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Purification and Modes of Antifungal Action by *Vicia faba* cv. *Egypt* Trypsin Inhibitor

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A new 15 kDa Bowman−Birk type trypsin inhibitor (termed VFTI-E1) from fava beans (*Vicia faba* cv. *Egypt 1*) was isolated using liquid chromatography. Though it exhibited substantial homology in *N*-terminal amino acid sequence to other protease inhibitors, VFTI-E1 showed antiproteolytic activity against trypsin (K_i 11.9 × 10^{-9} M) but hardly any activity against chymotrypsin. It demonstrated antifungal activity toward the filamentous fungus *Valsa mali* with an IC_{50} of 20 μM. The mechanism of its antifungal action toward *V. mali* included (1) induction of alteration of hyphal morphology, (2) growth inhibition by chitin deposition at hyphal tips, and (3) permeabilization of fungal membrane. The antifungal activity of VFTI-E1 was dependent on the ambient ionic strength as increasing concentrations of NaCl, $CaCl_2$, and $MgCl_2$ diminished the activity. The membranolytic action of VFTI-E1 was confined to fungus, but not exerted on human and rabbit erythrocytes. This study sheds light on the mode of hyphal growth inhibitory activity of protease inhibitors with antifungal activity. The antifungal activity of VFTI-E1 amplifies the scope of its potential applications.

KEYWORDS: Protease inhibitor; *Vicia faba*; innate immunity; antifungal protein; *Valsa mali*

INTRODUCTION

Fava bean (*Vicia faba* L.), also commonly named as fava bean, broad bean and field bean, is a species of bean which belongs to the family of Fabaceae (*1−3*). It could be mainly categorized into spring and winter ecotypes. As a leguminous crop cultivated worldwide for thousands of years, fava bean is a popular crop with multiple applications. Its seeds are a staple food in many countries including China and Egypt (*2*, *3*). Besides, it is often grown as a cover crop to prevent erosion due to its ability of cold resistance. Furthermore, fava bean forms root nodules with nitrogen-fixing *Rhizobium leguminosarum* bv. *Viciae* (*1*). Thus nitrogen in the soil can be fixed.

Except for genetic defects such as nosogenesis of the disease favism for people with glucose-6-phospate dehydrogenase (G6PD) deficiency (*4*), fava beans serve as welcome foods or ingredients in cuisines (*2*,*3*). Furthermore, fava beans might be of value in treating conditions such as hypertension, heart failure, renal failure, liver cirrhosis, tumor, Parkinson's disease and AIDS (*5−11*). In order to know more about the nutritional values of fava beans at the molecular level, a considerable amount of work has been done. Useful components comprising a hemagglutinin (*6*), trypsin inhibitors (*10−12*), a *Vicia faba* alpha-galactosidase (EC 3.2.1.22) (*13*), tannins (*14*), L-3,4-dihydroxyphenylalanine (L-dopa) (*7*), and others have been purified from fava beans.

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Protease inhibitors from fava beans with significant antifungal activity have been reported, but antifungal mechanisms have not been elucidated (*10*, *11*). The multitude of antifungal proteins reported to date have been classified according to their structural features and/or their amino acid compositions into main families such as cathelicidins, defensins, and antimicrobial peptides (*15*, *16*). Current knowledge concerning the mode of antifungal action of plant antifungal proteins includes the following aspects: (1) morphological alterations of hyphae, (2) Ca^{2+} uptake into hyphae, (3) K^+ efflux from hyphae, (4) alkalinization of the medium, (5) hyphal membrane potential changes, (6) inhibition of fungal chitin synthesis, (7) interference with fungal cell division, and others (*15−21*).

In the present study, a new Bowman−Birk type trypsin inhibitor with a molecular weight of about 15 kDa was purified from seeds of *Vicia faba* cv. *Egypt 1* by liquid chromatography techniques. Its biological and chemical characteristics were evaluated. It exerted antifungal activity toward the filamentous fungus *Valsa mali* by induction of hyphal morphological alterations, growth inhibition at hyphal tips, and hyphal membrane permeabilization. This study sheds light on the mode of hyphal growth inhibitory activity of protease inhibitors with antifungal activity. The antifungal activity of VFTI-E1 amplifies the scope of its potential applications.

MATERIALS AND METHODS

Materials. Seeds of *Vicia faba* cv. *Egypt 1* were purchased from Agricultural Research Center in Giza, Egypt. All columns including

