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A novel chitinase isolated from *Vicia faba* and its antifungal activity

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ABSTRACT

A novel chitinase with antifungal activity was isolated from fava bean (*Vicia faba*) seeds. The protein exhibited a molecular mass of 21.5 kDa in reduced condition while 25.5 kDa in oxidized condition on SDS-PAGE, indicating that there are disulfide bonds inside the molecule. Its N-terminal amino acid sequence was determined to be D-D-V-G-S-V-I-S-A-S-L-F-E-Q-L-L-K-H, showing homologous to those of chitinase and chitinase precursors from leguminous plants. The optimum pH and the optimum temperature for activity toward N-acetyl-D-glucosamine were 5.4 and 50 °C, respectively. The *p*I was determined to be 8.7 by isoelectric focusing electrophoresis. The chitinase was thermostable up to 58 °C in both enzymatic reaction and antifungal activity. It showed chitin-binding activity, suggesting that the catalytic domain is involved in the binding of chitinase to a certain extent. In addition, it exerted potent antifungal action toward a variety of fungal species including *Pythium aphanidermatum*, *Fusarium solani*, *Physalospora piricola*, *Alternaria alternate*, *Botrytis cinerea*, and *Fusarium oxysporum* f. sp. *melonis*. The present findings demonstrated a novel chitinase with disulfide bonds inside the molecule and show antifungal significance in agriculture.

1. Introduction

Like any other living organism, plants are exposed to many pathogens including fungi and bacteria during the growth period. Plants do not possess an immune system and thus they are vulnerable to pathogens, and fungal and bacterial infection which can result in serious damage to plants such as leguminous seeds. However, a series of defense compounds is synthesized by plants to protect themselves from pathogenic organisms. One class of these defense compounds is antifungal proteins. Antifungal proteins are structurally diverse and comprise glucanases (Mauch, Hadwiger, & Boller, 1988; Vogelsang & Barz, 1993), chitinases (Ye & Ng, 2005), protease inhibitors (Wang et al., 2006), thaumatin-like proteins (Ye, Wang, & Ng, 1999), miraculin-like proteins (Ye, Wang, & Ng, 2000a), cyclophilin-like proteins (Ye & Ng, 2000a), allergen-like proteins (Ye & Ng, 2000b), and ribosome inactivating proteins (Lam, Wang, & Ng, 1998).

Chitinases (EC 3.2.1.14) are listed as a class of antifungal proteins, and they are of particular interest in studies of defense compounds owing to their resistances both against predators such as insects as well as pathogens such as fungi (Vogelsang & Barz, 1993; Ye & Ng, 2005). The natural substrate of chitinases, chitin, is the second most abundant polysaccharide in nature next to cellulose (BeMiller & Whistler, 1965). Chitin is actually a β-1,4-linked homopolymer of N-acetyl-D-glucosamine (GlcNAc) that is widely distributed in the green algae, fungi, protozoan, crustaceans, molluscs and coelenterates (Konagaya, Tsuchiya, & Sugita, 2006). It is often present in fungi hyphae as the main component of fungal cell wall but is absent in plants (Collinge et al., 1993; Graham & Sticklen, 1994). Although plants lack endogenous chitin, they do express chitinases (Brunner, Stintzi, Fritig, & Legrand, 1998). The function of chitinase in plants appears to be a defense against attack by chitin containing fungal pathogens and insect pests. Chitinases, together with β-1,3-glucanases, break down fungal cell, thereby inhibiting fungal growth (Mauch et al., 1988).

Chitinases are classified into two types of exochitinase and endochitinase based on mode of action. The most extensively studied plant chitinases are endochitinases, which randomly hydrolyze internal β-1,4-linkages of chitin to release N,N′-diacetyl-chitobiose and cut them into shorter segments (Wang et al., 2006). Chitinases are further divided into seven classes including classes I through VII, distinguished by their amino acid sequences, structure and mechanism (Wang, Zhou, Shao, Lu, & Rao, 2008). However, most leguminous chitinases belong to classes I through IV, information pertaining to other three classes is lacking. During the previous decades, chitinases have received increased attention because of their wide range of applications. Practical applications of chitinases include use in the preparation of protoplasts from fungi, as a protective agent against plant-pathogenic fungi (Taira, Toma, & Ishihara, 2005; Wang, Shao, Fu, & Rao, 2009; Wang, Shao, Rao, Lee, & Ye, 2007; Ye & Ng, 2002, 2005).

The fava bean is very popular in the Asian countries for use in cakes and soups and as bean sprouts. It serves as a health food because of its abundant nutrients and proteins. The fava bean seeds harvested in

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2009, from a severely fungi-infected area located in Zhaoyuan, Shandong Province, China, which was reported by local television and confirmed by local farmers, were chosen as our investigation materials. It is to be expected that some pathogenesis-related proteins containing chitinases are induced and expressed in certain quantities and exhibit unique characters when compared to those derived from other beans. As a result, we herein reported a novel protein in fava bean, which exerts both chitinase activity and antifungal activity against a variety of fungal species. Its N-terminal amino acid sequence bears some resemblance to those of chitinases and chitinase precursors from other leguminous plants, but has not previously been reported from the fava bean (Chaieb, González, López-Mesas, Bouslama, & Valiente, 2011).

