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High-level expression and purification of active scorpion long-chain neurotoxin BjαIT from *Pichia pastoris*

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ABSTRACT

As an insect-selective neurotoxin, scorpion long-chain BjαIT is a promising prospect for insecticidal application; however, the difficulty of obtaining natural BjαIT represents the major obstacle preventing analysis of its insecticidal activity against agricultural insect pests. Here, we screened recombinant *Pichia pastoris* transformants showing high levels of secretory recombinant (r)BjαIT. Secreted rBjαIT was expressed at levels as high as 340 mg/L following methanol induction in a fed-batch reactor, with ~21 mg of pure rBjαIT obtained from 200-mL fed-batch culture supernatant by Ni^{2+}-nitriloacetic acid affinity chromatography and CM Sepharose ion-exchange chromatography. Injection of purified rBjαIT induced neurotoxicity symptoms in locust (*Locusta migratoria*) larvae, and the half-lethal dose of rBjαIT for locusts at 24-h post-injection ranged from 11 to 14 μg/g body weight. These results demonstrated that large amounts of active rBjαIT were efficiently prepared from *P. pastoris*, suggesting this system as efficacious for determining rBjαIT insecticidal activity against other agricultural insect pests.

1. Introduction

Insect predatory animals are natural sources of active insecticidal substances [1–4]. The scorpion is a carnivorous animal that preys on invertebrates, such as crickets, small centipedes, a variety of insect larvae, and nymphs. The scorpion venom gland contains polypeptides and small molecular proteins that exhibit highly selective activity against the insect nervous system [4–6]. These proteins are called anti-insect toxins and include excitatory contractive toxins and flaccid depressant toxins. Scorpion neurotoxins usually comprise 60 to 70 amino acids with four pairs of cross-linked disulfide bonds [7,8]. Because scorpion insect neurotoxins are insect-specific and harbour little or no toxicity to mammals, they can potentially be used as important pest-control agents against agricultural insects [9–12]. This application would require genetic modification of plants or insecticidal microbes to produce specific neurotoxins [9,13].

BjαIT is an excitatory, contractive, polypeptide neurotoxin isolated from the venom of the Judean black scorpion (*Buthotus judaicus*) [14]. This protein consists of 64 amino acids and four intra-molecular disulfide bridges and is specifically targets insect voltage-gated sodium channels. BjαIT does not cause toxicity in mice; however, the half-paralysis dose (PD50) is 50 ng/g body weight in locusts or cockroaches and 130 ng/g body weight in blowflies [14]. Due to its high specificity and toxicity, BjαIT has important potential for controlling agricultural insects. Recent studies have sought to improve microbial insecticides using the entomopathogenic fungus *Metarhizium acridum*, which targets locusts (*Locusta migratoria manilensis*) [15]. The gene encoding BjαIT was expressed in *M. acridum* to enhance its pathogenicity; however, pure recombinant BjαIT (rBjαIT) was not obtained due to purification difficulties and low levels of expression. Because studying neurotoxin bioactivity against insect pests requires large quantities of proteins, the lack of an avenue for obtaining high levels of BjαIT protein represents the major obstacle preventing its use as an insecticide. The amount of protein required to assay the half-lethal dose (LD50) of BjαIT is > 10-fold the PD50. Moreover, the lack of sufficient BjαIT protein might be the main reason why its LD50 has not been assayed. Therefore, preparation of active rBjαIT using other efficient heterologous gene-expression systems might represent the only method for generating large quantities of BjαIT.

As an efficient eukaryotic protein-expression system, *Pichia pastoris* has many advantages for recombinant protein expression. One advantage is that recombinant proteins can be secreted, precluding the necessity for yeasts to be lysed prior to protein purification [16]. *P. pastoris* is also frequently used to overexpress heterologous proteins,

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especially low-molecular-weight proteins. Additionally, *P. pastoris* is easy to use in low-cost salt medium for high-density fermentation and can produce up to gram-levels of secretory recombinant protein per liter from fermentation cultures [17]. Moreover, *P. pastoris* gene-expression systems often produce proteins harbouring the correct disulfide bonds, ensuring that the expressed product exhibits the correct spatial structure and biological activity. Therefore, it is possible that *P. pastoris* can achieve high levels of active expression and secretion of rBjαIT, which is a low-molecular-weight, secretory protein containing multiple disulfide bonds. In this study, we described a method for efficient secretory expression and purification of active rBjαIT from *P. Pastoris* (strain X33) by fed-batch fermentation.

