Cloning, expression, and purification of recombinant Enterotoxin B and Cholera toxin B fusion protein in *Lactobacillus plantarum*

Running title: Cloning, expression and purification of rSEB-CTB fusion protein

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Abstract

Background: Lactic acid bacteria (LAB) are promising platforms for mucosal vaccine development. *Staphylococcus aureus* enterotoxin B (SEB), a potent superantigen, is associated with to widespread food poisoning and toxic shock syndrome. Similarly, cholera toxin is the primary virulence factor in *Vibrio cholerae* infections, with its B subunit (CTB) serving as a well-established immune adjuvant that enhances antigen-specific responses in recombinant vaccines. This study aimed to engineer recombinant *Lactobacillus plantarum* as a dual-purpose vaccine candidate targeting *V. cholerae* and *S. aureus* by expressing of CTB and SEB antigens

Methods: A modified gene sequence encoding SEB (lacking superantigenic activity) and CTB was successfully designed, synthesized, and cloned for secretory expression in *Lactobacillus plantarum*. The resulting recombinant protein, tagged with His, was purified using Ni-NTA agarose ion-exchange chromatography and confirmed through Western blot analysis.

Results: Enzyme digestion and PCR analysis confirmed successful cloning of the SEB-CTB fusion gene into both the pBlueScript II SK (+) and pNZ7021 expression vectors, as evidenced by the expected band on agarose gel. SDS-PAGE revealed a ~49 kDa protein band, indicating expression of the recombinant rSEB-CTB protein, which was further validated by Western blot using an anti-His tag antibody.

Conclusion: The construct LP-pNZ7021–SP-seb-ctxB shows a promise as a candidate for recombinant vaccine development targeting *Vibrio cholerae* (cholera toxin-producing) and *Staphylococcus aureus* (SEB-producing), providing dual protection against both pathogens.

Keywords: rSEB, *Lactobacillus plantarum*, Cloning, Vaccines, CTB

Introduction

Staphylococcus aureus is a significant global cause of foodborne illnesses, producing various virulence factors that contribute to its pathogenicity (1-5). Among these, enterotoxin B (SEB) is a potent superantigen linked to congenital poisoning, toxic shock, and septic shock syndrome, especially in pregnant women, making it a potential vaccine antigen (6). In many developing countries, cholera remains a leading cause of child morbidity and mortality. Its symptoms are primarily driven by cholera toxin (CT), an 85 kDa AB5-type protein composed of CTA and CTB subunits. The CTB subunit, a non-toxic homopentamer, facilitates cell binding and acts as a mucosal adjuvant, enhancing both humoral and mucosal immunity (7, 8). It is a mucosal supplement for oral and nasal vaccines (9), and is a mucosal delivery or carrier system (10). By fusing *Staphylococcus aureus* enterotoxin with cholera toxin subunit B (CTB), it is hypothesized that CTB enhances the immune response against enterotoxin and enables immunogenicity against both pathogens. This study explored a chimeric construct combining SEB and CTB to develop a dual-target vaccine candidate. Lactic acid bacteria (LAB) were used as a delivery system, capable of directing heterologous proteins to the mucosal immune system. A 2003 study by Boles JW demonstrated successful cloning and expression of a mutant SEB protein in LAB, resulting in immunogenicity in mice (11). LAB has been widely employed for antigen expression and delivery of therapeutic proteins to mucosal tissues (12). Their growing use in mucosal secretion systems is supported by their role in the gut microbiota and their probiotic benefits (13-17).

In addition, to produce minimal immune responses against itself, LAB can induce a high level of systemic and mucosal antibodies against foreign antigens expressed on its surface following absorption by the mucosal immune system (2, 18). Unlike the currently used vectors, LAB, due to its probiotic and anti-inflammatory attributes, can stimulate the host immune system and is also considered an inactive microorganism. Among the lactic acid bacteria as vectors of delivery, lactobacilli are considered to be a good option due to their stimulating immunity and greater ability in surviving in the mucosal layer. Vaccination is the best way to control enterotoxaemia (3), and due to the problems of conventional vaccines in production processes and quality control stages, its production is not recommended (19). Therefore, the use of lactic acid bacteria in vaccine delivery is approved as an effective method. Advantages of recombinant toxin vaccines include lack of toxicity, greater stability, and greater immunogenicity (19-21). Since very few studies have been conducted in this field in Iran and considering the importance of foodborne diseases in developing countries and around the world, this study was designed to use a potential native probiotic Lactobacillus. In this study, we seek to design a recombinant r*seb-ctxB* fusion gene vaccine in Lactobacillus and protein purification. Vaccination with it in future studies could lead to a reduction in the incidence of infectious diseases of the gastrointestinal tract and reduce the use of antibiotics in the country. Therefore, in the present study, the r*SEB-CTXB* fusion gene was expressed in *L. plantarum* host in the secretory model, by pNZ7021 vector to be used as a candidate for the chimeric vaccine.

