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Full Length Research Paper

Primary purification of two antifungal proteins from leaves of the fig (*Ficus carica* L.)

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Low-molecular-weight extracts of fig (*Ficus carica* L.) leaves has antifungal and antibacterial activities against several types of microorganisms. In this work, two high-molecular-weight fractions with antifungal activity, termed figinI and figinII were obtained from leaves of fig using a procedure including ion-exchange chromatography (SP-Sepharose Fast Flow), hydrophobic-interaction chromatography (Phenyl Sepharose 6 Fast Flow and RESOURCE ISO) and ion-exchange chromatography (Mono S). By matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis (MALDI-TOF MS), the molecular mass of figinI was 21531Da and figinII was 31957Da. This is the first report on isolation of antifungal proteins from *F. carica* L., and it shows their potential for further investigation.

Key word: Fig, antifungal protein, chromatography, natural food preservative.

INTRODUCTION

Ficus carica Linn. is commonly referred to as "fig". It has many pharmacological effects, including anti-tumor, antioxidant, the ability to mediate body metabolism, hyperglycemia, hyperlipidemia and cholesterol levels, enhancement of oxidation resistance, antibiotic effects, antiviral properties, and the ability to mediate immunity and activate blood coagulation (Zhang and Jiang, 2006; Gilani et al., 2008). It has been traditionally used for its medicinal benefits as laxative, cardiovascular, respiratory, antispasmodic and anti-inflammatory remedies (Guarrera, 2005).

There are several reports about the antimicrobial effects of *F. carica*. In 2005, Zhao et al. reported that *F. carica* leaves extract has excellent antibacterial activity, and the minimal inhibitory concentrations (MIC) against *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcustet ragenus* and *Proteus vulgaris* is 0.025 g/ml and that against *Escherichia coli*, *Erwinia uredovora*, *Botrytis cinerea* and *Pestalotiopsis mangiferae* is 0.050 g/ml (Zhao et al., 2005). Antibacterial constituents were identified as bergapten and psoralen by spectral analysis (Zhao et al., 2005). In 2007, psoralen with high fungicidal activity was purified from *F. carica*. The half maximal effective concentrations (EC_{50}) of psoralen against *Valsa mali*, *Gibberella zeae* and *Fusarium oxysporum* is 0.07, 0.23 and 0.12 mg/ml, respectively (Hu et al., 2007). In 2009, antimicrobial potential of *F. carica* aqueous lyophilized extracts were tested against *Bacillus cereus*, *Staphylococcus epidermidis*, *S. aureus*, *E. coli* and *Pseudomonas fluorescens*, however no activity was noticed (Oliveira et al., 2009). Balestra et al. (2009) showed that *F. carica* extracts had antibacterial effects against tomato bacterial pathogens (*Pseudomonas syringae pv. Tomato*, *Xanthomonas vesicatoria* and *Clavibacter michiganensis subsp. Michiganensis*) and reduced disease incidence by 30% and disease severity by 22%. Recently, the antimicrobial proprieties against resistant human pathogens of methanolic, hexanoïc,

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Abbreviations: TL, Thaumatin-like proteins; **RIPs,** ribosome-inactivating proteins; **LTPs,** lipid-transfer proteins; **EDTA,** ethylenediaminetetraacetic acid; **DTT,** DL-dithiothreitol; **PVPP,** polyvinylpyrrolidone; **FPLC,** fast protein liquid chromatography; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **MALDI-TOF MS,** matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

chloroformic and ethyl acetate extracts of *F. carica* latex were investigated by Aref et al. (2010). The result showed that for the bacteria, the methanolic extract had effect only against *Proteus mirabilis*, while the ethyl acetate extract had inhibition effect on the multiplication of five bacteria species (*Enterococcus fecalis*, *Citobacter freundei*, *P. aeruginosa*, *E. coli* and *P. mirabilis*); for the opportunist pathogenic yeasts, ethyl acetate and chlorophormic fractions showed a very strong inhibition (100%); methanolic fraction had a total inhibition against *Candida albicans* (100%) at a concentration of 500 mg/ml and a negative effect against *Cryptococcus neoformans*. *Microsporum canis* was strongly inhibited with methanolic extract (75%) and totally with ethyl acetate extract at a concentration of 750 mg/ml. Hexanoic extract showed average results (Aref et al., 2010).

