Characterization and purification of a chitinolytic enzyme active against *Sesamia cretica* (pink borer)

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ABSTRACT

Corn borers are serious insect pests in many corn growing areas in Egypt and are responsible for significant yield losses. The ability of insect chitinases for attacking and digesting insect chitin raises the idea of using it for controlling insects such as corn borers. In this study, insect chitinases have been extracted from molting fluid as well as from the integument of Sesamia cretica (pink borer) and subjected to protein purification techniques, through three steps of precipitations 40, 60 and 80% of ammonium sulfate saturation. Protein Gel electrophoresis of the purified protein showed that the expected 88 kDa chitinase band is obtained with 40% ammonium sulfate precipitation. The purified protein was eluted from the gel and tested against Sesamia cretica. The LC100 was conducted and revealed 5 mg/ ml. The positive reaction of the 88 kDa purified protein in western blot analysis with monoclonal antibody against Manduca sexta (tobacco hornworm) chitinase protein, confirms its identity to be a chitinase. The obtained results indicate the ability of using the pink borer chitinase protein as a toxic biological reagent for controlling the insect.

Key word: *insect chitinases - Manduca Sexta (tobacco hornworm) - chitin.*

INTODUCTION

Billions of dollars are lost every year due to inadequate control of pests. It is evident that the world food supply depends on effective protection of crops, animals and humans from pests. The chemical control of pests was efficacious and attractive during the forties and fifties; however the adverse effects of such chemicals quickly began to show accumulation of chemical pesticides in soil, water, air, agricultural products and animals, and the development of resistance in target organisms which necessitated the use of more selective and environmentally acceptable agents for pest control. The critical need for safe and effective alternatives to chemical pesticides has stimulated considerable interest in using pathogens and predators as biological control agents for agriculturally and medically important pests. For instance, destruxins of the entomopathogenic fungus (*Metarhizium anisopliae*) are being used to perturb crucial biochemical targets related to growth and development in insects such as cuticle

(Fargues and Robert, 1986). Cuticle is an eukaryotic extracellular biopolymer produced by most invertebrates, including insects. It is composed of chitin (amino sugars), lipids and protein. Entomopathogenic fungi can infect an insect host by penetrating its cuticle. Additionally, chitinolytic fungi affect the larval peritrophic membrane (PM) that forms a chitinous sheet overlaying the gut epithelium. Regev *et al.* (1996) demonstrated that the scanning electron microscopy examination proves the ability of the *S. marcescens* chitinase to perforate the midgut (PM) of the cotton leaf larvae in vitro at the concentration of 0.1-10 mg/ml. In addition to fungi, all kinds of insects produce chitinases, which are essential to cuticle turnover mobilization. For instance, insects periodically shed their old exoskeleton and either continuously or periodically shed their PM and resynthesizes new ones, (Lehan, 1997).

Chitinases are among a group of proteins that insects use to digest the structural polysaccharide chitin in their exoskeletons and gut linings during the molting process (Fukamizo, 2000). Their pesticidal activity is due to their ability to bind with the chitin component of the insect gut and of the pathogenic fungi, causing degradation of chitin containing tissues and death to the organism. It is possible that the chitinase gene transfer technology will become as effective as the Bt gene transfer for the production of a pesticide free environment. In fact, chitinase gene transfer technology may ultimately prove to be more important, since chitinase affects both insect and fungal pathogens. It is generally accepted that bacterial and fungal chitinases are ineffective against some insects when they are fed diets containing these enzymes. Due to the higher effect of the insect chitinases the bacterial and fungal chitinase, were chosen to be a good alternative for insect control.

Millions of dollars are lost every year due to the pink borer infestation in the Middle East region. Corn is considered as the most important cereal crop after wheat and rice all over the world. In Egypt, pink borer significantly affects production of corn as well as sugar cane. In this study we isolated, characterized and purificied insect chitinase protein and proved its insecticidal activity against pink borer indicating its ability to be used as a new biopesticide against pink borer.

MATERIALS AND METHODS

Insects

Sesamia cretica (pink borer)-Noctuidae-Lepidoptera insects were kindly provided by insectary of Agricultural Genetic Engineering Research Institute (AGERI), ARC-Egypt.

Chitinase preparation from the integument

After dissection of the 4th instar larvae of *Sesamia cretica* to a small pieces on ice, integuments and tissues were washed in Ringer's solution (130 mM NaCl, 5 mM KCl, 2 mM CaCl2) three times, then homogenized in three volumes 1X PBS at pH 7, followed by adding protease inhibitors cocktail (5 mg/ml pepstatin, antipain, aprotonin, leupeptin, 1 mM PMSF, and 5 mM benzamidine).

