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ORIGINAL PAPER

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Further studies on the structural analysis of the cuticle of *Litomosoides chagasfilhoi* (Nematoda: Filarioidea)

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Abstract In order to obtain further information on the structural organization of the cuticle of nematodes, this structure was isolated from adult forms of the filariid *Litomosoides chagasfilhoi*. The purity of the fraction was determined by light and transmission electron microscopy, deep-etching, high resolution scanning electron microscopy, atomic force microscopy, immunocytochemistry, gel electrophoresis (SDS-PAGE) and Western blot. The epicuticle presented a rugous surface with parallel rows and several globular particles that could be involved in the absorption of nutrients and secretion of products. Analysis by SDS-PAGE of purified cuticles revealed five major polypeptides corresponding to 151, 41, 28, 13 and 11 kDa. A polyclonal antibody against a synthetic 18 amino-acid peptide that corresponds to the sequence of domain E of the *Haemonchus contortus* 3A3 collagen gene recognized several protein bands on the Western blot of purified cuticle, and labeled all cuticular layers, as shown by immunocytochemistry.

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Introduction

The cuticle of filarial nematodes is the portion of the organism which establishes direct contact with the immune system of the host and is also physiologically important as a site of nutrient acquisition. In addition, it is an elastic extracellular matrix synthesized and secreted by the underlying syncytial hypodermis (Selkirk and Blaxter 1990). Although great diversity in morphology is exhibited by cuticles from different species or between stages of the same organism, the basic biochemical composition appears to be well conserved (Wright 1987; Selkirk et al. 1989; Bird and Bird, 1991; Moraes Neto et al. 2001, 2002).

The major structural components of the cuticle are collagens which appear as monomers, dimers or trimers, via non-reducible bonds (Betschart and Wyss 1990; Cox et al. 1990; Politz and Philipp 1992). They are a complex and unique set of molecules, smaller than vertebrate collagen (Kramer 1994; Johnstone 1994) and differ in structure, containing shorter lengths of the proline-rich trimeric helical domains characteristic of the vertebrate collagens (Cox 1992).

The application of extrinsic iodination techniques has resulted in numerous surface antigens from nematode parasites (Selkirk et al. 1990; Selkirk 1991) including the 15 kDa protein and Gp 29 in *Brugia* spp., which do not appear to contribute to the structural integrity of the cuticle, and the 55 kDa protein in *Litomosoides carinii*, which corresponds to a collagenous protein (Philipp et al. 1984).

Previous studies using a rabbit antisera against an 18 amino acid-long peptide, which corresponds to the predicted sequence of the carboxy-terminal non-triple helical region of the *Haemonchus contortus* 3A3 collagen, have reported the presence of this specific collagen,

in various nematodes (Cox 1990; Cox et al. 1990; Peixoto et al. 1995).

Transmission electron microscopy (TEM), freeze-fracture and deep-etching techniques have been used to study the epicuticle and the cuticle of various nematodes (De Souza et al. 1993; Araújo et al. 1994; Peixoto and De Souza 1994, 1995; Martinez and De Souza 1995, 1997) but the results are contradictory.

Atomic force microscopy (AFM) has been applied with success for the investigation of certain biological surfaces such as chitin microfibrils in the cuticle of the shrimp *Xiphopenaeus kroyeri* (Andrade et al. 2002) and for studies of soft biomaterials, particularly polysaccharides such as scleroglucan strands (Vuppu et al. 1997), individual biopolymers and their supramolecular assemblies (McIntire and Brant 1997).

In order to obtain more information on the organization of the cuticle of nematodes, we decided to isolate this structure and subsequently to analyze (1) its structure, using light microscopy and TEM, quick-freezing followed by freeze-fracture, deep-etching and rotatory replication, high resolution scanning electron microscopy (SEM) and AFM; (2) the protein composition, by SDS-PAGE gel electrophoresis; and (3) the localization of collagen, using an immunocytochemical approach.

Materials and methods

Nematodes

Adult *L. chagasfilhoi* were collected as described by Moraes Neto et al. 1997. The filariids were washed three times in a sterile PBS solution (150 mM NaCl in 10 mM phosphate buffer), pH 7.2 and stored at −70°C until used.

Isolation of the cuticle

The cuticle from adult *L. chagasfilhoi* was isolated essentially as described by Cox et al. (1981) for *Caenorhabditis elegans*. Three adult females were washed three times in sterile PBS solution, pH 7.2 at 4°C, suspended in 5 ml of sonication buffer 10 mM Tris-HCl, pH 7.4, containing 1 mM ethylene diamine tetra acetic acid and 1 mM phenylmethyl sulphonyl fluoride), cut with a razor blade into pieces of about 0.5 mm length and disrupted by sonication on ice with three cycles of 10 s at 50% of the maximum amplitude of an ultrasonic apparatus (Sigma, model GEX 600) using the standard probe (13 mm radiating diameter). For biochemical purposes, cuticle pieces were recovered in the same solution at 4°C and washed five times with 10 ml of sonication buffer. The cuticle was then transferred to a 1.5 ml Eppendorf tube, suspended in 1 ml of ST buffer (1% SDS, 0.125 M Tris-HCl, pH 6.8) and heated for 2 min at 100°C. After several hours of incubation at room temperature, the cuticle was spun down in a Sorval RMC14 refrigerated microcentrifuge (2 min, 1,000 g, 28°C) and extracted again with ST buffer, as described above. The disulfide cross-linked proteins of the cuticle were solubilized by heating purified cuticles for 2 min at 100°C in 0.5 ml ST buffer with 5% ö-mercaptoethanol and agitating gently for several hours at room temperature. The insoluble cuticle material was extracted again with ST buffer, as described above, washed several times with distilled water and resuspended in sample buffer (Laemmli 1970).

Light microscopy

The purified cuticle was washed three times in PBS solution, pH 7.2, at room temperature and fixed in AFA (solution containing glacial acetic acid, 37% formalin, and 70% ethanol) at 70°C.

