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The role of the sheath in resistance of *Haemonchus contortus* infective-stage larvae to proteolytic digestion

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Abstract

Surface iodinated larvae of *Haemonchus contortus* were incubated in the presence of the fungal protease, proteinase K, and proteolysis quantified by scintillation counting of released radioactivity. No radioactivity was released from live ensheathed infective-stage larvae ($L_{3(2M)}$). In contrast, 58% of the radioactivity was released from ecdysed, second molt (2M) cuticles (sheaths) of $L_{3(2M)}$ and 48% from live exsheathed third-stage larvae (L_3). When $L_{3(2M)}$ larvae were killed by heat (80°C for 10 min) prior to proteinase K incubation, 61% of the radioactivity was released, whereas less than 7% was released from larvae killed by the metabolic inhibitors NaN_3 or KCN. Proteinase K released 44% of the radioactivity from live $L_{3(2M)}$ larvae which had been preincubated with 1% sodium dodecylsulfate (SDS), whereas no radioactivity was released from $L_{3(2M)}$ larvae preincubated with either 1% Triton X-100, 0.2% CTAB, 50% methanol, 50% ethanol, or water. Following incubation with proteinase K, only $L_{3(2M)}$ larvae which had been heat-killed or preincubated with SDS showed visible damage to the sheath. Material released from $L_{3(2M)}$ larvae by exposure to either heat or SDS contained a 98 000 M_r protein by SDS-PAGE autoradiography. These results indicate that viable $L_{3(2M)}$ larvae are resistant to attack by proteinase K and that this resistance is dependent on structural properties of the sheath.

Keywords: Nematode; Surface; Protease; Cuticular proteins; Parasite; Helminth

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1. Introduction

The nematode cuticle, a multilayered proteinaceous structure, has several important functions including protecting the nematode from harmful elements in the environment (Bird and Bird, 1991). Cuticular proteins, particularly those forming the external cortical regions, are extensively cross-linked thus creating a highly stabilized structure (Fetterer and Rhoads, 1990; Fetterer et al., 1993). Although many details are now known about the properties of cuticular proteins and the genes that control them (Cox, 1992; Fetterer and Rhoads, 1993), there is limited knowledge of functional aspects of the cuticle including its role in protecting the nematode from environmental factors. In particular, the free-living stages of nematodes must withstand physical factors such as temperature, moisture and UV radiation, as well as attack by a variety of microorganisms. A number of nematode predacious fungal species have been identified and indicate the potential of fungi as biological control agents (Waller and Faedo, 1993; Waller and Larsen, 1993; Gronvold et al., 1993). The killing mechanisms employed by microorganisms may vary widely, but proteolytic enzymes may be important components in the effector mechanism of fungi (Gupta et al., 1992; Persson and Friman, 1993). A purified protease from the nematode trapping fungi *Arthrobotrys oligospora* has been demonstrated to digest isolated nematode cuticle fragments (Tunlid et al., 1994) thus implicating proteolytic digestion in nematode killing.

The free-living, infective-stage larvae of trichostrongyles are distinguished by their retention of the second-stage cuticle which is loosened, but uncast, during the second molt (2M). This 2M cuticle or sheath undoubtedly contributes to the resistance of the infective-stage larvae to physical and biological insults prior to ingestion by the host (Elenby, 1968; Wharton, 1986). Once ingested, the sheath is shed during a process which involves the activation of a metallo-protease with specificity for degrading a defined area of the sheath (Gamble et al., 1989a; Gamble et al., 1989b).

Preliminary studies in this laboratory have shown that isolated sheaths as well as extracted cuticular proteins from infective-stage larvae of *H. contortus* are readily digested by the fungal protease, proteinase K. In the present study, the susceptibility of infective-stage larvae of *H. contortus* to digestion by proteinase K was assessed.