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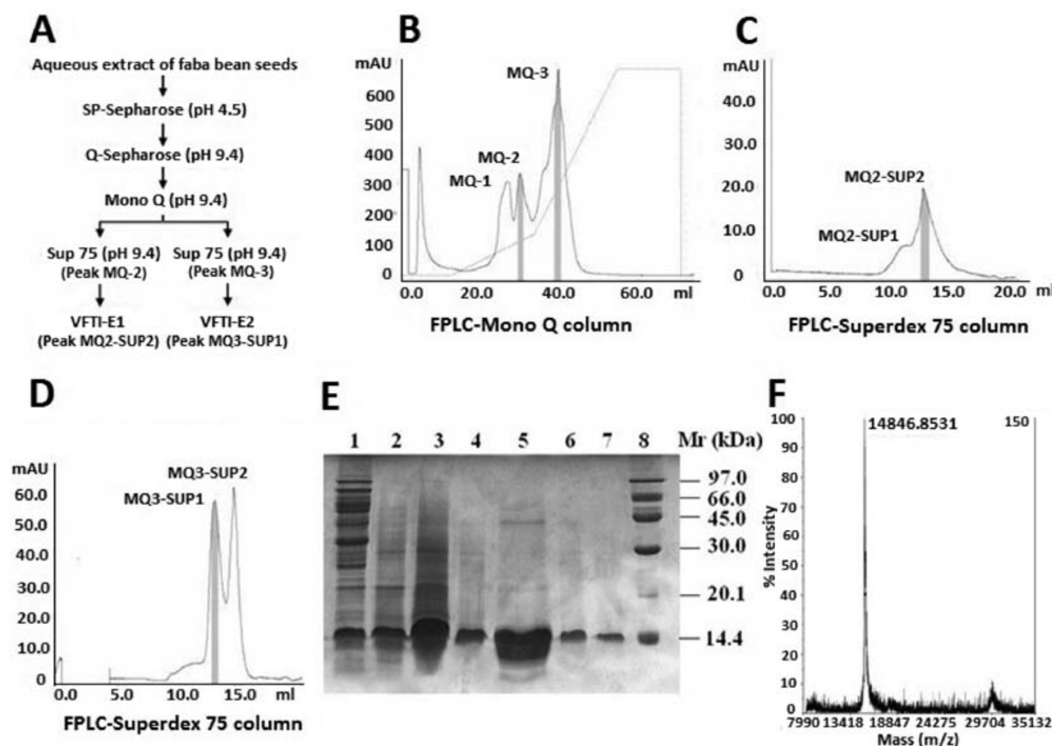

Figure 1. Isolation of VFTI-E1 and VFTI-E2. Elution profiles for VFTI-E1and VFTI-E2 were shown in **A**−**D**. (**A**) Schematic representation of the chromatographic steps used for the purification of VFTI-E1 and VFTI-E2. Aqueous extract of fava beans was first applied to SP-Sepharose. The adsorbed part was then run on Q-Sepharose (data not shown). (**B**) The Q-Sepharose bound fraction was followed applied to a Mono Q 5/50 GL column and three bound fractions appeared. (**C**) Fraction MQ-2 was subsequently loaded on a Superdex 75 10/300 GL column to yield a major peak MQ2-SUP2 which contained the purified trypsin inhibitor VFTI-E1. (**D**) Similarly, fraction MQ-3 was applied to a Superdex 75 10/300 GL column and yielded the peak MQ3-SUP1 which contained the purified trypsin inhibitor VFTI-E2. The dashed lines represented the concentration of NaCl employed. The fraction in which trypsin inhibitory activity resided was shaded. (**E**) SDS−PAGE recording purification procedures, purity and molecular weight of VFTI-E1 and VFTI-E2. Lane 1, aqueous extract; lane 2, fraction of extract adsorbed on SP-Sepharose; lane 3, fraction initially adsorbed on SP-Sepharose and subsequently adsorbed on Q-Sepharose; lane 4, fraction MQ-2 from FPLC-Mono-Q column; lane 5, fraction MQ-3 from FPLC-Mono-Q column; lane 6, fraction MQ2-SUP2 from Superdex 75 column; lane 7, fraction MQ3-SUP1 from Superdex 75 column; lane 8, protein ladder (GE Healthcare). (**F**) MALDI-TOF mass spectrometry results of VFTI-E1 showing a peak with a molecular mass of 14847 Da.

SP-Sepharose, Q-Sepharose (Fast Flow), Mono Q 5/50 GL, and Superdex 75 10/300 GL columns were obtained from GE Healthcare, China. Bovine pancreatic trypsin, α-chymotrypsin and soybean trypsin inhibitor were from USB Corporation (Cleveland, OH). All other chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated in the text. Filamentous fungi including *Rhizoctonia solani*, *Valsa mali*, *Helminthosporium maydis*, and *Bipolaris maydis* were provided by Professor H. X. Wang at Department of Microbiology of China Agricultural University.

Purification of Trypsin Inhibitors from *Vicia faba* cv. *Egypt 1*. First, the supernatant derived from centrifugation of the fava seed homogenate (250 g) was applied to a SP-Sepharose column, and then a Q-Sepharose column. The fraction adsorbed sequentially on the two aforementioned columns was then loaded on a Mono Q 5/50 GL column to yield fractions MQ-2 and MQ-3 manifesting trypsin inhibitory activity. The two fractions were then applied separately to a Superdex 75 10/300 GL column. Purified VFTI-E1 (from peak MQ2-SUP2) and VFTI-E2 (from peak MQ3-SUP1) were dialyzed extensively against distilled water, freeze-dried, and then stored at −20 °C for further studies.

Molecular Mass and *N*-Terminal Amino Acid Sequence Determination. Both sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE, 15% polyacrylamide gel) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) for molecular weight determination were carried out according to methods described by Lin and Ng (*22*). *N*-Terminal amino acid sequence analysis was performed using a HP 1000A Edman degradation unit and a HP 1000 HPLC system from Hewlett-Packard, Palo Alto, CA (*22*). The percentage of sequence identity with protease inhibitors from related plants was determined using BCM search launcher, the software ClustalX 1.83, and the on line BOXSHADE 3.21 server (*23*).

