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Effect of pressure on refolding of recombinant pentameric cholera toxin B

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ABSTRACT

The production of recombinant proteins is an essential tool for the expansion of modern biological research and biotechnology. The expression of heterologous proteins in *Escherichia coli* often results in an incomplete folding process that leads to the accumulation of inclusion bodies (IB), aggregates that hold a certain degree of native-like secondary structure. High hydrostatic pressure (HHP) impairs intermolecular hydrophobic and electrostatic interactions, leading to dissociation of aggregates under non-denaturing conditions and is therefore a useful tool to solubilize proteins for posterior refolding. Cholera toxin (CT) is composed of a non-toxic pentamer of B subunits (CTB), a useful adjuvant in vaccines, and a toxic subunit A (CTA). We studied the process of refolding of CTB using HHP. HHP was shown to be effective for dissociation of CTB monomers from IB. Posterior incubation at atmospheric pressure of concentrated CTB (1 mg/ml) is necessary for the association of the monomers. Pentameric CTB was obtained when suspensions of CTB IB were compressed at 2.4 kbar for 16 h in the presence of Tween 20 and incubated at 1 bar for 120 h. Soluble and biologically active pentameric CTB was obtained, with a yield of 213 mg CTB/liter of culture. The experience gained in this study can be important to improve the refolding of proteins with quaternary structure.

1. Introduction

Protein oligomers are essential for life, thereafter the folding of oligomeric proteins has important implications for both biotechnology and medicine (Jaenicke and Lilie, 2000).

Cholera is an acute diarrheal disease that can kill within hours, if left untreated. The clinical symptoms of cholera are mainly induced by the cholera toxin (CT) released by the bacterium *Vibrio cholerae* in the small intestine of the host (Holmgren, 1981). The CT is an AB_5 holotoxin with molecular mass of 85 kDa composed of five B subunits (CTB) noncovalently linked to the A subunit (CTA). The CTB is a 58 kDa non-toxic pentameric subunit of CT that binds specifically to the membrane lipid ganglioside GM1 on the surface of target human intestinal epithelial cells, promoting CT endocytosis. CTA is a 27-kDa protein with an enzymatic toxic ability that is proteolitically cleaved within the host cell in the domains A1 and A2 (Lhoir et al., 1990; Spangler, 1992). Integral CT travels from the plasma membrane of the epithelial cells to the endoplasmic reticulum (ER) where the toxic subunit A1 crosses the membrane into the cytosol and ADP-ribosylates a trimeric G protein to activate adenylyl cyclase (Fujinaga et al., 2003; Lencer and Tsai, 2003). The consequent stimulated production of cyclic AMP by intoxicated cells initiates a metabolic cascade that results in the excessive secretion of fluids and electrolytes into the intestinal lumen, characteristic of the disease (Bernardi et al., 2008).

The CTB consists of five identical peptides that form a pentamer through interactions between the β-sheets of adjacent monomers. Each monomer of CTB has 103 residues and a disulfide bond between Cys9 and Cys86. A portion from 11 to 15 Å is present at the center of pentamer and is composed of five amphipathic α helices which are involved in the stabilization of the pentamer (Mekalanos et al., 1983; Merritt et al., 1994; Sanchez and Holmgren, 2011; Spangler, 1992).

Several studies have described the immune properties of CTB. Recombinant forms of CTB have been successfully used as an adjuvant in vaccines for human use, such as cholera vaccine (Quiding et al., 1991) and the vaccine against *Escherichia coli* that cause diarrhea (Peltola et al., 1991; Qadri et al., 2000). Similarly, CTB

Abbreviations: CTB, cholera toxin B; HHP, high hydrostatic pressure; IB, inclusion bodies; GndHCl, guanidine hydrochloride.

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has proven to be a good adjuvant model for vaccine development against *Streptococcus pneumoniae* (Malley et al., 2004) and SARS-CoV virus (acute respiratory syndrome-associated coronavirus) (Qu et al., 2005) when administered intranasally in mice.

