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Biochemical characterization of fruit-specific pathogenesis-related antifungal protein from basrai banana

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ABSTRACT

Pathogenesis-related/thaumatin like (PR-5/TL) antifungal protein from basrai banana was purified by using a simple protocol consisting of ammonium sulphate precipitation, affinity chromatography (Affi-gel blue gel), Q-Sepharose chromatography and gel filtration on Sephadex G-75. The purified protein with acidic character (pI 6.67) has molecular weight of 21.155 kDa, as determined by MALDI-TOF mass spectrometry. The purified protein shared N-terminal sequence homology with other TLPs. Crude banana extract inhibited the growth of *Fusarium oxysporum*, *Aspergillus niger*, *Aspergillus fumigatus* and *Trichoderma viride* with IC_{50} values (determined by Probit analysis) 15 μM (slope = 0.086, χ^2 = 17.843, P = 0.033), 17 μM (slope = 0.183, χ^2 = 61.533, P = 0.011), 6.5 μM (slope = 0.211, χ^2 = 14.380, P = 0.023) and 29.11 μM (slope = 0.072, χ^2 = 45.768, P = 0.014).

The purified antifungal protein repressed the growth of *F. oxysporum*, *A. niger*, *A. fumigatus* and *T. viride* with IC_{50} values 9.7 μM (slope = 0.056, χ^2 = 11.538, P = 0.021), 11.83 μM (slope = 0.127, χ^2 = 42.82, P = 0.00), 4.61 μM (slope = 0.150, χ^2 = 10.199, P = 0.017) and 21.43 μM (slope = 0.053, χ^2 = 33.693, P = 0.00), respectively. The IC_{50} values of antifungal activity of crude banana extract were higher than the purified antifungal protein. It indicated that proteins in crude banana extract have antagonistic effect on the fungal growth. White bread is particularly vulnerable by fungal pathogens. Purified antifungal protein suppressed the growth of *Aspergillus phoenicis* and *Aspergillus flavus* on white bread suggesting that this protein can be used as a preservative in the bakery industry as well as in other relevant food processing industries.

1. Introduction

Banana, the second largest fruit crop of Pakistan, was introduced in Sindh in 1913. There are approximately 300 species but only 20 varieties are commercially cultivated in the world. In Pakistan and especially in Sindh, the Cavendish variety, locally known as basrai gives the most outstanding results by holding about 98% of plantation. The banana crop is known to be much more vulnerable to diseases than to insect pests. There are various diseases which attack the crop and cause huge losses every year. Its major disease is called Panama, as it first originated in that area. Now it is known as *Fusarium* wilt a highly destructive and the most notorious of all plant diseases which affects the vascular system of banana. The causal organism is a soil-borne fungus, *Fusarium oxysporum* (Jeger et al., 1996).

Plants do not have an immune system as vertebrate do, they do possess a very sophisticated system to monitor and react when facing a biotic stress (Jones and Dangl, 2006). Coevolution of plants and pathogens has created a multifaceted relationship, resulting from the exchange of molecular information between the species (Benhamou, 1996). Based on this, plants have developed a complex surveillance system with an array of defense mechanisms. Pathogens, on the other hand, possess strategies to overcome the defense system and colonize plants. The difference between success and failure of plant defense is most likely dependent on the time it takes for the plant to recognize a potential pathogen and subsequently activate the defense system (Garcia-Brugger et al., 2006). Once the defense is alarmed a wide range of proteins is induced in the plant, among these the pathogenesis-related proteins (PR) (van Loon et al., 2006).

PR proteins are classified into 17 families (PR1–PR17) based on serological and amino acid sequence analyses (van Loon et al., 2006). When the host plant is infected by a pathogen, PRs accumulate at the infection site (Antoniw and White, 1983). The production of PRs is considered to be a biochemical mechanism of plant-induced resistance, with their expression being a symbol of plant disease resistance. Systemic acquired resistance (SAR) is a mechanism of induced defense that confers long-lasting protection against a broad spectrum of microorganisms. Molecularly, SAR induction is generally accompanied by the activation

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of a set of defense-related genes involved in the formation of chemical barriers. Of these, the pathogenesis-related genes have become a central issue of plant disease resistance research in recent years, with most studies focusing on *Arabidopsis* (Uknes et al., 1992), tobacco (Uknes et al., 1993), rice (Masuta et al., 1991; Agrawal et al., 2001; Mitsuhara et al., 2008), and wheat (Niu et al., 2007).

Based on their sequence similarity with the sweet-tasting thaumatin, all PR-5 proteins are designated thaumatin like proteins (TLPs), although none of these proteins has been described to have a sweet taste. According to their molecular mass, TLPs are grouped into two groups: one group of proteins has a size ranging from 22 to 26 kDa whereas the other group comprises proteins of 18 kDa. Members of the first group usually accumulate in cell vacuoles as opposed to members of the second group which are mainly found extracellularly (Chan et al., 1999). TLPs have also been discovered in animals, more specifically in nematodes and insects (Shatters et al., 2006), and in fungi (Greenstein et al., 2006). TLPs could play a defense role against pathogens in these organisms.

