Immunological evaluation of some antigens of *Lucilia sericata* larvae

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Abstract The present study aimed to select an antigen of *Lucilia sericata* larvae showing both high antigenicity and cross-reactive binding abilities with other related antigens of *L. sericata* larvae for obtaining a promising candidate vaccine antigen. The ELISA results primary concluded that among the excretory secretory (ES) and midgut (MG) antigens of the different larval instars of *L. sericata*, MGL2 could be characterized as antigen which was able to reflect the highest level of antigenicity and cross-reactivity with the other tested *L. sericata* antigens. The results were extended to spot the light on the relation between different protein bands in MGL2 and rabbit hyper-immune sera (HIS) raised against the other tested antigens using SDS-PAGE and Western blot technique. Analysis by SDS-PAGE of ES and MG antigens of the different larval instars of *L. sericata* revealed common protein bands at molecular weights of about 10, 12, 16, 20, 28, 33 and 46 kDa. Western blotting of MGL2 antigen transferred to nitrocellulose sheet revealed reaction by MGL2 HIS to five polypeptide bands; 20, 28, 33, 46 and 63 kDa. Three bands of 28, 33 and 63 kDa were the most prominent bands detected whereas; there was a weak reaction with bands of 20 and 46 kDa. But what was apparent in Western blot was a strong reaction of all tested HIS with a polypeptide band of 63 kDa. This band might be considered to be the main cause of cross reactive binding ability of MGL2 antigen that had been recorded previously in ELISA technique.

Keywords *Lucilia sericata* larvae · Excretory secretory · Midgut · Antigens · ELISA · Western blot

Introduction

The larvae of *Lucilia sericata* (Meigen) (Diptera: Calliphoridae), is the main causative agent of cutaneous myiasis (fly strike) in sheep which constitutes a serious problem in all the major sheep-producing countries throughout the world (Phillips 2009). They feed on the host's living or dead tissue, usually at the surface of the skin or inside body orifices causing serious damage which if untreated may lead to host mortality (Talari et al. 2004). Fly strike control is important, and has relied on the use of organophosphate and synthetic pyrethroid dips and sprays (French et al. 1994; Tellam and Bowles 1997) as well as insect growth regulators (Levot and Sales 2004). However, usage of chemical insecticides promotes widespread contamination of the environment, toxicity to non-target organisms, resistance development and destructive effects on human and animal health (Khater and Khater 2009). Thus, controlling of insects larvae required alternative methods without harming to the environment. Successful vaccination against an ectoparasite would be an attractive alternative to insecticides. This immunological control of the parasite has been shown to be a promising alternative method in the control of myiasis-causing larvae (Otranto 2001). Control through vaccination has focused on two types of antigens; larval excretory-secretory products that elicit an immune response in the host and peritrophic membrane antigens of midgut which are physiological targets internal to larvae, not seen by the host (Tellam et al. 2001). These antigens are thought to be potential targets for

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vaccines that disrupt establishment of larval stages on/or within the host, and interfere with the digestion of food in the gut of larval stages (Tellam and Bowles 1997). The large amounts of IgG released at the wound site early in the infection provide encouragement for humoral mediated control of fly strike. Infestation of sheep with *L. cuprina* larvae led to the rapid development of antibodies to larval excretory and secretory products (Sandeman et al. 1985; Eisemann et al. 1990). The observation that larvae ingest antibodies when feeding on exudates on the skin surface and that these antibodies remain biologically active within the larval gut (Eisemann et al. 1993) led to a search for antigens derived from the peritrophic membrane of midgut that may be susceptible to immunological attack by the host (East et al. 1993). It was found, when sheep were immunized with peritrophin antigens, that larval growth in vivo was negatively correlated with antibody titer in serum and that larval growth in an in vitro feeding bioassay was suppressed by antibody in a dose dependent manner (Colditz and Eisemann 1994). Thus, it seemed probably that the quantity of antibody ingested by larvae influenced the efficacy of vaccines. Based on the previous facts, the present study focused on selection of an antigen of *L. sericata* larvae showing both high antigenicity and cross-reactive binding abilities with other related antigens of *L. sericata* larvae for obtaining a promising candidate vaccine antigen.

