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Cloning, expression, purification and characterization of the cholera toxin B subunit and triple glutamic acid decarboxylase epitopes fusion protein in *Escherichia coli*

Zhaohui Gong [a,*], Xiang'e Long [a], Lin Pan [a], Yanping Le [a], Qiong Liu [a], Shaomin Wang [a], Junming Guo [a], Bingxiu Xiao [a], Mi Zhou [a], Disen Mei [b]

[a] School of Medicine, Ningbo University, 818 Fenghua Road, Ningbo 315211, China
[b] College of Life Science and Biotechnology, Ningbo University, 818 Fenghua Road, Ningbo 315211, China

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ABSTRACT

Induction of specific immunological unresponsiveness by oral autoantigens such as glutamic acid decarboxylase 65 (GAD65) is termed oral tolerance and may be a potential therapy for autoimmune diabetes. However, the requirement for large amounts of protein will limit clinical testing of autoantigens, which are difficult to produce. Mucosal adjuvants such as cholera toxin B subunit (CTB) may lower the level of autoantigens required. Here we describe cloning, expression, purification and identification study of the CTB and triple $GAD_{531-545}$ epitopes fusion gene. The fusion gene was ligated via a flexible hinge tetrapeptide and expressed as a soluble protein in *Escherichia coli* BL21 (DE3) driven by the T7 promoter. We purified the recombination protein from the cell lysate and obtained approximately 2.5 mg of CTB–$GAD_{(531-545)3}$ per liter of culture with greater than 90% purity by a Ni–NTA resin column. The bacteria produced this protein as the pentameric form, which retained the GM1-ganglioside binding affinity and the native antigenicity of CTB and GAD65. Further studies revealed that oral administration of bacterial CTB–$GAD_{(531-545)3}$ fusion protein showed the prominent reduction in pancreatic islet inflammation in non-obese diabetic mice. The results presented here demonstrate that the bacteria bioreactor is an ideal production system for an oral protein vaccine designed to develop immunological tolerance against autoimmune diabetes.

Oral administration of food antigens can induce a state of specific immunological unresponsiveness to that antigen. This physiological response is known as oral tolerance [1]. Oral tolerance may be a potential therapeutic strategy for preventing and treating autoimmune diseases including diabetes, which has specific postulated autoantigens such as insulin and glutamic acid decarboxylase 65 (GAD65)[1]. Indeed, in animal models, the oral administration of pancreatic tissue-specific antigens has been shown to prevent the development of spontaneous autoimmune diabetes [2–4]. It has also been demonstrated that GAD65 appears to be a critical autoantigen in oral immune tolerance. However, the application of oral tolerance is limited by the requirement for repeated administration of large amounts of autoantigen. The cholera toxin B subunit (CTB) has been used to overcome such limitations by serving as a mucosal carrier molecule for genetically conjugated autoantigens for the induction of oral tolerance [5–7].

CTB is a pentameric non-toxic portion of cholera toxin, responsible for the holotoxin binding to the GM1-ganglioside receptor present on most nucleated cells. When conjugated to autoantigens, the CTB dramatically increases their tolerogenic potential after oral administration [8–14]. This effect is probably mediated by the ability of CTB to act as a mucosal carrier system [9], although CTB might also have direct effects on the immune system [15,16]. Recent studies have showed that CTB is an effective mucosal adjuvant in potentiating immune responses or increasing immunological tolerance to corresponding antigens [13,17–21]. These investigations indicate that CTB is a powerful edible vaccine if expressed in large-scale production in an edible tissues or organism.

On the other hand, a single intranasal administration of GAD65 peptides (T cell target determinants $GAD_{509-528}$ and $GAD_{524-543}$) to 2- to 3-week-old non-obese diabetic (NOD) mice induces high levels of IgG1 antibodies to GAD65. GAD65 peptide treated mice display greatly reduced IFN-γ responses and increased IL-5 responses to GAD65, confirming the diversion of the spontaneous GAD65 Th1 response toward a Th2 phenotype. Furthermore, GAD65 peptide treatment reduces insulitis and long-term type 1 diabetes (T1D) incidence in NOD mice [22]. Another finding has shown that both

* Corresponding author. Fax: +86 574 87608638.
E-mail address: zhgong@yahoo.com (Z. Gong).

