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Construction of Engineered Bacteria and Biological Activity Analysis of Fusion Proteins: Postprint

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Abstract

Objective: To construct engineered bacteria carrying the Staphylococcal Enterotoxin-like K (SElK) and green fluorescent protein (GFP) fusion gene, and to conduct preliminary biological activity analysis of the SElK-GFP fusion protein. Methods: The SElK-GFP fusion gene was cloned using PCR and overlap PCR, and inserted into the pET28a expression vector. After verification by colony PCR, plasmid double enzyme digestion, and sequencing, the successfully constructed pET28a-SElK-GFP plasmid was transformed into E. coli BL21 strain for induced expression. The SElK-GFP fusion protein was purified using a Ni affinity magnetic bead kit. The MTT assay was used to detect SElK-GFP-stimulated proliferation of mouse splenic lymphocytes, and ELISA was used to detect the secretion levels of cytokines IL-2 and IFN- in mouse serum after tail vein injection of SElK-GFP. Results: Engineered bacteria capable of expressing the SElK-GFP fusion protein were successfully constructed. The purified high-purity SElK-GFP fusion protein exhibited obvious green fluorescence. Biological activity analysis of the fusion protein demonstrated that SElK-GFP could significantly stimulate mouse splenic lymphocyte proliferation in a dose-dependent manner. Meanwhile, ELISA detection revealed that SElK-GFP could significantly increase the secretion levels of cytokines IL-2 and IFN- in mouse serum. Conclusion: This study successfully cloned, expressed, and purified high-purity SElK-GFP fusion protein, which not only retained the superantigen activity of SElK but also possessed the visualizability of GFP green fluorescence, providing a favorable tool for in-depth research on the biological activity of SElK.

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Full Text

Construction and Functional Analysis of a Staphylococcal Enterotoxin-like K and GFP Fusion Protein

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Abstract

Objective: To construct genetically engineered bacteria carrying the full-length fusion gene of Staphylococcal enterotoxin-like K (SElK) and green fluorescent protein (GFP), and to examine the biological activity of the SElK-GFP fusion protein.

Methods: The SElK-GFP fusion gene was obtained by overlap PCR and cloned into the pET28a expression vector. After verification by colony PCR, double digestion, and sequencing, the successfully constructed pET28a-SElK-GFP plasmid was transformed into *E. coli* BL21 for induced expression. The SElK-GFP fusion protein was purified using a Ni -affinity magnetic bead kit. The proliferation of mouse spleen lymphocytes stimulated by SElK-GFP was examined by MTT assay, and the secretion levels of cytokines IL-2 and IFN- in mouse serum after tail vein injection of SElK-GFP were detected by ELISA.

Results: We successfully constructed SElK-GFP-producing engineered bacteria and purified high-purity SElK-GFP fusion protein that exhibited obvious green fluorescence. Biological activity analysis showed that SElK-GFP could significantly stimulate mouse spleen lymphocyte proliferation in a dose-dependent manner. ELISA detection revealed that SElK-GFP significantly increased the secretion levels of cytokines IL-2 and IFN- in mouse serum.

Conclusion: This study successfully cloned, expressed, and purified high-purity SElK-GFP fusion protein, which not only retained the superantigen activity of SElK but also maintained the visualizability of GFP green fluorescence, providing a valuable tool for in-depth investigation of SElK biological activity.

Keywords: Staphylococcal Enterotoxin-like K; Superantigen; T lymphocyte; Prokaryotic expression

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Introduction

Staphylococcal enterotoxins (SEs) are a group of bacterial exotoxin proteins with superantigen activity. Unlike conventional antigens, SEs do not require processing by antigen-presenting cells (APCs). Instead, they directly bind to MHC class II molecules on the APC membrane in an unrestricted manner and are presented to T cells as intact molecules, thereby activating large populations (5%-20%) of T cells with specific V regions (including both CD4 and CD8 T cells). This activation triggers massive cytokine release, including IL-2, IFN- , and TNF- , ultimately eliciting strong immune responses [1, 2]. Human T lymphocytes are extremely sensitive to SEs, with effective immune activation occurring at very low concentrations (1-10 ng). Based on serological analysis, SEs can be classified into SEA-SEE, TSST-1, and TSST-2 [1, 3], with novel enterotoxins such as SER [4], SEG [5], SET [6], and SEM [7] being discovered more recently.