Materials and methods

Insect culture

L. sericata larvae were obtained from a laboratory colony maintained at the Department of Parasitology and Animal Diseases, Veterinary Research Division, National Research Centre, Egypt. The colony was reared under laboratory conditions of room temperature ranged between 14.6 and 32.1 °C and mean relative humidity ranged between 32.5 and 53.5%. Laboratory temperature and humidity were recorded daily by means of minimum and maximum thermometer and a hair sensitive hygrometer. Adults were fed on sucrose and water but larvae were reared on bovine meat (El-Khateeb 1999).

Obtaining and identification of 1st, 2nd and 3rd larval instars

In the laboratory, 1st stage larvae were obtained after hatching of eggs by 8–12 h according to temperature. 2nd and 3rd stages larvae were obtained after 31 and 72 h, respectively, according to temperature and humidity. For identification, 20 specimens of each larval stage were

mounted according to Pritchard and Kruse (1982), and then were identified following the key and morphological characters mentioned by Zumpt (1965).

Preparation of antigens

Excretory secretory (ES) antigen

The three larval instars were washed several times with saline before antigens preparation. 500 larvae from 1st, 2nd, and 3rd instars were incubated in PBS, pH 7.2 containing antibiotic at 25 °C overnight in a dark bottle. The supernatant was collected and centrifuged at 13,000 rpm for 20 min using cooling centrifuge, then divided into aliquots and stored at −80 °C until used (Tabouret et al. 2001).

Midgut (MG) antigen

Also, 500 larvae from 1st, 2nd and 3rd instars were dissected alive in ice-cold PBS medium under a stereoscope for removal of midgut then preserved in PBS pH 7.2. They were subjected to grinding using a homogenizer after chilling with ice and sonicated for 5 min at 5 pulse rate 60–80 amplitude value using Cole Parmer ultrasonic sonicator, 230 VAC. The homogenate was centrifuged at 10,000 rpm for 30 min at 4 °C and the supernatant fluid containing the antigenic material of larval stages was obtained and stored at −80 °C until used (Angulo-Valadez et al. 2007).

Determination of protein content of prepared antigens

The protein content of antigenic material was measured using the modified Lowry's methods (Lowry et al. 1951). All chemicals were purchased from Sigma-Aldrich, USA.

Reference rabbit hyper-immune sera (HIS)

Rabbit hyper-immune sera were raised against the six larval antigens (1st, 2nd and 3rd ES as well as 1st, 2nd and 3rd MG), via initial subcutaneous injection with 100 µg protein emulsified in 1 ml mineral oil and two consecutive intramuscularly injections during 35 days. The level of specific antibodies in sera of immunized rabbits was evaluated before slaughter. Rabbits were bled before immunization as negative control sera. The experimental protocol was approved by Medical Research Ethics Committee, National Research Centre, Egypt. The experiments were conducted in accordance with the guidelines laid down by the International Animal Ethics Committee and in accordance with local laws and regulations.

Enzyme-linked immunosorbent assay (ELISA)

Antibody levels in the previous HIS were measured by an ELISA using the tested antigens according to the procedure of Voller et al. (1976). The assay was performed in 96-well-flat bottom microtiter plates; which were coated by overnight incubation at 4 °C with 100 µl aliquots (per well) of test antigen (20 µg per ml of 0.1 M sodium carbonate buffer, pH 9.6). HIS raised against ES and MG antigens of the different larval instars of *L. sericata* were serially diluted (1:50–1:6400). The sera were added in duplicate wells (100 µl per well) and incubated at 37 °C for 1 h. Peroxidase—conjugated anti-rabbit IgG (Sigma) was diluted 1000-fold, 100 µl aliquots added per well and incubated as described. Reagents were diluted in 0.01 M PBS, pH 7.4 containing 0.05% Tween 20 (Sigma) (PBS-T). Wells were washed between incubations with PBS-T. The absorbance of the peroxidase reaction (determined with orthophenylenediamine 340 µg/ml citrate/phosphate buffer, pH 5.0 with 0.03% hydrogen peroxide solution) was measured at 450 nm using ELISA reader.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot techniques

Protein fractions of each tested larval antigens (ES of L1, L2, L3 and MG of L1, L2, L3) were demonstrated using 10% SDS-PAGE (40 µg/lane) according to Laemmli (1970) with the aid of molecular weight standard of pre-stained protein ladder (Pharmacia Biotech). Silver staining of the gel was done according to Wray et al. (1981). *L. sericata* MGL2 antigen was analyzed by SDS-PAGE then transferred onto nitrocellulose (NC) sheet for Western blot technique. Strips of the blotted NC were allowed to react with rabbit hyper-immune sera raised against ES and MG antigens of the different larval instars of *L. sericata* as well as normal rabbit sera. The molecular weights of reacted polypeptides were determined using Molecular Imager Gel DocTM XR System (Bio-Rad, USA).

