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Prokaryotic Expression and Purification of Heat-labile Toxin B Subunit Gene and EtpA Adhesion Gene from Enterotoxigenic *Escherichia coli*

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Enterotoxigenic *Escherichia coli* (ETEC) is an important pathogen causing diarrheal disease in humans and animals in developing countries. EtpA adhesin and heat-labile enterotoxin B subunit (LT B) are the main virulence factors of ETEC. The purpose of this study is to construct a prokaryotic expression vector for EtpA and LT B and to purify these two proteins. The cloning of the EtpA and LT B genes from ETEC using polymerase chain reaction (PCR) was performed. The fragments were then identified and cloned into the prokaryotic expression vector pET-32a. The recombinant plasmids were transformed into *E. coli* BL21(DE3) and induced to express with IPTG. The products of expression were analyzed by SDS-PAGE and western-blot. We obtained the optimum expression conditions of pET-EtpA and pET-LT B. The maximal expression quantity of EtpA was observed at 5 h of induction by 1 mM IPTG at 37 °C, and, whereas that of LT B was observed at 6 h of induction by 1 mM IPTG at 37 °C. The expression of EtpA and LT B provides the material basis for the further development of novel ETEC vaccine.

Key words: Enterotoxigenic *Escherichia coli* (ETEC); EtpA; LT B; Prokaryotic Expression; Purification.

Enterotoxigenic *Escherichia coli* (ETEC) is an important pathogen causing diarrheal disease in humans and animals in developing countries. ETEC leads to great financial loss in stock farming[1]. The major pathogenic factor of ETEC includes its adhesion and enterotoxin. Adhesion colonizes in the intestine, which is the prerequisite of diseases caused by bacterial infection.

The recently identified EtpA is a high-molecular-weight glycosylated extracellular protein belonging to the two-partner secretion family[2]. This protein has significantly immunogenicity[3]. Vaccination with EtpA can induce significant protection against intestinal colonization[4-5]. The secreted enterotoxin of ETEC is an external toxin in periplasmic bacteria. Enterotoxins can be divided into two groups, namely, heat-labile (LT) and heat-stable (ST) toxins. LT toxins are composed of an A subunit (LTA) and five B subunits (LT B). A subunit has normal GM1 ganglioside and glycoprotein receptors on cell membrane-binding activity[1]. B subunits are LT receptor-binding sites that are significantly immunogenic. The channel formed through B subunits specifically combines with GM1 ganglioside on the mammalian cell membrane. This channel can pass through A subunits to the target cell[6-7]. LT effectively results in the humoral immunity response and cellular immunologic response in part or in whole[8–10]. Thus, LT can

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effectively enhance the immunogenicity of an antigen because it is an efficient immunogen and mucosal adjuvant[11-12]. However, the application of LT is restricted because of its strong diarrheal effects[13]. Similar to LT, LT B is an immunogen and mucosal immune-adjuvant. Thus, LT B is an important adjuvant in the development of mucosal vaccines[14].

ETEC-caused diseases are generally treated using drugs and vaccines. However, the treatment of bacteria has become a problem because of the abuse of antibiotic and drug residues in recent years. Vaccine inoculation continues to be the optimum method of disease control. ETEC strains were discovered more than 40 years ago, but the development of ETEC vaccines has been hampered by the heterogeneity of known molecular targets. Existing vaccine strategies cannot provide broad-based sustained protection[15-16]. In this paper, wedescribed the successful expression and purification of EtpA and LT B proteins. SDS-PAGE analysis and western blot showed that the EtpA and LT B proteins demonstrated specific immunological activity against *E. coli* antiserum. This research provides a foundation on DNA vaccines and subunit vaccines for the development of an ETEC vaccine.

MATERIALS AND METHODS

Construction of plasmid pET-EtpA and pET-LT B

The EtpA DNA sequence was amplified by polymerase chain reaction (PCR) using a plasmid template (pJY 019, purchased from China Scientists Ordering Instruction). The specific primers of EtpA were designed based on the sequence of the plasmid template pJY 019 (GenBank acession number:AY920525). The following oligonucleotide primers were used for PCR amplification. The forward primer for EtpA amplification contained a *Bam*HI restriction site positioned before the initiator codon at the 5'-end of the coding sequence, whereas the reverse primer contained an *Xho*I restriction site at the 3'-end after the stop codon. PCR was performed with primers: EtpA-F,and EtpA-R (Table. 1).The gene fragment corresponding to the LT B subunit was amplified by PCR independently. Similar to EtpA, the primer of the LT B subunit used in PCR was ligated through the *BamH*I and *Xho*I sites. PCR was performed with primers: LT B-F, and LT B-R (Table 1).