Methods

Bacterial strains and growth conditions

Lactobacillus plantarum (*L. plantarum*) 299v. (purchased from the Pasteur Institute of Iran) and recombinant *L. plantarum* 299v were grown in M17 (Merck) medium containing 0.5% glucose (GM17) medium under anaerobic conditions at 30° C. The cultures was then incubated for 48 hours without shaking (22).

Sequence analysis, segment selection, gene synthesis, and cloning

The ctxB and SEB sequences were obtained from GenBank and stored in FASTA format. A linker repeat (EAAAK) was used to maintain protein structure and prevent Dominic interference.(23)The rseb variant was obtained after the mutations L45R, Y89A, and Y94A, respectively.(24) The Canadian Biometrics Company synthesized and optimized a fusion gene for recombinant Chimer protein, which was then cloned into the pBlueScript II SK (+) vector. Enzyme digestion confirmed the correct synthesis of the desired gene fragments. The pBlueScript II SK (+) and pNZ7021 vectors were digested with SphI and HindIII, and a purified rseb-ctxB gene was ligated to the pNZ7021 vector. This vector is used to express genes in LAB bacteria, overexpress homologous and heterogeneous genes, and perform metabolic engineering.

The recombinant expression vector pNZ7021-Sp-rSeb-ctxB was transformed into *L. plantarum* Electrocompetnent, used in this study to express extracellular rSEB-CTB in *L. plantarum*.

Making electrocompetent cells

The overnight *L. plantarum* culture was diluted in fresh MRS medium and incubated for 4 hours at 30°C. The pellets were washed twice with MgCl2, sucrose, and glycerol, and then suspended in the same solution and stored in an ice bath.

Electrification of cells

2 µL pNZ7021 vector was added to competent cells, homogenized, and transferred to pre-cooled cuvettes. Compound bacteria were poured into cuvettes and electroporated in a Gene Pulser device.(25). The bacteria were cultured on MARS agar medium, and chloramphenicol-resistant colonies were obtained. Clones were examined using PCR and primers synthesized by Sinaclone. The results confirmed the presence of a 976-bp amplified fragment in the PCR colonies.

Purification of recombinant proteins using nickel resin chromatography column

The recombinant protein sequence was purified using a nickel column (N-NTA) with a histidine sequence affinity. The process involved pouring prepared nickel-resin into an empty column, regenerating it with DBB, and adding the extracted protein sample. The column was washed with DWB denaturing Wash Buffer, NWB buffer, and imidazole buffers. The process was repeated, and the collected samples were stored at room temperature.

Western blotting analysis

The protein extract was prepared from recombinant *L. plantarum* culture medium. It was centrifuged, precipitated, and then centrifuged again. The supernatant proteins were then mixed with NaOH, PMSF, and DTT-LB.(22) Proteins from LP-pNZ7021 -Sp-rseb-ctxB and *L. plantarum* were analyzed using SDS-PAGE and Western blotting, with anti-His tag antibody detection for target protein presence.

Results

Sequence analysis and segment selection

The gene sequences for *ctxB* (372 bp) and *SEB* (798 bp), both referenced under GenBank accession number AB462486.1, were retrieved for this study. These segments encode a total of 390 amino acids—124 from CTB and 266 from SEB. To create a synthetic antigenic construct (SC), the two antigenic fragments were fused using specific linker sequences. In this design, repetitive linkers such as AEAAAKEAAAKEAAAKEAAAKA were employed to ensure proper structural flexibility and separation between domains.

