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Molecular Characterization and Evaluation of The Antimicrobial Activity of Phenoloxidase Purified from *Spodoptera littoralis* against Different Array of Pathogenic Bacteria

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ABSTRACT

Phenoloxidase (PO) is an important enzyme for various insect processes, such as defensive encapsulation and melanization of foreign organisms as well as wound healing. Here, we characterize phenoloxidase extracted from the hemolymph of the cotton leaf worm, *Spodoptera littoralis*, for the first time. We obtained a cDNA sequence of 600 bp as a new record, which has elevated homology with known PO sequences of other lepidopterous insects. Moreover, we evaluated the antibacterial activity of PO induced by synthesized antimicrobial peptides (AMPs) against Gram-positive *Staphylococcus aureus* and *Enterococci*, and Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumonia*. Standardized concentrations (0.5 McFarland) of bacterial suspensions were used. The highest antibacterial activity expressed as the "growth-inhibition zone," was 2.0 ± 0.2 Cm observed against *Staphylococcus*. For the Gram-negative bacteria, a growth-inhibition zone (1.5 ± 0.15 Cm) was observed only for *E. coli*. Based on these results, future studies evaluating the effect of AMPs compared with existing antibiotics are warranted.

INTRODUCTION

Antibiotics are widely used for microbial disease prevention. In 2010, the global antibiotic consumption for food animal production was conservatively estimated at 63,151 tons (Boeckel *et al.*, 2015). Concurrent with the success of antibiotics for treating infections, the emergence and rapid dissemination of antibiotic-resistant bacteria put a substantial risk to human health. Antibiotic resistance has become a serious global problem with human deaths resulting from antibiotic-resistant infections predicted to reach 10 million by 2050, which is more than the current death toll associated with cancer (Neill, 2014). In addition, the number of approvals of new antibiotics has significantly and steadily decreased over the past three decades (Alanis, 2005). In contrast, a growing list of diseases, such as pneumonia, tuberculosis, blood harming, gonorrhea, and foodborne infections are becoming harder to treat (WHO, 2018).

Thus, there is an urgent need to expand novel, effective antimicrobial agents. Antimicrobial peptides (AMPs) constitute an attractive alternative to classical antibiotics. AMP action relies on the interaction with the bacterial cell membrane (Kumar *et al.*, 2018). It targets metabolic forms within the bacteria including cell divider synthesis, nucleic corrosive, or protein blend, which are essential to the organism (Ebbensgaard *et al.*, 2015).

Insects depend exclusively on a well-developed natural resistant framework to protect themselves against microbial diseases (Franssens, 2006). They combat diseases by mounting robust immune reactions that are interceded by hemocytes, the fat body, the midgut, the salivary glands, as well as other tissues (Hillyer, 2016). AMPs and polypeptides are created primarily in the insect fat body (functional analog of mammalian liver) and are released into the hemolymph, where they play an

MATERIALS AND METHODS

1. Insect Rearing and Immunopathology Assays:

Adults *Spodoptera littoralis* were obtained from the Central Agribusiness Pesticides Investigate office (CAPL), Dokki, Giza, Egypt and allowed to oviposit. The emerging larvae were reared on an artificial diet (Poitout *et al.*, 1970) at $23 \pm 1^\circ\text{C}$ with a photoperiod of 16:8 light: dark hours and $40 \pm 5\%$ relative humidity.

Pathogenicity experiments were performed by injecting a suspension of *Bacillus thuringiensis* kurstaki (Bt) in an exponential growth phase (2×10^{10} cells/ml of LB broth) into fifth-instar larvae of *Sp. littoralis* (Bisch *et al.*, 2015). Six independent pathogenicity assays were performed to determine the sublethal dose. Ten larvae were selected for each concentration using a 10 μl Hamilton small scale (Insulin syringe) fitted with a 26-gage needle.

2. In Silico Analysis and Primer Design:

Forward and reverse primer sets specific for PO were designed for each

important role in the innate immune system and host defense mechanism. They reveal a broad spectrum of activity against both Gram-positive and Gram-negative bacteria as well as against fungi (Hoffman, 1995; Hoffmann *et al.*, 1996; Januszani *et al.*, 2012).

