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FRACTIONATION AND PURIFICATION OF BIOACTIVE PEPTIDES IN EXCRETORY/SECRETORY PRODUCTS OF THIRD INSTAR LARVAE OF *CHRYSOMYA MEGACEPHALA* (CALLIPHORIDAE : DIPTERA)

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Abstract

The beneficial effects of maggots on wounds have been attributed to various mechanisms notably the debridement (degradation) of necrotic tissue.It was demonstrated that the maggots ES exhibit antibacterial actions against both Gram-positive and Gram-negative bacteria, including MRSA, Escherichia coli and Pseudomonas aeruginosa.The present study revealed the presence of 40 protein fractions in excretory/secretory products of third instar larvae of Chrysomya megacephala, only 3 protein fractions showed antibacterial activity against Bacillis subtilis, number 22,23 and 24.The separated protein fraction number 22 showed protein bands at 28 and 31 KDa protein, separated protein fraction number 23 showed protein bands at 17, 24 and 28 KDa and separated protein fraction number 24 showed protein band at 26 KDa.

Key Words: ***Chrysomya megacephala, antibacterial peptides, Excretory/Secretory products***

I. INTRODUCTION

Maggot therapy has been traditionally practiced for debridement of necrotic wounds as well as for curing bacterial infections at the wounds site [1]. It has been reported to have advantages over the conventional methods, especially for treatment of wounds infected by multi-drug resistant methicillin-resistant *Staphylococcus aureus (S. aureus*) or MRSA [2]**.** Their antiseptic action has been investigated by many researchers in the past for specialized antibacterial properties or presence of antimicrobial factor(s). The beneficial effects of maggots on wounds have been attributed to various mechanisms notably the debridement (degradation) of necrotic tissue. It was originally believed that this debriding action of maggots was restricted only to their mechanical wriggling, but recently many proteolytic enzyme classes have been isolated from maggot excretions and secretions (ES) which are able to specifically dissolve the laminin and fibronectin of the extracellular matrix in the necrotic tissue. This liquifies the dead tissues enabling the maggot to take it up by suction [3**].** Finally, maggots promote wound healing, stimulate granulation and promote the formation of human fibroblasts [4].

In particular, the mechanism of action of maggot disinfection on wounds was studied [5]. They found that the excretion of maggots exhibited a strong and rapid disinfection action on *S. aureus*. Subsequently, several other groups collected the excretions/secretions (ES) of maggots and screened it on various microbes [6]. Many species of blowfly maggots were investigated against both gram negative and gram-positive bacteria [7]. Several recent studies have demonstrated that the maggots ES from aseptically-raised *Lucilia sericata* larvae exhibit antibacterial actions against *both* Gram-positive and Gram-negative bacteria, including MRSA, *Escherichia coli* and *Pseudomonas aeruginosa* [6]. In addition, maggots can ingest bacteria as part of their normal feeding process [3]. The antibacterial activity of *Lucilia cuprina* maggot ES was previously shown to have partial growth inhibition on gram-positive, *S. aureus* [8].

Recently, a low molecular weight insect peptide was found mainly responsible for the antimicrobial activity of the maggots, when exposed to an infectious environment of a wound The peptide, lucifencin,that belongs to the insect antimicrobial peptides (AMP), defensin family, was reported to be secreted in the hemolymph, body fat and ES of the maggots. The maggots were raised in an environment simulating a wound that increased the production of the AMP as an innate immune response of the insect [9].

Chrysomya megacephala (F.), the Oriental latrine fly, is a common blow fly species of medical importance in many parts of the world, including Egypt. Adults may feed on food sources including nectar, animal carcasses, garbage, and other filth materials, or even human food. Therefore, it is possible that mechanical transfer of potential disease causing pathogens, such as bacteria, viruses, protozoa, and helminthes eggs, to human food may occur [10]. Larvae of this species are known to cause myiasis in several mammal species, including humans [11]. Another facet of medical importance of this blow fly is its association with human corpses and its relevance to forensic entomology. Many researchers have reported that specimens of *C. megacephala* were found connected with cases of human death [12]. Recent molecular and biochemical studies on third instar larvae of *C. megacephala* revealed the presence of serine proteases especially trypsin and chymotrypsin in excretory/secretory products [13 , 14 **].**Also the excretory/secretory products of third instar larvae of *Chrysomya megacephala* showed antibacterial activity against *Escherichia coli*, *Staphylococus aureus* and *Bacillis subtilis* with nearly more activity against *Bacillis subtilis* followed *by Staphylococus aureus and Escherichia coli* [14] .