In view of the potential as a regulator of the immune response, CTB and CTB fusion proteins have been produced in yeast cells (Miyata et al., 2012), plants (Arakawa et al., 1998; Hamorsky et al., 2013; Nochi et al., 2007) and in prokaryotic cells, such as *Lactobacillus* spp. (Slos et al., 1998). Recombinant CTB were also expressed in *E. coli* as insoluble IB that were solubilized in denaturing condition (8 M urea and 10 mM β-mercaptoethanol) and refolded by dilution (Areas et al., 2002, 2004) or as soluble protein in bacterial cytoplasm (Arimitsu et al., 2009; Slos et al., 1998).

The refolding of proteins is a difficult task, frequently hampered due to reaggregation of misfolded proteins on the withdrawal of the denaturing reagent, often utilized for dissociation of the aggregates. The refolding of oligomers present the additional drawback that is the necessity of high protein concentration, a condition that potentially induce the association of misfolded monomers and that can favor reaggregation instead of the proper association of the monomers. HHP was previously described to be a useful tool for solubilization of aggregated proteins, enabling for the refolding of monomeric (Chura-Chambi et al., 2008, 2013; Fraga et al., 2010; Malavasi et al., 2011) and oligomeric proteins (Foguel et al., 1999). In the present study we present the refolding of CTB expressed as IB in *E. coli* to the pentameric and biologically active state utilizing HHP to solubilize the aggregates. We show that the association of the monomers occurs at atmospheric pressure after the treatment at HHP with a relatively high yield (45%).

2. Materials and methods

2.1. Expression of CTB, growth conditions, cell fractionation, and IB isolation

BL-21(DE3) was transformed with the vector pAE-ctxB containing a DNA sequence coding for CTB (Areas et al., 2002). For the expression of CTB, a colony was randomly picked from transformants that were grown on LB plates (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) containing 100 μg/ml ampicilin and inoculated in 2-HKSII rich medium (Jensen and Carlsen, 1990). Cells were grown at 37 °C, and the expression of CTB was induced by addition of isopropyl-β-D-thiogalactopyranoside (0.5 mM) when the O.D. at $A_{600\,nm}$ reached approximately 3. The culture was separated into flasks that were then incubated at different temperatures (20 °C, 30 °C or 37 °C). After incubation with constant orbital agitation (150 rpm) for a 16 h period, bacteria were collected by centrifugation at 2500 × *g* for 10 min at 4 °C. The pellet was resuspended in 50 ml of 100 mM Tris–HCl, pH 7.5, and 5 mM EDTA. Lysozyme, at a final concentration of 50 μg/ml, was added to the suspension, followed by incubation for 15 min at room temperature. The suspension was sonicated in the presence of 0.1% sodium deoxycholate and centrifuged at 8000 × *g* for 10 min. The supernatant was discarded, and the pellet was suspended in 100 mM Tris–HCl, pH 7.5, with 5 mM EDTA and 0.1% sodium deoxycholate. The pellet was washed twice in 50 mM Tris–HCl, pH 8.5, suspended in 40 ml of the same buffer and stored at −20 °C.

2.2. Sample pressurization

Suspensions of IB were diluted in refolding buffer (50 mM Tris–HCl, pH 8.5, 1 mM EDTA). IB suspensions (1 ml) were placed into plastic bags, which were sealed and then placed into a larger plastic bag that was vacuum/heat sealed. The bags were placed in a pressure vessel (R4-6-40, High-Pressure Equipment), and oil was used as a pressure-transmitting fluid. The samples subjected to HHP were centrifuged at 12,000 × *g* for 15 min. The supernatants were dialyzed and centrifuged. The pellets were discarded and the soluble fractions were stored at −20 °C.

