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Cloning and analysis of a cDNA encoding a putative serine protease comprising two trypsin-like domains of *Trichinella spiralis*

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Abstract The cDNA encoding a putative serine protease, *TsSerP*, was cloned by degenerative polymerase chain reaction and screening of the cDNA library from *Trichinella spiralis* adult-newborn larvae stage. Sequence analysis revealed the presence of two trypsin-like serine protease domains flanking a hydrophilic domain, with the catalytic triad residue histidine in the alpha domain substituted by an arginine residue. Southern blots indicated that this was a single copy gene in the parasite genome. Northern blots demonstrated a single 2.3 kb transcript during the muscle larvae and adult stages of *T. spiralis*. The recombinant protein from the *TsSerP* beta domain (β SerP) was produced but not recognised by *T. spiralis*-infected swine serum. An anti- β SerP polyclonal serum detected a 69 kDa polypeptide in the soluble antigens of *T. spiralis* muscle larvae. Immunolocalisation analysis located *TsSerP* on the inner layer of the cuticle and oesophagus of the parasite, suggesting a potential role in its moulting and/or digestive functions.

Keywords *Trichinella spiralis* · Serine protease · Trypsin-like domain · cDNA library · Cloning

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Introduction

Proteases appear to be required for critical events in the life cycle of numerous protozoan and helminth parasites. Parasite proteases may be involved in varied aspects of host-parasite interactions, such as host tissue invasion, parasite nutrition, anti-coagulation and the evasion of host immune responses (McKerrow 1989; Trap and Boireau 2000). These proteins have therefore been investigated as potential targets for chemotherapy and vaccines against parasite infections (Coombs and Mottram 1997; Dalton et al. 2003).

Parasitic nematodes of the *Trichinella* genus are widespread in nature, infecting virtually all mammals and some carnivorous birds when these hosts ingest parasite-laden muscle tissues. The life cycle of *Trichinella* is reproduced in the same host and can be divided into three main stages: muscle larvae, adult worms and newborn larvae. After digestion by the host, muscle larvae migrate through the enterocytes, leaving an empty path behind them (ManWarren et al. 1997) like a tunnel in the cytoplasm of contiguous cells, suggesting the possibility of enzymatic activities. The transformation of muscle larvae into adult worms occurs within a couple of days in enterocytes. Newborn larvae are released a few hours after fecundation and invade the bloodstream of the host, to penetrate striated muscle cells. One striking characteristic of *Trichinella* is the transformation of host-striated muscle cells into nurse cells. The addition of excretory/secretory (E/S) products from newborn larvae or muscle larvae into a culture of myocytes elicits morphological changes in myotubes, such as the formation of nodular structures that contain numerous cavities, probably due to enzymatic digestion by parasitic proteases (Leung and Ko 1997).

Protease activities have been identified in E/S products and crude extracts of *Trichinella spiralis* (Criado-Fornelio et al. 1992; de Armas-Serra et al. 1995; Todorova et al. 1995; Todorova and Stoyanov 2000; Todorova 2000;

Moczon and Wranicz 1999; Ros-Moreno et al. 2000). These proteases probably play a crucial role in the development and survival of *Trichinella*. All these data indicate the importance of the cloning and characterisation of *Trichinella* proteases to studying their function. Recently, a serine protease (Romaris et al. 2002; Nagano et al. 2003) and a metalloproteinase (Lun et al. 2003) were cloned and identified from E/S products of *T. spiralis* muscle larvae. In the present study, we report on the cloning and analysis of a putative serine protease gene with two protease domains from *T. spiralis* (*TsSerP*). Our findings show that *TsSerP* is expressed during all three antigenic stages, and its potential role in host-parasite interactions, particularly with respect to moulting and digestion, is discussed.

Materials and methods

Parasite preparations

Muscle larvae (ML) of *T. spiralis* (ISS 534) were recovered from infected OF1 mice by the acid-pepsin digestion of striated muscles at 45 days post-infection (dpi). For Northern blot analysis, ML were recovered from infected mice at days 26, 31, 35, 41 and 47 dpi. Adult worms (Ad) were isolated from the small intestine of infected rats at 3 and 5 dpi. Newborn larvae (NBL) were isolated from adult worms at 5 dpi, and the pool of adult-newborn larvae (Ad-NBL) was concentrated by centrifugation.

