Developmental and Comparative Immunology 65 (2016) 330–339

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

A single-CRD C-type lectin is important for bacterial clearance in the silkworm

Ming-Yue Zhan [a], Toufeeq Shahzad [a], Pei-Jin Yang [a], Su Liu [a], Xiao-Qiang Yu [b], Xiang-Jun Rao [a, *]

[a] School of Plant Protection, Anhui Agricultural University, Hefei, Anhui 230036, China
[b] Division of Molecular Biology and Biochemistry, School of Biological Sciences, University of Missouri-Kansas City, Kansas City, MO 64110, USA

ARTICLE INFO

Article history:
Received 9 June 2016
Received in revised form
7 August 2016
Accepted 8 August 2016
Available online 10 August 2016

Keywords:
Insect immunity
Silkworm
C-type lectin

ABSTRACT

C-type lectins (CTLs) depend on the carbohydrate-recognition domain (CRD) to recognize carbohydrates by a Ca^{2+}-dependent mechanism. In animals, CTLs play critical roles in pathogen recognition, activation of the complement system and signaling pathways. Immulectins (Dual-CRD CTLs) in lepidopteran are involved in recognizing pathogens. However, little is known about the immune-related functions of insect single-CRD CTLs. Here, we reported the characterization of C-type lectin-S3 (*CTL-S3*), a single-CRD CTL from the domesticated silkmoth *Bombyx mori* (Lepidoptera: Bombycidae). The ORF of *CTL-S3* gene is 672 bp, which encodes a putative protein of 223 amino acids. *CTL-S3* gene was expressed in a variety of tissues. Levels of *CTL-S3* mRNA in fertilized eggs and whole larvae were elevated upon bacterial challenges. CTL-S3 was secreted to larval hemolymph. The recombinant protein (rCTL-S3) binds to bacterial cell wall components and bacteria. CTL-S3 inhibited the growth of *Bacillus subtilis* and caused agglutination of *Staphylococcus aureus*. More importantly, CTL-S3 facilitated the rapid clearance of *Escherichia coli* and *Staphylococcus aureus* from the body cavity of larvae. Taken together, our results suggested that CTL-S3 may function as an opsonin in larval hemolymph to enhance the clearance of pathogens.
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1. Introduction

Invertebrates rely on the efficient innate immune system to defend themselves against invading pathogens (Iwanaga and Lee, 2005; Loker et al., 2004). Humoral and cellular immunity are the two major branches of innate immunity (Jiravanichpaisal et al., 2006). Humoral immunity includes pro-phenoloxidase activation and the production of antimicrobial effectors (Lu et al., 2014; Zänker, 2010). Cellular immunity includes encapsulation, melanization, and phagocytosis (Smith, 2010). Both types of immunity are mediated by the interaction between pattern recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs) (Janeway, 1989; Janeway and Medzhitov, 2002). Therefore, it is important to understand how PRRs interact with PAMPs and the underlying mechanisms.

C-type lectins comprise a large family of PRRs that bind to mannose-type or galactose-type carbohydrates in the presence of $Ca2^{+}$ (Drickamer, 1992). C-type lectin receptors (CLRs) are a family of receptors with at least one C-type lectin-like domain (CTLD). They are important not only for pathogen recognition but also for sensing dead and cancerous cells (Dambuza and Brown, 2015; Iborra and Sancho, 2015). Invertebrate C-type lectins have been shown to mediate immune responses and development (Huang et al., 2015; Li et al., 2014; Ling et al., 2008; Maeda et al., 2016; Yu et al., 1999, 2005, 2006; Yu and Kanost, 2004; Zhu et al., 2016).

