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Enhanced soluble production of cholera toxin B subunit in *Escherichia coli* by co-expression of SKP chaperones

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ABSTRACT

The cholera toxin B subunit (CTB) is a nontoxic portion of the cholera toxin that retains mucosal adjuvant properties. Expression of CTB in *Escherichia coli* is difficult as CTB aggregates and accumulates as insoluble inclusion bodies. To remedy this problem, the periplasmic chaperone, SKP, was investigated as possible co-expression partner to increase the solubility of recombinant CTB (rCTB) in *E. coli*. The result showed co-expression of SKP enhanced the soluble expression of rCTB in *E. coli*. Moreover, soluble rCTB was successfully expressed and secreted into the periplasmic space through the direction of the LTB leader signal. rCTB in periplasm was purified using an immobilized D-galactose resin; GM1-ELISA experiments showed that rCTB retains strong GM1 ganglioside-binding activity. Intranasal administration of ovalbumin (OVA) with rCTB significantly induced both mucosal and humoral immune responses specific to OVA. These data indicate that co-expression of the molecular chaperone SKP with CTB increased the solubility of rCTB while maintaining its function.

1. Introduction

Cholera toxin (CT) is the soluble toxin secreted by the gram-negative bacteria *Vibrio cholerae*, which can severely affect children and the elderly. The cholera toxin is composed of two proteins, subunit A (CTA), which is a monomer, and subunit B (CTB), which forms a pentamer [1]. CT is one of the most powerful mucosal adjuvants, but the use of CT for vaccine development is precluded by its strong toxicity [2]. However, the CTB contains the mucosal adjuvant properties and has been shown to improve protective immunity against infectious diseases [3–5]. CTB becomes fully biologically active when it adopts a pentameric form [6], and enters the body by binding GM1 on the gut epithelium.

Molecular chaperones, which are present in eukaryotic and prokaryotic organisms, influence both initial protein folding and subsequent maintenance. The 17 kDa protein (SKP), a molecular chaperone in the *Escherichia coli* periplasm, has an important role in folding and assembling outer membrane proteins [7]. Co-expression of SKP with rCTB could be used to increase the solubility of CTB and therefore increase the yield of rCTB. To our knowledge, there is currently no report using SKP to increase rCTB expression; however co-expression of SKP was used to improve the solubility and affinity of single chain variable fragment (scFv) previously [8–10].

In this study, we investigated the effects of the molecular chaperone SKP on expression and solubility of CTB. The pET-Duet vector containing two multiple cloning sites (MCS) was used for the construction and expression of the two target genes. The results indicate that co-expression of SKP enhanced the soluble expression of the rCTB in *E. coli*.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

Escherichia coli str. K-12 was used to clone the SKP gene. *E. coli*

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DH5α and BL21 (DE3) were purchased from Invitrogen (USA). The plasmid vector pETDuet-1 was purchased from Novagen (Germany) pET-CTB, which contains CTB fused with an LTB (heat-labile enterotoxin B subunit) signal peptide was constructed for this paper.

2.2. Cloning of the SKP gene and construction of the expression plasmid

The SKP gene sequence was obtained by PCR from the *E. coli* str. K-12. PCR amplification was conducted in a 50 μL reaction mixture, each containing 1 μL of *E. coli* K-12 culture as template, 25 μL master mix (Takara Japan) and 1 μL of forward and reverse primers. The PCR cycling parameters were as follows: an initial denaturing at 95 °C for 5 min, followed by 30 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and one final extension at 72 °C for 5 min. The following primers were used: upstream primer: 5′- AAACGCATATGAAAAAGTGGTTATTAGCTGCAG -3′, downstream primer: 5′-AAACGCTCGAGTTATTTAACCTGTTTCAGTACG -3′. The amplified PCR products, after clean-up and digestion with NdeI and XhoI, were inserted into the second MCS of pET-Duet-1 and the resulting construct was named pET-SKP. To construct the dual expression vector pET-CTB/SKP, CTB gene, fused with LTB signal peptide, was digested with NcoI and HindIII and inserted into the first MCS of pET-SKP.

2.3. Plasmid transfection and protein expression

PET-CTB, pET-SKP and pET-CTB/SKP were plasmid transformation into BL21 (DE3) for protein expression. Single colonies on LB plates with the ampicillin (50 μg/mL) were inoculated into 5 mL of CAYE medium and incubated at 37 °C until OD_{600} reached 0.6, and then IPTG (1 mM) was added to induce protein expression. After an additional 20 h of cultivation at 20 °C, 500 μL of bacterial culture was harvested by centrifugation at 6000 × g for 5 min at 4 °C, washed twice in distilled water, and resuspended in PBS (pH 7.4). Cells were broken by sonication and centrifuged at 12,000 × g for 10 min at 4 °C, after which the proteins were analyzed by SDS-PAGE. To analyze the localization of rCTB, cell fractions were prepared as described in the article and were analyzed by SDS-PAGE [11].

