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Comparative Study on Characterization of Recombinant B Subunit of *E. coli* Heat-Labile Enterotoxin (rLTB) Prepared from *E. coli* and *P. pastoris*

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***Escherichia coli* (*E. coli*) heat-labile enterotoxin B subunit (LTB) was regarded as one of the most powerful mucosal immunoadjuvants eliciting strong immunoresponse to co-administered antigens. In the research, the high-level secretory expression of functional LTB was achieved in *P. pastoris* through high-density fermentation in a 5-l fermentor. Meanwhile, the protein was expressed in *E. coli* by the way of inclusion body, although the gene was cloned from *E. coli*. Some positive yeast and *E. coli* transformants were obtained respectively by a series of screenings and identifications. Fusion proteins LTB-6× His could be secreted into the supernatant of the medium after the recombinant *P. pastoris* was induced by 0.5% (v/v) methanol at 30°C, whereas *E. coli* transformants expressed target protein in inclusion body after being induced by 1 mM IPTG at 37°C. The expression level increased dramatically to 250–300 mg/l supernatant of fermentation in the former and 80–100 mg/l in the latter. The LTB-6× His were purified to 95% purity by affinity chromatography and characterized by SDS–PAGE and Western blot. Adjuvant activity of target protein was analyzed by binding ability with GM1 gangliosides. The MW of LTB-6× His expressed in *P. pastoris* was greater than that in *E. coli*, which was equal to the expected 11 kDa, possibly resulted from glycosylation by *P. pastoris* that would enhance the immunogenicity of co-administered antigens. These data demonstrated that *P. pastoris* producing heterologous LTB has significant advantages in higher expression level and in adjuvant activity compared with the homologous *E. coli* system.**

Keywords: rLTB, characterization, expression and purification, *P. patoris*, *E. coli*

To date, most vaccines have been given parenterally. The development of vaccines delivered at mucosal surfaces could provide a safe method for inducing modulated systemic immune responses without injection-related hazards [16, 28]. *E. coli* heat-labile enterotoxin B subunit (LTB) has been regarded as one of the most powerful mucosal immunogen and mucosal adjuvant, and elicits a strong immunoresponse to co-administered antigens [5, 17, 24]. The non-toxic LTB has been found to be a more potent adjuvant than CTB [4, 13]. However, LTB preparation has been very difficult because of its structural complexity, heat-lability, inclusion forms, and stability of rLTB [1, 11]. Therefore, recently much more emphasis has been put on the preparation of LTB.

Although there have been a few reports that native or large-scale recombinant rLTB produced by *E. coli* have mucosal immunoadjuvant activity [5, 6], the lower soluble yields and bioactivity of rLTB expressed in *E. coli* are not satisfactory [24, 28]. The methylotrophic yeast *P. pastoris*, used as a host for industrial production of recombinant proteins [2, 25], has advantages such as high expression of heterologous proteins [22], efficient secretion of recombinant proteins into a low-protein medium [26], and high growth density in fermenter cultures, which are very useful for biotechnological purposes [20]. *P. patoris* harboring an expression vector can efficiently secrete heterologous protein in the correctly folded soluble and biologically active form [21]. In previous studies, the recombinant cholera toxin B subunit (rCTB) secreted by *Bacillus brevis* carrying pNU212-CTB has been reported to exhibit excellent adjuvant activity when intranasally co-administered with bovine serum albumin (BSA), tetanus toxoid (TT), and diphtheria toxoid (DT) in mice [10, 12]. There are previous reports concerning genes coding for bacterial proteins such as enzyme and endotoxin from *E. coli*, expressed with high efficiency in the yeast system [7, 14]. This showed it was possible to transcript, translate, and secrete the heterologous protein LTB from the bacterium in yeast.

Taking the above reasons into account, we have tried to construct an efficient expression–secretion system of rLTB and investigate the difference in expression of rLTB

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Table 1. Primers used in this study.

