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Expression of *Escherichia coli* Heat-labile Enterotoxin B Subunit (LTB) in *Saccharomyces cerevisiae*

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Heat-labile enterotoxin B subunit (LTB) of enterotoxigenic *Escherichia coli* (ETEC) is both a strong mucosal adjuvant and immunogen. It is a subunit vaccine candidate to be used against ETEC-induced diarrhea. It has already been expressed in several bacterial and plant systems. In order to construct yeast expressing vector for the LTB protein, the *elt*B gene encoding LTB was amplified from a human origin enterotoxigenic *E. coli* DNA by PCR. The expression plasmid pLTB83 was constructed by inserting the *elt*B gene into the pYES2 shuttle vector immediately downstream of the *GAL1* promoter. The recombinant vector was transformed into *S. cerevisiae* and was then induced by galactose. The LTB protein was detected in the total soluble protein of the yeast by SDS-PAGE analysis. Quantitative ELISA showed that the maximum amount of LTB protein expressed in the yeast was approximately 1.9% of the total soluble protein. Immunoblotting analysis showed the yeast-derived LTB protein was antigenically indistinguishable from bacterial LTB protein. Since the whole-recombinant yeast has been introduced as a new vaccine formulation the expression of LTB in *S. cerevisiae* can offer an inexpensive yet effective strategy to protect against ETEC, especially in developing countries where it is needed most.

Key words: enterotoxigenic *Escherichia coli*, LTB, *Saccharomyces cerevisiae*, gene expression, immunoblotting

ETEC is the most common cause of diarrhea, especially in young children, travelers and military personnel in developing countries (Katz *et al.*, 2003; Steinsland *et al.*, 2003). The major disease agent of ETEC is the heat-labile enterotoxin (LT). The LT is a plasmid-encoded, high-molecular weight toxin, which is immunologically and physicochemically related to cholera toxin (CT) (Guidry *et al.*, 1997; Fleckenstein *et al.*, 2000).

The crystal structure of LT revealed that it is composed of one A subunit (LTA) (27 kDa) and five non-covalently associated B subunits (LTB) (11.6 kDa each) forming a ring-like pentamer. LTA has ADP-ribosilating activity that causes constitutive activation of adenylate cyclase, an increase in the intracellular cAMP and subsequent severe diarrhea (Nataro and Kaper, 1998; Kozuka *et al.*, 2000). LTB is able to bind to ganglioside GM1 [Gal(β1-3)GalNAc(β1-4)(NeuAc(α2-3))Gal(β1-4)Glc(β1-1)ceramide], a glycosphingolipid found ubiquitously on the cell membranes of mammals and to other related receptors, such as GD1b-ganglioside, asialo-GM1, lactosylceramide and certain galactoproteins (Williams *et al.*, 1999). The LT and its related cholera toxin (CT) are extremely potent immunogens following mucosal or systemic delivery.

It has been shown that LT acts as a strong mucosal adjuvant, which enhances serum and local immune responses to co-administered antigens, where most antigens are unable to induce immune responses. Therefore, it is not surprising that LT has been incorporated into putative mucosal vaccines to guard against a range of infectious agents. However, its inherent toxicity and allergenicity have hampered progress for human use (Williams *et al.*, 1999).

One approach to overcome these problems is the use of a non-toxic derivative of LT, like LTB in isolation. Several studies with animal models and one human trial demonstrated that recombinant LTB (rLTB) can stimulate strong serum and mucosal immune responses against LT. Many studies have indicated that LTB could be used as a potent adjuvant (Tochikubo and Yasuda, 2000). Other investigations have also suggested that the rLTB can increase tolerance to heterologous antigens, a finding that has led to its further application in attempts to prevent autoimmune

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disease (Williams *et al.*, 1999).

At the present time, LTB is among the very few effective and non-living mucosal immunogens. Generally, the results of numeruos investigations have shown that LTB is a promising candidate to be a vaccine antigen against LT-producing ETEC. LTB is highly resistant to proteolytic degradation, and retains its pentameric quaternary structure in a pH as low as 2.0. This is additional evidence that supports LTB as a candidate antigen to be used in edible vaccines.

