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A novel isolation and purification of antifungal chitinase from cowpea (*Vigna unguiculata*) and its possible biotechnological applications

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ABSTRACT

A novel chitinase enzyme with molecular weight of approximately 50KDa was isolated from cowpea seeds. It was purified using ammonium sulphate and chitin affinity binding techniques. The enzyme was purified to 1.84-fold protein with specific activity of 2.017U/mg using ammonium sulphate precipitation and the purification increased to 7.76-fold protein and specific activity of 8.479 U/mg after chitin affinity binding technique. The enzyme showed strong antifungal activity against *Fusarium oxysporum*, *F. solani* and *Alternaria alternata* and moderate activity against *Penicillium frequantes*. The new enzyme may represent an additional element for constitutive defense in cowpea plant. The enzyme also showed moderate antifungal activity against *Saccharomyces cerivisiae* and very strong activity against *Candida Albicans*. This finding suggests its possible role as antifungal agent against some phytopathogenic fungi in the field and also its possible usage in food industry and food preservation. Besides, its anticandidal activity suggests its possible pharmaceutical applications.

Keywords: *Vigna unguiculata*, chitinase, chitin affinity binding, antifungal, biotechnological applications.

INTRODUCTION

Cowpea (*Vigna unguiculata*) is an important crop in tropical and subtropical areas around the world (Steel *et al*., 1985). It is considered as important source for human consumption and animal feeding (Steel *et al*.,1985; Steel and Mehre,1980). Cowpea seeds are heavily affected by a great number of pathogens and this may be due to the low level of defenses, either constitutive or induced, in this plant (Gomes and Xavier-Filho, 1994; Xavier-Filho *et al*.,1989). But several varieties or cultivars of cowpea can express defense activity against viruses, bacteria, fungi and insects (May *et al*., 1988). Several of these defenses are proteinsous compounds (Carvalho *et al*., 2001; Gomes *et al*.,1996; Sales *et al*., 2000; Xavier-Filho,1991).

Few studies have focused on the defense proteins from cowpea seeds.Conditions for extraction of proteins from cowpea were described by Sefa-Dedeh and Stanley (1979) followed by characterization of these proteins in the same year. It was also reported that antimicrobial peptides (Carvalho *et al*.,2001), pathogenesis related (PR) proteins that inhibit growth of fungi and insects (Gomes *et al*.,1996) and proteins with antiviral and antifungal (Ye *et al*.,2000) have been found in seeds of this plant.

The role of chitinase during infection of cowpea plant seeds by fungi is not understood. One reason for this lack of understanding is the low levels of this enzyme found in these organs. Therefore, present study aimed to investigate the presence of novel chitinase enzyme in cowpea that has antifungal activity which can describe its role in plant defense and also its possible role in various industrial applications.

MATERIALS AND METHODS

1.Plant material:

Dry seeds for cowpea (*Vigna unguiculata*) were obtained from the local market.

2.Extraction of seed proteins:

50 g of cowpea seeds were soaked in water at 4°C for 48 hours. The soaked seeds were homogenized in 500ml of 50mM sodium phosphate buffer (PH 6.4). The extract was centrifuged at 14000rpm for 15 min at 4°C. The supernatant was used for further experimentation.

3. Purification of Chitinase enzyme:

a. Ammonium sulphate precipitation:

150 ml of protein extract were precipitated with 80% ammonium sulphate saturation and the mixture was stored at 4°C overnight. The precipitate was collected by centrifugation at 4°C for 10 min at 14000 rpm. The precipitate was dissolved in 30 ml of 50 mM sodium phosphate buffer (PH 6.4) and dialyzed overnight against the same buffer at 4°C.

b. Chitin affinity binding:

