Indian Journal of Animal Sciences **87** (8): 947–950, August 2017/Article

An analysis of matrix metalloproteinases (MMPs) activity in excretory/secretory antigens of *Oestrus ovis* larvae from sheep

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Received: 24 May 2016; Accepted: 27 March 2017

ABSTRACT

The present study was carried out to ascertain the presence of matrix metalloproteinases activity in excretory/secretory (E/S) antigens of larvae of *Oestrus ovis* by gelatin zymography. The larvae of *O. ovis* were collected from sheep slaughtered at local abattoirs in Orathanadu and Thanjavur. Live, intact larvae were washed thoroughly with phosphate buffered saline (PBS, pH 7.4) and suspended in PBS at 37°C in an incubator for 16 h. After incubation, the fluid was collected, centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was used as E/S antigen. On gelatin zymographic analysis, presence of 220 kDa MMP-9 (pro form) and 210 kDa (active form) homodimeric forms was observed as a fainter band at the top of the gelatin zymogram. A clear, bright gelatinolytic activity was observed at 20 kDa level at the bottom of the zymogram. The other forms of MMP-9, viz. 135 heterodimeric form was observed at 125 kDa size (active form) as a fainter band and the 72 kDa MMP-2 was detected as a fainter bands at 72 kDa (pro form) and 62 kDa (active form). The strong gelatinolytic activity detected at 20 kDa level of E/S antigens of *O. ovis* was the catalytic breakdown products of the MMPs found in the fluid. It was concluded that the activity of the MMPs in the E/S antigens of *O. ovis* was very strong, helping in the migration of the larvae from one site to the other.

Key words: Excretory/secretory antigens, Gelatin zymography, MMP, *Oestrus ovis*

The larva of *Oestrus ovis* is a cosmopolitan obligate parasite of the nasal cavities and frontal sinuses of sheep, causing the clinical picture known as oestrosis. Larval feeding activity involves secretion of enzymes into the upper respiratory mucosal substrate and such enzymes degrade the substrate into smaller units that are then swallowed to support larval growth and development (Tabouret *et al*. 2003). These parasitic larvae use proteolytic enzymes for larval migration, establishment, feeding, growth and development (Muharsini *et al*. 2000). Excreted/secreted products of *O. ovis* larvae comprise a complex array of enzymes, including proteases that are associated with Type I hypersensitivity reaction.

Matrix metalloproteinases (MMPs) are the major group of enzymes with ability for degrading extracellular matrix proteins and this process of ECM remodeling is responsible for all these necessary processes (Shapiro 1998, Balamurugan *et al.* 2015a). The MMPs constitute a part of large family of zinc-dependent endopeptidases. These enzymes play various functional roles and touch many aspects of physiological (Balamurugan *et al.* 2015b, Prakash Krupakaran *et al*. 2015a,b, Pandiyan *et al*. 2015) and pathological processes (Prakash Krupakaran *et al*. 2016a,b,c). Many of these parasites have to migrate or invade through various organs of their hosts to complete their life cycle. During this journey, expression and release of MMPs by various parasitic species have been associated with pathology resulting from histolysis (Lai *et al*. 2005, Williamson *et al*. 2006, Arunkumar *et al.* 2016). However, the work on MMPs activity in excretory/secretory (E/S) antigens of larvae of *O. ovis* is very limited. Hence, the present investigation was aimed to ascertain the presence of MMPs activity in excretory/secretory (E/S) antigens of larvae of *O. ovis* by gelatin zymography.

MATERIALS AND METHODS

The *O. ovis* larvae were collected from sheep slaughtered at local abattoirs in Orathanadu, Pattukkottai and Thanjavur, Tamil Nadu. The larvae were washed thoroughly with PBS (pH 7.4) and were identified on the basis of morphological keys (Zumpt 1965). Twenty five live, intact larvae were washed thoroughly with PBS (pH 7.4) and suspended in 10 ml of PBS at 37°C in an incubator for 16 h. After incubation, the fluid was collected, centrifuged at 10,000 rpm for 15

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min at 4°C and the supernatant was used as E/S antigen. The supernatants thus collected were stored at –20°C till further use. The protein content was estimated by Lowry's method (1951). Gelatin zymography of E/S products was carried out as per the method of Heussen and Dowdle (1980) with some modifications.