Because of many advantages, the yeast *Saccharomyces cerevisiae* is increasingly being employed for expression of recombinant proteins. Unlike bacterial hosts like *E. coli*, "Bakers yeast", *S. cerevisiae* lacks detectable endotoxins and because of a long history of use in food and pharmaceuticals, it is generally recognized as safe (Schreuder *et al.*, 1996).

Genetic manipulation of *S. cerevisiae* has been simplified by a greatly improved understanding of the yeast's biology and genetics. Furthermore, the process technology for a yeast culture is well established and it can be easily adapted to produce recombinant proteins.

Other advantages of yeast *S. cerevisiae* include its fast cultivation, low nutrition requirements, less tendency for inclusion body formation, its kinetic stability by glycosylation, high transformation efficiency and high growth rate (Zhang *et al.*, 1996). Previous studies also support the use of the yeast expression system for recombinant vaccine formulations, because yeast derivatives have adjuvant properties, which enhance the immune response. Thus, production of recombinant proteins in transgenic yeast is a competitive and safe alternative to the traditional protein expression systems, like prokaryotic or plant systems.

In this research, we cloned the *elt*B gene into a construct for expression of LTB mature peptides in intracellular location of the yeast, *S. cerevisiae* as a recombinant protein. The recombinant yeast expressing LTB protein can be used to stimulate anti-LT mucosal and systemic immune responses as a vaccine candidate against ETEC.

Materials and Methods

Strains, Plasmids and Media

A human isolate enterotoxigenic *Escherichia coli* (ETEC) producing LT (E20738A) (McConnell *et al.*, 1990) was provided by Dr. H. Steinsland. Yeast experiments were performed with *S. cerevisiae* 2802 (*MAT∝ pep4:His3 prb-Δ1.6Rcan1 his3-20 ura3-52*). The yeast was cultured in a rotary incubator (200 rpm) at 30°C in SC-Ura, uracil-deficient complete synthetic medium containing 2% glucose (Difco, USA). *Escherichia coli* DH5α (Stratagene, USA) (*f- gyr A96 Nlar, recA1Thi-1 hsdR17 r- k m+k*) was used for the cloning, sequencing and maintenance of various DNA fragments. pYES2 (Invitrogen, USA) was used as a yeast shuttle and expression vector. It is a 2 μm-based multicopy yeast plasmid and contains the *URA3* gene and the *GAL1* promoter for selection and expression in *S. cerevisiae*, as well as *ori* and *Amp* for the selection and propagation in *E. coli*. The medium Luria Bertani (LB) (QUELAB, UK) was used for culturing *E. coli*.

Construction of the yeast expressing vector encoding LTB

General DNA manipulation was performed using typical recombinant procedures (Sambrock and Russell, 2001) with enzymes prepared from Roche (Germany). The DNA fragments were analyzed by electrophoresis on a 0.8% (w/v) agarose gel and purified from the agarose gel using a gel extraction kit according to the manufacturer's guide (Viogen, USA).

PCR was used to amplify the *BamH*I-*Eco*RI *elt*B cloning cassette. Oligonucleotide primers: 5'-TGTGGATCC**ATG**-GCTCCTCAGTCTATTACA-3' (Forward) and 5'-GCTTT-TAAGAATTC**CTA**GTTTTCCATACTG-3' (Reverse) were designed according to the *elt*B mature peptide gene sequence (Accession M17874) and synthesized by the MWG Company (Germany).

The first oligonucleotide contains: 1) a three-base leader followed by a *BamH*I site (underlined); 2) an ATG start codon (bold); and 3) 18 nucleotides that being in the sequence encoding LTB. The second oligonucleotide contains: 1) an eight-base leader; 2) an *Eco*RI site (underlined); and 3) 16 nucleotides complementary to the distal end of the LTB coding sequence including the complement of the CTA stop codon (bold).

Plasmid DNA was extracted from ETEC strain and was used as template for gene amplification. PCR conditions were as follows: denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and finally an extension at 72°C for 1 min for a total of 30 cycles. PCR was carried out with a model FGEN02TD (Techneh, UK) termal cycler.