In this study, two protein fractions, isolated as single peaks by Mono S chromotography, were found to have antifungal activity against *Trichoderma viride*, and purified from fig leaves. The fractions were termed figinI and figinII. This work provides a foundation for further investigation.

MATERIALS AND METHODS

Materials

F. carica L. leaves were collected from the courtyard of the Institute of Plant Protection, Chinese Academy of Agricultural Sciences. Fungi were obtained from the National Key Laboratory for Biology of Plant Diseases and Pests, China.

Isolation procedure

The fig leaves (1177 g) were submerged in liquid nitrogen and ground to powder. Proteins were extracted from the powder after 3 h incubation with 4.0 L of extraction buffer (30 mM Na_2HPO_4, 15 mM NaH_2PO_4, 150 mM NaCl, 25 mM EDTA, 1.30 mM DTT, and 0.05 mM PVPP), pH 7.2 at 4°C. After centrifugation (12000 ×g, 20 min, 4°C), proteins in the supernatant were precipitated by adding ammonium sulfate to 40% saturation and centrifuging (12000 ×g, 20 min, 4°C). The precipitate was dissolved in, and dialyzed against 25 mM sodium acetate buffer (pH 5.0), and subjected to ion-exchange chromatography by fast protein liquid chromatography on a SP-Sepharose Fast-Flow (2.6 × 30 cm) column (GE Healthcare) equilibrated with the sodium acetate buffer. After eluting unabsorbed proteins, the column was eluted with 130 mM NaCl in sodium acetate buffer to yield adsorbed fractions. Fractions with antifungal activity were diluted with sodium acetate buffer with 1 M $(NH_4)_2SO_4$, and subjected to hydrophobic-interaction chromatography on a Phenyl Sepharose 6 Fast Flow (1.6 × 34 cm) (GE Healthcare) column equilibrated with 2 M $(NH_4)_2SO_4$ in sodium acetate buffer (pH 5.0). The column was eluted with a linear concentration (2-0 M) gradient of $(NH_4)_2SO_4$ in sodium acetate buffer. All fractions were analyzed for antifungal activity and active fractions were subjected to hydrophobic-interaction chromatography on a Resource ISO column (1 ml) (GE Healthcare). The column was eluted with a linear concentration (2-0 M) gradient of $(NH_4)_2SO_4$ in sodium acetate buffer. Fractions with antifungal activity were dialyzed against sodium acetate buffer and subjected to ion-exchange chromatography on a Mono S column (1 ml) (GE Healthcare), equilibrated with sodium acetate buffer and eluted with a linear concentration (0 - 0.12 M) gradient of NaCl in the sodium acetate buffer. Fractions with antifungal activity were resubjected to ion-exchange chromatography on the Mono S column to yield a single peak.

Molecular mass determination

The homogeneity of the purified antifungal proteins and their molecular mass were estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were revealed by 0.1% Coomassie Brilliant Blue staining. Molecular masses of figinI and figinII fractions were determined by comparing electrophoretic mobilities with molecular mass marker proteins from GE Healthcare.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was employed to determine the molecular mass of homogeneous proteins using an Applied Biosystems 4700 Proteomics Analyzer.

Antifungal activity assay

Antifungal activities against six plant fungal pathogens (*T. viride*, *B. cinerea*, *Phoma asparagi* Sacc, *Magnaporthe grissea*, *F. oxysporum f.sp. vasinfectum*, and *Sclerotinia sclerotiorum*) were assessed using sterile Petri plates (9 × 15 cm) with 15 ml potato dextrose agar. After mycelial colonies developed, an Oxford cup was laid 10 cm from the mycelial colony rim. Protein aliquots in 10 mM Tris-HCl buffer (pH 7.2) were applied to the cup, and plates were incubated at 28°C for 48 h until mycelial growth enveloped the periphery of the cup containing the control, and had produced crescents of inhibition around the cup with the antifungal samples.