2.3. Intrinsic fluorescence and light-scattering (LS) measurements

The light scattering (LS) and fluorescence measurements of the sample were recorded on a Cary Eclipse spectrofluorometer (Varian). Data were collected using a 1 cm path length cuvette at atmospheric pressure. Fluorescence measurements of Trp emissions were carried out with an excitation wavelength of 285 nm. The emission fluorescence spectra were collected between 300 and 400 nm by using a response time of 1 s and scan speed of 240 nm/min. The LS measurements were performed at 320 nm, and the emission was recorded from 300 to 350 nm at an angle of 90° relative to the incident light. For studies under pressure, round quartz cuvettes filled with sample and sealed with flexible polyethylene caps were placed into a high-pressure cell equipped with three optical sapphire windows (ISS) and connected to a pressure generator (High Pressure Equipment). Ethanol was used as a pressure-transmitting fluid.

2.4. Circular dichroism

CD spectra were obtained using a Jasco-J810 spectropolarimeter equipped with a temperature-controlled liquid system and a 0.1 cm-light path cuvette. The reported curves of ellipticity are the averages of five measurements that were collected over a 3 min period. The measurements were carried out at 20 °C.

2.5. Determination of the percentage of solubilized CTB

SDS-PAGE analysis was performed on 12% SDS-polyacrylamide gels using the method described by Laemmli, and gels were stained with Coomassie Blue G-250. Suspensions of IB were heated at 95 °C for 5 min in SDS-PAGE sample buffer (Tris HCl 50 mM pH 8.5 containing 2% SDS, 0.1 M dithiotreitol (DTT), 0.01% bromophenol blue, and 10% glycerol) for complete solubilization of the protein applied in the SDS-PAGE. The respective bands in the SDS-PAGE were used as references for the total amount of CTB within IB. The same volume of soluble fractions of the HHP-treated suspensions of IB were applied to SDS-PAGE gels under non-reducing conditions (samples not heated and in the absence of DTT). Image J software (http://www.ncbi.nlm.nih.gov) was used to analyze the bands in digital photographs of the gels to determine the percentage of CTB pentamer in HHP-treated samples in comparison to the total amount of CTB in IB.

2.6. ELISA for the determination of the binding of CTB to the GM1 receptor

The ability of the CTB pentamers to bind to the GM1 receptor was assessed by ELISA, as described (Areas et al., 2004). Briefly, 96-well plates were coated with 2 μg/ml GM1 ganglioside in PBS, pH 7.2, or BSA in 0.05 mol/L carbonate–bicarbonate buffer, pH 9.6 at 37 °C for 4 h. After washing each well for two times with PBS-T, the wells were blocked by incubation with PBS containing 0.05% Tween 20 (PBS-T) and 2% BSA (BSA–PBS) for 16 h at 4 °C and the wells were washed twice again. Refolded CTB, diluted in BSA–PBS were added to the wells and incubated for 2 h at 37 °C. Unbound proteins were removed by washing the wells four times with PBS containing 0.05% Tween 20 (PBS-T). The binding of CTB to the receptor was determined by incubation with 100 μl per well of a rabbit polyclonal anti-CTB antibody (Sigma) diluted 1:1000 for 90 min. After washing three times with PBS-T, a peroxidase-conjugated goat anti-rabbit

