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Research Article

Protein profile of alimentary canal and secretions of second instar larvae of *Cephalopina titillator* (Oestridae: Diptera)

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Abstract

The protein content of salivary glands extract, anterior midgut extract, middle midgut extract, posterior midgut extract, hindgut extract and excretory/secretory products of second instar larvae was 31.7 mg/ml, 25.5mg/ ml, 12 mg/ml, 14 mg/ml, 13.2 mg/ml and 11 mg/ml respectively. The salivary gland extract and anterior midgut extract displayed a more complex protein composition At least five major and two minor protein bands were detected in the salivary glands extract. The molecular masses of these protein bands were distributed from 171 to 29 kilodaltons (KDa), mainly those of 171, 43, 36, 33, 32, 30 and 29 KDa. The anterior midgut extract protein profile showed six bands ranging from 149 to 32 KDa (149, 46,41,38,35 and 32 kDa). The middle midgut extract , posterior midgut extract and hind gut extract showed less complex protein profile The middle midgut extract protein profile showed three bands estimated at 41, 38, and 34 KDa .The posterior midgut extract protein profile showed two bands estimated at 41, and 38 KDa. The hind-gut protein extract profile showed three bands estimated at 155,128 and 42 KDa. The excretory/secretory products protein profile showed only one band estimated at 32 KDa.

Keywords: *Cephalopina titillator*, excretory/secretory products, proteases, alimentary canal.

1. Introduction

The nasal bot fly *Cephalopina titillator* has been known for a long time as a common obligate parasite of Oestridae family that attacks only camels, *Camelus dromedarius* in Egypt [Soliman, 1965], as well as in different countries in Africa and Asia, [Hussein *et al.*, 1982; Higgins, 1985]. The adult fly is widely distributed in areas where camels are found [Higgins, 1985].

During part of its life-cycle, the female fly darts towards the head and deposits its larvae directly into the mucous membrane of the upturned nostrils. From there the larvae crawl up to the nasopharynx and sometimes the paranasal sinuses and molt twice while attached to the naso pharyngeal and paranasal mucous membranes and cause extensive irritation and tissue damage. They remain attached to the mucous membrane of these organs for up to 11 months; during which time they feed and cause extensive irritation and tissue damage [Hussein *et al*., 1982]. The mature white or grey third stage larvae grow up to 35 mm and up to 15 mm in the second stage, but the first larval stage is only about 0.7 mm long.

These infestations impair animals' welfare, reduce host physiological functions, destroy host tissues and cause significant economic losses to livestock through abortion, reduction of milk production and losses in terms of weight gain, fertility [Hall & Wall, 1995; Otranto, 2001].

The presence of the larvae, often in large numbers, causes a number of pathological problems to camels. Infested camels lose their appetite, show difficulty in

breathing, snort, sneeze, expel the larvae from their nostrils and may show abnormal behavior resembling cranial coenuriasis and they often become restless and may even stop feeding [Zumpt, 1965]. They infrequently may finally die from meningitis caused by secondary bacterial or viral infections [Burgemeister *et al*., 1975; Musa *et al.,* 1989]. The intensity of clinical signs depends on the amounts of damage by migrating larvae.

Most of the previous studies on this parasitic insect in Egypt and the Arab World were based mainly on the morphology, incidence and some biological aspects, besides prevalence and monthly variations of the second and third instars [Hussein *et al*.,1982, and Fatani and Hilali, 1994], neglecting the study of the structure and biochemistry of the alimentary canal which is of basic importance for understanding the host parasite relationship.

The midgut in insects comprises the longest and functionally most important part of the digestive tract, dealing primarily with the digestion of food stuffs [Dimitriadis and Kastritsis, 1984].

The insects' protein digestion is achieved by means of digestive proteases. The majority of blood-sucking insects produce a range of alkaline digestive proteases, the exceptions occurring in the Hemiptera where digestive enzymes are active at acid pH [Lehane.1994]. In myiasis producing agents such as *Lucilia cuprina, Chrysomya bezziana* or *Hypoderma lineatum*, several proteases are secreted and exported into contact with the host [Sandeman *et al.,* 1990; Muharsini *et al.,* 2000; Boulard and Garrone, 1978].

The aim of the present study was to describe preliminary biochemical analysis including determination of total protein content and the simple proteolytic pattern of secretions produced by second instar of *Cephalopina titillator* from their salivary glands and alimentary canal during their feeding.

Materials and Methods

2.1 Larval collection

Cephalopina titillator larvae 2nd instars were recovered from naturally infected camel heads (*Camelus dromedarius*) of both sexes collected from a local slaughter house (Shebeen Al Kanater – Qalubia). Heads were split longitudinally using a hand saw. The cut started at the nose tip, then through the nasal maxillary and frontal sinuses till the base of the skull including the brain meninges. The larvae were collected from the pharyngeal region in plastic vials, second instar larvae were identified on the basis of morphological keys [Zumpt, 1965].