The resultant *elt*B cassette was confirmed by *Cla*I. This restriction enzyme produced two 100 and 235 bp fragments. The confirmed *elt*B was digested with both *BamH*I and *Eco*RI and recovered in pYES2 to create pLTB83. PCR fidelity was verified by a complete sequencing of the *elt*B portion (MWG Company, Germany). The resulting plasmid, pLTB83, contained the *elt*B cassette downstream of the yeast inducible *GAL1* promoter (Fig. 1). Its *BamH*I site is located downstream of native *GAL1* transcriptional start site but upstream of *GAL1* translational start site. Thus, the *elt*B cassette was translated beginning with the first ATG codon. Accordingly, under inducing conditions (i.e. galactose present and glucose absent) our construct should lead to the direct production of mature LTB.

Yeast transformation and maintenance

Yeast cells were transformed by the modified lithium chloride method (Hill *et al.*, 1991). The transformants were selected on SC-Ura, synthetic complete medium,

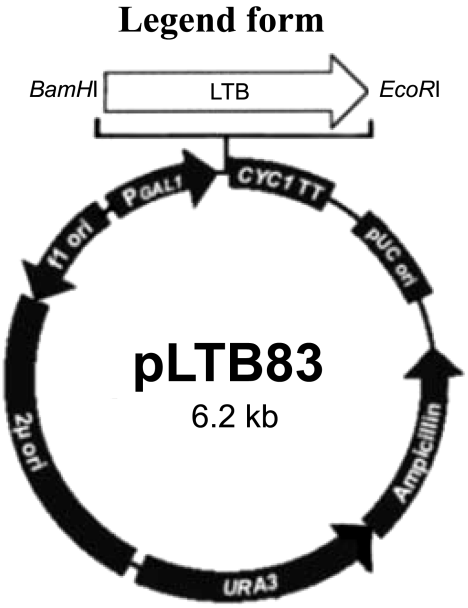

Fig. 1. Construction of pLTB83 yeast expressing vector containing *elt*B gene in downstream of *GAL1* promoter.

lacking uracil and containing 2% glucose and 2% agar. The strains were maintained as glycerol stocks at -70°C.

Plasmid recovery from transformants

To confirm the presence of recombinant plasmids in the transformed yeast, the plasmids were recovered from the yeast by a modified method of Smash and Grab (Hoffman and Winston, 1987). Briefly, the yeast cells were cultured overnight at 30°C in 5 ml of selective broth containing 2% glucose and, subsequently, pelleted in microcentrifuge. The cell pellets were resuspended in 200 µl of buffer (1% SDS, 2% Triton X-100, 100 mM NaCl, 10 mM Tris, pH 8.0, and 1 mM EDTA); 200 µl of phenol-chloroform and 0.3 g of acid-washed glass beads were added. The mixture was agitated at maximum power in a desktop vortex mixer for 2 min. The crude lysate was spun for 5 min and the aqueous phase was harvested into an equal volume of TE (10 mM Tris, pH 7.5 and 1 mM EDTA). The resultant mixture was extracted once each with phenol, phenol-chloroform, and chloroform, and then precipitated with ethanol. The recovered plasmids were further analysed by PCR to confirm the presence of the *elt*B cassette in the recovered plasmids.

Growth condition and preparation of cell extract

To assess whether transformed *S. cerevisiae* strain produced immunoreactive protein, the recombinant yeast were grown in a buffered (0.1 M potassium phosphate buffer, pH 6.4) SC-Ura medium containing 2% glucose and supplemented with appropriate amino acids at 30°C under shaking conditions (250 rpm) for 48 h. The cells were separated by centrifugation (1300 ×*g*, 3 min) and resuspended in the same volume of induction medium (SC-Ura medium supplemented with 2% galactose instead of glucose). The induced cells were collected by centrifugation (1300 ×*g*, 3 min) in 12 h intervals till 36 h after induction, washed once with fresh media, and resuspended in 400 µl of TPE (10 mM Tris-Cl, pH 8.0; 0.1 M sodium phosphate pH 8.0; 1 M EDTA and 30 µl of phenylmethylsulphonyl fluoride (PMSF)). The cells were then ruptured by vortexing with acid washed glass beads (0.3 g) at maximum power for seven 1-min increments. Then, the cell extract was centrifuged to recover a clear supernatant referred to as the total soluble protein (TSP) (Deresiewicz *et al.*, 1994).