2. Materials and methods

2.1. Materials

Plasmid pPICZαA and *P. pastoris* strain X33 were purchased from Invitrogen (Carlsbad, CA, USA). LBZ and YPD plates, BMGY, YPG and BMMY media were prepared according to standard operating instructions of Invitrogen. Ni^{2+}-NTA resin was from Qiagen (Valencia, CA, USA). Dialysis bags and filter devices (MWCO: 3 kDa) were from Millipore (Guangzhou, China). Rabbit anti-His × 6 IgG and goat anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (HRP-IgG) were from CST (Guangzhou, China). HiTrap CM Sepharose FF columns (5 mL) were purchased from GE (Guangzhou, China). One liter fed-batch basal salts medium contained 40 g glycerol, 27 mL H_3PO_4, 18 g K_2SO_4, 14.9 g $MgSO_4{\cdot}7H_2O$, 4.13 g KOH, 0.9 g $CaSO_4$, 0.3 mL antifoam silicone and 4.6 mL trace metal solution (TMS), adjusted pH to 6.0 with 30% ammonium hydroxide. Feed solution contained 100 mL 50% glycerol solution plus 1.2 mL TMS. Induction solution contained 100 mL methanol plus 1.2 mL TMS. One liter TMS contained: 65 g $FeSO_4{\cdot}7H_2O$, 24 g $MoNa_2O_4{\cdot}2H_2O$, 20 g $ZnCl_2$, 6 g $CuSO_4{\cdot}5H_2O$, 3 g $MnSO_4{\cdot}H_2O$, 0.5 g $CoCl_2$, 0.2 g biotin, 0.09 g KI, 0.02 g H_3BO_3 and 5.0 mL H_2SO_4. All analytical grade reagents were from Sangon, Sigma or Promega (Shanghai, China).

2.2. Plasmid construction and transformation

BjαIT with an 6 × His in the C-terminus was synthesized according to the amino acid sequence and *P. pastoris* codon bias (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species = 4922). The synthetic cDNA was: 5′-*CTCGAG* aaa aga GGT AGA GAC GCT TAC ATC GCT GAC AAC TTG AAC TGT GCT TAC ACC TGT GGT TCT AAC TCT TAC TGT AAC ACT GAA TGT ACT AAA AAC GGT GCT GTT TCT GGA TAC TGT CAA TGG TTG GGA AAA TAC GGA AAC GCC TGT TGG TGT ATC AAT TTG CCT GAC AAA GTT CCT ATC AGA ATC CCT GGA GCC TGT AGA CAT CAC CAT CAC CAT CAC TAA *TCTAGA*-3′. To obtain natural N- terminal rBjαIT, a Kex2 signal cleavage site (aaa aga) was fused upstream of the synthetic cDNA. DNA fragments of BjαIT with an 6 × His (CAT CAC CAT CAC CAT CAC) tag was digested with XhoI (*CTCGAG*) and XbaI (*TCTAGA*), and the digested fragment was inserted into the pPICZαA vector. The expression vector was linearized by digestion with SacI and transformed into *P. pastoris* X33 using LiCl, as recommended (Invitrogen, 2008). *P. pastoris* transformants were grown on YPD plates containing 100 μg/mL zeocin. Resulting colonies were transferred to YPD plates containing 200, 500, 1000, 1500 and 2000 μg/mL zeocin. After incubation at 28 °C for 3 days, large colonies from YPD plates containing 2000 μg/mL zeocin were picked and subjected to PCR analysis.

