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Changing patterns of vitellin-related peptides during development of the cattle tick *Boophilus microplus*

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ABSTRACT

The major components of protein extracts from the cattle tick *Boophilus microplus* eggs and larvae of various ages were characterized by molecular sieving chromatography, ion exchange chromatography and SDS-PAGE. The fractions analysed showed a changing chromatographic pattern development. A serum raised against the components of a fraction showing characteristics of vitellin strongly reacted in Western blots with the major peptides of extracts from eggs, larvae, gut and ovary. Comparison of patterns obtained by electrophoresis in non-denaturing PAGE, stained with Coomassie blue or with benzidine/hydrogen peroxide, revealed that the major proteins of these extracts are haemoproteins, possibly in different aggregation states or heterogeneous in composition.

Key words: *Boophilus microplus*, cattle tick, vitellin, haemoproteins, development.

INTRODUCTION

The ixodid tick, *Boophilus microplus* (Canestrini, 1887), is a blood-sucking, *Babesia* spp.-and *Anaplasma* spp.-transmitting ectoparasite of cattle, which causes significant economic losses in tropical and subtropical areas. In attempts to protect cattle against this arthropod, protein extracts have been used as experimental vaccines which resulted in inhibition of tick development (Johnston *et al.*, 1986; Opdebeeck *et al.*, 1989). Immunogenic and protection-related antigens were identified on the gut epithelium (Willadsen *et al.*, 1989) and in salivary glands (Brown *et al.*, 1984). Identification of the antigens involved in protective immune responses is an essential step in the development of a molecular vaccine. Such a

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vaccine can be produced by the use of recombinant DNA technology (Rand *et al.*, 1989; Turnbull *et al.*, 1990).

For the identification of immunogenic, tissue-specific antigens, one has to take into account the fact that some organs and tissue extracts contain significant amounts of vitellin-derived peptides. Vitellogenins are storage glycolipohaemoproteins which are synthesized in the fat body cells (Chinzei and Yano, 1985b) and the gut (Araman, 1979), released into the haemolymph and transported to the ovary where they are selectively taken up and stored by the oocyte (Diehl *et al.*, 1982). These proteins are the origin of several metabolites throughout embryogenesis and can still be detected in larvae 4 months after hatching (Chinzei and Yano, 1985a). In this paper the complex pattern of vitellin-related peptides present in the gut, ovary, egg, and larval protein extracts is described.

MATERIAL AND METHODS

Ticks

Boophilus microplus of the Porto Alegre strain, free of *Babesia* spp., were reared on calves obtained from an area free of ticks. After engorgement, adult females were kept in Petri dishes at 28°C and 80% relative humidity (RH) until completion of oviposition. Eggs were then collected and incubated in test-tubes under the same conditions.

Preparation of tick homogenates

Tick homogenates for chromatography were prepared either from eggs on different days after oviposition or from non-feeding larvae on different days after hatching. They were cooled on ice, triturated with a mortar and pestle and homogenized in 0.1 M Tris–HCl buffer, pH 8.1, in a glass–Teflon homogenizer. The suspension was then filtered through paper and centrifuged at 27000 *g* for 20 min at 4°C. The fat layer and pellet were removed and the supernate was either analysed immediately or frozen at −20°C. Protein assays were performed by the Lowry's method using, as a standard, a protein mixture extracted from tick eggs. Nitrogen content was measured by the Kjeldahl method.

Homogenates for PAGE, SDS–PAGE and Western blots

Homogenates for PAGE, SDS–PAGE, Western blots and animal inoculations were prepared from eggs, larvae, gut, ovary and fraction from agarose 1.5 chromatography. Five day old eggs or larvae were frozen in liquid nitrogen, triturated with mortar and pestle and sonicated in phosphate-buffered saline (PBS), in ice, at 50 W per 4 ml for ten 1 min

intervals with 1 min resting periods between each. The ovaries and guts were obtained by the dissection of engorged females, washed several times in PBS and sonicated in the same manner. All the extracts were centrifuged at 10000 *g* to remove debris and the protein contents of the supernates were measured.

