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Cloning, characterisation and heterologous expression of an astacin metalloprotease, *Sc*-AST, from the entomoparasitic nematode *Steinernema carpocapsae*

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ABSTRACT

Steinernema carpocapsae is a parasitic nematode that is high virulent to insects. The parasitic juvenile reaches the insect haemocoelium by passing through mid-gut barriers and develops there. During invasion, the nematode was predicted to express a large set of proteases, including metalloproteases, one of which was sequenced and expressed in this work. A 1583-nucleotide cDNA encoding a putative metalloprotease containing a 28-aa signal peptide, a 79-aa propeptide and a 311-aa mature protease with a predicted molecular mass of 35.2 kDa and a theoretical p*I* of 5.9 was cloned from the parasitic stage of the nematode. Sequence analyses predicted signature sequences of the astacin metalloprotease family, an astacin domain, a zinc-binding motif and a methionine turn motif; therefore, this protein was identified as an astacin and designated as Sc-AST. The astacin domain of Sc-AST has an amino acid sequence homology of 46% to prototypical astacin from *Astacus astacus* and 82% to *Caenorhabditis elegans* NAS-8. Like NAS-8 of *C. elegans*, *Sc*-AST has a C-terminal ShK toxin domain. Recombinant *Sc*-AST was produced in an *Escherichia coli* system and was purified by affinity chromatography. Maldi-MS/MS analysis of purified recombinant protein matched the Sc-AST sequence with a significance score of 499. Sc-AST was produced in the correct folding conformation, showed activities against gelatin and azocasein substrates and was inhibited by divalent metal-chelating agents. Sc-AST presented an optimum pH of 7.5 and temperature of 37 °C and K_m, V_{max} and k_{cat} values of 1.86 mM, 0.281 μM/min and 27.9 s^{-1}, respectively. Expression analyses indicated that Sc-AST is up-regulated in the parasitic stage and is strongly induced *in vitro* by insect tissues, thus suggesting that it plays a role in the parasitic process.

1. Introduction

Steinernema carpocapsae is an insect parasitic nematode symbiotically associated with the bacterium *Xenorhabdus nematophila*. This complex has emerged as an excellent biological control agent against insect pests and is produced and used all over the world to control a wide spectrum of insects [1]. The efficacy of these nematodes in controlling insect pests is mainly due to the virulence factors toward insects that this complex produces, which modulate invasion, evasion of self-defenses and insect death shortly after contamination. These virulence factors are toxins and proteases released by the bacteria [2] but they also include diverse compounds released by the nematode. The latter include proteins that are able to destroy insect antimicrobial peptides [3], proteins that cause insect death [4–6] and proteases [7]. Studies of the proteases expressed by the parasitic nematode received a great deal of support from expressed sequence tags (EST) generated with RNA. Analysis of these tags allowed the prediction of a large set of proteases, with a few of them predicted to be secreted and excreted [8]. So far, three serine proteases have been proven to modulate parasitic mechanisms. A serine protease was shown to induce cell disruption, thus facilitating parasite invasion [9] and another two serine proteases were shown to be interacting with insect self-defenses by inhibiting prophenoloxidase activity and by disturbing cellular encapsulation [10,11]. The second most important family of proteases predicted by EST analysis was metalloproteases, which all have homologues in parasitic nematodes [8]. Metalloproteases expressed by parasitic nematodes seem to have diverse roles, which range from feeding and digestion to tissue degradation and immune evasion, facilitating establishment [12]. Among the predicted secreted metalloproteases in *S. carpocapsae* there was an

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astacin protease. The astacin proteases are zinc metalloproteases found in various organisms [13]. In parasitic nematodes, astacins have been identified in *Trichinella spiralis*, *Strongyloides stercoralis* and *Ostertagia ostertagi* [12,14,15]. It has been suggested that astacin proteases of parasite worms disrupt the extracellular matrix and aid penetration of the parasite into host tissue and movement inside the host. Therefore, it was assumed that the astacin proteases family might be important molecules in the parasitic process of nematodes. Here, we report the identification, cloning, recombinant expression, purification and biochemical characterisation of an astacin protease expressed by *S. carpocapsae*.

2. Materials and methods

2.1. Nematode induction of the parasitic stage

S. carpocapsae Breton strain was grown *in vitro* according to [16], and infective juveniles (IJs) were stored in tap water at 10 °C for 2–4 months before use in assays. To induce recovery to the parasitic stage, IJs were surface sterilised with 0.5% sodium hypochloride, washed with 0.8% NaCl solution, transferred to Tyrod solution plus antibiotics (0.05 mg penicillin, 0.1 mg streptomycin, 50 U and neomycin; Sigma) with 10% of heat denatured insect homogenate and incubated at 23 °C with agitation for different defined times.

The life cycle stages were obtained from parasitized *Galleria mellonella* larvae after exposure to IJs. Parasitic stage nematodes were collected from parasitized larvae after 12 h of exposure. Fourth juveniles, adults, and first and second juveniles were harvested later, after inspection for the desired developmental stage.

2.2. Total RNA isolation and cDNA synthesis

Total RNA was isolated from the desired nematodes using the TRIzol reagent (Invitrogen, Germany) according to the manufacturer's instructions. cDNA was synthesised using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Germany) according to the manufacturer's instructions.