After the PCR reaction, the PCR products of EtpA and LT B were respectively subcloned into a pEASY-Blunt cloning vector (Transgen, Beijing, China) and subsequently subjected to automated sequencing reaction (Invitrogen, Beijing, China). The positive recombinant plasmid pEB-EtpA and pEB-LT B were respectively purified using a TIANprep mini plasmid kit (TIANGEN, beijing, China). The verified plasmid and prokaryotic expression vector pET32a were digested via *BamH*I and *Xho*I restriction enzyme. DNA fragments of EtpA (25 ng) or LT B (25ng) and linearized pET32a were incubated at 22 °C overnight with T4 DNA ligase (Invitrogen, Carlsbad, CA, USA) in a total volume of 10 µL according to the manufacturer's instructions. The positive recombinant plasmid pET-EtpA and pET-LT B were respectively purified using a TIANprep mini plasmid kit (TIANGEN, beijing, China). The purified plasmid was confirmed by double-restriction enzyme digestion, and plasmids of prokaryotic expression EtpA or LT B were sequenced to confirm that the right sequences had been inserted.

Protein expression and purification

After sequence verification, the final constructs were used to chemically transform competent *E. coli* expression strain BL21. We optimized several variables to acquire the optimal conditions, such as expression time, concentration of isopropyl beta-a-1-thiogalactopyranoside (IPTG), and expression temperature. LB media culture containing ampicillin with a final concentration of 100 µg/mL was inoculated with a single verified colony of BL21 cells containing the EtpA or LT B expression plasmid at 37 °C overnight with shaking. These cultures were used to inoculate 400 mL of LB media culture containing a certain antibiotic concentration used for small cultures. The cultures were then incubated at 37 °C with shaking. When OD_{600} reached 0.5 to 0.7, the culture was added with IPTG. To determine the optimum IPTG concentration, expression temperature, and expression time, we divided the cultures of OD_{600}=0.5 to 0.7 into three groups. The first group comprised seven samples. A sample was obtained every hour for 7 h at 37 °C with agitation, and the final concentration of IPTG was 1 mM. The second group comprised five samples. At 1 mM IPTG, the cultures of OD_{600}=0.5 to 0.7 were incubated at

different temperatures (28 °C, 30 °C, 32 °C, 34 °C, and 37 °C) for 7 h. The third group comprised six samples. The cultures of OD_{600}=0.5 to 0.7 were cultured at 37 °C for 7 h with different IPTG concentrations (i.e., 0.1, 0.2, 0.4, 0.6, 0.8, and 1 mM). The results were analyzed by SDS-PAGE.

Under the optimal conditions, we extensively incubated BL21 strains containing the EtpA or LT B expression plasmid. The slurry was stored at –20 °C until purification. The extensive culture was centrifuged at 8,000 rpm for 10 min at 4 °C, and the supernatant was discarded. The precipitate was resuspended completely in 10 mL of PBS (0.5 M NaCl, 100 mM KCl, 50 mM Na_2HPO_4, 50 mM KH_2PO_4, pH 7.4). The cell pellets were lysed by ultrasonication on ice using 20 cycles of 30 s on and 30 s off.