2.4. Immunoblot analysis

Expressed proteins were separated by 15% SDS-PAGE and then transferred by electroblotting onto PVDF transfer membranes (Millipore, USA) using a semidry transfer cell (Bio-Rad, USA) per the manufacturer's manual. Membranes were blocked with 5% BSA in TBST (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20). The membrane was incubated first with a 1:1000 dilution of an anti-cholera toxin rabbit antibody (Sigma, USA) overnight at 4 °C. After washing, the membrane was incubated with anti-rabbit IgG goat antibody (1:5000) conjugated to peroxidase at room temperature for 1 h. The washing procedure was repeated, and the reactions were visualized with metal-enhanced diaminobenzidine (DAB).

2.5. Purification of rCTB

The periplasmic fraction from above was collected and filtered with a 0.45 μm filter. Immobilized D-galactose resin (Thermo, USA) was prepared according to the manufacturer's instructions. It was first equilibrated with 10 bed volume binding buffer (20 mM Tris, pH 7.4 and 0.2 M NaCl). The sample was then loaded onto the column and then washed with 20–30 column volumes of binding buffer. The sample was eluted with elution buffer (0.1 M D-galactose in binding buffer). The purified protein was analyzed by SDS-PAGE and quantified using Bradford assays.

CTB forms a very stable pentamer that resists dissociation by SDS. Nonetheless, CTB pentamers can be denatured by heat and by DTT. Non-denaturing SDS–PAGE that DTT was omitted from the loading buffer and the samples were not boiled was conducted for determining subunit associations [12–15].

2.6. The GM1-ganglioside binding assay

CTB binding to GM1-ganglioside was measured using an enzyme-linked immunosorbent assay (ELISA) as described previously [11,16]. Microtiter plates were coated with monosialoganglioside-GM1 (5 μg/mL, 50 μL per well, Sigma) in bicarbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6), and incubated overnight at 4 °C. The plates were washed with PBST (10 mM Na_2HPO_4, 3 mM KH_2PO_4, pH 7.2, 100 mM NaCl, and 0.05% Tween-20) and blocked with 5% fat-free milk in PBST for 1 h at 37 °C. After washing the plates with PBST, purified rCTB, bovine serum albumin (BSA) (negative control), and commercial CTB (positive control, Sigma) were then added, and the plates were incubated for 2 h at 37 °C.The plates were then washed three times with PBST before the addition of the rabbit anti-cholera toxin antiserum (1: 2000, Sigma) for 1 h at 37 °C.The plates were washed three times with PBST and then incubated with goat anti-rabbit HRP-IgG (1: 5000, Sigma) for 1 h at 37 °C. The binding was detected by measuring the absorbance at 450 nm.

2.7. Immunization and sample collection

Twelve C57BL/6 mice (6-week old) were divided randomly into three groups. For nasal vaccination delivery, mice in one group were immunized with 100 μg OVA alone, while mice in other groups were immunized with 100 μg OVA combined with 10 μg rCTB or commercial CTB. All mice were immunized on days 0 and 14. The mice were sacrificed 14 days after the final immunization, and serum and a nasal wash were collected.

2.8. Detection of OVA-specific antibodies (Ab)

Antibody titers in the serum and nasal wash samples were determined by enzyme-linked immunosorbent assay (ELISA) as described previously [17]. The ELISA plate was coated with OVA (1 mg/mL), and blocked with 5% BSA. After washing with PBST, serially diluted serum and nasal wash samples were added to the wells in duplicate. Following a 1 h incubation at 37 °C, the plates were washed with PBST. Peroxidase conjugated goat anti-mouse IgG and IgA (Sigma) was added, and the plate was incubated for 1 h at 37 °C. All wells were incubated with o-phenylenediamine dihydrochloride substrate (Sigma), and the OD_{492} of the wells were measured. End-point titers were expressed as the reciprocal $\log_2$ of the highest dilution, which gave an optical density in a plate reader at 492 nm that was 0.1 units greater than the back ground level.

2.9. Statistical analysis

Data are expressed as the mean ± standard deviation (SD) and are analyzed by ANOVA using SPSS (Version 10.0). The comparison of the sample mean between immunized and control group was evaluated by Student's *t*-test, and *p*-values less than 0.05 were considered statistically significant.