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ScienceDirect
Journal of Invertebrate Pathology 96 (2007) 230–236
Journal of INVERTEBRATE PATHOLOGY
www.elsevier.com/locate/yjipa

Differentially-expressed glycoproteins in *Locusta migratoria* hemolymph infected with *Metarhizium anisopliae*

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Received 7 February 2007; accepted 21 May 2007
Available online 20 June 2007

Abstract

Glycoproteins play important roles in insect physiology. Infection with pathogen always results in the differential expression of some glycoproteins, which may be involved in host–pathogen interactions. In this report, differentially-expressed glycoproteins from the hemolymph of locusts infected with *Metarhizium anisopliae* were analyzed by two-dimensional electrophoresis (2-DE) and PDQuest software. The results showed that 13 spots were differentially expressed, of which nine spots were upregulated and four were downregulated. Using MS/MS with *de novo* sequencing and NCBI database searches, three upregulated proteins were identified as locust transferrin, apolipoprotein precursor, and hexameric storage protein 3. These proteins have been reported to be involved in the insect innate immune response to microbial challenge. Due to the limited available genome information and protein sequences of locusts, the possible functions of the other 10 differentially-expressed spots remain unknown.
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Keywords: Apolipoprotein precursor; Hexameric storage protein 3; *Locusta migratoria*; *Metarhizium anisopliae*; Transferrin

1. Introduction

The locust, *Locusta migratoria* migratorioides, is susceptible to the insect pathogenic fungus, *Metarhizium anisopliae* var. *acridum* (Lomer et al., 1997). Indeed, there has been considerable interest in the use of fungi in the biocontrol of locusts and grasshoppers in recent years (Lomer et al., 2001; Elliot et al., 2002). However, the slow speed of killing is perceived as a potential drawback for fungal insecticides. Further studies on mechanisms of fungal pathogenesis and host defense are imperative and may suggest new strategies for the development of more efficient anti-locust mycoinsecticides (Gillespie et al., 2000).

For fungal entomopathogens, pathogenesis is a complicated interaction between fungus and host. Pathogenic fungi kill insects by producing proteases to degrade the insect cuticle (Gillespie et al., 1998; Tiago et al., 2002), releasing destruxins to reduce the impact of the cellular immune response (Kershaw et al., 1999), or secreting glucosidase, trehalase, and acid phosphatase to utilize nutrition from insects (Xia et al., 2000; Zhao et al., 2006). On the other hand, insects will actively defend themselves against fungal infection. Their first-line defense consists of structural barriers that include the outer exoskeleton, the peritrophic matrix of the midgut epithelium, and the chitinous linings of the trachea. Once in the hemocoel, the infectious fungi fight the insect's humoral and cellular responses. Humoral immune responses in insects are carried by proteins in hemolymph (Royet, 2004). Some hemolymph proteins that directly or indirectly participate in immune responses have been identified and characterized, and their genes have been cloned from many insect species (Hoffmann, 1995; Chung and Ourth, 2002; Park et al., 2005).

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0022-2011/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2007.05.012

Glycoproteins are proteins that have been post-translationally modified by glycosylation. They are abundant in various tissues and play important physiological roles. Most work on immune diseases and pathogen infection has focused on glycoproteins, especially for the study of human diseases (Varki, 1993; Perez et al., 1996; Wang et al., 2001). Research on glycoproteins that are differentially expressed in healthy insects and pathogen-mycosed insects may identify proteins involved in pathogenesis.

Recently, proteomic technology based on two-dimensional electrophoresis (2-DE) has been shown to be an efficient approach to explore changes in protein expression during the complicated interactions of organisms responding to various agents. This approach has been used successfully to study proteins specifically induced by pathogens (Tsuji et al., 1999; Yeon et al., 1999; Adriana et al., 2004; Ouchia et al., 2005; Jones et al., 2004; da Fonseca et al., 2001). Some glycoproteins involved in human diseases were also studied using the 2-DE technique associated with glycoproteomic technology. However, few studies using 2-DE have been reported on the interactions between insects and entomopathogenic fungi, especially for glycoproteomics. At present, few pathogen-induced specific glycoproteins in insects have been described, such as drosocin, drosomycin, diptericin, and prophenoloxydase-activating enzyme in *Drosophila* (Levy et al., 2004).