Colloidal chitin was prepared according to method of Lee *et al*. (2009):1 g chitin powder was added slowly to 20 ml Hcl (concentrated) and kept at 4°C overnight with stirring. Mixture was then added to 200 ml ice-cold 95% ethanol with vigorous stirring and left at 4°C overnight. Precipitate was collected by filtration and dialyzed against distilled water till nutrient PH using dialysis tubing, and then the final volume was raised to 100 ml using ddH_2O. For chitin affinity 20ml of protein dialysate were mixed with 10ml of colloidal chitin prepared using 50mM sodium phosphate buffer PH 6.4. Mixture was left overnight at 4°C with continuous stirring. Mixture then was centrifuged at 10000rpm for collecting colloidal chitin for 10 min at 4°C. The precipitate was washed four times with 25 ml of 50 mM phosphate buffer for removal of unbound proteins. Then the protein (chitinase) was eluted from the precipitate with 25 ml of 50mM phosphate buffer containing 1M Nacl at PH 6.4. Mixture was stirred overnight at 4°C for elaboration the enzyme from colloidal chitin. After centrifugation at 10000rpm the supernatant was precipitated with 100% ammonium sulphate saturation. Pellet was dialyzed and dissolved in 5 ml of 50mM sodium phosphate buffer PH 6.4.

4. Measurement of chitinase activity:

The reaction mixture consisted of 750 ul of 1% (w/v) colloidal chitin suspended in 0.1mM acetate buffer and 750 ul of enzyme solution. After incubation at 40°C for 60 min, the reaction mixture was centrifuged at 14000rpm for 5 min and the supernatant was used for reduced sugar analysis using the dinitrosalicylic acid (DNS) method. One unit (U) of chitinase activity was defined as the amount of chitinase enzyme necessary to release 1umol GlcNAc per minute under the above mentioned conditions (Nawani and Kapadnis, 2005).

5.Determination of chitinase molecular weight:

Molecular mass of purified chitinase enzyme was determined by (SDS-PAGE) according to method of Laemmli (1970). Samples were mixed 1:4 with sample buffer and boiled for 10 min. Standard protein size marker (Geneaid) was used. Gels were stained in 0.1% (w/v) Coomassie brilliant blue R-250 (Sigma), 30% (v/v) methanol and 10% (v/v) glacial acetic acid and destaining was performed using solution of 30% (v/v) methanol and 10% glacial acetic acid in distilled water. Chitinase molecular weight was determined by comparing to those of the marker proteins.

6.Total protein estimation:

The protein concentration of samples was determined according to the method of Bradford using bovine serum albumin (Sigma) as standard Bradford (1976).

7. Antifungal activity:

Fungal isolates (*Fusarium oxysporum, Fusarium solani, Alternaria alternate, penicillium frequantes, Saccharomyces cerevisiae* and *Candida albicans*) were obtained from the fungal collection present in Botany and Microbiology department, Minia University. For antifungal activity test the filamentous fungi were subcultured on potato-dextrose agar medium (PDA) for 7 days and for test 5mm plug of fungi were inoculated to the center of petri dishes containing PDA medium and dishes were incubated for 48 h at 30^{o}C, then wells of 5mm were made in the agar infront of fungal colonies and 20ul of purified enzyme or buffer (as control) were added into the wells and plates then incubated for 24h at 30^{o}C. In this manner if the enzyme has antifungal activity then a crescent-shaped zone of fungal growth inhibition will be observed around the well. For yeasts, both *Saccharomyces cerivisiae* and *Candida albicans* were subcultured in potato-dextrose (PDA) for 24h and the optical density of the fungal suspension was adjusted to reading OD600=0.2 and 1ml of this suspension was spread on PDA plates, then two sterile filter paper discs were placed on the top of the agar surface and 20ul of the purified chitinase enzyme or of buffer (as control) were applied to the discs. Plates were incubated for 24h at 30^{o}C and clear zones formation around discs were checked.

RESULTS

Total proteins of cowpea seeds were extracted using 50mM sodium phosphate buffer (PH 6.4). And after ammonium sulphate precipitation (80% saturation) the chitin affinity binding technique was used for purification of a new chitinase enzyme. The purification method results showed that the enzyme was purified 1.84-fold with a specific activity of 2.017 U/mg protein after ammonium sulphate precipitation and after the chitin affinity binding purification step the enzyme purified to 7.76 fold with a specific activity of 8.479 U/mg protein (Table 1).

