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Steinernema glaseri surface enolase: Molecular cloning, biological characterization, and role in host immune suppression

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ABSTRACT

Entomopathogenic nematodes are widely used as biological control agents that can suppress or evade the host immune defense upon entry into insects. The surface coat of *Steinernema glaseri* has been shown to play important roles in defeating the host immune system. In this work, a protein fraction with antiphagocytic activity was separated by electro-elution and further analyzed by two-dimensional electrophoresis (2-DE). LC–MS/MS analysis of one protein spot from a 2-DE gel gave five peptides that were highly similar to enolases of many organisms. A 1311 bp cDNA was cloned that encodes a 47 kDa protein with high sequence identity to enolases from different species of nematodes. The deduced protein, Sg-ENOL, was expressed in *Escherichia coli*, and its glycolytic activity was demonstrated by the conversion of 2-phospho-D-glycerate to phosphoenolpyruvate. Recombinant Sg-ENOL significantly reduced the LT_{50}s of *Xenorhabdus poinarii* and *Metarhizium anisopliae* when co-injected into *Galleria mellonella* and *Locusta migratoria manilensis* Meyen, respectively. Using immuno-gold transmission electron microscopy, native Sg-ENOL was confirmed to be localized to both the nematode cuticle and the surface coat. *In vitro*, secretion of Sg-ENOL was inducible rather than constitutive. *In vivo*, Sg-ENOL was detected in the host hemolymph after infection of *G. mellonella* with *S. glaseri*, indicating that Sg-ENOL was secreted into the insect hemocoel and was involved in infection. This is the first report of the cloning and characterization of a surface coat protein in an entomopathogenic nematode. Our findings provide clear evidence for an important role for a cell surface enolase in *S. glaseri* infection and host immune suppression.

1. Introduction

Entomopathogenic nematodes (EPNs) from the families *Steinernematidae* and *Heterorhabditidae* are of considerable interest because of their potential as biological control agents of insect pests [1,2]. The infective nematode juveniles (IJs) search for insect hosts and enter the host hemocoel by enzymatic and mechanical means and then release symbiotic bacteria. The bacteria produce toxins, antibiotics, proteases, and lipases that kill the host within 24–48 h after nematode infection [3–5]. However, certain species of nematodes do not release symbiotic bacteria immediately upon entry into a host. *Steinernema glaseri* releases *Xenorhabdus poinarii*, which is susceptible to the host immune response [6], within 4–6 h after the nematode enters the host hemolymph [7]. To survive within the first few hours after penetrating the host and to create a lower stress environment for the symbiotic bacteria, *S. glaseri* must suppress the host immune defense. The success of insect colonization by EPNs depends on their ability to subvert the host immune system.

Insect hosts have developed defense mechanisms against nematode infections, despite the lack of T- and B-cells, which are found in vertebrates. The larvae can recognize intruders, initiate a rapid and effective immune response, and discriminate them from self [8–10]. The insect immune system is subdivided into cellular and humoral defenses. Cellular defenses include hemocyte-mediated immune responses, such as phagocytosis and encapsulation [11–13]. Humoral defenses are biological responses induced by invading organisms, including the production of antimicrobial peptides, lysozyme, lectins, and phenoloxidase that regulate melanization in the hemolymph [12,14]. In the insect immune system, the most important defense mechanism against metazoan parasites is cellular encapsulation or phagocytosis [15].

Abbreviations: SCP, surface coat protein; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-propane-sulfonate; 2-DE, two-dimensional electrophoresis; LC–MS/MS, liquid chromatography/mass spectrometry/mass spectrometry; IPTG, isopropyl β-D-thiogalactoside; GST, glutathione S-transferase; 2-PGE, 2-phospho-D-glycerate; PEP, phosphoenolpyruvate; HEPES, N-(2-hydroxyethyl) piperazine-N′-(2-ethane sulfonic acid); PVDF, polyvinylidene fluoride; TBST, tris buffered saline containing 0.1% Tween 20; TEM, transmission electron microscopy.

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Host cellular or humoral immune responses can be suppressed by nematodes. *S. glaseri*, which was originally isolated from the larvae of the Japanese beetle (*Popillia japonica*), has evolved mechanisms to evade immune encapsulation. Live axenic *S. glaseri* nematodes not only avoid host melanotic encapsulation but also protect freeze-killed nematodes from encapsulation by *P. japonica* larvae [7,16]. In all types of nematodes, including free-living and plant and animal parasitic forms, the cuticle is overlaid with an amorphous surface coat [17–20]. As an interface between the parasitic nematode and the host immune system, the surface coat of IJs is believed to play important roles in host-parasite interactions [20,21]. Surface coat shedding [22], surface antigen switching [23], and coat structure reorganization [24] help parasitic nematodes avoid host immune recognition during the early phase of infection. By lysing hemocytes or depleting hemolymph components, proteins [16,25,26], coat glycoproteins [27] and lipids [10,28–30] help IJs to suppress or evade the host immune response.

The surface coat proteins (SCPs) of *S. glaseri* have been reported to protect co-injected *Heterorhabditis bacteriophora* from encapsulation and melanization by *P. japonica* [16] and *Exomala orientalis* (the oriental beetle) [25]. Two isolated 38 kDa and 56 kDa *S. glaseri* SCPs have been shown to have an immunosuppressive effect. SCP peptides were sequenced using LC–MS/MS, but no significant matches were found in available databases [26]. The study of nematode surface coat components and secretions released into the insect hemocoel has provided significant insights into the evasion mechanisms of EPNs; nevertheless, there is limited understanding of the molecular aspects of the insect–nematode interaction. Thus far, little information has been available on the SCPs involved in the parasitic life of EPNs.

