

# Single-stranded endonuclease activity in the excretory–secretory products of *Trichinella spiralis* and *Trichinella pseudospiralis*

C. H. MAK, Y. Y. Y. CHUNG and R. C. KO\*

Department of Zoology, The University of Hong Kong, Pokfulam, Hong Kong, China

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## SUMMARY

A novel acidic extracellular single-stranded endonuclease was demonstrated for the first time in the excretory–secretory (E–S) products of 2 species of *Trichinella*. Unlike the double-stranded endonuclease reported earlier, the single-stranded molecule is divalent cation independent and is detected in both *T. spiralis* and *T. pseudospiralis* E–S products. It hydrolysed single-stranded DNA and RNA at comparable rates. The single-stranded endonuclease was sensitive to inhibition by  $Zn^{2+}$  and to high concentrations of NaCl. Zymographic analysis indicated that it was encoded by at least 3 peptides of  $M_r \sim 50$ –60 kDa. The rate of hydrolysis of single-stranded targets by the E–S products was substantially higher than that of the double-stranded molecule. Due to the differences in peptide profile, divalent cation dependence, and species-specific expression, the single and double-stranded endonucleases are likely to be encoded by different proteins and may have different functions.

Key words: endonuclease, *Trichinella spiralis*, *Trichinella pseudospiralis*, single-stranded endonuclease, excretory–secretory products.

## INTRODUCTION

*Trichinella spiralis* is a pathogenic nematode which parasitizes the striated muscles of mammals. The worm can reorganize the host muscles into a new syncytium known as the nurse cell complex which is circumscribed by collagenous fibres. *Trichinella pseudospiralis* is a closely related species which can infect both mammals and birds. However, the latter does not induce the formation of nurse cells and the site of the worm is not encapsulated. The worm can move freely along the myofibres, eliciting little inflammatory response.

In an earlier study, we detected the activity of double-stranded endonuclease in the excretory–secretory (E–S) products of the infective-stage larvae of *T. spiralis* maintained at 37 °C, but much weaker in the E–S products of *T. pseudospiralis* (Mak & Ko, 1999). Since endonucleases have not been found previously in parasitic organisms, their functions are completely unknown.

In other microorganisms, such as *Mycoplasma*, endonuclease is well known to play a significant role in modulating the genomic expression of host cells or act as a potential pathogenic determinant (Bendjennat *et al.* 1997). In Epstein–Barr virus, it provides nucleotides for DNA synthesis, and in herpes simplex virus, it pertains a host genome-shut off

function (Feighny, Henry & Pagano, 1981; Krikorian & Read, 1991). Similar to these microorganisms, *Trichinella* may also possess an endonuclease that is directly or indirectly involved in the transformation of host cells (Ko & Mak, 1999). Our discovery of endonuclease in a parasite may have broad biological implications. It now appears that during evolution, parasitic nematodes may have adopted the same strategy as viruses, bacteria and other microorganisms in the colonization of cells.

Recently, we have discovered that, besides the double-stranded endonuclease, a single-stranded molecule also occurs in the E–S products of *T. spiralis* and *T. pseudospiralis*. Since the two endonucleases are distinct in their peptide profile, cation dependence and species-specific expression, it would be worthwhile to report on the characterization of the novel single-stranded endonuclease.

## MATERIALS AND METHODS

### *Trichinella* E–S products

Infective-stage larvae of *T. spiralis* or *T. pseudospiralis* were isolated from muscles of experimentally infected ICR mice by the standard pepsin digestion method. After recovery, the larvae were washed twice with DMEM medium supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml), and counted under a stereo-microscope. E–S products from *T. spiralis* (TSES) or *T. pseudospiralis*

\* Corresponding author. Tel: +852 2299 0835. Fax: +852 2559 9114. E-mail: rcko@hkucc.hku.hk

(TPES) were obtained by incubating viable larvae (10000 larvae/ml) at 37 °C for 24 h in DMEM medium supplemented with antibiotics and complete proteases inhibitor (Boehringer Mannheim). Fifteen millilitres of E-S products were concentrated 100-fold by ultrafiltration using Centriplus-10 (Amicon). Protein concentrations were determined by the Bradford dye binding procedure (BioRad). Concentrated E-S products were then analysed by SDS-PAGE and visualized by Coomassie blue staining.