RNA purification from *T. spiralis* and cDNA library construction

Total RNA was extracted independently from approximately 150,000 ML, 20,000 Ad (3 dpi), 15,000 Ad (5 dpi) plus 100,000 NBL using a standard protocol as previously described (Vayssier et al. 1999). The poly (A) RNA was purified using the Oligotex Direct mRNA Midi Kit (Qiagen). A lambda ZAP II cDNA library of Ad-NBL was constructed according to the manufacturer's instructions (Stratagene).

Degenerative PCR and screening of the Ad-NBL cDNA library

Primer 1 (5'-GCT CAT GTT GGG CWK TC-3') and primer 2 (5'-CCA YGA GTT RGC AAY RAK CC-3') derived from conserved protease sequences (Harrop et al. 1995) were used to amplify a cDNA fragment from the *T. spiralis* Ad-NBL cDNA library. After gel purification, the amplified DNA was radioisotope labelled and used to screen the cDNA library by plaque hybridisation. Positive clones were sequenced on both strands, and the nucleotide sequence and deduced amino acid sequence were analysed.

Southern blot and Northern blot analysis

Genomic DNA was isolated from *T. spiralis* ML, according to the protocol for the DNeasy Tissue kit (Qiagen). Approximately 10 µg of genomic DNA was digested with *EcoRI* or *HindIII* restriction enzymes and separated on a 1% agarose gel before being denatured and transferred onto a Hybond N+ membrane (Amersham). The DNA fragment of *TsSerP* (nucleotides 6-1126) amplified by polymerase chain reaction (PCR) was labelled using the AlkPhos Direct Labelling and Detection System (Amersham) and hybridised overnight with the membrane at 55°C. The membrane was washed under high stringency conditions, and then CDP-Star (Amersham) was added to enable direct exposure to x-ray film for 1 h at room temperature. Total RNA from Ad and ML was denatured in 10× MOPS, 40% formaldehyde and deionised formamide at 65°C for 10 min, before being separated on a 1% agarose gel prepared under native conditions (1× MOPS) and transferred onto Hybond N+ membranes (Amersham). The membranes were hybridised and washed as described for Southern blot analysis.

Expression of recombinant protein and production of antibody

The *TsSerP* beta domain was cloned in frame with the thioredoxin protein (Trx) tag into the pET32 Xa/LIC vector (Novagen) using the following primers: SERLIC1 (5'-GGT ATT GAG GGT CGC TCA AAC AGA GTG TCT GGT GGA TGG-3') and SERLIC2 (5'-AGA GGA GAG TTA GAG CCT AGG CAG AGG AGT CAT AAA TCT GC-3') and then expressed in the AD 494 strain of *E. coli*. The Trx-βSerP fusion protein was purified using the Ni-NTA Spin Columns kit (Qiagen) under denaturing conditions. The immunization protocol consisted of two subcutaneous injections at an interval of 3 weeks in mice, using 50 µg of the purified Trx-βSerP fusion protein emulsified with Montanide ISA 50V adjuvant (SEPPIC). Sera were collected 6 weeks after a booster injection and stored at -20°C until use. Negative control serum samples were obtained from non-immunized animals.

Western blot analysis

Somatic antigens and E/S products of *Trichinella* were prepared as previously described (Boireau et al. 1997). The antigen preparations, including *T. spiralis* ML soluble and E/S antigens and the recombinant proteins, were separated on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before being electrophoretically transferred onto Hybond C extra membranes (Amersham). The membranes were then blocked and incubated with 1:100 diluted anti-βSerP mice sera or swine sera

Indirect immunofluorescence antibody test

Trichinella Ad and ML were embedded in tissue-tek (Miles Scientific) and frozen at -20°C . Sections of $10\ \mu\text{m}$ were prepared with a cryostat (Cryo-cut, American Optical Corporation) and fixed for 15 min in cold acetone. Cryostat sections were incubated for 1 h with polyclonal sera (1:50) against recombinant Trx- β SerP or with sera (1:50) obtained from non-immunized animals. The preparations were washed three times in phosphate-buffered saline (PBS) pH 7.2, 1% Tween 20 before being incubated for 1 h with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG (Interchim) (1:50).

