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Recombinant production of the insecticidal scorpion toxin BjαIT in *Escherichia coli*

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ABSTRACT

Scorpion long-chain insect neurotoxins have important potential application value in agricultural pest control. The difficulty of obtaining natural toxins is the major obstacle preventing analyses of their insecticidal activity against more agricultural insect pests. Here we cloned the insect neurotoxin BjαIT gene into the pET32 expression vector and expressed the resulting thioredoxin (Trx)-BjαIT fusion protein in *Escherichia coli*. Soluble Trx-BjαIT was expressed at a high level when induced at 18 °C with 0.1 mM isopropyl β-D-1-thiogalactopyranoside, and it was purified by Ni^{2+}-nitriloacetic acid affinity chromatography. After cleaving the Trx tag with recombinant enterokinase, the digestion products were purified by CM Sepharose FF ion-exchange chromatography, and 1.5 mg of purified recombinant BjαIT (rBjαIT) was obtained from 100 ml of induced bacterial cells. Injecting rBjαIT induced obvious neurotoxic symptoms and led to death in locust (*Locusta migratoria*) larvae. Dietary toxicity was not observed in locusts. The results demonstrate that active rBjαIT could be obtained efficiently from an *E. coli* expression system, which is helpful for determining its insecticidal activity against agricultural insect pests.

1. Introduction

Among various other components, scorpion venoms contain neurotoxins comprising 60–70 amino acids with four pairs of cross-linked disulfide bonds that possess high affinity for nerve and muscle sodium channels, which kill soft-bodied insect prey [1–3]. According to their modes of action, insect-specific neurotoxins are further divided into excitatory and depressant toxins [4,5]. Because of their strict selectivity, insect-specific neurotoxins have great potential value as candidates for biological pesticides, and possibly for use in transgenic plants [6–9]. It has been reported that the insecticidal properties of insect viruses is enhanced by introducing genes encoding insect neurotoxins, and that transgenic plants expressing such toxins become more resistant to insects [8–12]. A novel insect-selective toxin, BjαIT, a 64-amino-acid neurotoxin, was isolated from the venom of the Judean black scorpion (*Buthotus judaicus*) [13]. As an α-toxin, BjαIT affects voltage-gated sodium channels. Because of high toxicity to insects, BjαIT is believed to be potentially useful an agent for controlling agricultural insect pests [14,15].

Studying bioactivity against insect pests requires large quantities of insect neurotoxins. In previous studies, the gene encoding BjαIT was synthesized based on its amino acid sequence and expressed successfully in *Pichia pastoris* [16]. Because of the low expression level and the presence of large amounts of contaminating proteins, rBjαIT protein was not purified. Recently, studies have sought to improve microbial insecticides using an entomopathogenic fungus (*Metarhizium acridum*) that targets locusts (*Locusta migratoria manilensis*) [14]. Although BjαIT was expressed in *M. acridum*, pure rBjαIT was not obtained, mainly because of purification difficulties and a low level of expression. A lack of sufficient amounts of rBjαIT is the major obstacle preventing analyses of their insecticidal activity against more agricultural insect pests. Both prokaryotic and eukaryotic systems are used commonly for heterologous protein expression, and these systems have their own advantages and disadvantages [17–20]. A lack of rBjαIT is the major obstacle preventing structural studies, as well as analyses of its insecticidal activity against more agricultural insect pests. Therefore, establishing a high-level protein expression system and purification protocols is very important. Here we report the expression and purification of soluble rBjαIT in *Escherichia coli*.

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2. Materials and methods

2.1. Materials

All analytical grade reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Promega (Madison, WI, USA). *Escherichia coli* strains and the expression vector pET32a(+) were purchased from Novagen (Shanghai, China). Ni^{2+}-nitriloacetic acid (NTA) resin was purchased from Qiagen (Guangzhou, China). Recombinant bovine enterokinase (rEK) was purchased from the GenScript Corporation (Shanghai, China). A HiTrap CM Sepharose FF column (1 ml) was obtained from GE (Guangzhou, China). Centrifugal filter units were purchased from Millipore (Guangzhou, China). Dialysis bags were purchased from Union Carbide (Shanghai, China). An endotoxin assay kit was purchased from Genscript (Nanjing, China).

2.2. Optimized expression of Trx-BjαIT

BjαIT cDNA was amplified by polymerase chain reaction according to previous reports [16]. Using the forward primer 5′–GCCCATGGCAGGTAGAGACGCTTAC ATC–3′ and the reverse primer 5′–GCAAGCTTTTATCTGCAGGCTCCAGGGATT C–3′, the amplified BjαIT gene was cloned into pET32 such that it contained a Trx tag followed by a six-histidine tag containing an rEK cleavage site, and the constructed pET32/BjαIT plasmid was transformed into *E. coli* strain BL21(DE3). Several single colonies containing pET32/BjαIT were inoculated into 5 ml of Luria–Bertani broth containing 50 μg/ml ampicillin, and they were grown at 37 °C with shaking to an optical density at 600 nm of approximately 0.5. Different final concentrations of isopropyl β-D-1-thiogalactopyranoside (IPTG) (0, 0.1, 0.2, 0.5 and 1.0 mM) were added, and the cultures were induced for 8 h at 37, 25, and 18 °C. All harvested bacteria were resuspended in bacterial lysate buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5% Triton X-100, and 0.5 mM ethylenediaminetetraacetic acid) and lysed by sonication. Supernatants were collected by centrifugation and analyzed by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. Purification of Trx-BjαIT