Some TLPs have been shown to display antifungal potential *in vitro* (Roberts and Selitrennikoff, 1990). Thaumatin-like antifungal protein from Emperor banana demonstrates antifungal activity against *F. oxysporum* and *Mycosphaerella arachidicola*. Its antifungal activity is more potent than the thaumatin-like proteins isolated from French bean legumes and kiwi fruits (Vincent et al., 2007). Zeamatin, a TLP isolated from *Zea mays* acts quickly as an antifungal agent by rupturing hyphal membranes in as little as 15 s (Roberts and Selitrennikoff, 1990). However, the precise mechanism behind the antifungal activity of some TLPs still remains unclear. A 20 kDa TLP, isolated from French bean acts as antifungal agent against *F. oxysporum*, *Pleurotus ostreatus* and *Coprinus comatus* but not against *Rhizoctonia solani* (Ye et al., 1999). Three isoforms, belonging to the TLP family, were identified in peach as principal allergens (Palacín et al., 2010). CkTLP antifungal protein isolated from *Cynanchum komarovii* Al Iljinski and showed antifungal activity against *Verticillium dahliae*, *F. oxysporum*, *Rhizoctonia solani*, *Botrytis cinerea* and *Valsa mali* (Qinghua et al., 2011).

The role of antifungal proteins as preservatives is significant in food processing industries. Fungi are able to destroy all kinds of food including cereals, meat, fruit, vegetables, nuts and fats. Especially fungi of the genera *Penicillium*, *Aspergillus* and *Fusarium* are involved in food spoilage in particular by secretion of highly poisonous mycotoxins. Extract of oat seeds is used as an antifungal agent in food preservation (Hans et al., 2010). We report here a protein in Cavendish variety, locally known as basrai, which exerts antifungal activity against a variety of fungal species and its use in food preservation.

2. Materials and methods

2.1. Isolation and purification of antifungal protein

Banana fruits were selected from Jinnah Garden Lahore, Botanical Garden University of the Punjab, Lahore and kept at 25 °C until the pulp was completely softened. Peeled ripe banana (2 kg) were immersed in 50 mM acetic acid, soaked overnight at 4 °C and homogenized with a mixer in a total volume of 4 L of the same solution. The homogenate was kept at 4 °C for 24 h to allow the foam to separate from the liquid. After removal of the foam, the extract was passed through muslin cloth, adjusted to pH 3.0 with 1 M acetic acid and centrifuged at 9000 × *g* for 15 min. The supernatant was filtered through Whatman 3MM paper.

2.2. Ammonium sulphate precipitation

The banana supernatant was subjected to ammonium sulphate fractionation. Ammonium sulphate was added to 85% saturation and stirred overnight at 4 °C. Tris–HCl buffer (pH 7.2) was added to the supernatant obtained after centrifugation of the extract until the concentration of Tris attained 20 mM before subjected to affinity chromatography.

2.3. Affinity chromatography

The crude banana extract, after ammonium sulphate precipitation, was fractionated on a (1.5 × 10 cm) column of Affi-blue gel (Cibacron Blue F3GA, BioRad, USA). The adsorbed proteins were eluted with 1.5 M NaCl in 10 mM Tris–HCl buffer (pH 7.2). The active fractions were dialyzed and subjected to ion exchange chromatography for further purification.

2.4. Ion-exchange chromatography

Q-Sepharose (Sigma chemical Co., USA) was packed in column (1.6 × 16 cm) and equilibrated with 50 mM Tris–HCl (pH 8.0). The bound proteins were eluted by using a linear NaCl concentration gradient (0–1 M) in 50 mM Tris–HCl buffer (pH 8.0) at 0.5 ml/min.

2.5. Gel filtration

The Sephadex G-75 (Sigma chemical Co., USA) was packed in column (1.6 × 70 cm) and equilibrated with 0.1 M phosphate buffer (pH 8.0). Elution was done with 0.05 M phosphate buffer (pH 7.0) at a flow rate of 35 ml/h. Fractions (0.5 ml) were analyzed for protein contents by 12.5–25% (w/v) SDS-poly acrylamide gel electrophoresis. Active fractions were concentrated by ultrafiltration in an Amicon ultrafiltration cell fitted with 10,000 MW cut off membrane (PTGC 043 Millipore Company Co., USA) under 50 pounds/sq. inch nitrogen pressure.

2.6. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted according to the method of Laemmli (1970) with 5% stacking gel and 15% resolving gel using a vertical electrophoresis system (Bio-Rad). After electrophoresis, the gel was stained in Coomassie brilliant blue R-250 solution prepared by dissolving 50 mg dye in methanol:acetic acid:H_2O (45:9:45) and distained with a mixture of 5% methanol and 7% acetic acid in water. The molecular mass of the antifungal protein was determined by comparison of its electrophoretic mobility with those of unstained protein ladder markers from Fermentas.

2.7. Amino acid sequence analysis

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and Mascot data base analysis (www.matrixscience.com) were employed to determine the molecular mass of the purified protein. The target protein was excised directly from the gel and submitted to Saint Andrews University, Scotland, UK for amino acid sequence analysis.

2.8. Antifungal activity assay

The assay for antifungal activity toward *F. oxysporum*, *Aspergillus niger*, *Aspergillus fumigatus* and *Trichoderma viride* was carried out in 100 × 15 mm Petri plates containing 10 ml of malt extract agar. After the mycelial colonies had developed, sterile blank paper disks (0.62 cm in diameter) were placed at a distance of 0.5 cm away from