Results

Molecular cloning and sequence analysis of a cDNA encoding a new serine protease of *T. spiralis*

Five micrograms of poly (A) RNA purified from the mixture of *T. spiralis* Ad-NBL was used to construct a cDNA library consisting of 1.1×10^6 independent clones. Degenerate primers based on the conserved region of proteases (Harrop et al. 1995) allowed the amplification of a 610 bp DNA fragment, with the Ad-NBL cDNA library as template. Sequence analysis of this cDNA fragment revealed part of a consensus active site belonging to the trypsin group. Using this target sequence as a probe to screen 150,000 plaques from the Ad-NBL cDNA library, four positive clones were selected and sequenced on both strands. The resulting cDNA consisted of 2,126 bp (Fig. 1) with one major open reading frame (ORF) of 2,001 bp, with the initiation codon ATG at position 6–9 and a 3' untranslated region of 101 bp between the stop codon TGA and the poly (A) tail (GenBank accession number AF331156). A polyadenylation signal, AATAAA, was identified 9 bp upstream of the poly (A) tail.

The predicted protein, named *TsSerP*, comprised 667 amino acid residues, with a molecular weight of 71.6 kDa and an isoelectric point of 8.6. SignalP analysis predicted an N-terminal signal peptide with the cleavage site between residues serine 18 and aspartic acid 19. Surprisingly, SMART analysis revealed two trypsin-like domains of serine protease. The first domain, called the α domain, was situated between positions 38 and 282 and displayed substitution of the conserved histidine residue to an arginine residue in the catalytic triad (arginine 88, aspartic acid 142 and serine 233). The second domain, designated the beta domain, was located between positions 339 and 582 of *TsSerP* and exhibited the characteristic trypsin domain of serine proteases consisting of a catalytic triad of histidine 389, aspartic acid 444 and serine 533. A hydrophilic domain from positions 265 to 376 was flanked by these two domains, and two putative *N*-glycosylation sites were identified at positions 78 and 379. Analysis of the deduced amino acid sequence homologies using the BLAST

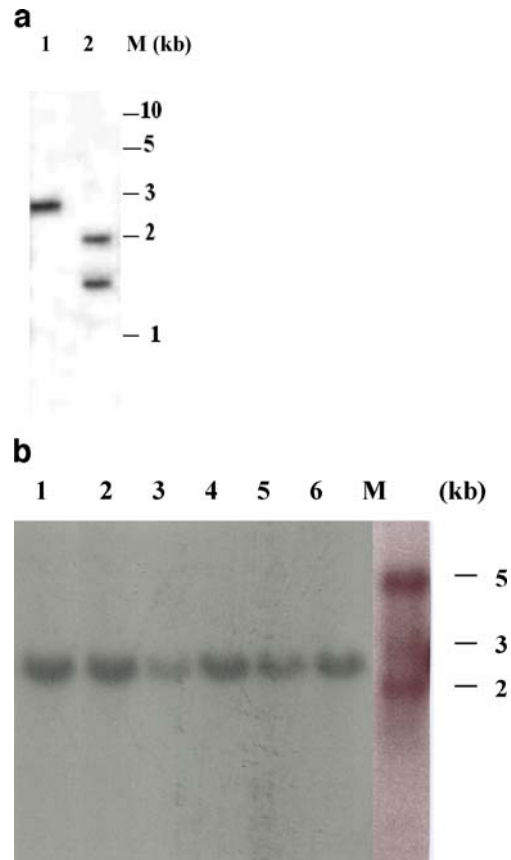


Fig. 2 **a** Southern blot analysis of *Trichinella spiralis* genomic DNA. *T. spiralis* genomic DNA was digested with *Eco*RI (lane 1) or *Hind*III (lane 2) and hybridised with the alkaline phosphatase-labelled PCR fragment of *TsSerP* cDNA (nucleotides 6–1120). *M* Molecular weight markers (kb). **b** Northern blot analysis of *T. spiralis* RNA. Total RNA from *T. spiralis* adult worms (lane 1) and muscle larvae isolated at 26, 30, 35, 41 and 47 dpi (lanes 2–6) were migrated under non-denaturing conditions and transferred onto Hybond N+ membranes before being hybridised with alkaline phosphatase-labelled DNA probe. *M* Molecular weight markers (kb)