We purified protein using the pre-equilibrated nickel agarose beads in a 6xHis-tagged protein purification kit (Kangwei, Beijing, China). The cell pellets were lysed completely in 10 mL of PBS (0.5 M NaCl, 100 mM KCl, 50 mM Na_2HPO_4, 50 mM KH_2PO_4, pH 7.4), and centrifuged at 1000 rpm for 15 min at 4 °C. The supernatants and sediments were stored separately at different containers. The precipitates were resuspended in 5 mL of binding buffer (20 mM Tris-HCl, 5 mM imidazole, 0.5 M NaCl, and 8 mM carbamide), and centrifuged at 1000 rpm for 20 min at 4 °C in a cold room. Finally, the supernatant containing protein was recovered. This liquid was filled in 5 mL polypropylene columns and dried by gravity. The binding buffer with 15 bed volumes was washed with the agarose beads. The binding protein with agarose beads in the column was eluted by elution buffer (20 mM Tris-HCl, 500 mM imidazole, 0.5 M NaCl, and 8 mM carbamide). Purification was conducted at 4 °C. The eluted fractions were analyzed by 15% SDS-PAGE. Moreover, the majority of pure EtpA or LT B proteins were found in the right location. The pure EtpA or LT B protein was preserved at –80 °C until further use.

Western blots for identifying EtpA and LT B protein

Immunoblotting was performed according to standard protocols. Polyclonal anti-LT B and EtpA antibodies were respectively generated by immunizing a rabbit with overall 0.7 mg of SDS-PAGE-purified LT B or EtpA protein.

RESULTS

Construction of the plasmid pET-EtpA and pET-LT B

According to the sequence of the plasmid template pJY 019 (GenBank number: AY920525), PCR was used to amplify the EtpA gene, which was 2751 bp in length (Fig. 1). The gene fragment of the LT B subunit was amplified by PCR, and the length of the PCR product was 375 bp (Fig. 1). The EtpA and LT B genes were separately constructed in the pEASY-Blunt cloning vector. These recombinant plasmids were named as pEB-EtpA and pEB-LT B. The recombinant plasmid of pEB-EtpA and pEB-LT B were confirmed by double-restriction enzyme digestion, and observed in the expected location (Fig. 2A). The EtpA and LT B gene fragments verified from pEB-EtpA and pEB-LT B were linked to the prokaryotic expression vector pET32a, and formed the recombinant prokaryotic expression plasmid pET-EtpA. and pET-LT B. The recombinant plasmid of pET-EtpA and pET-LT B was confirmed by double-restriction enzyme digestion, and observed in the expected location (Fig. 2B).

Optimized Condition of Expression

To obtain an optimal level of protein expression, several expression conditions were

Table 1. Primers used for this study

Primer[a]	*Sequence (5'-3')*[b]	USE
EtpA-F	CGCGGATCCATGAACCGTATATATAAACTGAAG(*Bam*HI)	EtpA amplification
EtpA-R	CCGCTCGAGCTATTGCCAGTACACCTCAC (*Xho*I)	
LT B-F	CGCGGATCCATGAATAAAGTAAAATGTTA (*Bam*HI)	LT B amplification
LT B-R	CCGCTCGAGCTAGTTTTTCATACTGATTG (*Xho*I)	

a F denotes a forward PCR primer; R denotes reverse transcription or a reverse PCR primer.
b Restriction sites introduced by PCR are underlined and specified in parentheses at the end of the sequence.

tested. The results by SDS-PAGE showed that the maximal expression quantity of EtpA protein was obtained at 5 h (Fig. 3A), and that of LT B protein at 6 h (Fig. 4A). Second, the optimal expression temperature was tested. However, no clear difference was observed in all five expression temperatures tested, indicating that the temperature range did not affect protein expression (Fig. 3B and 4B). Third, different IPTG concentrations were tested. The maximal quantity of EtpA protein expression was observed at 1.0 mM IPTG (Fig. 3C), whereas that of LT B protein was observed at 0.8 mM IPTG (Fig. 4C). The quantity of protein expression of EtpA and LT B in different IPTG conditions was poor compared with those in different expression times. Therefore, the expression time produced the greatest effect on protein expression among all the conditions.

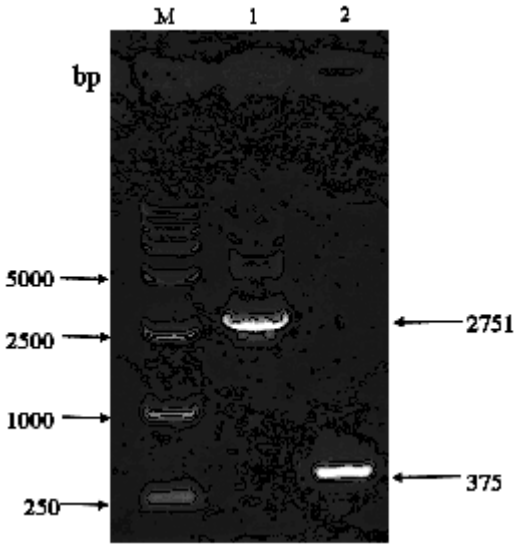

Fig. 1. PCR products were analyzed by agarose gel electrophoresis. Lane 1: The EtpA gene; lane 2: LT B gene. M: 15000bp DNA Marker.

