Molecular cloning and characterization of a serine proteinase inhibitor from *Trichinella spiralis*

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SUMMARY

We produced a recombinant protein from a cDNA library from muscle larvae of *Trichinella spiralis* which had proteinase inhibitory activity. The predicted amino acid sequence of the clone had an identity of only 30% to the serine proteinase inhibitors (serpins) from *Caenorhabditis elegans* or *Brugia malayi*. At the putative reactive region, however, the identity was about 50%. The recombinant protein expressed in *Escherichia coli* inhibited 82% of the activity of the serine proteinase (trypsin). Stage-specific expression of this protein was suggested from the following experiments. Antibody against the recombinant protein could stain proteins migrating at about 42 kDa (which is the expected size from the sequence) in crude extracts from newborn larvae and 18-day post-infection (p.i.) muscle larvae, but it failed to stain any proteins in crude extracts from 30-day p.i. muscle larvae. Production of mRNA transcript for the serpin gene was restricted largely to the newborn larvae and to 18-day p.i. muscle larvae. The antibody reacted with the stichocytes of the larvae at 18 days p.i., but did not react with the muscle larvae at 24 days and 30 days p.i.

Key words: *Trichinella spiralis*, serine proteinase inhibitor, serpin, cDNA cloning, gene expression.

INTRODUCTION

The genus *Trichinella* is a parasitic nematode in skeletal muscle cells of a wide variety of vertebrate hosts. Infection occurs by eating contaminated muscles which contain infective larvae. In the host stomach, infective larvae, which are released with the aid of host gastric juice, develop into adult worms in the host intestine in 2–3 days. From 5 days post-infection (p.i.), the gravid female begins to produce a second generation of larvae, which penetrate the host tissue and migrate through the body of the host through the blood and lymphatic vessels. The newborn larvae parasitize the muscle cells forming the cyst. One unique feature of *Trichinella* infection is that the muscle cell transforms to the nurse cell in the cyst (Despommier *et al*. 1990; Lee *et al*. 1991). Muscle cell transformation is likely initiated by excretory–secretory (E–S) products released from the larvae (Ko *et al*. 1994). Cyst formation is complete at about 18 days p.i., and the larvae in the cyst are infective for new hosts.

The major antigen recognized by the host during *Trichinella* infection is the larval TSL-1 antigen in the E–S products (Appleton *et al*. 1991; Denkers, Hayes & Wassom, 1991). The E–S products are known to contain some functional proteins including heat shock proteins, DNA-binding proteins and endonuclease and serine protease (Ko & Fan, 1996; Mak & Ko, 1999; Moczon & Wranicz, 1999; Ko & Mak, 1999). As a result, bioactive substances from *T. spiralis* are attracting a great deal of attention.

Serine proteinase inhibitors (serpins) regulate the complex cellular functions such as sequential activation of proteinases in the coagulation system. Serpins have been identified in a wide range of organisms including helminths (Macen *et al*. 1993; Blanton, Licate & Aman, 1994; Yenbutr & Scott, 1995; Pszenny *et al*. 2000). This paper deals with a serpin molecule which was cloned from the cDNA of *T. spiralis* muscle larvae.

MATERIALS AND METHODS

Crude extracts of T. spiralis

Muscle-stage larvae of *T. spiralis* (ISS413) were isolated by pepsin–HCl digestion from mice at 18 days and 30 days p.i. Adult worms were isolated from infected mouse intestines at 6 days p.i., and newborn larvae were isolated from the female adult worms according to the method described by Takada & Tada (1988). Crude saline extracts were prepared by conventional methods (Wu, Nagano & Takahashi, 1998; Wakelin *et al*. 1994).

Preparation of antisera

Infection sera were obtained from BALB/c mice infected with 300 larvae of *T. spiralis* for 2 months.

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Antiserum against the recombinant protein was collected from BALB/c mice injected intradermally with approximately 100 μg of recombinant protein with complete Freund's adjuvant followed by 4 booster injections of 50 μg of protein mixed with incomplete Freund's adjuvant at 2-week intervals.