program revealed that both the alpha and beta domains displayed up to 39% identity to serine proteases from a broad range of organisms including *T. spiralis* (newborn larvae-specific serine protease SS2, AAK16520; adult stage-specific serine proteinase, AAD09211; muscle larvae stage-specific serine proteinase, AAK31787).

Southern blot and Northern blot analysis

When digested with *Eco*RI, a single band of genomic DNA was recognised, while two bands were observed after *Hind*III restriction due to the presence of a cutting site in the probe, indicating that the selected sequence was present as a single copy in the genome of *T. spiralis* (Fig. 2a). Moreover, hybridisation of the total RNA from Ad and ML with the *TsSerP* probe demonstrated a single transcript of 2.3 kb at both stages (Fig. 2b) whatever the day of ML purification (from 26 to 47 dpi). A similar result was

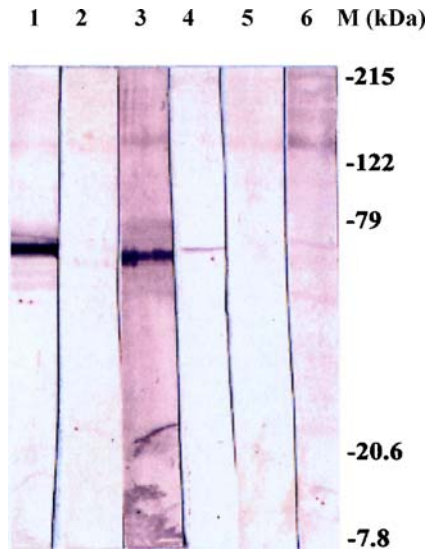
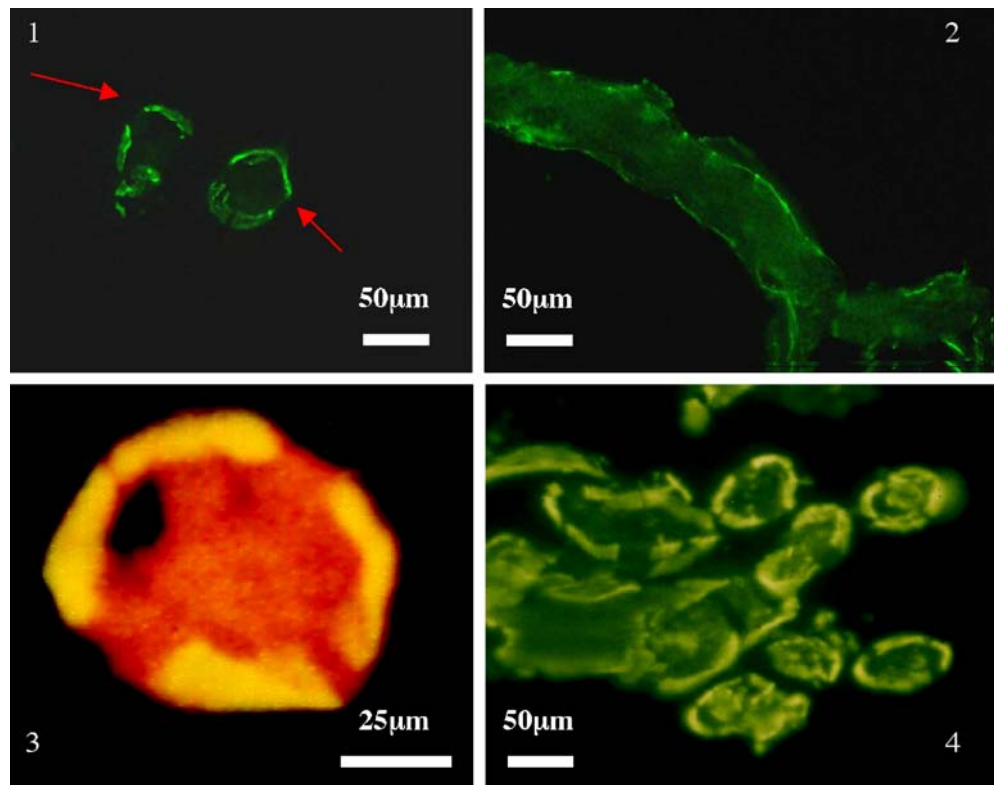


