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Study of vitellogenin in *Boophilus annulatus* tick larvae and its immunological aspects

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Abstract. *Boophilus annulatus* is an important one-host tick in the Mediterranean regions and Iran. It can transmit the *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* to cattle. Nowadays, immunization programs by tick proteins is one of the potential methods for the control and prevention of tick infestations. Therefore, the characterization and identification of various tick proteins are necessary. Vitellogenin is a precursor of vitellin that is produced in mid gut cells and fat bodies in ticks. In this study, we characterized vitellogenin protein of *B. annulatus* unfed larvae using one- and two-dimensional electrophoresis and immunoblotting. In one-dimensional immunoblotting, 48, 70, 100, 130 and >250 kDa protein bands positively reacted with immune sera. In two-dimensional immunoblotting many protein spots positively reacted with immune sera. Six of them were analyzed by MALDI-TOF and MALDI-TOF- TOF mass spectrometry. The results showed that amino acid sequences of four immunogenic proteins with molecular weights of 38, 43, 85 and 97 kDa had identity to tick vitellogenin and its homologues (GP80), based on the Mascot search results. It seems that more knowledge on tick proteins including vitellogenin and their characterization could be useful for the development of anti-tick vaccines.

INTRODUCTION

Boophilus annulatus is an important one-host tick in the Mediterranean regions and Iran. It can transmit the protozoans such as *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* to cattle (Estrada-Pena *et al.*, 2004). Vaccination by tick proteins is a new strategy for the control and prevention of tick infestations (Willadsen *et al.*, 1996). Therefore, more characterization and identification of tick proteins are necessary. Vitellogenin is a vitellin precursor produced by the tick mid gut cells and fat bodies. Vitellogenin is exported to hemolymph and then accumulates in oocysts as vitellin (Sappington & Raikhel, 1998). This phosphorilated heme-lipoglycoprotein is the main component of the tick eggs and the main source of amino acids and energy as well as heme for the development of tick embryos and unfed larvae (Logullo *et al.*, 2002). Furthermore, this protein acts as hormone carrier associated with embryogenesis (Bownes *et al.*, 1988). Reduced oviposition of *Boophilus micropolus* feeding on sheep vaccinated with vitellin was observed by Tellam *et al.* (2002). Similar results were obtained for *Ornithodoros moubata* fed on vitellin immunized rabbits (Chinzeie & Minoura, 1988). Therefore it could be a potential candidate for the development of anti tick infestation strategies.

In the current study, some unfed tick larva immunogenic proteins including vitellogenin have been identified.

MATERIAL AND METHODS

Tick collection, culture and infestation

Tick collections and culture were used based on an original protocol by Brown *et al.* (1984). Engorged female ticks were obtained from infested cattle in Mazandaran Province. Ticks were kept in glass tubes in incubator at 28°C and 85% relative humidity (RH) for oviposition. Two Holstein calves at three months of age were infested by using 10000 tick larvae four times at one month intervals.

Protein extraction

Ten-day hatched larvae were homogenized in phosphate buffer solution (pH = 7.2) and centrifuged at 3000 g for 5 min, and then the supernatant was separated. Protein concentration was measured by Bradford assay (Bradford, 1976).

One-dimensional gel electrophoresis

SDS-poly acrylamid gel (12%) electrophoresis on larval extracts was carried out as described by Lamelli (1970). Ten µg of larval extracts were dissolved in SDS-PAGE sample buffer (with or without mercaptoethanol) and mini gels were electrophoresed at a constant voltage (90V) for 2–3 h in 0.01 M Tris-glycine (pH=8.3) with the addition of 0.1% SDS to the electrophoresis buffer. Gels were stained with Coomassie blue and silver.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was carried out according to the method described by Yatsuda *et al.* (2003). Isoelecteric focusing (IEF) was done using immobilized pH gradient (IPG) 7-cm strips (non-linear pH range 3–10). Samples containing 300 µg proteins diluted in 120 µl rehydration buffer used to rehydrate strips for 18 h at room temperature. IEF was performed in a PROTEAN IEF Cell (Bio Rad) under conditions recommended by the manufacturer. Then, IPG strips were equilibrated for 20 min at room temperature in equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris pH = 8.8) containing 30 mM dithiotheritol for 20 min, followed by another 20-min incubation in equilibration buffer containing 135 mM iodoacetamide. Electrophoresis in second dimension SDS-gel (12%) was carried out in a BioRad electrophoresis system (USA). Coomassie brilliant blue R-250 was used to visualize proteins.