[1] *Abbreviations used:* GAD65, glutamic acid decarboxylase 65; CTB, cholera toxin B subunit; GALT, gut-associated lymphoid tissue.

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the p316–335 and p531–545 peptides of GAD65 bind weakly to I-Ag7. Some peptides with amino acid substitutions have antagonistic activity, and administration of a large amount of wild-type peptides reduces the severity of insulitis in NOD mice [23]. Moreover, Quinn et al. finds that there are at least two I-Ag7-restricted determinants present in the GAD65 sequence 524–543 (p524), each capable of recruiting unique T cell repertoires characterized by distinct T cell receptor V-β gene use. CD4 (+) T cells arise spontaneously in young NOD mice to an apparently dominant determinant found within the GAD65 peptide 530–543 (p530). The results also show i.p. injection with p524/incomplete Freund's adjuvant is very effective in providing protection from cyclophosphamide-induced T1D [24]. In total, these findings suggest that the regulatory T cells elicited by immunizing with GAD65 peptides are unique and distinct from those that arise from spontaneous endogenous priming, and the peptide vaccine therapy may be useful in autoimmune diseases, including T1D.

The above findings suggest that the CTB and GAD65 peptide can function in T1D together. However, studies to date have not demonstrated that CTB–GAD65 fusion protein has been expressed in *Escherichia coli*. Moreover, there is a lack of functional data related to the biological activity and therapeutic application of this fusion protein vaccine on T1D development. Here, we report the expression of the CTB and triple GAD65 peptide 531–545 (3p531) fusion gene in *E. coli* BL21 (DE3). Biochemical and immunological characterizations of the purified protein showed that the bacterial-derived CTB–$GAD65_{(531–545)3}$ fusion protein exhibited the biochemical and antigenic properties necessary for the purposes of this study. Furthermore, the GM1 binding ability also suggested proper folding of fusion protein molecules resulting in the functional pentameric structure. This work provides a method to produce a large amount of therapeutic CTB–$GAD65_{(531–545)3}$ fusion protein vaccine for T1D treatment.

Materials and methods

Reagents, E. coli and mice

DNA manipulation and PCR amplification kit were purchased from *TaKaRa Biomedicals* (Japan). The pET-28a expression vector and *E. coli* BL21 (DE3) were obtained from *Novagen* (USA). The rabbit anti-cholera toxin primary antibody, bacterial CTB peptides and monosialoganglioside-GM1 were provided by Sigma–Aldrich (USA). The goat polyclonal to GAD65 primary antibody was purchased from Abcam (USA). The Western blotting kit and Ni–NTA resin column were supplied by *Roche Diagnostics* (Germany). The recombinant plasmid pBlue-CTB was provided by Dr. Shengwu Ma. NOD mice were purchased from Shanghai Laboratory Animal Center (SLAC, CAS, China) and housed at the central animal facility, where they were screened for bacterial and viral pathogens.