2. Materials and methods

2.1. H.contortus

Infective-stage larvae ($L_{3(2M)}$) of *H. contortus* were recovered from cultured feces obtained from experimentally-infected sheep as previously described (Gamble et al., 1989a). In some experiments, $L_{3(2M)}$ were exsheathed in vitro, and the exsheathed larvae (L_3) and the ecdysed 2M cuticles (sheaths) were separated by centrifugation on a discontinuous Percoll gradient (30% v/v in Earle's balanced salt solution (EBSS); Sigma, St. Louis, MO) (Fetterer, 1989). The L_3 were washed extensively with EBSS and maintained at 37°C. The sheaths were collected and washed in distilled water.

2.2. Iodination

Infective-stage larvae, L_3, and sheaths were separately radiolabeled with ^{125}I using slight modifications of a previously described method shown to preferentially label the cuticular components (Rhoads and Fetterer, 1990). Two washed Iodobeads (Pierce, Rockford, Il) were added to 1 ml of phosphate buffered saline (PBS) containing 500 μCi of Na ^{125}I (New England Nuclear, Boston, MA) and incubated at room temperature for 5 min. A 1–2 ml aliquot of gravity sedimented $L_{3(2M)}$, L_3, or sheaths were added and incubated on ice with repeated mixing for 10 min. The suspensions of larvae or sheaths were separated from the Iodobeads, and washed extensively with PBS containing 1 mM tyrosine and 1 mM NaI. The larvae and sheaths were then washed additionally with PBS without supplements until radioactivity in the wash solutions approached background values.

2.3. Proteinase K incubation

Iodinated $L_{3(2M)}$ and L_3 (both viable), and sheaths were suspended in 0.1 M Tris–HCl buffer, pH 7.2, containing 5 mM $CaCl_2$ (Tris–Ca). Aliquots (0.4 ml) containing $(2–4) \times 10^5$ $L_{3(2M)}$, L_3, or $(1–2) \times 10^5$ sheaths were placed in 1.5 ml microfuge tubes. Proteinase K (BRL, Gaithersburg, MD) (5 mg ml^{-1} Tris–Ca) was added to a final concentration of 1 mg ml^{-1}. Control tubes received Tris–Ca in place of enzyme. Tubes were placed on a rotator (8 rev min^{-1}) and incubated at room temperature for 18 h. Following centrifugation, the supernatants were collected and the pelleted larvae or sheaths were resuspended in 0.25 ml water and recentrifuged. The initial supernatants and subsequent water washes were combined and radioactivity was determined by liquid scintillation counting (LS6000 Beckman Instruments, Fullerton, CA). The pelleted larvae or sheaths were incubated with 100 μl tissue solubilizer (NCS, Amersham, Arlington, IL) for 18 h, neutralized with 25 μl acetic acid and radioactivity determined. Results were expressed as the $\%^{125}I$ released by proteinase K minus the $\%^{125}I$ released by control (buffer) and calculated as follows: (cpm in supernatant/cpm in pellet + supernatant) × 100. The $\%^{125}I$ released by both proteinase K incubations and control (buffer) incubations are included in the figures. Incubations were performed in replicates of five and results expressed as means.

2.4. Killed $L_{3(2M)}$

Iodinated $L_{3(2M)}$ were suspended in water and heated in a water bath at 80°C for 10 min. Alternatively, $L_{3(2M)}$ were incubated in an aqueous solution of 10 mM NaN_3 or 10 mM KCN for 12 h at room temperature. The larvae were then washed three times with 10 ml water, suspended in Tris–Ca, and incubated with proteinase K as described.

2.5. Detergent and alcohol-treated $L_{3(2M)}$

Iodinated $L_{3(2M)}$ were suspended in water containing one of the following; 1% sodium dodecylsulfate (SDS), 1% Triton X-100 (TX100), 0.25% cetyltrimethylammo-

nium bromide (CTAB), 50% methanol (MeOH), 50% ethanol (EtOH), or water (control), and then incubated at 37°C for 30 min.

