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Identification and expression analysis of the *Steinernema carpocapsae* elastase-like serine protease gene during the parasitic stage

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ABSTRACT

A cDNA encoding elastase was isolated from *Steinernema carpocapsae* by suppression subtractive hybridization and rapid amplification of 5′ cDNA ends. The predicted protein contained a 19-aa signal peptide, a 44-aa N-terminal propeptide, and a 264-aa mature protein with a predicted molecular mass of 28,949 Da and a theoretical pI of 8.88. BLAST analysis showed 27–35% amino acid sequence identity to serine proteases from insects, mammals, fish and other organisms. The *Sc-ela* gene contains three exons and two introns with at least two copies in the *S. carpocapsae* genome. Expression analysis indicated that the *Sc-ela* gene was upregulated during the initial parasitic stage. Sequence comparison and evolutionary marker analysis revealed that Sc-ELA was a member of the elastase serine protease family with potential degradative, developmental and fibrinolytic activities. Homology modeling showed that Sc-ELA adopts a two β-barrel fold typical of trypsin-like serine proteases, and phylogenetic analysis indicates that Sc-ELA branched off early during elastase evolution.

1. Introduction

Entomopathogenic nematodes (EPNs), including *Steinernema carpocapsae*, are widely used as commercial bioinsectcides against many insect pests (Kaya et al., 2006). Infective-stage juveniles (IJs) live in soil and infect suitable insect hosts primarily by gaining entry through natural body openings (Koppenhofer et al., 2007). They then undergo a recovery process, become parasitic and release their symbiotic bacteria. The nematodes penetrate digestive tract tissues and invade the hemocoel of hosts. Both pathogens kill the host within 2–3 days and multiply within the insect cadaver. When food reserves are depleted, nematode reproduction ceases, and offspring develop into infective juveniles, which disperse from the dead host and are able to survive in the environment to seek new hosts. In order to complete their life cycle, parasite nematodes must overcome host immune attacks, penetrate the intestinal wall, and migrate into the hemocoel. Connective tissues, basement membranes and epidermis all represent major barriers to parasite invasion and migration. Therefore, degradation of host tissues is critical for parasite survival (Rhoads et al., 1997). Studies of specific molecules that facilitate the migration and penetration of infective-stage nematodes are important for a better understanding of the host-parasite relationship and to improve the parasitic capacities of beneficial nematodes.

Evidence has shown that extracellular proteases secreted by invasive helminth larvae help to mediate host invasion and migration through host tissues (McKerrow, 1989; Knox and Jones, 1990). Hypothetical tissue-destructive proteases have been reported to be present in excretory–secretory (ES) products of *Ancylostoma caninum* (Hotez et al., 1985; Hawdon et al., 1995), *Anisaki simplex* (Morris and Sakanari, 1994), *Brugia malayi* (Petralanda et al., 1986), *Dirofilaria immitis* (Richer et al., 1992), *Nippostrongylus brasiliensis* (Healer et al., 1991), *Strongyloides stercoralis* (McKerrow et al., 1990), *Toxocara canis* (Robertson et al., 1989) and *Trichuris suis* (Hill et al., 1993). Elastinolytic enzymes associated with tissue penetration by parasitic helminthes have been reported from ES products of parasites, including *Onchocerca volvulus* (L3) (Haffner et al., 1998), *S. stercoralis* (L3) (McKerrow et al., 1990), *Haemonchus contortus* (Gamble and Mansfield, 1996), and *Nippostrongylus brasiliensis* (L3) (Knox and Jones, 1990). However, far few studies have unequivocally attributed tissue penetration and migration to specific gene products. Thus far, recombinant protein studies have confirmed that elastase secreted from *Schistosoma mansoni* cercariae is the only gene product from a parasitic helminth with a proven role in skin penetration (Newport et al., 1988).

Elastase is a small serine protease that can degrade a wide variety of protein substrates, including elastin, the insoluble and highly cross-linked protein of connective tissue. As an endopeptidase, elastase catalyzes the hydrolysis of internal peptide bonds located adjacent to small non-polar residues within the protein. Based on

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differences in primary structure and specificity with synthetic substrates, elastases have been categorized into four different types: pancreatic elastase I and II, leukocyte elastase and pancreatic endopeptidase E.

Here, we describe the identification and characterization of a cDNA encoding an elastase-like serine protease from the parasitic stage of *S. carpocapsae*. Expression pattern analysis demonstrated that *Sc-ela* is an inducible gene that is upregulated in esophageal cells during the initial parasitic stage. Taken together these findings indicate this newly identified Sc-ELA protease may be crucial mediator of parasite migration.

2. Materials and methods

2.1. Nematode collection and induction

Infective juveniles (IJs) were produced in *Galleria mellonella* larvae, harvested in a white trap (Dutky, 1959) and stored at 10 °C for 1–2 months. Prior to use, IJs were surface-disinfected with 2% bleach for 10 min and washed three times in sterile water. Parasitic nematodes were obtained *in vitro* by induction with 1% insect hemolymph in 7 ml Tyrode solution plus 1% antibiotic (penicillin–streptomycin–neomycin, Sigma) and incubated under agitation at 25 °C (Hao et al., 2008). Hemolymph was obtained from *G. mellonella* larvae by bleeding the first pro-leg under cool conditions. Sterilized infective juveniles without any further treatment were used as controls. After a defined time, induced and control nematodes were harvested by centrifugation, washed three times in sterile water, separated using a filter, and immediately used for RNA extraction.