The aim of the present study is to fractionate the excretory/secretory products of third instar larvae of *Chrysomya megacephala,* to screen these protein fractions against *Bacillis subtilis* (as ES products has more activity against it) and finally to purify different peptides from the active protein fractions in order to clarify the usefulness of ES from *Chrysomya megacephala* in maggot therapy.

II. MATERIALS AND METHODS

2.1 REARING OF INSECT

The laboratory colony of C. megacephala used in this study was established in the Department of Entomology, Faculty of Science, and Helwan University. C. megacephala was reared according to following protocol [15]. They were identified according to reported method [16]. Adults from the stock colony of C. megacephala were kept in cages (38×38×56 cm) at 25±3°C, 14h photoperiod and 60–70% R.H. The cages were made with a wooden floor, a glass roof, and wire gauze on three of the sides. The fourth side was wooden with a circular hole fitted with a cloth sleeve to facilitate daily feeding, cleaning of the cage, and removal of eggs. Adults were supplied daily with granular sucrose, water, and pieces of liver.

Water was supplied by dipping a piece of cotton as a wick in a bottle filled with water, and the liver was provided in a Petri- dish. Egg batches were removed daily and transferred to a fresh piece of chicken placed in a rearing enamel bowl (35 cm in diameter) covered with muslin secured with a rubber band. At the prepupal stage, dry autoclaved sawdust was added to the bowl as a medium for pupation. Pupae were sieved from the sawdust and transferred to adult cages described above for adult emergence.

2.2 COLLECTION OF LARVAL SECRETIONS

The ES products were collected according to modifications of reported method [17]. Native excretions/secretions (nES) were collected by incubating third instars larvae of *C. megacephala* in (50,000 larvae/ 500 ml) sterile distilled water for 1h at 30°C in darkness. The sterile liquid was siphoned from the containers and centrifuged at 10,000×g for 5 min to remove particulate material,

after which the supernatant was collected and separated.

2.3 FRACTIONATION OF NATIVE EXCRETIONS/SECRETIONS (NES)

Gel filtration technique was used to purify antimicrobial active compounds from the crude sample investigated. The Pharmacia column (40×2 cm) was packed by Sephadex G l50 after its soaking with phosphate buffer (pH 7.2) overnight and the samples were mixed with blue dextran and bromophenol blue dyes, loaded on the top of the column and fractions were collected (5 ml of eachfraction), fractionation was performed using LPLC system (pump model MINI Puls3, detector
UV/VIS-151 FC 203B, Gilson).

2.4 SCREENING ANTIBACTERIAL ACTIVITY OF PROTEIN FRACTIONS

AGAR WELL DIFFUSION METHOD

The antibacterial activity of protein fractions was determined using agar well diffusion method [19]. All the protein fractions were tested *in vitro* for their antibacterial activity against Gram positive bacteria *Bacillus substilis* using nutrient agar medium. Ampicillin was used as standard drugs for *Bacillus substilis*. DMSO was used as solvent control. The protein fractions were tested at a concentration of 1 mg/ml against the bacterial strain.

The sterilized media was poured onto the sterilized Petri dishes (20-25 ml, each petri dish) and allowed to solidify. Wells of 6 mm diameter was made in the solidified media with the help of sterile borer. A sterile swab was used to evenly distribute bacterial suspension over the surface of solidified media and solutions of the test compounds were added to each well with the help of micropipette. The plates were incubated at 37°C for 24 hrs for antibacterial activity. This experiment was carried out in triplicate and zones of inhibition were measured in mm. scale.

2.5 SODIUM DODECYLE SULPHATE (SDS- PAGE) FOR ANTIMICROBIAL PEPTIDES

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to Separate proteins according to their molecular weight.

2.5.1 Reagent and buffers

Acrylamide Monomer Stock Solution (30%): 29.2 gm acrylamide and 0.8 gm N,Nó – methylene bis-acrylamide were dissolved in 100 ml distilled water. (**Resolving Buffer**: 1.5M Tris-HCl, pH 8.8.;**Stacking Buffer**: 0.5M Tris-HCl, pH 6.8., **Resolution Buffer**, pH 8.3: 0.192 M Glycine, 0.02 Trisbase and 0.5 % SDS, **Sample Buffer:** 12 ml 1.0 m Tris-HCL pH 6.8, 8 ml glycerol, 4 ml 2-mercaptoethanol, 16 ml 10% SDS and 2 ml 0.05% bromophenol blue were made up to 80 ml with distilled water).

2.5.2 Prepration of resolving gel (10%)

The gel was prepared from: 4.0 ml distilled water, 2.5 ml resolving buffer, 3.7 ml acrylamide monomer, 100 µl 10% SDS, 50 µl 10% ammonium persulphate and 5 µl Tetramethylethylene Diamine (TEMED).