Extraction of the insect chitinase from molting fluid

The extracted fluid was obtained by tiding of the 4th instar larvae before and after the prothoracic gland at molting time. After it for 3 days. The secreted fluid was withdrawn by a syringe.

Protein analysis by SDS-PAGE

The integument or the extracted fluid was dissolved in sample buffer (0.06 M tris HCl pH 6.8, 2% SDS, 10% glycerol, 5% B-

mercaptethanol and bromophenol blue). Samples were heated for 5 min in boiling water bath and separated in 10% polyacrylamide gel as described by Laemmli (1970). Gels were stained with 0.1 % coomassie blue R-250 and then destained in 7% (V/V) acetic acid containing 50% methanol.

Protein purification

The prepared integument protein was spin at 5000 rpm for 5 min; the supernatant was taken and then subjected to protein purification as described by Sambrook et al. (1987). The supernatant was precipitated with ammonium sulfate at 40%, 60% and 80% saturation and the precipitated protein was centrifuged at 5000 Xg for 15 min. Each precipitated pellet was then resuspended in the original volume of 20 mM tris-HCl pH 7.5 and dialyzed against 1X PBS buffer (10 mM Tris and 1 mM EDTA) overnight at 4 C. The purified protein was bioassayed against pink borer larvae. Protein concentration was determined as described by Bradford (1976).

Protein elution

The 88 kDa target protein was eluted from the protein profile obtained from SDS-PAGE, according to the method described by Hunkapiller et al. (1983). The gel was stained to visualize the band of interest then incubated in cooled 1M potassium chloride for 10 min. The band was chopped into small pieces with a razor blade and then transferred to a dialysis bag with 1 ml 1X TAE (4.84 g tris base, 0.744 g sodium EDTA) buffer without acetic acid. The dialysis bag was placed in an electrophoresis apparatus for 20 min using 2.5-5 mA (about 200 V). This process was repeated at least three times to assure complete protein elution.

Western blotting analysis

Western blot analysis of protein allows immunological confirmation of the homology and identity of the protein produced in *Sesamia cretica* with that reported for *Manduca sexta*. The intensity of the stained band by western blotting analysis depends on the total amount of protein in the band as well as the ability of the antibodies to bind to the protein. The method as modified by Towbin *et al.* (1979) and Burnette (1988). Western blotting was performed using monoclonal antirabit Chi 535 antibody from *Manduca sexta* .The antibody was kindly provided by Prof. Krishnan, Department of Biochemistry at Kansas State University, U.S.A. The protein was run on a gel containing 10% Polyacrylamide, and then transferred to a polyvinylidene difluoride (PVDF) membrane using a semi-dry blotting unit (Bio-Rad) overnight at 40 volt. After protein blotting, the membranes were blocked by soaking in milk based blocking buffer (5 % powder milk in 0.5% Tween 20) for two hours at room temperature, then probed with the antiChi-535 antibody (1:1000 dilution in blocking buffer) as a primary antiserum for 4 hr at room temperature. The membrane was washed three times with TBS (50 ml/wash for 15 min) to remove excess antibodies, then the immunoreactive protein was visualized using alkaline phosphates conjugated antirabbit (IgG-AP) as a secondary antibody (1:10,000 dilution in blocking buffer) for one hour. The membrane was washed three times with TBS (50 ml/wash for 15 min) without NaZ to remove excess antibodies and then with PBS three times (50 ml/wash for 15 min). The alkaline phosphatase activity was determined by incubating the membrane with adding equal volumes of Nitro Blue Tetrazolium (NBT) and 5-bromo, 4-chloro, 3-indolyl phosphate (BCIP) at room temperature for 10 minutes.

Bioassay

The eluted protein was conducted to bioassay tests against the 2nd instar larvae of *Sesamia cretica* (pink borer). The semi-artificial diet was reconstituted as following (3.6 L water, 72 g agar, 162 g senn flour, 320 g corn leaf, 36 g sucrose, 36 g glucose, 36 g casein, 7.2 g nibagene, 3.6 g sorbic acid and 144 g yeast) according to the method described by Metwally (1972) and cooked at 55(C. The bioassay cups were filled with 2.5 ml diet and allowed to cool and dry for 5 hr, then the eluted protein was added drop wise on the diet surface. A volume of 500 ml of 5 mg/ml was added to each cup as recommended by Bradford (1976). In designing the bioassay experiment, three replicas, with eluted protein containing 10 larvae per each cup, were carried out. Cups prepared for bioassay were kept at 28°C, and mortality was recorded after 5 days. Control consisted of an untreated diet with the same number of larvae and replicates. The LC100 was determined from Probit analysis plot (Finny, 1962).

