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New Chitinase Gene from the Desert Locust, Schitocereca Gregaria and its Possible Use in Biological Control

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Abstract: Chitin is one of the most naturally abundant polysaccharides. Chitin is not only a major component of the insect integument but also extends to cover fore- and hindguts of the insect digestive system. Chitin degrading enzymes, chitinases, are also found in a wide variety of organisms including insects, fungi, bacteria, plants and crustaceans. In this study, RT-PCR was successfully used to amplify the chitinase gene from a mobile wave induced desert locust, Shcistocerca gregaria. The chitinase gene was subsequently cloned using a TOPO TA cloning kit, sub-cloned into pUC18 and sequenced. The identity of this sequence with class III chiItnases does not exceed 64%. For *in vitro* transcription, the gene was subcloned into pET32a vector and expressed in the E. coli expression host. The extracted protein was purified, giving high activity on the solid plate containing purifed chitin. Additionally, the purified protein was visualized on SDS- PAGE and a 35 KDa protein was observed. Our results suggest the presence of a novel chitinase gene in Schistocerca gregaria. Contributing to the novelty of this gene may be that it has a transmembrane helicase region enabling the encoded enzyme to move from cell to cell, presumably to play a part in chitin hydrolysis. The presence of the transmembrane domain in such enzymes may increase their control activities, highly desirable for insect or fungal biocontrol. In addition, the deduced amino acid serine/theronine-rich, which may contribute to anchoring the enzyme tightly onto the substrate to facilitate the hydrolytic process. The phylogenetic analysis for the class III chitinases showed that chi-Hafez is one of the new chitinases sharing a common ancestor with the other chitinases.

Key words: Affinity chromatography · deset locust · Schistocerca gregaria · chitinase · biological control · chitinase purification

INTRODUCTION

More than 300 species of locusts and grasshoppers are known to exist in the African continent [1], but fortunately only a few of them are major pests; most species are sedentary, inhabiting a rather confined area throughout their life cycle. Only few species are highly mobile, migrating from one location to another with ecological conditions more suitable for feeding and reproduction [1]. Furthermore, locusts are used as models in many fields of biology especially in the field of neurophysiology due the ease of growing them [2].

Chitin is not only a major component of the insect integument but also extends to cover fore- and hindguts of the insect digestive system. Chitin degrading enzymes, chitinases, are also found in a wide variety of organisms including insects, fungi, bacteria, plants and crustaceans. Because chitin is one of the most abundant natural polymers, found in a wide variety of organisms (bacteria, viruses, invertebrates, plants, higher vertebrates and humans) [3]. Chitinases (glycosyl hydrolases which catalyze the degradation of chitin) play important physiological and ecological roles [4]. The roles of chitinase in different organisms are diverse, including assimilation of chitin, defense against fungal pathogens and separation of dividing cells. In insects, chitin associates with proteins to form the cuticle exoskeleton and the gut lumen peritrophic matrix. During moulting, the

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old cuticle is digested while a new one is deposited [5]. In addition to the integument, a chitinase has been found in the midgut of the mosquito Anopheles gambiae, where it is believed to be involved in hydrolysing chitin of the peritrophic matrix [6]. Chitinolytic enzymes are now being used for biotechnological applications in agriculture and health care [7]. Moreover, the interest in chitinases has increased in view of their possible uses as selective biopesticides [8]. Endochitinases (EC .2.1.14) are found in chitin-containing organisms, as well as in organisms that do not contain chitin [9,10]. The corresponding genes of many chitinases have been cloned and characterized [11]. Insect chitinases have been isolated in the last two decades from Manduca sexta [12], the silkworm, the fall webworm [13], the common cutworm [14], the yellow mealworm and the spruce budworm [15].

In this paper, we describe the cloning and sequencing of a chitinase gene from the desert locust, Schitocereca gregaria. Cloning and expression of such genes were performed to obtain the purified enzyme and to examine its activity on chitin solidified medium. The sequence analysis and phylogenetic context based on deduced amino acid sequence were also performed.