To inhibit the worm from producing E-S products, freshly isolated larvae were inactivated by freezing/thawing 3 times in liquid nitrogen/37 °C water bath. The treated larvae were washed with DMEM medium and incubated as described above.

E-S products were pre-cleared by immunoprecipitation. Anti-E-S IgG antibodies were immobilized on Protein A-agarose as described (Mak & Ko, 1999). Pre-immune serum and antiserum raised against the *T. spiralis* crude extract were used as a control. IgG-Protein A-agarose was incubated with 50 µg TSES at room temperature for 1 h. Protein A-antibody-antigen complexes were removed from the E-S products by centrifugation. Pre-cleared E-S products were used in the nuclease assays.

#### Preparation of endonuclease substrates

M13mp19(+) single-stranded circular DNA and 7.5 kb poly(A)-Tailed RNA were purchased from Life Technologies. Single-stranded salmon sperm DNA was generated by boiling the native DNA for 10 min, followed by snap-cooling on ice. Linear plasmid DNA was obtained by digesting the pBluescript SK II plasmid with restriction enzyme *EcoR* V.

#### pH, monovalent and divalent cation dependence

Standard mixtures containing 0.5 µg M13 single-stranded DNA or 0.5 µg poly(A) RNA were incubated for 10 min with 1 µg *T. spiralis* E-S products in reaction buffer (10% glycerol, 40 mM Tris-HCl/Tris, pH 4.0–9.0, 50 mM NaCl, 1 mM DTT) supplemented with 10 mM EDTA. To determine the divalent cation dependence of the endonucleases, the reaction buffer at pH 6.0 was supplemented with 2.5 mM different divalent cations ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ), 5 mM  $\text{Zn}^{2+}$  ion or 2.5/10 mM EDTA at conditions as described. To determine the monovalent cation dependence, the reaction buffer at pH 6.0 with 10 mM EDTA was supplemented with NaCl at concentrations from 10 to 500 mM. Proteins were removed from the mixture by adding 20 µg proteinase K and 0.5% SDS and incubated for 10 min at 37 °C. The digested products were resolved in 1% agarose gel and visualized by staining with ethidium bromide.

#### Kinetics of single and double-stranded endonuclease

One microgram of linearized pBluescript SK II plasmid was incubated with 1 µg TSES or TPES in reaction buffer (10% glycerol, 40 mM Tris-HCl, pH 6.0, 50 mM NaCl, 1 mM DTT, 2.5 mM  $\text{MnCl}_2$ ) at 37 °C for 60–240 min. Finally 0.5 µg M13 single-stranded DNA or poly(A) RNA was incubated with 1 µg E-S products in the reaction buffer supplemented with 10 mM EDTA at room temperature for 2.5–10 min.

#### Zymographic analysis of endonuclease activity

The pattern of the endonucleases detected was examined using established procedures, with several modifications (Rosenthal & Lacks, 1977). First, 20 µg/ml of denatured salmon sperm DNA, and secondly, 10 µg/ml bovine serum albumin were incorporated into 12% separating gels (containing 0.1% electrophoresis grade SDS) (Boehringer Mannheim). *T. spiralis* crude extracts, E-S products of *T. spiralis* and *T. pseudospiralis* were loaded into the gels and resolved by electrophoresis at 40 mV until the dye reached the bottom. SDS was then removed by overnight incubation at room temperature in 2 changes of 250 ml of renaturation buffer (40 mM Tris-HCl, pH 7.0, 0.04% 2-mercaptoethanol, 2 mM EDTA, 1% casein), with constant agitation. The nuclease activity of the resolved peptides was reactivated by rinsing the gel twice with distilled water, before it was transferred to 250 ml of activation buffer (0.04 M Tris-HCl, pH 6.0, 50 mM NaCl, 1 mM DTT, 10% glycerol and 10 mM EDTA). The gel was incubated statically at room temperature for 24 h. DNA was visualized by staining with ethidium bromide at the concentration of 2 µg/ml for 30 min. Degraded DNA would not be stained by ethidium bromide and appeared as dark bands in the gel. After destaining in 250 ml of autoclaved water for 30 min, the pattern of nuclease activity was recorded by the Gel Doc 100 system (BioRad).