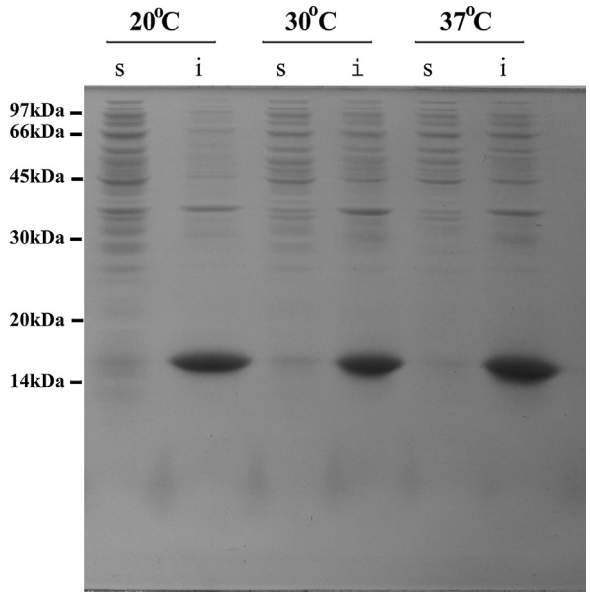

Fig. 1. CTB expression in different temperatures. Analysis of the cellular extract of *E. coli* transformed with the plasmid pAE-ctxB. Soluble (s) and insoluble (i) fractions of the activated cells cultivated at 20 °C, 30 °C and 37 °C. Equal volumes of supernatants and suspended pellets were analyzed by SDS-PAGE.

IgG (Sigma), diluted 1:2000, was added to each well and the plate was incubated for 1 h at room temperature. The wells were washed and the presence of the secondary antibody was revealed by the addition of 200 μl of 8 mg *o*-phenylenediamine (OPD) in 20 ml of a 0.2 mol/L citrate-phosphate buffer, pH 5.0 and 10 μl H_2O_2. The reaction was stopped by the addition of 25 μl of 2 M H_2SO_4. The absorbance was measured at 492 nm.

2.7. Purification of CTB by immobilized metal affinity chromatography (IMAC) and by size exclusion chromatography

Refolded CTB was purified by IMAC using a 1 ml column His Trap HP (GE Healthcare) charged with $Ni2^+$. The sample was loaded in buffer Tris HCl 50 mM pH 8.5 containing 0.5 M NaCl and 40 mM imidazol with a flow of 1 ml/min in the column pre-equilibrated in the same buffer. The CTB was eluted in the buffer containing 0.5 M imidazol and dialyzed. Purified CTB was further concentrated and applied on a size exclusion chromatography column (Superdex 75 10/300 GL, GE Healthcare), pre-equilibrated with buffer Tris HCl 50 mM pH 8.5 with a flow of 0.8 ml/min. Elution of proteins was monitored by determination of absorbance at 280 nm.

3. Results and discussion

3.1. Expression of CTB at different temperatures

The expression of CTB in the form of IB has been previously reported (Areas et al., 2002; Lhoir et al., 1990). To verify if the temperature of bacterial host cultivation affects the recombinant CTB fractionation into soluble/insoluble fractions, the transformed bacteria induced for CTB expression at different temperatures of cultivation were analyzed (Fig. 1). The CTB is largely expressed in the form of insoluble precipitates regardless of the temperature of production, in the range 20–37 °C. The refolding of proteins from IB produced by bacteria cultivated at low temperature was shown to produce proteins presenting structure that resemble the corresponding native state (Vera et al., 2007). Additionally, cultivation at high temperature promotes the formation of more stable aggregates, as this condition favors intermolecular interactions at the expense of native intramolecular contacts (de Groot and Ventura, 2006). Therefore, in order to produce CTB aggregates as similar as possible to the native protein and requiring milder conditions for solubilization, we chose to express this protein in the lowest temperature tested (20 °C).

3.2. Refolding of CTB at HHP in the presence of additives

High hydrostatic pressure (HHP) impairs intermolecular hydrophobic and electrostatic interactions. Thus, moderate pressures (1–3 kbar) are generally effective for dissociating protein oligomers and aggregates, whereas protein denaturation generally requires higher pressure levels (>3 kbar) (Silva et al., 2006). The solubilization of insoluble aggregates is the first step in the refolding processes. The refolding of proteins presenting secondary native-like structures can be improved in comparison to proteins denatured by the presence of high levels of urea or guanidine hydrochloride (GdnHCl), a step required for IB solubilization in the traditional refolding process at atmospheric pressure. Thus, the advantage of using HHP is to solubilize aggregated proteins in a relatively mild condition for posterior refolding. Hydrogen bonds are insensitive to pressure and low levels of urea or GdnHCl may be necessary to improve the solubilization of the IBs exerted at HHP (St John et al., 1999).

