An efficient strategy for the expression of Jingzhaotoxin-III in *Escherichia coli*

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ABSTRACT

Jingzhaotoxin-III (JZTX-III), a 36-residue peptide cardiotoxin containing three pairs of disulphide bonds, has been characterized from the venom of the Chinese tarantula *Chilobrachys jingzhao*. JZTX-III is a promising target for drug development and clinical application, due to its specific inhibitory effects on the human voltage-gated potassium channel subtype hKv2.1 and sodium channel subtype hNav1.5, which are mainly expressed in the cardiac myocytes. The most direct way to obtain JZTX-III is by extraction from the native venom of the tarantula *jingzhao*, but the amount is often insufficient to meet research and clinical demands. Therefore, there is need for an efficient expression system that can provide a larger quantity of JZTX-III. In this paper, we utilized a galactose auto-induction system to assist the *Escherichia coli* strain SHuffle T7 Express to express recombinant JZTX-III, followed by *in situ* purification on a Ni-nitrilotriacetic acid (Ni-NTA) column. Subsequent experiments were performed to demonstrate the advantages of the galactose auto-induction method and to optimize the incubation conditions. Under the optimal expression conditions, the product of the purified bioactive recombinant JZTX-III reached 12.1 mg/L. Furthermore, it is expected that this expression method can be widely applied to the heterologous expression of other disulphide-bond-rich peptides.

Abbreviations

JZTX-III Jingzhaotoxin-III; SHuffle strain *E. coli* strain SHuffle T7 Express; IPTG isopropyl β-D-thiogalactoside; IMAC capture immobilized metal affinity chromatography; HEK293 cells human embryonic kidney 293 cells; SUMO small ubiquitin-like modifier; ICK inhibitor cystine knot; MALDI-TOF/TOF matrix-assisted laser desorption/ ionization time of flight/ time of flight; Ni-NTA Ni-nitrilotriacetic acid

Introduction

Jingzhaotoxin-III (JZTX-III) is a cardiotoxin that was originally isolated from the Chinese earth tiger tarantula *Chilobrachys jingzhao*. It is a 36-residue peptide that includes 6 cysteine residues that form three pairs of disulphide linkages in a pattern of I–IV, II–V and III–VI [1]. It has been found that JZTX-III can specifically inhibit the activation of the hNav1.5 channel, which is mainly expressed in cardiac myocytes [1,2]. According to the alanine scanning analysis, an exposed hydrophobic patch formed by two acidic residues (i.e. Asp1 and Glu3) and four Trp residues (i.e. Trp8, Trp9, Trp 28 and Trp30) is the key residue that facilitates the affinity of JZTX-III to hNav1.5 [3]. Moreover, JZTX-III has shown a significant inhibitory effect on the hKv2.1 channel by modifying the gating of the channel [4]. The way JZTX-III acts on the hKv2.1 channel is similar to that of hanantoxin-I [2]. Based on a previous analysis, certain residues, especially Ser281, can affect the affinity of JZTX-III to hKv2.1 and its functional surface bound to hKv2.1, which is composed of four hydrophobic residues (i.e. Trp8, Trp28, Trp30 and Val33) and three charged residues (i.e. Arg13, Lys15 and Glu34) [5]. The solution structure of JZTX-III has been previously determined by nuclear magnetic resonance spectroscopy, showing that JZTX-III exhibits an inhibitor cystine knot (ICK) motif and its main feature is a hydrophobic patch surrounded by several charged residues [2]. If this peptide is simply isolated from native venom, 0.05 mg of the JZTX-III can be obtained in 1 mg of crude venom [1]. However, if the peptide is expressed in yeast by its secretory pathway, 5 mg of target protein can be purified and obtained from 1 L of culture medium [3]. A bacterial expression system for the expression of JZTX-III remains undetermined.

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The expression of bioactive peptides rich in disulfide bonds has been proved difficult in the cytoplasm of some *Escherichia coli* strains, including BL21 (DE3), because these strains possess reducing cytoplasmic environments [6,7]. However, the *E. coli* strain SHuffle T7 Express (SHuffle strain), whose two cytoplasmic reductive pathways are engineered, is capable of overcoming this problem [8] and has already been utilized to express another spider toxin peptide, Huwentoxin-IV [9].

Traditionally, isopropyl β-D-thiogalactoside (IPTG) is added to induce the expression of the proteins of interest in the SHuffle strain. But compared with the induction by IPTG, the auto-induction system is superior for improving protein yield [10]. When applying the auto-induction method, it is unnecessary to monitor the cell culture density, making it a preferable expression strategy. In the auto-induction system, lactose traditionally acts as the precursor for the inducer allolactose. However, it has been theoretically shown that lactose is unable to transform into allolactose in the cytoplasm of the SHuffle strain, whose *lac Z* gene is reported to be dysfunctional, causing the absence of β-galactosidase in its cytoplasm [8].