2. Materials and methods

2.1. Materials

Fava bean (*Vicia faba*) seeds were obtained from a local farm. The fungi *Physalospora piricola*, *Alternaria alternate*, and *Botrytis cinerea* were kindly provided by the Department of Biochemistry, Chinese University of Hong Kong, China. The fungi *Fusarium solani*, *Pythium aphanidermatum*, and *Fusarium oxysporum* f. sp. *melonis* were obtained from the Department of Plant Pathology, Fujian Agricultural University, Fujian, China. Chitin was purchased from Shanghai Biotechnology Company, Shanghai, China.

CM-Sephadex C-50, POROS HS-20, and Sephadex G-75 were purchased from Amersham Biosciences (Sweden), PerSeptive Biosystem (PB, USA), and TOSOH (Japan). All chemicals were of the highest purity available.

2.2. Extraction, isolation and purification of chitinase

2.2.1. Extraction

100 g of fava bean seeds was soaked in distilled water for 24 h at 4 °C and homogenized in 0.2 mol/L sodium acetate buffer (pH 5.4). The homogenate was centrifuged at 10,000 ×*g* for 20 min at 4 °C. The supernatant (800 mL) was designated as the crude extract for further investigation.

2.2.2. Ammonium sulfate precipitation

The crude extract was precipitated with ammonium sulfate 20%–80%. After precipitation, the crude chitinase extract was separated by centrifugation at 10,000 ×*g* for 20 min, suspended in 300 mL of 0.02 mol/L sodium acetate buffer (pH 5.4), and dialyzed against the same buffer with five changes.

2.2.3. Ion-exchange chromatography on CM-Sephadex C-50

30 mL of the dialyzed solution was applied to an open column of CM-Sephadex C-50 column (2.5 × 55 cm) previously equilibrated with the starting buffer (0.02 mol/L sodium acetate buffer, pH 5.4) for 2 column volumes. Following removal of a large amount of unadsorbed proteins, the column was eluted with a linear gradient of NaCl (0–0.35 mol/L, 300 mL) in the same buffer. The flow rate was 0.5 mL/min and the absorbance of the eluate was monitored at 280 nm. The eluate fraction was named as first, second and third fractions according to the elution order (Wang et al., 2009). Chitinase activity was determined for all the fractions. The third fraction (P3) demonstrating chitinase activity was pooled, dialyzed against 0.02 mol/L sodium acetate buffer (pH 5.4) at 4 °C for 24 h with five changes, and subsequently concentrated the sample to 1.00 mol/L for further purification.

2.2.4. Gel filtration on Sephadex G-75

A glass column (2 cm × 100 cm) was packed with Sephadex G-75 and equilibrated with 0.02 mol/L sodium acetate buffer (pH 5.4) for 2 column volumes. The concentrated P3 fraction was then applied to the Sephadex G-75 column. Protein elution was carried out with 0.02 mol/L sodium acetate buffer (pH 5.4). The flow rate was 0.3 mL/min and the eluate was monitored at 280 nm. The chitinase activity of each fraction was also determined. The second fraction (P2) demonstrating chitinase activity was pooled and stored at 4 °C.

2.2.5. High-performance liquid chromatography (HPLC) on POROS HS-20

The second fraction with chitinase activity from G-75 chromatography was afterwards chromatographed on a column of POROS HS-20 (1.25 × 7.5 cm) (Ye & Ng, 2005), which had been equilibrated with 0.02 mol/L sodium acetate buffer (pH 5.4) for 2 column volumes. After elution of a small quantity of unadsorbed materials, the column was eluted with a gradient of NaCl (0–0.15 mol/L, 100 mL) in the same buffer. The flow rate was 0.8 mL/min and chitinase activity was determined for all the fractions collected. The main fraction (P1) demonstrating chitinase activity was pooled for further investigation.

2.2.6. Capillary liquid chromatography

The purified chitinase was chromatographed on a C18 capillary reversed phase high performance liquid chromatography (CLC) column using an analyzer (Applied Biosystems Model ABI 140D, Perkin-Elmer Co., MA, USA).

2.3. Characterization of the purified chitinase

2.3.1. Protein determination

Protein concentrations were measured by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as standard.

2.3.2. Enzyme assays

Chitinase activity was determined by measuring the reducing end group N-acetamino-glucose produced from colloidal chitin according to the method of Boller, Gehri, Mauch, and Vogeli (1983). Preparation of colloidal chitin was performed by the method of Li, Zhao, and Li (2002). In a typical reaction, the reaction mixture consisting of 1 mL enzyme solution, and 1 mL of 1% (w/v) colloidal chitin (pH 5.4) was incubated at 50 °C for 60 min. The reaction was terminated by adding 2 mL dinitrosalicylic acid reagent and heating in boiling water for 5 min, then cooled to room temperature, and centrifuged at 6000 ×*g* for 10 min. The supernatant was subjected to spectrophotometric measurement at 530 nm. One unit of chitinase activity was defined as the amount of enzyme that liberates 1 μg N-acetamino-glucose per minute at pH 5.4 and 50 °C.