Transmission electron microscopy

The purified cuticle was washed three times in PBS solution, pH 7.2, at room temperature and fixed for 2 h at room temperature or overnight at 4°C in 2.5% glutaraldehyde, with 4% freshly prepared paraformaldehyde and 5 mM calcium chloride in 0.1 M cacodylate buffer, pH 7.2. The fixed purified cuticle was washed twice in 0.1 M cacodylate buffer, pH 7.2, post-fixed in a solution containing 1% osmium tetroxide, 5 mM calcium chloride and 0.8% potassium ferrocyanide in 0.1 M cacodylate buffer, pH 7.2. It was then dehydrated in an acetone series and embedded in Spurr's resin. Thin sections were collected on copper grids, counterstained with uranyl acetate and lead citrate and observed in a Zeiss 900 transmission electron microscope.

High resolution SEM

Purified cuticle was fixed and post-fixed as described for TEM purposes. It was dehydrated in an ethanol series, processed in a critical-point drier with CO_2, and sputter-coated with platinum in a Balzer's apparatus, and examined in a Jeol JSM 6340F field emission scanning electron microscope operating at 10 kV.

Deep-etching

Purified cuticle was washed three times in PBS solution, pH 7.2, at room temperature and fixed for 2 h at room temperature or overnight at 4°C in 2.5% glutaraldehyde, with 4% freshly prepared paraformaldehyde and 5 mM calcium chloride in 0.1 M cacodylate buffer, pH 7.2 before being rinsed three times in distilled water. It was placed on a specimen support disk that was designed for a Balzer's freeze-etch apparatus. Specimen disks were fixed to the plunger of a Med-Vac rapid freezing device assembled for impact freezing against a copper block, that was cooled by liquid nitrogen to −196°C. Rapidly frozen purified cuticle was stored in liquid nitrogen before freeze fracturing in a Balzer's apparatus. After fracturing at −115°C, the temperature was raised to −100°C for 20 min. for etching. Platinum was evaporated onto the sample at an angle of 25° and carbon at an angle of 90° as the sample was rotated. The replicas obtained were cleaned with sodium hypochloride and distilled water, mounted on 300 mesh nickel grids and observed by TEM. Micrographs were examined in negative contrast by photographically reversing them before printing, making platinum deposits look white and the background look dark. Heuser et al. (1979) have shown that this contrast-reversal enhances the three-dimensional appearance of the images.

Atomic force microscopy

A topometrix TMX 2010 Discoverer (Topometrix, Santa Clara, USA) instrument, equipped with a non-contact AFM probe head and a 70 μm Tripot scanner, was used to obtain scanning probe microscope images. The tips (Topometrix 1660) were made of silicon, with a spring constant of about 40 N/m and resonance frequencies in the 100–150 kHz range. Scanning was carried out at the free cantilever oscillation frequency and different amplitudes, depending on the stability and contrast obtained. The set point was fixed at 45–55% of the free oscillation amplitude. Purified cuticle was fixed on double-sided adhesive tapes and the AFM images

were obtained in the air. Changes in the sample's vertical position are presented as a height image. Changes in the phase angle of probe oscillation are presented as phase images.

Immunocytochemistry

Intact adult female *L. chagasfilhoi* were collected as described by Moraes Neto et al. (1997), washed three times in PBS solution, pH 7.2 and fixed for 2 h at 4°C in a solution containing 0.1% glutaraldehyde, 4% formaldehyde (freshly prepared from paraformaldehyde), 2% picric acid in 0.1 M cacodylate buffer, pH 7.2. After fixation, they were washed in PBS and dehydrated at progressively lower temperatures in 30–90% methanol. The infiltration was done in Unycril at −20°C for 2 weeks, and polymerization was performed under ultraviolet light at −20°C for 1 week. Ultrathin sections were collected on 300 mesh nickel grids and incubated for 30 min. at room temperature in 50 mM ammonium chloride, pH 8.3. They were then washed twice in PBS containing 3% BSA and 0.01% Tween 20, pH 8.3. For the localization of 3A3-collagen sites, the sections were initially incubated for 50 min at room temperature in the presence of a polyclonal antibody against the 3A3 collagen sequence (kindly supplied by G.N. Cox, Synergen, Boulder Colo.) diluted 1:500 in PBS containing 1% BSA, pH 8.3. The grids were washed twice in the same buffer, and incubated for 40 min. at room temperature in the presence of gold-labeled goat anti-rabbit IgG (Sigma) diluted 1:100 in PBS containing 1% BSA pH 8.3. After incubation, the sections were subjected to successive washes in 3% BSA-PBS, in 1% BSA-PBS and PBS, pH 8.3, followed by distilled water, counterstained with uranyl acetate and lead citrate, and examined in a Zeiss 900 transmission electron microscope. Two different controls were used: (1) the sections were treated as described above, but a rabbit non-immune serum (1:500) was used as primary antibody, and (2) the sections were incubated in the presence of the gold-labeled antibody only.

SDS-PAGE and Western blot

SDS-PAGE was performed as described by Laemmli (1970). Samples were analyzed in 7.5% (w/v) polyacrylamide gel. The molecular weight of the cuticle proteins was evaluated by comparison with Full-range Rainbow molecular weight marker (Amersham-Pharmacia) run at the same gel.

SDS-PAGE profiles were transferred to nitrocellulose sheets and submitted to Western blot as previously described (Towbin et al. 1979). Primary antibody against the 3A3 collagen sequence was used and detected with the ECL system (Amersham Pharmacia).

Results

Cuticle structure

By light microscopy, purified adult cuticle showed a transparent aspect. Our experimental conditions allowed the selective removal of the internal organs from worm fragments, with minimal damage to the cuticle (Fig. 1 A).