Primers	5'–3' primer sequences (RE sites introduced)	Description of utility (F/R)[a]	F or R
P_{pic-1}	5'-gacctcgagaaaagagctccccagactattacagaa-3' XhoI	Cloning of LTB into pPICZαA	F
P_{pic-2}	5'-gcggccgcctagttttccatactgattgccgc-3' NotI	Cloning of LTB into pPICZαA	R
P_{AOX1}	5'-gactggttccaattgacaagc-3'	Verification of yeast transformants	F
P_{AOX2}	5'-gcaaatggcattctgacatcc-3'	Verification of yeast transformants	R
P_{pet-1}	5'-ctgcatatgaataaagtaaaatgttatg -3' NdeI	Cloning of LTB into pET32a (+)	F
P_{pet-2}	5'-gtcctcgaggttttccatactgattgccgc-3' XhoI	Cloning of LTB into pET32a (+)	R

F, forward primer; R, reverse primer; Restriction enzyme (RE) sites are underlined.

between yeast and *Escherichia coli*. In this study, we focused on the efficient production of rLTB by methylotrophic yeast *P. pastoris* X-33 bearing the pPICZαA-LTB vector. A simple purifcation method, identification, and GM1-binding properties of rLTB expressed in yeast and *E. coli* were also investigated.

MATERIALS AND METHODS

Agents and Host Strains

Standard CTB was purchased from Sigma. IPTG, Zeocin, and other chemicals used in this study were of analytical or higher grade. The goat anti-CTB polyclonal antibody (primary antibody) and rabbit anti-goat HRP–IgGFc conjugate (second antibody) were purchased from Santa Cruz Biotechnology. Restriction enzymes, T4 DNA ligase, RNase, and Pyrobest DNA polymerase were purchased from Dalian Takara Biotech. The LTB gene was cloned, by the method described in a previous study [28], from human pathogenic *E. coli* O6:H16 (LT^+, ST^-) strain that was kindly provided by Prof. Zhijiang Zhou (University of Tianjin, China). *E. coli* strain DH5α, Top 10F' *E. coli* strain BL21 (DE3), *P. pastoris* strain X-33, and vector pPICZaA were from Invitrogen. *E. coli* and yeast were cultivated in accordance with the manufacturer's instructions.

Construction of Eukaryotic and Prokaryotic Expression Vectors

The 309-bp fragment without ss-LTB was amplified from pGEM-T-LTB plasmid constructed in a previous study by high-fidelity PCR using P_{pic-1} and P_{pic-2} primers (Table 1). After being digested with restriction enzymes, the fragment of interest without stop codon was ligated into the *Xho*I/*Not*I-digested pPICZαA in frame to the α-factor secretion signal, downstream of the alcohol oxidase I promoter, or the *Nde*I/*Xho*I-digested pET32a (+) in frame between downstream of the T7 promoter and upstream of the 6× his tag. The constructed plasmid pET32a (+)-LTB (Table 2) and the constructed plasmid pPICZαA-LTB (Fig. 1A) were multiplied in *E. coli* TOP10F and DH5α, respectively. The constructed plasmid pET32a (+)-LTB (Fig. 1B) that carried the desired foreign gene in the right read frame was transformed into the host *E. coli* BL21 (DE3).

Transformation and PCR Screening of Transformants of *P. pastoris*

The *P. pastoris* X-33 strains were transformed with *Sac*I-linearized pPICZaA-LTB by electroporation using a Bio-Rad Gene Pulser according to the manual of the *Pichia* Expression Kit v.3.0 (Invitrogen) and grown for 2 days at 30°C on YPDS plates containing 0.1 mg/ml Zeocin. *P. pastoris* strain X-33/pPICZaA (X-33 transformed with pPICZaA) was used as a control. Zeocin-resistant colonies were plated on YPDS containing 0.1 mg/ml Zeocin. To isolate multicopy recombinants *in vivo*, a quick and direct way was to replate the putative transformants above on YPDS containing 0.5, 1, and 2 mg/ml Zeocin.

A single colony was picked and resuspended in 25 μl of TE and incubated for 3 min in boiling water, and the supernatant after centrifugation was used as a template for identification. The PCR was performed in a 25-μl PCR reaction mixture with P_{AOX1} and P_{AOX2} primers (Table 1) to identify the AOX1 gene, and with P_{pic1} and P_{pic2} primers to identify the specific LTB gene.

Expression of the rLTB in *P. pastoris* and *E. coli*

Five yeast transformants resistant to 2 mg/ml Zeocin (clones A1–5 from strain X-33) were selected for small-scale expression. These *P. pastoris* X-33 selected clones were inoculated into 5 ml BMGY medium and grown at 28°C with shaking until the OD_{600} of the culture reached 2–6. Subsequently, the yeast pellets were resuspended in 25 ml of BMMY medium with 0.5% methanol for induction and were cultured at 28°C for a total of 120 h with shaking. Methanol was added to a final concentration of 0.5% every 24 h to maintain induction. Protein expression in 50-ml supernatants was analyzed by 15% SDS–PAGE and Western blotting.