Western blotting

Western blotting was carried out according to Wang & Nuttall (1994) with some modifications. Larval tick protein extracts were separated in 12% SDS-PAGE gels and transferred to nitrocellulose membranes using a wet system (BioRad, USA) in transfer buffer. The membrane blocked 45 min by PBS containing 2.5% Tween 20 and all the washing steps were carried out with PBS containing 0.05% Tween 20 (3 x 5 min). Membrane was incubated in hyperimmune sera derived from cattle experimentally infested with *B. annulatus* larvae (1/50 in PBS containing 0.05% Tween 20) one hour at room temperature; then, washed and incubated with anti-bovine IgG coupled to horse radish peroxidase (1/1000 in PBS) for 30 min at room temperature. After washing step, the colour development step was performed with diamino benzidine containing H_2O_2.

Mass spectrometry

Some immunogenic proteins selected for mass spectrometry analysis. These proteins were separated from parallel 2-D gels, stained with Coomassie blue and were subjected to Sinaclon Co. for MALDI-TOF and MALDI TOF TOF mass spectrometry analysis.

Mascot software was used to search spectra data against the NCBI database. Variable peptide modifications were considered including methionine oxidation and cysteine carbamidomethylation.

RESULTS

One-dimensional SDS-PAGE

In one-dimensional SDS-PAGE under reducing condition, six bands with molecular weights of 10, 28, 33, 63, 72 and 95 kDa were detected (Fig. 1A). Under non-reducing

condition, ten bands were detected including 26, 27, 33, 58, 72, 80, 85, 100, 150 and > 200 kDa bands (Fig. 1B).

One-dimensional western blotting

In Fig. 2, 1-D Western blotting, five protein bands including 48, 70, 100, 130 and >250 kDa bands positively reacted with bovine immune sera.

Two-dimensional electrophoresis

After Coomasie staining, numerous spots were detected (Fig. 3). Six immunogenic protein spots were submitted for mass spectrometry (Fig. 4). Four of them matched with vitellogenin and its homologues (GP80). Two other spots with molecular weight of 36 and 48 kDa were not identifiable, suggesting that they may be novel molecules.

Boophilus annulatus larval extract was reacted with sera of cattle infested with tick experimentally six times. Immunogenic proteins between 14 and 97 kDa were detected.

Mass spectrometry

Protein analysis by MALDI- TOF- TOF mass spectrometry analysis showed that amino acid sequences of four immunogenic proteins with molecular weights of 38, 43, 85 and 97 kDa had identity to tick vitellogenin and its homologues (GP80) (Table 1).

DISCUSSION

Ticks are important because of their ability to injure their host skin directly and transmit a variety of infectious agents (Sonenshine, 1993). Tick control is based mainly on the use of acaricides, but the use of these chemicals cause some problems such as selection of resistance in ticks and presence of residuals in meat and milk (Willadsen *et al.* 1996). Novel control method such as vaccination was introduced recently. Vaccine development also depends on the characterization and identification of tick proteome, of which some can induce appropriate immune responses in hosts (Untalan *et al.*, 2005). In the current study, vitellogenin and its homologues were characterized. These immunogenic proteins are most abundant in unfed *B. annulatus* larval extract.

Vitellogenin (vitellin) has been studied in *B. micropolus*, *Dermacentor andersoni*, *O. moubata*, *Rhipicephalus appendiculatus*, *Argas hermanni*, *Hyalomma dromedary* and *Dermacentor variabilis* (Tatchell, 1971; Boctor & Kamel, 1976; Chinzei *et al.*, 1983; Dhadialla, 1986; Shanbaky *et al.*, 1990; Rosell & Coons, 1991; Schriefer, 1991), but there is no information on vitellin or vitellogenin in *B.annulatus*. In this study, we present some information on larva *B. annulatus*

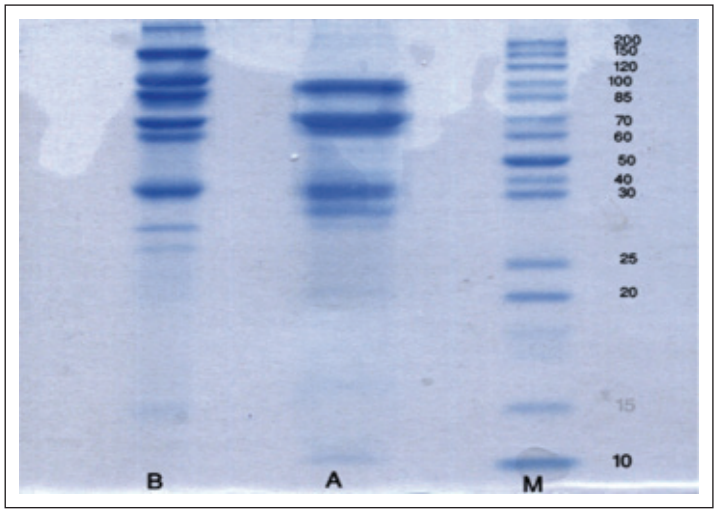

Figure 1. *Boophilus annulatus* unfed larval extract 1-D SDS-PAGE: A (reducing), B (non-reducing), M (marker)