2.2 Organ contents

Alimentary canal and salivary glands were dissected and separated; midgut was divided to three regions anterior midgut, middle midgut, posterior midgut. Hindgut also was separated. The different regions were collected in epindorfs. The contents of organs were expelled by centrifugation (5 min, 1000 x g) and nominated: salivary gland extract, anterior midgut extract, middle midgut extract, posterior midgut extract and hindgut extract. The supernatants were filtered through 0.8/0.2µm sieves and stored at (-70ºC) until used. Protein concentration in supernatants was determined.

2.3 Execrtory secretory products

Twenty alive larvae of 2nd instar were washed with distilled water to remove any debris. Larvae were incubated in 1ml PBS pH 7.3 for one hour at 30ºC in a dark incubator , removed and the remaining liquid collected and centrifuged (5min,10000 x g) and termed execrtory secretory products. The supernatants were filtered through 0.8/0.2µm sieves and stored at (-70ºC) until used. Protein concentration in supernatants was determined.

2.4 Protein concentration measurement

Total protein content was estimated by using the (Protein–Biuret Method) kit from Biodiagnostic according to Gornal *et al.* (1949).

2.5 Determination of molecular weights by sodium dodecyl sulphate acrylamide gel electrophoresis (SDS-PAGE)

The individual components of organ contents and the execrtory secretory products were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the reported method of Laemmli (1970). The different midgut regions and ES products were concentrated by

ultra- filtration followed by precipitation in 80 % (v/v) ice-cold acetone. The precipitate was redissolved in reducing sample buffer and boiled for 5 minutes prior to electrophoresis on 12 % polyacrylamide gel. Proteins bands were detected by staining with 0.25 % Coomassie brilliant blue R250 in 25 % ethanol, 10% acetic acid followed by distaining in the same solvent without the Coomassie stain. Proteinase components were examined after separation on substrate gels.

3. Results

3.1 Total protein content

The protein contents of salivary glands extract, anterior midgut extract, middle midgut extract, posterior midgut extract, hindgut extract and excretory/secretory products of second instar larvae were quantified as follow:

Organ	amount of protein
salivary gland extract	**31.7 mg/ml**
anterior midgut extract	**25.5mg/ ml**
middle midgut extract	**12 mg/ml**
posterior mid-gut extract	**14 mg/ml**
hind-gut extract	**13.2 mg/ml**
excretory /secretory products	**11 mg/ml**

3.2 Gel-electrophoretic studies

Total proteins in the extractions of the various micro-dissected morphological regions of salivary gland ,different middle midgut regions and hindgut and in the excretory/secretory products , were examined in CBB stained SDS polyacrylamide gels (Fig.1).The salivary gland extract and anterior midgut extract displayed a more complex protein composition (Fig. 1, Lane 2 and 3) . At least five major and two minor protein bands were detected in the salivary glands extract (Fig. 1, Lane 2). The molecular masses of these protein bands were distributed from 171 to 29 kilodaltons (KDa), mainly those of 171, 43, 36, 33, 32, 30 and 29 KDa. The anterior midgut extract protein profile showed six bands (Fig. 1, Lane 3) ranging from 149 to 32 KDa (149, 46,41,38,35 and 32 kDa). The middle midgut extract, posterior midgut extract and hind gut extract showed less complex protein profile (Fig.1, Lane 4, 5and 6). The middle midgut extract protein profile showed three bands estimated at 41, 38, and 34 KDa (Fig.1, Lane 4) .The posterior midgut extract protein profile showed two bands estimated at 41, and 38 KDa (Fig.1, Lane 5). The hind-gut protein extract profile showed three bands estimated at 155,128 and 42 KDa (Fig. 1, Lane 6). The excretory/secretory products protein profile showed only one band estimated at 32 KDa. (Fig.1, Lane 7).