Table 1. Purification of chitinase from *Vigna unguiculata.*

Step	Volume (ml)	Protein (mg)	Activity (Units)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	150	118.5	129.54	1.092	1	**100**
80% $(NH_4)_2SO4$	30	50.46	101.81	2.017	1.84	**78.59**
Chitin affinity Binding	5	0.98	8.31	8.479	7.76	**6.41**

The purified enzyme showed one intense band on SDS-PAGE of approximately 50 KDa (Fig 1). The purified enzyme was tested for its antifungal activity, since it showed strong antifungal activity against *F. oxysporum. F. solani*, and *A. alternata* and moderate activity against *P. frequantes* (Fig 2). The purified enzyme was also tested against two yeast isolates, namely *Saccharomyces cerivisiae*and *Candida albicans*and it showed moderate effect on *S. cerivisiae* and very strong effect against *C. albicans* (Fig 2).

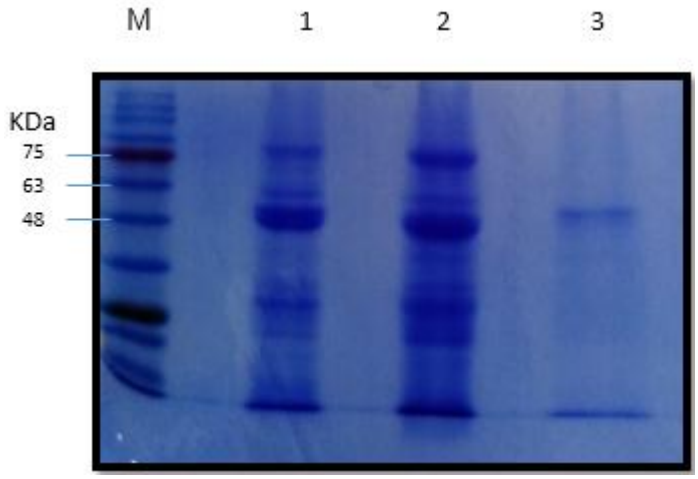

Fig 1. Purification of chitinase from *Vigna unguiculata* by SDS-PAGE. Lane M: molecular mass standards; lane 1, Crude enzyme; lane 2, 80% ammonium sulfate pellet; lane 3, chitin affinity binding.