2.3.3. N-terminal amino acid sequence analysis

The N-terminal amino acid sequence of the purified peptide was performed by Edman degradation using a protein sequencer (Applied Biosystems Model 476A, Perkin-Elmer Co., MA, USA). Phenylthiohydantoin derivatives were separated and identified by capillary reversed phase high performance liquid chromatography in a C18 column with an analyzer (Applied Biosystems Model ABI 140D, Perkin-Elmer Co., MA, USA).

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

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% T, 4% C) was performed according to the method of LaemmLi and Favre (1973). Gels were stained in 0.1% (w/v) Coomassie blue–30% (v/v) methanol–10% (v/v) acetic acid in water. The destaining solution was 30% (v/v) methanol–10% (v/v) acetic acid in water.

2.3.5. Isoelectric focusing electrophoresis

The isoelectric focusing (IEF)-PAGE was performed using a 2-dimensional electrophoresis and data analysis system (Investigator™ 5000, Tokyo, Japan). The PhastGel IEF for standard proteins was

bought from BIO-RAD Company, USA, covering the pH range 3–10. Proteins on the gel were stained with Coomassie blue R-250.

2.3.6. Determination of optimum pH and temperature

The effects of pH on the enzymatic activity of the purified chitinase preparation were investigated at 50 °C within a pH range between pH 4.0 and 8.0 using 0.2 mol/L sodium acetate buffer (pH 4.0, 5.0, and 5.4), and 0.2 mol/L sodium citrate buffer (pH 6.0, 6.8, 7.4 and 8.0), while the effects of temperature on the enzymatic activity were determined at pH 5.4 within a temperature range from 30 to 80 °C. The temperature curve was made using optimum pH 5.4.

2.3.7. Thermal stability

The thermal stability of the purified chitinase was estimated by determining the residual activity of the enzyme solution after incubation at various temperatures. Chitinase was treated at 50, 55, 60, 65, 75, 80 and 85 °C for 30 min in the experiment.

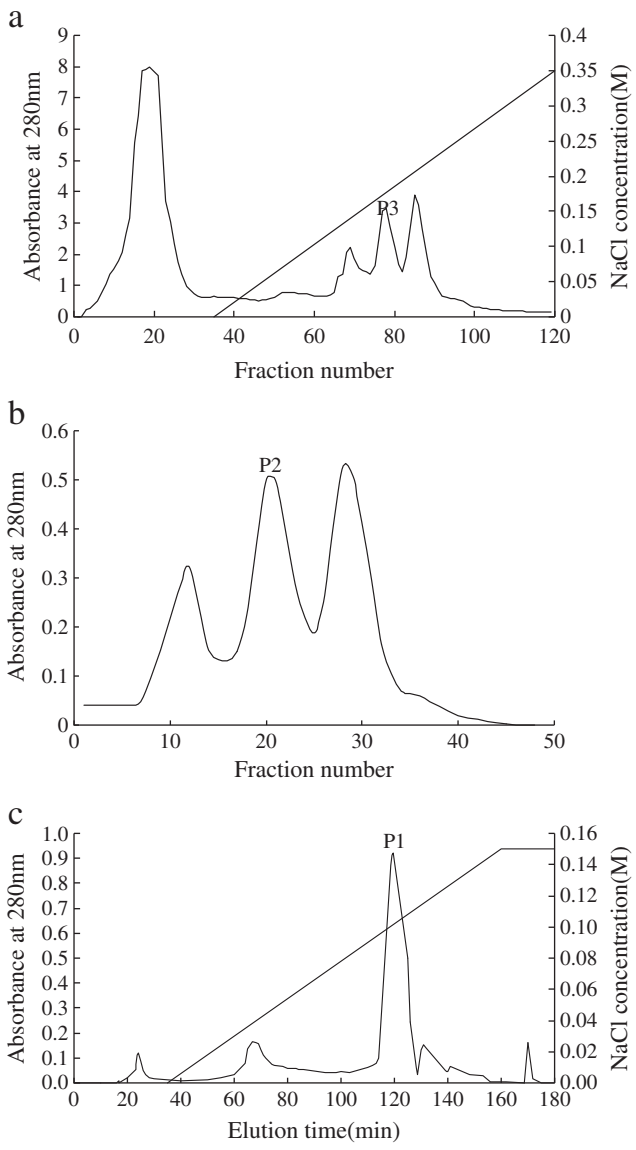

Fig. 1. (a) Fractionation on a CM-Sepharose column equilibrated with the binding buffer (0.02 mol/L sodium acetate buffer, pH 5.4). The gel was washed with the binding buffer and eluted with a linear gradient of 0–0.35 mol/L NaCl in the same buffer. (b) The fraction P3 with chitinase activity from CM column chromatography was pooled, concentrated and applied to gel filtration chromatography on a Sephadex G-75 column. Protein elution was carried out with 0.02 mol/L sodium acetate buffer (pH 5.4). (c) The second peak from the Sephadex G-75 column then applied to a POROS HS-20 column in 0.02 mol/L PBS buffer (pH 5.4). The column was then washed with the binding buffer. Adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.15 mol/L in the same buffer.