Preparation of T. spiralis *cDNA library*

Muscle larvae of *T. spiralis* were isolated from mice at 30 days p.i. and washed extensively with diethylpyrocarbonate–phosphate buffered saline. Poly (A)-rich RNA was isolated and purified using a Quickprep micro mRNA purification kit (Amersham Pharmacia Biotech, Tokyo, Japan), and cDNA was prepared using a Timesaver cDNA synthesis kit (Amersham Pharmacia Biotech). After addition of an *Eco*RI adaptor, the cDNA was ligated into a λ ZAP II vector (Stratagene, La Jolla, CA, USA) with dephosphorylated *Eco*RI overhangs, packaged in Gigapack Gold III packaging extract (Stratagene).

Immunoscreening of cDNA library and clone isolation

The cDNA library was immunoscreened with a 1:100 dilution of infection sera obtained at 2 months p.i. according to the conventional methods (Sambrook, Fritsch & Maniatis, 1989). To produce recombinant protein, some of the positive cDNA clones were converted to a plasmid by *in vivo* excision, and each plasmid was propagated in an *E. coli* SOLR strain. The *Eco*RI (New England BioLabs, Beverly, MA, USA) restriction fragments of the purified plasmid from the clones were subcloned into the pTrcHis expression vector (Invitrogen, Carlsbad, CA, USA).

Expression and purification of recombinant protein

The recombinant plasmid was transformed into an *E. coli* DH5α strain and the expression of polyhistidine-containing recombinant proteins was induced by adding β-D-thiogalactopyranoside (IPTG) to 1 mM at 37 °C for 3 h. The induced cells were harvested and disrupted by sonication in 20 mM phosphate buffer (pH 7·4) with 10 mM imidazole. Fusion proteins in the inclusion bodies were solubilized completely with 8 M urea in 20 mM phosphate buffer (pH 7·4) with 10 mM imidazole, and then subjected to a His Trap kit (Amersham Pharmacia Biotech) for affinity purification of histidine-tagged proteins according to the manufacturer's instructions. Urea was removed from the samples with a PD-10 column (Amersham Pharmacia Biotech). Recombinant proteins thus obtained were analysed with 11% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie Brilliant Blue to assess their purity.

DNA sequencing

Sequences were obtained using a *Taq* cycle sequencing Kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA). The products were sequenced using an automatic sequencer (Model 373S, Perkin Elmer Applied Biosystems). The DNA sequences were assembled and analysed using DNASIS software (Hitachi Software Engineering, Tokyo, Japan). The BLAST network service was used to search the DNA and protein database at the National Center for Biotechnology Information (Bethesda, MD, USA).

Western blotting analysis

Samples included crude extracts from adult worms, newborn larvae, 18-day p.i. muscle larvae, 30-day p.i. muscle larvae, and the recombinant protein. Twenty μg of crude extracts, or 0·4 μg of recombinant protein were electrophoresed on 11% SDS–PAGE then electro-transferred to nitrocellulose sheets. Antigen was detected using either the infection sera or antiserum against the recombinant protein pre-absorbed with *E. coli* lysate, diluted 1:100 (see *Preparation of antisera*). Goat anti-mouse IgG alkaline phosphatase-conjugate was used as the second antibody.

Immunocytolocalization

Skeletal muscle tissues from mice at 18, 24 and 30 days p.i. with *T. spiralis* were frozen and cryosectioned. Sections of 4 μm thickness were incubated in a humid chamber with the anti-recombinant protein antibody (1:100 dilution) for 1 h, washed and further incubated with anti-mouse IgG coupled to fluorescein isothiocyanate. Observations were made under a fluorescence microscope.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIZOL (Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. The isolated RNA was treated with DNase (Promega, Madison, WI, USA). RT-PCR was performed to assay gene expression using a Ready-To-Go RT-PCR Kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Primers for detection of the serpin gene were designed as follows: 5′-CGT TCG TTT CAT CGC TCA CCA-3′ (forward), 5′-TCA GCA CCG CTA ACT TTG GAT ACC-3′ (reverse). Primers for a constitutively expressed standard gene (18S rRNA) were designed as follows: 5′-TGA CGA AAA ATA ACG AGA CGA T-3′ (forward), 5′-AAT CGC TCC ACC AAC TAA GAA C-3′ (reverse). The same amount (1 μg) of total