RESULTS AND DISCUSSION

The pink borer (*Sesamia cretica*) is considered to be the most dangerous and deadly pest of corn and sugar-cane in Egypt; it causes serious damage to these crops. Females lay their eggs on the lower surface of the leaves, when eggs hatch, and larvae penetrate the stem and continue feeding inside the stem, it is the difficult to control them. The aim of this study is to control pink borer using highly selective and environmentally friendly biopesticide such as insect chitinase.

Protein purification

The protein content of the integument and molting fluid were fractionated in denatured SDS-PAGE by electerophoresis and their protein profiles are shown in Figure (1 panels A&B). It was expected that the integument protein preparation will be crude and has different kinds of unwanted proteins. Therefore the integument protein was further purified and fractionated in order to pinpoint the exact protein responsible for insecticidal activity. The integument protein extract was subjected to ammonium sulfate precipitations at 40, 60 and 80% saturation and purified by dialysis against TE (10 mM tris HCl, 1 mM EDTA, pH8) buffer. The putative chitinase band (~88 kDa) was present in all the protein preparations from the integument (panel A), however precipitation with 40% ammonium sulfate (lane 2) gave the highest yield of the desired protein, while the non-precipitated protein showed numerous protein bands with variable molecular weight sizes in addition to the putative chitinase band (lane 5). On the other hand, the protein content of the molting fluid is more specific than that of the integument as it mainly reveal the 88 kDa band but with low concentration as indicated in panel (B lane 2).

Protein elution

The 88 kDa protein was eluted in cooled 1 M potassium chloride according to the method described by Hunkapiller et al. (1983). For identifying the ability of eluting the protein with this method and to identify the degree of protein loss, the eluted protein was rerun in denatured SDS-PAGE by electerophoresis. Results shown in Figure (2) illustrate the purity and high concentration of the eluted band, indicating the high efficiency of this technique in protein elution.

