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Research paper

Bombyx mori cecropin A has a high antifungal activity to entomopathogenic fungus *Beauveria bassiana*

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ABSTRACT

A cDNA encoding cecropin A (CecA) was cloned from the larvae of silkworm, *Bombyx mori*, using RT-PCR. It encodes a protein of 63 amino acids, containing a 22 amino acid signal peptide and a 37 amino acid mat peptide of functional domain. The CecA secondary structure contains two typical amphiphilic α-helices. Real-time qPCR analysis revealed that *CecA* was expressed in all the tissues tested, including cuticle, fat body, hemocytes, Malpighian tubule, midgut and silk gland in the silkworm larvae with the highest expression in the fat body and hemocytes. The gene expression of *B. mori CecA* was rapidly induced by *Beauveria bassiana* challenge and reached maximum levels at 36 h after inoculation in third instar larvae. In the fifth instar larvae infected with *B. bassiana*, the relative expression level of CecA was upregulated in fat body and hemocytes, but not in cuticle, Malpighian tubule, midgut and silk gland. The cDNA segment of the *CecA* was inserted into the expression plasmid pET-30a(+) to construct a recombinant expression plasmid. Western blot results revealed that his-tagged fusion protein was successfully expressed and purified. Then the mat peptide of CecA was chemically synthesized with C-terminus amidation for *in vivo* antifungal assay and purity achieved 93.7%. Mass spectrometry and SDS-PAGE showed its molecular weight to be 4046.95 Da. Antifungal assays indicated that the *B. mori* CecA had a high antifungal activity to entomopathogenic fungus *B. bassiana* both *in vitro* and *in vivo* in the silkworm larvae. This is the first report that the CecA is effective to inhibit *B. bassiana* inside the body of silkworm.

1. Introduction

The entomopathogenic fungi *Beauveria bassiana* is a soil-borne pathogen of insects (Butt et al., 2001). *B. bassiana* is a wide host range, strong pathogenicity and adaptability entomopathogenic fungus, especially has a strong virulence on lepidopteran insects. Agostinio Bassi first discovered *B. bassiana* is the cause of the devastating muscardine disease of silkworms (Steinhaus, 1957). *B. bassiana* was usually used as a biological insecticide (Wang et al., 2004; Fan et al., 2007; Lu et al., 2008; Fang et al., 2009; Shang et al., 2012). But in sericulture, *B. bassiana* is a troublesome pathogenic fungus (Hou et al., 2013; Chen et al., 2015). *B. bassiana* usually infects insects through penetrating the cuticle and colonizes in vivo. Then the fungus fights with the innate immune system of insects, based on both cellular and humoral mechanisms (Ratcliffe, 1985; Lavine and Strand, 2002). Many antimicrobial peptides (AMPs) are produced and play a major role in humoral immune system.

In recent years, a variety of AMPs have been isolated and classified from different organisms, such as cecropins, histatins, defensins and gloverins (Koczulla and Bals, 2003; Cheng et al., 2006). These peptides were synthesized mainly in the fat body and secreted into the hemolymph to combat invading microbes (Cheng et al., 2006). Histidine-rich cationic histatins exert a potent killing effect *in vitro* on *Candida albicans* (Helmerhorst et al., 1997). Cysteine-rich polypeptide defensin was first detected in the flesh fly, *Sarcophaga peregrine* (Matsuyama and Natori, 1988). Defensins have been found in a broad range of organisms. They play an important role in *Bombyx mori* immune reactions against infection by bacteria and fungi (Kaneko et al., 2008; Wen et al., 2009). Glycine-rich gloverins are inducible antibacterial insect protein which inhibits the synthesis of vital outer membrane proteins leading to a permeable outer membrane (Kaneko et al., 2007).

The *B. mori* cecropins were first isolated from the fat body of immunized larvae (Yamano et al., 1994). They always have an open reading frame of 63 amino acids containing a mature peptide of 35 amino acids and two amphipathic α-helices. The α-helices probably urge them to target non-polar lipid membranes and then form ion-

Abbreviations: AMPs, antimicrobial peptides; BbHN6, *Beauveria bassiana* HN6; ORF, open reading frame; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; cDNA, complementary deoxyribonucleic acid; IPTG, isopropyl β-D-thiogalactoside; OD, optical density; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; hpi, hours post-inoculation; LT_{50}, the median lethal time; PBS, phosphate buffered saline; PDA, potato dextrose agar; LB, Luria–Bertani; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; qRT-PCR, quantitative reverse transcription polymerase chain reaction; HPLC, high-performance liquid chromatography; ESI, Electrospray ionization; MS, mass spectrometry; Co., Ltd, Company Limited.