Construction of expression vector

Prior to the construction of the CTB–$GAD65_{(531–545)3}$ fusion gene, the corresponding gene encoding triple GAD65 peptides 531–545 (3p531) was chemically synthesized and inserted into the pUC57 vector. Three copies gene of p531 were interconnected by a 6-bp oligonucleotide sequences encoding a flexible hinge bipeptide (GS). In order to introduce the 3p531 gene fragments at the 3′ end of the CTB gene, triple primer polymerase chain reaction (TP-PCR) was employed. Based on two templates pBlue-CTB and pUC57-3p531, three primers for PCR amplification were designed as follows: forward primer 5′-CG*GGATCC*ATGATTAAA TTAAAATTTGG-3′ (BamHI), anchor primer 5′-TTGCCGCAATTA GTATGGCAAATGGCCCCGGCCCCCCGGTTATCAAAGCTCGTATGAT-3′ (the underline sequence encoding a flexible hinge tetrapeptide (GPGP) was fused at the 3′ end of the CTB gene and at the 5′ end of the 3p531 gene fragments) and reverse primer 5′-GG*GAATTC*T-TAAACCATGGTAGTACCGTA-3′ (EcoRI). The reaction was carried out under the following conditions: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 45 s, annealing for 1 min at 55 °C and extension at 72 °C for 1 min. The PCR product was purified and digested with BamHI and EcoRI, and then subcloned into the expression vector pET28a previously cut with the same restriction enzymes. The identity of the insert was confirmed by sequencing, and the expression construct was designated pET–CTB–$GAD65_{(531–545)3}$.

Expression in E. coli

E. coli BL21 (DE3) were transformed with plasmid pET–CTB–$GAD65_{(531–545)3}$. A single colony was cultured overnight in LB broth containing kanamycin (50 μg/mL). The culture was diluted 1:100 with LB broth and subjected to further incubation at 37 °C for 1–6 h. The recombinant protein was induced by 0.5 mM IPTG in 1 L of culture, then the cells were collected by centrifugation (6.5 g of wet weight cell pellet), resuspended in 100 mL lysis buffer pH 8.0 (300 mM NaCl, 100 mM Tris, 1 mM PMSF) and sonicated in ice bucket 3 × 10 s. Cellular lysates were centrifuged at 26,000*g* for 15 min at 4 °C. Inclusion bodies were washed twofold with 15 mL of binding buffer (50 mM Tris and 500 mM NaCl) containing 10 mM β-mercaptoethanol and 2 M urea. Then, the inclusion bodies were dissolved in 10 mL binding buffer containing 10 mM β-mercaptoethanol and 8 M urea. The solubilized pellet was slowly diluted in 2 L of binding buffer containing 5 mM imidazole for recombinant protein refolding and incubated for 24 h at room temperature.

Purification of recombinant protein

The purification of recombinant protein was performed as described by Arêas et al. [25]. In brief, after the adsorption of the protein, the Ni–NTA resin was washed with 10 volumes of binding buffer containing 60 mM imidazole. Recombinant CTB–$GAD65_{(531–545)3}$ was eluted with five volumes of the same solution containing 500 mM imidazole. Then the recombinant protein was dialyzed in two steps. First, the equilibrium was established using 2 L solution containing 2 mM Tris, 20 mM NaCl, 0.1% glycine and 10 mM EDTA, to eliminate imidazole and $Ni^{2+}$from the solution. In the second step, the same buffer was used, without EDTA. Besides, an aliquot of purified CTB–$GAD65_{(531–545)3}$ was subject to an additional purification using Centricon YM-30 (Millipore, USA), according to the procedure described by the manufacturer, to separate the monomeric and active pentameric forms. In brief, 3.5 mL of the sample was centrifuged for 15 min at 3000*g*. The washes were performed with PBS for 10 times. All centrifugations were done for 15 min at 3000*g*. The 6× His-tagged recombinant CTB–$GAD65_{(531–545)3}$ was collected and analyzed by 12% SDS–PAGE according to our previous work [7]. The purity of recombinant protein was determined with a thin-layer chromatography scanning machine (Shimadzu, Japan) according to the manufacturer's instructions.

Western blot analysis

For detection of the presence of recombinant protein, the cell-lysed supernatant and the purified samples were electrophoresed in a 12% SDS–PAGE for 45–60 min at 20 mA in Tris–glycine buffer (25 mM Tris, 250 mM glycine, pH 8.3, 0.1% SDS). Prior to electrophoresis, samples were boiled for 5 min and then loaded directly on the gel. The separated proteins were transferred from the gel to Hybond-P membrane (GE, USA) by electroblotting on a wet blot-