MATERIALS AND METHODS

Insect materials and chitinase induction using mobile waves: A laboratory colony of the desert locust, Schistocerca gregaria was maintained in the insectary of the department of Entomology, faculty of Science, Cairo University under highly controlled conditions. Approximately50 insects were subjected to a single (acute) discontinuous radio frequency (RF) signal produced by a GSM multiband Ericsson mobile phone (about 900/ 1900 MHz and power approximately 0.03 mW/ cm2) for 4 hours. Two mobile phones were hung in the middle of a fiber cage (40x 40x 40 cm) containing the insects. A control group was placed in a fiber cage with mobile phones turned power off. A pool of five insects were killed by freezing at 0 hour post-exposure and kept at-20°C until used.

RNA extraction, chitinase gene amplification and cloning: Total RNA was isolated from insect tissues according to Chomczynski and Sacchi [16]. cDNA was prepared with a first strand DNA synthesis kit (Pharmacia) and subjected to RT-PCR amplification. A degenerate sense primer, CHI15 5` GGYGGYTGGAATGATGG`3 and an anti-sense primer, CH125 5` GAYTTAGATTGGGAATAYCC`3 were designed corresponding to insect chitinases class III. PCR amplification was performed using the following conditions: 94°C for 3 min for one cycle; 94°C for 30 s, 53°C for 30 s, 72°C for 1 min. for 35 cycles; 72°C for 7 min for one cycle. PCR products were separated in a 1% agarose gel and the major band of about 1100 bp was excised and eluted using QIA gene Gel extraction kit according to the manufacturer's instruction.

Band isolation, purification, sequencing and sequence accession number: A ~1.1 kbp band was eluted and cloned into a TOPO TA cloning kit (Invitrogen). The recombinant clone was sub-cloned into pUC18 and submitted to sequencing (Macrogene Company, Korea). The nucleotide sequence data reported here have been submitted to GenBank under accession number EU106867.

***In vitro* transcription, chitinase purification and activity assays:** The gene was released from the TOPO TA vector using EcoRI enzyme, sub cloned into pET32a and expressed in the Escherichia coli expression host Origami (Novagen). A 1.2 liter culture containing 0.5 M sorbitol and 100 μg ampicillin ml/ ml was inoculated and grown at 37°C until the OD550 reached 0.5. The culture was induced with 0.1 mM IPTG and incubated at 16°C for 16 h. The cells were harvested, resuspended in 5 ml lysis buffer [20 mM Tris/HCl (pH 7.5), 0.3 M NaCl, 10 mM imidazole, 0.5 mg lysozyme mg/ ml] and sonicated. The soluble fraction of the cell lysate was applied to an Ni-NTA column (Qiagen). The resin was washed twice with 50 mM NaHPO , 300 mM NaCl, 20 mM imidazole (pH 8), twice 2 4 with cleavage buffer [20 mM Tris/ HCl (pH 7.4), 50 mM NaCl, 2 mM CaCl] and then incubated for 16 h at room 2 temperature with 12.5 U enterokinase (Novagen). The released chitinase was collected and visualized on 10% SDS-PAGE. Also, the enzyme activity was examined according to Pan *et al*. [17] on multi-well plates (20mm). Hydrolysis diameters were measured and compared with the commercial chitinase (Sigma). SDS-PAGE and molecular weight determination: The purified protein was subjected to SDS-PAGE [18]. Approximately 2 to 10 μg of purified protein were analyzed by SDS-PAGE under reducing conditions on pre castBio-Rad 4 to 20% Tris-HCl gradient gels, using a Mini PROTEAN II electrophoresis system (Bio-Rad) and following the manufacturer's instructions. Bio-Rad SDS-PAGE broad-molecular-weight-range proteins were used as a standard.

Protein estimation and amino acid analysis: Protein concentration was measured by the method of Bradford [19]. Bovine serum albumin was used as the standard. Samples (50 mg) of powdered purified protein were resuspended in 1 ml double-distilled H O. Aliquots of the 2 extracts were analyzed for free amino acids with a Beckman 7300 amino acid analyzer.