2.6. Analysis of $L_{3(2M)}$

Following the various treatments, $L_{3(2M)}$ were sedimented by centrifugation and the treatment solutions were collected. The larvae were washed five times with water, resuspended in Tris–Ca, and incubated with proteinase K as described. Some of the larvae were examined at 100 × magnification under a light microscope, both before and after proteinase K incubation, to determine motility and assess morphological changes.

2.7. Analysis of treatment solutions

Cold trichloroacetic acid (TCA) was added to treatment solutions to a final concentration of 10% (v/v). The precipitates were washed twice with 10% TCA and once with water. The radioactivity of each precipitate was determined with a gamma counter (Beckman 5000, Beckman Instruments, Fullerton, CA). The precipitates were then analyzed by Tris/Tricine sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% separating gel overlaid with a 4% stacking gel (Schagger and von Jagow, 1987). Samples and ^{14}C-labeled molecular weight marker proteins were boiled for 1 min in 1.5 M Tris–HCl, pH 8.45, containing 4% SDS and 2.5% 2-mercaptoethanol. Following electrophoresis, the gels were incubated in 7% acetic acid, 10% methanol for 15 min to fix the proteins, dried and then exposed to X-omatic AR X-ray film in an X-omatic cassette with a regular intensifying screen (Kodak) at −70°C.

3. Results

3.1. Release of radioactivity by proteinase K

Proteinase K released 58% of the radioactivity from isolated sheaths and 48% from live L_3 (Fig. 1). In contrast, proteinase K did not release radioactivity from live $L_{3(2M)}$. In addition, proteinase K did not release radioactivity from $L_{3(2M)}$ pretreated with metabolic inhibitors. These larvae were completely nonmotile and appeared dead. However, proteinase K released 61% of the radioactivity from heat-killed $L_{3(2M)}$ (Fig. 2). Furthermore, proteinase K released 44% of the radioactivity from SDS-treated, 20% from CTAB-treated, and 8% from TX100-treated $L_{3(2M)}$ (Fig. 3). Proteinase K did not release radioactivity from MeOH- or EtOH-treated $L_{3(2M)}$.

3.2. Viability and morphology of treated $L_{3(2M)}$

Heat-, NaN_3-, KCN-, and MEOH-treated $L_{3(2M)}$ were nonviable (Table 1). EtOH-treated $L_{3(2M)}$ had greatly reduced viability, while the viability of larvae treated with SDS, TX100, and CTAB was moderately reduced compared to untreated controls. The morphology of $L_{3(2M)}$ following all treatments appeared normal. Incubation of heat- or

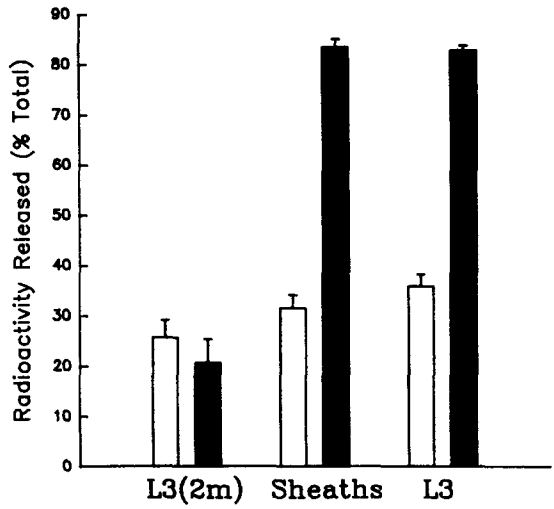

Fig. 1. The release of ^{125}I from live *Haemonchus contortus* infective-stage larvae ($L_{3(2M)}$), exsheathed larvae (L_3) or sheaths by incubation in the presence (solid bars) or absence (open bars) of proteinase K. Values are means of five replicates. Vertical lines represent one standard error.