We previously reported the genome-wide analysis of C-type lectin domain proteins (CTLDPs) in *Bombyx mori* and the tobacco hornworm *Manduca sexta* (Lepidoptera: Sphingidae) (Rao et al., 2015a, 2015b). Based on their domain architectures, we proposed that CTLDPs could be classified into three types: 'CTL-S' type (single-CRD), 'Immulectin' type (dual-CRD) and 'CTL-X' type (complex domains). The 'CTL-S' type has a single CRD without other known motifs. The 'Immulectin' type is generally found in lepidopteran, a few members of this type are also found in crustaceans and Coleoptera (Yu and Kanost, 2001; Zou et al., 2007). Immulectins mediate the defense against pathogens by promoting agglutination, melanization and encapsulation (Kim et al., 2003; Yu and Kanost, 2003, 2004; Yu et al., 1999, 2005, 2006). The 'CTL-X' type

* Corresponding author. Department of Entomology, School of Plant Protection, Anhui Agricultural University, Hefei, Anhui 230036, China.
E-mail address: rxj@ahau.edu.cn (X.-J. Rao).

http://dx.doi.org/10.1016/j.dci.2016.08.004
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carries at least one CRD along with other motifs that are involved in different functions. CTL-S and CTL-X have more diversified functions in development and immunity. A 26-kDa Periplaneta lectin appeared transiently in the regenerating cockroach leg (Kubo et al., 1993). The heterodimer of CTL4 (CTL-S) and CTLMA2 (CTL-S) cooperate to defend *Anopheles gambiae* against Gram-negative bacteria (Schnitger et al., 2009). Mutations in four CTL-X orthologs in *Drosophila melanogaster* led to different phenotypes. *Drosophila* Furrowed/Selectin gene encodes a transmembrane orthologs of selectin, which is required for the proper establishment of planar cell polarity (PCP) in a tissue requires coordination of directional signals (Chin and Mlodzik, 2013; Leshko-Lindsay and Corces, 1997). *Drosophila* uninflatable (uif) gene encodes a large transmembrane receptor with a CTLD, and it is required for the development of the tracheal system (Zhang and Ward, 2009). The genome of *B. mori* presumably encodes at least 5 CTL-X, 6 immulectins and 12 CTL-S (Rao et al., 2015b; Tanaka et al., 2008). The functions of most of them haven't been explored.

In the present study, we identified and characterized the *B. mori* C-type lectin-S3 (*CTL-S3*). *CTL-S3* was expressed in various larval tissues and secreted to hemolymph. The levels of *CTL-S3* mRNA were elevated in challenged eggs and whole larvae. The recombinant protein binds to bacteria cells and to various bacterial cell wall components. CTL-S3 is also important for efficient bacterial clearance.

2. Materials and methods

2.1. Insects, bacteria and microbial components

Silkworms ('Dazao') were maintained in our lab. Larvae were reared on fresh mulberry leaves. *Escherichia coli* (*E. coli)* DH5α strain HM01 and BL21 strain GZEC-3, *Staphylococcus aureus* (*S. aureus*) strain RCB1010, *Bacillus subtilis* (*B. subtilis*) strain MPF_80, *Serratia marcescens* (*S. marcescens*) strain FZSF02 were given by Dr. Erjun Ling in the Institute of Plant Physiology and Ecology (Shanghai, China). Laminarin from *Laminaria digitate* (L9634), lipopolysaccharide (LPS) from *E. coli* 055:B5 (L2880), Lipoteichoic acid (LTA) from *B. subtilis* (L3265) were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO). Peptidoglycans (PGNs) PGN-EK from *E. coli* K12 (tlrl-pgnek) and PGN-SA from *S. aureus* (tlrl-pgns2) were purchased from InvivoGen (San Diego, CA).

2.2. CTL-S3 full-length cDNA cloning

A silkworm cDNA sequence predicted to encode a 'CTL-S' type protein was identified by us previously (GeneID: BGIBMGA005977) (Rao et al., 2015b). The coding sequence was cloned from cDNA using a forward primer with the NdeI site (5′-GGAATTC**CATATG**CAGAAGCCCGGTCGTTTCCTGTC-3′) and a reverse primer with the XhoI site (5′- CCG**CTCGAG**TAGACGGAGACCGGGATTATTGCTC-3′). The PCR product was digested with NdeI/XhoI (Thermo Scientific™) and ligated to the pET-30a (+) vector (Novagen). The cDNA of eGFP (enhanced green fluorescent protein) was cloned with a forward primer with the NdeI site before the His-tag codon (5′-CCC**CATATG***CACCATCATCATCATCAT*GTGAGCAAGGGCGAGGAGC-3′) and a reverse primer with the BglII site (5′-CCC**AGATCT**TTACTTGTACAGCTCGTCCATGCC -3′). The eGFP PCR product was digested with NdeI/BglII and ligated to the pET-30a (+) vector. *E. coli* BL21 (DE3) competent cells were transformed with the correct expression vectors.