Results

Antigenic relationship between ES and MG antigens of different larval instars of *L. sericata*

ES and MG antigens of the different larval instars of *L. sericata* were allowed to react with their rabbit hyper-immune sera (HIS) at different levels of serum dilutions (1:50–1:6400) using ELISA technique. Concerning ES antigens, the ability of ES antigens of the different instar larvae of *L. sericata* (ESL1, ESL2 and ESL3) in binding

with specific antibodies of the three related HIS revealed a strong reaction between the three selected antigens and sera; this reaction decreased with increasing serum dilution (Fig. 1). ESL1 antigen reacted specifically with its homologous HIS at different levels of serum dilution with decreasing mean ELISA O.D. value from 1.285 to 0.692 on increasing serum dilution from 1:50 to 1:6400, respectively. At a time, ESL2 and ESL3 showed lower mean ELISA O.D. values on reaction with their homologous HIS ranging from 0.681 to 0.190 and 0.732 to 0.094, respectively. On the other hand, ESL1 antigen showed strong reaction with heterologous HIS raised against ESL2 and ESL3 antigens, where the mean ELISA O.D. values ranged from 1.550 to 0.468 and 1.595 to 0.674 on increasing

Fig. 1 Antigenic reactivity of ES antigens of different larval instars of *L. sericata* versus hyper-immune rabbit sera; raised against ES L1, L2 and L3, using ELISA technique. **a** ES L1 antigen, **b** ES L2 antigen, **c** ES L3 antigen

serum dilution from 1:50 to 1:6400, respectively. On contrary, ESL3 antigen showed lower mean ELISA O.D. values on reaction with heterologous HIS at different levels of serum dilution ranged from 0.516 to 0.083 and 0.379 to 0.062 with ESL1 and ESL2 HIS, respectively. ESL2 antigen showed strong reaction with ESL3 HIS, where the mean ELISA O.D. values ranged from 1.560 to 0.355 however, it recorded lower mean ELISA O.D. values with ESL1 HIS ranged from 0.392 to 0.116. The data in Fig. 1 characterized ESL1 as antigen of the highest antigenicity and reactivity toward HIS raised against ES antigens of the other instar larvae of *L. sericata*.

On reaction of MG antigens of the different instar larvae of *L. sericata* (MGL1, MGL2 and MGL3) with their HIS using ELISA technique, a rate of strong reaction, decreased with increasing serum dilution, was occurred (Fig. 2). MGL2 antigen reacted specifically with its homologous HIS at different levels of serum dilution with decreasing mean ELISA O.D. value from 1.442 to 0.145 on increasing serum dilution from 1:50 to 1:6400, respectively. At a time, MGL1 and MGL3 showed lower mean ELISA O.D. values on reaction with their homologous HIS ranging from 1.047 to 0.303 and 0.366 to 0.063, respectively. On the other hand, MGL2 antigen showed strong reaction with heterologous HIS raised against MGL1 and MGL3 antigens, where the mean ELISA O.D. values ranged from 1.282 to 0.414 and 0.516 to 0.068 on increasing serum dilution from 1:50 to 1:6400, respectively. On contrary, MGL1 antigen showed lower mean ELISA O.D. values on reaction with heterologous HIS at different levels of serum dilution ranged from 0.823 to 0.154 and 0.213 to 0.133 with MGL2 and MGL3 HIS, respectively. While, MGL3 antigen showed strong reaction with HIS raised against MGL1 and MGL2 antigens, in spite of being the lowest antigenic one, with mean ELISA O.D. values ranged from 0.943 to 0.526 and 1.145 to 0.116, respectively. The data in Fig. 2 characterized MGL2 as antigen of the highest antigenicity and reactivity toward HIS raised against MG antigens of the other instar larvae of *L. sericata*.