Spodoptera littoralis, the Egyptian cotton leafworm, is prevalent in Africa, southern Europe, and the Middle East, where it is a particularly destructive pest of cotton and economically important vegetables and ornamentals (Ellis, 2004). We examined hemolymph antimicrobial activity for one of the AMP components, phenoloxidase (PO), as a primary step for a new class of antibiotics. We evaluated the resistance of various pathogenic Gram-negative and Gram-positive bacteria against PO. In addition, we characterized a candidate PO gene that is potentially involved in the melanization process of *Sp. littoralis* against invading organisms.

transcript using Invitrogen\Vector NTI Advance software, version 10. Primers were designed based on an alignment between the isoforms of the PO gene in a species closely related to *Sp. littoralis*.

3. RNA Extraction and RT-PCR:

Total RNA was extracted from whole larvae of *Sp. littoralis* using the Thermo Scientific GeneJET RNA purification kit (#K0731, #K0732) according to the manufacturer's instructions. RNA was isolated from aliquots consisting of three moth larvae stimulated with *Bt.* as described. RNA was quantitated by absorbance at 260 nm. First-strand cDNA was synthesized from template RNA using a High-Capacity cDNA Reverse Transcription Kit with an RNase inhibitor (Thermo Scientific, Cat No. 4368814). The 10 μl reaction contained 2.0 μL 10X RT Buffer and 5 μg of total RNA and was incubated at 25°C for 10 min. PCR conditions for the PO primers were 95°C for 3 min, followed by 35 cycles of 94°C for 30 s, 70°C for 30 s, 72°C for 1 min, followed by a final extension of 72°C for 5 min. The PO primer sequences were forward primer: 5'

TACTTCCGCGAGGACATCGG-3' and reverse primer: 5'-TGCCAGCGGTAG AACACGGGGTC-3'. Direct sequencing was done in both directions by the Sanger method with primers used for DNA amplification. Sequence alignment was performed with the CLUSTAL W program which was included in the MEGA-X software (Kumar *et al.*, 2018).

4. Hemolymph Collection:

Larvae from each of two groups (naïve control larvae and larvae stimulated with *Bt*) were first flooded in a hot water shower at 60°C for 2–5 min, then allowed to dry on a paper towel. Hemolymph was collected by piercing the 2nd thoracic segment with a 50 µl glass capillary tube and oozing hemolymph was aspirated into a micropipette. The hemolymph of the inoculated larvae was collected 24 h after treatment with *Bt*.

5. PO Purification:

The refinement of PO was done by HiTrap CM Sepharose FF according to the manufacturer's instructions. Collected hemolymph was centrifuged at 12,000 for 10 min. Hemolymph from aliquots of larvae was washed with 5 ml of bis-Tris pH 5, followed by 5 ml of an elution buffer. The protein concentration of the hemolymph was determined using a nanodrop instrument (Thermo Scientific).

6. Preparation of Microbial Cells:

Standardized concentrations of bacterial suspensions (0.5 McFarland) were obtained from the Infection Control Unit at Ain Shams Specialized Hospital. Bacteria were prepared and measured by a densitometer (BIOMERIEUX, Densi CHEK plus).

Two Gram-positive bacterial suspensions were prepared including *Staphylococcus aureus* and *Enterococci*, as well as four Gram-negative bacterial suspensions: *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*. Bacterial cultures were grown for 48 h at 40°C in Luria Bertani's rich nutrient medium until a stationary phase was reached.

7. Antibacterial Activity of Tested Hemolymph:

Microbial growth inhibition was tested by the agar disc diffusion method (Radwan *et al.*, 2019; Valgas *et al.*, 2007). Briefly, the bacteria were grown on an agar plate as described, then the agar was punched aseptically by a sterile tip into a 5 mm diameter well. Twenty microliters of the PO extract were added to each well. Three replicates were used for each treatment and control wells were filled with 20 µl of double-distilled water. Petri-dishes were incubated at 40°C for 48 h. Generally, antimicrobial agents including PO spreads in agar and prevent germination and bacterial growth. The diameter of the growth inhibition zone was measured.

