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Isolation of an antifungal thaumatin-like protein from kiwi fruits

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Abstract

A single-chain 21 kDa protein exhibiting antifungal activity against *Botrytis cinerea* and some suppressive effects on *Mycosphaerella arachidicola* and *Coprinus comatus* was isolated from kiwi fruits. The protein, designated kiwi fruit thaumatin-like protein, did not inhibit translation in the cell-free rabbit reticulocyte lysate system but inhibited HIV-1 reverse transcriptase. It was purified to apparent homogeneity using a procedure involving saline extraction, $(NH_4)_2SO_4$ precipitation, ion exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel, ion exchange chromatography on SP-Sepharose and gel filtration on Superdex 75, respectively.

Keywords: Kiwi fruit; *Actinidia chinensis*; Actinidiaceae; Thaumatin-like protein; Antifungal

1. Introduction

Antifungal proteins are structually diverse. Thaumatin-like proteins (Del Campillo and Lewis, 1992; Vu and Huynh, 1994; Ye et al., 1999), chitinases (Vogelsang and Barz, 1993; Ye et al., 2000a), ribosome inactivating proteins (Leah et al., 1991), cyclophilin-like proteins (Ye and Ng, 2000) and miraculin-like protein (Ye et al., 2000b) are members of the family of antifungal proteins. In addition, there are antifungal proteins with other structures such as cysteine protease inhibitor (Joshi et al., 1998) and peptides (Lee et al., 1995).

The kiwi fruit is a popular fruit very rich in vitamin C. There are two varieties, one with green flesh and another with yellow flesh. The intent of the present study was to ascertain whether kiwi fruit elaborated any antifungal proteins; this paper describes the isolation of an antifungal protein designated kiwi fruit thaumatin-like protein from the variety with green flesh.

2. Results

Throughout the course of protein purification, the relative absorbance of the column fractions was monitored at 280 nm. The fractions were pooled according to absorbance and assayed for antifungal activity. When the kiwi fruit extract was applied to a DEAE-cellulose column, only the unadsorbed fraction contained antifungal activity. The fraction was then fractionated by affinity chromatography on Affi-gel blue gel into an unadsorbed fraction without activity and an adsorbed fraction with antifungal activity (data not shown). Chromatography of the adsorbed fraction from Affi-gel Blue gel on SP-Sepharose yielded a small unadsorbed inactive peak (SP1) and a large adsorbed peak with antifungal activity (fraction SP2) (Fig. 1). After removal of a trace amount of unadsorbed inactive material (fraction SU1) by chromatography on Superdex 75, a large peak (SU2) was obtained (Fig. 2), which appeared as a single band with a molecular mass of ca. 21 kDa on SDS-PAGE (Fig. 3). The TLP yield was 96 mg from 3 kg kiwi fruits. The N-terminal sequence of this antifungal protein showed substantial homology (65–80% identity) to monocot and dicot thaumatin-like proteins (TLP) (Table 1).

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Kiwi fruit TLP did not inhibit translation in rabbit reticulocyte lysate and lacked RNase activity when tested up to 50 μM but manifested modest antifungal activity against *Botrytis cinerea* (Fig. 4) and a weaker inhibitory activity toward *Mycosphaerella arachidicola* (Fig. 5) and *Coprinus comatus* (Fig. 6). The IC_{50} values of its antifungal activity against *B. cinerea* and *M. arachidicola* were respectively 0.43 μM (Fig. 7) and 8 μM (data not shown). Kiwi fruit TLP also exerted suppressive action on the growth of *Physalospora piricola* but there was no effect on *Fusarium oxysporum* and *Rhizoctonia solani* (data not shown).

At a concentration of 0.57 mg/ml (27 μM), kiwi fruit TLP caused (30.6±1.67)% inhibition of HIV-1 reverse transcriptase ($N=3$). No inhibition was observed at a concentration of 0.06 mg/ml (2.7 μM).