E. coli BL21 (DE3) harboring pET32a (+)-LTB was grown in 5 ml of LB medium containing 100 μg/l ampicillin at 37°C overnight. Each culture was then diluted 1:50 into 5 ml of 2× YTG (1.6% tryptone, 1% yeast extract, 0.4% glucose) medium supplemented with a final concentration of 100 μg/l ampicillin and cultivated and induced with 1 mM IPTG when OD_{600}=0.8 at 37°C. The cells were

Table 2. Plasmids constructed in this study.

Construct name	Transfer vector	Promoter	Signal sequence	C-Terminal fusion protein	Name of expressed protein
pET32a (+)-LTB	pGEM-T	T7	ss-LTB	-6His	rLTB -6His (I)*
pPICZαA-LTB	pGEM-T	AOX1	α-factor	-6His	rLTB-6His (II)*

ss-LTB, LTB with signal sequence; α-factor, secretion signal sequence from *S. cerevisiae* α-factor prepro peptide; * Proteins purified in this study.

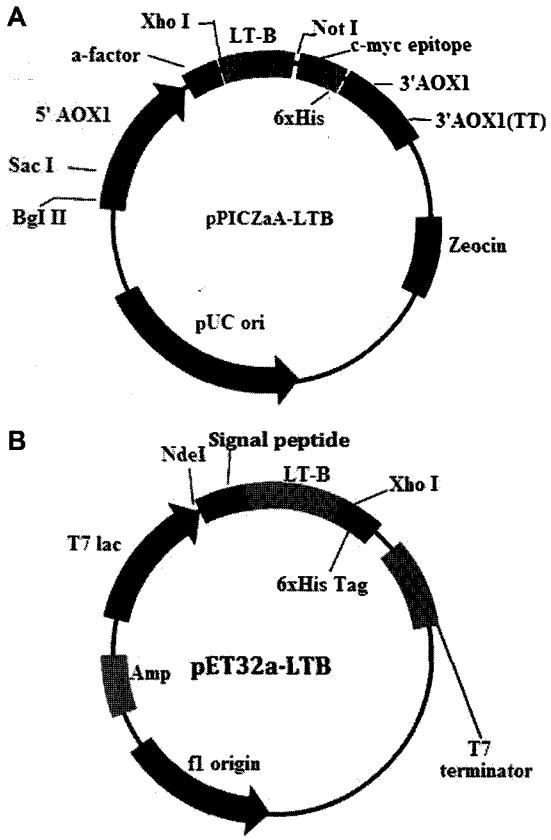

Fig. 1. Construction of eukaryotic and prokaryotic expression vectors.
A. Schematic diagram of the plasmid pPICZαA-LTB for expression in yeast. **B.** Schematic diagram of the plasmid pET32a (+)-LTB for expression in *E. coli*.

harvested after 3 h of induction and the total cell lysate was analyzed by 15% SDS–PAGE and Western blotting.

High-Density Fermentation of Engineering Strain and Production of rLTB

For high cell-density fermentation, recombinant *P. pastoris* X-33 was cultured in a 500-ml shaking flask containing 100 ml of BMGY at 30°C until an OD_{600} value reached 20, and then 100 ml of seed culture was transferred to and cultured in a 5.0-l fermentor (Gouqiang, China) containing 3.5 l of BMGY. During fermentation, the temperature and pH were maintained at 30°C and 6.0, respectively. The stirring speed was set to 600 rpm to maintain airflow at approximately 2–3 vvm. The cells were then collected by centrifugation and induced in 4 l of fresh BMMY containing 0.5% (v/v) methanol when the glycerol in the medium had been completely exhausted, as indicated by a sudden increase in the level of dissolved oxygen. During the induction period, methanol feeding (100% methanol) was controlled and maintained at 0.5% concentration. Samples were taken periodically throughout this phase for rLTB and protein analyses.