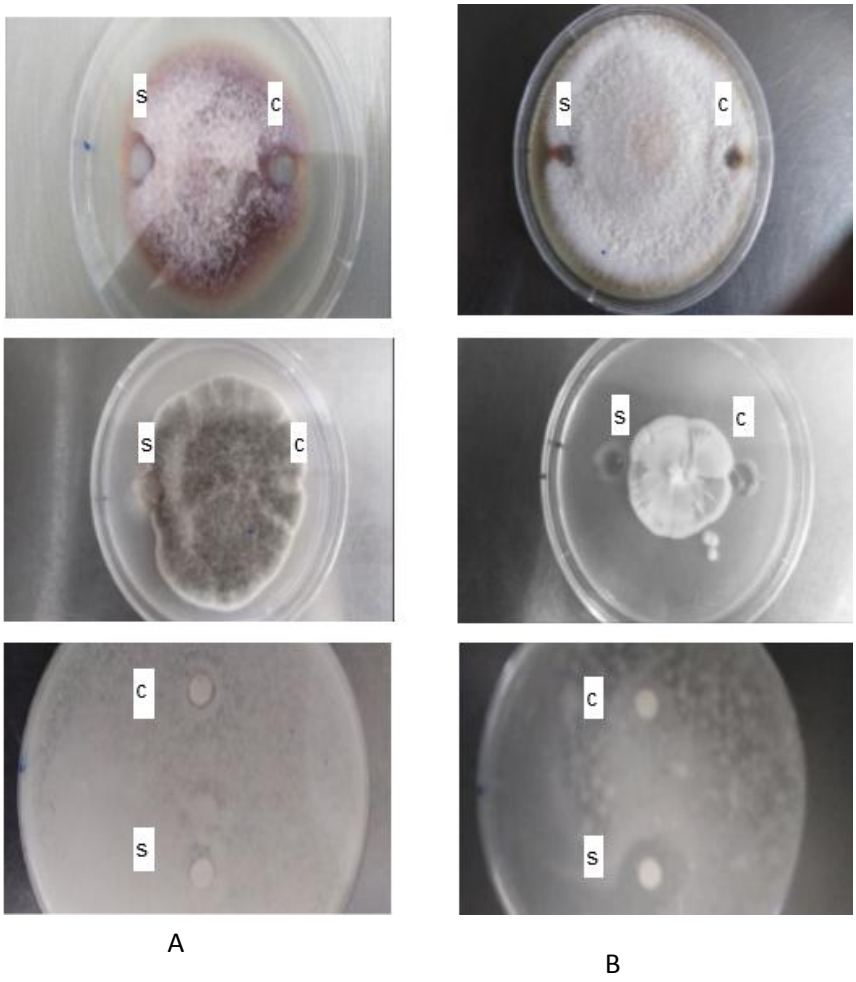

Fig 2. Antifungal activity against different fungi (A) *F. oxysporum*, (B) *F. solani*, (C) *A. alternata*, (D) *P. frequantes*, (E) *S. cerevisiae*, (F) *C. Albicans* c :control (Buffer), s: sample(purified chitiase)

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DISCUSSION

As mentioned in many reports for purification of chitinase enzymes from different sources, the chitin affinity binding technique was used here for purification of a new chitinase from cowpea seeds. The results indicated the effectiveness of the purification method in this research and the chitinase was purified to 7.76-fold with a 6.41% yield. Many other researchers have reported multistep procedure for purification of chitinase. Ye and Ng (2005) purified a chitinase throw three steps of chromatography (CM-Sephadex C-50, POROS HS and Sephadex G-75 column chromatography) from mung beans to 3.9-fold and Taira *et al*., (2005) purified chitinase from pineapple leaf throw four chromatography steps such as chitin affinity, Butyl-Toyopearl 650 column, Resource Q column and HPLC on phenyl superpose column.

The current results indicated the molecular weight of the noval isolated chitinase enzyme of approximately 50 KDa. Chitinases from plants are generally have molecular masses in the range of 25 to 35 KDa (Sahai and Manocha, 1993). But many other researchers have reported higher molecular weight chitinases i.e., *Beta vulgaris* (64 KDa) (El sayed *et al*., 2000) and leaf rachises of Cycas (40 KDa) (Taira *et al*., 2009).

There is great interest to understand the role of chitinases in plants since substrates for these enzymes are not present in plant tissues. Most reports are based on assumption that these enzymes are involved in defense mechanisms against fungi, since these enzymes act on the growing tips of fungal hyphae with newly deposited chitin when the plant is attacked (Chrispeels and Raikhel, 1991). In the present study we tested the growth inhibition of some selected phytopathogenic fungi, namely *Fusarium oxyeporum* (causing stem and root rot in cowpea), *F. solani* (causing damping of cowpea), *Alternaria alternata* (causing destructive foliar disease of cowpea) and *Penicillium frequantes* (pectinolytic fungus). We observed very strong inhibition of the purified chitinase against *F. oxyeporum*, *F. solani* and *Alternaria alternata* and less inhibition effect on *P. frequantes* (Fig 2). Not very much is known about the role of chitinases in seeds, but the absence of their substrates in seeds and its presence as constituent of fungal cell strongly suggests a protective role of these hydrolases in these plants.