SDS-PAGE was carried out as described by the method of Laemmli (1970). The resolving gel (8%) was co-polymerized with 0.3% gelatin solution (final concentration of gelatin in gel was 0.15%) and the electrophoretic run was carried out at 100 V until tracking dye reached the bottom. Then, renaturation was carried out with renaturation solution (2.5% Triton X-100) for 3 h on a mechanical shaker with mild agitation. Then, developing was carried out by incubating the gel in developing buffer (10 mM $CaCl_2$, 0.15 M NaCl and 50 mM Tris (pH 7.5)) for 18 h at 37°C and then stained with 0.2% Coomassie blue for 2 h, followed by destaining for 1 h with destaining solution and then further destaining was carried out with distilled water. Thereafter, calibration of the gelatin zymogram was carried out with human capillary blood gelatinases as per the procedure suggested by Makowski and Ramsby (1996). A drop of human capillary blood (15–20 µl) was obtained by fingerstick puncture and placed in a tarred polypropylene tube. The weight of the blood was determined in an analytical balance and 20 volumes of non-reducing Laemmli buffer was immediately added. The sample was then vortex mixed (30 sec) and aliquots were stored (–20°C), and the preparations were found to be stable for at least 3 months.

RESULTS AND DISCUSSION

In the present investigation, the protein content of excretory/secretory (E/S) antigens of larvae of *O. ovis* was 1.84 mg/ml. The presence of gelatinases activity in excretory/secretory (E/S) antigens of larvae of *O. ovis* was assessed by gelatin zymography as shown in Fig.1.

On gelatin zymographic analysis, presence of 220 kDa MMP-9 (pro form) and 210 kDa (active form) homodimeric forms was observed as a fainter band at the top of the gelatin zymogram. A clear, bright gelatinolytic activity was observed at 20 kDa level at the bottom of zymogram. The other forms of MMP-9, viz. 135 heterodimeric form was observed at 125 kDa size (active form) as a fainter band and the 72 kDa MMP-2 was detected as a fainter bands at 72 kDa (pro form) and 62 kDa (active form). The strong gelatinolytic activity detected at 20 kDa level of E/S antigens of *O. ovis* was the catalytic breakdown products of the MMPs. To our knowledge, this is the first report demonstrating gelatinolytic activity in *O. ovis* larval excretory/secretory products. Literature available on MMPs activity in excretory/secretory products of *O. ovis* larvae is very scanty.

Tabouret *et al*. (2001) reported a 28 kDa protein band in excretory/secretory products of *O. ovis* larvae by SDS-PAGE analysis and they assessed humoral immune response to this protein in sheep. Tabouret *et al*. (2003) demonstrated serine proteases activity in excretory/secretory products of