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permeable channels, resulting in cell depolarization, irreversible cytolysis, and finally cell death (Boman, 2003). *B. mori* cecropins contains three classes, cecropin A, cecropin B and cecropin D (Yamano et al., 1994; Yamano et al., 1998; Yang et al., 1999). Some researches demonstrated that cecropins had a considerable activity to bacteria and fungi in vitro (Cavallarin et al., 1998; Xia et al., 2013; Lee et al., 2015). But there are no reports about AMPs tested *in vivo* on antifungal activities.

Our previous study demonstrated by transcriptome analysis that expression of cecropin A gene was up-regulated in the silkworm larvae challenged with *B. bassiana* (Hou et al., 2014). In this paper, the cDNA of the CecA gene was cloned from the silkworm using RT-PCR. The structure of CecA protein was analyzed. Fluorescent quantitative realtime PCR was performed to analyze the expression characteristics of CecA in the whole larvae, fat body, hemolymph and other tissues at different time points after *B. bassiana* inoculation. The recombinant protein of CecA was expressed in prokaryotic system for testing its antifungal acitivity against *B. bassiana*. And the mat peptide of CecA was chemically synthesized with C-terminal amidation. Antifungal assays indicated that *B. mori* CecA had a high antifungal activity to entomopathogenic fungus *B. bassiana* both *in vitro* and *in vivo* in the silkworm larvae.

2. Materials and methods

2.1. Silkworm and fungal strain

In this study, the silkworm strain P50 was provided by Sericultural Research Institute of Chinese Academy of Agricultural Sciences. They were raised to second and fourth motling under a standard condition of a photoperiod of 12 h light and 12 h dark at 25 °C. *B. bassiana* Strain BbHN6 (preserved in the Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, China) used in this study was originally isolated from the cadaver of infected silkworm. BbHN6 was cultured on potato dextrose agar (PDA) for 14 days at 25 °C before being inoculated to the silkworm larvae.

2.2. B. bassiana inoculation and tissues collection

The spores of BbHN6 was suspended in distilled water containing 0.01% (vol vol^{-1}) Tween-20 to a concentration of 1×10^8 conidia ml^{-1}. The newly exuviated third or fifth instar silkworm larvae were immersed in conidia suspension for 15 s, then removed out with a plastic tweezer and reared on fresh mulberry leaves at standard rearing environment. The control larvae were treated with distilled water containing 0.01% (vol vol^{-1}) Tween-20.

The different tissues of the fifth instar silkworm were collected from both BbHN6-infected and control larvae at 8 h post-inoculation (hpi). The isolated tissues were quickly washed with diethylpyrocarbonate-treated water to remove attached leaf pieces and then were collected in storage tubes with 0.5 ml Trizol reagent (TaKaRa). The hemolymph was directly inpoured into the storage tubes in which phenylthiourea antioxidant was added. Samples were immediately frozen in liquid nitrogen and stored at −80 °C. The tissues included cuticle, fat body, hemocytes, Malpighian tubule, midgut and silk gland. Tissues from 10 larvae of fifth instar were pooled as one sample.

2.3. Quantitative real-time PCR (qRT-PCR)

The qRT-PCR method was used to detect the expression level of CecA in silkworm between BbHN6-infected and control groups. Total RNAs were isolated from five third instar larvae respectively at 9, 12, 15, 18, 24, 30, 36, and 42 hpi with a RNApure total RNA rapid extraction kit (Beijing Bolingkewei Biotechnology Co. Ltd.) according to the manufacture's procedures, then quantified by measuring the absorbance at 260 nm. DNase I was inactivated at 42 °C for 2 min in a 10 μl reaction volume containing 1 μl of gDNA Eraser (Takara), 2 μl 5 × gDNA Eraser buffer and 2 μg of each RNA sample. The resulting RNAs were used for the synthesis of the cDNAs using PrimeScript™ RT Reagent Kit (TaKaRa). Total RNAs of different tissues were also extracted respectively from cuticle, fat body, hemocytes, Malpighian tubule, midgut and silk gland collected above at 8, 12, 16, 20, 24, 30, 36, 42, 48, and 54 hpi and the cDNA templates were synthesized as described above.