TEM of thin sections (Fig. 1B) of the isolated cuticle showed that it presents a trilaminate epicuticle and four other layers of variable electron densities: cortical, intermediate, fibrous and basal. Striations were limited to the outermost cortical layer, which was relatively homogeneous and had a thickness of 0.6–1 μm at the mid-body region. The intermediate layer (2.6–2.7 μm) was relatively electron-lucent and contained a densely stained line. The fibrous layer was the thickest (5.4–6.1 μm) and most electron-dense layer. The innermost basal layer, 0.3–0.5 μm thick, was intimately associated with the hypodermis.

By high resolution SEM, the epicuticle showed deep transverse striations, with a periodicity of about 0.2 μm (Fig. 1C, D). The striations had a rugous surface with transverse parallel strings, with a thickness varying between 67 and 83 nm and separated by 16–34 nm (Fig. 1D). The innermost basal layer showed a rugous surface (Fig. 1C), with irregularly shaped and randomly distributed protuberances the width of which varied from 33 to 200 nm (Fig. 1E).

Longitudinal views of quick-frozen, freeze-fractured, deep-etched and rotatory shadowed replicas of purified adult cuticle, revealed that it presented deep transverse striations (1.5–2 μm wide) with a periodicity of about 0.2 μm. In the central area of each striation, there was a 200–240 nm wide small projection (Fig. 2A), with a smooth surface. The external surface of the epicuticle was granular, presenting numerous irregularly shaped globular particles with diameters varying from 40 to 100 nm (Fig. 2A). The external face of the inner lamina of the epicuticle (Fig. 2B) was also granular and presented several globular particles with diameters ranging between 42 and 122 nm. The fibrous layer (Fig. 2C) was composed by two types of fibers: thick (12–50 nm) and thin (6–11 nm), which seemed to be connected to each other in a particular form. The distance between the thick and the thin fibers was 87–300 nm and 12–60 nm, respectively. The internal surface of the innermost basal layer was rugous, as seen by high resolution SEM, with irregularly shaped and randomly distributed protuberances whose width varied from 30 to 210 nm (Fig. 2D). Traversing the cuticular layers were channels spaced at intervals of 2.4–2.8 μm, each being 130–250 nm wide (Fig. 2C). These channels were more evident in the fibrous layer and apparently connected the epicuticle to the underlying muscular layer, extending through the hypodermis. Several transverse pores were seen along these channels.

Purified adult cuticle of the filariid *L. chagasfilhoi* was imaged by AFM. No modification of the surface structure was observed even when scanned for a long time. Figure 3A shows a nanometer scale AFM image that reveals the granular aspect and fibrillar nature of the epicuticle. The external surface of the epicuticle was organized in parallel microfibrils (60–300 nm wide) that were quite straight and aligned. Numerous irregularly shaped globular particles, with diameters over 40–160 nm, were visualised on those microfibrils. Figure 3B shows the cuticular striations with a periodicity of about 0.2 μm and 1.2–1.3 μm wide. The height of the cuticular striations varied from 141 to 146 nm. Figure 4A shows a micrometer scale AFM image which reveals that the internal surface of the innermost basal layer of the cuticle