SDS-treated larvae with proteinase K resulted in the distention and malformation of the sheath; visible holes and tears were often apparent (Fig. 4). Incubation of TX100-treated $L_{3(2M)}$ with proteinase K showed slight damage to the sheath which was observable on

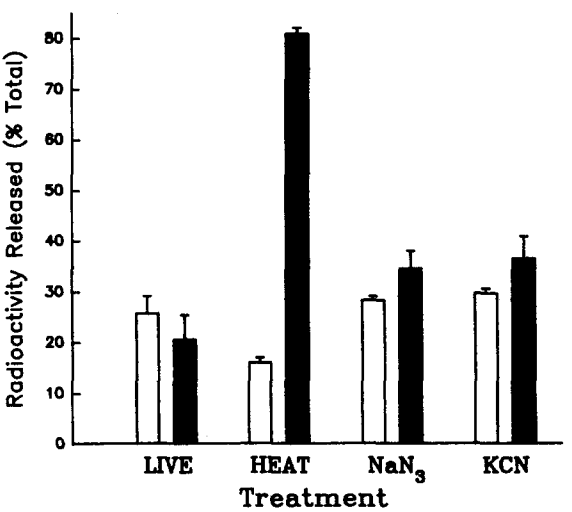

Fig. 2. The release of ^{125}I from live, heat killed or metabolically poisoned *Haemonchus contortus* $L_{3(2M)}$ in the presence (solid bars) or absence (open bars) of proteinase K. Larvae were killed by heating at 80°C for 5 min. NaN_3 and KCN were used at a concentration of 10 mM. Values are means of five replicates. Vertical lines represent one standard error.

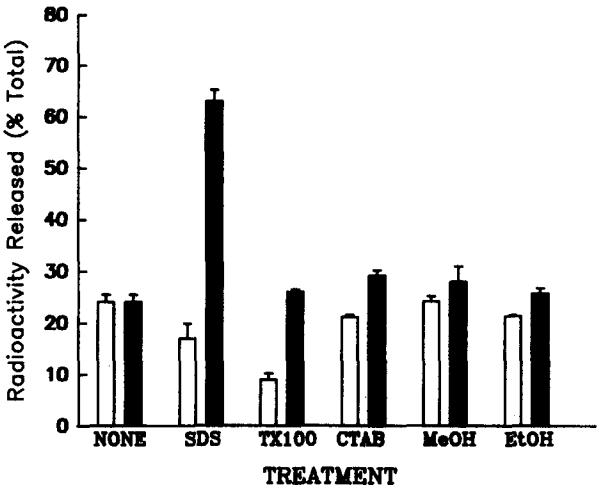

Fig. 3. The release of ^{125}I from *Haemonchus contortus* infective-stage larvae ($L_{3(2M)}$) treated with detergents and alcohols in the presence (solid bars) or absence (open bars) of proteinase K. Treatments were employed as described in the text. Con, control; SDS, 1% sodium dodecylsulfate; TX100, 1% Triton X-100; CTAB, 0.25% cetyltrimethylammonium bromide; MeOH, 50% methanol, EtOH, 50% ethanol. Values are means of five replicates. Vertical lines represent one standard error.

about 20% of the larvae. Incubation of CTAB-, MeOH-, or EtOH-treated $L_{3(2M)}$ with proteinase K did not cause any visible damage to the sheath.

3.3. Analysis of radioactivity released from $L_{3(2M)}$ by treatments

TCA-precipitable radioactivity was released from $L_{3(2M)}$ by heat (41 800 cpm), SDS (19 900 cpm), and TX100 (16 800 cpm). Compared with radioactivity released by water (control) (8800 cpm), no additional TCA-precipitable radioactivity was released by

Table 1
The effect of various treatments on viability and sheath morphology of *Haemonchus contortus* infective-stage ($L_{3(2M)}$) larvae

Treatment	Viability	Cuticle damage by proteinase K
Control	+ + +	None
Heat	−	Extensive
NaN_3 (10 mM)	−	None
KCN (10 mM)	−	None
MEOH (50%)	−	None
ETOH (50%)	±	None
SDS (1%)	+ +	Extensive
CTAB (0.25%)	+ +	Slight
TX100 (1%)	+ +	Slight

+ + +, great effect; −, no effect; ±, marginal effect.