Previous research has primarily focused on classical enterotoxin molecules such as SEA-SEC and TSST-1, investigating their antitumor activity and mechanisms through numerous basic and preclinical studies. These studies have demonstrated that SEs can induce strong cell-mediated cytotoxicity and exhibit high killing efficacy against MHC II-positive tumor cells [8-13]. However, research indicates that high-dose SEs exhibit immunosuppressive activity by inducing T cell apoptosis, thereby reducing antitumor efficacy [14]. Furthermore, the inherent enterotoxicity of SEs and their ability to induce toxic side effects such as emesis, diarrhea, and fever at high doses have severely limited their clinical application [15, 16].

With deeper investigation into SEs, it has become clear that emetic toxicity is not an essential property of all staphylococcal enterotoxins. In 2004, the International Nomenclature Committee designated staphylococcal superantigens lacking verified emetic toxicity as SEl (enterotoxin-like) [17]. Although studies have demonstrated that early SEl members (SElK [18, 19], SElL [20], and SElQ [21]) possess potential emetic toxicity, their toxic potency is significantly weaker than that of typical SEs, making them more suitable for clinical applications [22]. Green fluorescent protein (GFP) has been widely used as a reporter gene for detecting target gene expression, studying intracellular metabolism, and tracking cell differentiation. Therefore, this study aimed to construct engineered bacteria expressing the SElK-GFP fusion gene through genetic engineering techniques and to preliminarily characterize the biological activity of the SElK-GFP fusion protein. This work provides a valuable tool for in-depth investigation of SElK biological activity and promotes the clinical application of SElK in tumor therapy.

Materials and Methods

1.1 Strains and Plasmids

The *Staphylococcus aureus* strain carrying the SElK gene and the strain carrying the GFP gene, *E. coli* BL21, *E. coli* DH5 , and the pET28a plasmid were preserved in our laboratory. Female BALB/c mice (6-8 weeks old, weighing 24±2 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (License No. SCXK (Beijing) 2012-0001).

1.2 Reagents

Bacterial genome extraction kits, plasmid extraction and purification kits, and BCA protein quantification kits were purchased from Beijing CoWin Biotech Co., Ltd. The BeaverBeads™ IDA-Nickel kit was purchased from BeaverNano (Suzhou) Co., Ltd. The one-step competent cell preparation kit was purchased from Shanghai Beyotime Biotechnology Co., Ltd. Restriction endonucleases, PrimeSTAR Max Premix (2×), and T4 DNA ligase were purchased from TaKaRa Bio (Dalian) Co., Ltd. Bovine serum albumin (BSA), IPTG (isopropyl -D-1-thiogalactopyranoside), and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma-Aldrich. Fetal bovine serum (FBS) and RPMI1640 medium were purchased from Hyclone. ELISA kits were purchased from Neobioscience Technology Co., Ltd.

1.3.1 Cloning of SElK-GFP and Construction of Prokaryotic Expression Vector

Staphylococcus aureus carrying the SElK gene and the GFP-carrying strain were inoculated into LB liquid medium and cultured overnight at 37°C for genomic DNA extraction. Using the extracted genomes as templates, SElK and GFP gene fragments were amplified by PCR (primers listed in). The SElK-GFP fusion gene was obtained by overlap PCR as shown in [Figure 1: see original paper]A, with primers listed in ; underlined sequences indicate EcoR I and Xho I restriction sites. PCR amplification was performed as follows: 95°C for 5 min; 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min; followed by 72°C for 10 min. The purified SElK-GFP gene fragment was double-digested with EcoR I and Xho I and ligated into similarly digested pET-28a expression vector. The ligation product was transformed into *E. coli* DH5 competent cells. Positive clones were verified by colony PCR, plasmid double digestion, and sequencing.

1.3.2 Induced Expression and Purification of SElK-GFP Fusion Protein

The successfully constructed pET-28a-SElK-GFP plasmid was transformed into the expression strain *E. coli* BL21. Positive single colonies were inoculated into LB liquid medium containing kanamycin (60 g/mL) and cultured overnight at 37°C. The culture was then inoculated at 1% (V/V) into fresh medium (500 mL)

and incubated at 37°C with shaking until OD reached 0.5. IPTG was added to a final concentration of 1.0 mmol/L, and expression was induced at 30°C. At induction time points of 0 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, and 8 h, 1 mL of bacterial culture was collected, centrifuged to harvest cells, washed twice with PBS, and resuspended in 100 L PBS before transfer to a 96-well plate. Based on the intrinsic green fluorescence of the fusion protein, GFP fluorescence signals (490 nm) were measured using a microplate reader to optimize the induction time for SElK-GFP expression. After induction, cells were harvested by centrifugation, lysed by sonication, and SElK-GFP was purified using the BeaverBeads™ IDA-Nickel magnetic bead kit. The purified protein was dialyzed against PBS at 4°C for 48 h (with PBS changes every 12 h) and stored at -80°C. SDS-PAGE was used to verify expression and purification efficiency, and protein concentration was determined by the BSA method.