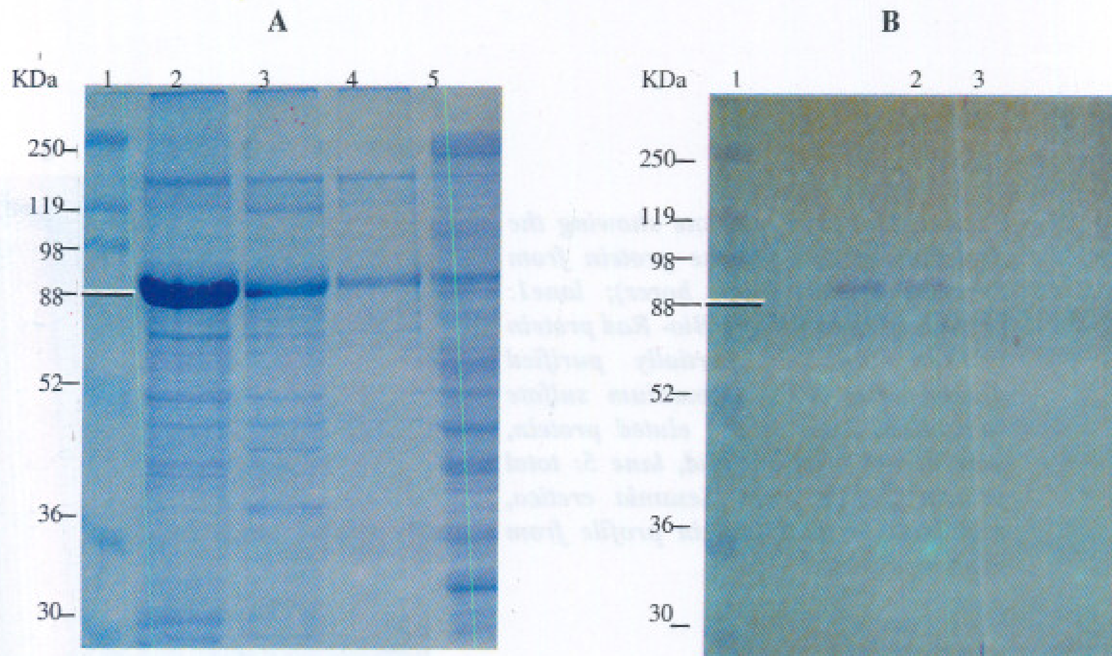

Fig. (1): Polyacrylamide gel electrophoresis SDS-PAGE stained with coomassie R-250 brilliant blue for protein extracted from integument (panel A) and molting fluid (panel B). Lane 1 represents prestained broad range Bio-Rad protein marker. In panel A, lanes 2, 3 and 4 show the 88kDa expected protein after 40%, 60% & 80% ammonium sulfate saturation, respectively, while lane5 represents total protein from Sesamia cretica before saturation. Lanes 2 & 3 in panel B represents unique but low concentrated 88kDa bands obtained from the molting fluid of Sesamia cretica (pink borer).

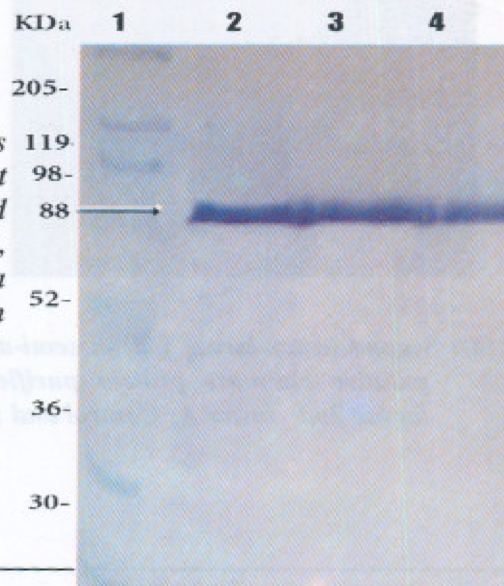

Fig. (2): SDS-Polyacrylamide gel electrophoresis stained with coomassie R-250 brilliant blue showing; lane1: prestained broad range Bio-Rad protein marker, lanes 2, 3, and 4 the eluted protein of the ~ 88 kDa protein band after 40% ammonium sulfate saturation.

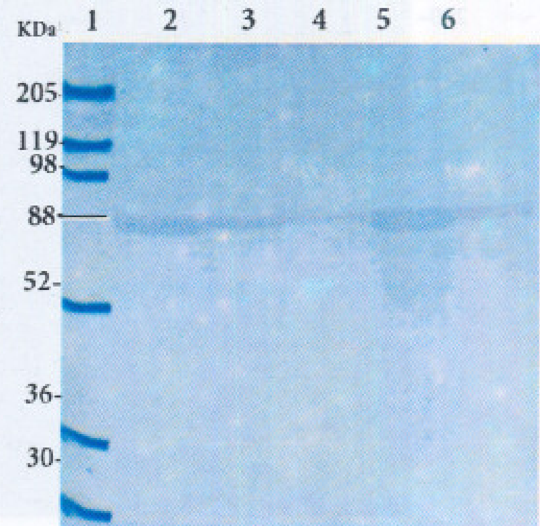

Fig. (3): Western blotted membrane showing the detection of the citinase protein from Sesamia cretica (pink borer); lane1: prestained broad range Bio- Rad protein marker, lane 2: partially purified protein after 40% ammonium sulfate saturation, lane 3: the eluted protein, lane 4: the molting fluid, lane 5: total protein profile from Sesamia cretica, and lane 6: total protein profile from Manduca sexta.

Fig. (4): Second instar larvae fed on semi-artificial diet supplemented with 5 mg/ml putative chitinase protein purified from Sesamia cretica (pink borer). larvae 2nd instar A: Control and B: Treated.

Western blotting analysis

This test was carried out to confirm that the purified protein is an insect chitinase protein. Therefore, extracts from the integument precipitated protein after 40% saturation, molting fluid, and the eluted protein from pink borer in addition to the protein profile from *Manduca sexta* were subjected to SDS-PAGE and immunoblotting using antibody raised against the 88 kDa chitinase protein extracted from *Manduca sexta*. The 88 kDa protein from the pink borer reacted strongly with the *Manduca sexta* antibody as shown in Figure (3).

Insect toxicity assay

Pink borer larvae fed on protein extracted from the integument and molting fluid showed a high degree of mortality after 72 hr, indicating their insecticidal activity. The 40% precipitated protein from the integument was further purified and eluted against TE (10 mM Tris-HCl, 1 mM EDTA) buffer. The eluted protein was then used in bioassay against pink borer larvae 2nd instar. The LC_{100} was proven to be 5 mg/ ml after 72 hr causing insect mortality as shown in Figure (4). Protein concentration used in the bioassay tests was determined according to Bradford (1976). The results of this study are consistent with that obtained by Kramer and koga (1986) as they proved that *M. sexta* chitinase harbor a chitin binding domain capable of recognizing of the insoluble chitin polymer and can be used as biopesticide.