The SYBR Green RT-PCR assay was run on an ABI PRISM 7300 Sequence Detection System (Applied Biosystems) by three-step real-time qPCR according to the SYBR Premix Ex Taq™ Kit (Takara) protocol. The thermal profiles for SYBR Green RT-PCR were 95 °C for 2 min, followed by 45 cycles of 95 °C for 5 s, 50 °C for 15 s and 72 °C for 40 s using primers CecA-1-F (CCAAAGACGCATTTAGCTTG) and CecA-1-R (ACTCGGTCACTCTGAGAAAG). Every sample was run in triplicates along with the internal housekeeping gene *β-actin* using primers Bmactin-F (CCGTATGCGAAAGGAAATCA) and Bmactin-R (TTGGAAGG TAGAGAGGGAGG). The *β-actin* fragment was used as an internal control to calibrate the cDNA templates. All data of relative quantification expression were analyzed by an opticon monitor analysis software (MJ Research). The expression difference of *CecA* gene between BbHN6-infected and control groups was calculated by $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001). The data were also figured out terms of relative mRNA expression as means ± SE. Through analysis of t-test, the P values of less than 0.05 were considered indicating statistical significance.

2.4. Cloning the cDNA of CecA

Total RNA was extracted from the third instar silkworm strain P50 using a RNApure total RNA rapid extraction kit (Beijing Bolingkewei Biotechnology Co. Ltd.) according to the giving protocol. Then the first-strand cDNA was synthesized from the total RNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara) according to the standard protocol. The antimicrobial peptide *CecA* gene was amplified from the first-strand cDNA using the primers CecAF (GGATCCATGAATTTCGTACG TATT, the underlined is restriction site) and CecAR (GCGGCCGCTTCTAT TTTCCTAAGG) by gradient PCR following: 40 cycles of denaturing for 30s at 95 °C, annealing for 15 s at 40–68 °C, and extension for 90s at 72 °C. The DNA products were cloned into pMD19-T vector (Takara) and sequenced.

2.5. Expression and purification of the CecA protein

The sequence of the *CecA* gene was amplified from the plasmid pMD19-T-CecA by PCR using the upstream primer CecA30aF (GGAT CCGCTCCTGAGCCCAGGTGG) and the downstream primer CecA30aR (AAGCTTG**CTA**TTTCCTAAGGATT). The PCR product and the expression plasmid vector pET-30a(+) were digested with *Bam*H I and *Hind* III and ligated. The resulted recombinant plasmid pET-30a(+)-CecA was transformed into *E. coli* BL21. The positive clone of *E. coli* BL21-pET-30a(+)-CecA was used for expression of CecA after being cultured overnight in Luria-Bertani (LB) broth containing 30 μg/ml kanamycin. The expression was induced by adding 0.5 mM isopropyl β-D-thiogalactoside (IPTG). After 5 h of induced culture at 37 °C, the cells were harvested.

The cell pellet was washed by 1 × PBS (pH 7.4) three times and subjected to sonication at 300 W for 30 min (10 s working, 10 s free) on ice. After centrifugation at 12,000 g for 10 min at 4 °C, the supernatant was filtered through a 0.22 mm Millipore filter and loaded onto a Ni-NTA Agarose column (Beijing Bolingkewei Biotechnology Co. Ltd.). At the same time, the precipitate was washed and lysed in dissolution buffer (20 mM Tris–HCl, 500 mM NaCl and 8 M urea, pH 8.0) at 4 °C for 12 h, subjected to centrifugation at 12,000 g at 4 °C for 20 min, then the supernatant was filtered and loaded onto a Ni-NTA Agarose column as above. The recombinant CecA was purified by the 6 × His Fusion Protein Purification Kit (Beijing C&N International Sci-tech Co., Ltd.). His-tagged proteins were eluted with elution buffer (20 mM Tris–HCl, 500 mM NaCl, 8 M urea and 250 mM imidazole, pH 8.0). The flow-through liquid