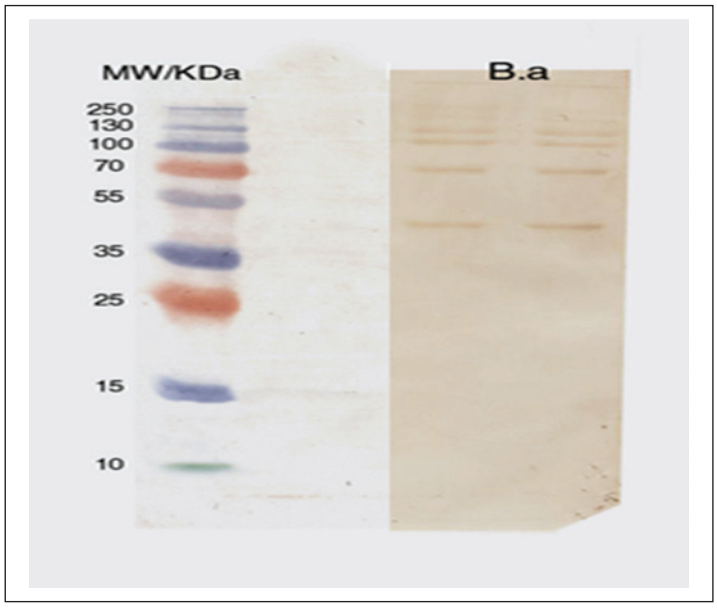

Figure 2. *Boophilus annulatus* unfed larval extract 1-D Western blotting

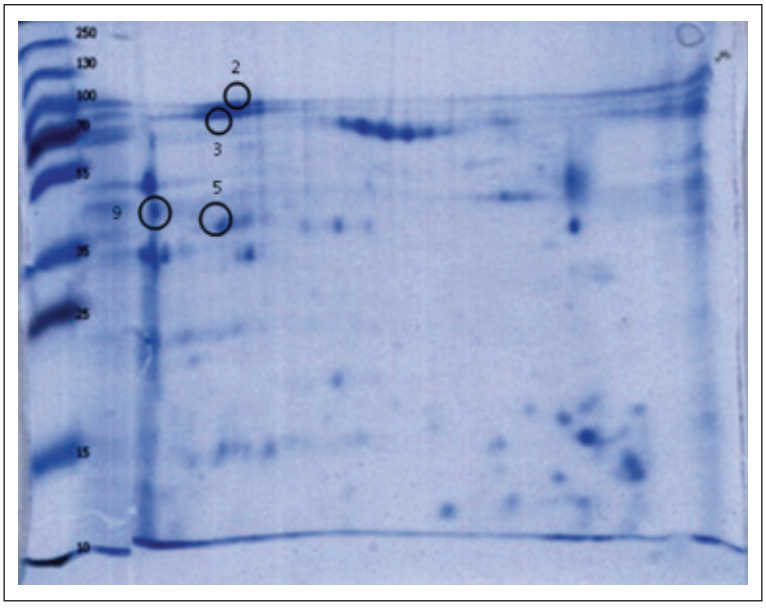

Figure 3. Two-dimensional electrophoresis of *B. annulatus* unfed larval extract

Figure 4. *Boophilus annulatus* unfed larval extract 2-D Western blotting

Table 1. *B. annulatus* unfed larval protein extracts using Mascot search

Spots No.	Peptide sequences[a]	Score[b]	Coverage[c]	Mr (KDa)[d]	PI[e] theoretical	Accession No[f]	Protein ID[f]
5	TPVYEELFEK, AVYGHFVYGTSFDK	146	1	213.8/38	7.92	gi.194319795	Vitellogenin-2 precursor [*Dermacentor variabilis*]
1	YLVPNLVLSGLDR, LIVTVYPTSGPHDPPVVK, YPTHHEYPTRHEYPTHHEYPTR	324	8	70.3/97	7.14	gi.154940364	Vitellogenin [*Rhipicephalus microplus*]
4	ASTTGPLTTHLVR, VFVPAELTEESPSVVHTSVK	92	1	209/43	7.01	gi.82878755	Vitellogenin [*Dermacentor variabilis*]
4	VFVPADLTEESPSVLHTSVK, LVNVEVESASHGPLLPLPLYGKLYQQPSER, YEHNLDFLAK, VYTPTKPLAYDESQETPYER	445	6	147.7/43	8.88	gi.1224123	GP80 precursor [*Rhipicephalus microplus*]
2	TDVNPYVTVAHGELLLPGEK, YLVPMLVLSGLVR, HDFAVVAQPLDLTVGTK, LLVTVYPTSGPHDPPVVK	436	11	70.3/85	7.14	gi.154940364	Vitellogenin [*Rhipicephalus microplus*]
2	TDVNPYVTVAHGELLLPGEK, HDFAVVAQPLDLTVGTK, LLVTVYPTSGPHDPPVVK, EPLVSTLPVHYLEELK	429	5	147.7/85	8.88	gi.1224123	GP80 precursor [*Rhipicephalus microplus*]