The seed *E. coli* BL21 (DE3) harboring the vector pET32a-LTB was cultured in 2× YT at 37°C. Fermentation was carried out at 37°C and pH 7.5 in a 5.0-l fermentor (Guoqian, China). Recombinant protein expression was induced with 0.5 mM IPTG for 5 h at OD_{600}=1.0. The cell pellets were harvested by centrifugation and resuspended with the sonication buffer (20 mM phosphate buffer, 0.5 M NaCl, 20 mM glycerol, pH 8.0) of 1/10 culture volume. Soluble protein in the supernatant after sonication for 1 h at 4°C was recovered by centrifugation (15,000 ×*g*, 15 min), and analyzed or purified further.

Purification of rLT-B Expressed from *E. coli* and *P. pastoris*

The supernatant from *E. coli* was loaded to a Ni-NTA agarose column. Then, the column was washed with buffer A (20 mM Tris-HCl, 0.5 M NaCl, 20 mM glycerol, pH 7.9) to remove the unbound proteins. A linear gradient with buffer A and buffer B (20 mM Tris-HCl, 0.5 M NaCl, 20 mM glycerol, 1 M imidazole, pH 7.9) was performed from 5% to 60% B at 4 ml/min for 30 min. Every 4-ml eluate was collected and analyzed by 15% SDS–PAGE. The fusion protein rLTB-6His (I) was desalted with a desalting column (Amersham Pharmacia Biotech, Sweden) in buffer C (20 mM Tris-HCl, 20 mM NaCl, pH 7.2) at 10 ml/min as recommended.

The supernanant from X-33/pPICZaA-LTB medium was concentrated and loaded onto a Ni-NTA agarose column (Qiagen) that was pre-equilibrated with 50 mM sodium phosphate buffer (PB), pH 8.0, containing 300 mM NaCl and 5 mM imidazole. The column was washed with PB-8.0 containing 300 mM NaCl and 20 mM imidazole. Finally, the column was eluted with PB-8.0 containing 300 mM NaCl and 250 mM imidazole. Fractions containing rLTB-6His (II) were pooled and concentrated.

Ganglioside Binding Assay of Purified rLTB-6× His

GM1–ELISA was performed to determine the affinity of the rLTB protein with the GM1-ganglioside receptor. Microtiter plates were coated with 100 µl of 3 µg/ml monosialoganglioside-GM1 (Sigma G-7641, U.S.A.) in bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) per well at 4°C overnight. Negative-control wells were coated with 100 µl of 3.0 µg/ml BSA per well. Then, 100 µl of increasing concentrations of the soluble purified rLTB proteins expressed in *E. coli* and *P. pastoris* in bicarbonate buffer, respectively, was added to the well of the above plate and incubated at 37°C for 2 h. A 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma G-7641) in 0.01 M PBS containing 0.5% BSA was added into the wells and incubated at 37°C for 2 h. The wells were then washed three times with PBST. Color development was performed using *p*-nitrophenyl phosphate (Sigma, N-3129). Absorbance values were measured at 405 nm (550 nm as reference wavelength). Bacterial CTB (Sigma C-1655) and rabbit anti-CTB (Sigma, C-3062, 1/8000 in PBS) were the positive controls.

Analytical Techniques of Proteins

The soluble supernatant of cell lysate from *E. coli* BL21 (DE3) was analyzed with a 15.0% (w/v) polyacrylamide gel. The MW of target protein in SDS–PAGE gel was estimated by a BIO-RAD gel image analysis system. For Western blotting analysis, the proteins on the gel were transferred onto a PVDF (polyvinylidene difluoride) membrane (Bio-Rad) in a semi-dry blotting apparatus (120 min at 120 mA). The membrane was blocked with 0.3% (w/v) BSA in Tris-buffered saline (TBS, pH 7.4), and then incubated with goat anti-CT-B antibody and HRP-conjugated rabbit anti-goat IgG in turn, according to the manufacturer's protocol. After treatment with the Western Blotting Luminol Reagent (Santa Cruz Biotechnology), the membrane with a positive signal was exposed to a film. The images of gels and membranes were scanned by a GEL-DOC 2000 gel documentation

system (Bio-Rad). The Bradford protein assay was used for quantitative analysis of protein [23].

RESULTS

Construction of the Expression Strain of *E. coli* and *P. pastoris* with the LTB Gene

The sequencing results and construction analysis of plasmid pPICZaA-LTB to *P. pastoris* are shown in Fig. 2A. A series of *P. pastoris* transformants with LTB gene were obtained by screening in plates and identification of strain PCR and sequencing to amplified products.