RESULTS AND DISCUSSION

Protein isolation

Fig leaf extracts subjected to ion-exchange column chromatography on SP-Sepharose Fast Flow, yielded three fractions, an unabsorbed fraction and two adsorbed fractions S1 and S2 (Figure1a). The antifungal activity resided in fraction S1. Hydrophobic-interaction column chromatography of this fraction on phenyl-Sepharose 6 Fast Flow yielded five adsorbed fractions (h1-h5) (Figure 1b). Antifungal activity resided in h2 and h3, so h2 was subjected to hydrophobic-interaction column chromatography on RESOURCE ISO, yielding adsorbed fractions i1 and i2 (Figure 1c). Ion-exchange chromatography of i2 on a Mono S column and elution with a linear NaCl gradient yielded five adsorbed fractions (Figure 1d). Antifungal activity resided in the second fraction, named figinI. Hydrophobic-interaction chromatography of the h3 fraction yielded fractions i3 and i4 (Figure 1e). Ion-exchange chromatography on i4, as above, yielded four fractions (Figure 1f). Antifungal activity resided in the second, named figinII.

Molecular mass determination

FiginI had a molecular mass of approximately 20 kDa by SDS-PAGE (Figure 2). Molecular mass by MALDI-TOF

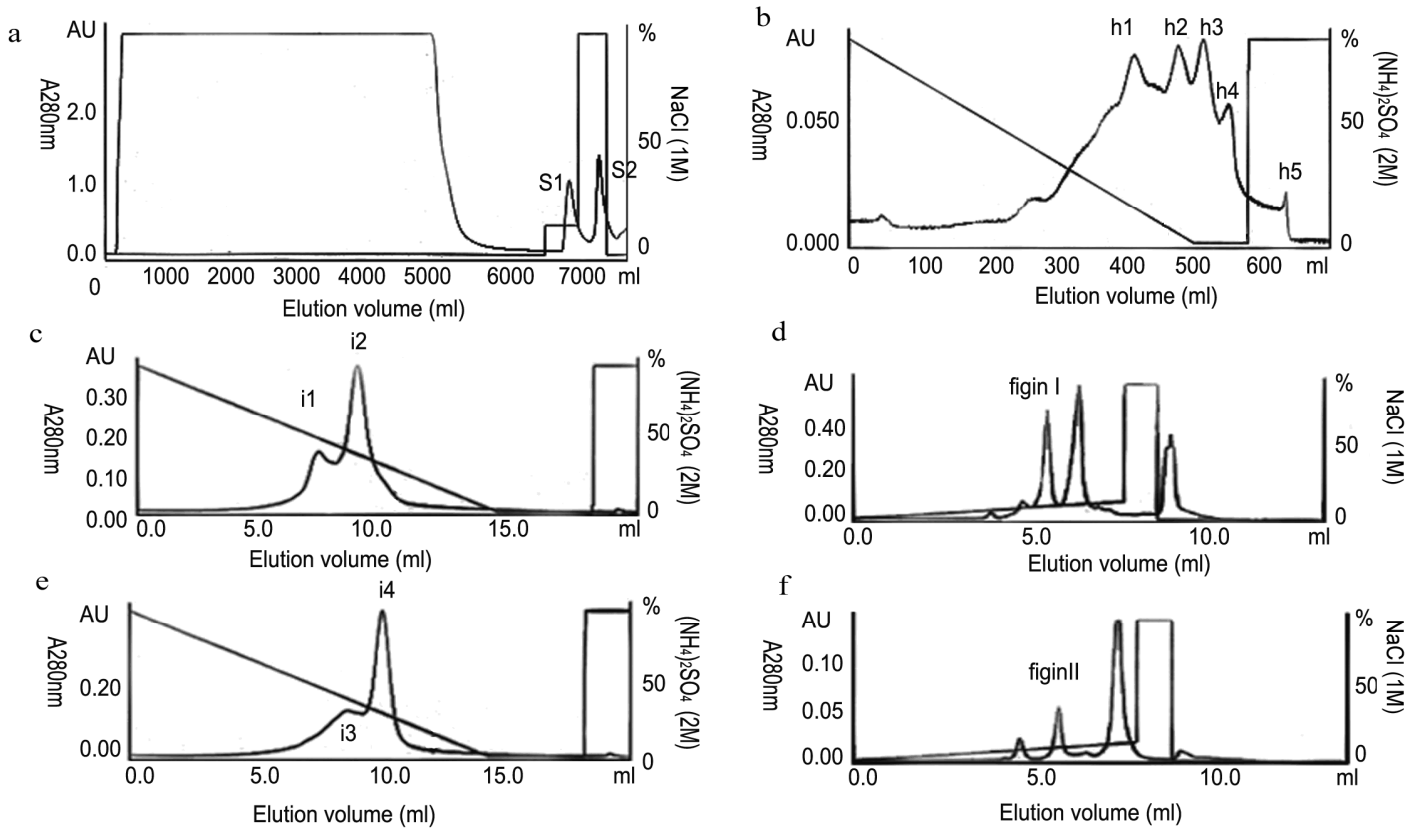

Figure 1. (a) Ion-exchange chromatography of dialyzed fractions from a SP-Sepharose Fast Flow column (2.6 × 30 cm). Samples were 40% ammunonium sulfate precipitates of fig leaf extracts dialyzed against sodium acetate buffer (pH 5.0). Starting chromatography buffer: 25 mM sodium acetate buffer (pH 5.0). Elution buffer: 0.13 M NaCl in starting buffer. Antifungal activity was detected in fraction S1; (b) hydrophobic-interaction chromatography of S1 on phenyl-Sepharose 6 Fast Flow column (1.6 × 34 cm). Sample was fraction S1 from SP-Sepharose Fast Flow diluted with sodium acetate buffer with 1 M $(NH_4)_2SO_4$. Starting buffer: 2 M $(NH_4)_2SO_4$ in sodium acetate buffer (pH 5.0). Elution was a linear gradient (from 2 to 0 M) of $(NH_4)_2SO_4$ in sodium acetate buffer. Antifungal activity was detected in fraction h2 and h3; (c) hydrophobic interaction chromatography of fraction h2 on