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Trichinella spiralis: proteinases in the larvae

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Abstract Under in vitro conditions, muscle larvae of *Trichinella spiralis* secreted minute amounts of a cysteine proteinase into the outer environment from the stichosome. The proteinase hydrolyzed azocoll at pH 5.0 but not a number of synthetic *N*-blocked and *N*-unsubstituted proteinase substrates at this pH. The reducing compound dithioerythritol enhanced the enzyme activity, but the thiol-blocking reagent sodium-*p*-hydroxymercuribenzoate (0.1 m*M*) was without effect. Phenylmethylsulfonyl fluoride (PMSF) (2 m*M*) and leupeptin (100 m*M*) produced partial and complete inhibition, respectively, whereas soybean trypsin inhibitor, pepstatin A, and 1,10-phenanthroline were non-inhibitory. Calcium (1 m*M*) produced a slight decrease in the activity that was reversed by 1 m*M* EGTA. Although multiple proteinase activities were detected histochemically in the somatic muscles, stichosome, midgut, and genital primordium of the muscle larvae, none of these enzymes appeared to be the one secreted. Several histochemically demonstrable proteinases were also found in the cells of 48- to 72-h-old juveniles of the parasite. One was localized in the esophageal lumen and at or around the anterior esophagus of the larvae, where developing stichocytes are believed to occur. The proteinase hydrolyzed *N*-acetyl-L-methionine-1-naphthyl ester and was sensitive to the metal cation-complexing compound EGTA as well as to PMSF, an inhibitor of serine proteinases.

Abbreviations *E/S* Excretions/secretions · *PBS* Phosphate-buffered saline · *SDS* Sodium dodecylsulfate · *EGTA* Ethylene glycol-*O*,*O*′-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid · *PMSF* Phenylmethanesulfonyl fluoride · *pHMB* Sodium *p*-hydroxymercuribenzoate · *DTE* Dithioerythritol · *STI* Soybean trypsin inhibitor · *Z-Ala*$_2$*-Lys-NHnapMeO* *N*-Benzyloxy-carbonyl-L-alanyl-L-alanyl-L-lysine-4-methoxy-2-naphthylamide · *Ala*$_2$*-Phe-NHnapMeO* L-Alanyl-L-alanyl-L-phenylalanine-4-methoxy-2-naphthylamide · *Gly-Pro-NHnap* Glycyl-L-proline-2-naphthylamide · *Lys-Ala-NHnapMeO* L-Lysyl-L-alanyl-4-methoxy-2-naphthylamide · *Z-Gly*$_2$*-Arg-NHnapMeO* *N*-Benzyloxycarbonyl-glycyl-glycyl-L-arginine-4-methoxy-2-naphthylamide · *Ac-Gly-Lys-Onap* *N*-Acetyl-glycyl-L-lysyl-2-naphthyl ester · *Ser-Tyr-NHnapMeO* L-Seryl-L-tyrosine-4-methoxy-2-naphthylamide · *Z-Pro-Ala-Gly-Pro-NHnapMeO* *N*-Benzyloxycarbonyl-L-prolyl-L-alanyl-glycyl-L-proline-4-methoxy-2-naphthylamide · *Ac-Met-Onap* *N*-Acetyl-L-methionine-1-naphthylester · *His-Ser-NHnapMeO* L-Histidyl-L-serine-4-methoxy-2-naphthylamide · *Z-Arg*$_2$*-NHnapMeO* *N*-Benzyloxycarbonyl-L-arginyl-L-arginine-4-methoxy-2-naphthylamide · *Z-Ala-NHnapMeO* *N*-Benzyloxycarbonyl-L-alanine-4-methoxy-2-naphthylamide · *Z-Phe-Arg-NHnapMeO* *N*-Benzyloxycarbonyl-L-phenylalanyl-L-arginine-4-methoxy-2-naphthylamide · *Bz-Phe-Val-Arg-NHnapMeO* *N*-Benzoyl-L-phenylalanyl-L-valyl-L-arginine-4-methoxy-2-naphthylamide · *Z-Gly-Pro-Arg-NHnapMeO* *N*-Benzyloxycarbonyl-glycyl-L-prolyl-L-arginine-4-methoxy-2-naphthylamide · *Z-Ala-Onap* *N*-Benzyloxycarbonyl-L-alanine-2-naphthylester · *Glt-Gly*$_2$*-Phe-NHnap* *N*-Glutaryl-glycyl-glycyl-L-pheny lalanine-2-naphthylamide · *Bz-Phe-Onap* *N*-Benzoyl-DL-phenylalanine-2-naphthylester · *Pro-Lys-Ala-NHnapMeO* L-Prolyl-L-lysyl-L-alanine-4-methoxy-2-naphthylamide · *Z-Arg-NHnap* *N*-benzyloxycarbonyl-L-arginine-2-naphthylamide · *Bz-Leu-NHnap* *N*-Benzoyl-L-leucine-2-naphthylamide · *Leu-Gly*$_2$*-NHnapMeO* L-Leucyl-glycyl-glycine-4-methoxy-2-naphthylamide.

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Introduction

Muscle larvae of the parasitic nematode *Trichinella spiralis* invade hosts by penetrating small intestine enterocytes (Wright 1979; Dunn and Wright 1985). Fol-

lowing maturation and mating, the female worms produce their offspring, newborn larvae, that migrate through the bloodstream and lymphatics (Wang and Bell 1986a, b) to reach skeletal muscle cells. Striated muscle cells invaded by newborns undergo profound changes in their morphology and physiology, resulting in their transformation into nurse cells. The parasite can survive at least 30 years within the nurse cell (Gullota and Fröscher 1983).

The question whether proteolytic enzymes play a role in the penetration of enterocytes by the muscle larvae has not been extensively studied to date. Using an electrophoretic technique, Rupova-Popjordanova et al. (1996) detected three proteinase activities in extracts from the larvae. According to Criado-Fornelio et al. (1992) and Armas et al. (1993), the muscle larvae secrete and/or excrete minute amounts of proteinases into the outer environment, but it is not yet known which organ in the parasite produces these enzymes and in which way the proteinases are expelled.

The migratory behavior of newborn *T. spiralis* larvae suggests participation of a proteolytic enzyme in tissue penetration. There is evidence for their extravascular migration out of local capillaries and then back to the circulation (Wang and Bell 1986a, b). Moreover, when applied parenterally to a host, newborns find their way into peritoneal and skeletal muscles (Matoff 1943; Dennis et al. 1970). However, the stichosome, a potential candidate for the organ that would produce a proteolytic enzyme, is either absent (Gold et al. 1990) or relatively weakly developed in newborns (Bruce 1974). The aim of the present study was to examine the distribution of proteolytic enzymes in the muscle larvae and in the newborns by means of histochemical methods to find structures that synthesize and release proteinases into host tissues.

Materials and methods

Three-month-old muscle larvae of *T. spiralis* were obtained from the carcasses of infected mice by a standard peptic digestion method. The larvae were separated from undigested particles of host tissues by allowing them to pass through three layers of cotton gauze and by a repeated sedimentation in phosphate-buffered saline (PBS). Subsequently, a dense suspension of the larvae was loaded onto and sedimented in a 20-cm-long, 2-cm-wide column of 0.3 *M* sucrose-0.02 *M* phosphate buffer at pH 7.4. The heavy fraction comprising undigested particles of host tissues was removed before the parasites settled at the bottom of the tube. Following sedimentation and sucking off the supernatant, the material was washed by sedimentation in cold PBS supplemented with 0.02% Triton WR-1339 (w/v), an injectable, non-ionic detergent. After extensive washing with PBS and rinsing with unbuffered saline, the purified larvae were used for microscopic observations and for histochemical and biochemical study.