2.2. RNA and DNA isolation

Total RNA was isolated using the TRIzol reagent (Invitrogen, Portugal) according to the manufacturer's instructions. Genomic DNA for cloning and Southern blot was extracted from pooled *S. carpocapsae* nematodes using the method described by Sulston and Hodgkin (1988).

2.3. Suppression subtractive hybridization (SSH) and gene identification

Suppression subtractive hybridization (SSH) and differentially expressed gene screening were performed using the PCR-Selected cDNA Subtraction Kit (BD Biosciences Clontech, Palo Alto, USA) and reverse Northern blot, respectively (Hao et al., 2008). Plasmids from positive clones were purified and sequenced (Stabvida, Oeiras, Portugal). Homology searches were carried out using the Blast program (Altschul et al., 1990) against GenBank database sequences (http://www.ncbi.nlm.nih.gov/).

2.4. Full-length cDNA cloning

Full-length elastase cDNA was synthesized by SMART RACE (rapid amplification of cDNA ends) technology (Clonteck-Takara, Portugal). 5′-RACE cDNA pools were synthesized from 2 μg of total RNA isolated from parasitic stage nematodes using Superscript III reverse transcriptase (Invitrogen, Porugal). The *Sc-ela* gene-specific primer (5′TGCCACGGTATGTGTAGGTTCCAAAGC3′) was designed using the cDNA sequence isolated by SSH. PCR conditions were as follows: 30 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min, with a final extension at 72 °C for 5 min. PCR products were cloned into the pCR4-TOPO vector, then transformed into *E. coli* DH5α cells by heat shock. DNA inserts isolated from positive clones were sequenced, and full-length cDNA was obtained by joining the two fragments.

2.5. Bioinformatic analysis of Sc-ELA

Identification of protein motifs was carried out by SMART (http://smart.embl-heidelberg.de/), Conserved Domain Database search from NCBI and MotifScan analysis (http://myhits.isb-sib.ch/cgi-bin/motifscan). The signal peptide was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/), and the theoretical isoelectric point and molecular weight were predicted using ProtParam and Compute pI/Mw (http://expasy.org/tools/protparam.html), respectively. Disulfide bonds were identified with ScanProsite (http://expasy.org/tools/scanprosite/), and hydrophobicity profile analyzes was performed using the Kyte-Doolittle method (http://www.expasy.org/cgi-bin/protscale.pl).

Sequence similarity was analyzed by BLAST, searching in the non-redundant protein databases at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). Multi-sequence alignment was generated using CLUSTAL W, and the sequence logo alignment was created online using WebLogo (http://weblogo.berkeley.edu/logo.cgi). Phylogenetic analysis was conducted using MEGA 4.0 (Tamura et al., 2007). An unrooted tree was inferred by the Neighbor-Joining (NJ) method with Poisson correction for amino acids, complete deletion of gaps, and 2500 bootstrap replications. *S. carpocapsae* chymotrypsin was used as an outgroup.

2.6. Genomic sequence isolation and Southern blot analysis

Partial *Sc-ela* genomic sequences were PCR amplified using primer Sc-elaF1 (5′GGCTTCTTTTCACCTTCGTCTG3′) and Sc-elaR1 (5′GTTAGAAGTAAATGCAAGGGGC3′) designed according to the full-length cDNA sequence. Each reaction contained 50 ng of genomic DNA, 2.5 μl 10 × PCR buffer, 10 mM of each dNTP, 0.4 μM of each primer, 1.6 mM $MgCl_2$, and 0.2 μl *Taq* polymerase (5U/μl) in a final volume of 25 μl. Amplification conditions were as follows: 94 °C for 4 min, followed by 25 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 3 min. PCR products were cloned and sequenced as described above. Comparisons between cDNA and gDNA were made to assess similarities in coding regions and to identify intron–exon boundaries.

For Southern blot analysis, a 357 bp fragment was amplified using gene-specific primers [Sc-elaF2 (5′GTTCCCTCAGCAGGCGTTC C3′) and Sc-elaR2 (5′AGGAAGGCATTGTGGGATTG3′)] and labeled with digoxingenin (Roche, Germany). Genomic DNA (10 μg) was digested with *Blg*II, *Eco*R V, *Hind*III, *Xba*I or *Xho*I before separation by 0.8% agarose gel electrophoresis and transferred onto Hybond-N^+ nylon membranes (Amersham-Pharmacia). Membranes were UV-crosslinked and hybridized with labeled probe. After prehybridization at 65 °C for 1 h in 5 ml Express Hybridization solution, membranes were hybridized with denatured cDNA probe at 65 °C overnight. Hybridized membranes was then washed (i) three times with 2 × SSC and 0.1% SDS at 65 °C for 10 min each; (ii) twice with 0.1 × SSC and 0.5% SDS at 62 °C for 15 min each; (iii) with detection buffer for 5 min; and (iv) developed with NBT/BCIP substrates for color detection overnight.

2.7. Sc-ela gene expression analysis

Total RNA from nematodes in the juvenile infective-stage, parasitic stage (6-, 12-, 24- and 48-h treatment) and post-parasitic stage (72-h treatment) were isolated and reverse-transcribed with random hexamers (as described above), and the resulting cDNA products were PCR amplified.

Sc-ela RT-PCR analysis was performed using Sc-elaF2 and Sc-elaR2 gene specific primers. Amplification of 18S rRNA was used as an internal control [primers 18SF (5′GCTAATCGGAAACGAAAGT C3′) and 18SR (5′CATCCACCGAATCAAGAAAG3′)]. PCR reactions were performed using the following program: 94 °C for 4 min,