RESOURCE ISO. Sample was h2 from phenyl-Sepharose 6 Fast Flow. Starting buffer and elution as in (b). Antifungal activity was detected in fraction i2; (d) ion exchange chromatography of fraction i2 on Mono S. Sample was fraction i2 from RESOURCE ISO dialyzed against 25 mM sodium acetate (pH 5.0). Starting buffer: 25 mM sodium acetate (pH 5.0). Elution buffer was a linear gradient (from 0 to 0.13 M) of NaCl in sodium acetate. The second fractions had antifungal activity (figinI); (e) hydrophobic interaction chromatography of fraction h3 on RESOURCE ISO. Sample was h3 from phenyl-Sepharose 6 Fast Flow. Starting buffer and elution as in (b). Antifungal activity was detected in fraction i4; (f) ion-exchange chromatography of i4 on Mono S. Sample was i4 dialyzed against 25 mM sodium acetate (pH 5.0). Starting buffer and elution as in (d). The second fraction showed antifungal activity (figinII).

MS was 21,531 Da (figure 3a). FiginII had a molecular mass of approximately 30 kDa by SDS-PAGE (Figure 2), with a molecular mass by MALDI-TOF MS of 30,957 Da (Figure 3b).

Antifungal activities test

The water extracts of fig leaves and purified proteins (figinI and figinII) were tested against 6 plant fungal pathogens (*T. viride*, *B. cinerea*, *P. asparagi* Sacc, *M. grissea*, *F. oxysporum f.sp. vasinfectum* and *S. sclerotiorum*). These exhibited potent antifungal activity against *T. viride* (Table 1). We assume this was because *T. viride* is widespread in the environment, and may have been on the fig leaves collected, stimulating the tree to produce the antifungal compounds.

50 µl samples were used for determination of antifungal activity. Because the hyphal extension-inhibition assay was used, it is difficult to report the antifungal activity in numerical terms. The activity of the protein in this work was determined by the distance from the centre of the Oxford cup to the growth of the fungi. Active (+), moderately active (++), strongly active (+++) and very strongly active (++++) denote distances less than 1 mm, between 1 and 3 mm, between 3 and 6 mm and more than 6 mm, respectively.