Newborn larvae, 48–72 h old, were obtained from in vitro maintenance of mature *T. spiralis* females isolated from intestinal mucosa of infected mice. Following separation from the females, newborns were washed by sedimentation in F14 tissue culture medium (GibcoBRL) and used for histochemical studies.

Two experiments were performed to demonstrate the presence of proteinases in excretions/secretions (E/S) produced by the muscle larvae under in vitro conditions. The first consisted of suspending the material in a sterile, immobilizing medium at 40 °C, comprising 0.8% agarose, 1% gelatin, 5 m*M* KCl and 0.1 *M* 3,3′-dimethylglutaric acid-NaOH buffer at pH 5.0. The suspension was smeared over microscope slides preheated to 40 °C. The slides were then incubated in a moist chamber for 18 h, at 37 °C. Following incubation, the smears were stained with 0.1% Coomassie brilliant blue R-250 in 7% acetic acid, differentiated in 1% acetic acid and examined under a microscope for the presence of transparent areas indicating gelatinolytic activity. The second experiment was performed in a similar way, except that gelatin was replaced by fine-grained azocoll obtained by sonication of the coarse-grained commercial product. Following 18 h of incubation at 37 °C, the smears were examined under a microscope for the presence of clear areas indicating hydrolysis of the cloudy mass of particles in the insoluble substrate.

Newborn larvae were examined in a similar way, except that agarose and gelatin were melted in the F14 medium.

Histochemistry

Drops of a dense suspension of muscle larvae in hypotonic, unbuffered saline were deposited on microscope slides. The parasites were chopped with a razor blade and dried for 30 min at room temperature. Newborn larvae were dried on microscope slides. Following fixation and defatting in an acetone-chloroform mixture (2:1 by volume) for 1 min at 4 °C, the material was sealed with incubation media for visualization of proteinase activities. The media comprised synthetic proteinase substrates, a diazonium salt (fast garnet GBC·BF_4), and either a 0.07 *M* 3,3′-dimethylglutaric acid-NaOH buffer at pH 5.0 or a 0.07 *M* Tris-HCl buffer at pH 7.5. Since diazonium salts are strong, irreversible inhibitors of cysteine proteinases, the fluorescence method of Dolbeare and Vanderlaan (1979) was employed to search for the activity of these enzymes. However, the fluorescence method has limited application, since it works well only in a narrow pH range (pH 4.0–5.5), utilizes only 4-methoxy-2-naphthylamine derivatives of proteinase substrates and requires dithioerythritol (DTE) in the incubation medium. The differentiation between cysteine proteinases and other proteolytic enzymes was, therefore, done by comparing reaction intensities in tissues pretreated with 1 m*M* *N*-ethylmaleimide (an irreversible inhibitor of cysteine proteinases) with those obtained in tissues not treated with the inhibitor i.e., in which both thiol-dependent and thiol-independent proteinases were active. Results observed in histochemically processed material were evaluated against those in control specimens, which were incubated in the absence of any substrate.

Gelatinolytic activity in extracts

This experiment was performed on newborn larvae only. Approximately 10 mg (fresh weight) of 48- to 72-h old, pelleted newborns were suspended in 100 µl of 0.01% Triton X-100 (w/v). The larvae were disrupted by application of five 3-s ultrasonic bursts at 100 W (Braun sonifier, model Labsonic L) with intervening cooling at 0 °C. Following centrifugation for 15 min at 16,000 *g*, at 4 °C, the supernatant fraction was collected and used as a source of proteolytic enzymes. A number of aliquots of the extract, 3 µl volume each, were loaded onto a strip of a slightly exposed and developed, double-sided X-ray film. The drops were left for almost complete evaporation of water before another 3-µl portion was added. Following the fifth series of extract droplets, the moist spots comprising concentrated extract proteins were supplemented with 3 µl of 0.05 *M* 3,3′-dimethylglutaric acid-NaOH buffer (pH 5.0–7.2) or 0.05 *M* Tris-HCl buffer (pH 7.4–8.4). The strip was then kept in a moist chamber for 20 h at 30 °C prior to being washed and examined under a dissection microscope for transparent spots indicating proteolytic activity. In another X-ray film test, a number of proteinase inhibitors was empolyed. Control tests were performed in a similar way except that 0.01% Triton X-100 was used instead of the extract.

Proteolytic activity in E/S

Samples of purified muscle larvae, approximately 0.6 g each, were resuspended in 3-ml volumes of a sterile incubation medium comprising 0.04 *M* NaCl, 2 m*M* KCl, gentamicin (50 μg ml^{-1}), and 0.1 *M* 3,3′-dimethylglutaric acid-NaOH buffer at pH 5.0. Some of these mixtures were supplemented with 10 mg of azocoll, whereas others were not. Controls contained no larvae. Both experimental and control mixtures were incubated for 18 h at 38 °C and then centrifuged. The supernatants that contained the dye liberated as a result of enzymic degradation of azocoll were examined spectrophotometrically against controls. Other supernatants were either supplemented with azocoll and incubated for another 18 h at 38 °C, or were used as a source of proteolytic enzymes for studies on inhibition. An absorbance produced by 1 ml of a buffer that contained products of a complete hydrolysis of 1 mg of azocoll by 0.01 mg of papain served as a basis for calculating the amount of substrate hydrolyzed by a proteolytic enzyme present in the E/S collected.

Studies on inhibition were performed at pH 5.0. Assay mixtures comprised 0.07 *M* 3,3′-dimethylglutaric acid-NaOH buffer at pH 5.0, azocoll, E/S, and proteinase inhibitors. Incubation time was 18 h at 37 °C. Control mixtures were supplemented with E/S aliquots just at the end of the incubation. The results were expressed as a percentage of reduction in absorbance compared with an inhibitor-free control.

Another type of assay was carried out using several synthetic substrates, some of which were intensely hydrolyzed by proteinases histochemically detected in the muscle larvae (Table 1). These were *Z*-Ala_2-Lys-NHnapMeO, Ala_2-Phe-NHnapMeO, Gly-Pro-NHnap, Lys-Ala-NHnapMeO, *Z*-Gly_2Arg-NHnapMeO, Ser-Tyr-NHnapMeO, *Z*-Pro-Ala-Gly-Pro-NHnapMeO, *Ac*-Met-Onap, *Bz*-Phe-Onap, and *Z*-Arg_2-NHnapMeO. A number of 50-mg samples of muscle larvae were incubated for 8 h at 38 °C in 300-μl volumes of 0.05 *M* 3,3′-dimethylglutaric acid-NaOH buffer (pH 5.0), each comprising one of the above-mentioned substrates. Control mixtures contained no larvae. Collected supernatants that contained naphthols and naphthylamines liberated from substrates by a proteinase expelled by the larvae were supplemented with fast-black K salt (a naphthol- and naphthylamine-binding reagent), sodium dodecylsulfate (SDS) (that solubilized the reaction products), and a phosphate buffer at pH 7.0 (that optimized the rate of azo-coupling reactions and prevented a pH-dependent shift in absorbance). The resulting colored products were quantified with the aid of a spectrophotometer and calibration curves. The sensitivity of the method was 1 nmol ml^{-1} for 2-naphthylamine, 4-methoxy-2-naphthylamine, 1-naphthol, and 2-naphthol, the corresponding absorbances being centered around 0.04 (with a lightpath of 1 cm).