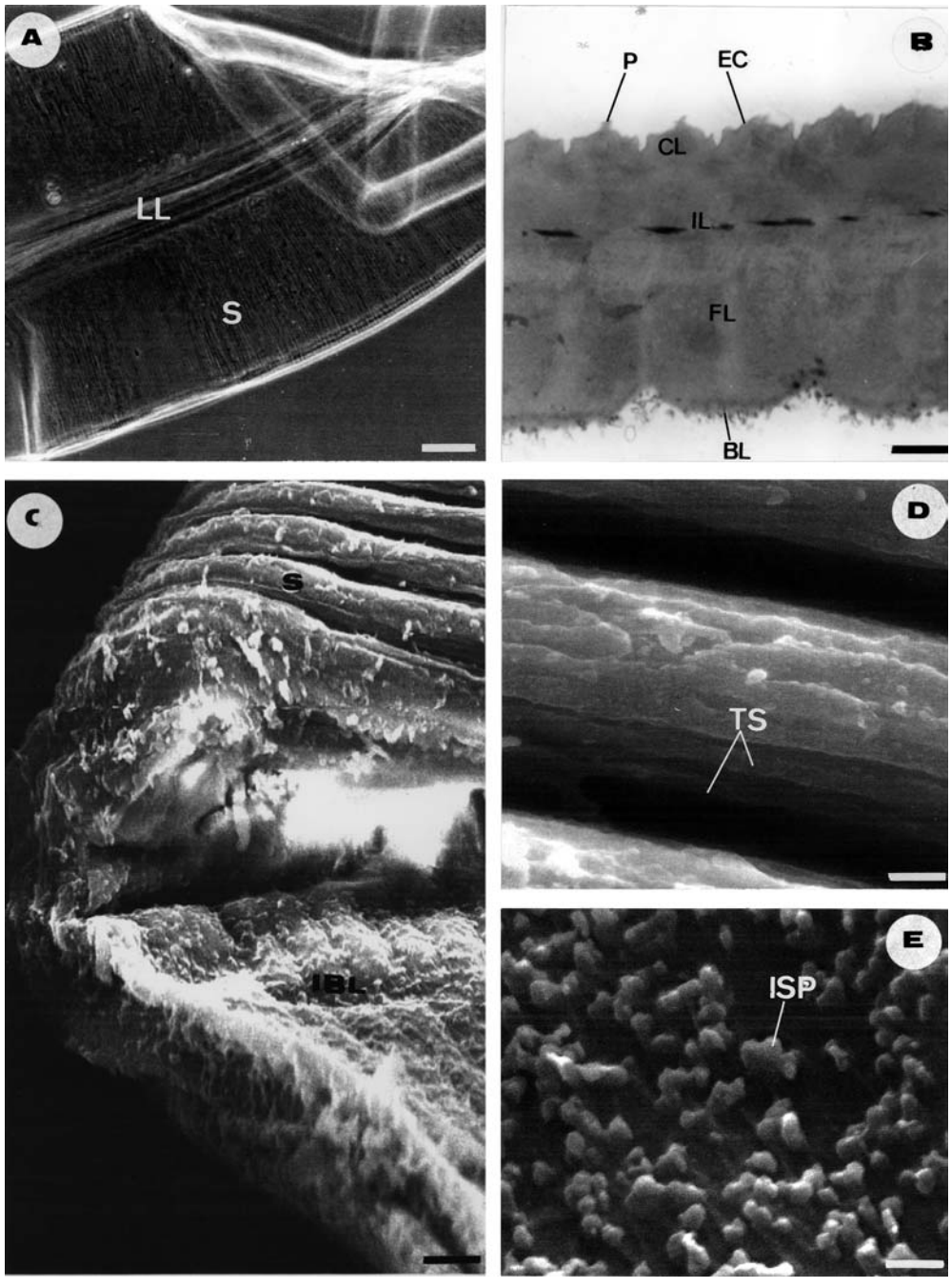

Fig. 1A–E Light microscopy, transmission electron microscopy (TEM) and high resolution scanning electron microscopy (SEM) of the purified adult cuticles of *Litomosoides chagasfilhoi*. **A** Light microscopy of the purified adult cuticle showing the cuticular striations (*S*) and the lateral line (*LL*). *Bar* 46μm. **B** TEM view of a longitudinal thin section of the purified adult cuticle showing the striations with a projection (*P*) and the cuticular layers. Epicuticle (*ec*), cortical layer (*cl*), intermediate layer (*il*), fibrous layer (*fl*), and basal layer (*bl*). *Bar* 1.7μm. **C** High resolution SEM of the purified adult cuticle showing the cuticular transverse striations (*S*) and the rugous internal surface of the innermost basal layer (*IBL*). *Bar* 1.2μm. **D** High resolution SEM of the purified adult cuticle showing the rugous surface of an striation with parallel transverse strings (*TS*). *Bar* 167 nm. **E** High resolution SEM of the purified adult cuticles showing the rugous internal surface of the innermost basal layer with irregularly shaped and randomly distributed protuberances (*ISP*). *Bar* 250 nm

was rugous and irregular, presenting several irregularly shaped and randomly distributed protuberances. The phase image revealed that these protuberances were constituted of the same material of the internal surface. A tracing (Fig. 4B) across the protuberances observed in Fig. 4A was produced to estimate their width and the height. This varied from 254 to 380 nm and 227 to346 nm, respectively.

Figures 3C and 4C show the AFM three-dimensional topographic image of the epicuticle and of the internal surface of the innermost basal layer of *L. chagasfilhoi*.

Immunocytochemical localization of collagen

We used a polyclonal antibody against a synthetic, 18 amino-acid peptide, to immunolocalise this specific collagen in the intact cuticle of *L. chagasfilhoi*. The 3A3-collagen was readily demonstrated in all layers of the cuticle, as well as in the hypodermis of adult *L. chagasfilhoi* worms, when compared to the controls (Fig. 5A–C). Except for the epicuticle, all areas showed intense labeling (Fig. 5C). Some gold particles were also observed at the hypodermis and striated muscle cells. In