In the present study, we characterized a protein from *S. glaseri* SCPs and cloned its encoding gene. Our data demonstrate that this cuticle protein has a high similarity to α-enolases of many organisms, including *Caenorhabditis elegans*. Furthermore, we expressed this protein in *Escherichia coli* and confirmed its immuno-suppressive activity. This study increases our understanding of the mechanisms of host immune suppression by EPNs.

2. Materials and methods

2.1. Nematodes, microbes and insect species

Galleria mellonella was grown on artificial culture medium (a mixture of wax, honey, corn powder, milk powder, wheat bran and yeast extract) in the dark at 28 °C. Last-instar larvae (250–300 mg in weight) were used throughout this study.

The NC strain of *S. glaseri* was originally obtained from Professor Randy Gaugler (Rutgers University, USA) and cultured in last-instar *G. mellonella* at room temperature. IJ-stage nematodes were harvested from White traps [31] and were washed and stored in distilled water at 8 °C.

X. poinarii was obtained by sampling the hemocoel of *G. mellonella* larvae infected with the *S. glaseri* NC strain [32]. The primary form of *X. poinarii* was purified on nutrient agar with 0.004% (w/v) triphenyltetrazolium chloride and 0.025% (w/v) bromothymol blue (NBTA). For bioassays, single colonies were selected and inoculated into a yeast extract-based medium (10 g of yeast extract, 5 g of glucose, 0.5 g of K_2HPO_3 and 2 g of NaCl per L). After culturing at 180 rpm for 48 h at 28 °C, *X. poinarii* was harvested and washed twice with PBS (137 mM NaCl, 2.6 mM KCl, 6.4 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4).

Metarhizium anisopliae var. *acridum* strain CQMa102 was a gift from Professor Yuxian Xia (Chongqing University, China) and was cultured on ¼-strength Sabouraud's dextrose agar at 28 °C for 12 days or until sporulation occurred. Conidia were harvested in distilled water, and the resulting spore suspension was filtered through sterile lens paper to remove any mycelia and medium debris.

Locusta migratoria manilensis Meyen were fed on fresh wheat shoots and maintained at 26 °C under a 12 h light:12 h dark photoperiod. They were used 1 day after molting to the fourth larval stage.

All experimental insects were untreated to prevent pharmacological effects on the immune system.

2.2. Purification and bioassay of SCPs

Crude SCPs were extracted as previously described [16,26]. Briefly, IJs were washed four times with distilled water and placed in ice-cold 35% ethanol for 1 h. The suspension was filtered, and the resulting solution was frozen at −80 °C and lyophilized. The crude protein powder was dissolved in PBS, separated on an 8% preparative native PAGE, and electro-eluted at 85 mA for 25 min in elution buffer (25 mM Tris, 19.2 mM glycine, pH 8.3) using the Mini Whole Gel Eluter (Bio-Rad, USA). The eluted fractions were collected and ultrafiltered against PBS using an Amicon ultrafiltration device with a cutoff of 10 kDa (Millipore, USA). The protein purity and concentration of each fraction were determined by SDS-PAGE analysis and by the BCA™ assay kit (Pierce, USA), respectively, and aliquots were stored at −20 °C.

The antiphagocytic activity of purified SCPs was tested as described previously [16] with some modifications. Protein fractions collected from the gel eluter were adjusted to 50 μg/ml and covalently coupled to carboxylate-modified fluorescent microsphere beads (F-8821, Invitrogen) following the manufacturer's protocol, and BSA was used as a control. Last-instar *G. mellonella* larvae were injected with 5 μl of protein-coupled microsphere beads (approximately 1×10^6 beads per larva). At 2 h post-injection, 5 μl of hemolymph was collected by cutting the forelegs of the larvae and was added to anticoagulant buffer. Phagocytosed and free beads were examined under a BX51 fluorescence microscope (Olympus, Japan).

2.3. Two-dimensional electrophoresis (2-DE)

The electro-eluted fraction with the highest antiphagocytic activity was precipitated with 10% trichloroacetic acid in acetone (containing 2% β-mercaptoethanol) at −20 °C overnight. After centrifugation at 10,000 × *g* for 30 min at 4 °C, the pellet was washed with ice-cold acetone three times, air-dried and dissolved in rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 64 mM dithiothreitol, 0.5% 3–9.5 ampholytes). Subsequently, 250 μl of solubilized protein was loaded onto non-linear 13-cm IPG strips, pH 3–10 (GE Healthcare, USA). After 12 h of active rehydration at 50 V, the IPG strips were subjected to isoelectric focusing using an Ettan IPGphor3 system (GE Healthcare) programmed as follows: 1 h at 500 V, a 1-h gradient to 1000 V, a 3-h gradient to 8000 V and constant at 8000 V for a total of 20,000 V h. The strips were subsequently incubated for 15 min in equilibration buffer I (6 M urea, 75 mM Tris, pH 8.8, 2% SDS, 30% glycerol, 1% dithiothreitol) followed by 15 min in equilibration buffer II (6 M urea, 75 mM Tris, pH 8.8, 2% SDS, 30% glycerol, 2.5% iodoacetamide). The second-dimension of electrophoresis was performed on a 12% SDS-PAGE using a PROTEAN II xi cell (Bio-Rad). After separation, proteins were visualized by silver staining, which is compatible with mass spectrometry [33].

2.4. Mass spectrometry analysis

Liquid chromatography/mass spectrometry/mass spectrometry (LC–MS/MS) was performed to identify proteins from 2-DE gels