Results

General observations

Muscle larvae of *T. spiralis* immobilized in agarose-gelatin gels at pH 5.0 released relatively large amounts of proteinaceous substances from their natural body openings (Fig. 1). The staining intensity of the secreta and excreta surpassed that of copolymerized gelatin, thus making impossible to demonstrate the gelatinolytic activity that eventually took place in corresponding areas in the gels. Another experiment performed on the material immobilized in agarose-azocoll gels at pH 5.0 revealed poorly recognizable areas of proteolytic degradation of azocoll around oral openings of the larvae. No proteolytic activity was detected around natural body openings of newborn larvae, presumably due to a relatively low sensitivity of the methods employed.

Microscopic observations revealed a rhythmic, reverse peristaltic activity of the esophagus in the muscle larvae. An initial contraction of esophageal muscles always appeared at the posterior part of the organ (Figs. 2, 4). The resulting peristaltic wave moved toward the mouth and reached the anterior part of the esophagus in about 1 s (at 23 °C). Consequently, the liquid substance released from stichocytes was pumped toward the mouth of the larva and stored for five to six pumping cycles in a short part of the esophagus, situated posterior to the mouth and assuming an ovoid shape. The structure rapidly collapsed after the secretion was expelled through the mouth to the outer environment. These observations indicate that the esophagus of the muscle larva plays no role in feeding, at least while the parasite remains immature, but acts as a permanently working pump that evacuates the proteinaceous secretion produced by stichocytes.

No peristaltic activity of the midgut or hindgut was noticed. Under visible light, the midgut of freshly isolated larvae appeared empty. However, about 0.1% of the specimens that were incubated for 12 h in the medium at pH 5.0 showed the presence of a substance in their midguts that produced strong pale-gold autofluorescence under UV light (Fig. 3). It seems that the appearance of this substance in the midgut of some larvae was associated with degenerative changes rather than with structural and functional transformation that might overwhelm the organ several hours after parasite decapsulation.

Microscopic observations also revealed the presence of two intestinal "accessory" cells situated symmetrically at both sides of the esophago-intestinal junction. The cells were triangular in shape and possessed prominent nuclei and nucleoli (Figs. 5, 6). Their opaque cytoplasm exhibited an intense silver autofluorescence under UV light, although no secretory granules were seen in the cytoplasm (Fig. 9). Since the chemical nature of the fluorescing substance synthesized and stored within the cells is unknown, one can only presume that the substance comprised relatively large percentages of tyrosine, tryptophan, and phenylalanine.

In the vicinity of the two above-mentioned cells was another cell, the cytoplasm of which comprised numerous secretory granules showing no autofluorescence under UV light (Figs. 7, 8). It was probably the cell type that was examined at the electron microscope level by Takahashi et al. (1990), who suggested its endo(exo)-crine nature.

According to Kozek (1975) and Takahashi et al. (1987), the female muscle larvae of *T. spiralis* have a short hindgut and PAS-positive spots in the genital primordium, whereas male specimens have a long hindgut and their genital primordium shows no PAS-positive spots. We found that under UV light, the differentiation between the sexes was very easy. The spot in the female genital primordium (Fig. 10) exhibited strong pale-gold autofluorescence (Fig. 11), whereas no fluorescing spots were observed in the organ of male larvae.

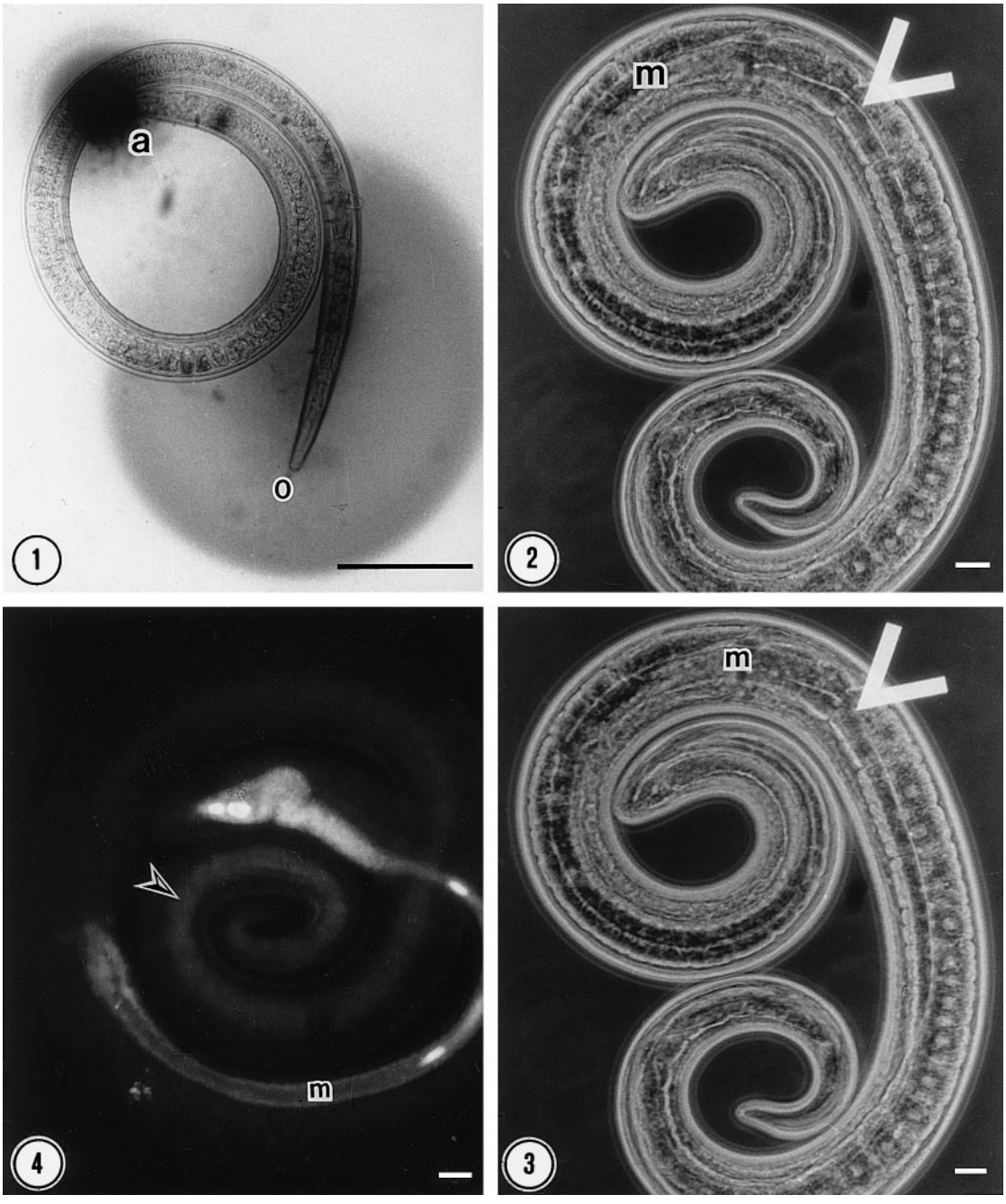
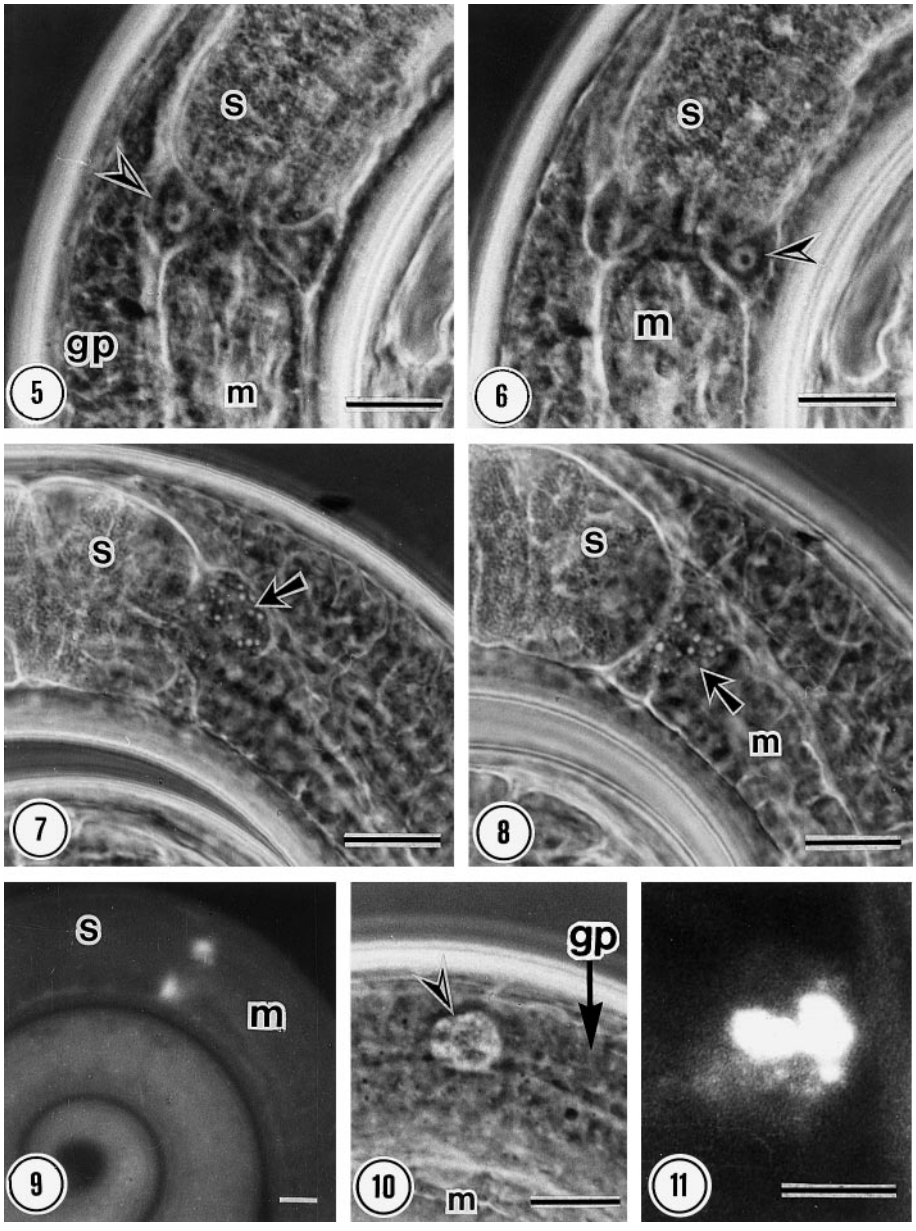