Concentration of recombinant protein

The extract of yeast cell contains some proteins that complicate the assessment of the recombinant protein, especially when the recombinant protein level is low. SDS-PAGE analysis of TSP (boiled and unboiled) showed only a thin band of recombinant LTB in monomeric form in the expected position. Thus, to facilitate evaluation of the recombinant protein in the immunoblotting assay, the yeast TSP was partially purified by ultrafiltration. Briefly, the recovered clear supernatant passed through a 0.2 µm microbiological membrane and then filtered by a 100 kDa cut-off ultrafiltration membrane (Sartorius, Germany). This pore-size membrane could pass the majority of recombinant proteins and retain most of the high-molecular weight proteins. Those filtrates were concentrated with an acetone precipitation method (Powell, 2003).

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting analysis were used for evaluation of the presence and antigenicity of the new recombinant protein. Yeast concentrated protein extracts were prepared as described earlier. The amount of concentrated yeast protein was estimated by a Bradford assay (Hirst, 1991). An aliquot of 20-25 µl concentrated protein containing 20 µg protein were separated by 15% sodium dodecylsulfat polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V for 45-50 min in Tris-glycin buffer (25 mM Tris, 250 mM glycine, pH 8.3, 0.1% SDS). Samples of bacterial CTB (identical to LTB) and concentrated protein from non-induced yeast were loaded on the gel as well. The resolved proteins were transferred from the gel to a 0.45 µm nitrocellulose membrane (Sigma, USA) by blotting at 300 mA for 1 h with active cooling. Non-specific antibody binding sites were blocked by incubation of the membrane in 30 ml of 3% non-fat dry milk in TBS (20 mM Tris-HCl, pH 7.5 and 500 mM NaCl) for 1 h at room temperature on a rotary shaker (35 rpm). Subsequently, the membrane was washed in TBST (TBS with 0.05% Tween-20) for three 5-min increments with gentle agitation. The membrane was incubated for 2 h in 25 ml

of 1:1000 dilution of rabbit anti-cholera toxin B subunit antiserum (a donation from Dr. Yasuda, Nagoya City University, Japan) in TBST. Followed by washing three times for five min with TBST, the membrane was further incubated with 1:2000 sheep anti-rabbit IgG, peroxidase conjugate (Sigma, USA) in TBST for 2 h at room temperature with gentle agitation.

The membrane was washed three times in TBST, but only once with TBS, followed by incubation in 1 mg/ml DAB (diaminobenzidine-H_2O_2) (Sigma, USA) in TBS buffer, and finally placed in a dark chamber. Color developed after 15 min.

CTB and LTB are antigenically related and showed a high degree of homology with an 85% conservation rate of amino acids in the mature proteins. There is also a high degree of immunological cross-reactivity between the two molecules. Based on these facts, instead of anti-LTB, anti-CTB uses for immunological assay of LTB generally, and CTB could be used as a positive-control protein.

ELISA quantification of LTB

Induced and non-induced yeast TSPs were prepared as described previously. The amount of yeast total soluble protein content was estimated by a Bradford assay (Hirst, 1991). Certain amounts of TSP in the range of 100-800 ng were bound to a 96-well microtitration plate (Linbro/Titertek, Flow Laboratories, USA) overnight at 4°C. The plate was washed three times in PBST (phosphate buffered saline (PBS) containing 0.05% Tween-20). The background was blocked by incubation in 5% (w/v) non-fat dry milk in PBS (300 µl per well) for 2 h at 37°C followed by washing three times with PBST. The plate was incubated in a 1:4000 dilution of polyclonal rabbit anti-CTB antibody in 0.01 M PBST (100 µl per well) for 2 h at 37°C, followed by washing the wells three times with PBST. The wells were washed and incubated with 1:2000 sheep anti-rabbit IgG peroxidase conjugate in 0.01 M PBST (100 µl per well) for 2 h at 37°C, followed by washing five times with PBST. Plates were developed with TMB (tetramethylbenzidine-H_2O_2) substrate (Bio-Rad, France). The reaction was stopped by the addition of 2 M H_2SO_4 and the plates were read at 450 nm. Comparison of the absorbance at 450 nm of a known amount of bacterial CTB-antibody complex (liner standard curve) and that of the absorbance differences between a known concentration of the induced and non-induced *S. cerevisiae* TSPs were used to estimate the recombinant LTB expression levels.