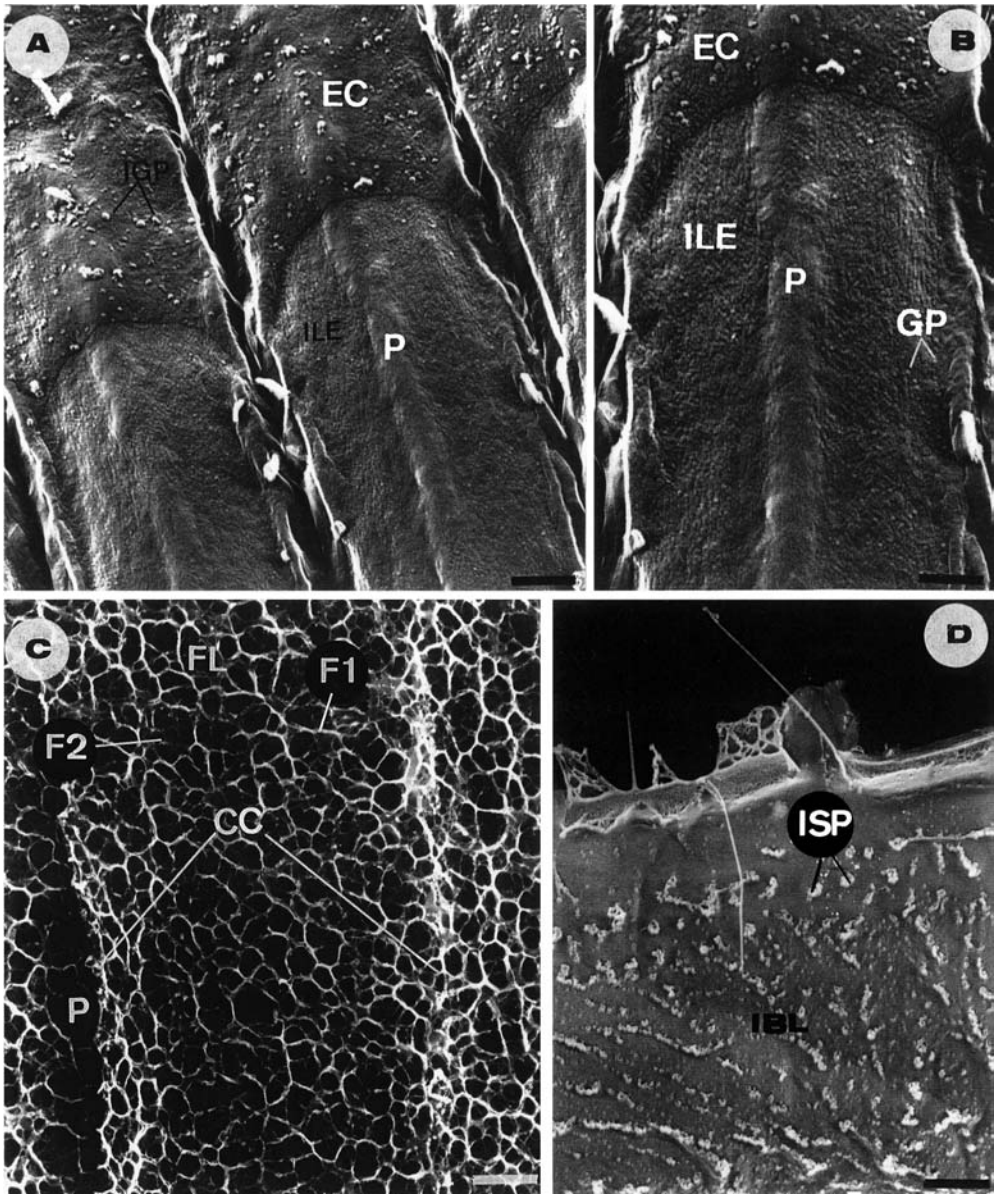

Fig. 2A–D Deep-etch longitudinal views of the purified adult cuticle of *Litomosoides chagasfilhoi*. **A, B** Deep-etch longitudinal view of the purified adult cuticle showing the striations with a projection (*P*) as viewed in thin sections; the granular external surface of epicuticle (*EC*) presenting numerous irregularly shaped globular particles (*IGP*) and the granular external face of the inner lamina of epicuticle (*ILE*) with numerous globular particles (*GP*). *Bars* (**A**) 0.4μm, (**B**) 0.3μm. **C** Deep-etch longitudinal view of the purified adult cuticle showing the cuticular channels (CC) presenting pores (*P*) and the meshwork of fibers of the fibrous layer (*FL*) composed of two types of fibers: thick (*F1*) and thin (*F2*). *Bar* 0.4μm. **D** Deep-etch longitudinal view of the purified adult cuticle showing the rugous internal surface of the innermost basal layer (*IBL*), as seen by high resolution SEM and AFM, with irregularly shaped and randomly distributed protuberances (*ISP*). *Bar* 0.7μm

contrast, few or no gold particles were seen labeling the sheath and the cuticle of the mature intrauterine microfilariae (Fig. 5D).

SDS-PAGE electrophoresis and Western blot

Analysis by SDS-PAGE of purified adult cuticles of *L. chagasfilhoi* revealed a complex protein composition, with molecular weights ranging from 150 to 11 kDa. Five major proteins were identified, corresponding to 151, 41, 28, 13 and 11 kDa (Fig. 6A). SDS-PAGE profiles were transferred to nitrocellulose sheets and submitted to Western blot, probed with the same polyclonal antibody used in the immunocytochemical experiments. The primary antibody against the 3A3 collagen sequence reacted with the proteins of higher molecular weights than 30 kDa (Fig. 6B).

Discussion

Our experimental conditions permitted the selective removal of the internal organs and the hypodermis and muscular layers from worm fragments with minimal damage to the cuticle, as seen in Fig. 1A and B. This approach allowed us to further analyze the organization of the cuticle, facilitating the visualization of its innermost basal layer. Our observations are in agreement with our recent description of the cuticle of intact worms of *L. chagasfilhoi* (Moraes Neto et al. 2002) and those of Vincent et al. (1975) for *Brugia malayi*, and Storey and Ogbogu (1991) for *L. carinii*.

High resolution SEM showed that the cuticular striations have a rugous surface, with parallel central projections, which probably increase the absorption surface, facilitating the passage of nutrients and secretion of

