بسم الله الرحمن الرحيم

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Department of Entomology

Evaluation of the antimicrobial activity of purified *Spodoptera littoralis* Phenol Oxidase in vitro

A Thesis submitted for the degree of Doctor of Philosophy in Science in Entomology

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CONTENT

LIST OF ABBREVIATIONS

AGERI	Agricultural Genetic Engineering Research Institute
AMP	Antimicrobial peptide
B. cereus	*Bacillus cereus*
bp	Base pair
Bt	Bacillus thuringiensis
C	Celsius
CAPL	Central Agriculture Pesticides Research
cDNA	Complementary Deoxyribonucleic acid
Cm	Centimeter
CuA	copper molecule A
CuB	copper molecule B
Dist.	Distilled
DSCAM	Down syndrome cell-adhesion molecule
DTT	Dithiothreitol
E.coli	*Escherichia coli*
ESBL	Extended-spectrum beta-lactamase
Fig.	Figure
G-ve	Gram negative
G+ve	Gram positive
Hr	Hour
KDa	Kilodalton
L	Liter
LPS	Lipopolysaccharide
MDR	Multi drug resistant
MRSA	Methicillin resistant *Staph aureus*
Min	Minute
mg	Miligram
ml	Mililiter
ng	nanogram

P. aeruginosa	*Pseudomonas aeruginosa*
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PGN	Peptidoglycan
PGRP	Peptidoglycan recognition protein
PO	Phenol oxidase
PPAE	prophenoloxidase-activating enzyme
PPO	Prophenol oxidase
PRP	Pattern recognition protein
PRR	Pattern recognition receptors
RNA	Ribonucleic acid
RPM	Revolution per minute
S. aureus	*Staphylococcus aureus*
Sp. litorallis	*Spodoptera litorallis*
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Tab	Table
TBE	Tris+Boric acid+ EDTA
µl	Microliter
UV	Ultraviolet
VRE	vancomycin-resistant *Enterococci*

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Abstract

Hanan Sayed Amer. Evaluation of the antimicrobial activity of purified Spodoptera littoralis Phenol Oxidase in vitro. Faculty of Science, Ain Shams University, 2022.

Insect innate immune system comprises humoral and cellular defense mechanisms organized differently depending on the insect species and the type of microbial invasion. Humoral defenses against pathogens depend on pathogen receptors that activate the immune response, including the production of defense peptides as antimicrobial peptides (AMPs) and enzymes such as prophenoloxidases (PPOs) and lysozymes.

Phenoloxidase (PO) is the primary enzyme involved in melanin biosynthesis thus, plays an important role in insect immunity. In the present study, larval hemolymph of *spodoptera littoralis* had been immunized by serial dilutions of *Bacillus thuringiensis* (Bt). We recorded $2X10^{60}$ as a sub lethal concentration of the bacterial cells (LC_{20}), that induce immune induction of the larval hemolymph to enable us to complete the subsequent experiments. Significant developments have been made in the purification methodology. In the present study we select fast and rapid purification technique using 1ml HiTrap CM FF as a prepacked with CM Sepharose Fast Flow, a weak cation exchanger for small-scale protein purifications. We perceived the presence of PO enzyme at 65 kDa compared with its native enzyme PPO at 70KDa using SDS gel electrophoresis technique. The antimicrobial activity of the purified enzyme was tested against different types of pathogenic bacteria;

Staphylococcus aureus and *Enterococcus* as grams- positive strains and *E. coli*, *Pseudomonas Enterococci*, *Acinetobacter baumannii*, and *Klebsiella pneumonia* as gram- negative strains. Data revealed that the potential of PO as antimicrobial peptide against Gram-positive strains is higher than that of Gram-negative strains. The cDNA of PO was detected in *S. littoralis* larvae 24 hours post induction. Sequence analysis of detected PO showed high similarity to homologous sequences in other lepidopterous species, particularly *S. litura*. These results established that prophenoloxidase activation is an integral component of the insect defense which immobilizes and kills invading microorganisms.

Thus, the study needs more investigations to confirm the activity of PO against human and animal tissues to prove its antimicrobial application as antibiotics alternative.

Keyword: Insect immune response, Phenol-oxidase, Spodoptera littoralis, antimicrobial peptides.

I. Introduction

The abuse of antibiotics in medicine, agriculture and animal husbandry, especially in developing countries, antimicrobial resistance has become a serious problem. The Kenyan study detected a large number of antibiotic residues in edible meat (**Ayukekbong *et al.,* 2017**). The prevalence of vancomycin-resistant *Enterococci* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) in clinical medicine is increasing, so counter measures are urgently needed to solve these bacterial infections. But from the perspective of pharmaceutical companies, the development of new antibiotics has resulted in lower profitability. Therefore, the substitution of antibiotics has become a consideration in the pharmaceutical, farming, animal rearing and food industries (**Huan *et. al.* 2020**).

Insects have been remarkably successful in evolution. Current estimates are that they account for 90% of all known species within the animal kingdom. With exception of the oceans, insects colonize all ecological niches on earth. Consequently, they are confronted by an extremely large variety of potentially harmful microorganisms. The evolutionary success of the insects can be attributed to various reasons, among which is the presence of a highly efficient immune system. In contrast to vertebrates, insects lack true antibodies and, hence, also an adaptive immune response. They rely solely on a well-developed innate immune system to defend themselves against microbial infections (**Franssens, 2006)**.

Insects depend on innate immune responses to defend themselves against foreign microorganisms. Its cellular defense mechanism is mediated by blood cells, which are the immune cells of insects. Blood cells play a key role in phagocytosis, nodulation, and encapsulation of invading pathogens. Humoral immunity involves antimicrobial peptides and the phenoloxidase

(PO) system (**Lemaitre and Hoffmann 2007**). The PO system is responsible for melanization, which is a process in which the insoluble brown-black pigment, melanin, is synthesized and deposited.

Antimicrobial peptides represent research and clinical care in the modern antimicrobial field. Many AMPs show incredible safe effects an antimicrobial resistant microorganisms and have a low probability of developing resistance (**Kendurkar and Sengupta, 2018**). The action of activated antimicrobial peptide (AMP) on larval hemolymph showed a significant antimicrobial action against various Gram-negative (G-ve) and Gram-positive (G + ve) bacteria (**Lei *et al* 2019**). The gene-encoded AMPs are activated immediately after infection and act against a broad spectrum of microbes. These peptides kill bacteria (including strains resistant to conventional antibiotics), fungi, enveloped viruses, and even tumors. These properties make them excellent candidates for therapeutic agents **(Bals, 2000**). It is obvious that the PPO of different insect's species can be used for defense against attacking pathogens due to its rapid action (**Hillyer *et al*., 2003, 2004; Cerenius *et al*., 2008**). After using several purified protein, **Zhao *et al* 2007** proved for the first time that the reactive compound produced by PO binds, aggregates and kills various bacteria and fungi. Proteolytic activation of proPO has been recorderd in various insect species. The activation process mediates the production of intermediates that form the integral part of the insect immune system against microbial invasion (**Gillespie *et al*., 1997; Ashida and Brey, 1997; Cerenius and So¨derha¨ll, 2004).**

Spodoptera littoralis is a genus of most important crop pests found throughout the Americas, Southeast Asia and countries around the Mediterranean (**Ellis, 2004; Meagher *et al*., 2008**). *S. littoralis*, the Egyptian cotton leafworm, is found in Africa, southern Europe, and the