The selected ESL1 and MGL2 antigens were allowed to react with heterologous HIS raised against MG and ES antigens of the different instar larvae of *L. sericata*, respectively, at different serum dilutions (1:50–1:3200) using ELISA technique (Fig. 3). The ELISA readings with MGL2 antigen showed mean O.D. values higher than that were recorded with ESL1 antigen. Where, the mean ELISA O.D. values with MGL2 antigen on reaction with HIS raised against ESL1, ESL2 and ESL3 antigens ranged from 0.480 to 0.183, 0.554 to 0.175 and 0.897 to 0.187, respectively, While they ranged from 0.366 to 0.146, 0.367 to 0.198 and 0.424 to 0.135; on reaction of ESL1 antigen with MGL1, MGL2 and MGL3 HIS, respectively.

Fig. 2 Antigenic reactivity of MG antigens of different larval instars of *L. sericata* versus hyper-immune rabbit sera; raised against MG L1, L2 and L3, using ELISA technique. **a** MG L1 antigen, **b** MG L2 antigen, **c** MG L3 antigen

Based on the previous ELISA results, MGL2 antigen might be considered as candidate vaccine antigen due to its high antigenicity and cross reactivity with other related antigens of the different instar larvae of *L. sericata*.

SDS-PAGE and western blot analysis

Analysis by SDS-PAGE of ES antigens of *L. sericata* L1, L2 and L3 revealed at least 8, 8 and 10 polypeptides in each antigen, respectively, while those of MG antigens were 9, 12 and 12, respectively (Fig. 4). These polypeptides bands were in similar pattern but not identical, especially between ES and MG antigens, and their molecular weights ranged from 10 to 169 kDa. The protein bands at molecular weights of about 10, 12, 16, 20, 28, 33

Fig. 3 Cross-reactivity of ESL1 and MGL2 antigens of *L. sericata* versus heterologous hyper-immune rabbit sera; raised against MG and ES antigens, respectively, of different larval instars of *L. sericata* using ELISA technique. **a** ES L1 antigen, **b** MG L2 antigen

Fig. 4 SDS-PAGE of ES and MG antigens of *L. sericata* larval instars

Fig. 5 Western blot reaction at various protein bands of MGL2 antigen against sera from rabbits hyper immunized against ES and MG antigens of the different larval instars of *L. sericata*. The arrow indicates cross-reactive band. MW molecular weight marker, NS normal rabbit sera

and 46 kDa were common among all tested antigens. Notably, five protein bands more in MGL2 antigen at molecular weight of 63, 100, 120, 149 and 169 kDa shared with MGL3 antigen.

L. sericata MGL2 antigen was analyzed by SDS-PAGE then transferred onto NC sheet for Western blot technique. The fractionated and transferred MGL2 antigen was allowed to react with rabbit hyper-immune sera raised against ES and MG antigens of the different instar larvae of *L. sericata* as well as normal rabbit sera (Fig. 5).

Western blotting of MGL2 antigen transferred to NC sheet revealed reaction by MGL2 HIS to five polypeptide bands; 20, 28, 33, 46 and 63 kDa. Three bands of 28, 33 and 63 kDa were the most prominent bands detected, whereas there was a weak reaction with bands of 20 and 46 kDa. All those five immunoreactive bands could be detected by ESL1, MGL1 and MGL3 HIS, While ESL2 and ESL3 HIS recognized only two polypeptide bands at molecular weights of 20 and 63 kDa. Their failure to identify bands of 28, 33 and 46 kDa might be attributed to their low antigenicity; as previously expressed by their low ELISA O.D. values on reaction with MGL2 antigen. No reaction was observed with normal rabbit sera. But what was apparent in Western blot was a strong reaction of all tested HIS with a polypeptide band of 63 kDa. This band might be considered to be the main cause of cross reactive binding ability of MGL2 antigen that had been recorded previously in ELISA technique.