Protein Purification

The proteins were purified using a 6xHis-tagged Protein Purification Kit. The EtpA protein and LT B protein were further analyzed by SDS-PAGE (Fig. 5A and 5B). The expressed fusion protein existed in the form of inclusion bodies, which could avoid protein degradation caused by bacterial protease in favor of purification

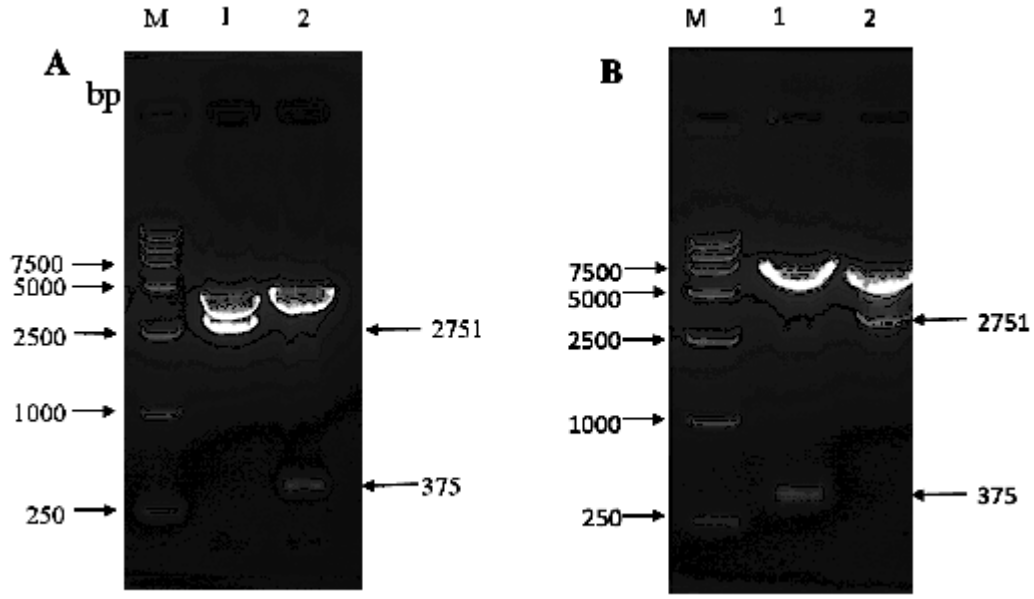

Fig. 2. Double-restriction enzyme digestion products were analyzed by agarose gel electrophoresis. The recombinant cloning plasmid pEB-EtpA and pEB-LT B are digested via the *BamH*I and *Xho*I restriction enzyme recognition sites at 37 °C for 3 h (A). The front stripe is the cloning vector stripe (middle or right). The final stripe is the expected EtpA gene (middle). The final stripe is the LT B gene corresponding to the projected size (right). Using the same measure, we identified the recombinant prokaryotic expression plasmids pEB-EtpA and pEB-LT B (B). The front stripe is the prokaryotic expression vector stripe (middle or right).

DISCUSSION

Although ETEC strains were discovered more than 40 years ago[17], they still cause diarrheal diseases in humans and animals in developing countries. Colonization factors and LT, a relatively small number of antigens, are important targets for ETEC vaccine development[18]. However, the development of an ETEC vaccine with broad-based sustained protection has been hindered by numerous factors[16], such as a wide range of drug resistance and the lack of anti-LT immunity complete sustained protection. The technological advances in genomics[19] and proteomics[20] can contribute to the successful construction of a broadly protective vaccine of ETEC that will include multiple antigens for broad representation.