It has previously been discovered that galactose can be used instead of lactose to auto-induce the expression of proteins in the *E. coli* strain BL21 (DE3) and its derivative strains [11]. This is because, similar to lactose, there is a glucose–galactose diauxie in the BL21 (DE3) strain [12], and galactose can also act as an inducer for the expression of proteins in the strain [13]. The SHuffle strain used in this study is a BL21 (DE3) derivative strain, resulting in the expression of rJZTX-III capable of being auto-induced by galactose. Thus, in this work, we utilized galactose as the alternative inducer to auto-induce the SHuffle strain to express a large amount of recombinant JZTX-III (rJZTX-III). Additional assessments highlight the significant advantages of this expression strategy.

**Materials and methods**

**Recombinant plasmid construction**

The JZTX-III amino acid sequence was acquired from GenBank (accession no. EU233832) and was then optimized based on the codon usage of *E. coli* strain K12 by using the software JCat. The gene sequence of JZTX-III was synthesized by Sangon (Shanghai, China). The JZTX-III gene sequence is

1 GACGGTTGAAT GCCGTGGTCT 21 CTGGTGGAAA TGGCGCTCGT

41 GTAAACGCGG GTGCTGAAAA 61 GGTTACGCTT GCTCTAAAA

81 CTGGGGTTGG TGCGCTGGT 101 AAGCTCCG,

and its amino acid sequence is

1 DGEFGFWWK CGRGKPCCCK 21 YGACSKTWGW CAVEAP.

A pair of primers (i.e. P-JZTX3-forward: 5'–GCTCA CCGTTGAACAGATCGTGACGTTGAATCGCTGG-3'; P-JZTX3-reverse: 5'–GGTACCGTCGACGTCCCGATATTACGAGCTTCAACAGCGC-3') were designed to clone the gene encoding JZTX-III into the in-house plasmid pWS-SUMO, which has a 6-histidine tag and a small ubiquitin-like modifier (SUMO) tag, by the one-step cloning method [14]. A terminal codon was inserted to the 3' end of the JZTX-III gene. The recombinant plasmid was sequenced and confirmed to be correct.

**Expression of rJZTX-III**

**Auto-induction**

The recombinant vector pWS-SUMO-JZTX-III was transformed into the selected *E. coli* SHuffle strain (NEB, Ipswich, MA, USA). The strain was cultured overnight on a fresh Luria–Bertani (LB) agar plate at 30 °C. According to the auto-induction protocol described in [11], a single colony of the transformants on the plate was inoculated in ZYM-505 medium (i.e. 1% N-Z-amine AS, 0.5% yeast extract, 25 mmol/L NaHPO<sub>4</sub>, 25 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 50 mmol/L NH<sub>4</sub>Cl, 5 mmol/L Na<sub>2</sub>SO<sub>4</sub>, 2 mmol/L MgSO<sub>4</sub>, 0.5% glycerol and 0.05% glucose) containing 100 ng/μL ampicillin at 30 °C at a shaking rate of 220 r/min for 8 h (the optical density at 600 nm (OD<sub>600</sub>) was approximately 2.5). A portion of the culture was transferred into the ZYM-5051 medium (i.e. 25 mmol/L Na<sub>2</sub>HPO<sub>4</sub>, 25 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 50 mmol/L NH<sub>4</sub>Cl, 5 mmol/L Na<sub>2</sub>SO<sub>4</sub>, 2 mmol/L MgSO<sub>4</sub>, 0.2 x metals, 0.5% glycerol, 0.05% glucose, 0.1% galactose and 0.25% aspartate) containing 100 ng/μL ampicillin in a volume ratio of 1:1000. The auto-induction culture was incubated at 30 °C at a shaking rate of 220 r/min for approximately 16 h (OD<sub>600</sub> was approximately 7.5). Cell pellets were harvested by centrifugation at 4000g for 10 min.

**IPTG induction**

To compare the expression efficacies of auto-induction and induction by IPTG, we also used IPTG to induce the expression of rJZTX-III. For cells induced by IPTG, when the OD<sub>600</sub> of the primary cultures reached 0.6, they were transferred to larger LB media at a volume ratio of 1:100.
Then, different concentrations of IPTG were added when the OD$_{600}$ of the transferred cultures reached 0.6. All the cultures induced by IPTG were incubated at 30 °C at a shaking rate of 220 r/min and their OD$_{600}$ values were monitored every 30 min. Cells were harvested when the OD$_{600}$ values of the cultures stopped increasing.