[a]Peptide sequence for matched protein from MALDI- TOF- TOF mass spectrometry as determined by Mascot search
[b]Protein score is derived from ion scores (sum of ion scores). Ions score is -10 log(P), where P is the possibility that observed match is a random event
[c]Percentage of the protein sequence covered by the matching peptides
[d]Theoritical molecular mass predicted from amino acid sequences in kDa, observed molecular mass estimated from position of protein spot on gel
[e]pH isoelecteric predicted from amino acid sequences
[f]Protein in NCBI database to which peptide sequence were matched with Mascot

vitellogenin and its imunogenesis. We obtained six and ten protein bands in 1-D SDS-PAGE under reducing and non-reducing conditions, respectively. The main cause of the absence of some protein bands in reducing condition can be related to use of mercaptoethanol, in comparison of non-reducing condition. Mercaptoethanol can reduce disulfide bands; therefore, both tertiary and the quaternary structures of some proteins can be disrupted. Because of its ability to disrupt the structure of proteins, mercaptoethanol was used in the analysis of tick larval proteins which contain monomeric and disulfide linked dimmers or higher order oligomers such as vitellin and vitellogenin.

As mentioned previously, five protein bands with molecular weights of 48, 70, 100, 130 and > 250 kDa were detected using 1-D Western blotting by hyperimmune sera from experimentally infested calves by ticks. In 2-D electrophoresis, numerous protein spots were separated. According to parallel 2-D Western blotting results and protein analysis by mass spectrometry, four immunogenic proteins with molecular weights of 38, 43, 85 and 97 kDa were identical to ticks vitellogenin and its homologues (GP80) based on Mascot search results (Table 1). Our data is similar to data reported by Tellam *et al.* (2002). They purified vitellin as the most abundant protein in *B. microplus* eggs and showed that vitellin consisted of six main polypeptides with molecular weights of 107, 102, 87, 67, 65 and 44 kDa using non-reducing SDS-PAGE. Furthermore, they obtained three lighter bands with 33, 35 and 18 kDa molecular weight. In another study, purified vitellin from *B. microplus* eggs was analyzed by native polyacrylamid gel electrophoresis and SDS-PAGE. In native PAGE, a single band with 490 kDa was observed. After submitting to SDS-PAGE, this fraction was split into nine different bands, with molecular weights between 18 and 205 (Logullo *et al.*, 2002). Purification and partial characterization of vitellin from *Ixodes scapularis* was done by James & Oliver (1997). They reported seven polypeptides between 35 to 154 kDa using SDS-PAGE under reducing condition.

Vitellogenin is a large precursor polypeptide which is synthetized in fat bodies as two similar primary products, P1 and P2 (215 and 205 kDa, respectively). This protein is processed during passage into hemolymph to smaller polypeptides (160, 140, 125 and 100 kDa). Excreted vitellogenin exists in both monomer (300 kDa) and dimer (600 kDa) forms and is composed of a mixture of primary peptides and their fragments. Circulating vitellogenin is taken up by the developing oocysts upon receptor mediated endocytosis. Endocytozed vitellogenin is converted to vitellin by partial proteolysis and some other modifications. In this stage, all traces of the primary polypeptides disappear and further processing will result in lower fragments (60 and 50 kDa) (Krischer *et al.*, 1970).

An immunological cross-reactivity between GP80 and vitellin exists and the common amino terminal sequence for GP80 and vitellin indicates that GP80 is a processed product from vitellogenin and is a component of vitellin. Vaccination of sheep with purified vitellin or GP80 induced immune responses that were effective against tick infestation (Tellam *et al.*, 2002). Ingested host antibodies by the tick may react with vitellogenin produced by mid gut epithelial cells and may lead to damage to the tick (Coons *et al.*, 1989).

CONCLUSION

This information suggests that anti-vitellogenin antibodies are induced in cattle after experimental exposure to tick and may play a role in protecting against tick infestation; therefore, it could be used as vaccine candidate, alone or in combination with other protective antigens. Further knowledge on tick proteins and their characterization can be useful for the development of anti tick vaccines.

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