Fig. 1 Staining of proteinaceous substances expelled from mouth (o) and *analpore* (a) of a muscle larva of *Trichinella spiralis* immobilized in an agarose-gelatin medium (*bar* 0.1 mm). **Fig. 2** Strong autofluorescence produced by the midgut (*m*) content in a muscle larva incubated for 18 h at pH 5.0. It is likely that the substance represents products of autolysis of epithelial cells of the organ. The stichosome (*arrowhead*) exhibits poorly visible autofluoresence (*bar* 10 μm). **Fig. 3** The *arrowhead* points at the lumen of a short, 10-μm fragment of the posterior esophagus just before its contraction that initiated the propagation of the peristaltic wave toward the mouth of the larva (*bar* 10 μm). **Fig. 4** The same fragment of the esophagus as in Fig. 3 that entered the phase of contraction, making the lumen invisible. The most posterior fragment of the esophagus, i.e., that visible between the *arrowhead* and the midgut (*m*) showed no peristaltic activity (*bar* 10 μm)

Histochemistry

Most histochemical reactions performed on chopped muscle larvae at pH 5.0 and pH 7.5 gave positive results in somatic muscles, stichosome, midgut, and genital primordium of the muscle larvae (Table 1, Figs. 12–19). The chemical diversity of hydrolyzed substrates indicated multiple proteinases in the larvae. Thus, fourteen *N*-blocked substrates were hydrolyzed by endopeptidases and six *N*-unsubstituted substrates were split by exopeptidases (and perhaps also by endopeptidases). The use of EGTA (an inhibitor of divalent metal-dependent proteinase activities) and PMSF (an inhibitor of serine proteinases) enabled differentiation



Figs. 5, 6 Two intestinal "accessory" cells (*arrowheads*) located at the esophago-intestinal junction in the muscle larva (*s* stichosome, *gp* genital primordium, *m* midgut). See also Fig. 9. (*bars* 10 μm). **Figs. 7, 8** A glandular cell (*arrow*) showing numerous secretory granules in its cytoplasm, at two focal levels. The cell adheres to the outer surface of the midgut and is situated "between" the two intestinal "accessory" cells seen in Figs. 5, 6 (*s* stichosome, *m* midgut; *bars* 10 μm). **Fig. 9** Intestinal "accessory" cells showing autofluorescence under UV light (*s* stichosome region). The glandular cell remains invisible due to the absence of UV-excitable constituents (*m* midgut region, *bar* 10 μm). **Fig. 10** Fragment of genital primordium (*gp*) with a characteristic spot (*arrowhead*) that seems to contain a semiliquid substance (*m* midgut; *bar* 10 μm). **Fig. 11** Strong autofluorescence produced by the substance contained in the spot in the genital primordium of a female larva (*bar* 10 μm)

between enzymes occupying various organs as well as between organ-specific enzymes (Table 1). None of the enzymes detected and shown in Table 1 was a cysteine proteinase, due to the presence of a diazonium salt in the incubation media. However, application of the fluorescence method that produces no inhibition of cysteine proteinases (but works at the pH range 4.0–5.5 only) failed to detect thiol-dependent enzymes in the material.

Proteinase activities in the cells of the genital primordium manifested as fine-grained precipitates dispersed throughout the cells (Figs. 12, 16). Two reaction patterns were observed in the stichosome: a banded and a uniform pattern. The banded pattern, characterized by alternation of heavily stained and weakly stained stichocytes (Figs. 12, 14), was most distinct in one half of the posterior region of the stichosome. Heavy precipitates deposited here resulted from hydrolysis of Ala_2-Phe-NHnapMeO and Gly-Pro-NHnap by two distinct exopeptidases that were active at pH 7.5 (Table 1, test no. 2 and 3). Observations at a high power revealed that these enzymes did not occur in secretory granules, which remained unstained, but were located in the surrounding cytoplasm. The uniform pattern was characterized by the occurrence of finely grained precipitates in the cytoplasm of all stichocytes. The deposits resulted from hydrolysis of the remaining substrates by proteinases whose activity was not associated with secretory granules (Figs. 16–18).