The recently identified EtpA, which is relatively conserved in the ETEC pathovar, is a high-molecular-weight glycosylated extracelluar protein belonging to the two-partner secretion family[21]. EtpA appears to be significantly immunogenic[22], and geographically dispersed and conserved among these known pathogens[23].

EtpA may be important for vaccine development. In China, in-depth studies about the mechanism or application of LT B should be conducted. Previous studies showed that LT-mediated activation of cAMP is strictly required in flagellar motility and the intimate interaction of ETEC with host cells [24]. The B subunits of LT are LT receptor-binding sites and confer LT with

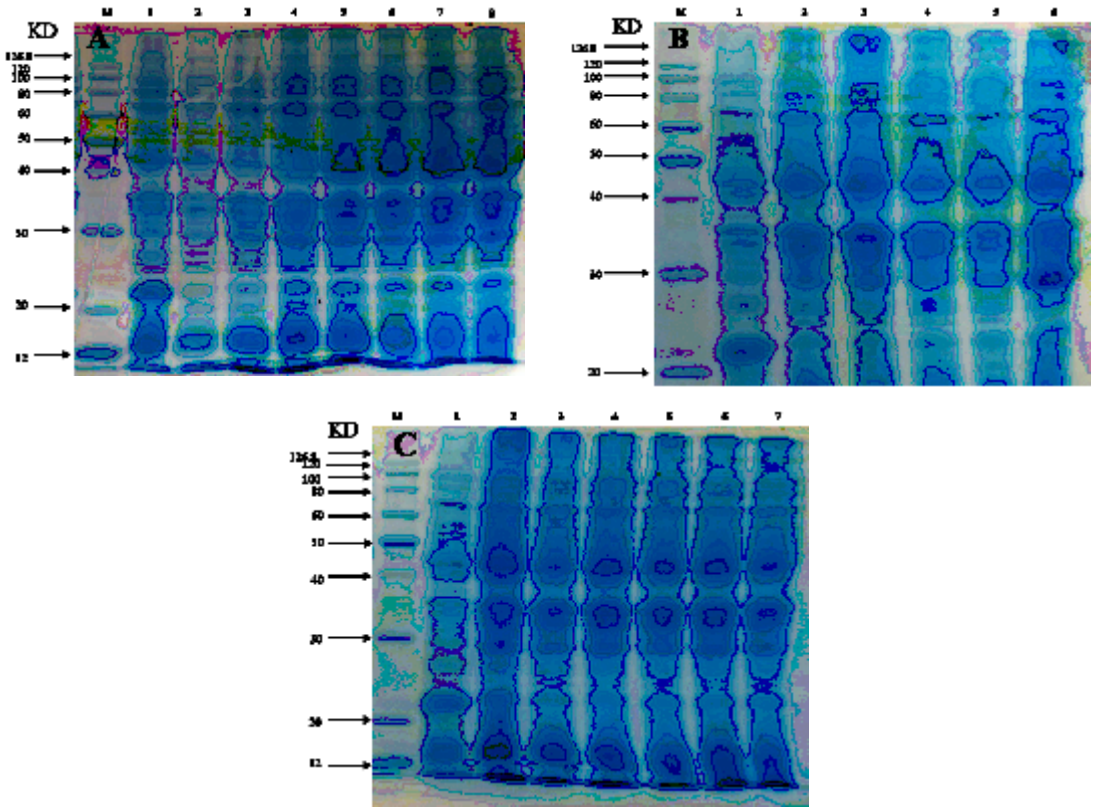

Fig. 3 SDS-PAGE (15%) analysis of EtpA at different conditions. Different expression times were tested (A), and the maximal expression quantity of the EtpA protein was observed at 5 h. M, protein standard; Lane 1 (A, B, and C) is in the control group of the vector. Lane 2 is not induced in the control group of the recombination expression plasmid. Lanes 3 to 8 are the induced products from 1 h to 6 h. The results were not observed upon production in all five expression temperatures tested (B). Lanes 2 to 6 show the different expression temperatures: 28 °C, 30 °C, 32 °C, 34 °C, and 37 °C. The maximal quantity of EtpA protein expression was obtained at an IPTG concentration of 1.0 mM. This conclusion was verified by SDS-PAGE (C). Lanes 2 to 7 are the different IPTG concentrations, namely, 0.1, 0.2, 0.4, 0.6, 0.8, and 1 mM