RESULTS

1. Sequence and Phylogenetic Analysis of PO:

In *Sp. littoralis* inoculated with *Bt*, initial amplification of the PO cDNA transcript using forward and reverse primers produced sequences of 362 and 155 bp long, respectively. The amplification of a fragment of PO produced a specific band of 600 bp (Fig. 1). Sequence reads of the PCR product were assembled into one contiguous sequence using a code aligner program (<http://www.codoncode.com/aligner/>).

Blast analysis revealed that the multiple sequence alignment of *Sp. littoralis* at the nucleotide level shared a high degree of similarity to species in the genus *Spodoptera* (Fig. 2). It revealed 96, 91, and 90% homology with *Sp. litura*, *Sp. frugipe*, and *Sp. exigue*, respectively. It also exhibited 80–85% identity to *Bombyx*, *Manduca*, and *Ostrinia* PO.

A phylogenetic tree of the moth PO family was generated at the nucleotide level. Phylogenetic analysis revealed that PO sequences from *Sp. littoralis* is the ancestral root for PO from the related lepidopterous species. Moreover, PO of *Sp. littoralis* exhibited a marked similarity to that of other species in the genus *Spodoptera* (Fig. 3).

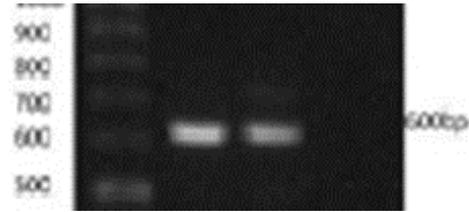


Fig. 1. PO cDNA amplification from *Sp. littoralis* using specific primers. A PCR product of ~600 bp (M: 100 bp marker, C: control, 1,2: replicant of treated hemolymph samples).