A number of proteinases of unexpectedly high activity were detected in the cells of the midgut (Table 1, Figs. 13, 15, 16, 19). No differences in the occurrence of these enzymes and their sensitivity to inhibitors were found between the cells of the midgut and the "acces-

Table 1 Results of histochemical reactions performed on muscle larvae of *Trichinella spiralis*. Substrates sparingly soluble in water were initially dissolved in *N*,*N*-dimethylformamide. The final concentration of substrates in the incubation media ranged from 0.2 m*M* to 0.5 m*M*. The final concentration of *N*,*N*-dimethylformamide in some media was 10–15%. Proteinase activities evaluated on the basis of the rate of deposition of reaction products are expressed in an arbitrarily established scale from + + + + (very high) to ± (very low), and – (no activity). The following typographic symbols provide further characteristics of respective enzyme activities: ● sensitive to 1 m*M* EGTA, ○ insensitive to 1 m*M* EGTA, ◆ sensitive to 2 m*M* PMSF, ◇ insensitive to 2 m*M* PMSF. Target proteinase refers to pure enzymes, mainly of mammalian origin, for assay of which the corresponding substrates are commonly used. This column is of limited informative value that may be helpful in planning detailed biochemical studies in the future. This is because the use of one substrate does not permit a clear-cut differentiation among various enzymes and because co-operative activity of various organ-specific proteinases under histochemical conditions cannot be excluded. Reaction intensity: when compared horizontally, the inhibitory data allow differentiation between proteinases that hydrolyzed the same substrate but occupied different organs. Vertical comparison of the inhibitory data allows differentiation between various organ-specific proteinases

Test no.	Substrate		pH	Reaction intensity			
	Name	Target proteinase		Somatic muscles	Stichosome	Midgut	Genital primordium
1	*Z*-Ala_2-Lys-NHnapMeO	Plasmin and plasmin-like	5.0	+ + ●◆	–	+ + + ○◆	–
		serine endopeptidases	7.5	+ +	±	+ + +	±
2	Ala_2-Phe-NHnapMeO	Tripeptidyl peptidase II, chymotrypsin,	5.0	±	–	±	–
		and chymotrypsin-like proteinases	7.5	± ○◆	+ + ○◆	+ + + ○◆	± ○◆
3	Gly-Pro-NHnap	Dipeptidyl aminopeptidase IV	5.0	–	–	±	–
			7.5	+ + ●◆	+ + + ●◆	+ + + ●◆	+ + ●◆
4	Lys-Ala-NHnapMeO	Dipeptidyl aminopeptidase II	5.0	–	–	–	–
			7.5	± ○◆	+ + ○◆	+ + + ○◆	± ○◆
5	*Z*-Gly_2-Arg-NHnapMeO	Trypsin, trypsin-like, and	5.0	±	±	±	±
		some cysteine endopeptidases	7.5	+ + + +	+	+ +	±
6	*Ac*-Gly-Lys-Onap	Trypsin, trypsin-like, and	5.0	+	+	+	+
		some cysteine endopeptidases	7.5	+ +	+	+	+
7	Ser-Tyr-NHnapMeO	Dipeptidyl aminopeptidase I	5.0	+ + ○◆	+ ○◆	+ + + + ○◇	+ + ○◆
			7.5	+ ●◆	+ ●◆	+ + + + ○◇	+ ●◆
8	*Z*-Pro-Ala-Gly-Pro-NHnapMeO	Collagenolytic endopeptidases	5.0	–	–	–	–
			7.5	+	+ +	+	+ +
9	*Ac*-Met-Onap	Esterproteinases, many serine,	5.0	–	–	±	–
		cysteine, and metalloendopeptidases	7.5	±	±	±	±
10	His-Ser-NHnapMeO	Dipeptidyl aminopeptidase I	5.0	±	–	±	±
			7.5	+	–	±	+
11	*Z*-Arg_2-NHnapMeO	Trypsin, trypsin-like, and	5.0	+	–	+	–
		cysteine endopeptidases	7.5	–	–	–	–
12	*Z*-Ala-NHnapMeO	Elastase and elastase-like	5.0	–	–	–	–
		serine endopeptidases	7.5	+ +	–	–	–
13	*Z*-Phe-Arg-NHnapMeO	Serine and cysteine endopeptidases	5.0	+	–	+	–
			7.5	–	–	–	–
14	*Bz*-Phe-Val-Arg-NHnapMeO	Thrombin, trypsin-like, and	5.0	–	–	–	–
		some cysteine endopeptidases	7.5	+	–	+	–
15	*Z*-Gly-Pro-Arg-NHnapMeO	Thrombin and trypsin-like	5.0	–	–	–	–
		endopeptidases	7.5	+	–	–	–
16	*Z*-Ala-Onap	Esterproteinases, elastase-like,	5.0	–	–	–	–
		and some cysteine endopeptidases	7.5	–	±	–	–
17	Glt-Gly_2-Phe-NHnap	A highly specific substrate for	5.0	–	–	–	–
		chymotrypsin	7.5	–	±	–	–
18	*Bz*-Phe-Onap	Chymotrypsin, and chymotrypsin-like	5.0	–	–	–	–
		endopeptidases	7.5	–	–	±	–

19	Pro-Lys-Ala-NHnapMeO	Tripeptide aminopeptidase	5.0	−	−	−	−	−	−
			7.5	−	−	±	−	−	−
20	*Z*-Arg-NHnap	Trypsin, trypsin-like and cysteine endopeptidases	5.0	−	−	−	−	−	−
			7.5	−	−	−	−	±	−
21	*Bz*-Leu-NHnap	Some "neutral" endopeptidases	5.0	−	−	−	−	−	−
			7.5	−	−	−	−	−	−
22	Leu-Gly_2-NHnapMeO	Tripeptide aminopeptidase	5.0	−	−	−	−	−	−
			7.5	−	−	−	−	−	−

sory" cells. This indicated that from a histogenetic point of view, "accessory" cells belong to the midgut cell line.

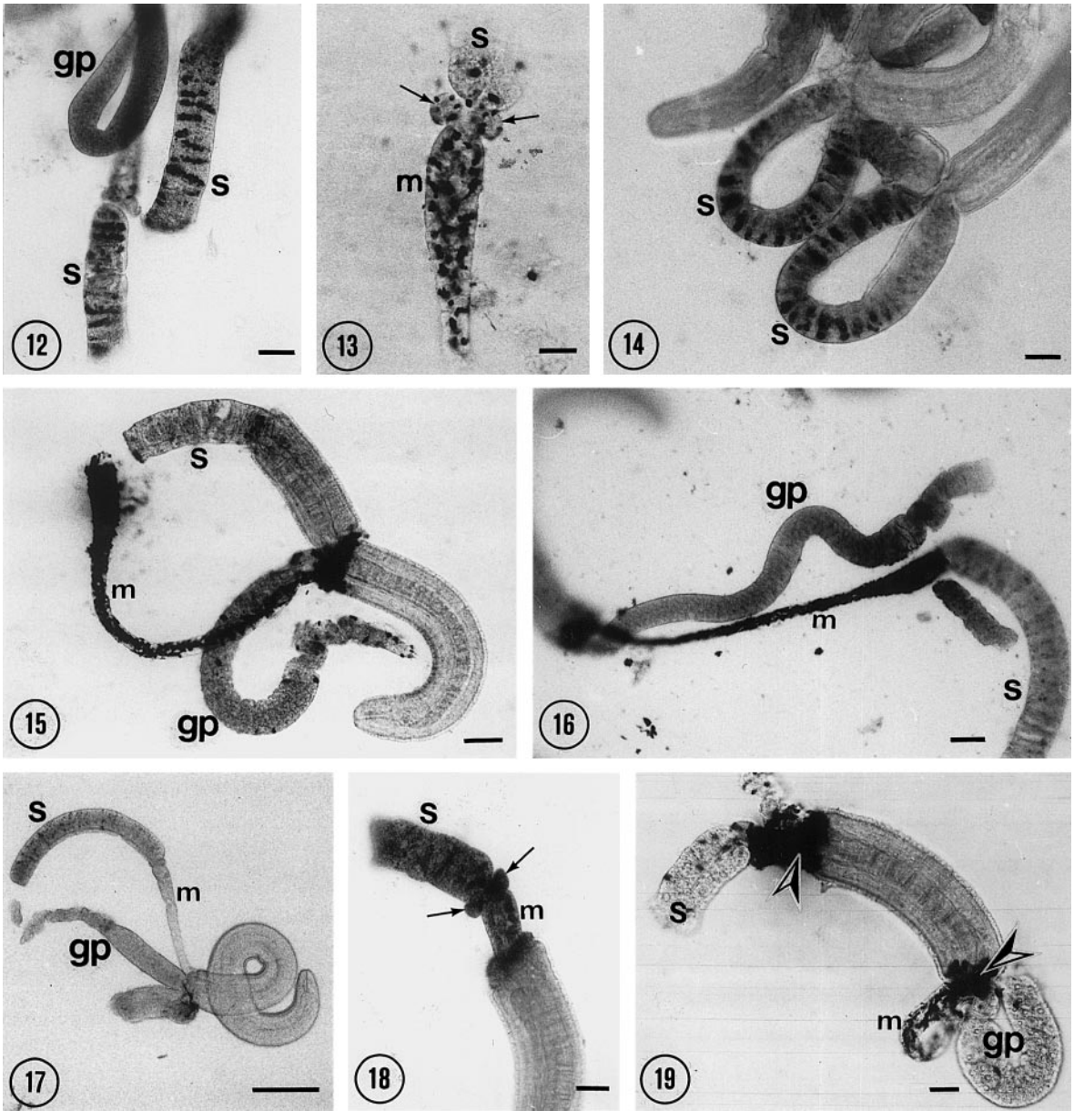
A number of proteinases were also detected in subcuticular (somatic) muscles of the larvae. However, results of particular reactions could only be observed in those muscle fragments which were evaginated as a result of mechanical damage to the cuticle. Larvae undamaged prior to their drying and fixation remained totally unstained due to the impermeability of their cuticles to the substrates present in the incubation media (Fig. 19).