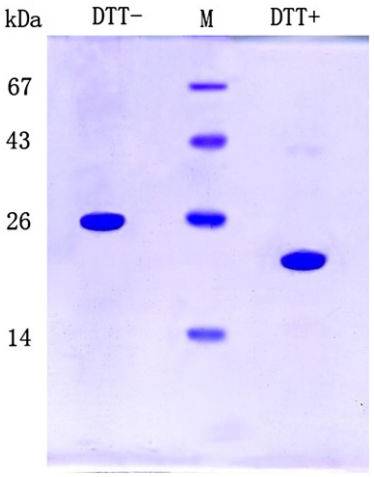

Fig. 2. SDS-PAGE of fava bean chitinase. From left to right: lane dithiothreitol− (DTT−) was loaded with 15 μg chitinase under nonreducing conditions (without addition of DTT); lane M was molecular mass standards; and lane DTT+ was loaded with 15 μg chitinase under reducing conditions (with DTT added).

2.3.8. Effect of metal ions on chitinase activity

Chitinase activity was determined by the standard assay method in the presence of Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, and Pb^{2+} at 1 mM and 2 mM. The relative activity was calculated with respect to the control where the reaction was carried out in the absence of any additive.

2.3.9. Chitin-binding assay

Chitin-binding assay was determined by the method described by Gilkes et al. (1992). The chitin used in this experiment as binding substrate was prepared according to the method of Molano, Duran, and Cabib (1977), and the degree of acetylation was more than 95%. The binding assay for these experiments was carried out at pH 5.4 for 3 h. The amount of bound protein was calculated from the difference between the initial protein concentration and the free protein

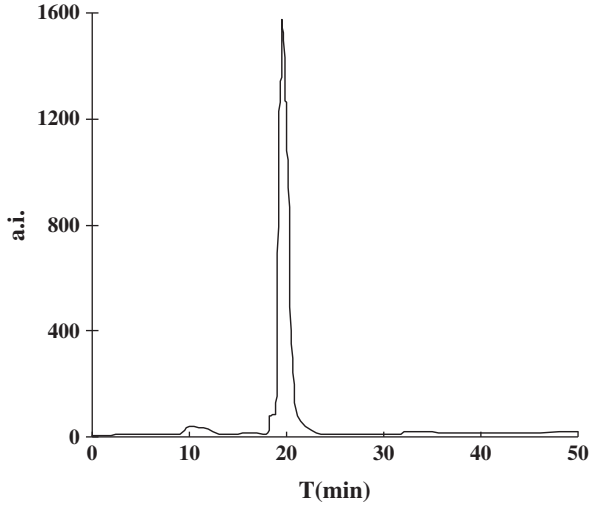

Fig. 3. Capillary reversed phase high performance liquid chromatography of the purified chitinase using a C18 column.

Table 1
Summary of purification of a chitinase from fava beans.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	2.03×10^4	1.57×10^4	0.77	1.0	100
Ammonium sulfate precipitation (20–80% saturation)	8.12×10^3	9.86×10^3	1.21	1.6	62.8
CM-Sephadex C-50 (P3, 3rd adsorbed peak)	9.75×10^2	7.95×10^3	8.15	10.6	50.6
Sephadex G-75 (P2) (2nd peak)	98.5	3.62×10^3	36.75	47.7	23.1
POROS HS-20 (P1) (P1 peak)	9.3	8.67×10^2	93.23	121.1	5.5

Note: protein obtained from 100 g fava beans (dry basis).

concentration after binding. The relative equilibrium association constants *Kr* ($L\,g^{-1}$) were determined from double-reciprocal plots of binding data described by Gilkes et al. (1992).

2.3.10. Assay for antifungal activity

Assay for antifungal activity was executed using the method of inhibition crescents (Wang et al., 2009). After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed around and at a distance of 0.5 cm away from the rim of the mycelial colony. An aliquot (8 μL containing 30 or 150 μg) of chitinase in 20 mmol/L sodium citrate buffer (pH 5.4) was introduced to a disk. The plates were incubated at 28 °C for 72 h until mycelial growth had enveloped peripheral disks containing the control (buffer) and had produced crescents of inhibition around disks containing samples with antifungal activity.

2.4. Statistical analyses

All data are presented as means (standard deviations, SDs) of three independent experiments. Statistical analysis was done using Student's *t* test. A value of $P<0.05$ was considered statistically significant.