The gel was poured between the glass plates immediately and carefully overlaid with a layer of distilled water, and then the gel was kept at room temperature to polymerize.

2.5.3 Preparation of stacking gel (4%)

The gel was prepared from: 3.06 ml distilled water, 2.5 ml stacking buffer, 0.65 ml acrylamide monomer, 100 µl 10% SDS, 50 µl 10% ammonium persulphate and 5 µl TEMED. The surface of the polymerized resolving gel was rinsed with 5-7 mL of stacking gel solution. The comb

was aligned in the proper position, then, the stacking gel solution was added up to 2 mm from the top edge of the resolving gel, then left for polymerization at room temperature.

2.5.4 Sample preparation

30 µl of each active fraction were mixed with 10 µl of loading sample dye. The samples were then boiled in a water bath for 2 minutes. The samples were then applied to the gel wells.

2.5.5 Running Conditions

Electrophoresis was carried out with constant volt of 60V, The run was terminated when the bromophenol blue marker reached to the bottom of the gel. The separated proteins on polyacrylamide gels were then stained with Coomassie blue R-250 according to the method described by Merril (18).

The electrophoresed gel was soaked in excess of staining solution for two hours, then the gel was rinsed with distilled water and destained with excess amount of destaining solution for several times until the excess stain was removed.

III. RESULTS

The present study revealed the presence of 40 protein fractions in excretory/secretory products of third instar larvae of *Chrysomya megacephala*, only 3 protein fractions showed antibacterial activity against *Bacillis subtilis*, number 22,23 and 24(Table 1). The separated protein fraction number 22 showed protein bands at 28 and 31 KDa protein(Fig.1 ,Lane 1), separated protein fraction number 23 showed protein bands at 17 , 24 and 28 KDa (Fig.1 ,Lane 2) and separated protein fraction number 24 showed protein band at 26 KDa (Fig.1 ,Lane 3).

Table (1):Mean zone of inhibition in mm ± Standard deviation against *Bacillis subtilis* using different fractions. Results are depicted in the following table:

Fraction number	Mean zone of inhibition in mm ± Standard deviation
22	**21.3 ±1.2**
23	**20.1± 1.5**
24	**17.6± 0.58**

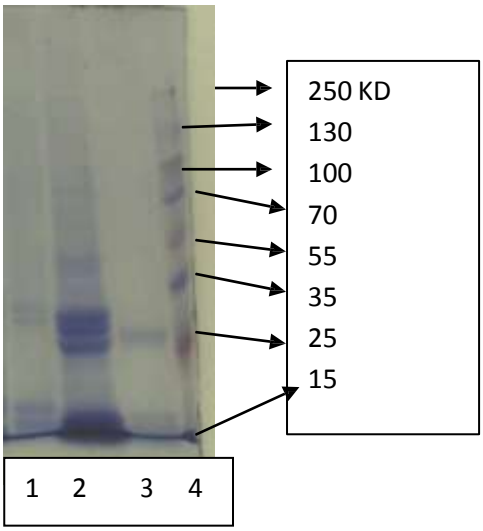

Fig : (1): SDS-PAGE of separated proteins compared with standard molecular weight proteins, protein fraction number 22(Lane 1),protein fraction number 23 (Lane 2),protein fraction number 24 (Lane 3),Marker molecular weight (Lane 4).

IV. DISSCUSSION

The present study revealed the presence of 40 protein fractions in excretory/secretory products of third instar larvae of *Chrysomya megacephala*, only 3 protein fractions showed antibacterial activity.On purification of these protein fractions, they showed protein bands between 17KDa and 31 KDa.

Since 2000, several research groups have been aiming to isolate and characterize such antimicrobial peptides from the ES by utilizing current methods of protein purification. In the laboratory of [19] the ES of maggots was fractionated using an ultra filtration device with a 10 KDa and 500 Da molecular weight cut-off membrane generating three fractions of molecular weights: >10 kDa,500 Da–10 KDa and <500 Da. The activity against *S. aureus* was detected in <500 Da fraction and 500 Da–10 KDa fraction, but not in the fraction above 10 KDa. Even though these fractions were investigated in further detail regarding their physicochemical properties and antimicrobial activities [19], their constituents were not identified. The antimicrobial properties of *L. sericata* larval ES and the attempts to characterize its components were independently studied in several other laboratories [6]. For example, the study of Kerridge *et al.* [20] revealed in the secretions the presence of small (<1 kDa) antimicrobial factors active against Gram-positive bacteria such as *S. aureus*, including both methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA), and *Streptococcus pyogenes*. However, Gram-negative *Pseudomonas aeruginosa* was less sensitive. This active factor passed through the filter of the 3 kDa cut-off when the secretion was fractionated by ultrafiltration procedure. In this case, anti-MRSA activity was also detected in the retenates of the 10 kDa and 5 kDa filters indicating the presence of at least one additional larger antimicrobial agent. The authors concluded that the activities in the secretions possess characteristics consistent with insect antimicrobial peptides and are considered to be of low molecular weight, highly stable and a systemic part of the larva [20].