2.3. Selection of transformants

Transformants selected from YPD plates containing 2 mg/mL zeocin were grown in 10 mL BMGY at 28 °C with shaking at 250 rpm for 24 h in 50 mL tubes. Cells were then harvested by centrifugation at 1500*g* for 10 min at 28 °C and resuspended in 2 mL BMMY cultured for 72 h. Methanol (100%) was added to cultures every 24 h to a final concentration of 1%. 1 mL cultures were collected by centrifugation at 15,000 g for 10 min at 4 °C, 0.9 mL supernatants of collected cultures were mixed with 0.1 mL 100% trichloroacetic acid (TCA) and incubated at −20 °C for 4 h. After centrifugation at 15,000 g for 30 min at 4 °C, precipitates were resuspended in 30 μL of 1 × loading buffer with 8 M urea; 10 μL samples were subjected to 15% sodium dodecyl sulfate–-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

2.4. Optimization of time course

Selected colonies with the highest expression of rBjαIT were grown in 200 mL BMGY to an OD_{600} up to 10. The cells were harvested by centrifugation at 1500*g* for 10 min at 28 °C, resuspended in 20 mL BMMY, and grown for 4 days at 28 °C with shaking at 240 rpm. Each day, methanol was added to a final concentration of 1%. Before adding methanol, 1 mL sample was collected at 0, 24, 48, 72, and 96 h after induction with methanol. After centrifugation at 15,000 g for 10 min at 4 °C, 0.9 mL supernatants of collected cultures were mixed with 0.1 mL 100% TCA and incubated at −20 °C for 4 h. After centrifugation at 15,000 g for 30 min at 4 °C, precipitates were resuspended in 30 μL of 1 × loading buffer with 8 M urea; 8 μL samples were subjected to SDS-PAGE analysis and Western blotting analysis using the anti-His × 6 antibody, the protocols of Western blot was carried out in Ref. [13]. The amount of secretory rBjαIT was assayed by 15% SDS-PAGE gel scanning method (soft Quantity One, BioRad, USA).

2.5. Fed-batch fermentation

A single colony of the most highly expressing X33 transformant was inoculated into 5 mL YPG media and grown for 20 h at 28 °C, and inoculated to 200 mL fed-batch basal salts medium with 2% (w/v) glycerol and shaken at 28 °C and 250 rpm to OD_{600} at about 10. Fed-batch fermentation was in a 5-L bioreactor (NCBIO, China) with 2 L basal salts medium. Compressed air was maintained at 5 L/min, dissolved oxygen (DO) was maintained higher than 30% saturation by stirring at 200–1000 rpm. Temperature was maintained at 28 °C. Fermentation was started by adding 200 mL fed-batch basal salts seed culture into 2 L basal salts medium, with pH maintained at 6.0 with 30% ammonium hydroxide. To maintain glycerol concentration in the glycerol fed-batch phase, feed solution was added automatically according to DO > 30% saturation for 12 h by series regulation of feeding and DO. After the feed solution was consumed, induction solution was added for 84 h, with medium pH maintained at 6.0 with 30% ammonium hydroxide. For methanol feeding, the feeding rate of methanol was controlled at 2 mL/h for 12 h, 4 mL/h for 24 h, 6 mL/h for 36 h, and 4 mL/h for 12 h. Cell cultures (10 mL) were collected at 0, 12, 24, 36, 48, 60, 72 and 84 h after methanol induction. For each time point, 0.9 mL supernatants of collected cultures were mixed with 100 μL 100% TCA and incubated at −20 °C for 4 h. After centrifugation at 15,000 g for 30 min at 4 °C, precipitates were resuspended in 90 μL 1 × SDS loading buffer with 8 M urea and 5 μL samples were subjected to 15% SDS-PAGE. The amount of secretory BjαIT was assayed by gel scanning and 10 μg BSA was used as the protein concentration standard. Wet weights of cultured cells were obtained by centrifugation and removal of supernatant. Total protein concentration in supernatants was estimated using Bradford assays.

2.6. Optimization the conditions of Ni affinity purification

Supernatants from bioreactor (40 mL) were adjusted to pH 7.5 with NaOH. After centrifugation at 15,000 g for 60 min at 4 °C all supernatants were loaded onto a 30-mL column containing 2 mL Ni^{2+}-NTA resin pre-equilibrated with buffer A (20 Na_2HPO_4, pH 7.5, 200 mM NaCl). The column was washed with buffer B (20 Na_2HPO_4, pH 7.5, 200 mM NaCl, 20 mM imidazole), and then eluted with buffer B