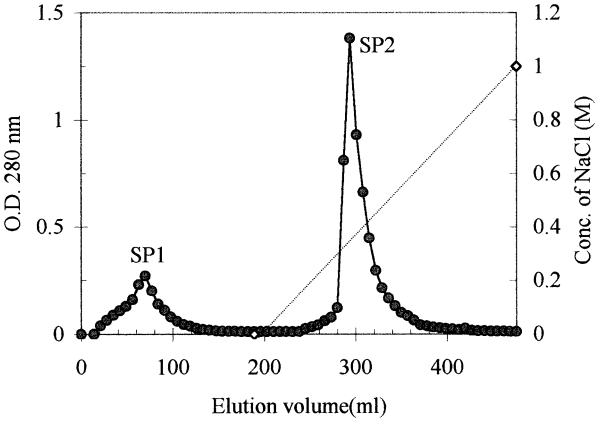

Fig. 1. Ion exchange chromatography of the fraction of kiwi fruit extract (unadsorbed on DEAE-cellulose and subsequently adsorbed on Affi-gel Blue gel) on an SP-Sepharose column (1.5×15 cm). The slanting line across the right half of the chromatogram represents the linear NaCl concentration gradient (0–1 M) in 10 mM NH_4OAc buffer (pH 4.5) used to elute fraction SP2 from the column. Antifungal activity was observed only in fraction SP2. Fractions corresponding to elution volume of 280–340 ml were pooled to form SP2.

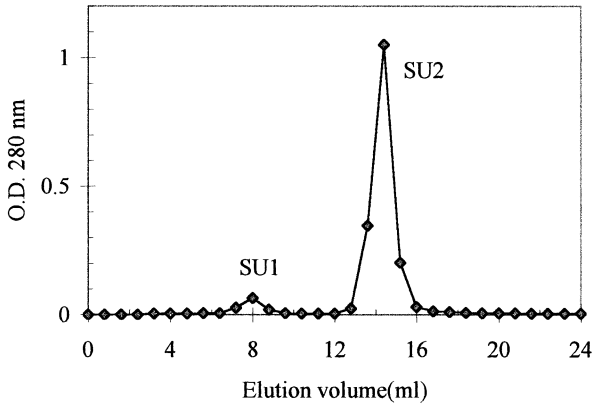

Fig. 2. Gel filtration of fraction SP2 by fast protein liquid chromatography on a Superdex 75 column in 100 mM NH_4HCO_3 (pH 8.8). Flow rate: 0.4 ml/min. Antifungal activity was observed only in SU2.

3. Discussion

Sequence analysis by Edman degradation revealed that the antifungal protein isolated from kiwi fruits in this investigation is a thaumatin-like protein. The N-terminal sequences of TLPs from monocots and dicots exhibit 65–80% identity to kiwi fruit TLP. A difference lies in the 5th residue (F) of kiwi TLP which is present only in this TLP but not in others. TLPs from

Fig. 3. SDS-PAGE of kiwi fruit TLP visualized by staining with Coomassie Brilliant Blue. Lane A: Pharmacia molecular weight markers, from top downward, phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa). Lanes B and C: 20 μg kiwi fruit TLP.

Table 1
Comparison of N-terminal sequences of kiwi fruit thaumatin-like protein (TLP) and other TLPs

	Amino acid residue number		% Identity
Kiwi TLP	1	ATFNFI·NNCPFTVWAAAVP·G	100
French bean TLP	1	ANFN·IVNNCPYTVWAAASP·G	80
Wheat TLP	26	ATFN·IKNNCPYTVWPAATPIG	80
Barley TLP	1	ATFTVI·NKCQYTVWAAAVPAG	75
Maize TLP	1	AVFTVV·NQCPFTVWAASVP·G	65
Rice TLP	32	ATF·AITNRCQYTVWPAAVPSG	70
Chickpea TLP	22	ANFE·IVNNCPYTVWAAASP·G	75
Flaxseed TLP	1	ARFD·IQNKCPYTVWAASVP·G	70
Grape TLP	25	ATFD·ILNKCTYTVWAAASP·G	70

The above sequences were obtained from a BLAST search and are aligned for maximal similarity. Amino acid residues identical to corresponding residues in kiwi fruit TLP are underlined. Amino acid residue number 26 for wheat TLP refers to A being the 26th amino acid residue in the TLP.