3. Results and discussion

3.1. Purification of the chitinase

Following removal of a large amount of unadsorbed proteins, the third adsorbed fraction (P3) on CM-Sephadex exhibiting chitinase activity was desorbed from the column with a linear NaCl concentration gradient (Fig. 1a). The active peak was pooled, dialyzed and subsequently concentrated. After that chromatography on a G75 column was carried out. The second peak (P2) demonstrated chitinase activity (Fig. 1b). The active fraction was further fractionated by HPLC on POROS HS-20 column (Fig. 1c). The only main peak, which is designated as the first peak (P1), displayed chitinase activity. Its SDS-PAGE pattern is shown in Fig. 2. The purified chitinase was shown by CLC (Fig. 3) to be of high purity.

The protein yield and enzymatic activity at each purification step are presented in Table 1. There was a 121 purification fold increase of the purified chitinase compared with that of the crude extract.

3.2. Characterization of the purified chitinase

3.2.1. Molecular mass and isoelectric point

The molecular mass of the chitinase obtained was estimated by SDS-PAGE to be 25.5 kDa under non-reducing and 21.5 kDa under reducing conditions, i.e., in the absence or presence of dithiothreitol (DTT), as shown in Fig. 2, indicating that the purified chitinase is a monomeric protein and that there are disulfide bonds inside the molecule. Fig. 2 indicates, in fact, that when DTT is added the molecular mass of chitinase is lowered and still a single band is observed. These two observations support the view that the purified enzyme is a monomeric protein with intra disulfide bonds. The isoelectric point (*p*I) of fava bean chitinase was determined to be 8.7 based on the results of isoelectric focusing electrophoresis (Fig. 4), demonstrating a basic property of newly reported chitinase.

There is a range of molecular masses reported for antifungal chitinases. Some chitinases have a molecular mass near 30 kDa, while others are well over 30 kDa in molecular mass (Ye & Ng, 2005). Delandin, the chitinase-like antifungal protein from rice bean, has a molecular mass of 28 kDa (Ye & Ng, 2002), similar to its counterparts from field bean and black turtle bean (Chu & Ng, 2005; Ye et al., 2000a; Ye, Wang, & Ng, 2000b). However, the chitinases from mung bean, peanut, cranberry beans and chickpea have a molecular mass of 30.8 kDa, 30.4 kDa, 30.6 kDa and 32.4 kDa, respectively (Li & Wang, 2006; Wang et al., 2007, 2009; Ye & Ng, 2005). This study's fava bean chitinase has a molecular mass (25.5 kDa) within the range of molecular masses (between 25 and 35 kDa) reported for chitinases (Ye & Ng, 2002). The results that the purified chitinase shows single band on SDS-PAGE either in the absence or presence of dithiothreitol experiment indicate that the enzyme is a monomeric protein as are the previously reported chitinases (Chu & Ng, 2005; Ye & Ng, 2002; Ye et al., 2000b).

At the same time, the molecular mass which the protein exhibited in reduced condition (21.5 kDa) is different from that in oxidized condition (25.5 kDa) on SDS-PAGE (Fig. 2), indicating that there are disulfide bonds inside the molecule. When DTT is added, the S – S bonds in a protein are expected to be reduced and hence the molecular mass of the protein is lowered under such conditions. In this case, the breakage of intra-disulfide bonds caused a significant loss of 16% in the enzyme's molecular mass, from 25.5 kDa to 21.5 kDa. Probably, the breakage of disulfide bonds caused the loose structure in intra-molecular and the migration velocity on SDS-PAGE was severely retarded. It is quite

Fig. 4. A profile of isoelectric focusing electrophoresis results, loaded with 10 μg chitinase. The PhastGel IEF for standard proteins covered the pH range 3–10.

Table 2
Comparison of N-terminal sequence of fava bean chitinase with those from other plants.

Protein name	Residue number	N-terminal sequence	Residue number	Identity (%)	Length
Fava bean chitinase	1	DDVGSVISASLFEQLLKH	18	100	
Chitinase precursor, *Canavalia ensiformis* (CAA07413)	28	DDVGSVIDASLFDQLLKH	45	88	270
Chain A, structure of the chitinase from jack bean (1DXJA)	1	DVGSVIDASLFDQLLKH	17	88	242
Putative chitinase, *Musa acuminata* (CAC81812)	76	VGSIISSSLFEQMLKH	91	81	317
Endochitinase, European chestnut, *Castanea sativa* (AAB01895)	76	DVGSLISASLFDQMLK	91	81	316
Putative chitinase, *Musa acuminata* (CAC81811)	69	VASIISSSLFEQMLKH	84	75	318
Endochitinase precursor, European hop, *Humulus lupulus* (AAD34596)	75	DVSSVISSALFEEMLKH	91	70	316
Endochitinase 4 precursor, potato, *Solanum tuberosum* (P52406)	77	DIGSVISNSMFDQMLKH	93	70	302
29 kDa chitinase-like thermal hysteresis protein, *Solanum dulcamara* (AAP32201)	16	DLGSVISNSMFDQMLKH	32	70	267
Basic chitinase, common tobacco, *Nicotiana tabacum* (AAB23374)	78	DLGSIISSSMFDQMLKH	94	64	328
Endochitinase, wood tobacco, *Nicotiana tabacum* (CAC17793)	74	DLGSIISSSMFDQMLKH	90	64	324
Endochitinase B precursor (CHN-B), common tobacco, *Nicotiana tabacum* (P24091)	74	DLGSIISSSMFDQMLKH	90	64	324