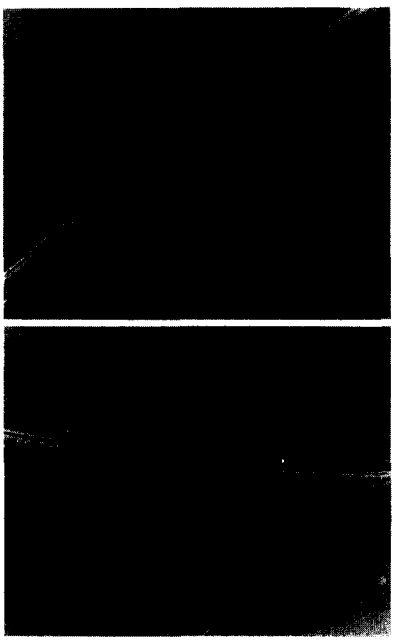

Fig. 4. Photomicrograph of mid-body region of heat-killed *Haemonchus contortus* infective-stage larvae ($L_{3(2M)}$) with (B) or without (A) exposure to proteinase K. Larvae were unstained and viewed with Nomarski optics at magnification of $\times 100$. Arrow indicates damaged portion of cuticle.

EtOH (8200 cpm), MeOH (4600 cpm), or CTAB (2600 cpm). ^{125}I radiolabeled proteins released by heat, SDS, and TX100 treatment were detected by SDS-PAGE autoradiography (Fig. 5). TX100-released components were poorly resolved, whereas SDS-released components contained a major band at 43 000 M_r and a minor band at 98 000 M_r.

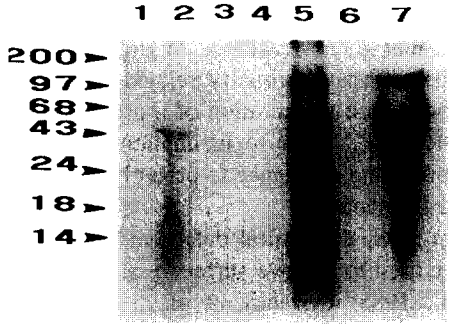

Fig. 5. SDS-PAGE autoradiographs of material released from ^{125}I labeled *Haemonchus contortus* infective-stage larvae ($L_{3(2M)}$) by heat, detergents or alcohols. Each batch of larvae contained approximately 2×10^6 cpm of ^{125}I prior to treatment. Lane 1, control (8880 cpm); Lane 2, TX100 (16 800 cpm); Lane 3, EtOH (9200 cpm); Lane 4, MeOH (4600 cpm); Lane 5, heat (41 800 cpm); Lane 6, CTAB (2600 cpm); Lane 7, SDS (19 900 cpm). The M_r of ^{14}C-labeled standard proteins are indicated.

Radioactive components with approximate M_rs of 29000, 43000 and 98000 were released from $L_{3(2M)}$ by heat, and showed a more distinct banding pattern than that observed with the detergent-released components.

4. Discussion

In the current study, the vulnerability of *H. contortus* infective-stage larvae to proteolytic degradation was examined. Proteinase K, a fungal protease available in pure form and with broad substrate specificity, was chosen because preliminary data indicated that this enzyme readily digested *H. contortus* sheaths as well as isolated collagenous and noncollagenous sheath proteins (R.H. Fetterer, unpublished observation). Other fungal or bacterial proteases may have similar properties.