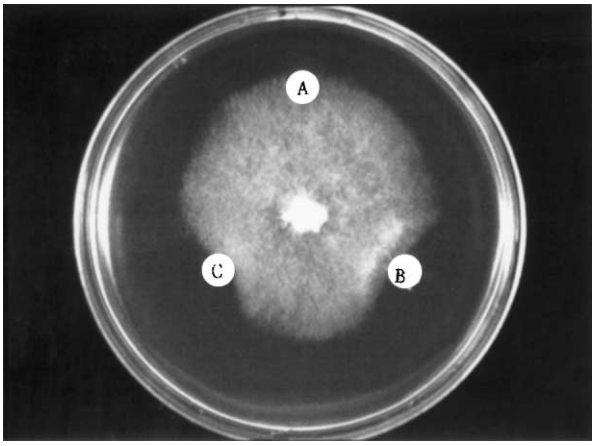

Fig. 4. Inhibitory activity of kiwi fruit TLP toward *Botrytis cinerea*. (A) 0.1 M NH_4OAc (pH 5.5), 10 μl, (B) 80 μg TLP in 10 μl, and (C) 12.8 μg TLP in 10 μl 0.1 M NH_4OA_c (pH 5.5), respectively.

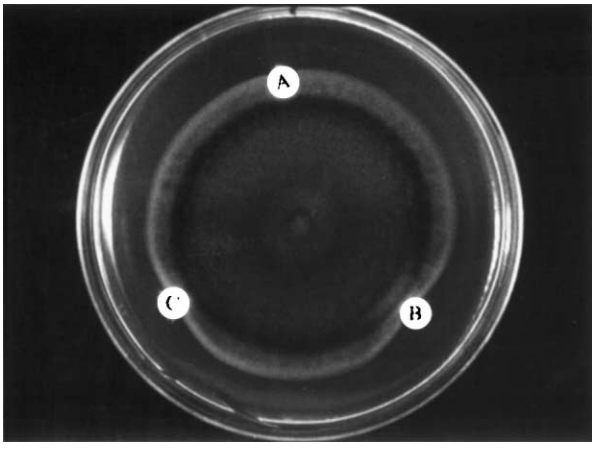

Fig. 5. Inhibitory activity of kiwi fruit TLP toward *Mycosphaerella arachidicola* (A) represents 0.1 M NH_4OAc (pH 5.5), 10 μl, (B) represents 80 μg TLP in 10 μl, and (C) represents 12.8 μg TLP in 10 μl 0.1 M NH_4OA_c (pH 5.5), respectively.

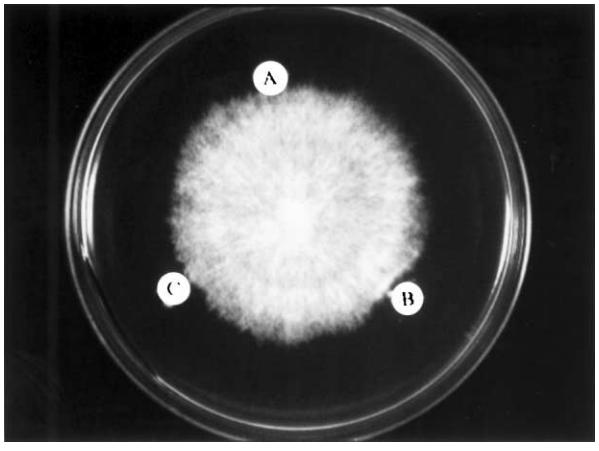

Fig. 6. Inhibitory activity of kiwi fruit TLP toward *Coprinus comatus*: (A) represents 0.1 M NH_4OAc (pH 5.5), 10 μl, (B) represents 80 μg TLP in 10 μl, and (C) represents 12.8 μg TLP in 10 μl 0.1 M NH_4OA_c (pH 5.5), respectively.