The recombinant pET32a-LTB plasmid in frame to downstream of the T7 promoter is shown in Fig. 2B. The plasmids carrying the desired foreign genes were prepared and transformed into *E. coli* BL21 (DE3) for rLTB expression.

Expression and Identification of rLTB Protein in *P. pichia* and *E. coli*

The protein of interest rLTB was secreted into the medium by the engineered *P. pichia* in high-density fermentation in a shaking flask and 5-l fermentor under the induction of 0.5% methanol. The protein rLTB-6× His reached 250–300 mg/l in the supernatant of the medium and came up to 20–25% of total proteins secreted into the medium, as shown in Fig. 3A.

However, the protein rLTB was expressed in *E. coli* by the way of inclusion body, although the LTB gene was cloned from *E. coli*, and constructed with signal sequence. Inclusion body accounted for 15–20% of the total proteins. SDS–PAGE and Western blotting analysis of rLTB expressed in *E. coli* are shown in Fig. 3B. The fusion protein rLTB-6× His produced by *E. coli* BL21(DE3) in a 5-l fermentor was up to 80–100 mg/l.

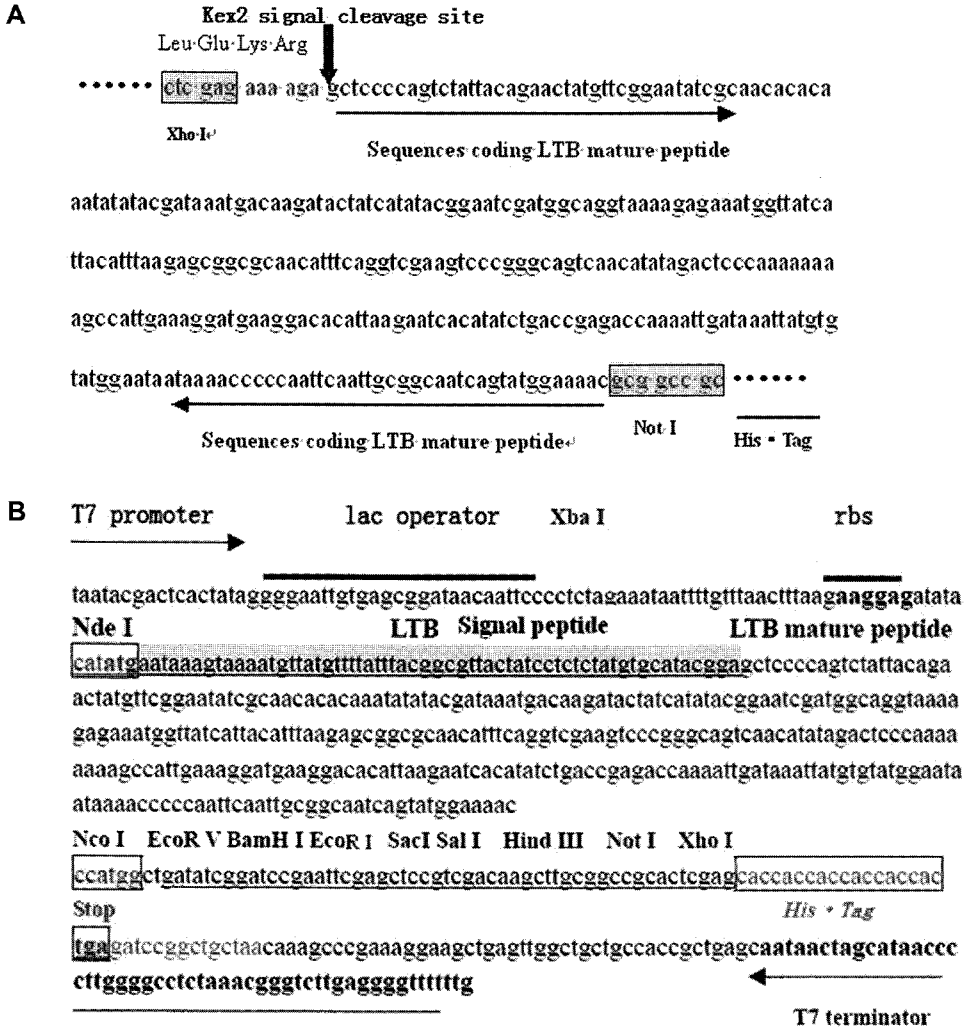

Fig. 2. Construction strategies and analysis of coding sequence of rLTB expressed in *P. pastoris* with pPICZaA-LTB (**A**) and *E. coli* with pET32a-LTB (**B**).