## RESULTS

#### Nuclease activity between E-S products of different species

To compare the mode of action of endonucleases between E-S products of *T. spiralis* and *T. pseudospiralis*, linear plasmid DNA, M13mp19(+) single-stranded circular DNA and 7.5 kb poly(A) RNA were incubated with various E-S products and the digested products were resolved in 1% agarose gel. Activities of both single-stranded and double-stranded endonucleases were detected in *Trichinella* E-S products. Within 10 min, E-S products from *T. spiralis* (TSES) and *T. pseudospiralis* (TPES) com-

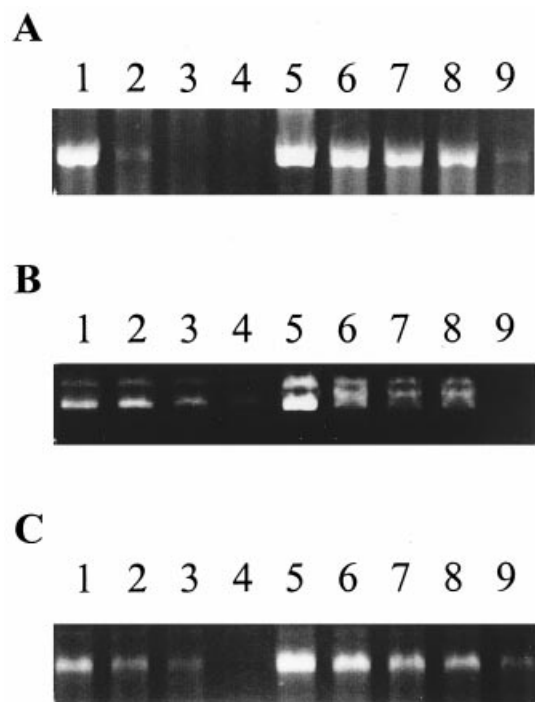


Fig. 1. Rate of hydrolysis of dsDNA, ssDNA and RNA by TSES and TPES. Double-stranded (ds) DNA was incubated with 1  $\mu$ g E-S products in pH 6.0 reaction buffer supplemented with 2.5 mM  $Mn^{2+}$  ion at 37 °C for 60–240 min. Single-stranded (ss) DNA and RNA were incubated with the E-S products in pH 6.0 buffer containing 10 mM EDTA for 2.5–10 min. (A) 1  $\mu$ g dsDNA was incubated with 1  $\mu$ g TSES (lanes 1–4) and TPES (lanes 6–9) for various time periods; lane 5: substrate alone; lanes 1 and 6: 60 min, lanes 2 and 7: 120 min, lanes 3 and 8: 180 min, lanes 4 and 9: 240 min. (B) 0.5  $\mu$ g ssDNA and (C) RNA were incubated with TSES (lanes 1–4) and TPES (lanes 6–9) for various time periods; lane 5: substrate alone; lanes 1 and 6: 2.5 min, lanes 2 and 7: 5 min, lanes 3 and 8: 7.5 min and lanes 4 and 9: 10 min. The digested products were analysed in 1% agarose gel, and stained with ethidium bromide.

pletely degraded the single-stranded DNA and RNA (Fig. 1B, C). The rate of hydrolysis of the single-stranded DNA and RNA was comparable in the 2 E-S products. However, only TSES degraded linearized plasmid after 120 min incubation. Similar incubations of TPES with dsDNA did not degrade a significant amount of DNA (Fig. 1A). A substantially weaker double-stranded endonuclease activity was expressed in TPES. As compared to the native dsDNA, all E-S products attacked the single-stranded polynucleotides at a markedly higher rate, indicating the substrate preference of the endonuclease for single-stranded DNA and RNA.