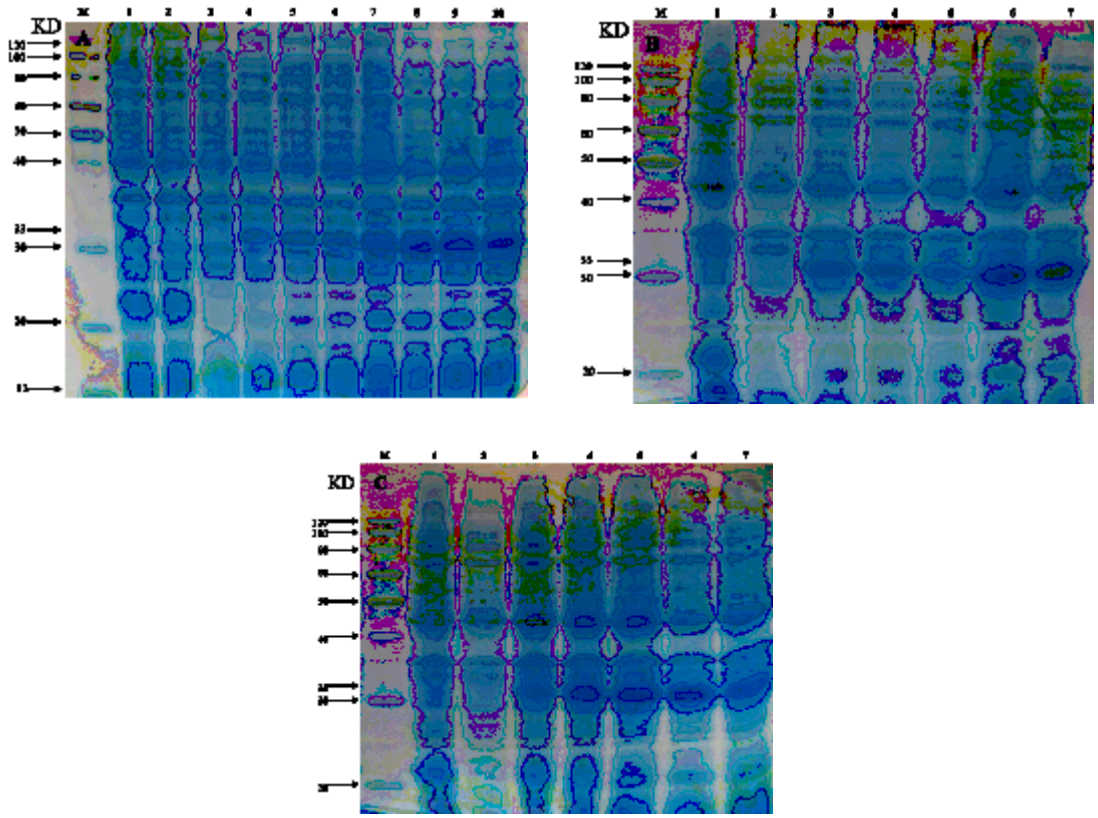

Fig.4 EtpA protein were analyzed by SDS-PAGE (15%) at different conditions. Different expression time of LT B protein was selected in experiment (A), and maximal expression quantity of LT B protein is 6 hours. The results is not observed upon production of LT B protein in all five expression temperature tested (B). LT B protein expression is maximal quantity in the IPTG concentration of 1.0mM. This conclusion is identified by SDS-PAGE (C). M, protein standard; Lane 1 (A, B, C) is in the control group of vector; Lane 2 (A) is induced expression vertor in the control group; Lane 3 (A) is not induced in the control group of recombination expression plasmid; Lane 4-10 (A) is the induced products from 1hour to 7hours. Lane 2 (B) is not induced in the control group of recombination expression plasmid. Lane 3-7 is the different expression temperatures of 28°C, 30°C, 32°C, 34°C, 37°C. Lane 2-7 (C) is select the different IPTG concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, and 1 mM.

unique traits, such as efficient immunogenicity and mucosal immune-adjuvant activity. Bacterial adhesion that targets the epithelium is not only the basis of colonization in the intestine but is also a crucial prerequisite for toxin delivery by ETEC. EtpA may have an essential function in the delivery of LT, which is similar to its function as a molecular bridge between ETEC flagella and host cell for adhesion[5]. Thus, LT B-adjuvant EtpA should be recognized as the candidate factor for the study of ETEC vaccines.