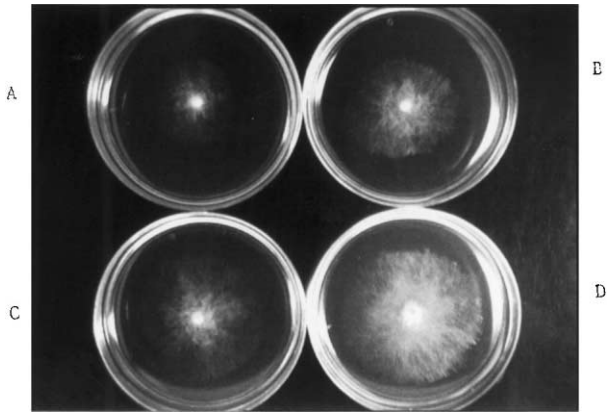

Fig. 7. Determination of IC_{50} value of antifungal activity of kiwi fruit TLP toward *Botrytis cinerea*. Upper left: 2.5 μM TLP; upper right: 0.5 μM TLP; lower left: 0.1 μM TLP; 0.1 м NH_y 0Az, respectively; lower right: control. IC_{50} value was determined to be 0.43 μM.

chickpea, grape, wild potato, wheat, rice, *Pseudotsuga menziesii*, *Musa acuminata* and *Arabidopsis thaliana* possess an additional N-terminal extension when compared to those from kiwi fruit, French bean, soybean, tobacco, barley, maize and flax seed. The molecular mass (21 kDa) of kiwi fruit TLP is also close to TLPs from maize (22 kDa) (Huynh et al., 1992), tomato (23 kDa) (Pressey, 1997) and grape (24 kDa) (Tattersall et al., 1997). In fact all TLPs isolated to date possess very similar sequences and molecular masses (Ye et al., 1999).

The chromatographic procedure used to purify kiwi TLP was similar to that employed for isolation of French bean TLP (Ye et al., 1999). The kiwi TLP, like French bean TLP (Ye et al., 1999), was adsorbed on Affi-gel blue gel and SP-Sepharose/CM-Sepharose. In the present investigation the additional steps included in the isolation protocol were $(NH_4)_2SO_4$ precipitation, ion exchange chromatography on DEAE-cellulose and gel filtration on Superdex 75. By comparison, other combinations of chromatographic procedures have been employed for purifying other TLPs. For instance, $(NH_4)_2SO_4$ precipitation, chitin extraction and Mono S column chromatography were used for maize TLP (Huynh et al., 1992); $(NH_4)_2SO_4$ precipitation, followed by chromatographic separations on S-Sepharose, hydroxyapatite and butyl-Sepharose for tobacco TLP; chromatography on S-Sepharose, DEAE-Sephadex and Mono S for tomato TLP (Pressey, 1997); $(NH_4)_2SO_4$ precipitation, DEAE-Sepharose chromatography and reversed phase HPLC on C18 column for *Diospyros texana* TLP (Vu and Huynh, 1994); and Q-Sepharose and Superdex 200 chromatography for grape TLP (Tattersall et al., 1997); and chromatography on P-60 and phenyl-Sepharose followed by HPLC using a polypore phenyl column for soybean TLP (Graham et al., 1992). Nevertheless, the purification procedure for kiwi

fruit TLP was a simple and efficient one and served its purpose.