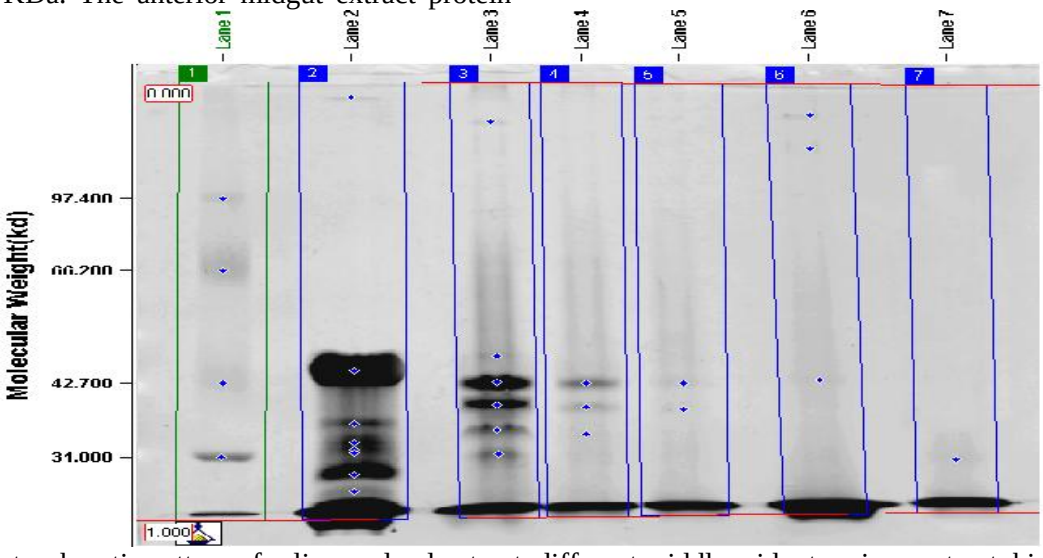

Fig.1: Electrophoretic pattern of salivary gland extract, different middle midgut regions extract, hindgut extract and excretory/secretory products of *Cephalopina titillator* under reducing conditions . Lane 1(marker), Lane 2 (salivary gland extract), Lane 3 (anterior midgut extract), Lane4 (middle midgut extract), Lane 5 (posterior midgut extract), Lane 6 (hindgut extract) and Lane 7 (excretory/secretory products).

4. Discussion

The preliminary electrophoretic protein separation of salivary glands, anterior midgut, middle midgut, posterior midgut, hindgut and excretory/secretory products (ESP) of second instar larvae of *C. titillator* produced bands between 171 KDa and 29 KDa. The protein analysis of salivary gland contents produced different number of bands ranging between 171 and 29 KDa, anterior-midgut bands ranges from149 KDa and 32 KDa, middle midgut bands ranges from 41 KDa and 34 KDa, posterior midgut bands were two bands 41 KDa and 38 KDa, hindgut were three bands at 155, 128 and 42 KDa and ESP band was at 32 KDa. The analyses of *C. titillator* confirm that the most active areas secreting secretory ESP are the salivary glands and anterior midgut regions. The total protein content was higher in the salivary gland, anterior and posterior midgut than in the middle midgut. However the number of protein bands in the middle was more than in the posterior midgut.

Insect trypsin have been characterized and purified from species of Coleoptera, Orthoptera, Lepidoptera and Diptera. Most insect trypsin is 20–30 KDa as determined by SDS-PAGE [Terra and Ferreira, 1994]. These insect trypsin are most active at alkaline pH, are not activated by calcium ions, and are sensitive to natural trypsin inhibitors [Terra and Ferreira, 1994]. Recently, workers in Nottingham, UK, demonstrated *in vitro* a range of enzymes secreted by *Phaneicia sericata* larvae [Chambers *et al*., 2003]. Four proteolytic enzymes, comprising two serine proteases, a metalloproteinase and an aspartyl proteinase, were detected, with molecular weights ranging from 20 to 40 KDa, with activity across a wide pH range.

Proteolytic enzymes are a major component of the digestive process of parasites and are presumed to be released to interact with host tissues [Rhoads and Fetterer, 1997]. Parasitic and microbial organisms utilize the digestive actions of proteases on proteins of cells, tissues and organs for the purposes of invasion and migration in host tissues.

Evidence from the larvae of several fly species is that trypsin and chymotrypsin-like serine proteases are the dominant digestive proteases [Terra and Ferreira, 1994]. A great number of trypsin and mainly chymotrypsin-like proteases were found in the sheep blowfly, Lucilia *cuprina*. These larval chymotrypsin-like proteases of digestive origin are involved in wound formation and nutrition [Casu *et al.,* 1996 and Sandeman *et al.,* 1990]. The existence of digestive serine proteases in larval ESPs of *Chrysomya bezziana* was reported [Sandeman, 1990]. Hypodermins, some proteases secreted by the cattle grub. *lineatum* were characterized and classified, also, as serine proteases [Boulard and Garrone,1978).These proteases play a fundamental role allowing *H. lineatum* L_1 larvae nutrition and migration through host tissues .Also the occurrence of serine proteases in *Oestris ovis* larvae ESPs was established by Tabouret *et al.* (2003) as well as in *Chrysomya megacephala* L_3 larvae [El-Ebiarie and Taha 2012].Angulo-Valadez *et al*. (2010) reported that Oestrid larvae cannot be fully reared in vitro because of many aspects related to larval nutrition and appropriate environmental conditions that still need to be investigated. Tabouret *et al.* (2001a) stated that the excretory /secretory products (ESP) proteins were produced mainly by digestive tube and salivary gland in larvae of *Oestrus ovis*. Tabouret *et al.* (2001b) showed that, in vitro, that ESP could stimulate nitric oxide production inducing plasma protein leakage. This up regulation is time and dose-dependent . The presence of proteases in the excretory/secretory products (ESP) of *O.ovis* larvae has been clearly identified (Tabouret *et al*., 2003). These proteases appear to originate mainly from the gut and are exported on the nasal or the sinusal mucosa. They also observed weak proteolytic activity in the salivary glands.

In the present study of *C.titillator* strong proteolytic activity was observed in studies of SDS-gel electrophoresis. Further investigations using molecular tools are needed to confirm whether all proteases are produced only from gut as *Oestris* or the salivary gland is responsible for a great part of them.

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