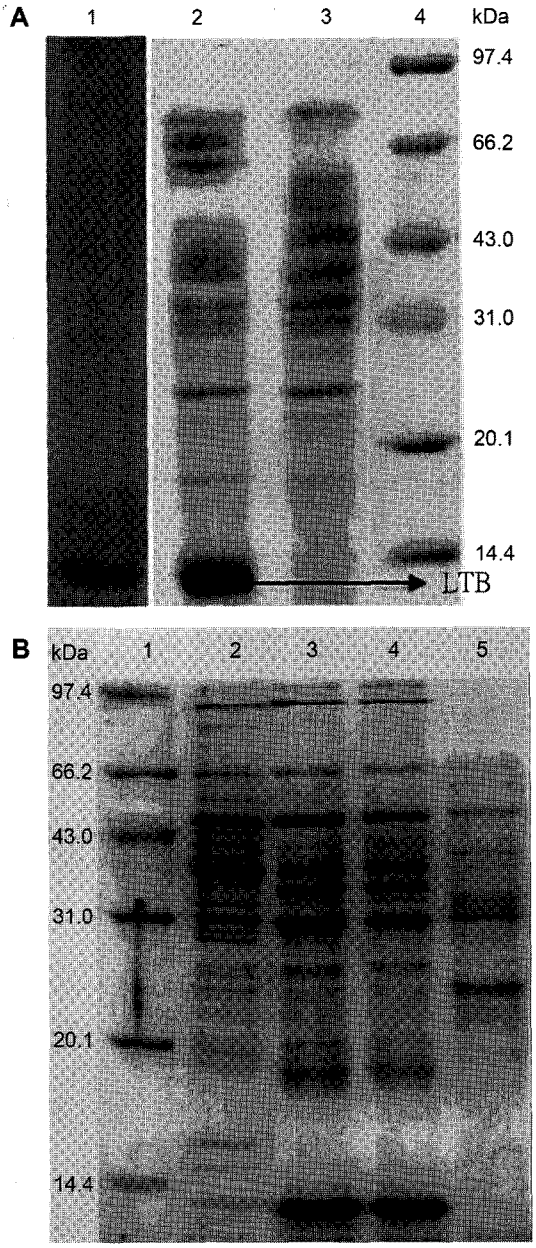

Fig. 3. SDS–PAGE and Western blotting analysis of expression results of fusion protein rLTB-6× His in *P. pastoris* X-33/ pPICZaA-LTB in supernatant (**A**) and *E. coli* BL21 (DE3)/ pET32a-LTB in inclusion body (**B**).
A: Lane 1: western blotting analysis; Lane 2: fusion protein rLTB-6×His expressed in *P. pastoris*; Lane 3: supernatant of medium of *P. pastoris* induced by methanol; Lane 4: protein marker. B: Lane1: protein marker; Lane 2, 5: supernatant of medium of recombinant *E. coli* BL21 induced by IPTG; Lane 3–4: inclusion body of fusion protein rLTB-6×His expressed in *E. coli* BL21.