Gene evolution and phylogenetic analysis: Analysis of nucleotide DNA sequence and deduced amino acid sequence was carried out using EditSeq-DNAstar Inc., Expert Sequence Analysis software, Windows 32 Edit Seq 4.00 (1989-1999) and the ExPasy database on the internet (us.expasy.org/tools). Blast search for alignment of the obtained sequence with pre-existing ones was done using the database of the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). Phylogeny construction was performed using MEGA-4 software.

Prediction of transmembrane proteins using Sosui system (TMHMM): The transmembrane protein (TM) is vital for chitinase cell entry. The presence of transmembrane domains in chitinase sequences was investigated using Sosui [20] and TMHMM [21]. SOSUI system distinguishes between membranes and soluble proteins from amino acid sequences and predicts the transmembrane helices for the former.

Prediction of the potential glycosylation and phosphorylation sites: Potential glycosylation and phosphorylation sites were identified using the NetOGlyc 2.0 [22], NetNGlyc1.0 [23] and the NetPhos2 [24] prediction servers. All programs used were available through the ExPASy (expert protein analysis system) proteomics servers of the Swiss Institute of Bioinformatics (SIB) (http://us.expasy.org/).

RESULTS

Chitinase gene cloning and sequencing accession number: About 1100 bp (Fig. 1A) of the chitinase gene was isolated and the amplified band was cloned into the TOPO TA cloning vector subcloned into pUC18 and sequenced. The sequence accession number is EU106867.

The sequence analysis and alignment: The sequence showed a single open reading frame of 860bp. The sequence showed similarity reaching 64% for both the chitinase gene NM_001039426 (Chi-3) and AY873915 (Chi-16), isolated from Tribolium castaneum (Fig. 2). Comparison of amino acid sequence of Chi-hafez with other enzymes: The protein sequence of chi-hafez was compared with available protein sequence of the chitinases class III (GenBank and EMBL) as well as those from the literature. The protein sequence of Chi-hafez was aligned with 31 different chitinase genes from different organisms. The examined genes clustered them into five main groups. Group I divided into two subgroups: subgroup one consists of angiosperms {Citrus sinensis, Beta vulgaris, Solanum lycoperscum, Hordeum vulgare, Allium sativum, Vitis vinfera and Brassica rapa}; subgroup two comprises Homarus americanus, Spodoptera frugiperda, Vibrio alginolyticus, Seerratis polymuttica, Samia cynthia and Spodoptra littoralis. Group II contains only Tribolium castaneum while Group III contains a mixture of insects and angiosperms including; Schistocera gregaria, Pseudomonas aerginosa, Medicago truncatula, Oryza sativa, Casuarina glauca, Fragaria xannassa, Sphenostylis stenocarpa, Cucumis sativus, Rehmannia glutinosa and Glycine max. Group IV contains only insects including; Chelonus sp, Aedes aegypti and Anopheles gambiae. Group V, on the other hand, is constituted by Tribolium castaneum (NM_001039426 & AY873915). Finally, Group VI contains only the frog: Xenopus tropicalis.

***In vitro* transcription, protein purification and enzyme activity:** The Chi-hafez clone was expressed in a pET32a expression vector and some of the resultant expressed clones were transferred into Petri dishes containing native chitin. In this process, many clear zones appeared. One of these clones was cultured in a 2 liter flask and collected cells subjected to lysis and protein purification; a Ni-column was used to capture the histidine tagged protein. The purified protein was loaded into an SDS gel; its molecular weight was estimated as approximately 35 kDa (Fig. 1B). The purified enzyme was then used in plate bioassay according to Pan *et al.* [17]; good results were obtained as big clear zones in the plates contain the chi-hafez chitinase compared with the other commercial chitinase.

Amino acid determination in purified chitinase: Amino acid analysis was performed for the purified chitinase and at least 18 amino acids were predicted (Table 1). The concentration of amino acids per milligram ranged from 15 mg/ml for Cysteine, 800 mg/ml for leucine (Table1).