Enzyme Kinetics. A modified protocol described by S. W. Dahl and his colleagues (*24*, *25*) was applied. Briefly, different doses of 20 μL of VFTI-E1 (or VFTI-E2) in 50 mM Tris-HCl buffer (pH 7.6, at final concentrations ranging from 1.25, 2.5, 5.0,7.5, to 10 nM), were incubated with 40 μL of trypsin/chymotrypsin (1 mg/mL in 50 mM Tris-HCl buffer, pH 7.6) for 5 min at 37 °C. The residual trypsin activity was measured by adding 0.5 to 1 mM trypsin-specific substrate *N*-α-benzoyl-L-arginine ethyl ester (BAEE, in 50 mM Tris-HCl buffer, pH 7.6) or chymotrypsin-specific substrate *N*-benzoyl-L-tyrosine ethyl ester (BTEE, in 50 mM Tris-HCl buffer, pH 7.6). Changes in absorbance per min were monitored at 253 and 256 nm, respectively. The trypsin inhibitory activity was calculated with an equation as reported previously (*26*): BAEE units/mg trypsin inhibitor = ($\Delta A_{253/\min}$ positive − $\Delta A_{253/\min}$ sample tested)/[(0.001)(0.04)(sample)]. For chymotrypsin inhibitory activity, the same equation was used. In addition, 1% casein was used as a substrate for ascertaining the dose-dependent relationship between purified trypsin inhibitor and trypsin inhibitory activity (*26*). Inhibition constants (K_i) of purified trypsin inhibitor against both trypsin and α-chymotrypsin were determined as described elsewhere (*27*). The K_i value was estimated by using Sigma Plot 10.0, Enzyme Kinetics Module 1.3 (Systat Software Inc., San Jose, CA).

Assay of Thermal, pH and Chemical Stability. Thermal and pH stability tests were performed as described earlier (*26*). The effect of the reducing agent dithiothreitol (DTT) on the stability of VFTI-E1 was also studied (*26*). Korean bean trypsin inhibitor (*26*) and soybean trypsin inhibitor (USB Corporation) were employed as positive controls.

In Vitro Antifungal Activity Assay. The assay was processed as described previously (*22*). Briefly, fungi including *Rhizoctonia solani*, *Valsa mali*, *Helminthosporium maydis*, and *Bipolaris maydis* were grown on 100 × 15 mm Petri dishes containing 10 mL of potato dextrose agar.

Table 1. Summary of Purification of VFTI-E1 and VFTI-E2 from 250 g of Faba Beans

name	chromatographic step (peak)	total protein (mg)	specific trypsin inhib act. (units/mg)[a]	purification fold[b]	yield (%)
	water extract	11200	102	1.0	100
	SP-Sepharose	7350	nd[c]	nd	65.6
	Q-Sepharose	4430	nd	nd	39.5
VFTI-E1	Mono Q (MQ-2)	890	1175	11.5	7.9
	Superdex 75 (MQ2-SUP2)	135	4520	44.3	1.2
VFTI-E2	Mono Q (MQ-3)	1240	<50	nd	11.1
	Superdex 75 (MQ3-SUP1)	205	<50	nd	1.8

[a] BAEE was used as substrate. [b] Purification fold equals value of specific trypsin inhibitory activity of the chromatographic fraction divided by the value of specific activity of water extract. [c] Not determined.

After loading different doses of VFTI-E1 on paper disks (1 cm away from the rim of the hyphal colony), the dishes were incubated at 25 °C until hyphae had surrounded the paper disk of the control (to which buffer instead of VFTI-E1 was added). Samples with antifungal activity would produce a crescent of inhibition of hyphal growth, i.e., an invagination around the paper disk. The IC_{50} of antifungal activity was defined as the concentration required for obtaining 50% inhibition (reduction of area of fungal colony by 50%), and the value was determined by measuring changes in area of the hyphal colonies between control and sample-treated group (*22*). Brassiparin, an antifungal peptide from *Brassica parachinensis* seeds, served as a positive control (*22*).

Congo Red Uptake Assay. After incubation with 20 μM VFTI-E1 for 12 h, hyphae of *V. mali* were stained with 0.025% Congo red for 5 min with agitation in the dark. After rinsing with phosphate buffered saline (PBS), hyphae from different treatments were examined with a NIKONTE 2000 microscope to assess the hyphal growth inhibitory affect of VFTI-E1. An excitation wavelength of 543 nm and an emission wavelength of 560−635 nm were used (*28*).

SYTOX Green Uptake Assay. It was carried out in an analogous manner as the Congo red uptake assay. Briefly, hyphae of *V. mali* were first treated with 20 μM VFTI-E1 for 12 h and then stained with 1 μM SYTOX green for 30 min. After rinsing with PBS, fluorescence was viewed with a NIKONTE 2000 microscope for fluorescence detection (excitation wavelength, 450 to 490 nm; emission wavelength, 520 nm) (*16*, *17*, *21*).

Effect of Salts on Antifungal Activity. Hyphae of *V. mali* were incubated for 12 h with 20 μM VFTI-E1 buffers containing NaCl, $CaCl_2$, or $MgCl_2$ at different concentrations. Aliquots of hyphae were planted on Petri dishes for 48 h. Areas of hyphal colony for each treatment were measured. Residual antifungal activity was calculated as percentage of the area of the negative control in quintuplicate (*29*).