Note. Identical corresponding amino acid residues are underlined. The BLAST accession number of the sequences is indicated in brackets.

dissimilar to monomeric chitinases without disulfide bonds from mung bean, peanut and cranberry beans mentioned above (Wang et al., 2007, 2009; Ye & Ng, 2005), while analogous to chitinases from jack bean and barley with three disulfide bonds in each molecule (Hahn, Hennig, Schlesier, & Hohne, 2000; Hart, Monzingo, Ready, Ernst, & Robertus, 1993).

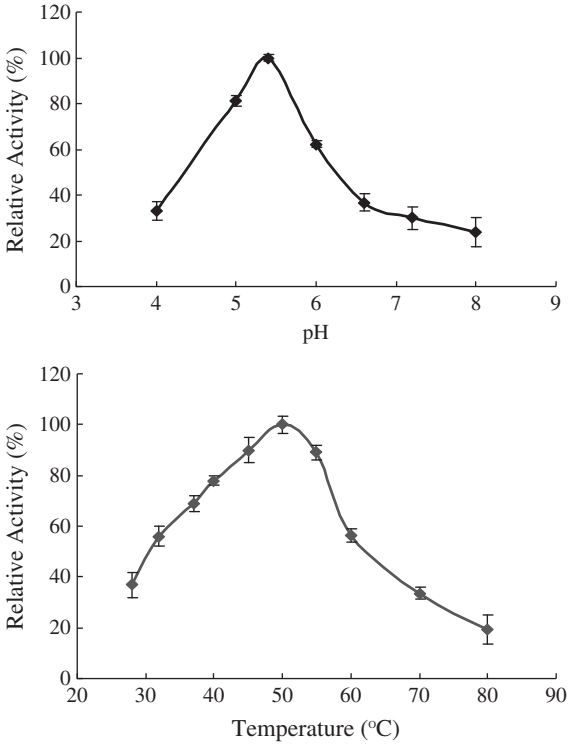

Fig. 5. Effect of pH and temperature on activity of the purified chitinase. Activity at pH 5.4 and 50 °C was used as standard.

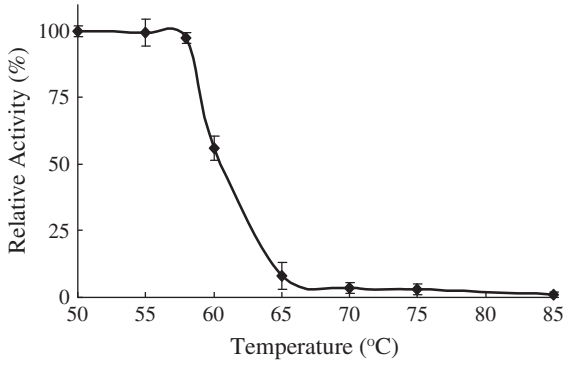

Fig. 6. Thermal stability of the purified chitinase.

3.2.2. N-terminal amino acid sequence

The N-terminal amino acid sequence of the purified fava bean chitinase was determined to be D-D-V-G-S-V-I-S-A-S-L-F-E-Q-L-L-K-H. This demonstrated some resemblance (up to 88%) to chitinases and chitinase precursors from other leguminous plants (Table 2).

The individual chitinases often different in their physical–chemical and biological properties represent the diversity of chitinases, even from the same species. It is noteworthy that the first two amino acid residues (D–D) of N-terminal in the fava bean chitinase existed in the jack bean chitinase precursor, while only one of the two D–D amino acid residues is present in jack bean chitinase (Hahn et al., 2000). This is probably because protease in jack bean cuts the precursor in the middle of D–D amino acid residues, while protease in fava bean cuts in front of D–D amino acid residues. However, this extra amino acid may play a minor role in enzyme activity.

3.2.3. pH and temperature optima

Relative enzyme activity as a function of pH and temperature is shown in Fig. 5. The optimum pH was 5.4 and the optimum temperature was 50 °C. The slight increase or decrease of pH around 5.4 would lead to a sharp reduction in the chitinase activity. The chitinase was almost

Table 3
The effect of metal ions on fava bean chitinase activity.