Jana et al., [21] partially purified a compound of maggot ES of *Lucilia sericata* with antibiofilm properties against *S. aureus* isolate with a size of around 25 KDa. This molecular weight is similar to those separated in the present study and this may indicate that these purified peptides from maggots ES of third instar larvae of *C.megacephala* with antibiofilm properties against *S. aureus.*

Insect immunity system is based on the recognition of microbial cell wall components, such as lipopolysaccharide (Gram-negative bacteria), peptidoglycans and lipoteichoic acids (Gram-positive bacteria), as well as □-1,3-glucans (fungi) by specific receptors. Insects' defense mechanisms generally are divided into two categories: non-specific and specific (innate immune system). In non-specific defense,wide varieties of mechanisms: including morphological, behavioral,developmental (like immunity at maturity), physiological,nutritional, biochemical and genetic are being rendered [22]. Specific mechanisms, which described above,are subdivided into cellular and humoral immune systems [23]**.**The humoral immune system, is based on the production of induced antimicrobial peptides (AMPs) against bacterial and fungal pathogens [23]. AMPs are synthesized in insect fat body and or in some blood cells, and secreted into haemolymph [24]. Antimicrobial peptides have mainly antifungal and antibacterial properties [25]. In insects, the constitutive AMPs are accumulated in blood cells and salivary glands. Upon a microbial challenge the AMPs get released into the haemolymph [26]. In contrast, induced AMPs are synthesized in fat bodies right after the microbial infection takes place and subsequent to their biosynthesis, AMPs release into the blood stream [27]. Meanwhile, it is noteworthy that other organs and tissues such as haemocytes might be involved in AMPs synthesis [28]. Insect antimicrobial peptides are classified into four major groups; cationic peptides, anionic peptides, aromatic dipeptides and also peptides derived from oxygen-binding proteins [29]. Most found AMPs are classified as cationic peptides

with 12-50 amino acids in length [30].

Cationic peptides bear 30-50% hydrophobic amino acid residues. These peptides have a wide range of activities such as antibacterial, antifungal, antiviral (AntiHIV, AntiHSV), anticancer, antiparasitc as well as antiendotoxin activities [30].

Cationic peptides can be subdivided into four broad classes on the basis of biochemical and structural features; a)linear □-helical peptides, b) cyclic peptides containing one or several disulfide bridges, c) proline and glycine-rich peptides,d) □- sheets peptides [30].

Of mode of action, proline-rich peptides are more active against Gram-negative bacteria than Gram-positive bacteria. The higher resistance of Gram-positive bacteria might be due to the peptide decomposition by activity of bacterial extracellular proteases. Glycine-rich peptides have molecular weight between 8-30kDa. These are mostly active against Gram-negative bacteria and including Attacin, Gloverin,Diptericin, and others [31].Although the peptides purified from ES products of third instar larvae of *C. megacephala* may be cationic peptides as the range of peptides purified in the present study is from 17KDa to 31KDa,but they are active against gram positive as *B.stibulis* and *S.aureus*.This may be due to that peptides purified from maggot ES of third instar larvae of *C.megacephala* are not decomposed by activity of bacterial extracellular proteases.

In general, mechanisms of action of antimicrobial peptides are mainly depended on both the molecular characteristics of the peptide and the metabolic states of the microbial cells [32]. Although mechanism of action of AMPs is a controversial subject, but the general consensus is that the majority of antimicrobial peptides, if not all, remarkably disrupt the cell membranes by making pores or channels, leading to the release of ions that may disrupt cell membrane. Consequently, the cell metabolisms may come to a halt and ultimately cell death to occur [33]. It seems ion channel or large pore formation (due to the lack of cholesterol in microbial cell membranes especially bacteria) within pathogen cell membrane is the key mechanisms of the most AMPs [34]. AMPs with the hydrophilic and hydrophobic tails manage their way through the phospholipids bilayer membranes of pathogens, leading to excess membrane permeation **[**35].

V. CONCLUSION

Finally, it is clear from the present study that *Chrysomya megacephala* may be also useful in maggot therapy as *Lucilia sericata*. This also may reinforce the study of other maggots and their validity for larval therapy due to the presence of a lot of proteases in these maggots.

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