Hemolytic Activity Assay. The hemolytic activity of VFTI-E1 was determined in both 4% (vol/vol in 1× PBS) suspensions of human and rabbit erythrocytes as described previously (*16*, *19*). Release of hemoglobin was estimated by absorbance at 540 nm. Controls included blank (erythrocytes in 1× PBS) and 100% hemolysis (erythrocytes in 1% Triton X-100).

Statistical Analysis. Results were taken from three independent experiments performed in triplicate or as mentioned, and data were expressed as mean ± standard deviation (SD). IC_{50} was determined using SPSS 11.0.1 statistical software (SPSS Inc., Chicago, IL). For between-group comparisons, Student's *t* test was used.

RESULTS

Purification of VFTI-E1 and VFTI-E2. To purify VFTI-E1 and VFTI-E2 from fava beans, the aqueous seed extract was subjected to a series of chromatographic steps as shown in **Figure 1**. The crude seed extract was first run on an SP-Sepharose column, then on Q-Sepharose (data not shown), followed by FPLC on a Mono Q column and at last on a Superdex 75 column (**Figure 1A−D**). The purified VFTI-E1 (135 mg from peak MQ2-SUP2, **Figure 1C**) and VFTI-E2 (205 mg from peak MQ3-SUP1, **Figure 1D**) were obtained to near homogeneity (**Figure 1E**, lanes 6 and 7). The purity of the VFTI-E1 and VFTI-E2 was confirmed by presence of a sharp single peak in both FPLC-gel filtration on Superdex 75 (data not shown) and mass spectrometry (**Figure 1F**). Data in **Figure 1E** and **Table 1** tracked the steps of purification of VFTI-E1. For VFTI-E1, a yield of 0.54 g/kg beans was obtained with a 44-fold purification.

Biological and Enzymatic Activities. SDS−PAGE disclosed a molecular weight of about 15 kDa for both VFTI-E1 and VFTI-E2 in accordance with the MALDI-TOF MS results (**Figure 1E**, **F**). The inhibitory activities of VFTI-E1 toward trypsin and α-chymotrypsin were 4520 BAEE units/mg and 37 BTEE units/mg, respectively. For VFTI-E2, its antiproteolytic activities toward trypsin (< 50 BAEE units/mg) and chymotrypsin (< 20 BTEE units/mg) were both very weak. A dose-dependent trypsin/chymotrypsin activity inhibition relationship is shown in **Figure 2A** with casein as substrate. Similar results were noticed: very potent inhibition of trypsin and meager suppression of chymotrypsin.

Since the antiproteolytic activity of VFTI-E2 toward both trypsin and chymotrypsin was negligible (**Figure 2A**, with potential reasons discussed later), special attention was focused on VFTI-E1. Dixon plots (**Figure 2B**) and Lineweaver−Burk double reciprocal plots (**Figure 2C**) unveiled the competitive nature of VFTI-E1 against trypsin with a K_i value of about 11.9×10^{-9} M. The sequence of the first 24 *N*-terminal amino acids of VFTI-E1 is shown in **Figure 3** (indicated by arrow); different extents of homology with other plant protease inhibitors were observed.

VFTI-E1 was relatively thermostable up to 50 °C for 30 min, and its optimum pH value was 8 (**Figure 4A** and **Figure 4B**). Furthermore, VFTI-E1 was sensitive to DTT treatment. Its trypsin inhibitory activity started to decrease at 1 mM DTT and underwent a significant loss when the DTT concentration was augmented (**Figure 4C**). At 100 mM DTT, less than 20% of the activity remained. Results of the two positive controls (trypsin inhibitors from soybean and Korean soybean) were consistent with previous reports (*30*).

Antifungal Activity and Potential Mode of Action. VFTI-E1 showed preferential antifungal activity against only *V. mali*, with an IC_{50} of about 22 μM (**Figure 5A** and **Figure 5B**). Under the light microscope, an increased number of swollen cells both at the terminus of hyphae and within the hyphae were noticed (data not shown). However, no hyphal growth inhibition was observed in *R. solani*, *H. maydis*, and *B. maydis*. Furthermore, we investigated the mode of action and inhibitory effects of VFTI-E1 on the fungus *V. mali*. Congo red uptake results showed that VFTI-E1 inhibited *V. mali* growth by inducing chitin deposition at hyphal tips. A marked enhancement in red fluorescence was found in VFTI-E1 treated hyphae (**Figure 6**, panels **A1−4**). In controls (**Figure 6**, panels **B1−4**) in which *V. mali* was incubated with Congo red without pretreatment with VFTI-E1, no appreciable (or just slight) fluorescence was discerned.

To investigate membrane permeation caused by VFTI-E1, the SYTOX green uptake assay was performed. The plasma membranes of VFTI-E1-treated fungi were permeabilized as indicated by a drastically increased fluorescence (**Figure 7**, panels **A1−3**), whereas no appreciable fluorescence was observed in