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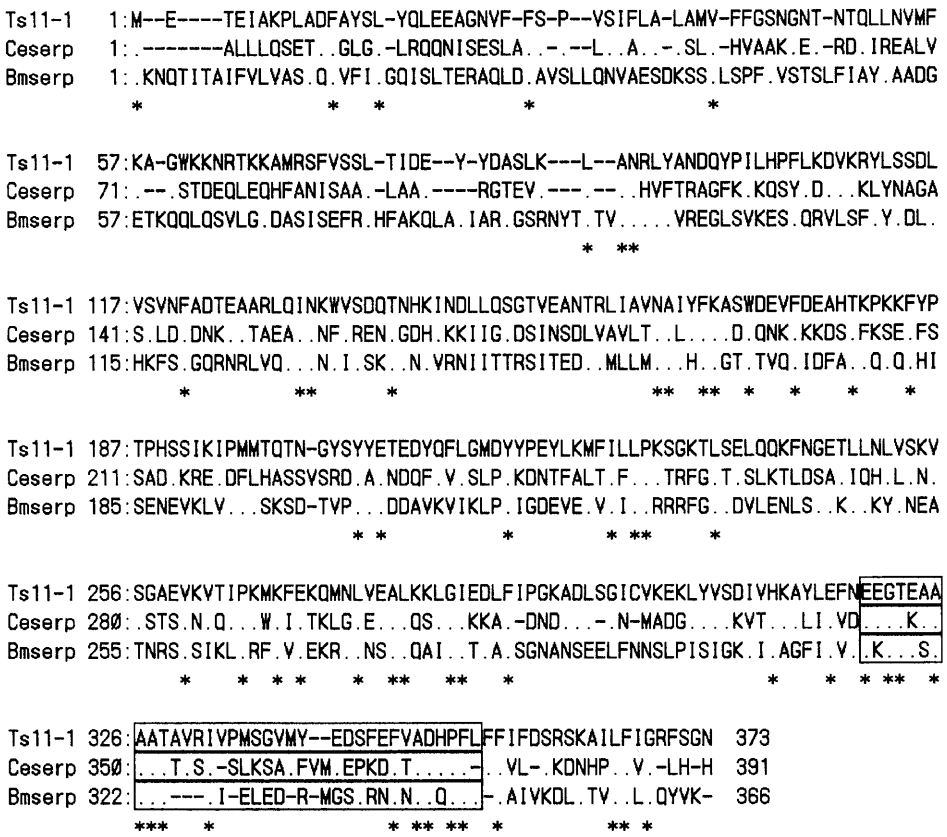

Fig. 1. Alignment of the deduced amino acid sequence of the Ts11-1 open reading frame, *Caenorhabditis elegans* serpin (Ceserp) (accession number U50301) and *Brugia malayi* serpin (Bmserp) (accession number U04206). The asterisks indicate amino acids that are shared by Ts11-1, Ceserp and Bmserp. Amino acid residue identities with Ts11-1 are represented by dots. Gaps are represented by dashed lines. The numbers along the margin designate the positions of amino acid residues. Boxed amino acids indicate the deduced amino acid sequence of the putative reactive regions of Ts11-1, Ceserp and Bmserp.

RNA from each sample and 5 μl of 5 μM each primer were added to the Ready-To-Go tube (total volume 50 μl). RT-PCR was performed using the condition of 1 cycle at 42 °C for 30 min and 95 °C for 5 min; 30 cycles of 95 °C for 30 sec, 54 °C for 30 sec, and 72 °C for 1 min.