In this paper we also tested the antifungal activity of the purified chitinase against two yeasts, namely *Saccharomyces cerivisiae* (saprophytic fungus) and *Candidaalbicans* (causing candidiasis in human) and we observed moderate inhibition effect of the enzyme against *S. cerivisiae* and a very strong inhibition effect against *C. albicans*. The antifungal activity of the purified chitinase (either against filamentous fungi or yeast) suggests the possibility of its use for food preservation against fungi, since food industry is still facing unsolved food spoilage problems because of incursion of molds and spores. The use of conventional chemical preservation in food has their own inherent disadvantages, so to avoid that, the natural plant products can be promising alternative. So the production of new antifungal products from plants materials has become an interesting research area in food industry (Irkin and Korukluoglu, 2007). Hans *et al*. (2010) reported that the antifungal activity of Oat (*Avena sativa*) seed extracts showed great potential to prevent the spore germination of *P. roqueforti*, when applied on rye bread. Also Fig leaves extracts showing antifungal and antibacterial activities against some microbes in food have been reported (Hu *et al*., 2007; Balestra *et al*., 2009; Oliveira *et al*., 2009; Aref *et al*., 2010). Also, the strong antifungal activity of the purified chitinase against *C. albicans* suggests the possible pharmaceutical application of this enzyme,

since the previous studies revealed that fungi are the major threat to immune-compromised patients and their infections lead to the patient death. So the production of new natural antifungal products can prevent such infections (Shoham and Levitz, 2005).

Chitinase enzyme isolated and purified in this paper can throw light on additional constitutive defense present in seeds of cowpea plant together with other previously detected compounds like cyanogenetic glucosides (Jaffe, 1950), tannins and lectins (Xavier-Filho *et al*., 1989), proteinase inhibitors (Xavier-Filho and Ventura, 1988; Fernandes *et al*., 1991), variant vicilins (Macedo *et al*., 1993) and many others indicted that seeds, in general, contain several chemical defenses against different pathogens especially phytopathogenic fungi.

The new isolated purified enzyme can be used in different industrial and pharmaceutical applications as mentioned above. Also gene of the new chitinase can be isolated and over expressed in the same plant or for production of transgene of other plants for resistance against phytopathogenic fungi.

Conclusions:

A new antifungal chitinase enzyme was isolated and purified from cowpea seeds. It may represent additional factor for constitutive defense in seeds of this plant. The enzyme can be used in several biotechnological applications.

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فصل و تعريف لانزيم كيتنيز جديد من نبات اللوبيا مع التطبيقات الحيوية الممكنة له

وليد نظير
قسم النبات و الميكروبيولوجى كلية العلوم جامعة المنيا

المستخلص

تم عزل انزيم كيتنيز جديد ذو وزن جزيئى حوالى 50 كيلو دالتون من نبات اللوبيا. الانزيم تم تنقيته باستخدام كل من تقنية الترسيب بكبريتات الامونيوم وتقنية قابلية الارتباط بالكيتن. تم تنقية الانزيم باستخدام كبريتات الامونيوم 1.84 ضعف مع نشاط نوعى 2.01 وحدة/ملجم، وزادت التنقية الى 7.76 ضعف مع نشاط نوعى 8.479 وحدة/ملجم بعد اجراء الارتباط بالكيتن. أظهر الانزيم نشاطا قويا مضادا للفطريات ضد *F. oxysporum* و *F. solani* و *A. alternata* ، ونشاطا متوسطا ضد *P. frequantes* . الانزيم ربما يمثل عنصرا اضافيا فى الدفاع ضد الفطريات لنبات اللوبيا. الانزيم أظهر نشاطا متوسطا مضادا للفطريات ضد *S. cerivisae* ونشاطا قويا ضد *C. albicans*. النتائج تشير الى أهمية دور الانزيم كمضاد للفطريات الممرضة للنباتات فى الحقل و كذلك امكانية استخدامه فى صناعة وحفظ الغذاء، كذلك نشاط الانزيم المضاد للخمائر يظهر امكانية استخدامه فى التطبيقات الدوائية.