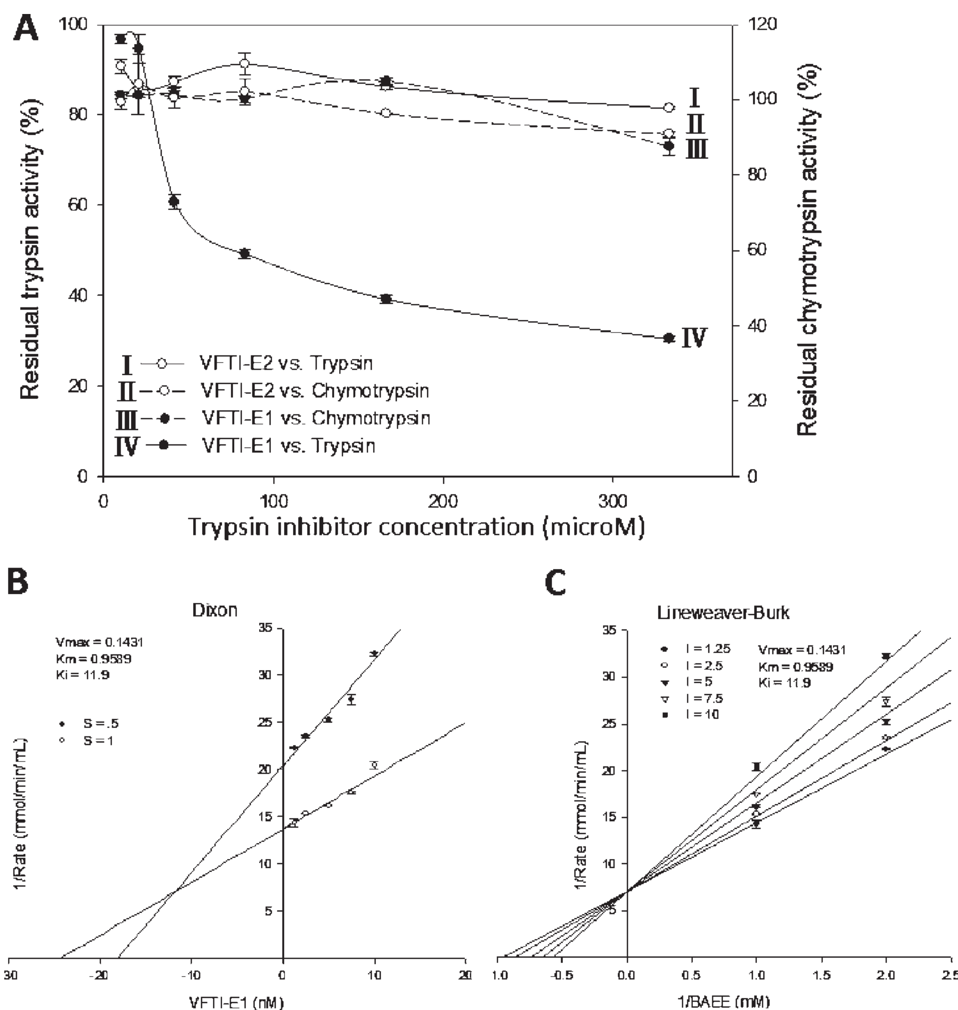

Figure 2. Protease inhibitory activities of VFTI-E1 and VFTI-E2. (**A**) After incubation with trypsin (curves I and IV, corresponding to left *Y*-axis) or α-chymotrypsin (curves II and III, corresponding to right *Y*-axis) with different concentrations of purified VFTI-E1 or VFTI-E2 (11 to 350 μM) at 37 °C for 30 min, their residual enzymatic activities were measured using 1% casein as a substrate. Experiments were carried out in triplicate with values as means ± SD. (**B**) Dixon plot was used for determination of the inhibition constant (K_i) of VFTI-E1 against trypsin. The reciprocal of reaction velocity was plotted against the corresponding VFTI-E1 concentration with the K_i value obtained from the intercept of two lines at two concentrations of the specified substrate BAEE. (**C**) Lineweaver−Burk plot showing competitive nature of trypsin inhibition by VFTI-E1 at two different concentrations of BAEE (0.5 and 1 mM). Results are expressed as means ± SD of triplicate measurements.

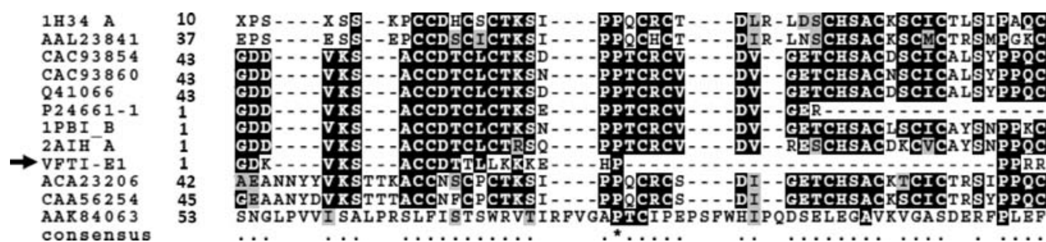

Figure 3. Alignment of *N*-terminal amino acid sequences of VFTI-E1 and related trypsin inhibitors. The abbreviations used are as follows: VFTI-E1 (this study, with bold black arrow); 1H34_A, *Phaseolus lunatus* (lima bean) trypsin inhibitor (containing totally 83 amino acids/aa); AAL23841, *Phaseolus vulgaris* trypsin inhibitor (107 aa); CAC93854, *Pisum sativum* (pea) trypsin/chymotrypsin inhibitor (104 aa); CAC93860, *Pisum sativum* trypsin/chymotrypsin inhibitor (90 aa); Q41066, *P. sativum* trypsin/chymotrypsin inhibitor (114 aa); P24661-1, *Vicia faba* (fava bean) Bowman−Birk type proteinase inhibitor (63 aa); 1PBI_B, *P. sativum* Bowman−Birk Inhibitor (72 aa); 2AIH_A, *Lens culinaris* Bowman−Birk type proteinase inhibitor (67 aa); ACA23206, *Glycine max* Bowman−Birk type protease inhibitor (117 aa); CAA56254, *Medicago sativa* serine proteinase inhibitor (113 aa); AAK84063, *Bauhinia variegata* trypsin inhibitor (175 aa).

the negative control group (without VFTI-E1 treatment, **Figure 7**, panels **B1−3**). In addition, a dose-related increase of membrane permeabilization (the influx of SYTOX green into the hyphae) as indicated by an increase in intensity of fluorescence was observed.