Molecular sieving and ion exchange chromatography

The supernates described above (50 mg of protein per experiment) were chromatographed in a Bio-Gel A-1.5 agarose (200–400 mesh, Bio-Rad, Richmond, CA) column (90 × 2.9 cm, flow rate 5 ml h^{-1}) at 4°C using a 0.1 M Tris–HCl, pH 8.1, buffer. the eluate was monitored photometrically at 280 and 410 nm and fractions containing haemoproteins were pooled and dialysed against 0.01 M Tris–HCl, pH 8.1. Further chromatography on a DEAE-cellulose column (20 × 2.2 cm) was performed with the same buffer. After elution of the unbound material, a linear salt gradient (1000 ml, from 0 to 0.5 M NaCl in the same buffer) was applied at a flow rate of 25 ml h^{-1}. Fractions of 6 ml were collected and monitored for absorbance at 280 nm and 410 nm.

SDS-PAGE

SDS-PAGE was performed on 10% polyacrylamide gels with 0.1% SDS. Samples were dissolved in ten times the volume of 0.1 M Tris–HCl buffer, pH 8.8, 2% SDS, 5% β-mercaptoethanol and 10% glycerol containing bromophenol blue and heated for 5 min in boiling water. Two gels were run simultaneously, one was stained with Coomassie blue, while the other was Western blotted. Samples from the DEAE-cellulose columns were treated with 1% SDS, 5% β-mercaptoethanol and 8 M urea for 24 h at 48°C. The reduced and dissociated samples were run on 14% SDS–PAGE and stained with Coomassie blue.

Haem detection on PAGE of undenatured protein extract

PAGE of undenatured material was performed on slabs according to the method of Laemmli (1970) in 7% polyacrylamide gels. Samples (30 μg of protein) were dissolved in ten times the volume of 0.1 M Tris–HCl buffer, pH 8.8, as well as 10% glycerol containing bromophenol blue and applied to the gel. Electrophoresis was carried out at 60 V for 6 h, at 4°C, in a 0.01 M Tris-glycine buffer, pH 8.3. After electrophoresis, gels were rinsed in distilled water and stained for peroxidase activity utilizing the procedure described by Thomas *et al.* (1976), using a 15 mM benzidine solution in methanol. Three parts of the benzidine solution were mixed with seven parts of 0.25 M sodium acetate, pH 5.0. Gels were then incubated in this

solution for 2 h with constant agitation in the dark. Then, hydrogen peroxide was added to a final concentration of 30 mM, with a few crystals of potassium ferrocyanide. The gels were developed for 1 h. To clear the background and enhance staining intensity, they were immersed in a solution of isopropanol: 0.25 M sodium acetate (3 : 7). A replica of this gel was stained for protein with Coomassie brilliant blue R-250.

Serum against tick vitellin

One hundred microgrammes of tick vitellin preparation derived from eggs 5 days after oviposition (E5, fraction II from the agarose 1.5 chromatography; see the Results) were emulsified with 0.5 ml complete Freund's adjuvant and then injected subcutaneously into various sites on the back of a White New Zeland rabbit. Three additional weekly injections were given with incomplete Freund's adjuvant. One week after the final injection, the rabbit was bled and the serum was separated and stored at −20°C. The titre was measured by enzyme linked immunosorbant assay (ELISA) (Johnstone and Thorpe, 1982; Harlow and Lane, 1988).

Western blotting

Western blotting was performed according to Towbin *et al.* (1979) and modified by Burnette (1981). After SDS–PAGE, the gel was electrotransferred on to a nitrocellulose membrane using a carbonate buffer, pH 9.9 (Dunn, 1986) at 70 V for 1 h. Immune detection of proteins was accomplished after treatment of the membrane with 5% non-fat dry milk and 0.5% Tween 20 in PBS for 1 h under constant agitation. The membrane was then successively incubated with the appropriate serum and with goat anti-rabbit IgG conjugated to peroxidase (Sigma, St Louis, MI). Colour was developed with diaminobenzidine (DAB), hydrogen peroxide and cobalt chloride.

RESULTS

Chromatographic pattern of major proteins from egg and larva extracts of B. microplus

Crude homogenates of both eggs and larvae yielded various fractions with molecular sieving chromatography on an agarose 1.5 column (Fig. 1). Fractions I and III, respectively represented elution volumes of major components found at the late larval stage (L21, Fig. 1). Fraction II, of elution volume between fractions I and III, corresponded to major components of egg extracts at the early stage (Ovi, Fig. 1). These components contained haem groups, as indicated by their absorption at 410 nm (Fig. 1,

hatched lines). Fraction IV showed both haem group and catalase activity at the late larval stage (L21, Fig. 1) and was not characterized further.