Discussion

Owing to the development of a vaccine, the ability to isolate and identify protective antigens from the parasite is needed. The isolated fractions must then be evaluated for their immunogenicity before testing their protective efficacy in vaccination (Tellam and Bowles 1997). Indeed, the results primary concluded that among the ES and MG antigens of the different larval instars of *L. sericata*, MGL2 could be characterized as antigen which was able to reflect the highest level of antigenicity and cross-reactivity with the other tested *L. sericata* antigens. In that sense, the parasitic larvae in the skin of the sheep presented excretory and secretory antigenic components to the host's immune system, and there was some evidence to suggest that repeated infections with blowfly larvae induced a degree of resistance in sheep (Eisemann et al. 1990). However, up to seven or eight repeated infections might be required to induce demonstrable acquired immunity. Some of the secretions or excretions produced by the parasitic larvae were powerfully immunosuppressive (Kerlin and East 1992) and this might, to some extent, explain why ESL1 antigen showed lower antigenicity in comparison with MGL2 antigen, as shown in the current study. Moreover, use of hidden antigens provided an opportunity to avoid the immuno-modulatory effect of ES antigens. This was particularly evident in attempts of Panadero et al. (2009) to induce immunity in animals infested with *Hypoderma* spp. Based on the ELISA results of the present study, MGL2 antigen might be considered as candidate vaccine antigen. It is known that the only region of the insect digestive tract without an impermeable cuticle lining is the midgut, which is responsible for the digestive functions of the insect. This region of the insect gut is lined with a semi-permeable matrix, the peritrophic matrix [or peritrophic membrane (PM)], composed of proteins, proteoglycans and chitin (Tellam 1996; Lehane 1997). The PM is closely associated with the insect digestive process. Molecules that bind to PM proteins such as specific antibodies can disrupt its permeability and functions that way affecting insect growth, especially during the larval stages (Tellam and Eisemann 2000; Wang and Granados 2000; Tellam et al. 2001). In the later, ingested antibodies bound to the PM, blocking the pores in this normally semi-permeable matrix and presumably inhibited movement of nutrients to the digestive epithelia thereby starving the larvae.

In order to clarify the main cause of cross reaction recorded in ELISA technique between MGL2 antigen and other tested antigens, the results were extended to spot the light on the relation between different protein bands in MGL2 and HIS raised against the other tested antigens using SDS-PAGE and Western blot technique. This was the first time that the ES and MG antigens of all instars larvae of *L.*

sericata were characterized and compared. Western blot reaction of fractionated MGL2 antigen of *L. sericata* revealed that a polypeptide band of 63 kDa might be considered to be the main cause of cross reactive binding ability of MGL2 antigen that had been recorded previously in ELISA technique of the present study. In this connection, El-Khateeb and Kutkat (2004) identified immunogenic antigens in different larval instars of *L. sericata* using SDS-PAGE and immunoblotting. They found that protein profile of the second and third larval instars revealed three common protein bands (27.5, 57.5 and 160 kDa) against one protein band only (27.5 kDa) in the first larval instar. Also, Western immunoblotting of the electrophoresed protein bands with polyclonal rabbit antisera prepared against mixture of the three larval protein antigens showed two common reactive bands located at 57.5 and 160 kDa in the second and third larval antigens. They added that the previous protein bands were specific for evoking antibodies against *L. sericata*. Besides, the present results appeared to be in line with some studies performed on the immune response of sheep to proteins from *L. cuprina*. Where, Seaton et al. (1992) recorded three molecules of ES antigen at molecular weights of 25, 30 and 35 kDa were recognized by sera from experimentally infested sheep. Fry et al. (1994) characterized a crude third instar midgut homogenous preparation by gel electrophoresis and immunoblotting. They found common reactive bands at 40, 50 and 80 kDa. Elvin et al. (1996) identified two major integral peritrophic membrane proteins with molecular weights of 44 and 48 kDa. On the other hand, Tellam et al. (2000) noted that immuno-blots and ELISAs showed strong recognition of peritrophin-95 with sera from sheep naturally or artificially infested with *L. cuprina* larvae. The results implied that the ovine immune system was exposed to peritrophin-95 even though this protein was strongly attached to the peritrophic membrane in the midgut of the larvae and seemingly hidden from the host's immune system. They suggested that the response was induced by regurgitation or excretion of digestive gut proteases into the myiasis site to facilitate the larval feeding process. This might explain the high cross reactive binding ability of MGL2 antigen toward hyper immune sera raised against ES antigens of the different larval instars of *L. sericata*. Besides, the 63 kDa protein band appeared to be highly antigenic in MGL2 antigen. It is necessary to carry out further in vivo studies with immunoprotective effect of 63 kDa protein against *L. sericata* larvae.

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