Additive	Concentration (mM)	Relative activity (%)
Control	–	100.0
KCl	1	99.2 ± 0.4
	2	100.6 ± 1.1
NaCl	1	100.5 ± 1.0
	2	101.2 ± 1.3
$CaCl_2$	1	97.5 ± 0.9
	2	99.4 ± 0.7
$MgCl_2$	1	95.8 ± 1.2
	2	103.0 ± 1.0
$MnCl_2$	1	97.6 ± 0.8
	2	98.2 ± 0.6
$ZnCl_2$	1	99.8 ± 0.6
	2	100.0 ± 0.5
$CuSO_4$	1	87.6 ± 0.7
	2	88.7 ± 1.2
$FeCl_2$	1	72.1 ± 0.8
	2	66.0 ± 0.7
$PbCl_2$	1	71.2 ± 0.6
	2	36.3 ± 0.5
$HgCl_2$	1	48.9 ± 1.3
	2	20.0 ± 0.8

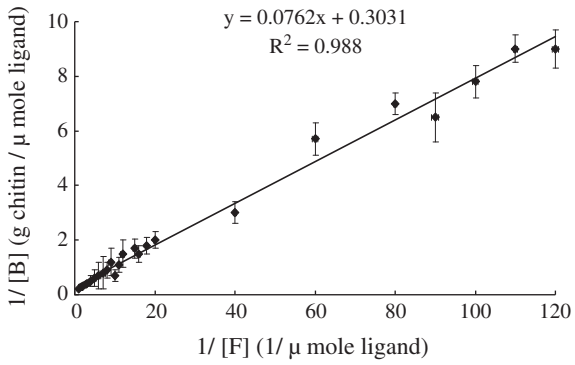

Fig. 7. Double-reciprocal plots of binding data for chitinase. The binding assay was performed by keeping the binding assay mixtures on ice for 3 h. [B], bound protein concentration; [F], free protein concentration.

completely inactivated at pH above 6.8 or below 4.0 and also at temperatures above 85 °C.

The optimum pH of the chitinases from *Bacillus brevis* (Li et al., 2002), bean leaves (Boller et al., 1983) and *Phaseolus vulgaris* (Yang & Luo, 1998) was pH 5.5, 6.5 and 6.6, respectively. Therefore optimum pH of the purified chitinase was similar to its counterpart obtained by Li et al. (2002), but different from chitinases acquired by either Boller et al. (1983) or Yang and Luo (1998). This chitinase from fava bean would undergo up to 80% loss of activity at pH 6.5. However, its optimum temperature (50 °C) was similar to all those chitinases mentioned above.

3.2.4. Thermostability

As shown in Fig. 6, the activity of the purified enzyme would not decrease at all while incubated for 30 min at the temperature under 58 °C. Namely, the chitinase was very stable at room temperature or in a hotter environment below 58 °C. Incubation at 60 and 70 °C for 30 min resulted in approximately half loss of the enzyme activity. The chitinase was completely inactivated after incubation at a temperature above 85 °C.

The thermostability of the fava bean chitinase is higher than that of the chitinases reported (Boller et al., 1983; Li & Wang, 2006; Yang & Luo, 1998). The function of protein is determined by structure, and the existence of disulfide bonds in molecular structure usually increases the molecular stability (Yin, Tang, Wen, Yang, & Yuan, 2010). Therefore we suggest that the reason for the relative stability of the chitinase probably is related with the disulfide bonds in its molecular conformation (Fig. 2). A relatively heat-resistant enzyme from fava seeds would certainly be significant in its potential application in agriculture. However, a chitinase from pomegranate shows more higher thermostability (Kopparapu, Liu, Yan, Jiang, & Zhang, 2011), it has optimal activity at pH 4.5 and 70 °C, and the literature is lacking in the structure information.

3.2.5. Effect of metal ions on chitinase activity

In the presence of Na^+, K^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, and Zn^{2+}, the chitinase activity was retained well. However, the activity was severely inhibited by Cu^{2+}, Fe^{2+}, Pb^{2+} and Hg^{2+}. General characteristics of the purified chitinase were shown in Table 3. The effect of metal ions on chitinase activity varies among different types. Hg^{2+} is a common inhibitor of most of the chitinases. The activity of a chitinase from *Microbispora* sp. V2 was just inhibited by Hg^{2+}, in the presence of which only 10% activity was retained (Nawani, Kapadnis, Das, Rao, & Mahajan, 2002). Among the two chitinases that were purified from the intestinal tract of the South American sea lion, one was inhibited with Cu^{2+}, Fe^{2+}, Hg^{2+} and Zn^{2+}, while the other one was inhibited with Fe^{2+} (Konagaya et al., 2006). The lima bean chitinase was inhibited by Pb^{2+} and Hg^{2+} rather than Cu^{2+} and Fe^{2+} (Wang et al., 2008).