Results

Construction of LTB expression vector

PCR was used successfully to amplify a gene fragment encoding the sequence of mature LTB. For preliminary confirmation, the fragment was subjected to digestion with *Cla*I. Digestion with *Cla*I yielded 100 and 235 bp fragments at the expected positions (Fig. 2). To construct the yeast expressing vector (pLTB83) and facilitate the sequencing, the fragment was cloned to pYES2 and transformed in *E. coli* DH5α for amplification. Then, the transformants were selected and the plasmid was extracted for further analyzing. The presence of *elt*B was approved by restriction analysis. The complete sequencing of the PCR-generated fragment in pYES2 revealed complete homology at the nucleotide level to *elt*B gene in NCBI.

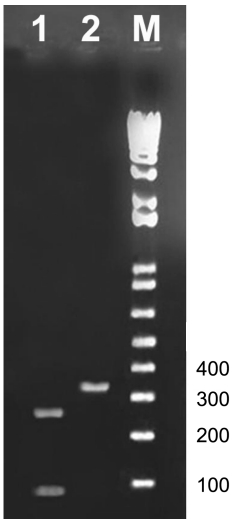

Fig. 2. PCR amplification of *elt*B from a human isolated ETEC E20738A (lane 2), *Cla*I digestion of *elt*B (lane 1) and molecular weight marker (M).

Transformation of S. cerevisiae

The plasmid, which incorporated *elt*B driven by the *GAL1* promoter, was constructed as described, used for transformation of *S. cerevisiae*. Out of several colonies that were raised on selective media, 10 colonies were selected and the presence of *elt*B was confirmed by PCR following plasmid recovery from transformants. Samples of pYES2 harboring *elt*B, intact pYES2 and untransformed yeast either carried out in the same condition. The results indicated the presence of the fragment (*elt*B) in the transformed yeast, but not in the untransformed and intact pYES2.

Intracellular expression and immunoblotting

Because no signal sequence was present, the product was expected to remain intracellularly. Expression of pLTB83 in *S. cerevisiae* was induced in the presence of 2% galactose. SDS-PAGE analyses of TSPs (boiled and unboiled) showed only a thin band of recombinant LTB in monomeric form with the expected molecular mass being close to 11.6 kDa. This band showed sharply in concentrated TSPs (Fig. 3).

The results showed that the newly synthesized recom-

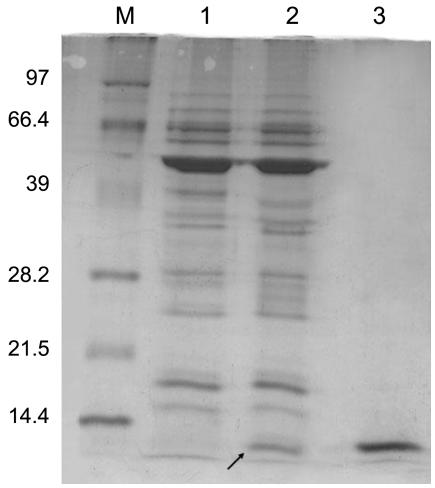

Fig. 3. SDS-PAGE analysis of concentrated TSPs from: transformed but non-induced (1), transformed and induced recombinant *S. cerevisiae* (2), monomeric bacterial CTB (3) and low range molecular weight marker (M). Arrow indicates the band (M~11.6 kDa) corresponding to recombinant LTB monomers. The gel was stained with Coomassie blue R250 to visualize the protein bands.

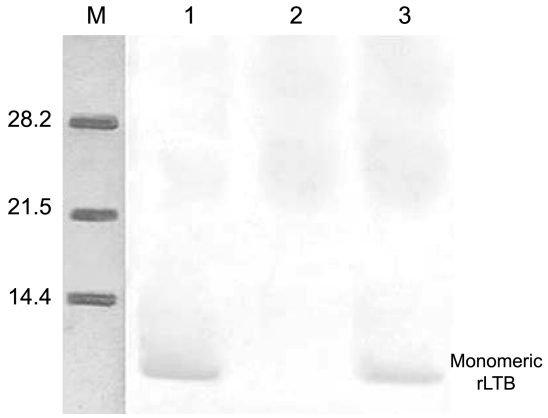

Fig. 4. Immunoblotting of transformed and induced (3), transformed but non-induced (2), yeast concentrated protein extract, bacterial monomeric CTB (1) and low range molecular weight marker (M).

binant protein could not assemble to its pentameric form (55.6 kDa) as native bacterial protein. However, immunoblotting analysis showed the yeast-derived LTB protein was antigenically indistinguishable from bacterial protein (Fig. 4). No immunoreactive band was found under the non-inducing conditions.