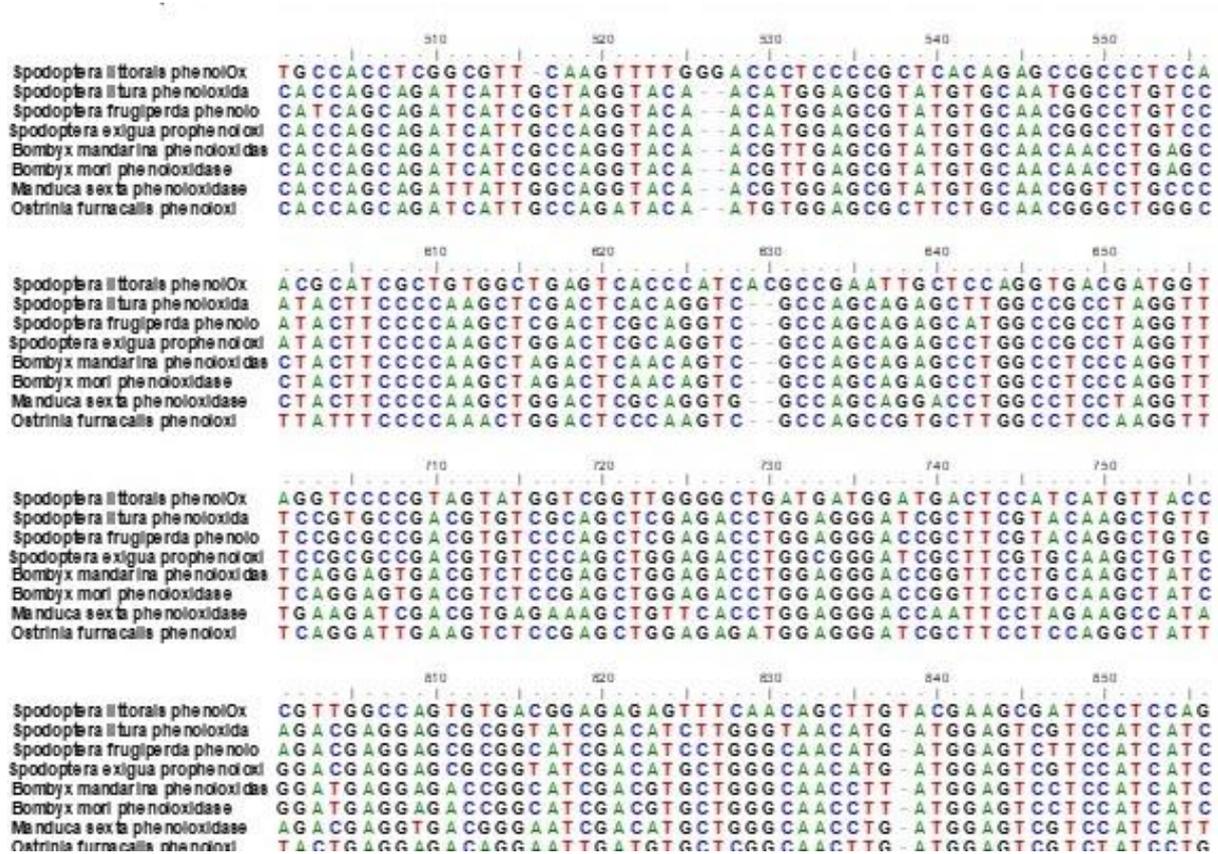


Fig. 2. CLUSTAL W multiple alignments at the PO nucleotide level for the contiguous sequence of *Sp. littoralis* treated with *Bt*, and seven PO transcripts in related lepidopterous species.

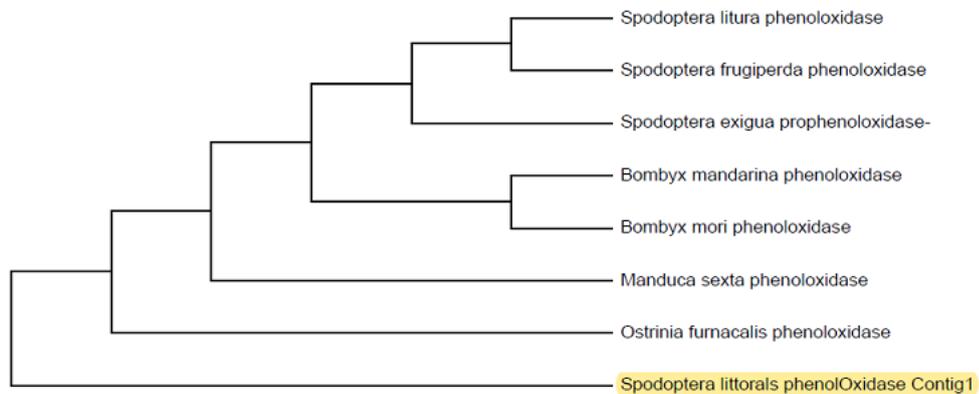


Fig. 3. Phylogenetic analysis of the PO sequences from various species of *Spodoptera* and other lepidopterous species.

2. Susceptibility of Pathogenic Bacteria to PO:

Data from Figures 4 and 5 indicate that the PO extract of *Sp. littoralis* hemolymph exhibited antibacterial activity as estimated by a growth inhibitory area against both pathogenic Gram-positive and Gram-negative bacteria. The growth-inhibition zone against Gram-positive

Staphylococcus and *Enterococci* were 2.0 ± 0.2 Cm and 1.0 ± 0.1 Cm, respectively. For Gram-negative bacteria, only *E. coli* presented a growth-inhibition zone of 1.5 ± 0.15 Cm in size (Fig. 4). After 60 min, neither Gram-negative nor Gram-positive bacteria exhibited susceptibility against PO extract.