1.3.3 MTT Assay for Mouse Spleen Lymphocyte Proliferation Activity

Mouse spleen lymphocyte suspensions were prepared aseptically and adjusted to a concentration of 1×10 cells/mL in RPMI1640 medium containing 10% FBS. The cell suspension (100 L) was added to each well of a 96-well plate. SElK, GFP, and SElK-GFP were diluted in RPMI1640 medium with 10% FBS and added to the 96-well plate (100 L per well) to achieve final concentrations of 0.01, 0.1, 1, and 10 g/mL. The negative control group was treated with 10 g/mL BSA, and the blank control consisted of RPMI1640 medium with 10% FBS. Each sample was tested in five replicates. After incubation at 37°C with 5% CO for 48 h, cell proliferation was measured by standard MTT assay. The proliferation index (PI) was calculated as: PI = (absorbance of experimental well) / (absorbance of negative control well) [19].

1.3.4 In Vivo Cytokine Detection

Mice were randomly divided into four groups (n=5): the SElK group received tail vein injection of 200 L SElK solution (1 g/mL in PBS); the GFP group received 200 L GFP solution (1 g/mL); the SElK-GFP group received 200 L SElK-GFP solution (1 g/mL); and the control group received an equal volume of PBS. Blood samples (50 L) were collected from the tail vein at various time points (0 h, 12 h, 24 h, 36 h, 48 h, 60 h, and 72 h), diluted in an equal volume of heparin-containing PBS, centrifuged at 3000 rpm for 10 min to obtain serum, and stored at -80°C. Serum IL-2 and IFN- levels were measured using appropriate ELISA kits according to the manufacturer’ s instructions.

Statistical Analysis

Experimental data were analyzed using SPSS 13.0 statistical software. Paired t-tests were used for comparisons between groups, and one-way ANOVA was used for mean comparisons. $P < 0.05$ was considered statistically significant.

Results

2.1 Construction and Identification of SElK-GFP Producing Strain

The GFP gene fragment (720 bp) and SElK gene fragment without stop codon (657 bp) were amplified by conventional PCR ([Figure 1: see original paper]B, lanes 1-4). The SElK-GFP fusion gene (1407 bp) was then obtained by overlap PCR and verified by agarose gel electrophoresis ([Figure 1: see original paper]A, lanes 5 and 6). Positive clones obtained after transformation into *E. coli* DH5 were screened by colony PCR, with products analyzed by agarose gel electrophoresis ([Figure 1: see original paper]C, lanes 1 and 2). After confirmation by colony PCR, randomly selected positive clones were cultured, and plasmids were extracted for double digestion verification with EcoR I and Xho I. Agarose gel electrophoresis results are shown in [Figure 1: see original paper]C (lanes 3 and 4). Following confirmation by both colony PCR and double digestion, plasmids were sent to Genewiz (Suzhou) for final sequencing verification to ensure the accuracy of pET28a-SElK-GFP vector construction.

2.2 Expression, Purification, and Fluorescence Detection of SElK-GFP Fusion Protein

Based on the intrinsic green fluorescence of the fusion protein, the induction time was optimized. As shown in [Figure 2: see original paper]A, bacterial cultures after IPTG induction exhibited obvious green fluorescence under UV illumination, which reached a stable plateau after 6 h of induction with no significant change upon further extension, indicating that the optimal induction time for SElK-GFP fusion protein was 6 h ([Figure 2: see original paper]B). SDS-PAGE analysis ([Figure 3: see original paper]A) demonstrated that SElK-GFP protein could be efficiently obtained after IPTG induction, with a molecular weight of approximately 54 kDa. High-purity (>95%) SElK-GFP fusion protein was subsequently obtained after purification with BeaverBeads™ IDA-Nickel magnetic beads.