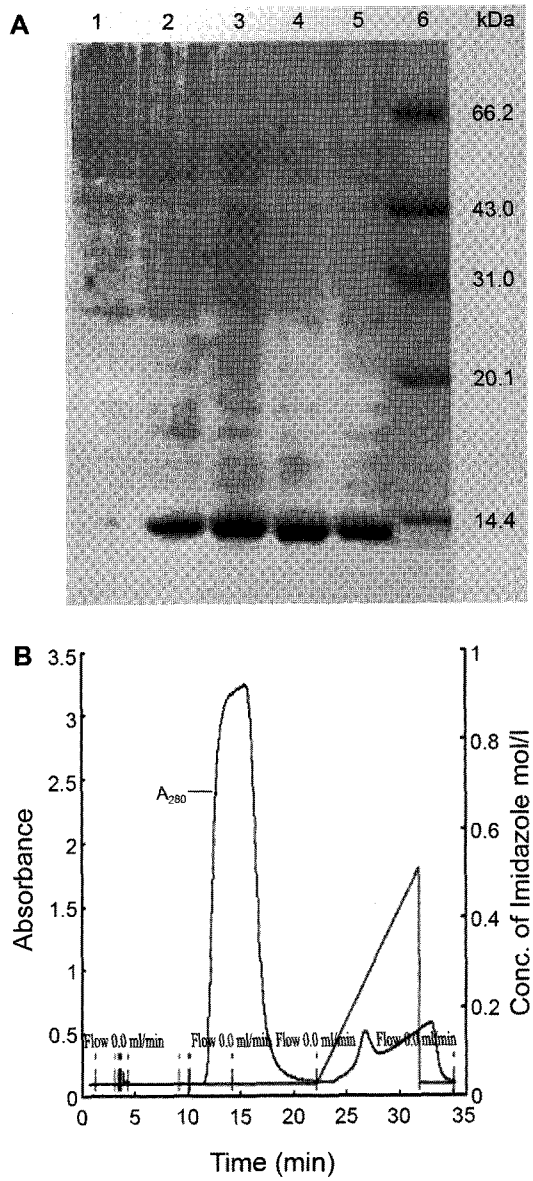

Fig. 4. SDS–PAGE analysis of rLTB-6× His purified with Ni+ Chelating Sepharose Columnn (**A**) and Elution profile for Ni-chelating chromatography of rLTB-6× His (**B**).
Lane 1: flow-through as a control. Lane 2–3: Purified rLTB-6× His expressed in *E. coli* BL21 (DE3) (named erLTB temporarily). Lane 4–5: Purified rLTB-6× His expressed in engineering *P. pastoris X-33*(named prLTB temporarily). Lane 6: Protein marker.

Purification and Characterization of rLTB Protein

The supernatant that contained protein rLTB-6× His harvested from different host strains was loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) agarose column, and the fusion protein rLTB-6× His was desalted with a desalting column and purified to 95% by thin layer scan. The yield rate was about 46%. Through calculating, the final output of rLTB protein was 120 mg per liter culture of *P. pastoris*. The elution profile for Ni-chelating chromatography of rLTB-6× His is shown in Fig. 4A, and SDS–PAGE analysis of purified rLTB-6× His is shown in Fig. 4B. In addition, the molecular mass of LTB-6× His expressed in *P. pastoris* was greater than that in *E. coli*, which was equal to the expected 11 kDa perceived in SDS–PAGE analysis.

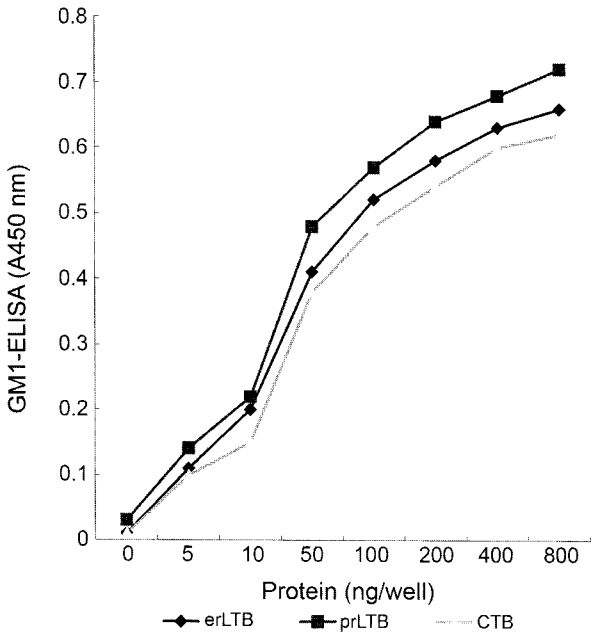

Fig. 5. Comparation on affinity with GM1 ganglioside of purified erLTB produced from engineered *E. coli* BL21 (DE3), prLTB from *P. pastoris* yeast, and commercially available CTB from cholera as a positive control.
The ELISA experiment was performed by incubation of coated GM1 ganglioside with purified rLTB or CTB as positive control, and BSA as the negative control.