Proteinase inhibitory activity of recombinant protein

Proteinase inhibition was measured by incubating the recombinant protein with 6 μg of trypsin (Sigma Chemical Co. St Louis, MO, USA) in 100 mM Tris-HCl with 1 mM $CaCl_2$ (pH 8·0) in a final volume of 1·0 ml for 20 min at 37 °C. To check dose dependency, the recombinant protein was 2-fold diluted serially (0, 0·039, 0·078, 0·156, 0·3125, 0·625, 1·25, 2·5, 5 and 10 μg). Chloramphenicol acetyl transferase recombinant protein (Invitrogen, Carlsbad, CA, USA) and trypsin inhibitor (Sigma Chemical Co. T9253, type II-O from chicken egg white) were included as negative and positive controls. Residual trypsin activity was determined by adding 3 mg of azocoll (Sigma Chemical Co.) for 16 h at 37 °C. The tubes were centrifuged, and degradation was determined from the absorbance of the supernatant at 540 nm.

Nucleotide sequence accession number

The nucleotide sequence reported in this article has been submitted to the GenBank™, EMBL and DDBJ databases and has the accession number of AF231948.

RESULTS

Molecular characterization of the Ts11-1 clone

Primary screening of the cDNA expression library (200000 plaques) with infection mouse serum resulted in 11 positive clones. Differences of length and restriction patterns using the restriction enzyme of the inserted fragments showed that 3 of the 11 positive clones overlapped each other (data not shown). One clone of the 3 clones, designated Ts11-1, was sequenced and its amino acid sequence was deduced. It consisted of 1274 bp including a 3′ and 5′-untranslated region, and the predicted open reading frame encoded a protein of 373 amino acid residues (Fig. 1) with a molecular mass of 42412 Da. The identity in the amino acid sequence, except for the deleted/inserted residues, between the Ts11-1

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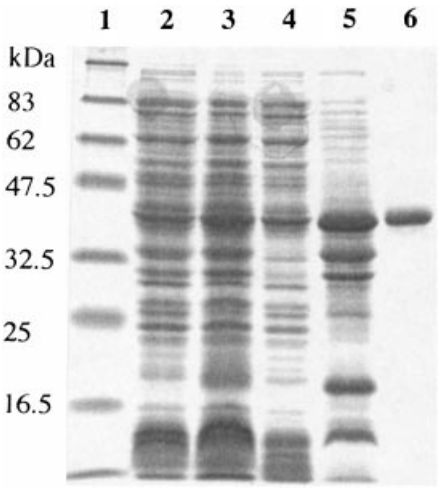

Fig. 2. SDS–PAGE (11 % gel) analysis of Ts11-1 recombinant protein. Lane 1, molecular weight standard, size in kDa is shown on the left side; lane 2, lysate of *E. coli* with the cDNA before induction; lane 3, lysate of *E. coli* with the cDNA after induction; lane 4, supernatants of lysate of *E. coli* with the cDNA after induction; lane 5, precipitants of lysate of *E. coli* with the cDNA after induction; lane 6, purified recombinant proteins by affinity purification method.