2.3. Expression and purification of the recombinant proteins

BL21 cells were grown from a single colony in LB media to OD = 0.5. 1 mM IPTG was used to induce CTL-S3 expression for 6 h at 37 °C on a shaking incubator. Bacteria were collected and sonicated in PBST buffer. The inclusion bodies were dissolved completely in buffer B (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH8.0) and run through Ni-NTA slurry. After two washes with buffer C (pH6.3), the recombinant protein was eluted by buffer D (pH5.9). The protein elute were dialyzed successively in the refolding buffers (20 mM Tris-HCl, 150 mM NaCl, 2 mM GSH, 0.02 mM GSSG, 10% glycerol) containing 6 M, 4 M, 2 M urea and finally in TBS with 10% glycerol. The purified recombinant CTL-S3 was used to immunize a rabbit to make antiserum (Huabio, China). EGFP was expressed at 25 °C in the soluble form, purified with Ni-NTA and eluted by the Elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH8.0). Recombinant proteins were separated on 15% SDS-PAGE gels and stained with Coomassie brilliant blue R250, or transferred to PVDF membranes, blotted with CTL-S3 antiserum (1:1000) or His-tag IgG (1:1,000, Affinity Biosciences, #T0051) and goat anti-rabbit IgG-HRP (1:10,000, Santa Cruz Biotech, #sc-2004). Bands were visualized with the DAB staining reagents (#AR1025, Boster Biotech, Wuhan, China).

2.4. Bioinformatic analysis

The cDNA and deduced protein sequences were analyzed with DNAMAN (Lynnon Corporation, Quebec, Canada). The conserved motifs were predicted on the SMART server (http://smart.embl-heidelberg.de/). The signal peptide was predicted by SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). The N- and O-glycosylated sites were predicted by NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 4.0 Server (http://www.cbs.dtu.dk/services/NetOGlyc/). Sequences of orthologs were analyzed with the BLASTP tool (http://www.ncbi.nlm.nih.gov/). The amino acid sequences were aligned by the MUSCLE module of the MEGA6 software. A neighbor-joining tree was generated with a bootstrap of 1000 replications using MEGA6 (Tamura et al., 2013).

2.5. Tissue expression profile and feeding assay

For the tissue expression analysis, tissues were collected from larvae injected with saline or formaldehyde-killed bacteria. For the feeding assay, larvae were starved for 12 h before feeding. Mulberry leaves were cut into 2 × 2 cm squares. Control leaves were soaked in PBS, other leaves were soaked in bacteria (4×10^6 cfu/μl) overnight. Larvae ate leaves to a similar degree. Samples were grinded in RNAiso plus for RNA extraction (#9108, Takara Biotechnology). cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo Scientific™). qPCR was performed to examine the relative expression levels of *CTL-S3* with the standard protocol (95 °C 30 s, 40 cycles of 95 °C 5 s, 60 °C 30 s). *CTL-S3* qPCR primers were S3-RTN (5′-CCTACATTTGGACATCGGGAC-3′) and S3-RTC (5′-CGGTTCGGTATCTGATTAGTGG-3′). *B. mori* Actin-A3 was used as the reference gene. qPCR was performed on the BioRad CFX96 with Thunderbird SYBR qPCR mix (QPS-201, TOYOBO CO., LTD.) The relative expression levels were obtained by normalizing samples to the corresponding calibrators. The results were calculated by the $2^{(-\Delta\Delta CT)}$ method where $\Delta\Delta CT = (CT_{target} - CT_{reference})_{sample} - (CT_{target} - CT_{reference})_{calibrator}$.

2.6. Bacterial growth inhibition assay

E. coli, S. aureus, B. subtilis and *S. marcescens* were chosen to test the effects of CTL-S3 on bacterial growth. Single colonies were cultured overnight in tryptic soy broth (TSB) at 37 °C on a shaking incubator. The next morning, bacteria were washed twice with PBS (5 min, 12,000*g*) and resuspended in fresh TSB at a concentration of

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