Live *H. contortus* $L_{3(2M)}$ were shown to be resistant to digestion by proteinase K, whereas isolated sheaths and live, exsheathed L_3 were not. Since it is unlikely that proteinase K could diffuse across the sheath of live, intact $L_{3(2M)}$, the enzyme would be unable to interact with either the internal surface of the sheath or with the L_3 cuticle, which is protected by the sheath. However, both the internal and external surfaces of isolated sheaths, which have been ruptured during ecdysis would be accessible to proteinase K. Further, since ecdysis of $L_{3(2M)}$ involves an enzymatic process (Gamble et al., 1989a), the structure of the isolated sheath may be altered increasing the susceptibility of both the inner and outer surfaces to digestion. The outer, cortical region of the sheath is composed of approximately 60% noncollagenous proteins, many of which are cross-linked by dityrosine residues imparting considerable chemical stability (Fetterer and Rhoads, 1990). Thus, an intact outer cuticular surface of the sheath may provide the primary barrier to proteolysis.

A fungal protease was reported to degrade purified cuticle fragments from the free-living nematode *Panagrellus redivivus* (Tunlid et al., 1994) and was suggested to be important in nematode killing. However, the current results suggest that degradation of cuticle fragments may not be a valid way to predict degradation of intact larvae.

Because the metabolic inhibitors NaN_3 and KCN rendered the $L_{3(2M)}$ completely non-motile yet did not alter their resistance to digestion, protection of $L_{3(2M)}$ from proteolysis appears to be a passive property related to cuticular structure and not the result of an active response such as secretion of a proteolytic inhibitor. Furthermore, of the various treatments to $L_{3(2M)}$ prior to incubation with proteinase K, there was no correlation between viability and resistance to proteolysis as measured both by release of ^{125}I or visual damage to the sheath.

The anionic detergent SDS, the neutral detergent TX100, and the cationic detergent CTAB have been widely used to remove surface-associated proteins from the nematode cuticle (Pritchard et al., 1985; Devaney et al., 1990; Rhoads and Fetterer, 1994). Alcoholic extraction has also been used to characterize lipophilic components of the cuticle (Page et al., 1992). Surface-associated proteins are also removed by hot aqueous solutions (Rhoads and Fetterer, 1990). In the present study, heat and SDS removed radiolabeled surface proteins from $L_{3(2M)}$ that were detected by SDS-PAGE autoradiography. TX100 released a similar amount of radioactivity as did SDS, but resulted in a

less defined banding pattern. Radioactivity detectable by scintillation counting was also released by the other treatments (CTAB, MeOH, EtOH, and water) but no bands were visible, suggesting that the extracted radioactivity was either not associated with protein or the molecular weights were outside the limits of the electrophoretic system.

It is reasonable to conclude that removal of protein components from $L_{3(2M)}$ by heat or SDS is related to the subsequent susceptibility of the larvae to proteolysis. Although the protein profiles obtained by the two treatments are different, the presence of a 98 000 M_r protein in both suggests that this protein may be involved in maintaining the structural integrity of the sheath in the presence of proteinase K. Interestingly, in a previous study, a 100 000 M_r protein was extracted from iodinated $L_{3(2M)}$ by homogenization with buffer containing 1% SDS, whereas no labeled proteins were extracted from iodinated L_3 or sheaths (Rhoads and Fetterer, 1990). Also, a 100 000 M_r protein was identified as the cuticle component hydrolyzed by the metallo-protease involved in ecdysis (Gamble et al., 1989a) and localized to a distinct area of the sheath. These data suggest that this specific area of the sheath may be critical in maintaining resistance to proteolytic attack.

The results of the current study support a hypothesis that the free-living infective-stage larvae of *H. contortus* are resistant to digestion by nonspecific proteases such as proteinase K, and that one or more structural protein components are integral for this resistance. This study describes a rapid and sensitive method to assess the effect of proteolytic enzymes on degradation of infective-stage larvae and to evaluate the mechanisms for avoidance of proteolytic digestion.

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