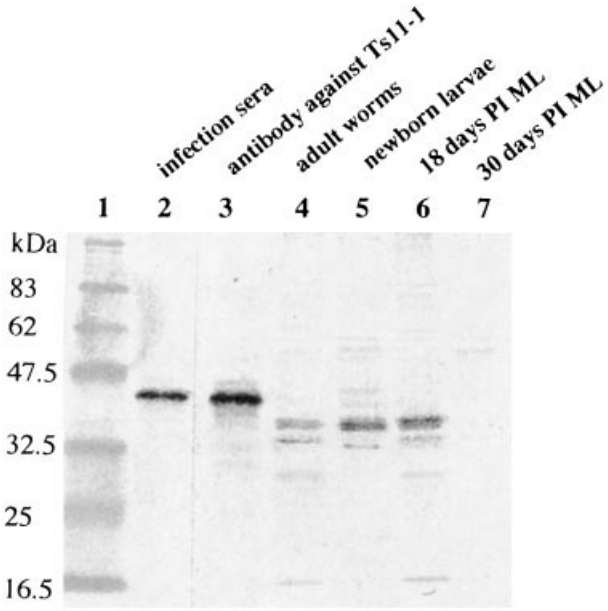

Fig. 3. Western blotting analysis of Ts11-1 recombinant protein with the infection sera (lane 2) and the antibody against Ts11-1 recombinant protein (lane 3). Western blot analysis of crude extracts from adult worms (lane 4), newborn larvae (lane 5), muscle larvae obtained 18 days post-infection (p.i.) (lane 6), muscle larvae obtained 30 days p.i. (lane 7) with the antibody against Ts11-1 recombinant protein. Lane 1, molecular weight standard, size in kDa is shown on the left side. ML, muscle larvae.

clone and the *Caenorhabditis elegans* or *Brugia malayi* serpin was about 30 % (Fig. 1). However, the level of identity between the Ts11-1 protein and *C. elegans* serpin over their presumed reactive region was 54 % and the level of identity between the Ts11-1 protein and *B. malayi* serpin was 49 %.

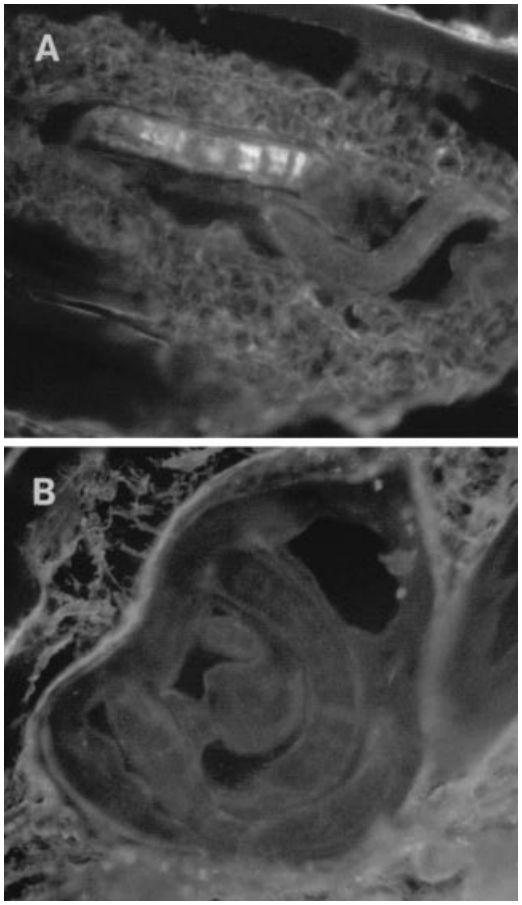

Fig. 4. Cytolocalization of the Ts11-1 recombinant protein in the infected skeletal muscle. Showing reactivity of anti-Ts11-1 antibodies to antigens localized within the stichocyte of *Trichinella spiralis* muscle larvae obtained 18 days post-infection (A) and 24 days post-infection (B).

Expression of the Ts11-1 clone

Although the Ts11-1 protein is 42412 Da, it migrated at 45 kDa on SDS–PAGE (Fig. 2) due to its possession of 3 kDa plasmid vector proteins. The Ts11-1 recombinant protein was much more highly expressed in inclusion bodies (lane 5 in Fig. 2) than in the supernatant of the induced cells (lane 4 in Fig. 2). Protein synthesis was induced more efficiently in the sample with 1 mM IPTG treatment (lane 3 in Fig. 2) than in the sample without treatment (lane 2 in Fig. 2). The recombinant protein could be purified at a single band level using a His Trap kit and eluted with 500 mM imidazole (lane 6 in Fig. 2).