ELISA quantification of LTB expression

The recombinant LTB protein was quantitatively estimated in TSP of transformed and induced *S. cerevisiae* by ELISA analysis. The amount of LTB was detected in different concentrations of the TSP and expressed in nanograms per sample (Fig. 5). As shown in Fig. 5, optimal concentrations of the TSP (100-400 ng) loaded in the wells of the microtiter plate, yielded optimal increases of

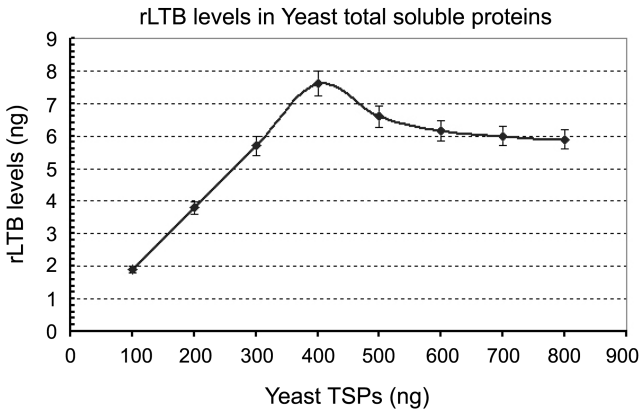

Fig. 5. Determination of recombinant LTB levels in the yeast TSP by quantitative ELISA. LTB protein levels (ng) were plotted against the dilutions of *S. cerevisiae* TSP. The ELISA detection indicated that LTB level increased optimally in 100, 200, 300 and 400 ng concentrations of the recombinant yeast TSP and reached to 1.9, 3.8, 5.4 and 7.6 ng, respectively. These amounts are equivalent to 1.9% of the recombinant yeast TSP. Error bars indicate SE.

LTB protein level (reaching to 1.9% of the TSP). When the concentration of the TSP deviated from optimal levels, the amounts of the detected LTB protein decreased. It may be due to the binding characteristics of microtiter plates to the LTB protein in a mixture of total yeast proteins. With increases of the total protein levels, the increasing amount of recombinant protein may be unable to bind to the wells and is eventually lost through washing. The maximum expression level was about 1.9% of TSP. This is more than rLTB produced in potato (0.02%), maize (0.01-0.3%) and in leaves of *Nicotiana benthamiana* (0.75%) but less than the amount produced in tobacco plants (2.5%) (Chikwamba *et al.*, 2002; Kang *et al.*, 2003).

Discussion

Up to now, because of limitations, such as glycosylations, there is less tendency to express prokaryotic proteins in *S. cerevisiae*. However, there are some examples, such as, RTEM-β-lactamases, *Staphylococcus aureus* nuclease A, TSST-1 and Shiga-like toxin I A-chain that are expressed in *S. cerevisiae* (Pines *et al.*, 1988; Pines and London, 1991).

In this study, we showed that the *S. cerevisiae* could produce the heat-labile enterotoxin B subunit of *Escherichia coli* (LTB). As indicated in the results, we detected the monomeric forms of LTB in recombinant yeast lysates. That is different from the result of previous study which reported that the expressed oligomeric complex of the hybrid protein of Lipo-LTB in *S. cerevisiae* (Schonberger *et al.*, 1991). Previous studies on the assembly of LTB in *E. coli* have revealed that enterotoxoid oligomerization occurs after liberation of the mature subunit into

the periplasm (Hirst, 1991).

