Insect Biochemistry and Molecular Biology 43 (2013) 555–561

Contents lists available at SciVerse ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Manduca sexta serpin-7, a putative regulator of hemolymph prophenoloxidase activation

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ARTICLE INFO

Article history:
Received 15 February 2013
Received in revised form
22 March 2013
Accepted 26 March 2013

Keywords:
Hemolymph
Immunity
Melanization
Protease inhibitor
Serpin
Tobacco hornworm

ABSTRACT

Serpins regulate various physiological reactions in humans and insects, including certain immune responses, primarily through inhibition of serine proteases. Six serpins have previously been identified and characterized in the tobacco hornworm *Manduca sexta*. In this study, we obtained a full-length cDNA sequence of another *Manduca* serpin, named serpin-7. The open reading frame of serpin-7 encodes a polypeptide of 400 amino acid residues with a predicted signal peptide of the first 15 residues. Multiple protein sequence alignment of the reactive center loop region of the *M. sexta* serpins indicated that serpin-7 contains Arg–Ile at the position of the predicted scissile bond cleaved by protease in the serpin inhibition mechanism. The same residues occur in the scissile bond of the reactive center loop in *M. sexta* serpin-4 and serpin-5, which are protease inhibitors that can block prophenoloxidase activation in plasma. Serpin-7 transcript was detected in hemocytes and fat body, and its expression increased in fat body after injection of larvae with *Micrococcus luteus*. Recombinant serpin-7 added to larval plasma inhibited spontaneous melanization and decreased prophenoloxidase activation stimulated by bacteria. Serpin-7 inhibited prophenoloxidase-activating protease-3 (PAP3), forming a stable serpin-protease complex. Considering that serpin-3 and serpin-6 are also efficient inhibitors of PAP3, it appears that multiple serpins present in plasma may have redundant or overlapping functions. We conclude that serpin-7 has serine protease inhibitory activity and is likely involved in regulation of proPO activation or other protease-mediated aspects of innate immunity in *M. sexta*.

1. Introduction

A key aspect of insect innate immunity is the ability of insects to melanize microbes and foreign objects (Ayres and Schneifer, 2008; Cerenius et al., 2008; Suwanchaichinda and Paskewitz, 1998). Melanization requires proteolytic activation of prophenoloxidase (proPO) to its active form (Kanost and Gorman, 2008; Vavricka et al., 2010). This activation is mediated by a cascade of multiple serine proteases, with proPO-activating proteases (PAPs) as a possible terminal component of the pathway. PAPs contain one or two regulatory clip domains at the amino terminus and a catalytic serine protease domain at the carboxyl terminus, which is activated by a specific proteolytic cleavage (Kanost et al., 2004). The activated proteases are regulated through their production as zymogens and rapid inhibition once they are activated, to prevent damage to the host's physiological systems.

Serpins are a superfamily of proteins folding into a conserved tertiary structure with a reactive center loop (RCL) near the C-terminus, which acts as bait for a target protease (Gettins, 2002). After a protease cleaves a scissile bond between amino acid residues P1 and P1′ of the RCL, the serpin undergoes a conformational change and traps the protease in an inactive state, resulting in irreversible inhibition of the protease. Multiple serpin genes have been identified in insect genomes (Reichhart, 2005; Suwanchaichinda and Kanost, 2009; Zou et al., 2009). Several of these insect serpins regulate innate immune responses. In *Manduca sexta*, with the exception of serpin-2 (Gan et al., 2001), serpin-1, -3, -4, -5, and -6 have been characterized and shown to be inhibitory and to regulate proteases that function in cascades leading to activation of proPO and the cytokine spätzle (An and Kanost, 2010; An et al., 2011; Christen et al., 2012; Jiang and Kanost, 1997; Jiang et al., 2003; Ragan et al., 2010; Tong and Kanost, 2005; Tong et al., 2005; Wang and Jiang, 2004; Zhu et al., 2003).

In this study, we cloned a cDNA for an additional *M. sexta* serpin, serpin-7 (GenBank: HQ149330), and biochemically characterized

Abbreviations: HP, hemolymph protease; PAP, prophenoloxidase-activating protease; proPO, prophenoloxidase.

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http://dx.doi.org/10.1016/j.ibmb.2013.03.015

the serpin-7 recombinant protein. Our findings indicated that serpin-7 can inhibit proPO activation in *M. sexta* plasma and it can inhibit PAP3.

2. Materials and methods

2.1. Insect rearing

Manduca sexta eggs were originally obtained from Carolina Biological Supply (Burlington, NC). The insect larvae were reared as described previously (Dunn and Drake, 1983).

2.2. cDNA cloning and sequencing

A partial 3′-end sequence obtained from an *M. sexta* EST (GenBank: CA798822) was used to design primers for the oligo-capping rapid amplification of cDNA ends (RACE) method to obtain the remaining sequence of the serpin-7 transcript. The 5′-RACE reaction was performed according to the manufacturer's protocol for a GeneRacer kit (Invitrogen). Fat body mRNA samples were prepared from day-2 fifth instar larvae 24 h after injection with 50 μg of *Micrococcus luteus*. Corresponding cDNA was produced and used as a template for amplification. The RACE products were cloned and sequenced, and the full-length cDNA was then amplified by RT-PCR. The product was cloned into a pGEM-T plasmid vector (Promega). Sequencing of plasmids and PCR products was carried out at the Iowa State University Sequencing Facility (Ames, IA). The deduced amino acid sequence was obtained by using the Translate tool provided by the Swiss Institute Bioinformatics (Gasteiger et al., 2003).

