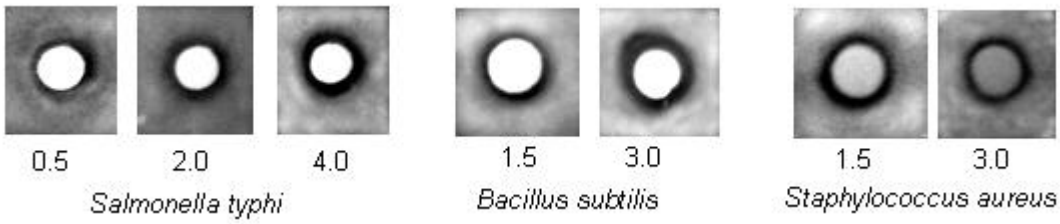

Figure 2: Antibacterial activities of SPCs towards *Salmonella typhi*, *Bacillus subtilis* and *Staphylococcus aureus* at different protein concentrations (0.5-4.0 mg/mL).

Figure 3: Antifungal activities of SPCs towards *Candida albicans*, *Fusarium vasinfectum* and *Penicillium sp.* at different protein concentrations (1.5 and 2.5 mg/mL).

most of them have shown the antifungal activity. It was reported that *Stinging nettle* rhizomes lectin, *Klaxveromyces bulgaricus* lectin, a lectin-like protein from *Amaranthus caudatus* and a chitinase-like lectin from *Urtica diocia* possessed the anti-fungal activities (Broekaert *et al.*, 1989; Verheyden *et al*., 1995). SPCs showed noticeable activities against *Candida albicans*, *Aspergillus fumigatus* and *Penicillium* sp. The anti-fungal activities of SPCs showed good correlation with the activities of chitinases studied previously (Broekaert *et al.*, 1989; Verheyden *et al.*, 1995; Campos-Olivas *et al.*, 2001; Rebers and Willis, 2001; Suzuki *et al.*, 2002; Van Dellen *et al.*, 2002). From the above study it is obvious that Shilbilati potato chitinases (SPCs) possessed antimicrobial activities.

CONCLUSIONS

In summary, a mixture of chitinases named as SPCs were isolated from the Shilbilati potato prototype. It possessed marked antimicrobial activity towards several bacterial and fungal species that is remarkable and hopefully it can be developed into an antimicrobial agent for the treatment of infectious diseases. Further studies might lead to the purification of novel lectins from this source and can be regarded as a more potent antimicrobial agent.

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