The relative protein concentration in fractions I–III, as estimated from the area below the peaks in the chromatograms shown in Fig. 1, varied with the developmental stage of the tick. Larval hatching was relative to the decrease in the proportion of fraction II and the increase in fractions I and III. Furthermore, the ratio A_{410} nm/A_{280} nm in fraction III varied during larval development, indicating changes in its composition.

Further chromatographic analysis on a DEAE-cellulose column for each of the three haem-containing fractions (I–III) was performed. Both fractions I (from L21, Fig. 1) and II (from Ovi, Fig. 1) each contained a single component in the chromatography (data not shown). Fraction III

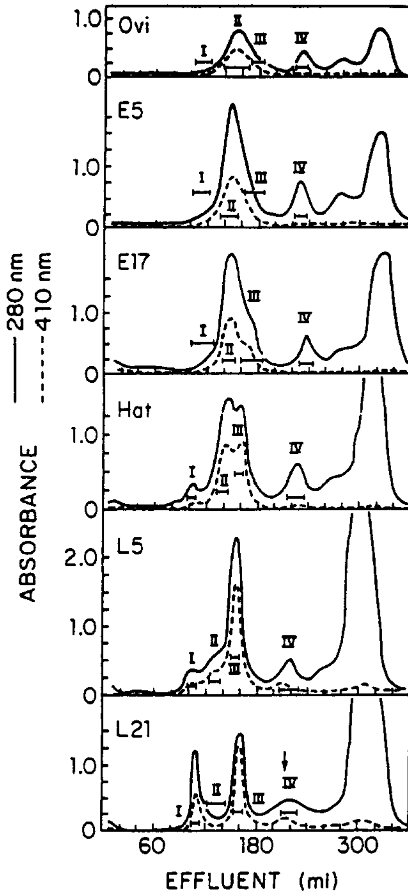

Fig. 1. Agarose 1.5 chromatography of egg and larval extracts from different developmental stages. Egg extracts at oviposition (Ovi), 5 days (E5), 17 days (E17), hatching (Hat): larval extracts at 5 days (L5) and at 21 days (L21) of age.

(from L21, Fig 1), however, separated into two components, III_a and III_b (Fig. 2). The isolated components contained haemoproteins based on their absorption at 410 nm, each showing a specific A_{410} nm/A_{280} nm ratio as estimated by their respective peak areas in Figs 1 and 2.

Composition of isolated haemprotein fractions

Reduction and dissociation of the isolated haemoproteins followed by SDS-PAGE (Fig. 3) showed the components of fractions I (L21, Fig. 1) and II (Ovi, Fig. 1) to be essentially the same, although there were differences in the relative intensities of some bands. Fraction III_a, clearly differed from fractions I and II, particulary in the protein bands of heavier molecular weight. Fraction III_b primarily displayed two components, which differ from those of haemoproteins I, II and III_a.

Cross-reactivity between components of isolated fractions in Western blots

In order to see the homology between the peptides in the isolated fractions, a Western blot with protein extracts from eggs, larvae, the gut, egg fraction II and larvae fraction I was screened with a serum raised against proteins present in fraction II (Fig. 4). Most major peptides were recognized in all extracts by the serum (Fig. 4B) when compared with the peptides visible in the gel stained with Coomassie blue (Fig. 4A). No peptides were recognized with the pre-immune serum (result not shown).

Haem association with major proteins

Figure 5 shows two non-denaturing PAGEs of tick extracts and fractions of the agarose 1.5 chromatography. In Fig. 5 A, the gel was stained with Coomassie blue while B was stained with benzidine/hydrogen peroxide for detection of haemoproteins. A common pattern can be noticed in both gels, with the major proteins detected by Coomassie blue reacting to benzidine/hydrogen peroxide.

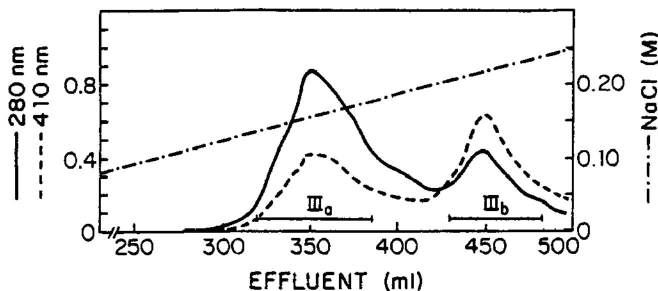

Fig. 2. Ion exchange chromatography on DEAE-cellulose of larval fraction III obtained from the agarose 1.5 column (L21, Fig. 1).