A number of substrates were hydrolyzed in the cells of the newborn larvae. Hydrolysis of *Z*-Arg-NHnap, *Z*-Ala_2-Lys-NHnapMeO, Ala_2-Phe-NHnapMeO, Gly-Pro-NHnap, Lys-Ala-NHnapMeO, His-Ser-NHnapMeO, *Bz*-Phe-Val-Arg-NHnapMeO, Glt-Gly_2-Phe-NHnap, and *Z*-Gly-Pro-Arg-NHnapMeO at pH 7.5 by an unknown number of proteolytic enzymes resulted in the appearance of fine-grained precipitates in some cells of the larvae (Fig. 20). An endopeptidase that hydrolyzed *Ac*-Gly-Lys-Onap at pH 6.8 (Fig. 21) occupied the subtegumental muscle system and was resistant to aldehyde fixation (2% formaldehyde-0.5% glutaraldehyde at pH 7.2, 60 min at 22 °C) as well as to 1 m*M* PMSF, 1 m*M* Sodium *p*-hydroxymercuribenzoate (pHMB), and 1 m*M* EGTA. A proteinase that hydrolyzed Ala_2-Phe-NHnapMeO at pH 7.4 and was aldehyde-sensitive occurred in relatively large cells situated in the middle part of the larvae (Fig. 22). Since the future product of the differentiation of these cells is unknown, we could only suppose that the cells represented the prospective genital primordium. The activity of another, probably "soluble," endopeptidase manifested itself as a non-particulate, diffuse mass of precipitates dispersed throughout the larva body resulting from hydrolysis of the collagenase substrate *Z*-Pro-Ala-Gly-Pro-NHnapMeO at pH 7.5. The enzyme was sensitive to 1 m*M* EGTA and to aldehyde fixatives.

Yet another proteinase hydrolyzed *Ac*-Met-Onap at optimal pH 6.8 and was most intriguing, since it was detectable at the mouth (Figs. 23, 24), in the lumen of the anterior esophagus (Fig. 25), and at or around the anterior esophagus (Figs. 25–28). The droplet-like pattern of the reaction, together with the localization of the reaction product, suggested the secretory nature of the enzyme. PMSF (1 m*M*) and 1 m*M* EGTA inhibited the enzyme, indicating its membership of the family of serine proteinases. Fixation in the acetone-chloroform mixture decreased its activity by approximately half. The enzyme was undetectable in specimens treated with aldehyde fixatives.

Gelatinolysis

The X-ray film test revealed the presence of three gelatinolytic enzymes in the extract from newborns. The first was presumably a cathepsin-B-like cysteine proteinase that was active at pH 5.0 in the presence of both 1 m*M*



EGTA and 1 m*M* DTE but became inactive in the presence of 0.1 m*M* pHMB. The second enzyme was a Ca^{2+}-dependent serine proteinase that was active at pH 5.0–6.0 but became inactive in the presence of either 1 m*M* EGTA or 2 m*M* PMSF. It was probably the same enzyme that hydrolyzed *Ac*-Met-Onap under histochemical conditions. The third proteinase that was detected at pH 7.6 represented a collagenase-like, Ca^{2+}-dependent enzyme that was activated by 0.1 m*M* pHMB but became inactive in the presence of 1 m*M* EGTA. It is possible that this proteinase and the histochemically detectable one that hydrolyzed *Z*-Pro-Ala-Gly-Pro-NHnapMeO were the same enzyme. The gelatinolytic activity of the three proteinases was relatively low, resulting merely in an erosion of the surface of the photographic emulsion after as long an incubation period as 20 h, instead of the expected removal of the gelatin layer.

Proteolytic activity

The best way to evaluate proteinase activity in E/S expelled by the muscle larvae was simultaneous collection of the E/S and the exposure of azocoll to a successively increasing amount of the enzyme in the incubation medium at pH 5.0. Under these conditions, as much as 0.6 g of muscle larvae produced an amount of proteinase that hydrolyzed approximately 4 mg of the substrate after 18 h incubation at 38 °C. In the absence of azocoll, the enzyme apparently underwent gradual inactivation, possibly by self-degradation, since post-collection assays (consisting of 18 h exposure of azocoll to the E/S that had previously been collected for 18 h) revealed small amounts of hydrolyzed substrate about 2.8 mg.

Inhibition studies revealed the presence of a cysteine proteinase in the E/S (Table 2). Thus, 1 m*M* $CaCl_2$ re-