Hundreds of antifungal peptides or proteins are known and produced by a multitude of organisms, including plants belonging to *Amaranthaceae*, *Araliaceae*, *Balsaminaceae*, *Basellaceae*, *Bignoniaceae*, *Brassicaceae*, *Chenopodiaceae*, *Compositae*,

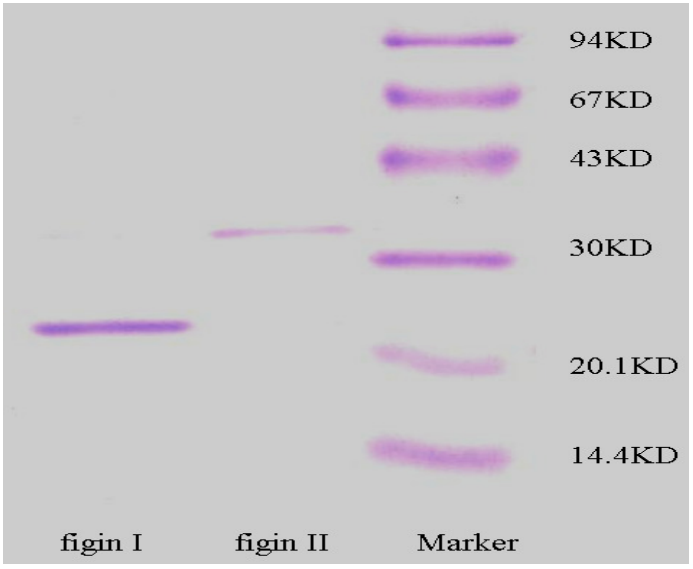

Figure 2. SDS-PAGE profile of figin I and figin II.

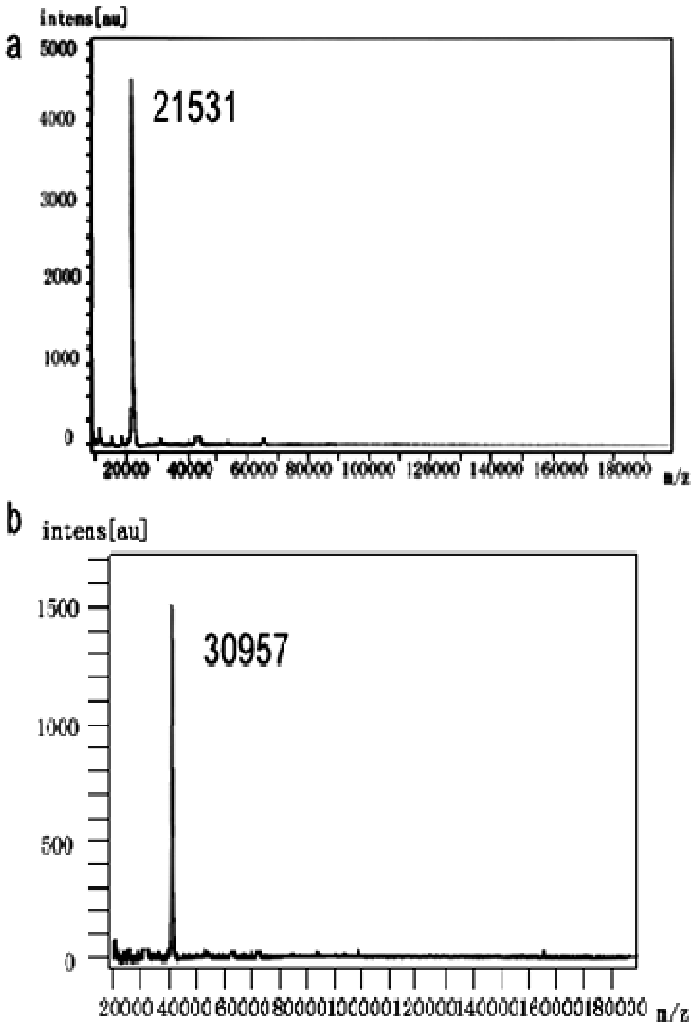

Figure 3. MALDI-TOF mass spectrum of figinI (a) and figinII (b).