French bean TLP exerted antifungal activity against *Fusarium oxysporum*, *Pleurotus ostreatus* and *Coprinus comatus* but not against *Rhizoctonia solani* (Ye et al., 1999). The kiwi fruit TLP similarly demonstrated antifungal action against *Coprinus comatus* but was ineffective toward *Rhizoctonia solani*. However, while French bean TLP produced some suppressive action on the growth of *Fusarium oxysporum,* kiwi fruit TLP did not affect the fungal pathogen. In addition, kiwi fruit TLP inhibited the growth of *Botrytis cinerea, Mycosphaerella arachidicola* and *Physalospora piricola*, with the inhibitory effect on *Botrytis cinerea* being the most potent.

The lack of translation-inhibitory and RNase activities in kiwi fruit TLP was in accordance with previous observations on other antifungal proteins showing very low, if any, of these activities (Leah et al., 1991; Ye et al., 1999).

It is interesting to note that kiwi fruit TLP manifested an inhibitory activity against HIV-1 reverse transcriptase. Similarly French bean TLP (Ye et al., 1999) exerted an inhibitory effect on this HIV enzyme. At a concentration of 5 mg/ml (59.3±3.6)% inhibition ($N=3$) was achieved. In general, most antifungal proteins examined displayed an inhibitory activity on HIV-1 reverse transcriptase at similar concentrations (Ng et al., unpublished data).

Assays of the kiwi fruit extract for hemagglutinating, ribonuclease and cell-free translation-inhibiting activities yielded negative results. This observation suggests that lectins, ribonucleases and ribosome inactivating proteins are either absent or present at very low levels in the kiwi fruit. Lectins, ribonucleases and ribosome inactivating proteins, just like antifungal proteins, are defense proteins which are protective against invading pathogens. Different organisms elaborate different sets of defense proteins. Only an antifungal protein is present in kiwi fruits at high enough a level to be isolated.

4. Experimental

4.1. Purification of kiwi fruit thaumatin-like protein

Kiwi (*Actinidia chinensis*) fruits (3 kg), purchased from a local supermarket, were extracted with a 0.9% saline solution. After centrifugation (15,000 rpm. 30 min) proteins were precipitated from the supernatant by addition of $(NH_4)_2SO_4$ to 30–80% saturation. The precipitate was dissolved in Tris–HCl buffer (10 mM, pH 7.2) and then dialyzed against the same buffer using dialysis tubing with an 8 kDa cut off, at 4 °C for 24 h with several changes of dialysis buffer, before application on a column of DEAE-cellulose (5×8 cm) previously equilibrated with the same buffer. The unadsorbed proteins were eluted from the column with the same buffer and adsorbed proteins were eluted with 1 M NaCl in the same buffer. The unadsorbed fraction had antifungal activity, and was applied to a column of Affi-gel blue gel (2.5×15 cm) in Tris–HCl buffer (10 mM, pH 7.2); the column was eluted with the same buffer, and after removal of unbound proteins, which were devoid of antifungal activity, adsorbed proteins with antifungal activity were eluted by addition of 1.5 M NaCl to the buffer. After dialysis the adsorbed active fraction was loaded onto a SP-Sepharose column (1.5×15 cm) in 10 mM NH_4OAc buffer (pH 4.5). After elution of unadsorbed inactive materials (fraction SP1), adsorbed materials with antifungal activity (fraction SP2) were eluted with a linear concentration gradient of NaCl (0–1 M) in 10 mM NH_4OAc (pH 5.5). Fraction SP2 was further fractionated on Superdex 75 in 50 mM NH_4HCO_3 (pH 8.6) to yield a small inactive peak SU1. A much larger peak (SU2) represented the purified antifungal protein which was designated kiwi fruit thaumatin-like protein (TLP). The Superdex 75 column had previously been calibrated with molecular mass markers including bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa) and cytidine (0.246 kDa).

4.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

This was conducted according to the method of Laemmli and Favre (1973). After electrophoresis the gel was stained with Coomassie Brilliant Blue. The molecular mass of kiwi fruit TLP was determined by comparison of its electrophoretic mobility with those of molecular mass marker proteins (Amersham Pharmacia Biotech).