Furthermore, results of the effects of different salts on antifungal activities of VFTI-E1 against *V. mali* are summarized in **Figure 8**. All salts used including NaCl (from 25 mM), $CaCl_2$ (from 0.5 mM), and $MgCl_2$ (from 2.5 mM) could reduce the antifungal activity of VFTI-E1 in a dose-dependent manner. VFTI-E1 was

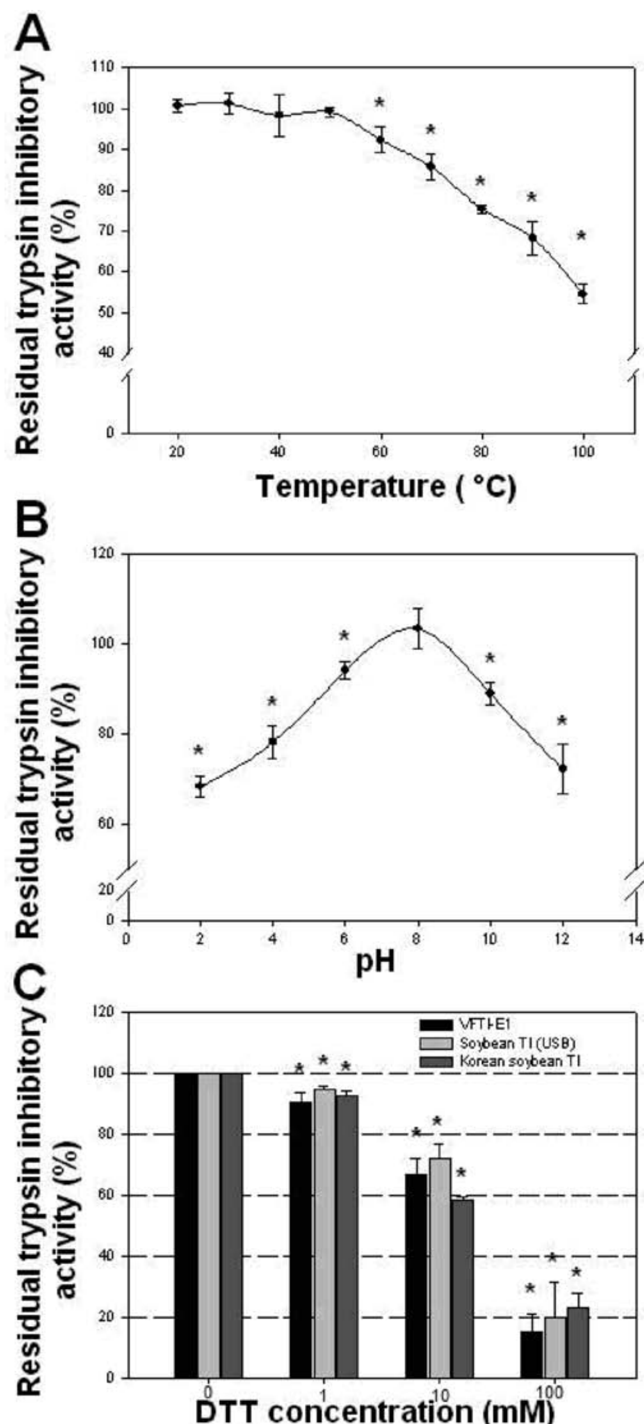

Figure 4. Thermal, pH and chemical stabilities of VFTI-E1. (**A**) Thermal stability of VFTI-E1 was carried out after it (10 μM in 0.02 M Tris-HCl buffer, pH 7.6) had been exposed to 20 to 100 °C for 30 min. (**B**) pH stability was measured after dissolving the lyophilized protein in buffers of different values to a final concentration of 10 μM, followed by incubation for 0.5 h at 37 °C. (**C**) For the chemical stability, VFTI-G1 was incubated with dithiothreitol (DTT) at dose-escalating concentrations (0, 1, 10, and 100 mM) for 30 min at 37 °C, and the residual trypsin inhibitory activity was tested. Soybean TI and Korean soybean TI were as positive controls. Residual trypsin inhibitory activity in all (**A**), (**B**) and (**C**) was measured by using BAEE as substrate. Means $\pm$ SD of three independent experiments are shown. *$p < 0.05$ versus the residual activity at 20 °C (**A**) or pH 7 (**B**) or no treatment group (**C**), respectively.

devoid of hemolytic activity toward both human and rabbit erythrocytes when tested up to 100 μM (data not shown).