Fig. 3 Western blot analysis of soluble and E/S antigens of *T. spiralis* muscle larvae using the anti-Trx- β SerP serum. Soluble (lanes 1 to 3) and E/S (lanes 4 to 6) antigens were migrated on polyacrylamide gel under denaturing conditions and blotted onto Hybond C filters. Lanes 1 and 4, the mouse serum raised against the recombinant *Ts* Δ HSP70 (Vayssier et al. 1999) was used as a positive control (recognition of *Ts*HSP70 being similar in size to full-length SerP). Lanes 2 and 5, mouse serum against Trx- β SerP collected at day 0. Lanes 3 and 6, mouse serum against Trx- β SerP collected 42 dpi. *M* Molecular weight markers (kDa)

obtained by the dot blot analysis of *T. spiralis* cDNAs at the Ad, ML and NBL stages (data not shown), suggesting continuous transcription of the *Ts*SerP gene.

Fig. 4 IIF test with anti-Trx- β SerP serum or the 2A6d3 monoclonal antibody. 1 and 2, cryostat sections of adult worms. 3 and 4, cryostat sections of muscle larvae. A peripheral immunostaining is visible on the entire length of the worm with anti-Trx- β SerP serum (2 and 4). A gap in the inner part of the cuticle and oesophageal fluorescence are indicated with arrows (1). The 2A6d3 monoclonal antibody raised against antigenic group 3 of the ML stage (Boireau et al. 1997) exhibits an identical IIF pattern (3)



Western blot analysis and localization of *Ts*SerP

The recombinant protein of the beta protease domain (β SerP) was successfully expressed, but exhibited no reactivity with the serum of pigs experimentally infected with 10,000 *T. spiralis* ML. Antiserum from mice collected 42 days after immunization with the β SerP recombinant protein allowed the detection of a polypeptide of 69 kDa in ML soluble antigens, but not in E/S products, corresponding to the expected size of the mature *Ts*SerP with two domains (Fig. 3). Specific sera against the recombinant protein β SerP were then used for an indirect immunofluorescence (IIF) test on cryostat sections of *T. spiralis* ML and Ad stages (Fig. 4). Narrow peripheral fluorescence extending the entire length of the parasite at both stages was located in the inner layer of the cuticle. On cross sections of the parasite, four peripheral symmetrical gaps were delineated, and a bright circular fluorescence in the oesophagus was identified. No differences were observed concerning *Ts*SerP immunolocalisation with *Trichinella britovi*, *Trichinella nativa* and *Trichinella pseudospiralis* cryostat sections (data not shown).

Discussion

The present study describes the cloning and analysis of a novel *T. spiralis* gene, *Ts*SerP, encoding a putative serine protease composed of two protease domains. Although the first of these protease domains did not possess the consensus proteolytic triad because of the substitution of

conserved histidine, it might still retain substrate-binding activity. The presence of several protease domains in a single translation product is very unusual, and this is the first description in parasites. Oviductin from *Xenopus laevis* (Lindsay et al. 1999a) and *Bufo japonicus* (Hiyoshi et al. 2002) contains two serine protease domains, one of which is catalytically inactive and require post-translational processing to generate a mature protease. Ovochymase from *X. laevis* (Lindsay et al. 1999b) and human polyserase-I (Cal et al. 2003) are mosaic proteins with three serine protease domains that are released after the translation of a single polyprotein product. Sequence comparisons with known serine proteases suggested that *TsSerP* was a protein precursor with a pro-region cleavage site between lysine 38 and isoleucine 39. The mature protein would thus be 629 amino acids in length with a predicted molecular weight of 67.5 kDa. Because the specific anti-Trx- β SerP serum only immunostained a 69-kDa polypeptide in soluble antigens of *T. spiralis* muscle larvae, we concluded that unlike the serine proteases mentioned above, the two serine protease domains of *TsSerP* were not separated after post-translational processing. Whatever the case, the enzymatic activity of *TsSerP* and the function of the first protease domain require further study.