It has been postulated that the periplasm of gram-negative bacteria provides an environment that would favor the spontaneous assembly of the B subunit monomers into oligomers (Hirst and Holmgren 1987a, 1987b). It is clear that the accumulation and concentration of proteins are important factors for the oligomeric assembly of them. Schonberger *et al.* (1991) have directed Lipo-LTB into endoplasmic reticulum (ER) lumen which acted in a manner similar to the periplasmic space of gram-negative bacteria, and provided an intracellular environment in which monomeric B subunits are concentrated and assembled into oligomeric form. In addition, there is the same concept about the oligomeric proteins expressed in plant systems. In plant expression vectors, usually LTB and CTB are expressed as fusion proteins together with the leader and SEKDEL fragments (Arakawa *et al*., 1997; Chikwamba *et al*., 2002). The leader peptide of the recombinant proteins directs newly synthesized protein in the ER lumen and SEKDEL helps to accumulate LTB within plant tissue.

Because the aim of this research was to produce native LTB intracellulary, and to avoid secretion and some unwanted modifications, such as glycosylation, we did not use a signal sequence. We used pYES2, the yeast shuttle vector. The DNA fragment carrying *elt*B gene is located downstream of *GAL1* promoter, and the start codon ATG is located upstream of the first codon of the mature protein. Thus, as expected, our recombinant protein is expressed at intracytoplasm. This is why our recombinant protein at could not retain its oligomeric form. It may be that so large space of yeast cytoplasm could not provide a limited environment to accumulate and assembly of monomeric LTB as that provided by ER.

Besides these assumptions, there was less tendency to form the inclusion body in yeast, which may conflict with this approach.

Recombinant LTB produced in *S. cerevisiae* retained its antigenicity as indicated by western blotting analysis. However, previous studies claimed that assembly of LTB monomers into an oligomeric (pentamer) structure is required for binding to its natural receptors (Tochikubo and Yasuda 2000). Thus, it seems that our recombinant protein could not bind GM1. However, recently, researcher show that a portion of the monomeric subunit LTB (the LT-B 20-mer peptide 26-45) is capable of binding to GM1 and eliciting mucosal and serum IgA responses in mice (Takahashi *et al.*, 1996).

As estimated by quantitative ELISA the maximum expression level of recombinant LTB was about 1.9% of TSP. This is more than rLTB that was produced in potato (0.02%), maize (0.01-0.3%) (Chikwamba *et al.*, 2002) and in leaves of tobacco plants (*Nicotiana benthamiana*) (0.75%). But, it is less than the one produced in other studies with tobacco plants (2.5%) (Kang *et al.*, 2003).

Aggregation of assembled pentamers similar to bacterial antigen should have resulted in the underestimation of recombinant protein in ELISA test (Daniell *et al*., 2001). However, our monomeric protein (rLTB) may not be subjected to this problem.

In bacterial and yeast systems, the level of gene expression is strictly related to specific codons (Yu *et al.*, 2004). Recently, chimeric CTB expressed significantly high levels (100 mg/l) according to the codon usage preferred by *H. polymorpha* (Song *et al.*, 2004). Thus, it would be possible to increase the expression levels of prokaryotic LTB in *S. cerevisiae* by specific codon optimization. Further studies must be conducted to confirm this idea.

In the last several decades, a great number of studies have been devoted to the use of adjuvants to potentiate the immune responses to antigens. This effort has been particularly important in recent years with the development of synthetic, purified, subunit and recombinant vaccines, which are generally poor immunogens. As indicated in introduction, whole-recombinant yeast has remarkable potential as a vaccine vector. It is well established that recombinant yeast confers a potent adjuvant property against recombinant antigens. The goal in designing a vaccine for induction of immune response is to develop a vector that ideally delivers whole protein antigens in to both the MHC class I and class II antigens processing pathways of antigen presenting cells (APCs). The vaccine formulation should be non-toxic and should function without the need for additional adjutants, but still be able to activate the APCs. Whole-recombinant yeast perfectly shows these properties.

A major problem in the development of new vaccines against ETEC will be to make them sufficiently inexpensive and to develop a formulation that can be readily distributed to huge population in high risk areas. Booster doses will probably be needed for each of new oral vaccines and the formulations will be need to be sufficiently simple that the vaccine might even be self-administrated at time of risk. All of this shows that whole-recombinant yeast can be used as a safe, effective and inexpensive vaccine delivery system and developing such formulation could possibly circumvent problems such as the cost of development, delivery and administration logistics of current vaccines especially in developing countries.

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