4.3. Amino acid sequence analysis

The N-terminal amino acid sequence of kiwi fruit TLP was analyzed by means of automated Edman degradation. Microsequencing was carried out using a Hewlett Packard 1000A protein sequencer equipped with an HPLC system (Lam et al., 1998).

4.4. Assay for ribonuclease activity.

The activity of kiwi fruit TLP toward tRNA was estimated by measuring the production of acid-soluble, UV-absorbing species using the procedure of Wang and Ng (1999). Yeast tRNA (200 μg) was incubated with kiwi fruit TLP (20 μg) in MES buffer (pH 6.0, 150 μl, 100 mM) at 37 °C for 15 min. The reaction was terminated by addition of ice-cold 3.4% perchloric acid (350 μl). After standing on ice for 15 min, the mixture was centrifuged (15,000 *g*, 15 min) at 4 °C. The absorbance of the

supernatant, after suitable dilution until the absorbance fell within the range 0.5–1.0, was measured at 260 nm. One unit of ribonuclease activity was defined as the amount of ribonuclease that produces an absorbance increase at 260 nm of one per min in the acid-soluble fraction per ml of reaction mixture under the specified conditions.

4.5. Assay for cell-free translation-inhibitory activity

Rabbit reticulocyte lysate was prepared from the blood of rabbits rendered anemic by phenylhydrazine injections, and an assay based on the rabbit reticulocyte lysate system was used. Kiwi fruit TLP (10 μl) was added to 10 μl of radioactive mixture (500 mM KCl, 5 mM $MgCl_2$, 130 mM phosphocreatine and 1 μCi-[4, 5-^{3}H] leucine) and 30 μl working rabbit reticulocyte lysate containing 0.1 μM hemin and 5 μl containing 5.2 μg rabbit muscle creatine kinase (250 units/mg). Incubation proceeded at 37 °C for 30 min before addition of 1 M NaOH (330 μl) and 1.2% aq H_2O_2. Further incubation for 10 min allowed decolorization and tRNA digestion. An equal volume of the reaction mixture was then added to 40% trichloroacetic acid (w/v) with 2% casein hydrolysate (w/v) in a 96-well plate to precipitate radioactively labeled protein. The precipitate was collected on a glass fiber Whatman GF/A filter, washed and dried with absolute alcohol passing through a cell harvester attached to a vacuum pump. The glass fiber filter was placed in a scintillation vial containing scintillant and counted in an LS6500 Beckman liquid scintillation counter (Lam et al., 1998).

4.6. Assay of antifungal activity

The assay of antifungal activity toward *Botrytis cinerea*, *Mycosphaerella arachidicola*, and *Coprinus comatus* was carried out in Petri plates (100×15 mm) containing of potato dextrose agar (10 ml). After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm away from the rim of the mycelial colony. An aliquot (6 μl containing 10 μg) of kiwi fruit TLP was added to a disk. The plates were incubated at 23 °C for 72 h until mycelial growth had enveloped disks containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity (Ye et al., 1999).

For quantitative assays, three doses of kiwi fruit TLP (0.1, 0.5 and 2.5 μM) were added to potato dextrose agar (4 ml) at 45 °C, these being mixed rapidly and poured into 3 separate 6-cm petri dishes. After the agar had cooled down to room temperature a small amount (1 ×1 mm) of mycelia, the same amount to each plate, was inoculated. Buffer only was employed for a negative control. After incubation at 23 °C for 72 h, the area of the mycelial colony was measured and the inhibition of fungal growth and hence the IC_{50} was determined.

4.7. Assay for HIV-1 reverse transcriptase inhibitory activity

HIV-1 reverse transcriptase activity was measured by ELISA as described (Collins et al, 1997) using a non-radioactive kit from Boehringer Mannheim (Germany). The inhibition assay was performed as described in the protocol included with the kit, except that each well contained 2 ng recombinant HIV-1 reverse transcriptase in a total reaction volume of 60 μl.

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