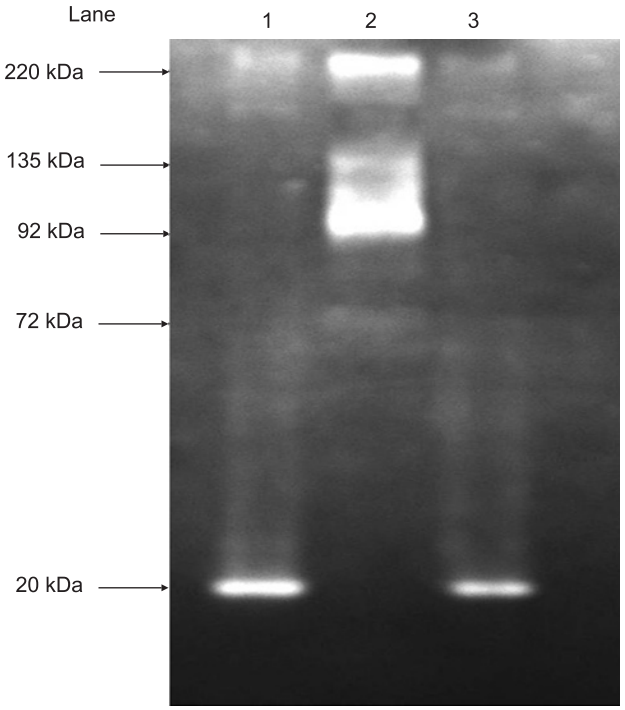

Fig. 1. Gelatin zymographic analysis of E/S antigens of *Oestrus ovis*. Lane 1&3, E/S antigens of *Oestrus ovis*; lane 2, Human capillary blood MMP-9 and MMP-2 as markers.

O. ovis larvae and reported serine proteases from 20 to 100 kDa molecular weights. Further, they observed that these proteases appear to originate mainly from the gut and are exported on nasal or sinusal mucosa.

Chambers *et al*. (2003) reported four proteolytic enzymes comprising two serine proteases, a metalloproteinase and an aspartyl proteinase with molecular weights ranging from 20 to 40 kDa, with activity across a wide pH range in excretory/secretory products of *Lucilia sericata* larvae. Musleh *et al*. (2015) analyzed the protein profile of excretory/secretory products of camel nasal bot fly, *Cephalopina titillator* by SDS-PAGE and observed a protein band at 32 kDa level. Further, they confirmed that the most active areas secreting E/S products are the salivary glands and anterior midgut regions.

Proteolytic enzymes are major components of the digestive process of parasites and are presumed to be released to interact with host tissues (Rhoads and Fetterer 1997). Parasites 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. A great number of trypsin and mainly chymotrypsin like proteases were found in sheep blow fly, *Lucilia cuprina*. These larval chymotrypsin like proteases of digestive origin are involved in wound formation and nutrition (Casu *et al*. 1996)

Excretory-secretory proteins are deposited onto the nasal and sinusal mucosa for partial substrate degradation and subsequently, pre-digested feed and larval enzymes are

ingested by the feeding larva for final digestion in the digestive system which occurs in other oestrid larvae (Angulo-Valadez *et al*. 2007). By *in-vitro*, Oestrid larvae cannot be fully reared because of many aspects related to larval nutrition and appropriate environmental conditions that still need to be investigated (Angulo-Valadez *et al*. 2010).

The pH optimum for proteases in excretory/secretory products from *O. ovis* was between 7.0 and 9.5 (Tabouret *et al*. 2003, Chambers *et al*. 2003). In the case of *O. ovis*, the frontal sinuses of sheep maintain a fairly constant temperature of 37°– 40°C. This would indicate that Oestrid species possess proteases that remain active above the normal host temperature, possibly relating to the functional physical-biochemical properties of these enzymes. On the other hand, proteolytic activity decreased at lower temperatures. Proteolytic enzymes found in *O. ovis* larval salivary gland products belong to the serine proteases subclass as do those in the excretory/secretory products of myiasis causing flies such as *Chrysomyia bezziana, Lucilia sericata, Lucilia cuprina* (Muharsini *et al*. 2000, Chambers *et al*. 2003). In the saliva of several haematophagous insects, enzymes responsible for hydrolyzation of IgGs have been identified. These enzymes preferentially cleave the hinge region of the heavy molecular chain, which is carried out during larval feeding and immuno-modulation of the host response (Nisbet and Huntley 2006). Likewise, proteases excreted and secreted by O. *ovis* can cleave IgG proteins (Tabouret *et al*. 2003). Based on this study, it was concluded that the activity of the MMPs in the E/S antigens of *O. ovis* was very strong, helping in the migration of the larvae from one site to the other.

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