Western blotting analysis of Ts11-1 recombinant protein

The Ts11-1 recombinant protein migrated at 45 kDa, which was positively immunostained with

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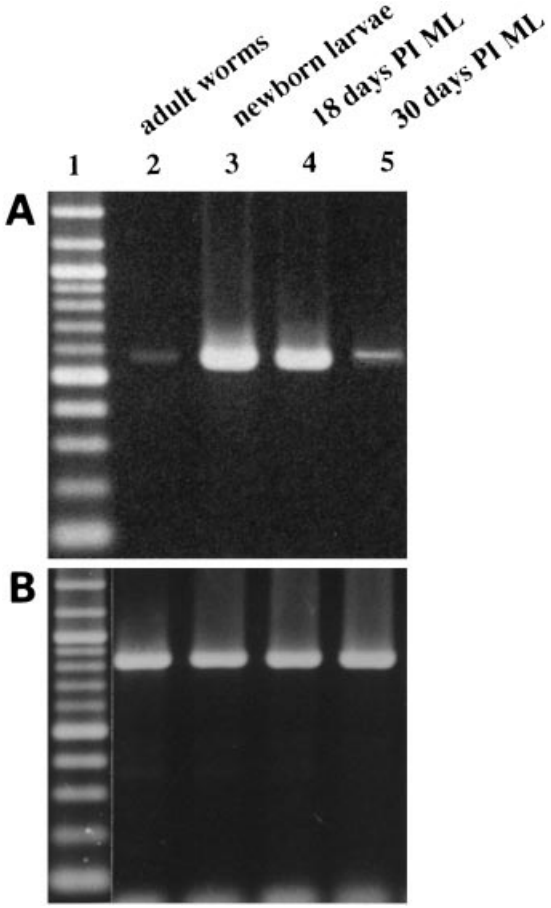

Fig. 5. Reverse transcription-polymerase chain reaction detection of serine proteinase inhibitor mRNA (A) and 18S rRNA (B) in adult worms (lane 2), newborn larvae (lane 3), 18-day post-infection (p.i.) muscle larvae (lane 4) and 30-day p.i. muscle larvae (lane 5). Lane 1, molecular size marker (100 base-pair ladder). ML, muscle larvae.

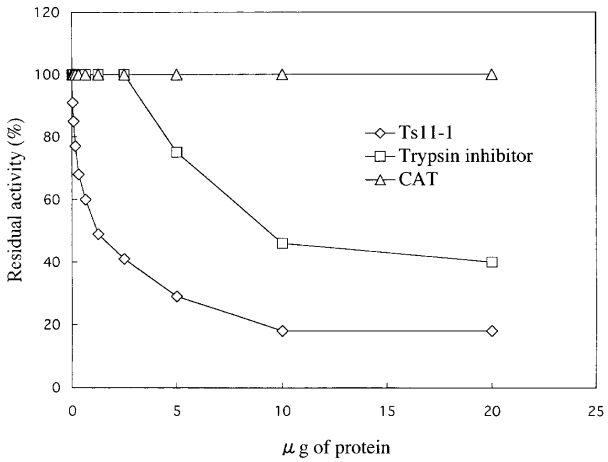

Fig. 6. Inhibitory activity of Ts11-1 recombinant protein against trypsin. CAT (chloramphenicol acetyl transferase) recombinant protein and trypsin inhibitor (type II-O from chicken egg white) were used as control. Results are expressed as the percentage of residual activity of the enzyme against the concentration of the inhibitor.

infection sera (lane 2 in Fig. 3) and the antibody against Ts11-1 recombinant protein (lane 3 in Fig. 3).

The antibody against Ts11-1 recombinant protein stained proteins migrating at about 42 and 37 kDa in crude extracts from newborn larvae (lane 5 in Fig. 3) and 18-day p.i. muscle larvae (lane 6 in Fig. 3), but failed to stain any proteins in crude extracts from 30-day p.i. muscle larvae (lane 7 in Fig. 3). Weak staining was detected in the crude extracts from adult worms (lane 4 in Fig. 3) which is likely due to the presence of fetus in the female adults. The positive staining of the 42 kDa protein is expected because it is the predicted size of a serpin from the amino acid sequence. However, we have no ready explanation as to why the 37 kDa protein was also immunostained. It may be a cleavage product of the 42 kDa protein.