**Cell lysis**

The cell pellet was re-suspended in a phosphate buffered saline (PBS) solution (i.e. 2 mmol/L KH$_2$PO$_4$, 10 mmol/L Na$_2$HPO$_4$, 137 mmol/L NaCl and 2.7 mmol/L KCl, pH = 7.4). The suspension was ultrasonicated on ice to lyse the cells. After the suspension was centrifuged at 15,000g at 4 °C for 30 min, the lysate pellet was spun down. The supernatant was preserved and filtered by a 0.2 μm filter (Pall Corporation, New York, MI, USA).

**Capture immobilized metal affinity chromatography (IMAC) and in situ purification**

A 5 mol/L imidazole stock solution was added to the cell lysate supernatant to achieve a final concentration of 20 mmol/L, and the Ni-nitrilotriacetic acid (Ni-NTA) column (Takara Bio Inc., Kusatsu, Shiga, Japan) was equilibrated by 20 column volumes (CV) of PBS containing 20 mmol/L imidazole. The lysate supernatant was then loaded onto a gravity Ni-NTA column at a flow rate of 1 mL/min. The supernatant was washed by 50 CV of PBS containing 50 mmol/L imidazole. Then, the wash buffer was exchanged to 50 CV of SUMO protease buffer (i.e. 50 mmol/L Tris-HCl, pH = 8.0, 0.2% Igepal, 150 mmol/L NaCl, 0.1 mmol/L dithiothreitol) to prepare the column for the SUMO protease cleavage reaction. Subsequently, 5 CV of protease buffer was used to suspend the resin, which was bonded to the fusion protein His6-SUMO-JZTX-III. SUMO protease (Sangon, Shanghai, China) was then added to the suspension to give a final concentration of 20 U/mg. The cleavage reaction was cultured overnight at 30 °C at a constant shaking rate of 220 r/min. After the sample was centrifuged at 600g and 4 °C for 10 min, the supernatant containing the rJZTX-III fractions was obtained. The cleavage product was desalinated by dialysis overnight utilizing a 1 kDa dialysis tube (Sangon, Shanghai, China) at 4 °C. The dialysate was lyophilized and re-suspended in deionized distilled water or stored in a −80 °C refrigerator.

**RP-HPLC and mass spectrometry**

The collected rJZTX-III fractions dissolved in water were subjected to reverse phase high performance liquid chromatography (RP-HPLC) using a C18 column (4.6 mm × 250 mm). A linear gradient of acetonitrile between 25% and 45% was used to wash the sample, and eluted proteins were collected and lyophilized. The molecular mass of the purified protein was determined by matrix-assisted laser desorption/ionization time of flight/time of flight (MALDI-TOF/TOF) mass spectrometry (Ultraflex™, Bruker Daltonics, Billerica, MA, USA).

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

The protein samples of the cell cultures, cell lysates and purified proteins were analysed via SDS-PAGE. Briefly, 10 μL of each protein sample was mixed with SDS buffer solution and boiled for 10 min before loading into the wells of a 12% acrylamide gel, which was subsequently treated with 10% formaldehyde fixative and Coomassie brilliant blue staining.

**Whole-cell patch-clamp experiments on rJZTX-III**

A plasmid possessing the sequence encoding hNav1.5 was transfected into cultured human embryonic kidney 293 (HEK293) cells. To perform the whole-cell patch-clamp experiment, cells emitting green fluorescence were chosen at 36–72 h following transfection. The purified rJZTX-III was dissolved in deionized distilled water to a concentration of 1 mmol/L. Before being used, the toxin stock solution was stored at −20 °C and was diluted by the bath solution to an appropriate concentration. The whole-cell patch-clamp assay was then performed utilizing an EPC-9 amplifier (HEKA, Lambrecht, Germany) at room temperature (20–25 °C). The HEK 293 cells were held at −80 mV and depolarized to 0 mV. The Pulse+pulsefit 8.0 (HEKA, Lambrecht, Germany) and Sigmaplot 9.0 (Systat Software Inc.) programs were used for data recording and analysis.

**Results and discussion**

**Optimization of the expression conditions**

To increase the yield of the desired peptides, the dependence of the recombinant expression yield on different incubation conditions was experimentally investigated for the recombinant protein rJZTX-III in SHuffle cells, because the optimal expression conditions in the SHuffle strain are protein specific [8]. To optimize the expression conditions of rJZTX-III through the galactose auto-induction method, a series of experiments were performed. When incubated in the 5051 medium containing...
galactose, the average culture densities and expression efficiency under various conditions (including 16 °C for 36 h, 25 °C for 20 h, 30 °C for 16 h, and 37 °C for 16 h, all at a shaking rate of 220 r/min) were compared and analysed (Figure 1; Table 1). All the cells prepared for SDS-PAGE analysis were re-suspended with distilled water to a standard OD$_{600}$ value of 10. Figure 1 indicated that efficient expression of rJZTX-III was achieved at both 30 and 37 °C for 16 h. Additionally, the data in Table 1 indicated that the cells incubated at 30 °C for 16 h achieved the highest average culture density. Therefore, we choose growth at 30 °C for 16 h as the best expression conditions.