#### Biochemical properties of endonuclease activity

TSES single-stranded endonuclease activity was divalent cation independent, and fully active in the

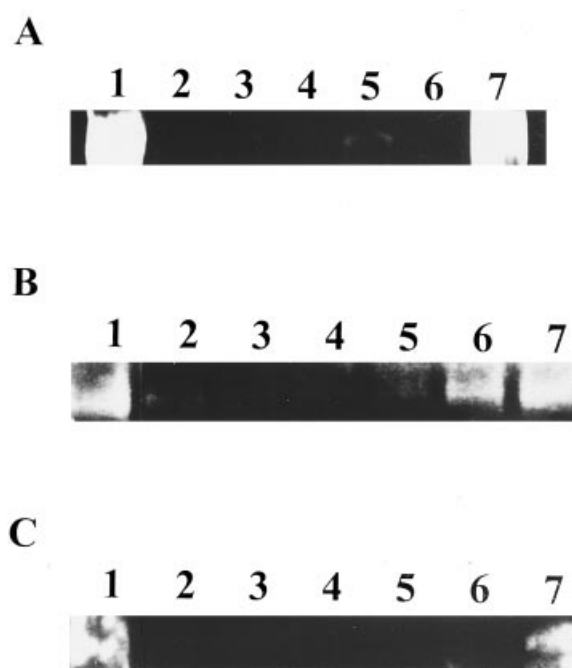


Fig. 2. Effect of cation, pH and ionic strength on the nuclease activity. Samples of 0.5  $\mu$ g circular single-stranded DNA were incubated with 1  $\mu$ g *Trichinella spiralis* E-S products in various reaction buffers for 10 min. (A) Effect of divalent cations. The experiment was performed in pH 6.0 buffer containing 50 mM NaCl. Lane 1, substrate alone; lanes 2–7: 2.5 mM EDTA, 10 mM EDTA, 2.5 mM  $Ca^{2+}$ , 2.5 mM  $Mg^{2+}$ , 2.5 mM  $Mn^{2+}$  and 5 mM  $Zn^{2+}$ , respectively. (B) Effect of pH. The experiment was performed in buffer containing 50 mM NaCl and 10 mM EDTA. Lane 1: substrate alone; lanes 2–7: pH 4, 5, 6, 7, 8 and 9, respectively. (C) Effect of ionic strength. The experiment was performed in pH 6 buffer supplemented with 10 mM EDTA. Lane 1: substrate alone; lanes 2–7: 10, 25, 50, 100, 250, 500 mM NaCl, respectively. Reaction products were separated by electrophoresis in 1% agarose gel, and visualized by ethidium bromide staining.

presence of 10 mM EDTA. Replacing the divalent cations with 5 mM  $Zn^{2+}$  completely abolished the nuclease activity (Fig. 2A). The optimal pH for single-stranded endonucleases was acidic (Fig. 2B). It was most active at pH below 8. Enzymatic activities of the single-stranded endonucleases were optimal in low ionic strength buffer. At 500 mM NaCl, the nuclease activities were inhibited (Fig. 2C). Similar results were obtained when *T. pseudospiralis* E-S products or 7.5 kb poly(A) RNA was used (data not shown).