Approximately 1.2 g of cell pellets expressing Trx-BjαIT were suspended in 30 ml of bacterial lysate buffer and sonicated thoroughly on ice. Following centrifugation, the supernatants were loaded onto a Ni^{+}-NTA affinity column. The column was washed with ice-cold lysate buffer containing 30 mM imidazole and 0.5% Triton X-114 to remove unbound proteins and endotoxin. Trx-BjαIT was eluted with increasing concentration imidazole of 50–300 mM in elution buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl). Forty microliter eluates were resuspended in 10 μl of 5 × SDS loading buffer and 10 μl samples were subjected to SDS-PAGE (15% gel) analysis. The amount of Trx-BjαIT was assayed by gel scanning density analysis method with software Quantity One (Biorad, USA) and 10 μg bovine serum albumin (BSA) was used as the protein concentration standard.

2.4. Removal of the Trx tag and purification of rBjαIT

A Trx-BjαIT solution was dialyzed in a dialysis bag (8–14 kDa cut-off) against ice-cold dialysis buffer A (20 mM Tris-HCl, 100 mM NaCl, pH 8.0). After concentration with centrifugal filter units (molecular weight cut-off of 10 kDa), rEK was added and incubated at 20 °C for 8 h. The digestion solution was dialyzed against buffer B (20 mM Tris-HCl, pH 6.8) at 4 °C overnight, then the dialyzed solution outside the dialysis bag was collected and loaded onto a 1-ml HiTrap CM FF column that was pre-equilibrated with buffer B. After washing with buffer C (20 mM sodium phosphate, pH 6.8, 20 mM NaCl), rBjαIT was eluted with buffer C containing 100 mM NaCl. The final solution was collected and concentrated with centrifugal filter units (molecular weight cut-off of 3 kDa). Forty microliter eluates were resuspended in 10 μl of 5 × SDS loading buffer containing or in the absence of reducing agent dithiothreitol (DTT) and 10 μl samples were subjected to SDS-PAGE (15% gel) analysis respectively. The amount of BjαIT was assayed by gel scanning density analysis method with software Quantity One (Biorad, USA) and 10 μg bovine serum albumin (BSA) was used as the protein concentration standard. The endotoxin of concentrated rBjαIT was detected using endotoxin assay kit.

2.5. Mass spectrometry (MS) analysis

The molecular weight of purified and concentrated target protein was verified by matrix-assisted laser desorption/ionization tandem time-of-flight MS (MALDI-TOF). To identify BjαIT, the corresponding protein band was cut out from the SDS-PAGE gel and further analyzed by MALDI-TOF-TOF MS following trypsin digestion. The MS data were analyzed using a Mascot database search (an MS/MS ion search).

2.6. Insect bioassay

To evaluate the bioactivity of purified rBjαIT, fifth-instar locust *(Locusta migratoria)* weighing 0.8 ± 0.1 g were used. Each larva was injected with 20 μg/g body weight of purified rBjαIT (2 mg/ml) or 10 μl phosphate-buffered saline (PBS) as a control using 10 μl sharp Hamilton syringes. The injection occurred in the soft junction between the third and fourth abdominal segments of locusts. Ten locusts were used as a group and tests were performed on three independent groups. Post-injection toxicity was monitored with a lethality test at 24 and 48 h. The bioactivity of rBjαIT provided in the diet was analyzed as follows: The rBjαIT protein solution (2 mg/ml) was added at a rate of 0.5 ml per gram artificial diet, dryed at room temperature after stirring. The artificial diet containing rBjαIT was fed to fifth instar locusts. The artificial diet containing rBjαIT was replaced every day. Locusts were fed for 5 days and the number of dead locusts was recorded. PBS was added to the artificial feed as control. Ten locusts were used as a group and tests were performed on three independent groups.

3. Results

3.1. Optimized expression of Trx-rBjαIT

As shown in Fig. 1, a recombinant protein with a molecular weight of 25 kDa was expressed after induction with IPTG at 18 °C, while no such protein was expressed without IPTG induction. The molecular weight of the 25-kDa protein is consistent with the theoretical mass of Trx-BjαIT (BjαIT is approximately 7 kDa and the Trx tag is approximately 16 kDa). Temperature had the greatest effect on the expression of soluble Trx-BjαIT, and the expression of soluble Trx-BjαIT was highest when induced at 18 °C. The effect of the IPTG concentration on the expression of Trx-BjαIT was not as significant as that of temperature. With increasing IPTG concentrations, the expression of Trx-BjαIT decreased obviously. At the optimum temperature of 18 °C, soluble Trx-BjαIT had the highest expression following induction with 0.1 mM IPTG.

3.2. Purification of soluble Trx-BjαIT

A soluble cell lysate containing Trx-BjαIT was loaded onto a