Convolvulaceae, *Cucurbitaceae*, *Fabaceae*, *Ginkgoaceae*, *Gramineae*, *Hydrangeaceae*, *Liliaceae*, *Linaceae*, *Malvaceae*, *Nyctaginaceae*, *Phytolaccaceae*, *Poaceae, Proteaceae*, *Rosaceae*, *Rubiaceae*, *Rutaceae*, *Solanaceae* and *Solanaceae* genera (Berrocal-Lobo et al., 2005; Flores et al., 2002; Ng, 2004; Parkash et al., 2002; Selitrennikoff, 2001; Xia and Ng, 2004; Ye and Ng, 2002a; Ye and Ng, 2002b; Ye and Ng, 2002c; Ye et al., 2002; Wang and Ng, 2001; Wang and Ng, 2002). However, to our knowledge, only three studies have reported antifungal proteins in the family *Moraceae*. An antifungal chitinase was purified from jelly fig (*Ficus awkeotsang*) achenes, which significantly inhibited the spore germination of *Colletotrichum gloeosporioides* (Li et al., 2003).Two chitinases (GLx, Chi-C) exhibiting strong antifungal activity were purified from the latex of gazyumaru (*Ficus microcarpa*) (Taira et al., 2005). Two novel chitin-binding lectins were purified from seeds of *Artocarpus integrifolia* (jackfruit) and *Artocarpus incisa* (breadfruit). They inhibited the growth of *Fusarium moniliforme* and *Saccharomyces cerevisiae* (Trindade et al., 2006). In this work, we described the primary isolation of two antifungal proteins from *F. carica* L., of the *Moraceae*.

Previous studies demonstrated that low-molecular-weight extracts of fig leaves had antifungal or antibacterial activities against several microorganisms (Zhao et al., 2005; Hu et al., 2007; Oliveira et al., 2009; Balestra et al., 2009; Aref et al., 2010). Two antifungal proteins termed figinI and figinII were obtained in this first report of high-molecular extracts from figs with antifungal activity.

The potential for natural food preservative

Figs are one of the earliest fruits to be cultivated, they are rich in fiber, copper, manganese, magnesium, potassium, calcium, and vitamin K, and are an excellent source of flavonoids and polyphenols. They are low in sodium and have no fat or cholesterol, making them excellent functional foods (Vayaa and Mahmooda, 2006; Fatemi et al., 2007; Vinson et al., 2005; Vinso, 1999; Solomon et al., 2006). In China, figs are found in 28 provinces; however, they are one of the least abundant fruits trees by planting area, because of low commercial value. This and previous works showed that extracts of fig leaves had antifungal or antibacterial activities and may inhibit harmful microbes in food (Zhao et al., 2005; Hu et al., 2007; Oliveira et al., 2009; Balestra et al., 2009; Aref et al., 2010), so they have potential use as an all natural food preservative. This gives a new potential for fig development, since the fruit is a good functional food, and the leaves may be sources of natural food presservative.

In summary, two proteins (figinI and figinII) with antifungal activity against *T. viride,* were isolated from fig leaves. This work provides a foundation for further study.

Table 1. Antifungal activity of extracts of fig leaves and purified proteins against various fungal pathogens.

Plant fungal pathogen	Water extract	figin I	figin II
T. viride	+	+++	+++
B. cinerea	-	-	-
P. asparagi Sacc	-	-	-
M. grissea	-	-	-
*F. oxysporum f.*sp. *vasinfectum*	-	-	-
S. sclerotiorum	-	-	-

Note: 50 µl samples were used for determination of antifungal activity. Because the hyphal extension-inhibition assay was used, it is difficult to report the antifungal activity in numerical terms. The activity of the protein in this work was determined by the distance from the centre of the Oxford cup to the growth of the fungi. Active (+), moderately active (++), strongly active (+++) and very strongly active (++++) denote distances less than 1 mm, between 1 and 3 mm, between 3 and 6 mm and more than 6 mm respectively.

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