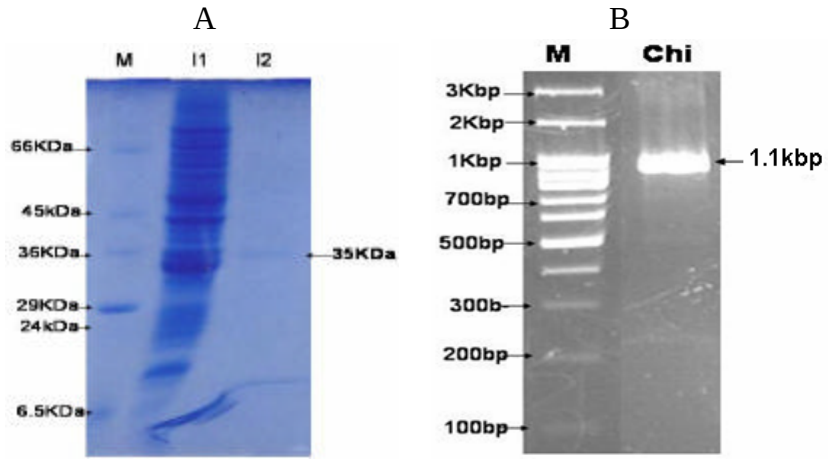

Fig. 1: A; 12% SDS-protein gel for the partial and purified Chitinase from the recombinant clone. Lanes; M: Low range protein marker. Lane 11: the partial protein and Lane 12: purified protein. (B): 2% agarose gel of the chitinase PCR product, lanes: M: 3Kbp DNA ladder and Lane chi, the chitinase PCR amplicone