Southern blot analysis indicated that the *TsSerP* gene was present as a single copy in the genome of *T. spiralis*. EST sequence data also confirmed that the sequences belonged to *TsSerP* (Mitrevic et al. 2004). Recently, a *T. spiralis* muscle larvae stage-specific antigenic serine protease was cloned and characterised as a secretory glycoprotein (Romaris et al. 2002; Nagano et al. 2003), and the proteinase activity of the recombinant serine protease was confirmed (Nagano et al. 2003). We also cloned a newborn larvae stage-specific putative serine protease gene (Mingyuan and Boireau, unpublished data). All these data support the existence of a superfamily of serine proteases in *T. spiralis*. It has been demonstrated that the C-terminal part of the protease domain is crucial to exerting influence on the active site and triggering certain conformational changes (Krem et al. 1999). According to this observation, serine protease genes should have different functions in *T. spiralis*. Multigene protease families have been found in numerous parasites such as *Fasciola hepatica* (Heussler and Dobbelaere 1994), *Haemonchus contortus* (Cox et al. 1990) and *Aspergillus flavus* (Ramesh et al. 1994). Each member of these protease families may have a specific role dependent on particular regulation (constitutive or stage-specific), location (intestinal tract or excreted), the presence of a regulatory domain (in N or C terminal part) or variations in the amino acid sequence.

It has been hypothesised that the hydrophilic region of *TsSerP* may contain some immunodominant epitopes, but neither of the recombinant proteins of the *TsSerP* beta domain and hydrophilic domain (data not shown) was recognised by *T. spiralis*-infected pig serum. Although some parasite proteases have been shown to be immunodominant antigens (Romaris et al. 2002; Nagano et al. 2003), *TsSerP* is poorly immunogenic. The detection of

TsSerP gene transcription in Ad, ML and NBL indicated its continuous expression during these stages. Proteolytic activities have been reported by Ros-Moreno et al. (2000) in crude extracts and E/S antigens throughout the life cycle of *T. spiralis*. *TsSerP* should not therefore be involved in the proteolytic activities of E/S products because *TsSerP* is not found in E/S products.

The anti-Trx- β SerP serum localised *TsSerP* on peripheral regions and the oesophagus of *T. spiralis* muscle larvae and adult worms. The characteristic IIF pattern observed is consistent with the antigenic group 3 of muscle larvae previously described for various *Trichinella* species with monoclonal antibodies (Boireau et al. 1997). The peripheral and oesophageal location of *TsSerP* and its continuous expression throughout all antigenic stages suggest two potential roles in the physiology of *Trichinella*. Firstly, *TsSerP* may be involved in the proteolysis of cuticle proteins during the moulting of first-stage *T. spiralis* larvae and may thereby play a critical role in antigen shedding. Proteases from other parasites have been shown to be involved in this essential process of moulting. Aminopeptidase activity was identified during in vitro development of the L3 to L4 larval stages of *Ascaris suum* (Rhoads et al. 1997), and peak secretion of the enzyme was correlated with moulting, thus demonstrating its involvement. Metalloproteases of *H. contortus* (Gamble et al. 1996) and metalloproteases associated with cysteine proteases of *Dirofilaria immitis* (Richer et al. 1992) have also been suggested to play a role in the moulting process and in the degradation of cutaneous tissue components. Secondly, *TsSerP* may be involved in parasite nutrition through its digestive function. The two functions may be linked if transcuticular digestion is important in *Trichinella* physiology, but verification of such a hypothesis requires determination of the roles of the intestinal tract vs the cuticle in the absorption of major nutrients. The constitutive expression and location of *TsSerP* suggest a function in moulting and/or digestion, and this protease may play a key role in the parasite.

The experiments performed in the context of this study complied with current laws in the countries where the experiments took place.

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