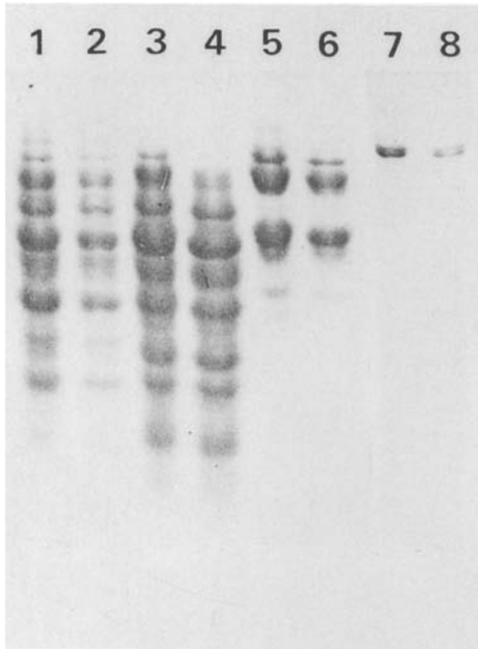

Fig. 3. SDS-PAGE 14% of proteins from fractions of chromatographic columns (1) and (2) fraction I and (3) and (4) fraction II from agarose 1.5 chromatography (L21 and Ovi, Fig. 1, respectively), (5) and (6) fraction III_a and (7) and (8) fraction III_b from DEAE-Cellulose column.

DISCUSSION

Sera produced against tick extracts at various stages recognized in Western blots, peptides in both the homologous and the heterologous extracts. Similar findings in different but related ticks (Chinzei *et al.*, 1983; Rosell and Coons, 1991) led to the hypothesis that these common peptides could be derived from vitellins. Indeed, proteins of fraction II from agarose 1.5 chromatography of tick extracts at various developmental stages, displayed typical vitellin behaviour in the sense that they were more prevalent in the early embyro stage and decreased in later stages (Fig. 1). Furthermore, a serum against fraction II (from eggs) reacted in Western blots (Fig. 4B) with the major detectable peptides in the Coomassie blue-stained protein gel (Fig. 4A). The presence of a haem group in the tick proteins, as observed in a native PAGE revealed with benzidine/hydrogen peroxide (Fig. 5B), reinforces the assumption that the major peptides in the protein extracts are vitellin related.

In addition to the vitellin present in fraction II, haemoproteins were

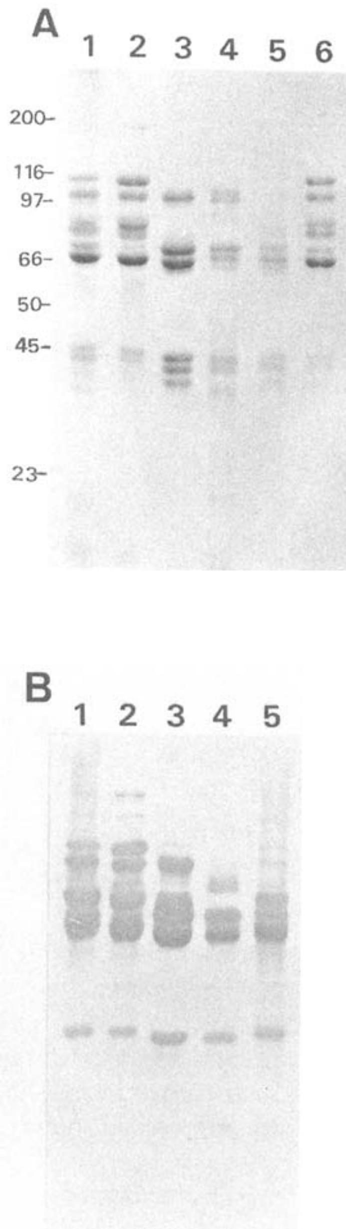

Fig. 4. Western blot with serum against fraction II. Extracts from (1) gut, (2) ovary, (3) eggs, (4) larvae and (5) fraction I from larvae and (b) fraction II from egg protein extracts obtained from the agarose 1.5 chromatography (L21 and E5, Fig. 1, respectively). (A) The gel was stained with Coomassie blue. (B) Western blot of a duplicate of gel A, using serum against fraction II.