#### Authenticity of nuclease activity in E-S products

The main purpose of freezing/thawing and immunoprecipitation experiments was to determine whether the detected endonuclease is a real E-S component or a leaky byproduct of the larval somatic enzymes. Examination under a microscope showed that after

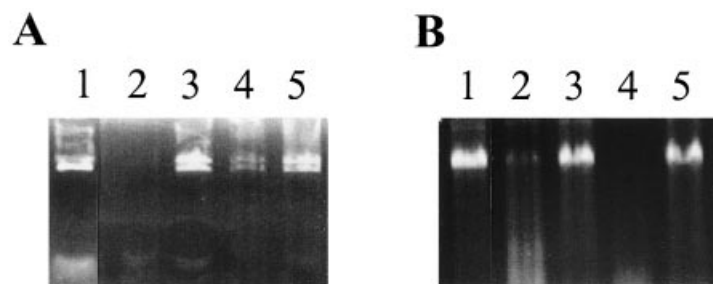


Fig. 3. Freezing/thawing of worms inactivates endonuclease activity. *Trichinella spiralis* and *T. pseudospiralis* secretory products were incubated with 0.5  $\mu$ g ssDNA (A) or 0.5  $\mu$ g RNA (B) in pH 6.0 reaction buffer containing 10 mM EDTA. Digested products were resolved by 1% agarose gel, and stained with ethidium bromide. Lane 1: control (incubated without E-S products); lane 2: 1  $\mu$ g TSES; lane 3: 0.3  $\mu$ g TSES-Freeze; lane 4: 1  $\mu$ g TPES; lane 5: 0.3  $\mu$ g TPES-Freeze.

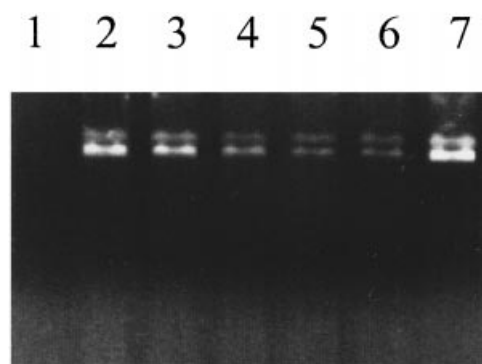


Fig. 4. Neutralization of TSES endonuclease activity by anti-E-S serum. Endonuclease neutralizing activities of various sera on single-stranded endonuclease were tested by immunoprecipitating TSES with various antibodies. The pre-cleared extracts were used in nuclease assays with single-stranded DNA as substrates. The digested products were resolved in 1% agarose gel, and stained with ethidium bromide. Lane 1: TSES without treatment; lane 2: TSES immunoprecipitated with anti-TSES antibodies; lane 3: TSES immunoprecipitated with anti-TPES antibodies; lane 4: TSES immunoprecipitated with anti-*Trichinella spiralis* crude extract antibodies; lane 5: TSES immunoprecipitated with normal rabbit antibodies; lane 6: TSES immunoprecipitated with Protein A-agarose; lane 7: control (substrate alone).

freezing and thawing, more than 90% of the worms were killed. The concentration of the E-S products produced by the treated larvae was only about 25% of the untreated sample (data not shown). Fig. 3 shows that after treatment, single-stranded endonuclease activity was abolished. Endonuclease activities were only observed in the untreated samples. Authenticity of the endonuclease was further analysed by immunoprecipitation using antibodies raised against the *Trichinella* E-S products. TSES single-stranded endonuclease was recognized by IgG from both anti-TSES and anti-TPES sera but not by normal or antiserum against *T. spiralis* crude somatic worm extracts (TSCE) (Fig. 4). After immunoprecipitation with the respective antiserum,

most single-stranded endonuclease activities were abolished. The slight decrease in enzymatic activity observed in the immunoprecipitation process (Fig. 4, lanes 4–6) was probably due to the non-specific binding of the E-S products to Protein A-agarose. However, the decrease was insignificant as compared to those of the experimental samples. The data indicate that the single-stranded endonucleases of both *T. spiralis* and *T. pseudospiralis* are authentic components of the larval E-S products. They may be encoded by similar proteins, or they share a common epitope.