Optimized gene constructs

The gene constructs were optimized for expression in *Lactobacillus plantarum 299v*, resulting in a codon adaptation index (CAI) of 0.8. Codon usage was appropriately modified, leading to a 33.5%

increase in GC content and a reduction in destabilizing motifs and repetitive sequences. Enzymatic digestion results for extraction of desired gene fragments from pBlueScript II SK vector (+)
Restriction enzyme digestion using KpnI and EcoRI was carried out on the *pBlueScript II SK (+)* plasmid by Canadian Biometrics Company to verify the accurate synthesis and insertion of the target gene fragments. (Figure 1)

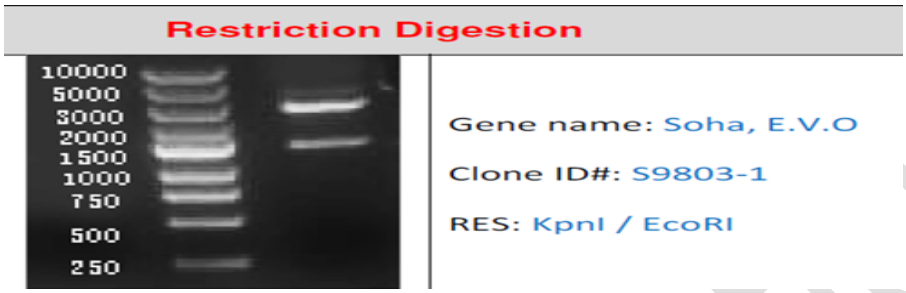

Figure 1. Enzymatic digestion results show successful synthesis of the target gene fragments, confirmed by the appearance of a 1500 bp band on agarose gel electrophoresis after restriction enzyme digestion. Presence of the recombinant expression vector in *L. plantarum* was confirmed.

The plasmid pNZ7021-*rseb-ctxB* was successfully introduced into *L. plantarum*, resulting in the recombinant strain *L.P. pNZ7021-SP-rseb-ctxB*. Transformation was confirmed through colony PCR and sequencing. Based on the target sequence, the expected distance between the forward and reverse primers was 976 bp, and a corresponding 976 bp band was observed on agarose gel electrophoresis (Figure 2).

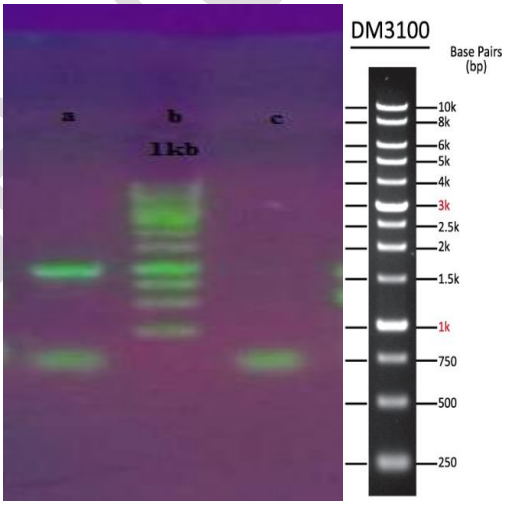

Figure 2. Confirmation of transformation by Colony PCR: Detection of a band with a molecular weight of 976 bp on agarose gel electrophoresis. From left to right a. The desired band b. Marker 1kb DM3100.c: Negative control.

Expression of rSEB-CTB in *L. plantarum*

The expression of the recombinant protein was analyzed by SDS-PAGE and subsequently confirmed via Western blot using an anti-His tag antibody. The Western blot revealed a distinct band at approximately 49 kDa, indicating successful expression of the *rSEB-CTXB* fusion gene in *L. plantarum*. (Figure 3**).**