2.3 SElK-GFP Promotes Mouse Spleen Lymphocyte Proliferation in a Dose-Dependent Manner

The superantigen activity of SElK-GFP was preliminarily evaluated by detecting mouse spleen lymphocyte proliferation using the MTT assay. As shown in [Figure 3: see original paper]B, after 48 h treatment with GFP, SElK, and SElK-GFP at concentrations of 0.01, 0.1, 1, and 10 g/mL, GFP showed no stimulatory effect on mouse spleen lymphocyte proliferation. In contrast, SElK-GFP fusion protein, consistent with SElK, significantly stimulated mouse spleen lymphocyte proliferation in a dose-dependent manner ($P < 0.05$), with no statistically significant difference in stimulatory efficacy between the two.

2.4 SElK-GFP Significantly Stimulates Cytokine Expression in Mice

After tail vein injection of SElK, GFP, SElK-GFP fusion protein, or PBS, serum IL-2 and IFN- levels were measured by ELISA at different time points. As shown in [Figure 4: see original paper], SElK-GFP fusion protein exhibited a similar trend to SElK: IL-2 secretion levels increased significantly compared with the control group after 60 h post-injection ($P < 0.05$) and showed a gradual upward trend throughout the detection period. IFN- secretion levels increased significantly to peak levels after 12 h ($P < 0.01$), then decreased rapidly, returning to normal levels by 36 h. GFP showed no promoting effect on IL-2 and IFN- secretion in mice.

Discussion

Recent research on SEs has primarily focused on the interactions among various lymphocytes and signal transduction pathways during SE-induced immune activation [23, 24], as well as the mechanisms underlying SE-induced diseases [15, 16]. In addition to studies on the prevention and treatment of SE-related diseases, the development of novel antitumor agents utilizing SE superantigen activity has attracted widespread attention [8-13]. Although SEs show strong potential for tumor therapy, their inherent enterotoxicity and emetic toxicity produce severe side effects that limit their application as novel antitumor agents. The discovery of SEls lacking or with low emetic toxicity provides candidate drugs for developing new antitumor preparations [25].

Our research group previously successfully cloned and obtained high-purity SElK protein [19] and systematically investigated its superantigen activity. However, the lack of effective antibodies and tracing methods has severely hindered research into SElK superantigen activity and antitumor mechanisms. Previous studies have shown that the binding affinity between SEs and their specific TCR V ranges between 10 -10 M, similar to the affinity of antigenic peptide/MHC II complexes for corresponding TCR V (10 -10 M) but significantly lower than antibody-antigen affinity (10 -10 ¹ M) [26-28]. We hypothesize that SElK binding to its specific TCR V follows this pattern, meaning that conventional research methods involving resuspension and centrifugation could easily disrupt the SElK-TCR V interaction. Moreover, the complexity and destructiveness of traditional tracing methods make them unsuitable for studying SElK interactions with relevant cells in vivo. Therefore, we propose that constructing a fluorescent protein-tagged SElK would provide a valuable tool for investigating the mechanisms underlying SElK superantigen activity and its potential antitumor applications.

GFP is one of the most widely used marker proteins in cell biology in recent years. Its small molecular weight, lack of significant toxicity when expressed in bacterial and fungal cells, ability to function in living cells, and stable fluorescence properties without requiring additional substrates or enzymes make it

an ideal stable soluble protein for functional studies [11]. Therefore, this study successfully constructed a prokaryotic expression vector for the SElK-GFP fusion gene using overlap PCR technology. In designing the fusion protein, we positioned the GFP tag at the C-terminus of the SElK fragment, considering the SElK binding sites for its specific TCR V and MHC II [29]. A short peptide linker consisting of 10 glycine and serine residues (GGGGSGGGGS) was incorporated, as previous studies have demonstrated that this linker effectively reduces steric hindrance between fusion protein domains, thereby maximizing the preservation of original conformations and functions of each component [13, 30].

The induced expression of SElK-GFP fusion protein validated this design, with obvious green fluorescence observed at different time points during induction. Based on fluorescence intensity, the optimal induction time was determined to be 6 h. Furthermore, after obtaining high-purity SElK-GFP fusion protein via Ni affinity magnetic beads, biological activity assays demonstrated that SElK-GFP fusion protein exhibited biological activity identical to SElK, significantly promoting mouse spleen lymphocyte proliferation in vitro and enhancing IL-2 and IFN- expression in vivo, thereby activating immune responses.

In summary, this study successfully constructed and expressed SElK-GFP fusion protein, which possesses both the superantigen activity of SElK and the green fluorescence property of GFP. This provides a valuable tool for investigating the mechanisms of SElK superantigen activity and its potential antitumor applications, and offers insights for the construction of subsequent fusion proteins.

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