Comparisons of inducers

Because of the toxic impact of IPTG to the E. coli culture, the production yield is often restricted and it is necessary to utilize an auto-induction system to enable the SHuffle strain to improve its expression product yield. To evaluate the induction efficacies of IPTG and galactose, different IPTG concentrations were used and compared with galactose auto-induction under the optimized incubation conditions (i.e. 30 °C, 220 r/min for 16 h) (Figure 2(A)). For cells induced by IPTG, their highest average OD$_{600}$ values were all recorded (Table 2). All the cells prepared for SDS-PAGE analysis were re-suspended with distilled water to a standard OD$_{600}$ value of 10. The results illustrate that, although the expression efficiencies per 10 OD$_{600}$ of these two induction methods are approximately similar (Figure 2(A)), the auto-induction system was so beneficial for the growth of the SHuffle cells that the OD$_{600}$ value of the auto-induction culture was much higher than that of the culture induced by IPTG (Table 2). This implies that the former induction approach improves the protein expression efficiency and the quantity of rJZTX-III auto-induced by galactose is consequently larger than that induced by IPTG.

Simultaneously, the efficacies of auto-induction by galactose and lactose in the SHuffle strain were compared. Because lactose cannot be converted to the

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**Table 1.** Highest average OD$_{600}$ values (each repeated 10 times) of cultures auto-induced by galactose under different expression conditions.

| Inducers | Galactose at 16 °C | Galactose at 25 °C | Galactose at 30 °C | Galactose at 37 °C |
|----------|--------------------|--------------------|--------------------|--------------------|
| OD$_{600}$ | 2.80               | 5.19               | 5.66               | 4.7                |

**Table 2.** Highest average OD$_{600}$ values (each repeated 10 times) of cultures induced by listed inducers.

| Inducers | Galactose at 30 °C | 0.2 mmol/L IPTG | 0.5 mmol/L IPTG | 0.8 mmol/L IPTG | Lactose at 30 °C |
|----------|--------------------|-----------------|-----------------|-----------------|-----------------|
| OD$_{600}$ | 5.66               | 2.49            | 2.45            | 2.41            | 5.68            |

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**Figure 1.** Accommodation of expression conditions.

**Figure 2.** Comparisons of inducers: IPTG induction vs. galactose auto-induction (A) and auto-induction by galactose vs. lactose (B).
by the arrow in lane 6 shows a protein with a molecular weight of approximately 4 kDa, similar to the native JZTX-III protein of 3919.4 Da. It is also observed from lane 6 that the obtained fractions through the in situ purification method were highly pure.

**Analysis of the results from RP-HPLC and mass spectrometry**

The fractions obtained during the IMAC and in situ purification steps were subjected to RP-HPLC. The graphic information in Figure 4(A) delineates the 280 nm ultraviolet absorption curve of the sample. The eluted rJZTX-III was collected and lyophilized. The total yield of the final purified rJZTX-III produced by the galactose auto-induction method under optimal conditions was approximately 12.1 mg/L. The mass spectrometry result shown in Figure 4(B) indicated that the molecular mass of the sample collected during RP-HPLC purification was 3919.13 Da, which is close to its theoretical value of 3919.4 Da.

**Electrophysiological properties analysis of rJZTX-III**

The whole-cell patch-clamp experiment was performed to analyse the electrophysiological activities of rJZTX-III. As demonstrated in Figure 5, 1 μmol/L rJZTX-III could significantly inhibit the current of hNav1.5 expressed in HEK293 cells, which was similar to the effects observed for the natural JZTX-III [15]. This result suggests that rJZTX-III expressed in the cytoplasm of the SHuffle strain through the galactose auto-induction method retains its bio-functional activity.

The functional expression of rJZTX-III using a prokaryotic system and the enhancement of the total yield of the small peptide toxin of interest are the two main issues emphasized in this paper. First, by comparison with IPTG, the galactose auto-induction system provides particularly high product yield. Second, the mass spectrometry result and electrophysiological analysis suggest that the rJZTX-III expressed in the cytoplasm of the SHuffle strain preserves its bioactivity. These results indicate that the expression strategy we proposed has addressed these problems effectively.

**Conclusions**

We employed the galactose auto-induction system to help the SHuffle strain functionally express the recombinant toxin peptide JZTX-III. It is experimentally proved that, by comparing the induction by IPTG, the galactose auto-induction system is capable of improving the protein expression efficiency in the SHuffle strain, whose
cytoplasm environment is more beneficial for the formation of disulphide bonds than that of BL21 (DE3) cells. After optimizing the incubation conditions, the total yield of the final purified bioactive rJZTX-III was as high as 12.1 mg/L. We believe that other disulphide bond-rich peptides can also be produced by this efficient prokaryotic expression strategy.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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