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Figs. 12–19 Examples of some histochemical reactions performed on the muscle larvae and summarized in Table 1. As a result of the pretreatment with hypotonic saline, the chopped larvae underwent an explosive "evisceration" that enabled substrates to pass into cells of particular organs. **Fig. 12** Test no. 3 performed at pH 7.5. High activity of a proteinase that hydrolyzed Gly-Pro-NHnap in some cells of the stichosome (*s*) and in the cells of the genital primordium (*gp*) (*bar* 50 μm). **Fig. 13** Test no. 3 at pH 7.5. High activity of a proteinase that hydrolyzed Gly-Pro-NHnap in the cells of the midgut (*m*) and in the intestinal "accessory" cells (*arrows*) (*s* a small fragment of the stichosome) (*bar* 10 μm). **Fig. 14** Test no. 2 at pH 7.5. Hydrolysis of Ala_2-Phe-NHnapMeO by a proteinase that was very active in some stichosome cells (*s*) (*bar* 50 μm). **Fig. 15** Test no. 7 at pH 7.5. Very high activity of a proteinase that hydrolyzed Ser-Tyr-NHnapMeO in the midgut (*m*). Another enzyme of very low activity split the same substrate in the stichosome (*s*) and genital primordium (*gp*) (*bar* 50 μm). **Fig. 16** Test no. 7 at pH 5.0. Very high activity of a proteinase that hydrolyzed Ser-Tyr-NHnapMeO in the midgut (*m*). Another "acid" proteinase that split the same substrate exhibited very low activity in the stichosome (*s*) and moderate activity in the genital primordium (*gp*) (*bar* 50 μm). **Fig. 17** Test no. 17 at pH 7.5. Very low activity of an endopeptidase that hydrolyzed Glt-Gly_2-Phe-NHnap in the stichosome (*s*). No proteolytic activity occurred in the midgut (*m*) and genital primordium (*gp*) (*bar* 50 μm). **Fig. 18** Test no. 8 at pH 7.5. Moderate activity of an enzyme that hydrolyzed *Z*-Pro-Ala-Gly-Pro-NHnapMeO in the stichosome (*s*), midgut (*m*), and intestinal "accessory" cells (*arrows*). The reaction product is uniformly dispersed throughout stichocytes (*bar* 50 μm). **Fig. 19** Test no. 5 at pH 7.5. An example of very high activity of a *Z*-Gly_2-Arg-NHnapMeO-splitting endopeptidase in somatic muscles (*arrowheads*). Note that the reaction product was deposited only in evaginated, naked muscle fragments, whereas those protected by the cuticle remained unreactive (*bar* 50 μm)

duced the azocollytic activity by about 20%, whereas 1 m*M* DTE and the mixture of 1 m*M* EGTA and 1 m*M* DTE increased it by about 60% and 70%, respectively. At 10 μ*M*, leupeptin, an inhibitor of thiol-dependent proteinases, was ineffective, presumably due to the inactivation of its aldehyde group by an excess of amines excreted by the larvae. However, 100 μ*M* leupeptin depressed the enzyme activity and 2 m*M* PMSF, a non-specific inhibitor of cysteine proteinases, reduced it by about 70%. For an unknown reason, 0.1 m*M* pHMB, a specific inhibitor of cysteine proteinases was without effect and STI (50 μg ml^{-1}) elevated the azocollytic activity by about 24%. The ineffectiveness of 5 μ*M* pepstatin A (an inhibitor of aspartyl proteinases) and 2 m*M* 1,10-phenanthroline (an inhibitor of zinc-dependent metalloproteinases) proved the absence of these enzymes in the E/S.

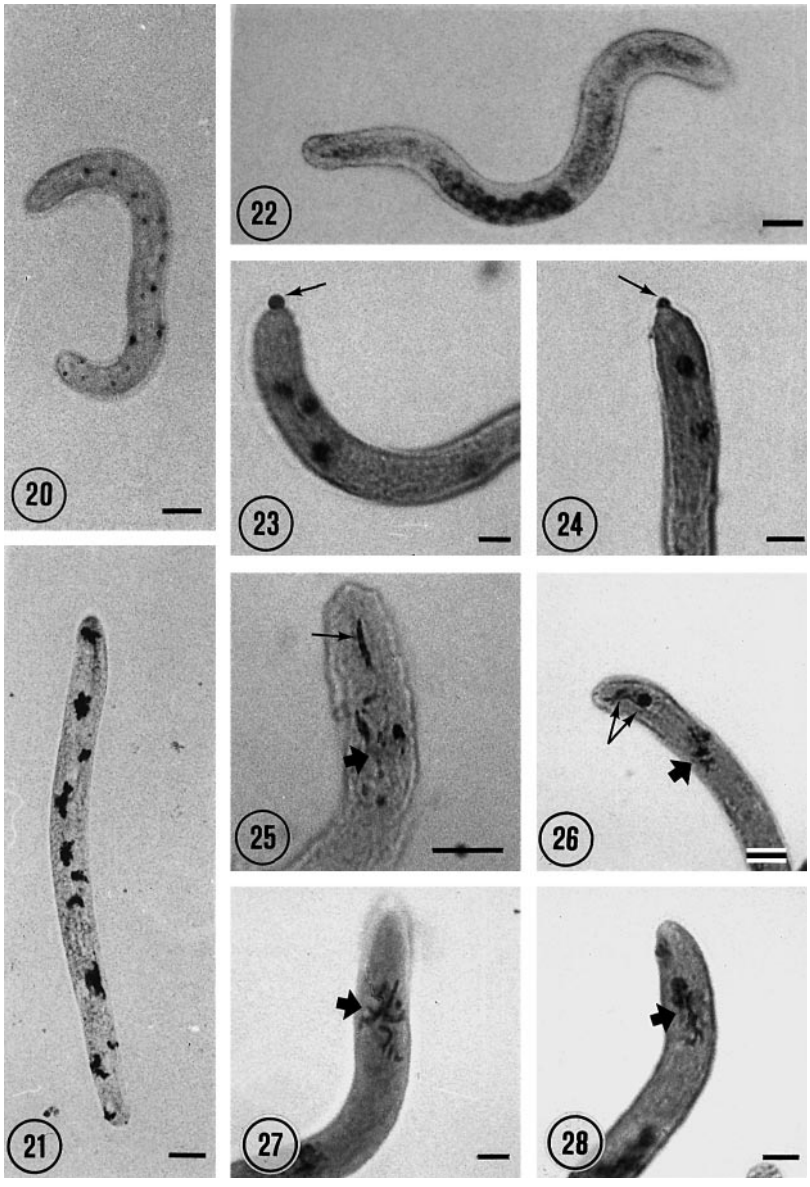
No proteolytic activity in the E/S was detected using a number of artificial proteinase substrates. This indicates that the secretory proteinase was unable to attack short-chain synthetic peptides.

Discussion

When ingested by a host, muscle larvae of *T. spiralis* enter gut enterocytes where they continue their development into mature worms (Wright 1979). The larvae do not settle themselves permanently within one or more cells but continuously migrate through the gut epithelium (Dunn and Wright 1985). However, the exact mechanism of enterocyte penetration by the larvae is unknown. Criado-Fornelio et al. (1992) suggested that proteolysis may facilitate the process of penetration, since under in vitro conditions, the muscle larvae secrete and/or excrete minute quantities of gelatinolytic enzymes. Later, Armas et al. (1993) reported that the only proteolytic enzyme expelled by the muscle larvae was a cysteine proteinase that lacked disulfide bonds.

The results of our biochemical studies support those reported by Armas et al. (1993). The azocollytic proteinase secreted by the muscle larvae was activated by the reducing compound DTE and was sensitive to leupeptin and to a non-specific cysteine proteinase inhibitor PMSF. The source of the enzyme was the stichosome as was shown by the experiment on larvae immobilized in the agarose-azocoll medium. Though the histochemical studies revealed the presence of multiple proteinase activities occupying particular organs of the muscle larvae, none of the synthetic substrates used in biochemical assays was hydrolyzed at a measurable rate by the enzyme secreted by the parasite. Searching for an elastinolytic activity in the E/S in the pH range 5.0–8.6 (at 0.4-pH intervals and using elastin-orcein as a substrate) was also unsuccessful.