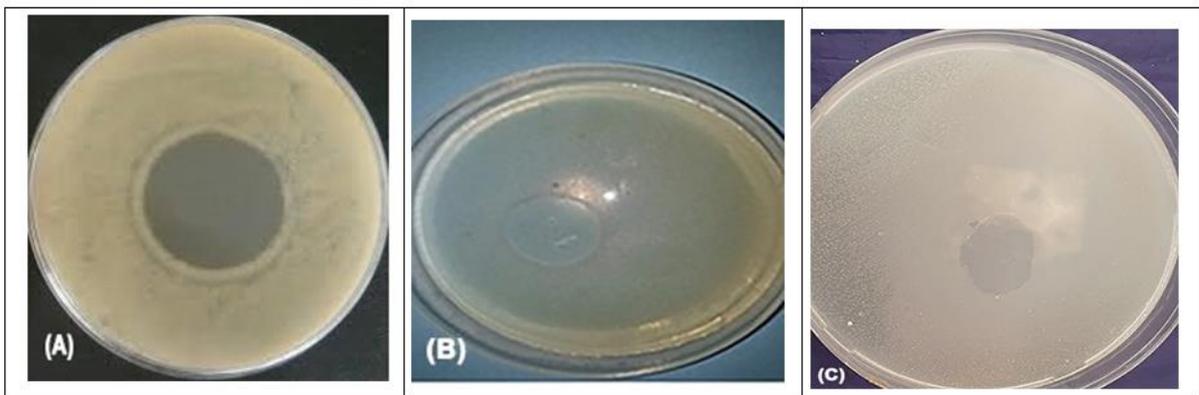


Fig. 4. Antimicrobial activity of *Sp. littoralis* phenol oxidase as demonstrated by a growth-inhibition zone against Gram-positive bacteria (a) *Staphylococcus aureus* (b) *Enterococci* and Gram-negative bacteria (c) *Escherichia coli*.

DISCUSSION

Insects are one of the most effective groups of organisms. Nearly one million species have evolved, and they inhabit almost all ecological habitats. They also survive despite a significant diversity of bacterial pathogens. The innate immune system of insects delivers an effective defense against pathogens and parasites. The activation of immune mechanism consists of specific and non-specific receptors that are triggered in the presence of pathogens.

Constitutive immunity in insects is generally characterized by background induction of the coli. (proPO) cascade and by the cellular immune response (Ashida *et al.*, 1995). In most species, humoral defenses against pathogens rely on pathogen receptors that activate the immune response as well as the induction of enzymes, such as lysozyme, pro-phenoloxidases, and AMPs. Although phenoloxidase is extensively dispersed in the animal kingdom, its relationship to the

stimulation of the proPO system remains controversial. The precursors of the microbial cell wall components belong to different phyla, which tend to indicate a unifying biochemical mechanism.

Spodoptera littoralis represent a useful model for the study of immunological effects in insects (Paterson *et al.*, 1987; Seufi *et al.*, 2011; Radwan *et al.*, 2019). They can be quickly maintained in large numbers using inexpensive media and materials and they have a large blood volume. The pathogenicity toward *Bt* depends on the mortality results obtained from laboratory tests as recorded LC₂₀ (2×10^6 cells/ml) values, which is a sublethal concentration of bacterial cells that induces an immune response of the larval hemolymph. The pathogen is administered directly by injection to susceptible haemocoel tissue to avoid variations induced by loss of dose and irregularities of invasion. For the *in vivo* study of the biochemical and physiological

changes triggered by pathogenic induction, several groups have applied this method using the same or different bacterial pathogens (Pereira *et al.*, 2015; De Viedma, and Nelson, 2017; Parthuisot *et al.*, 2018; Radwan *et al.*, 2019).

Although a common feature of insect phenoloxidases is that they all appear to be produced as inactive precursors and their activation occurs in response to stress and microbial invasion (Sukumaran *et al.*, 1993). Recently, improvement has been made in isolating, purifying, and characterizing various insect POs (Zibae *et al.*, 2011; Ajamhassani *et al.*, 2012; Mahmoud *et al.*, 2015; Kamalanathan *et al.*, 2020). The deficiency of effective tools to identify the quantitate distinct PO isoforms is considered a limitation to the study of PO. (Chase *et al.*, 2000) used a conventional chromatographic method in place of immunoaffinity chromatography. (Aspán *et al.*, 1991) used Blue Sepharose as an essential and effective method for the purification of PO from its components in crayfish hemocytes. (Mahmoud *et al.*, 2015) purified and characterized PO from the hemolymph of *Schistocerca gregaria* (Forsk.) by a combination of ammonium sulfate precipitation, blue Sepharose CL-6B, and phenyl Sepharose CL-4B chromatography to obtain a 209.97-fold purification and 54.75% recovery of activity. In the present study, we selected a fast and rapid purification technique using 1 ml HiTrap CM FF prepacked with CM Sepharose Fast Flow, a weak cation exchanger for small-scale protein purification.