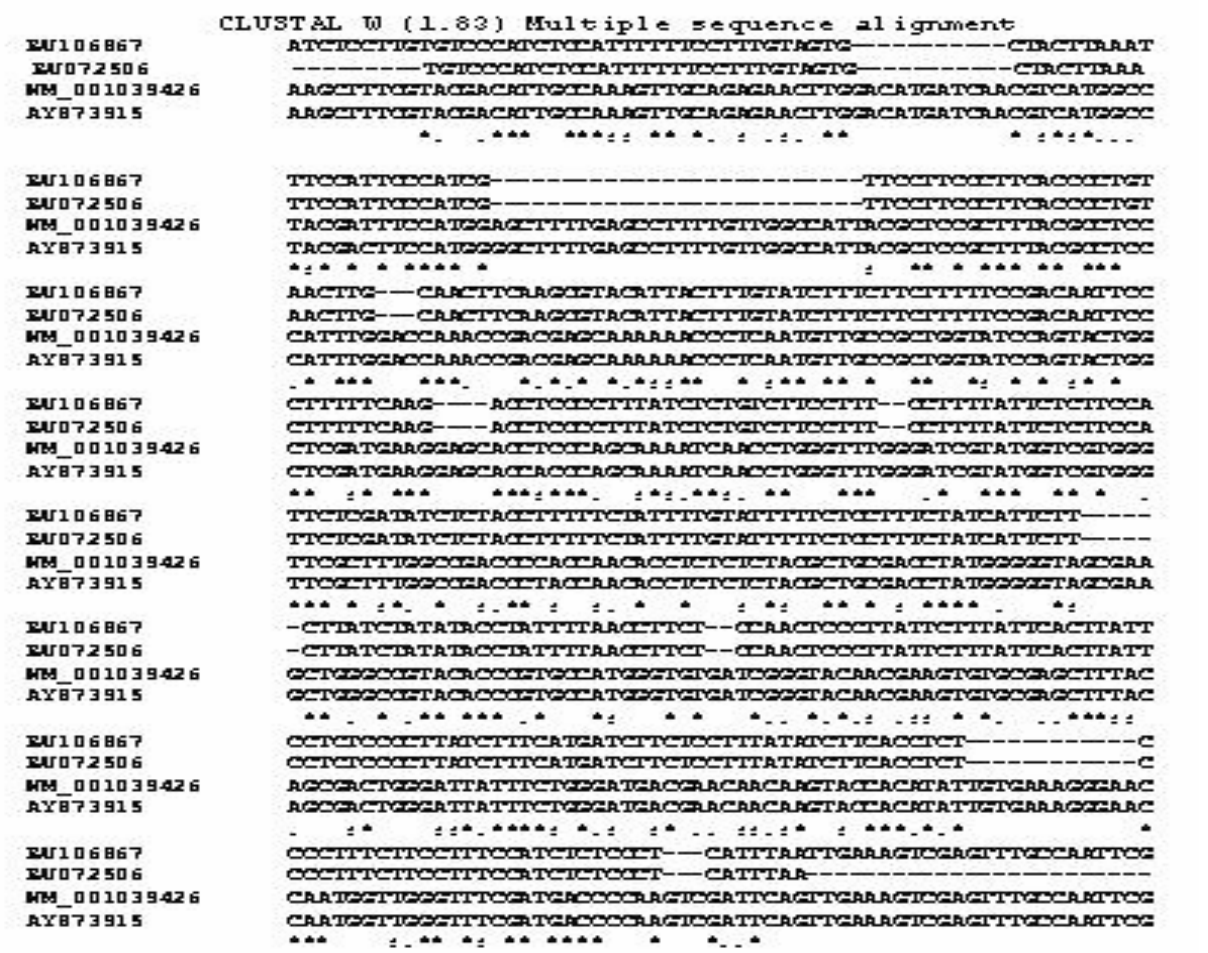

Fig. 2: Comparative DNA sequence analysis of chitinase genes using ClustalW(1.83). The accession numbers; EU106867 is *Schistocerca gregaria*, NM_001039426 is *Tribolium castaneum* chitinase 3 (Chi-3), and AY873915 is *Tribolium castaneum* chitinase 16 (Chi-16)