2.3. Expression and purification of recombinant serpin-7

Serpin-7 cDNA encoding the mature protein was amplified by PCR using high fidelity Platinum Taq DNA polymerase (Invitrogen), a forward primer (5′-ATCCATGGCGCCGAATGTGGGA-3′) containing an *Nco*I site, and a reverse primer (5′-ATGCGGCCGCTCACACAACTGAAG-3′) containing an *Not*I site. After digestion with the restriction enzymes *Nco*I and *Not*I (New England BioLabs), the PCR product and the plasmid H_6pQE60 (Lee et al., 1994) were purified by agarose gel electrophoresis. The purified PCR product was subsequently cloned into the plasmid vector at the corresponding restriction sites. The DNA construct was sequenced to confirm the correct reading frame, which included an amino-terminal hexahistidine tag.

The hexahistidine-tagged recombinant serpin-7 was expressed in *Escherichia coli* strain XL1-blue. The bacteria were grown in 1 L LB medium at 37 °C, 300 rpm. Carbenicillin was used as a selective agent, and isopropyl β-D-thiogalactoside (IPTG) was used to induce protein expression. The soluble portion of the expressed protein was efficiently purified under native conditions by Ni-NTA chromatography. Column elution fractions were dialyzed in Tris buffer (20 mM Tris·HCl, 50 mM NaCl, pH 8.0) overnight at 4 °C and concentrated before use in the experiments.

2.4. Multiple sequence alignments

The amino acid sequence of serpin-7 was aligned with other *M. sexta* serpins previously identified by using the ClustalW program. The prediction of the P1-P1′ residues and the reactive center loop region was manually identified. The three-dimensional structure of *Manduca* serpin-1K has been previously characterized (Li et al., 1999), and was used for comparison in this study.

2.5. Inhibition of spontaneous melanization of larval plasma

Hemolymph of day-1 fifth-instar larvae was collected into a chilled microcentrifuge tube from a cut in the dorsal horn. Hemocytes were removed by centrifugation at 5,000× *g* for 10 min. Six μl of the plasma was immediately mixed with 1, 2 and 3 μg of the purified recombinant serpin-7 in a total volume of 10 μl, adjusted with Tris buffer (0.1 M Tris·HCl, pH 8.0/0.1 M NaCl). The mixtures were incubated at room temperature and photographed after 30 and 60 min.

2.6. Detection of serpin-protease complexes

Active recombinant *M. sexta* PAP3 was kindly provided by Dr. Maureen Gorman at Kansas State University. Reaction mixtures of PAP3 with serpin-7 were prepared according to Michel et al. (2006) and separated by electrophoresis using gradient 4–20% Tris·HCl Ready gels (Bio-Rad, Hercules, CA). The resolved proteins were transferred onto a nitrocellulose membrane and subjected to immunoblot analysis using antisera against either PAP3 or His_{tag} (Qiagen) as primary antibodies (1:2500 dilution). Alkaline phosphatase-conjugated goat anti-rabbit IgG (1:3000 dilution) was used as the secondary antibody, and the antibody binding was visualized by using the alkaline phosphatase-conjugate substrate kit (Bio-Rad).

2.7. Inhibition of protease activities

Inhibition assays of PAP3 activity and larval prophenoloxidase activation were carried out as described previously (Michel et al., 2006). Briefly, for inhibition of PAP3 activity, recombinant serpin-7 at various concentrations was mixed with active PAP3 for 15 min at room temperature. After adding the *N*-acetyl-Ile-Glu-Ala-Arg-*p*-nitroanilide substrate solution, protease activity was monitored at 405 nm in a PowerWave340 microplate reader (Bio-Tek, Winooski, VT). Three replicates were performed (1 Unit = 0.001 ΔA_{405}/min). For inhibition of prophenoloxidase activation, recombinant serpin-7 at different concentrations was incubated with 1 μl of day-1 fifth instar larval plasma. After incubation at room temperature for 10 min, *M. luteus* suspension was added to the mixtures and further incubated for 5 min. After adding the dopamine substrate solution, PO activity was measured by monitoring absorbance at 470 nm. Three replicates with different larval plasma samples were performed.

2.8. Serpin-7 gene expression

Collections of hemocytes and fat body from larvae injected with either 0.85% NaCl (control samples) or *M. luteus* (immune-challenged samples) were prepared according to Tong and Kanost (2005). Total RNAs of the hemocytes and fat body samples were prepared as described. RNA samples in the amount of 2.5 μg were used as templates for cDNA synthesis by using the SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen). One μl of each cDNA sample was used as a template for PCR. The primers were: 5′-GCAGAGAGCGGAATTACCAG-3′ and 5′-ACACCAGCAAAGAGGACGAT-3′ for *serpin-7*, and 5′-TCAGGCCGAGTCTTTGAGAT-3′ and 5′-AGCACTCCTTGCCTGAGAAG-3′ for ribosomal protein S3 (*rpS3*). PCR products of *serpin-7* were amplified with 25 cycles for hemocyte samples and 28 cycles for fat body samples (94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s). The *rpS3* was used as an internal control (19 cycles; 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s). All PCR products were analyzed by agarose gel electrophoresis and detected by ethidium bromide staining.