Assay for Mucosal Adjuvant Activity

The purified rLTB-6× His proteins expressed in two different host strains were assayed to determine their mucosal adjuvant activity, which is the affinity to the GM1-ganglioside receptor by GM1–ELISA assay. The bacterial CTB acted as the positive control because CTB from cholera is highly homologous to LTB from enterotoxin *E. coli*. Absorbance values measured at 405 nm indicated that prLTB from *P. pastoris* and erLTB from *E. coli* had very high similar affinity to the GM1-ganglioside receptor as that of CTB from cholera, as shown in Fig. 5. Simultaneously, we could find that there was a bit of dissimilarity that the prLTB from *P. pastoris* showed higher affinity to GM1-ganglioside receptor than the others. It can be said that the mucosal adjuvant activity of the prLTB is the strongest.

DISCUSSION

Eukaryotic Expression of Mucosal Immunoadjuvant LTB from Prokaryotic Microbes

The non-toxic LTB is a potent mucosal adjuvant, which could be used in various vaccines. Therefore, much more emphasis has been put on the preparation of rLTB in the last 30 years. However, previous methods for rLTB preparation with a prokaryotic expression system were not efficient enough, and also costly, although natural LTB is derived from the prokaryotic microbe *E. coli* [20, 21]. The reasons for the inefficiency may involve structural complexity, heat-lability, stability of rLTB, and so on. Meanwhile, it may be the main factors that the *E. coli* expression system could easily form insoluble inclusion body and give rise to a lower yield of bioactive rLTB. The methylotrophic yeast *P. pastoris* has been used as a host for industrial production of recombinant proteins, and there are many advantages such as high expression of heterologous proteins, efficient secretion, and high growth density in fermenter cultures [20, 22, 26]. In the study, our results clearly demonstrated that protein LTB originated from *E. coli* was produced perfectly by *P. pastoris*. This system will be of considerable importance for using as an expression system of recombinant proteins from prokaryotic microbes.

Purification Techniques for rLTB from Different Expression Systems

In the present study, two different expression systems were used to express target protein rLTB, and rLTB was generated in a large amount in the culture of *P. pastoris* through high-density fermentation in a 5-l fermentor. The 6× His tags of rLTB from different expression systems made the purification more convenient. Protein samples in media were purified on a Ni-NTA agarose column after concentration. This method increased the purity and output of rLTB greatly, and also reduced the cost considerably compared with the previous techniques of GM1 receptor-affinity chromatography and hydrophobic interaction chromatography to purify rLTB, which were more costly and complicated [26]. The project has developed an efficient and simple purification technique of recombinant *E. coli* heat-labile enterotoxin B subunit (rLTB), which made the preparation procedures of rLTB more simple, convenient, and economical.

Identification and Difference of the Mucosal Adjuvant Activity rLTB from *P. pastoris* and *E. coli*

The rLTB protein prepared from *P. patoris* was proved to be a strong mucosal adjuvant by a comparative detection on affinity to GM1-ganglioside receptor. The high-affinity interaction of LTB with the GM1 ganglioside or other glycosphingolipids promotes the uptake of the toxic A subunit into eukaryotic cells [10]. Five identical B subunits, each of 11 kDa, formed the LTB oligomer pentameric ring, by noncovalent interactions. The intact receptor-binding site of the LTB pentamer is necessary for the potent immunogenicity and adjuvant activity of LTB [3, 15]. Nevertheless, in this study, heat treatment at temperatures over 70°C or sodium dodecylsulfate (SDS) led to the destruction of the pentameric ring and the release of the monomers. In order to confirm the mucosal adjuvant activity of purified rLTB from *P. pastoris* and *E. coli*, a

specific test method was performed to determine the affinity of the rLTB protein to the GM1-ganglioside receptor with GM1–ELISA [10]. The method was more simple and convenient than detection by IgG and IgA antibody levels in the serum or small intestine in experimental animals [27].

The results demonstrated that the molecular mass of rLTB expressed in *P. pastoris* was greater than that in *E. coli* equaling to the expected 11 kDa, which may be resulted from glycosylation by *P. pastoris* [20]. The rLTB from *P. pastoris* showed higher affinity to the GM1-ganglioside receptor than rLTB from *E. coli* and CTB, which means higher immunogenicity [19]. Hence, it could be concluded that the rLTB protein prepared from *P. pastoris* is better as a potent mucosal immunoadjuvant than that from *E. coli*. It can be said that the eukaryotic expression system is more suitable and advantageous in producing some particular proteins of interest derived from prokaryotic microbes.

Acknowledgments

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