3.2.6. Chitin-binding activity of purified chitinase

The double-reciprocal plots of chitin binding results suggest that the chitinase shows chitin-binding activity (Fig. 7). The relative equilibrium association constants (*Kr*) of chitinase toward regenerated chitin represent the binding activity of tested chitinase, and *Kr* was determined by the regression equation $y = 1/Kr\ x + b$, based on the double-reciprocal plots proposed by Gilkes et al. (1992). The regression equation for these experiments was estimated as $y = 0.0762x + 0.3031$ (Fig. 7), the corresponding *Kr* was calculated to be 13.1 L g^{-1}. The chitinase showed

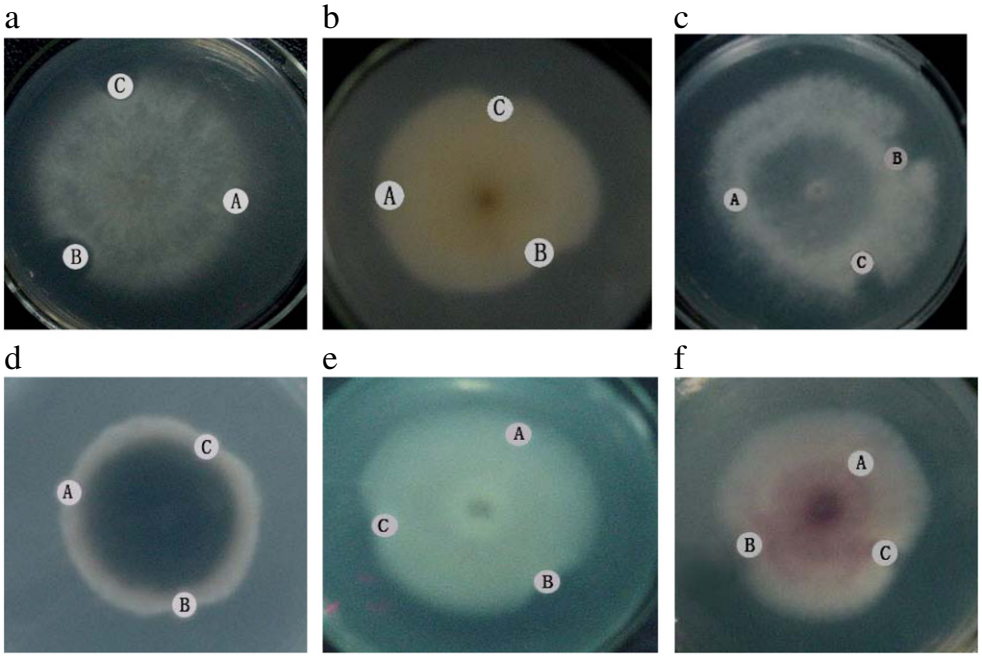

Fig. 8. Inhibitory activity of fava bean chitinase toward *Pythium aphanidermatum*, *Fusarium solani*, *Physalospora piricola*, *Alternaria alternate*, *Botrytis cinerea*, and *Fusarium oxysporum* f. sp. *melonis*, respectively. (A) 0.02 mol/L sodium acetate buffer (pH 5.4), (B) 150 μg purified chitinase, and (C) 30 μg purified chitinase.

comparable chitin-binding activity when its *Kr* is compared with those reported by Hashimoto et al. (2000) and Wang et al. (2008). The equation represented systematical linearity ($R^2 = 0.988$, Fig. 7), signifying that the model was a good fit to the behavior of chitin binding.

The chitin-binding capacity of purified chitinase varies upon variation of pH (Gilkes et al., 1992), therefore sharp variation of activity with pH (Fig. 5) suggests that the chitin-binding activity is involved in the catalytic process of chitinase. Loss of the chitin-binding domain deprived the enzyme of the ability to bind to chitin and significantly reduced the ability to hydrolyze colloidal chitin (Gilkes et al., 1992). Therefore, it appeared that the chitin-binding domain is important for efficient degradation of chitin.

3.2.7. Antifungal activity

Antifungal activity of chitinase was tested toward a series of fungi. Fig. 8a–f presents the antifungal activity of fava bean chitinase on fungal species. It can be seen that the purified protein showed antifungal activity toward *P. aphanidermatum* (Fig. 8a), *F. solani* (Fig. 8b), *P. piricola* (Fig. 8c), *A. alternate* (Fig. 8d), *B. cinerea* (Fig. 8e), and *F. oxysporum* f. sp. *melonis* (Fig. 8f). Based on a comparison of the zone/crescent of inhibition of mycelial growth, the inhibitory action of fava bean chitinase on fungal growth was more potent than that from the bean seeds (Yang & Luo, 1998), and the French bean thaumatin-like protein (Boller et al., 1983), while similar to that of the chitinase-like protein from adzuki bean (Ye & Ng, 2002). The observation that the purified protein showed antifungal activity toward different fungal species has potentially important implications in agriculture.

It is noteworthy that the purified chitinase retained almost 100% antifungal activity after treated by incubating at 20, 30, 40, 50 and 58 °C for 10 min, respectively (data not shown). However, a pomegranate chitinase did not exhibit any antifungal activity (Kopparapu et al., 2011). The thermal stability determination to antifungal activities demonstrated that the chitinase displayed a relative thermostability in antifungal activity.

4. Conclusions

In summary, a novel chitinase with both relative thermostability and antifungal activity against a variety of fungal species was isolated from fava bean seeds. The purified chitinase was rather distinct with those from bean leaves and bean seeds in molecular mass and conformation, and slightly different from these in temperature optima and thermostability. Its N-terminal amino acid sequence shows resemblance to those of chitinases and chitinase precursors from other leguminous plants.

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