#### Zymographic analysis of endonucleases

The patterns of renaturable endonuclease activities of TSES, TPES and TSCE were analysed in polyacrylamide gels containing heat-denatured salmon sperm DNA. The integrity and concentration of the samples were determined by SDS-PAGE and Coomassie blue staining (Fig. 5A). Single-stranded endonuclease activities were terminated after 24 h of incubation. *In situ* hydrolysis of the target DNA results in a reduction in ethidium bromide fluorescence.

Using heat-denatured salmon sperm DNA as substrate, comparable single-stranded endonuclease activity was detected in TSES and TPES. A much weaker activity was detected in TSCE, which may have originated from residual E-S products expressed in the larvae. Three prominent bands, with apparent molecular masses of ~50–60 kDa, were observed to be associated with the single-stranded endonuclease activity in TSES and TPES (Fig. 5B).

#### DISCUSSION

Little is known about endonucleases in parasitic organisms. Following our previous discovery of a specific, double-stranded endonuclease of *T. spiralis* (Mak & Ko, 1999), the present study documents a second non-specific, and faster action single-stranded molecule in the E-S products of *T. spiralis*

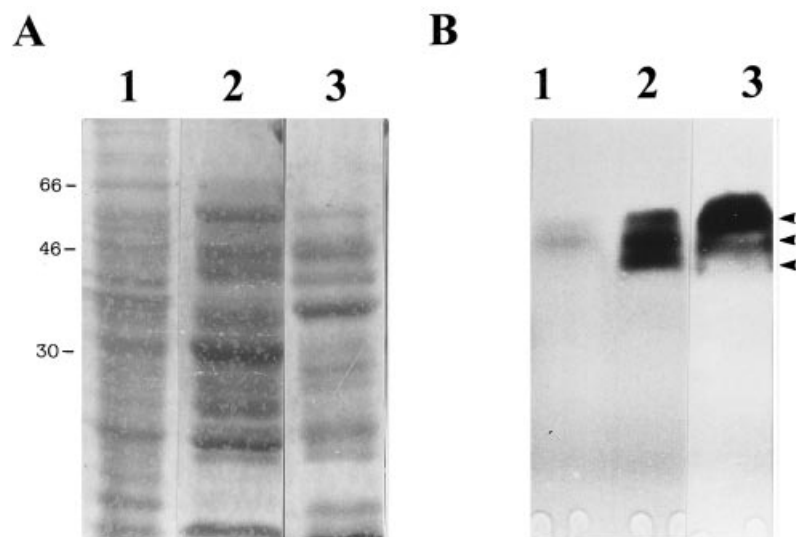


Fig. 5. SDS-PAGE nuclease band pattern of the endonuclease activity of various worm extracts. Samples of 40  $\mu$ g of *Trichinella spiralis* crude extract (lane 1), TPES (lane 2), and TSES (lane 3) were subjected to electrophoresis in 12% SDS-polyacrylamide gel (A) or polyacrylamide gel containing 10  $\mu$ g/ml bovine serum albumin and 20  $\mu$ g/ml heat-denatured salmon sperm DNA (B). After electrophoresis, gel was stained with Coomassie blue (A), or the nuclease activity in the gel was reactivated (B). Nuclease activity was identified by staining the remaining DNA with ethidium bromide and visualized by UV illumination. Degraded DNA which would not be stained appeared as dark bands.  $M_r$  markers are indicated on the left side. Arrows indicate the positions of the major endonuclease activities.

and *T. pseudospiralis*. The finding of 2 types of endonucleases in *Trichinella* indicates that the parasite-induced changes in host muscle cells may involve complicated pathways.

As in other eukaryotes, non-long terminal repeat retrotransposons are widespread in genomes of parasitic worms like *Schistosoma mansoni* and *Caenorhabditis elegans* (Bowen & McDonald, 1999; Drew *et al.* 1999). The retrotransposable element encodes an endonuclease that is crucial to the integration process (Yang, Malik & Eickbush, 1999). Since retrotransposition occurs in the nucleus, the endonuclease has been suggested to be basically an intracellular enzyme. However, the endonuclease observed in *Trichinella* is likely to be extracellular. It was detected in substantial quantity only in the E-S products. Freezing/thawing larvae (to break the cells) failed to increase the level of activity. The activity could also be blocked by antiserum against E-S products. Therefore, we believe that the endonuclease is an authentic component of the larval E-S products.