In the present study, 2-DE and mass spectrometry (MS) were utilized to identify specific differentially-expressed glycoproteins in locust hemolymph infected with *M. anisopliae* (CQMa102). Our results may provide clues for exploring the molecular mechanisms of fungal pathogenesis once the fungus infects the hemolymph.

2. Materials and methods

2.1. Insects

Locusta migratoria was reared in our lab under crowded conditions as previously described (He et al., 2006). Male and female insects were separated after adult emergence. Male adult locusts were used 5 days after final ecdysis.

2.2. Entomopathogenic fungus

Metarhizium anisopliae var. *acridum* strain CQMa102 (Li et al., 2006) was grown on 1/4 strength Sabouraud's dextrose agar medium (1% dextrose, 0.25% mycological peptone, 2% agar, and 0.5% yeast extract, w/v) for 12 days at 28 °C for the production of conidia.

2.3. Infection of insects

Conidia suspensions of CQMa102 were prepared in cottonseed oil at 1×10^7 spores/ml as previously described (Cao et al., 2007), and 5 μl were inoculated into the head-thorax junction of locusts. Control locusts were inoculated with 5 μl cottonseed oil. The infected and control insects were housed individually and returned to their normal diet and normal maintenance conditions.

2.4. Collection and treatment of hemolymph

On the 6th day after inoculation, about half of insects inoculated with pathogen died and the concentration of fungal blastospores in the hemolymph was about 10^8 per ml. Hemolymph collected from the arthrodial membrane of the hindleg of the locust was diluted 3-fold with ice-cold anticoagulant buffer (0.098 M NaOH, 0.18 M NaCl, 0.041 M citric acid). Samples were then centrifuged at 13,000*g* for 20 min to remove hemocytes. The supernatant was pipetted off and stored at −40 °C until use.

2.5. Protein concentration measurement

Protein concentration was determined with the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories). Bovine serum albumin (Fluka) was used as the standard.

2.6. Concanavalin A chromatography

The cell-free hemolymph sample was buffer-exchanged into binding buffer (20 mM Tris, pH 7.4 containing 0.5 M NaCl) using a G-25 desalting column. The binding buffer exchanged sample (5 ml) was loaded onto a Con A-Sepharose column (with caps at both ends) pre-equilibrated with binding buffer and then gently agitated at 4 °C for 30 min. The resin was washed with binding buffer until no protein was detected in the washing solution. The bound glycoprotein was eluted with 20 mM Tris buffer (pH 7.4) containing 0.5 M methyl-α-D-mannopyranoside. The elution fractions were collected and dialysed for 24 h against 1 liter Milli-Q water with three changes of water, lyophilized, and stored at −80 °C until 2-DE was performed.

2.7. Two-dimensional electrophoresis

For 2-D PAGE, lyophilized glycoprotein was resolubilized in sample buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholyte) for 3 h at 27 °C with vigorous shaking. Then, 1.8 mg protein sample was mixed with rehydration buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholyte, 0.001% bromophenol blue) up to a final volume of 350 μl. This mixture was used to rehydrate 17 cm, pH 5–8 linear RreadyStrip™ IPG Strips (Bio-Rad) for 11 h at 17 °C, with a constant voltage (50 V) applied across the gel strips, which were placed in the Protean IEF Cell focusing tray. The rehydrated gels were electrophoresed at 250 V for 30 min, subjected to a linear voltage ramp from 250 to 10,000 V for 5 h, and then focused at 17 °C until 60,000 V. The IPG strips were then incubated in SDS–PAGE equilibration buffer with 1% (w/v) dithiothreitol with gentle shaking. After 10 min, the procedure was repeated with SDS–PAGE

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