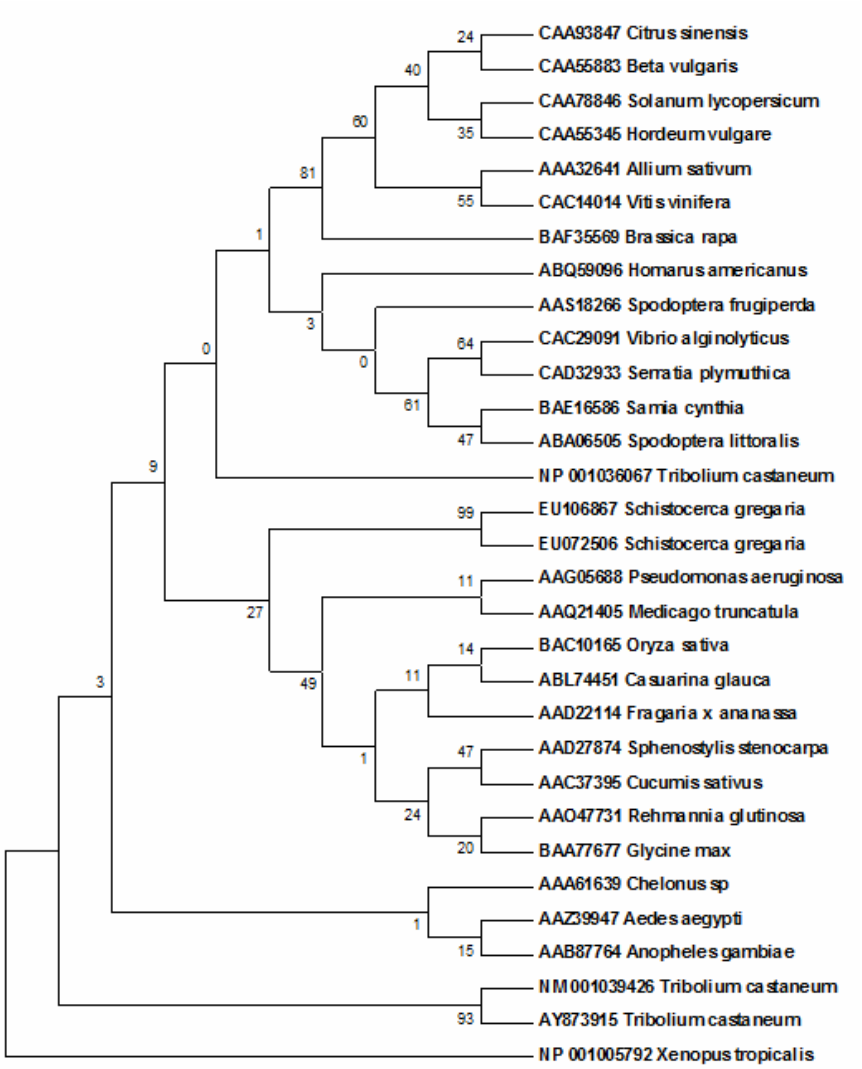

Fig. 3: Phylogenetic tree of amino acid sequences of chitinase genes. The accession number was combined with the source organism. The bold and under line is our chitinase. The numbers is indicating to the percentage of relatedness between the examined genes

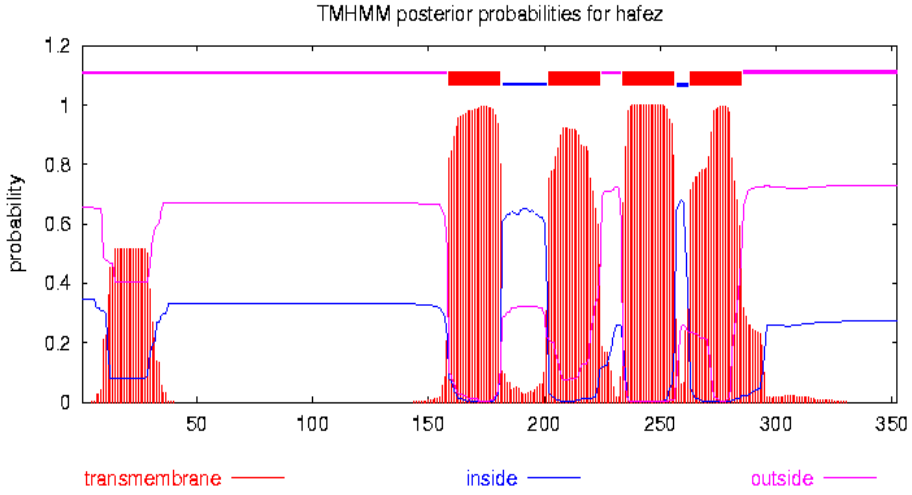

Fig. 4: Prediction of the transmembrane protein of chitinase recombinant protein using Sosui system (TMHMM)

Table 1: Amino acid content in the purified chitinase protein and their concentrations per (mg/ml)

Amino acid	Cysteic acid	Aspartic Acid	Serien	Glutamic acid	Praline	Glycine	Alanine	Cystine
Conc. (ug/ml)	148	120	224	64	274	212	145	15
Valine	Methionine	Leucine	Tyrocin	Phynlalanine	Norrleucine	Lycine	Histidine	Argianine
58	66	800	115	183	282	52	36	53

Table 2: Amino acid sequence of the chitinase and analysis of transmembrne protein (which have 2 transmembrane helices)

No.	N terminal	Transmembrane region	C terminal	Type	Length
1	100	SRNPPLYLCLPFLALFSSILDQS	122	SECONDARY	23
2	126	FSILYLWVLLPTLSFFLSILPTY	148	PRIMARY	23

Prediction of transmembrane proteins using the Sosui system (TMHMM): Membrane proteins play important roles in the cell as key components of cell-cell signaling mechanisms. The transmembrane protein (TM) is vital to chitinase cell entry and in our case it will play a part in insect cell chitin hydrolysis. The presence of transmembrane domains in chitinase sequences was investigated using Sosui [25] and TMHMM [21]. The SOSUI system distinguishes between membrane and soluble proteins based on amino acid sequences and predicts the transmembrane helices for the former. A polypeptide can become a membrane protein if it contains at least one transmembrane helix. The Sosui structural prediction algorithm effective for the TM domain strongly predicted the presence of hydrophobic, membrane spanning helices in our chitinase gene (Fig. 4 and Table 2).