The biochemical properties of the novel single-stranded endonuclease of *Trichinella* differ from those of other well-characterized molecules. This is the first report of an extracellular acidic single-stranded endonuclease whose activity is independent of divalent cations. Most well-known single-stranded endonucleases such as those from KB cells (a continuous line of human epithelial cell), aneuploid cells, and mouse plasmacytoma mitochondria are divalent cation dependent (Pedrini *et al.* 1976; Wang & Rose, 1981; Kouidou *et al.* 1987). Nucleases secreted by *Streptococcal* strain S43, *Staphylococcus*

*aureus*, basidiomycete fungus *Armillaria mellea*, and *Rhizopus stolonifer* require divalent cations as a cofactor (Citak & Gray, 1980; Udou & Ichik'awa, 1980; Chacko & Shankar, 1998; Healy, Doonan & McCarthy, 1999). Activities of other well-characterized extracellular nucleases such as *Serratia marcescens* nuclease, *Cunninghamella echinulata* var. *echinulata* nuclease C1 and barley nuclease are inhibited by EDTA (Brown & Ho, 1987; Benedik & Strych, 1998; Ho *et al.* 1998). Endonucleases with similar properties are secreted by *Dictyostelium discoideum*, *Aspergillus*, *Bacillus subtilis* and *Vibrio* (Maeda & Taga, 1976; Guyer, Skantar & Deering, 1985; Kafer, Tittler & Fraser, 1989; Nakamura *et al.* 1992). The molecular masses of nucleases produced by *S. marcescens*, *C. echinulata*, barley, *B. subtilis*, *D. discoideum*, *Aspergillus* and *Vibrio* are 30.6, 30, 35, 32, 44, 88 and 100 kDa, respectively. These are quite distinctive from those of the endonuclease detected in the present study.

Significant differences (especially in their peptide profile, cation dependence, and species-specific expression) occur between the single and double-stranded endonuclease of *Trichinella*. The double-stranded endonuclease is divalent cation dependent and is encoded by peptides with  $M_r \sim 25$ , 30 and 58 kDa. It is mainly expressed in *T. spiralis* E-S products (Mak & Ko, 1999). On the other hand, the single-stranded endonuclease is divalent cation independent, encoded by peptides with  $M_r \sim 50$ –60 kDa. It is expressed in both *T. spiralis* and *T. pseudospiralis*. Therefore, the two endonucleases are probably encoded by different E-S proteins and may play a distinct role in the reorganization of host

tissues or in pathogenesis. However, the expression pattern of the single-stranded endonuclease suggests that it may have a more general role.

In viruses, endonucleases are associated with replication/integration. Single-stranded endonucleases are also involved in restriction of foreign DNA, homologous recombination, RNA editing and DNA repairing (Maeda & Taga, 1976; Pham & Coleman, 1985; Panet & Baltimore, 1987; Trujillo *et al.* 1998). In *Mycoplasma penetrans*, it degrades host nucleic acids to acquire the precursors for nucleic acid biosynthesis, leading to chromosomal alterations (Bendjennat *et al.* 1997). In *E. coli*, RNase III can affect genomic expression by influencing post-transcriptional control of mRNA stability, or mRNA translational efficiency (Dasgupta *et al.* 1998). Many pathogen-associated endonucleases, e.g. the virion host shutoff protein, and some viral nucleases, can degrade the host mRNA selectively, and stop the metabolism of the host cell (Krikorian & Read, 1991). Also, the 20S proteasome with intrinsic endonuclease activity can alter the turnover of cellular proteins by regulating the post-transcriptional gene expression (Petit *et al.* 1997). As in the above microorganisms, the single-stranded endonuclease of *Trichinella* may also be involved in a variety of functions but further studies are required to substantiate this hypothesis.

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