Cytolocalization of the Ts11-1 recombinant protein in the infected skeletal muscle

Intense staining with the anti-Ts11-1 serum was found within the stichocyte of muscle larvae in the early stage of infection (18 days p.i.) (Fig. 4A). This positive staining, however, was not observed in the muscle larvae at the later stages including 24 days p.i. (Fig. 4B) and 30 days p.i.

RT-PCR analysis

The mRNA transcript (565 bp) for the Ts11-1 gene was detected strongly in newborn larvae (lane 3 in Fig. 5A) and in 18-day p.i. muscle larvae (lane 4 in Fig. 5A), and faintly in adult worms (lane 2 in Fig. 5A) and in 30-day p.i. muscle larvae (lane 5 in Fig. 5A). On the other hand, primers for a standard gene (18S rRNA) generated the expected size (843 bp) bands of the same intensity in all samples (lanes 2–5 in Fig. 5B).

Inhibitory activity of Ts11-1 recombinant protein against trypsin

The Ts11-1 recombinant protein inhibited the enzymatic activity of trypsin in a dose-dependent manner (Fig. 6). For example, 1 μg of the recombinant protein inhibited 50 % of the activity of trypsin, and 10 μg of the protein achieved 82 % inhibition, which was more efficient than the Sigma T9253 trypsin inhibitor.

DISCUSSION

In this study, we have established a cDNA clone named Ts11-1 that encodes the 42412 Da protein of *T. spiralis*. The recombinant protein encoded by the Ts11-1 clone was successfully produced in the *E. coli*

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expression system, and the resultant recombinant protein was used to define certain characteristics of the native gene product.

The recombinant protein is most likely a serpin because the predicted amino acid sequence of the Ts11-1 clone had an identity of 50 % to serpins of *C. elegans* or *B. malayi* at the putative reactive region. The hypothesis that Ts11-1 recombinant protein is a serpin was further strengthened by an *in vitro* test where the recombinant protein induced 82 % inhibition of proteinase activity. Over 40 proteins, derived from sources as diverse as humans, viruses, and plants, are members of the serpin superfamily (Gettings, Patston & Schapira, 1993). The majority of serpins are single-chain glycoproteins with molecular weights between 40 kDa and 100 kDa that contain a single reactive site located in the carboxyl terminus. Serpins inactivate proteinases by forming complexes with serine proteinase.

Western blotting analysis showed the presence of antigenic epitopes of Ts11-1 recombinant protein in crude extracts from newborn larvae and 18-day p.i. muscle larvae, but not in crude extracts from 30-day p.i. muscle larvae. Production of mRNA transcript for the serpin gene was restricted largely to the newborn larvae and to 18-day p.i. muscle larvae. Therefore, the serpin may not be synthesized after completion of cyst formation. The present immunocytochemical study identified Ts11-1 recombinant protein in stichocytes of muscle larvae of an 18-day specimen but not in those of 24 and 30-day specimens. It can be concluded then, that serpin is synthesized only in the early stage of the muscle larvae. The different expression of serpin in different developmental stages has been reported in other systems; serpin of *S. haematobium* (a 54–58 kDa glycoprotein) was located on the surface of adult worms only (Blanton *et al*. 1994). Another example is given by *B. malayi*, in which the concentration of serpin from infective 3rd-stage larvae was 10 to 16 times higher than the concentration of serpin from adults or microfilariae (Yenbutr & Scott, 1995).

Possible functions of serpin have been postulated in a number of other systems including modulation and inhibition of host immune responses (Ray *et al*. 1992; Macen *et al*. 1993). For example, it has been reported that *B. malayi* larvae can inhibit the initiation of thrombosis through a suppression of the intrinsic coagulation pathway by inhibiting the function of serine proteinase (Yenbutr & Scott, 1995). The ability to inhibit coagulation during the early stages of parasitism may be important for the survival of parasites in the host.

Mass production of serpins by genetic engineering may provide a new means for immunodiagnostics and vaccine production because proteinase inhibitors are known to provoke a strong immune response (Chandrashekar, Ogunrinade & Weil, 1996; Hong *et al*. 1996; Frank *et al*. 1998).

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