For the molecular characterization of PO, we amplified a cDNA transcript using forward and reverse primers to generate a sequence of 362 and 155 bp. Phenoloxidase sequence analysis using the Basic Local Alignment Search Tool (BLAST) revealed that the sequence has regions of similarity with *Sp. littoralis* at the nucleotide level and shared a high degree of similarity to species in the genus *Spodoptera*. There was 96%, 91%, and 90% identity to *Sp. litura*, *Sp. frugiperda*, and *Sp. exigua*, respectively. In

addition, there was 80–85% identity to *Bombyx*, *Manduca*, and *Ostrinia* PO. The percent homology declined when linked with various species in other insect orders. The sequence analysis of our amplified product showed remarkable homology with the enzymes that activate insect proPO as described by (Arora *et al.*, 2009), who used degenerate primers designed from conserved regions to generate a 600 bp PCR fragment from *S. litura* hemocyte cDNA.

The Phylogenetic analysis at the nucleotide level indicated that the PO sequences from *Sp. littoralis* represent the ancestral root of PO from related lepidopterous species. Moreover, PO of *Sp. littoralis* revealed a high similarity to that of other species in the genus Spodoptera.

AMPs can bypass common resistance mechanisms that reduce the effectiveness and safety of conventional antibiotics. Our data clearly demonstrate the susceptibility of Gram-positive bacteria to PO. (Zhang *et al.*, 2005) demonstrated that AMPs inhibit methicillin-resistant *Staphylococcus aureus* and multidrug-tolerant *Pseudomonas aeruginosa*. Therefore, the antimicrobial activity of PO-reactive compounds was evaluated directly by testing its antimicrobial efficiency against detected pathogenic strains using a growth inhibitory zone assay. The estimated zone against Gram-positive *Staphylococcus* and *Enterococci* were 2.0 ± 0.2 Cm and 1.0 ± 0.1 Cm, respectively, whereas, of the Gram-negative bacteria, only *E. coli* exhibited a growth-inhibition zone of 1.5 ± 0.15 Cm in size. After 60 min, neither Gram-ve nor Gram +ve bacteria showed susceptibility to the PO extract. This weak antibacterial response against Gram-negative bacteria is consistent with that of previous reports (Gerardo *et al.*, 2010; Burke *et al.*, 2011; Mahmoud *et al.*, 2015). In contrast, (Cerenius *et al.*, 2010) observed a strong antibacterial effect of PO isolated from freshwater crayfish (*Pacifistic leniusculus*) on the bacteria Gram -ve, although a weaker, still significant effect, against Gram +ve. (Buonocore *et al.*, 2021) explained the

antimicrobial efficiency against Gram-negative bacteria as a result of a mixed group of immune-related proteins. Our findings demonstrate the susceptibility of Gram-positive bacteria to PO. Indeed, the observed sensitivity of *Enterococcus* and *Staphylococcus aureus* to treatment with PO can be attributed to the fact that these Gram-positive bacteria are surrounded by a cytoplasmic lipid membrane, but do not have the outer cell membrane which does not exist in Gram-negative bacteria (Sewify *et al.*, 2017). The absence of this outer membrane renders these bacteria susceptible to the effects of PO. This is supported by other studies of bacterial resistance to antibiotics. Generally, our findings reflect a special focus on the antimicrobial efficiency of *Sp. littoralis* PO against bacterial pathogens and emphasizes its role as an alternative antimicrobial peptide.

In conclusion, a zymogen of phenoloxidase is contained in the larval hemolymph of *Spodoptera littoralis* and activated by proteolytic cleavage when exposed to foreign pathogens. In silico analysis including peptide length of the putative purified active peptide (phenoloxidase) revealed that the PO sequence of *S. littoralis* is of ancestral origin compared with related lepidopterous species. Phenoloxidase exhibits antibacterial activity. These findings indicate that PPO activation is an essential constituent of the insect defense system against microbial invasion. Further analyses using cloning and transcriptional or translational studies of PO are needed to further characterize the physiological function of these arthropod POs. Also, studies are required to confirm the antimicrobial activity of PO against living mammalian tissues.

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