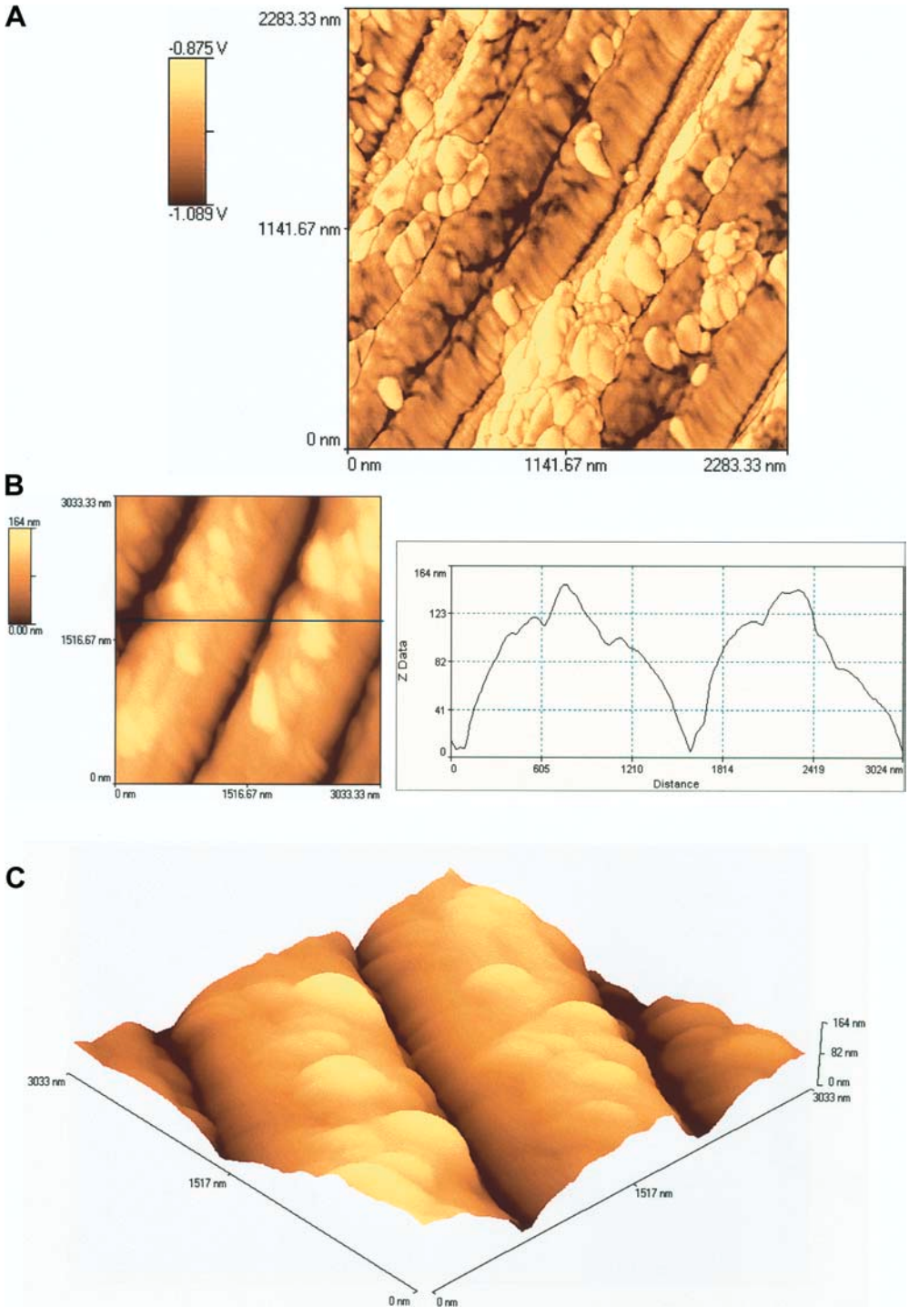

Fig. 3A–C Atomic force microscopy (AFM) longitudinal views of the cuticular striations and the external surface of the epicuticle of purified adult cuticles of *Litomosoides chagasfilhoi*. **A** Phase image showing parallel microfibrils that were quite straight and aligned, and numerous irregularly shaped globular particles. **B** Topographic image showing the cuticular striations and its topological measurements. **C** AFM three-dimensional topographic image showing the distribution of irregularly shaped globular particles on the external surface of the epicuticle

products. It may also help in the locomotion of the filariid. In contrast, replicas of quick-frozen, freeze-fractured, deep-etched and rotatory replicated purified adult cuticles revealed, especially in longitudinal deep-etch views, that the external surface of the epicuticle was granular, with numerous irregularly shaped globular particles, as seen by AFM. The external face of the inner lamina of the epicuticle showed the same aspect, with several globular particles with smaller diameters than those irregularly shaped globular particles seen on the external surface of the epicuticle. The internal surface of the innermost basal layer was rugous, with irregularly shaped and randomly distributed protuberances, as seen by high resolution SEM and AFM. As previously described, using the freeze-fracture and deep-etching technique to study the whole organism (Moraes Neto et al. 2002), the purified cuticle of *L. chagasfilhoi* was shown to contain channels. Although their function remains a matter of speculation, their association with the highly-invaginated membrane at the cuticle-hypodermis