In this study, the EtpA and LT B genes were amplified by PCR. Subsequently, the recombinant prokaryotic expression plasmids pET-EtpA and pET-LT B were successfully constructed. The maximal expression quantity of EtpA protein was obtained at 5 h, 37 °C, and 1 mM IPTG. The maximal expression quantity of LT B protein was obtained at 6 h, 37 °C and 1 mM IPTG. The EtpA and LT B protein existing in the form of inclusion bodies were expressed and purified. This study provides a foundation for the development of ETEC vaccine.

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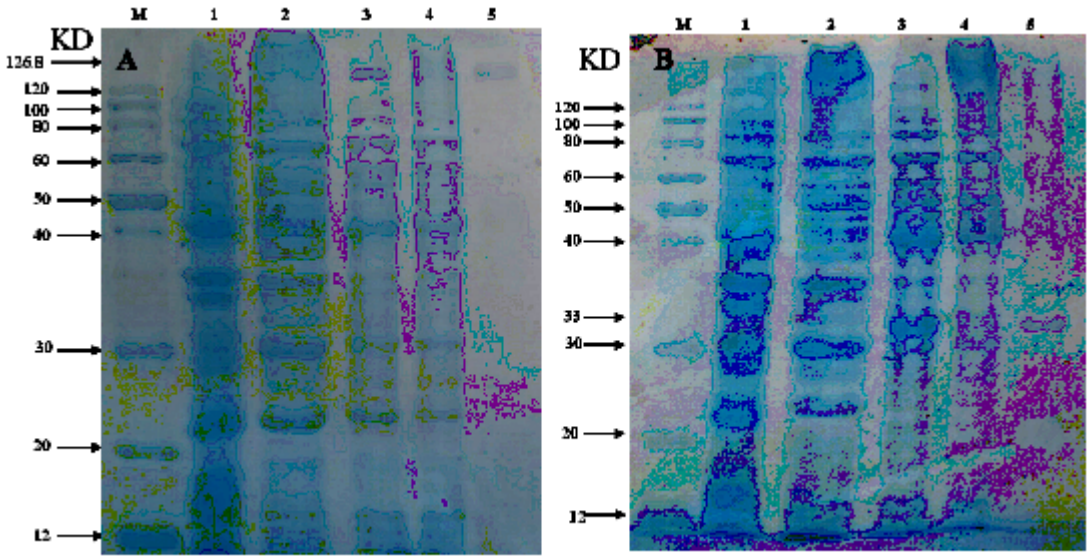

Fig. 5. Purified EtpA and LT B protein were analyzed by SDS-PAGE (15%). M, Protein standard; Lane 1 (A, B) is the control group of expression vector. Lane 2 (A, B) is the supernatant from centrifuged slurry mixture ultrasonic. Lane 3 (A, B) is the sediment from centrifuged slurry mixture ultrasonic. Lane 4 (A, B) is effluent liquid from Binding buffer containing purpose fractions through nickel agarose beads colum. Lane 5 (A, B) is represent fractions from nickel affinity washed by elution buffer

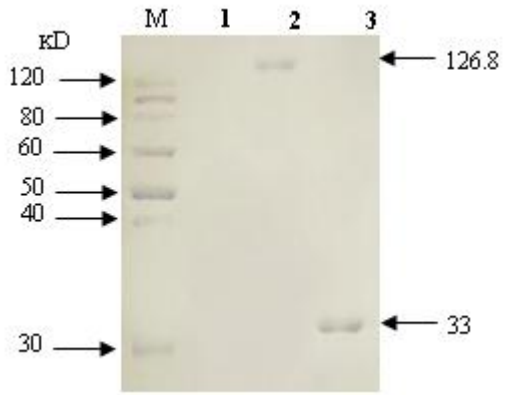

Fig. 6. Purified EtpA and LT B protein were analyzed by Western blots. M, Protein standard; Lane 1, Vector control; Lane 2, EtpA proteinÿLane 3, LT B protein

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