Prediction of the potential glycosylation and phosphorylation sites: The deduced proteins do not contain a cysteine-rich carboxyl domain that has been reported in chitinases from insects, nematodes and humans; but contain extensive serine/threonine rich domains which are involved in anchoring the enzyme tightly onto the substrate to facilitate the hydrolytic process. In addition, three O-linked glycosylation sites were also observed.

DISCUSSION

This work is based on the hypothesis that chitinase is one of the defense system genes in most organisms. Chitinase is also essential for chitin degradation in the mid gut of most insects that feed on plants. In this study we induce of the defense system in the desert locust to obtain a highly expressed chitnase gene in the total RNA extracted from the insects exposed to mobile waves. The first screening with PCR showed the presence of the chitinase gene both in the exposed insects and the non exposed insects, but a high amount of DNA was obtained with induced insects more than the non induced.

Chitin covers both the skin and the midgut of insects. Chitin is one of the most naturally abundant polysaccharides and its metabolism in insect tissues is not understood. Chitin degrading enzymes, the chitinases, are also found in a wide variety of organisms including insects, fungi, bacteria, plants and also crustaceans. A total of 1100bp of the chitinase gene was amplified from S. gregaria using degenerate primers and cloned in E.coli by using a TOPO TA cloning vector. The EcoRI fragment was subcloned both into pUC18 and pET32a and expressed in E.coli DH5á. The DNA sequencing revealed 64% identity with chi 3 and chi 16 of Tribolium castenium strain.

On the other hand, the putative amino acids showed a less similarity with chi 3 or chi 16 of Tribolium castenium. Among 31 different chitinase class III genes of different organisms were aligned with chi-hafez and the results indicated that chi-hafez genes were closer to some plants and bacterial chitinases than insects. Our results, also in agreement with those of Jin *et al.* [26] and Ward *et al.* [27], may be due to some adaptation that was carried out in the chitinase enzyme in the locust enabling the chitinase to be more specific for the digestion of the plant chitin. Bologensi *et al.* [28] postulated that the expression of chitin synthase and chitinase genes are precisely coordinated to control the synthesis of peritrophic membrane (PM) during feeding stage; and its degradation during larval-larval and larval-pupal molts, which was accomplished at the level of mRNA transcription. Phylogenetic analysis showed that all the chitinases of class III had only one ancestor, branched to six groups. These groups then were divided into 8 subgroups resulting from the five main groups. Xenopus

tropicalis is considered as the outgroup for all these chitinases based on the amino acid analysis.

Activity of the purified enzyme was carried out on solidified plates containing crustacean chitin; and a large clear zone was observed when compared with commercial chitinases. The activity of the purified enzyme may be due to the fact that it is rich in cysteine, which makes it highly effective for membrane binding [12]. Such cysteine rich domains are generally found in most chitinases [29]. The function of these domains in chitinases is presumably to anchor the enzyme tightly onto the large insoluble polymeric substrate, thereby facilitating the heterogenous hydrolysis process [28].

This trasnsmembrane protein (TM) enables the chitinase enzyme to bind with the midgut membrane, facilitating chitin digestion. These results are in agreement with Boot *et al*. [30] who detected a high chitinase activity in the extract of the intestines of mice and chicken. Also, the SOSUI structural prediction algorithm effective for the TM domain strongly predicted the presence of hydrophobic, membrane spanning helices in the putative amino acids of chitinase. It also increases the chance of using this gene as fungicide or insecticide.

In conclusion, we have a highly active new chitinase gene, that is promising for further testing as antifungal or as insect biocontrol.

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