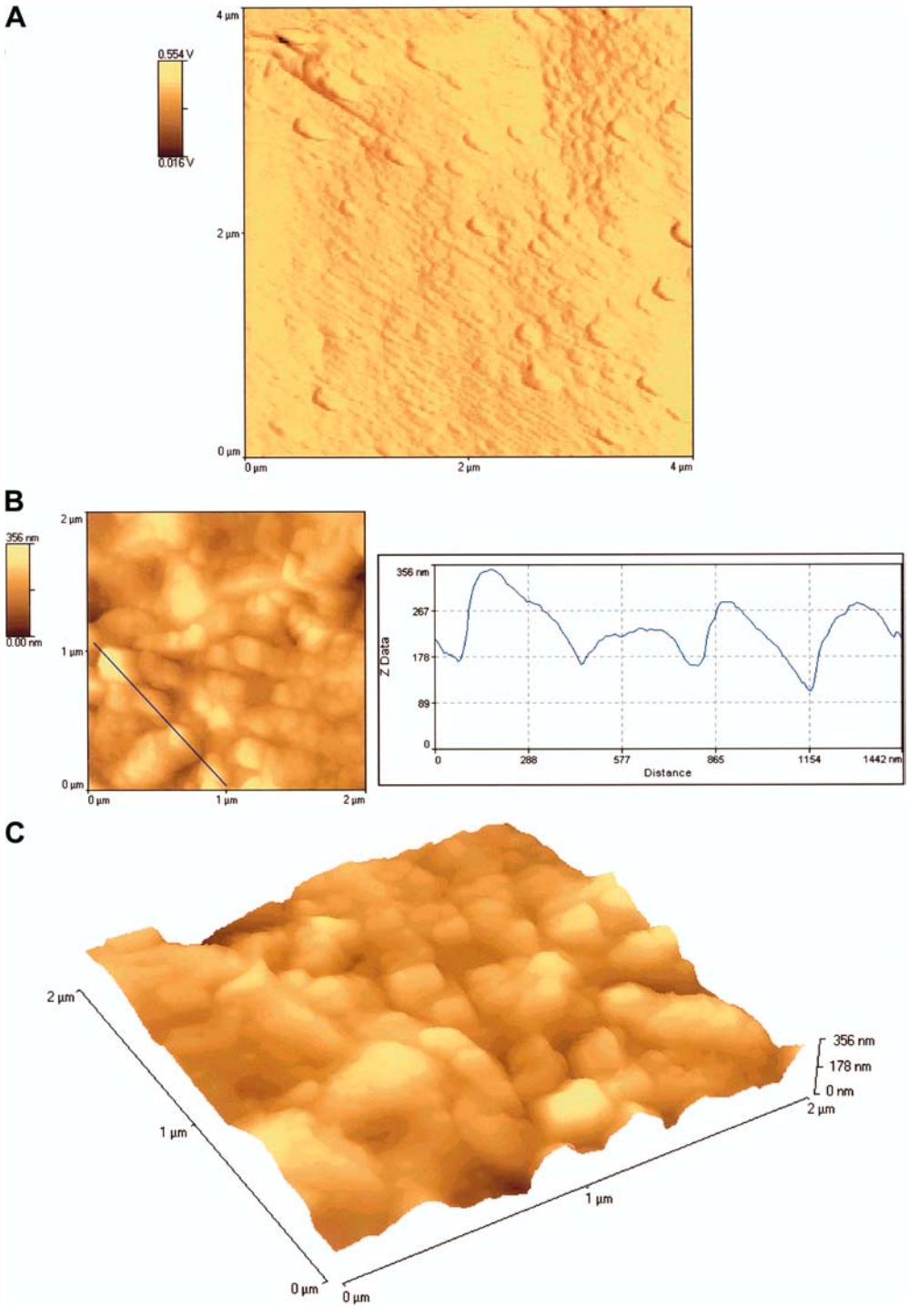

Fig. 4A–C Atomic force microscopy (AFM) longitudinal views of the internal surface of the innermost basal layer of purified adult cuticles of *Litomosoides chagasfilhoi*. **A** Phase image showing the rugous and irregular surface presenting several irregularly shaped protuberances. **B** Topographic image showing the surface, the irregularly shaped protuberances and its topological measurements. **C** AFM three-dimensional topographic image showing the distribution of irregularly shaped protuberances on the internal surface of the innermost basal layer

interface suggests an involvement in nutrient acquisition and transport.

AFM has the advantage of preserving molecular features, making the non-destructive imaging of these surfaces possible without special sample preparations. As far as we know, this is the first AFM study of the cuticle of a filariid. Our observations show that the parallel transverse strings of the cuticular striations seen by high resolution SEM were composed by several parallel microfibrils and numerous irregularly shaped globular particles (also observed by freeze-fracture and deep-etching techniques) that until now have not been observed on the epicuticle of this adult filariid. These particles have an aspect different from that observed in the epicuticle of the mature microfilariae of *L. chagasfilhoi*, seen by the freeze-fracture and deep-etching techniques (Moraes Neto et al. 2001), which we suggested could represent integral membrane proteins. Therefore, these particles may correspond to products of the excretion/secretion activity of the cuticle. Using