2.3. Full-length cDNA

5′ and 3′ RACE *Sc*-AST cDNA was obtained using the SMART™ RACE cDNA Amplification Kit (Clontech-Takara, UK). According to the sequence in the EST library constructed in our laboratory, the specific primers AST5′ (5′-TGGAGGAGGTAGCGGGAGGTTC-3′) and AST3′ (5′-GCAGCCGACCCTAAGTATTG-3′) were designed for 5′RACE and 3′RACE, respectively. PCR conditions were as follows: 25 cycles at 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 subcloned into the pCR4-TOPO vector (Invitrogen, Germany) and transformed for screening. The positive clones were sequenced by STAB VIDA, Portugal.

2.4. Analysis of Sc-AST gene expression

Total RNA from different nematode stages and from nematodes induced for 0, 6, 12, 24, 36, 48 and 72 h was isolated and reverse-transcribed into cDNA products. Relative gene expression of *Sc*-AST was quantified by real-time RT-PCR using 18S rRNA as an endogenous control. Primers for 18S rRNA were 18SF (5′-GCTAATCGGAAACGAAAGTC-3′) and 18SR (5′-CATCCACCGAATCAAGAAAG-3′). Primers for *Sc*-AST were ASTF1 (5′-TTCCTTGCTCCGCTTTACGATG-3′) and ASTR1 (5′-TCCTCGAATGTCTCCGCGATAG-3′). Real-time RT-PCR was performed using SYBR Green Mix according to the manufacturer's instructions (Applied Biosystems). Real-time RT-PCR conditions were as follows: 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and 60 °C for 60 s. qRT-PCR data from three replicate samples were analyzed with the RelativeManager Software (Applied Biosystems, USA) to estimate transcript levels of each sample using the $2^{-\Delta\Delta Ct}$ method [17].

2.5. Bioinformatics analysis

Protein motifs were identified using SMART (http://smart.embl-heidelberg.de/) and the Conserved Domain Database from NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). 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 Compute pI/MW (http://expasy.org/tools/protparam.html). Sequence similarity was analyzed by BLAST from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Multi-sequence alignment was generated using CLUSTAL W in BioEdit 7.0. Phylogenetic analysis was conducted using MEGA 4.1 [18]. Homology modeling was performed by the SWISS-MODEL server (http://swissmodel.expasy.org/) [19].

2.6. Construction of a heterologous expression system

The T7-based expression system, pET23a(+), which allows protein expression upon induction of the T7-polymerase in *Escherichia coli* cells by IPTG, was chosen to obtain strong and reproducible expression of the recombinant protein. The sequence of mature *Sc*-AST was amplified using the forward primer ASTF2 (5′-GGAATTCCATATGAAAAGGTGGCCGAACGGACG-3′) containing an NdeI restriction site (underlined) and the reverse primer ASTR2 (5′-CGCGGATCCCCGAATCGCTGGTAGAGCGGA-3′) containing a BamHI restriction site (underlined). The fragments were cloned into the NdeI and BamHI sites of vector pET23a(+), which was then designated AST/pET and transformed into *E. coli* Rosetta II (DE3). The transformants were designated AST/RII.

2.7. Expression and production of recombinant Sc-AST

A single colony of AST/RII was inoculated into 5 ml LB broth containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol, incubated overnight at 30 °C and then transferred to 1 l LB broth plus antibiotics and incubated at 37 °C. After 3 h of incubation, IPTG was added to the culture at a final concentration of 0.2 mM, and the cultures were incubated for another 3 h. Cells from broth culture were harvested by centrifugation (4000 × *g* for 15 min). The pellet was resuspended in 40 ml of 20 mM PB, pH 7.4, containing 0.5 M NaCl, 10 mM imidazole and 1 mg/ml lysozyme. After storage at −20 °C overnight, the sample was defrosted, 1 ml of 1 M $MgCl_2$ and 600 μl of 1 mg/ml DNAase were added, and the mixture was incubated for 2 h at room temperature. Cell debris was then removed by centrifugation (12,000 × *g* for 20 min), and the supernatant was collected.

2.8. Purification, SDS-PAGE and zymogram analysis of Sc-AST

The *Sc*-AST solution was filtered with a 0.22 μm filter and injected onto a 1 ml HisTrap column (GE Healthcare, Germany) equilibrated with 20 mM PB, pH 7.4, 0.5 M NaCl and 20 mM imidazole. After sample application, unspecific bound proteins were washed away with 5 ml equilibration buffer, and recombinant protein was eluted with 5 ml of 20 mM PB, pH 7.4, 0.5 M NaCl, and 500 mM imidazole. Purification was performed at 4 °C.

SDS-PAGE was performed on a 12% polyacrylamide resolving gel and a 5% stacking gel using a Mini-protean II gel system (Bio-Rad, USA). The gel was stained using colloidal Coomassie as described [20]. The zymogram was carried out according to Lantz and Ciborowski [21] on a 12% polyacrylamide gel with 0.05% gelatin. After electrophoresis, the gel was washed three times for 30 min in