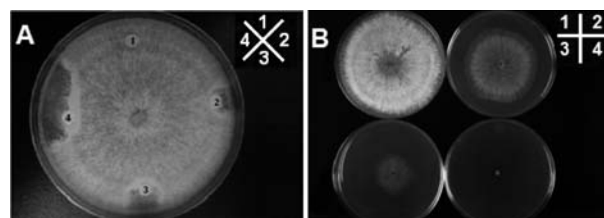

Figure 5. Antifungal activity of VFTI-E1 toward *V. mali*. (**A**) The samples applied to the paper disks including (**1**) 20 μL of 1× PBS as negative control, (**2**) 1 μg of brassiparin (an antifungal peptide from *Brassica parachinensis* seeds) as positive control, (**3**) 10 μg of VFTI-E1, and (**4**) 50 μg of VFTI-E1. (**B**) The IC_{50} of antifungal activity of VFTI-E1 toward *V. mali*. Concentrations of VFTI-E1 used including 0 μM (plate **1**), 10 μM (plate **2**), 50 μM (plate **3**), and 100 μM (plate **4**). The numbers on the upper right corner of each picture represent the numbers of the paper disks (panel **A**) or plates (panel **B**).

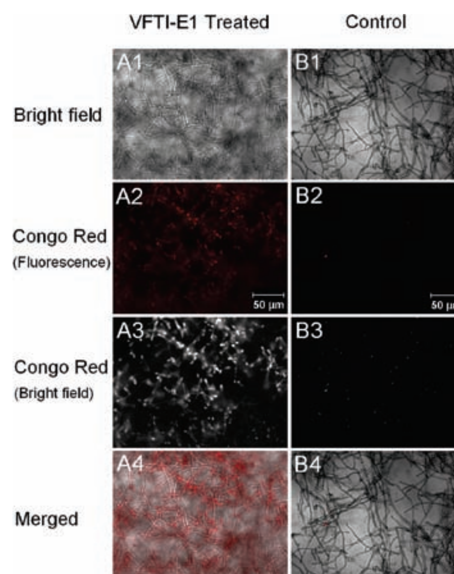

Figure 6. VFTI-E1 inhibited hyphal growth of *V. mali* as revealed by staining with Congo red. After incubation with (panels **A**) or without (panels **B**, as negative control) 20 μM VFTI-E1 for 12 h, hyphae of *V. mali* were stained with 0.025% Congo red for 5 min with agitation in the dark. After rinsing with phosphate buffered saline (PBS), hyphae from different treatments were examined by means of a NIKONTE 2000 microscope to assess the hyphal growth inhibitory effect of VFTI-E1.

DISCUSSION

The isolation method used to obtain VFTI-E1 and VFTI-E2 is satisfactory since the products exhibited homogeneity (confirmed by both SDS−PAGE and MALDI-TOF MS) with an acceptable harvest rate of approximately 1.2% and 1.8%, respectively. Owing to differential binding strength on the Mono-Q anion exchange column, VFTI-E1 and VFTI-E2 could be separated. Though they share the same molecular weight, their enzyme kinetics characteristics are grossly different reminiscent of other trypsin inhibitors previously described (*31*). Since VFTI-E1 manifests significant protease inhibitory activity specifically to trypsin, and just slight trypsin/chymotrypsin inhibitory activity is found in VFTI-E2, a focus was placed on VFTI-E1 (**Figure 2**). Reasons for the discrepancy in potency between VFTI-E1 and VFTI-E2 may be associated with differences in their structures. In contrast, two previously reported protease inhibitors from a Chinese cultivar of *Vicia faba* manifest trypsin inhibitory and

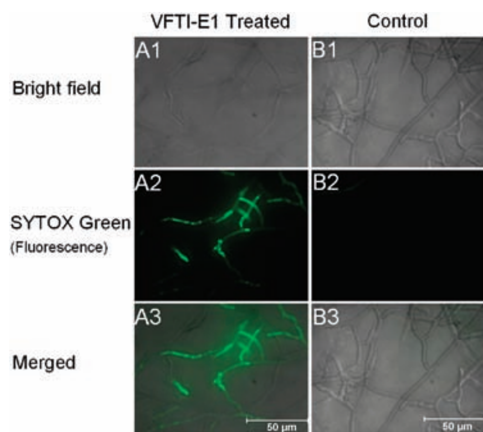

Figure 7. VFTI-E1-induced membrane permeabilization and subsequent influx of SYTOX green into *V. mali* hyphae. Hyphae of *V. mali* were first exposed (panels **A**) or not exposed (panels **B**, as negative control) to 20 μM VFTI-E1 for 12 h, and then stained with 1 μM SYTOX green for 30 min. After rinsing with phosphate buffered saline (PBS), hyphae from different treatments were examined by using a NIKONTE 2000 microscope to investigate hyphal membrane permeation.