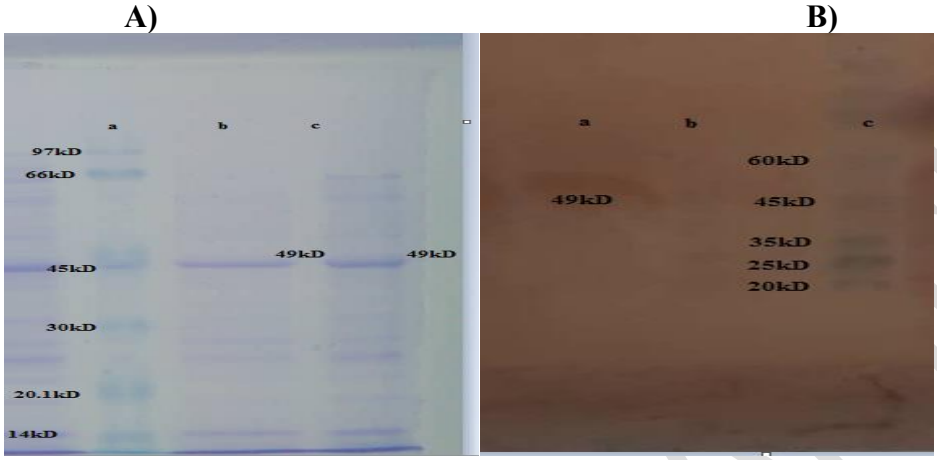

Figure 3. Shows the detection of the purified rSEB-CTB fusion protein by SDS-PAGE method and confirmation of recombinant protein by Western blotting method using an anti-His tag.
A) The rSEB-CTB fusion protein, with a molecular weight of 49 kDa, was detected after purification by a nickel column using SDS-PAGE in the supernatant of recombinant *L. plantarum*. a: Marker, b: Recombinant protein purified by elution buffer with imidazole 350 mM. B) The expression of recombinant protein purified from L.P-pNZ 7021-SP_rseb-ctxB was confirmed by Western blotting. a: Band 49 kDa, b: Negative control, c: PM1700 protein marker.

Discussion

The research aims to design an oral vaccine antigen using lactic acid bacteria as a safe carrier, focusing on chimeric proteins for increased immunogenicity and cellular and humoral immune response (26). Masoumeh Al-Rasoul et al. studied the effectiveness of CS3 antigen combined with LT toxoid, finding that chimer protein LTB and CstH produced a better immune response (27). This study explores the design of chimeric proteins, including rSEB and CTB antigen proteins, for their immunogenicity and CTB adjuvant properties, following a 2020 study using ctxB (28). This study showed that fusion antigens with CTxB offer superior stability and half-life. To preserve the structural integrity of each protein, coding sequences were joined using EAAAK-based linkers, which expose linear and structural epitopes for immune recognition (29). Khaloiee et al. used four repeats of this linker to design chimeric proteins targeting three intestinal pathogens, ensuring spatial separation of subunits and proper folding (30). Nazarian et al. used quadratic linker replication to separate chimeric protein subunits, enhancing folding and biological activity (23). Amani et al. showed that EAAAK linkers maintained distinct structures for EspA, intimin, and Tir. Due to rare codons in ctxB and seb genes, all chimeric sequences were optimized via online tools for high expression in *L. plantarum* (29).
Codon selection and optimization effectively enhanced expression efficiency, raising the chimeric CAI to 0.8—reflecting improved tRNA availability in *L. plantarum*. Lactic acid bacteria (LAB) are widely recognized as robust hosts for heterologous protein production due to their resilience

in gastrointestinal environments, ability to colonize intestinal tissues, and safe, beneficial characteristics (31, 32). LAB strains have been successfully employed to deliver bioactive molecules, including vaccines, to the gut mucosa. (33, 34) Prior studies have demonstrated recombinant lactobacilli expressing antigens such as *Streptococcus pneumoniae* PsaA and tetanus toxin fragments (35) has been reported. Notably, Guo (2020) utilized *L. plantarum* to produce and secrete rChIL17B, effectively inhibiting infectious bronchitis virus (IBV) propagation (36).
In a 2020 study by Jianzhong Wang, recombinant *L. plantarum NC8* was used to develop a safer and more effective oral rabies vaccine. This strain showed promise as a novel approach for rabies prevention in animals (37). Using safe bacteria like lactic acid bacteria (LAB) as antigen carriers offers a viable alternative due to their adjuvant properties and low immunogenicity. The concept of using live bacteria to deliver vaccine antigens dates back to the 1980s. *L. plantarum* was selected for its stronger innate immune-stimulating capacity compared to species like *L. casei* and *L. lactis*. Additionally, a 2007 study by Beatriz del Rio demonstrated that nasal immunization with the E7 antigen bound to *L. plantarum*'s cell wall triggered a robust systemic immune response (38,39).

Conclusion

This study examined the expression of the recombinant rSEB-CTB protein *using L. plantarum* as the host and pNZ7021 expression vector. The resulting LP-pNZ7021 - SP-rseb-ctxB is suitable for recombinant vaccines against *Vibrio cholera* and *Staphylococcus aureus*, and has potential immunogenicity applications.

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Ethical statement

Not applicable.

Conflicts of interest

There are no conflicts of interest, among the authors.

Author contributions

Each author contributed to the design and conceptualization of the study.

Data availability statement

The research data produced in this work is publicly accessible through PubMed and Scopus.

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