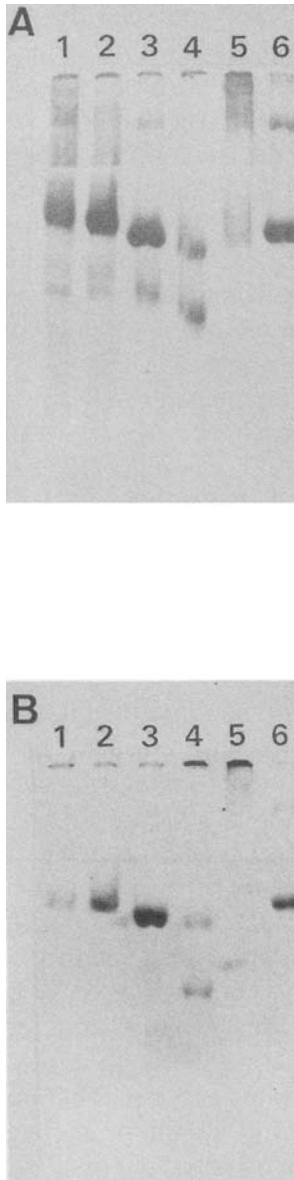

Fig. 5. Non-denaturing polyacrylamide gel electrophoresis for detection of haem in *B. microplus* proteins. PAGE 7% with protein extracts form (1) gut, (2) ovary, (3) eggs, (4) larvae and (5) fraction I from larvae and (b) fraction II from egg extracts obtained from the agarose 1.5 chromatography (L21 and E5, Fig. 1, respectively). (A) The gel was stained with Coomassie blue. (B) The gel was stained with benzidine/hydrogen peroxide.

found in three other tick fractions at different developmental stages. Fractions I and III_a (Figs 1 and 2) contain molecules with common peptides (Fig. 3), but with distinct chromatographic properties. Fraction III_b represents a haemoprotein distinct from I, II and III_a, as it contains different peptides (Fig. 3, lanes 7 and 8). Indeed, radioactive labels attached to fraction II of the egg at the time of vitellogenesis were traced only to larval fractions I and III_a (Del Pino *et al.*, 1989). We suggest that the three fractions represent metabolic steps of the major haemovitellin present in the egg of the cattle tick *B. microplus*. Similar haemoprotein interconversion was observed with *Hyaloma dromedarii* during its embryogenesis (Kamel *et al.*, 1982).

The presence of vitellin in different organs of *Rhipicephalus sanguineus* (Coons *et al.*, 1989) and *Dermacentor variabilis* (Rosell and Coons, 1990) as well as during distinct developmental stages of *D. variabilis* (Rosell-Davis and Coons, 1989) was noticed. Chinzei (1983) showed that 85% of the egg protein of *Ornithodoros moubata* is vitellin. Rosell and Coons (1992) reported that the vitellin is synthesized in the gut of ixodid ticks. Chinzei and Yano (1985a) revealed that nymphs of *O. moubata* survive for over 4 months without a blood meal, using its vitellin content. Our results suggests that the structural and temporal organization of vitellin in *B. microplus* is also similar. Another characteristic of vitellin, observed in the present study, is that the haemtophagous ticks studied until now have had a non-covalently attached haem group. Spectrophotometric analysis suggests that the haem is probably derived from the host (results not shown).

The haem group (protoporphyrin IX) contains a form of iron capable of performing reactions that led to the formation of oxygen radicals which causes lipid, DNA and protein harm (Tappel, 1955; Aft and Muller, 1983; Aft and Mueller, 1984). By attaching the haem group to proteins in the extracellular fluids an organism can arrest these deleterious effects (Muller-Eberhard and Morgan, 1975). A mechanism for this kind or protection was proposed in *Rhodnius prolixus* (Dansa-Petretski *et al.*, 1985). The arrest of haem reactions by a haem-binding protein could be the haem-detoxification mechanism present in haematophagous arthropods. The fact that vitellin generation and haemoglobin degradation take place at the same time while 74% of the protein intake is converted to vitellin, which comprises 80% of a recently laid egg protein, corroborates the hypothesis (Chinzei, 1983). However, the true biological significance of haem in tick vitellins is still to be determined.

Immunization of animals with vitellin of *O. moubata* or *Rhipicephalus appendiculatus* leads to some degree of host protection (Dhadialla *et al.*, 1985; Chinzei and Minoura, 1988). It was suggested that antibodies against vitellin cross the gut epithelium obstructing vitellin synthesis, transport

through haemolymph and ovary storage. The results indicate the possibility of intervening in the reproduction of feeding ticks by exploiting vitellin as an antigen in vaccines. Studies are under way in our laboratory to address this potential in *B. microplus*.

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