Both the rate of azocoll degradation and the data on inhibition varied from stock to stock of freshly decapsulated larvae. Therefore, a number of supplementary experiments had to be performed to account for the sources of these variations. The results of these experiments led us to draw the following conclusions that may be helpful for those interested. (a) The peptic digestion should be carried out under slightly hypertonic conditions that slow down the secretory activity of the decapsulated larvae. For this purpose, the digestion fluid may be supplemented either with mannitol or sorbitol, but not with sucrose, that add to the viscosity of the mixture, thus prolonging the digestion procedure. (b) Purification of the material should not be prolonged, since most proteinase activity is expelled 4–5 h after activation of the larvae. (c) To avoid contamination of the E/S with serine proteinases of host origin, the larvae must be perfectly separated from undigested particles of host tissues (mainly of adipose and connective tissue) and extensively washed with an appropriate, slightly hypertonic medium in order to remove host glycoconjugates and, particularly, phospholipid micelles that might retain active enzyme molecules of host origin and liberate them during the incubation of the parasites. Use of the non-ionic detergent Triton WR-1339 is therefore recommended. (d) When incubated in a non-nutritive isotonic medium at 38 °C, the muscle larvae secrete the proteinase for about 8 h, i.e., as long as their esophagi exhibit reversed peristalsis. When the peristalsis ceases, apparently due to the exhaustion of glycogen reserves of esophageal muscles, practically no more proteolytic activity is secreted by the larvae. If absolutely necessary,



Figs. 20–28 Patterns of some histochemical reactions for the presence of proteinases in newborn larvae of *T. spiralis*. **Fig. 20** Result of hydrolysis of *Z*-Arg-NHnap by an endopeptidase located in some cells of a newborn and active at pH 7.4 (*bar* 10 μm). **Fig. 21** Hydrolysis of *Ac*-Gly-Lys-Onap at pH 6.8 by an endopeptidase insensitive to aldehydes, 1 m*M* PMSF, 1 m*M* pHMB, and 1 m*M* EGTA. The reaction product is deposited around places of increased permeability of the cuticle. In mechanically damaged newborns, the reaction product was spread all over the subcuticular muscle system (*bar* 10 μm). **Fig. 22** Hydrolysis of Ala_2-Phe-NHnapMeO at pH 7.5 by a proteinase localized in a group of cells that might represent the prospective genital primordium (*bar* 10 μm). **Figs. 23, 24** Localization of the activity of an *Ac*-Met-Onap-splitting endopeptidase in unfixed newborns, at an optimal pH of 6.8. The reaction product is deposited in small drops of a liquid substance expelled from the mouth (*arrows*) (*bars* 10 μm). **Figs. 25, 26** Same Figs. 23, 24. Positive reaction in the esophageal lumen (*thin arrows*) and at or around the anterior part of the esophagus where glandular cells filled with secretory granules (i.e., the prospective stichosome) are believed to occur (*thick arrow*) (*bars* 10 μm). **Figs. 27, 28** Other aspects of enzymic reaction of hydrolysis of *Ac*-Met-Onap in the newborn larvae (*bars* 10 μm)

the incubation time may be prolonged for up to 18 h with a slight chance of contamination of the medium by non-secretory proteinases mainly deriving from midgut cells that underwent autolysis as a result of the exhaustion of their energy sources. (e) The use of an isotonic, glucose-containing medium at pH 5.0 to retard the glycogen exhaustion is of no special benefit.

The very low azocollytic activity of the proteinase that was continuously expelled by 0.6 g of the larvae maintained in vitro (approximately 4 mg of azocoll was hydrolyzed after 18 h of incubation at 38 °C) makes doubtful the supposition that the enzyme might facilitate the penetration of host enterocytes by the larvae. It also seems that the presumptive destructive effect of the enzyme on host enterocytes invaded by the parasite can be insignificant in comparison with cytotoxic effects produced by a number of amines (Haskins and Weinstein 1957), volatile fatty acids, products of glucose fermentation (Barrett 1981), and other waste metabolites secreted and excreted by the larvae.

Both the exact localization and the physiological role of the secretory proteinase remain unknown. Since the enzyme is secreted from stichocytes, it may be synthesized and stored there as one of at least five types (Takahashi et al. 1989) of secretory granules. But if it occurs in the cytoplasm, it may participate in the synthesis of certain secretory proteins and/or in the formation and maturation of secretory granules. In the latter case, the release of the proteinase would be a consequence of cell membrane recycling and relatively poor control over intracellular processing and compartmentalization of the enzyme. However, the presence of the proteinase in the secretion from stichocytes may be indispensable for converting some biologically inactive secretory products into active ones, by means of limited proteolysis.

Table 2 Effect of inhibitors/activators on azocollytic activity of the proteinase secreted by muscle larvae of *T. spiralis* and assayed at pH 5.0. Values represent means of duplicate assays. Results are expressed as a percentage of reduction/increase in absorbance compared with an additive-free control

Compound added	Inhibition/activation
None	100.0
$CaCl_2$ 1 m*M*	81.3
EGTA 1 m*M*	96.0
DTE 1 m*M*	161.0
EGTA 1 m*M* + DTE 1 m*M*	168.8
PMSF 2 m*M*[a]	31.0
pHMB 0.1 m*M*	95.6
Leupeptin 10 μ*M*	92.7
Leupeptin 100 μ*M*	5.4
STI 50 μg ml^{-1}	124.7
1,10-Phenanthroline 2 m*M*[b]	101.2
Pepstatin A 5 μ*M*[c]	99.4

[a] Prepared from a 0.2 *M* stock solution in anhydrous isopropanol
[b] Prepared from a 0.5 *M* stock solution in dimethylsulfoxide
[c] Prepared from a 1 m*M* stock solution in *N*,*N*-dimethylacetamide

A relatively intense and easily detectable hydrolysis of some proteinase substrates in muscle larvae both at pH 5.0 and 7.5 indicates rapid protein turnover in the cells of the examined organs. High proteinase activities in the stichosome can be explained by a high rate of synthesis of secretory proteins in this organ. Two proteinases that produced banded reactions in stichosomes (Figs. 12, 14; Table 1, test no. 2, 3) were probably located in α-stichocytes, being involved there in the synthesis and/or maturation of secretory granules of the α type. Similar banding that resulted from staining of muscle larvae with the AZAN method was observed by Takahashi et al. (1988a) and interpreted as being characteristic for the secretory granules of the α type. High proteinase activities in the genital primordium reflect high trophic requirements of proliferating and maturing cells in this organ. Unexpectedly high proteinase activities in the cells of the midgut are somewhat puzzling, since this organ plays no digestive or food-storing role in *Trichinella* muscle larvae. The wall of the midgut consists of a single layer of epithelial cells which show no signs of secretory activity (Takahashi et al. 1988b). Lack of muscle cells in the midgut wall (Takahashi et al. 1988b) precludes the interference of proteolytic activity of other origin with that existing in the epithelial cells. An apparently rapid protein turnover in the midgut cells suggests that the midgut plays a role resembling that of the liver in higher animals i.e., it processes simple nutritional compounds and (inter)converts peptide metabolites, supplying the whole organism via pseudocoelomic fluid-mediated transport, with peptide substrates indispensable for growth and renewal. Further work is required to solve this enigma.

The ultrastructural studies of Bruce (1974) revealed the presence of stichocytes (or stichocyte precursor cells) in in utero juveniles of *T. spiralis* as early as 12 h before parturition. The presence of secretory granules in these cells was clearly demonstrated by this author. The juveniles penetrating a muscle cell 5–6 days post-parturition had a well-developed stichosome, the secretory activity of which was evident due to the presence of the product of the secretion in the esophageal lumen. We have detected the activity of a serine proteinase at the mouth, in the esophageal lumen, and at or around the anterior part of the esophagus where stichocytes loaded with secretory granules are believed to occur in 48- to 72-h-old juveniles (Figs. 23–28). Our results indicate involvement of the *Ac*-Met-Onap-hydrolyzing enzyme in the synthesis of secretory proteins in stichocytes. Like the muscle larvae, the juveniles secrete the enzyme via their mouth. It remains to be established to what degree the proteinase activity is involved in the penetration of muscle cells by the larvae.

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