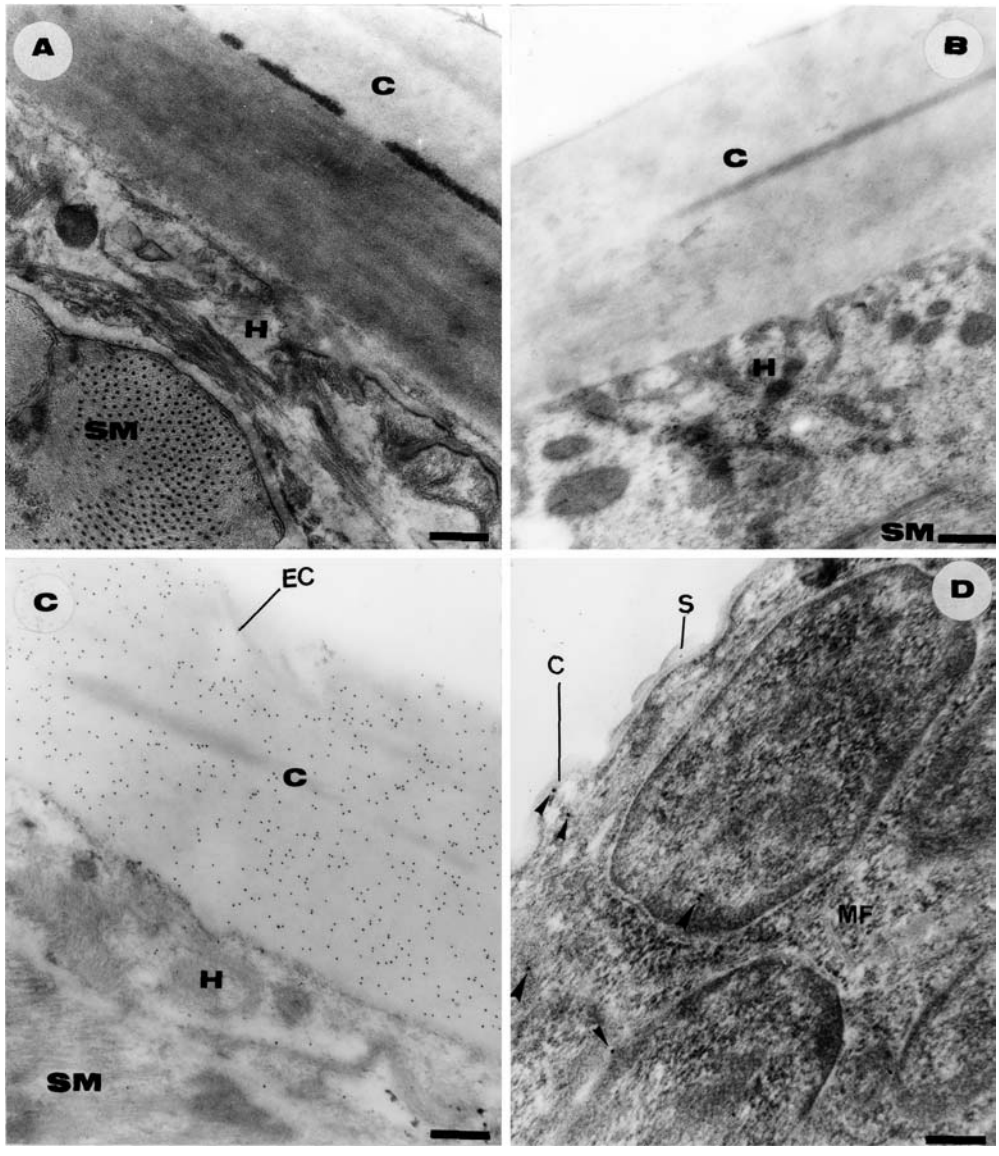

Fig. 5A–D Localization of antigenic sites in thin sections of Unicryl-embedded adult female and mature intrauterine microfilariae of *Litomosoides chagasfilhoi* using a polyclonal antibody against a synthetic 18 amino-acid peptide that corresponds to the sequence of the domain E of the *Haemonchus contortus* 3A3-collagen gene. **A** Control thin section of adult female incubated with a rabbit non-immune serum (1:500) and a gold labeled goat anti-rabbit IgG (secondary antibody, 1:100). **B** Control thin section of adult female incubated only in the presence of the gold labeled goat anti-rabbit IgG (secondary antibody, 1:100). **C** Localization of antigenic sites in thin section of adult female using a polyclonal antibody against the 3A3 collagen sequence (1:500) and a gold labeled goat anti-rabbit IgG (secondary antibody, 1:100). Except for the epicuticle (*EC*), all areas show intense labeling. Cuticle (*c*), hypodermis (*h*), somatic musculature (*Sm*). **D** Localization of antigenic sites (*arrowheads*) in a thin section of mature intrauterine microfilariae (*MF*) using a polyclonal antibody against the 3A3 collagen sequence (1:500) and a gold labeled goat anti-rabbit IgG (secondary antibody, 1:100). Few or no gold particles were seen labeling the sheath (*s*) and the cuticle (*C*). *Bars:* (**A**) 0.5μm, (**B**), (**C**) 0.4μm, (**D**) 0.2μm

AFM we observed that the internal surface of the innermost basal layer was rugous, and more irregular than when observed by high resolution SEM and freeze-fracture and deep-etching techniques, and that the distribution of the irregularly shaped protuberances was better visualized than in the other techniques used in this study. In addition, it was possible to observe the three-dimensional aspect of these surfaces as well as of the fibers and globular particles that compose it.

The intense labeling observed in all layers of the cuticle, except the epicuticle, and the hypodermis of adult *L. chagasfilhoi* incubated in the presence of antibody that recognized 3A3-collagen is in accordance with previous biochemical studies (Cox 1990; Selkirk and Blaxter 1990; Fetterer and Rhoads 1993) that demonstrated that the collagenous proteins are confined to the internal matrix of the cuticle. In contrast, few or no gold particles were seen labeling the sheath or the cuticle of the mature intrauterine microfilariae. These observations are in agreement with previous studies (Cox et al. 1981; Politz and Edgard 1984; Kramer et al. 1985; Selkirk and Blaxter 1990; Kingston 1991; Peixoto et al. 1995; Rhoads et al. 2001; Merriweather et al. 2001) that indicate that each stage in the life cycle of the parasite presents a distinct set of cuticular collagens.

The 11 and 13 kDa proteins observed by SDS-PAGE in the cuticle of *L. chagasfilhoi* may correspond to the one described as being 15 kDa previously identified in adult *Brugia* spp. and shown by autoradiography to be localized in the cuticle (Marshall and Howells 1985; Sutanto et al. 1985; Selkirk et al. 1986). This protein, as previously described in other filariids, is non-glycosylated and does not appear to perform any major structural role in the cuticular architecture (Selkirk and Blaxter 1990). Gp 29 may correspond to the major protein of 28 kDa (Selkirk et al. 1989). The other two proteins of 41 and 151 kDa represent the collagenous structural components of the cuticle. Using the same polyclonal antibody, Western blots of the purified cuticular proteins identified proteins with molecular weights

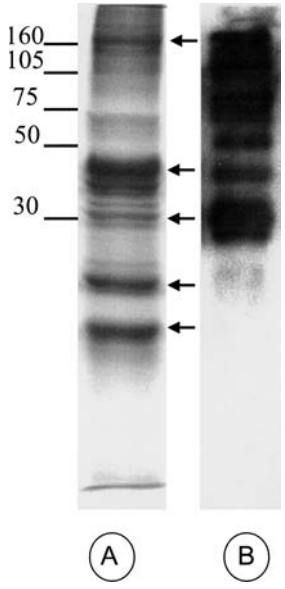

Fig. 6A, B Biochemical analysis of purified adult cuticle of *Litomosoides chagasfilhoi*. **A** SDS-PAGE analysis under reducing conditions of the purified cuticle. Molecular weight markers are shown and indicated at left. **B** Western blot of SDS-PAGE profiles transferred and probed with the polyclonal antibody against a synthetic 18 amino-acid peptide that corresponds to the sequence of the domain E of the *Haemonchus contortus* 3A3-collagen gene

higher than 30 kDa (Fig. 6B). These results are in agreement with those of Cox et al. (1990), who reported that the 3A3 polyclonal antisera reacted predominantly with multiple, high molecular weight (> 68 kDa) proteins on Western blots of worm extracts of several nematode species. All of these observations confirm previous suggestions that the 3A3 collagen, a member of the *col*-1 collagen gene family, appears to be ubiquitous in the phylum Nematoda, even though several collagens common to other animals have also been identified (Cox 1990; Cox et al. 1990; Peixoto et al. 1995).

Taking together the present work and previous studies carried out in other nematode species (Lee et al. 1986; Martinez and De Souza 1995, 1997; Peixoto and De Souza 1995; Peixoto et al. 1997), we can conclude that there is a significant difference in the structural organization of the cuticle of nematodes. Therefore, it is necessary to extend the study to other species in order to obtain additional information which may help us to understand the physiological meaning of the differences. We can also conclude that the use of complementary techniques, such as those used in this paper, are necessary for a better description of the structural components of complex structures.

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