Considerable interest in the chitinolytic enzymes has been stimulated by their possible involvement as defensive agents against the chitin containing pestiferous and pathogenic organisms such as insects, nematodes and fungi (Carr and Klessig, 1989; Linthorst, 1991 and Sahai and Manocha, 1993). The peritrophic membrane and exoskeleton of insects act as physicochemical barriers to environmental hazardous and predators. Both are composed of materials primarily of chitin and protein, however some pathogenic fungi such as *Metarahizium anisopliae*, *Beauveria bassiana*, *Nomuraea rileyi*, and *Aspergillusn flavus* have overcame these kinds of barriers by producing multiple extra cellular degradative enzymes, including chitinolytic and proteolytic enzymes that help the pathogens to penetrate the barriers and expedite infection (St Leger et al., 1986 and El Sayed *et al.*, 1989). Some types of insect venoms also contain hydrolytic enzymes which might serve to facilitate the entry of components of the venom into chitin/ protein – protected prey (Krishnan *et al.*, 1994). Chitinolytic enzymes which are used by insects, nematodes, fungi, viruses, and other organisms for molting or penetration of structural barriers are potentially useful in pest management as targets for inhibitors. In the late 1970s, chitinases were shown to degrade insect gut peritrophic membranes in vitro. Brandt *et al.* (1978) proposed that chitinases cause perforations in the membranes, thus facilitating entry of the pathogens into the tissues of susceptible insects. *Escherichia coli*-produced recombinant endochitinase ChiAll encoded by *S. marcescens* was found by scanning electron microscopy to perforate *Spodoptera* - larval midgut peritrophic membranes after *in vitro* incubations at concentrations of 0.1-10 µg/ml (Regev *et al.*, 1996). Moreover, perforation peritrophic membranes also occurred *in vivo* after the fifth-instar larvae were fed a diet containing recombinant ChiAll.Chitinases also facilitate the penetration of the host cuticle by entomopathogenic fungi (Coudron *et al.*, 1989 and St Leger *et al.*, 1991).

In conclusion, the chitinolytic enzyme purified from pink borer exhibited insecticidal activity against the 2ndinstar larvae of pink borer. The estimated molecular weight for this

chitinolytic enzyme is of 88 kDa and this agree with Kramer and Koga (1986). The data demonstrated that the insect chitinase isolated can be used as a biological control agent against pink borer. Finally, cloning of insect chitinase gene encoding for pink borer toxicity is one of our targets in the near future.

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الملخص العربي

توصيف وتنقية بروتين الكيتنيز القاتل لحشرة دودة القصب الكبيرة

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تعتـبر دودة القصـب الكبـيرة أحد أخطر الآفات الحشرية في الشرق الأوسط وأكثرها انتشارا في مصر مسببة خسارة فادحـة وانخفـاض واضـح في المحصول. وتحلل الكيتين عن طريق إنزيمات الكيتنيز الحشرية يعتبر مدخلاً هاماً ومؤثراً في مقاومـة مـثل هـذه الثاقـبات. ومـن خلا ل هذه الدراسة تم استخلاص إنزيم الكيتنيز الحشري من سائل الانسلاخ والأنسجة الخارجـية(طـبقة الجلد) من حشرة دودة القصب الكبيرة من خلال تكنيكيات تنقية البروتين المختلفة مثل تقنية الترسيب بسلفات الأمونـيوم ٤٠ – ٦٠– ٨٠% علـي التوالـي، وفي نفس الوقت إزاحة البروتين المرسب من علي الجيل. تم اختبار البروتين المنقـي عند وزن جزئيي ٨٨ كيلو دالتون تجاه الحشرة المستهدفة(دودة القصب الكبيرة). بالجرعة المميتة والتي تعطي عند ٥ مللـي جرام/ مللي. وهذه النتيجة تأكدت عن طريق نقل هذا البروتين المنقي إلى غشاء نيلون وتفاعله بقوة مع الأجسام المضادة لحشرة التبغ القرنية . يتضح من هذه النتيجة إمكانية استخدم الكتينيز الحشري كمبيد حيوي لمقاومة ثاقبات الذرة .