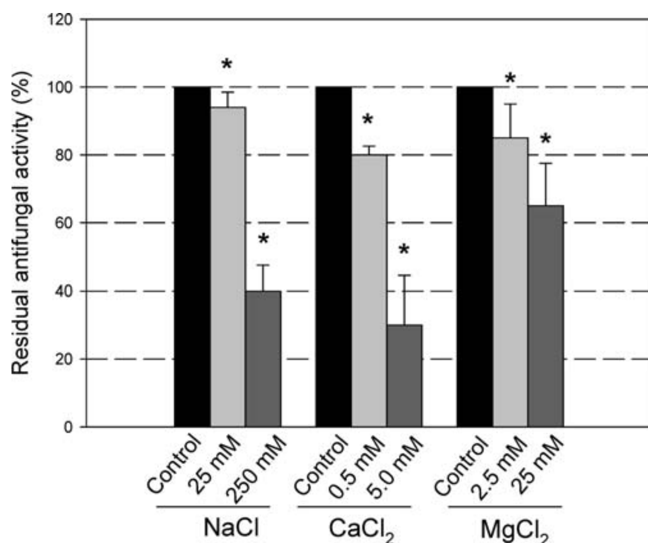

Figure 8. Effect of metallic chlorides on antifungal activity of VFTI-E1 against *V. mali*. Hyphae of *V. mali* were incubated in presence of 20 μM VFTI-E1 and also different concentrations of NaCl, $CaCl_2$, or $MgCl_2$ for 12 h. Aliquots were planted on Petri dishes for 48 h. Zones of hyphal colony in each treatment were measured, and residual antifungal activity was calculated as percentage of the zone of the negative control. Results are expressed as means ± SD of quintuplicate measurements. *$p < 0.05$ versus negative control.

also significant chymotrypsin inhibitory activities. Different antiproteolytic activities of the protease inhibitors from fava beans may be a consequence of genetic changes among different cultivars (*3*, *32*, *33*).

The biochemical characteristics of VFTI-E1 were examined in the present study. Its K_i value (11.9 nM) is comparable with values of other protease inhibitors as *Momordica cochinchinensis* trypsin inhibitor-1 (76.8 nM) (*31*) and *Bauhinia variegata* var. *candida* trypsin inhibitor (6.9 nM) (*34*). VFTI-E1 (tribe: Vicieae) displays pronounced resemblance in *N*-terminal sequence to another two trypsin inhibitors from fava bean and some lima bean (*Phaseolus lunatus* within the tribe of Phaseoleae) oriented trypsin inhibitors though they belong to different tribes (*10*−*12*). On the basis of characteristics including a small molecular weight (< 20 kDa), and *N*-terminal sequence, VFTI-E1 was subsumed into the Bowman−Birk trypsin inhibitor family (*35*, *36*).

In a previous report, a 7.5 kDa trypsin inhibitor from fava beans with antifungal activity toward *Mycosphaerella arachidicola*, *Fusarium oxysporum*, and *Botrytis cinerea* has been isolated (*10*). Another 13 kDa protease inhibitor from fava beans also exerted an inhibitory action toward mycelial growth of *M. arachidicola* and *Physalospora piricola* (*11*). In this study, VFTI-E1 exhibited antifungal activity against *V. mali*. By infecting apple trees through wounds such as pruning ends or fruit scars, *V. mali* can cause Valsa canker, one of the most important diseases of apple which has seriously limited apple production, especially in eastern Asia (*37*).

To investigate the mode of antifungal action of VFTI-E1 on *V. mali*, the dye Congo red was used to demonstrate that VFTI-E1 induced chitin deposition at hyphal tips. This is the same as the mode of antifungal action of other combinatorial and plant defensins (*19*, *28*). Though reported to be feasible as a microscopic fluorescence indicator of hyphal growth (*38*, *39*), Congo red itself could activate chitin polymerization (*40*). In the current study, a very low concentration (0.025%) of Congo red combined with a short incubation period (5 min) was applied to ensure a significant fluorescence for positive results, and a relative black background for negative findings (**Figure 6**). In addition, since hyphal membrane permeation is a common antifungal mode of action for many defensins (*15*, *16*, *19*, *20*), we investigated the permeation potential of VFTI-E1 by using the cationic dye SYTOX green (molecular weight 900 Da), which is incapable of entering into an intact cell unless its membrane has been disrupted by exogenous compounds (*16*). A significant fluorescence of SYTOX green indicating binding to the nucleic acids of VFTI-E1-treated fungal cells was observed. In contrast, no fluorescence was detected for the negative control group (**Figure 7**).

Besides membrane permeation, treatment of fungi with some plant defensins could also induce a rapid K^+ efflux and Ca^{2+} uptake which can adversely affect fungal growth (*17*, *18*). Salts including NaCl, $CaCl_2$ and $MgCl_2$ at low concentrations could curtail the antifungal activities of some human defensins (*29*). In the current study, parallel results were obtained using VFTI-E1, signifying that some cations may also participate in the antifungal process following membrane permeation (**Figure 8**). VFTI-E1 did not affect the integrity of human and rabbit erythrocyte membranes as demonstrated for some other antifungal proteins (*16*, *19*, *28*).

In conclusion, this study constitutes the first report of the isolation of a 15 kDa Bowman−Burk type trypsin inhibitor VFTI-E1 from the seeds of *Vicia faba* cv. *Egypt 1*. The biological and medicinal activities were to some extent different from those of previously reported trypsin inhibitors. VFTI-E1 shows specific antifungal activity against the filamentous fungus *V. mali* with modes of action including (1) alterations of hyphal morphology (increased swollen cells), (2) chitin deposition, (3) membrane permeation, (4) cation balance interruption. This study sheds light on the mode of inhibition of fungal growth caused by protease inhibitors with antifungal activity.

ABBREVIATIONS USED

VFTI-E1, *Vicia faba* cv. *Egypt 1* trypsin inhibitor; DTT, dithiothreitol; BAEE, *N*-α-benzoyl-L-arginine ethyl ester; BTEE, *N*-benzoyl-L-tyrosine ethyl ester.

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