The reaggregation from misfolded species after removal of the factor that promotes solubilization of the aggregates, such as high pressure or denaturing concentration of urea or GdnHCl, is the main cause of low levels of refolding yields. Therefore, inhibition of protein interactions that lead to intermolecular contacts and aggregation can be an effective strategy to suppress the formation of these states. The presence of chemical compounds, such as low concentrations of chaotropic agents, amino acids, surfactants and osmolytes can be a useful tool to avoid reaggregation and improve refolding yields (Qoronfleh et al., 2007; Shukla et al., 2011). However, for the refolding of oligomeric proteins, a balance must be found between favoring the native inter-monomer interactions and disfavoring of non-native contacts that lead to aggregation. In order to evaluate the utility of the presence of additives, we compressed suspensions of CTB IB in the presence of low levels of GdnHCl (1.5 M) and the other reagents (shown in Fig. 2A) at 2.4 kbar, a pressure level condition that usually promotes the dissociation of the aggregates, followed by step decompression and centrifugation to remove insoluble aggregates and dialysis of the soluble fraction to remove the additives. In the samples that were compressed in the absence of additives, no bands corresponding to the pentameric form of CTB are seen in the SDS-PAGE of the soluble fraction of the treated IB suspension. However, the presence of the surfactants that were analyzed, Triton X-100, and especially Tween 20, contributed for the obtainment of the CTB pentamer. The immunoblot (Fig. 2B) shows that in addition to the monomer and pentamer, an intermediate form, possibly a trimeric form of CTB, is also present in the soluble fraction. Our results indicate that Tween 20 leads to a productive CTB folding pathway, assisting in oligomerization of CTB to the pentameric form. Tween is a polyoxyethylene sorbitol ester, a nonionic surfactant that contains hydrophobic groups that was shown to reduce the extent of self-association and aggregation, improving the refolding yield of the human monomeric growth hormone, possibly driven by hydrophobic interactions with the protein (Bam et al., 1996). It was also previously shown that the presence of nonionic surfactants can assist for the refolding of the oligomeric proteins OmpA (Wang et al., 2013) and OmpF (Visudtiphole et al., 2005).

For the posterior assays, the suspensions were diluted in refolding buffer Tris HCl 50 mM, containing 1 mM EDTA and 1 mM Tween 20 in the absence of GdnHCl, because higher yields of

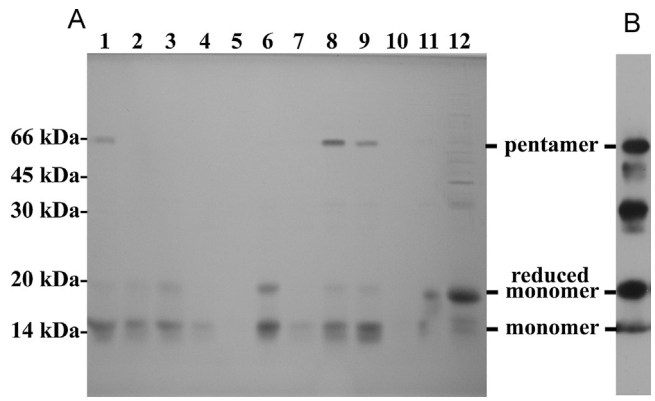

Fig. 2. Effect of the presence of additives on CTB refolding by HHP. (A) Suspensions of IB in refolding buffer (50 mM Tris–HCl, 1.5 M GdnHCl, 1 mM EDTA, pH 8.5) were submitted to HHP (2.4 kbar for 16 h, followed by decompression in steps of 0.4 kbar every 30 min) in the presence of different additives. After decompression, the samples were centrifuged, the supernatants were subjected to dialysis, centrifuged again and equivalent volumes of the supernatants were applied to the SDS-PAGE. Lane 1, NaCl (0.15 M); lane 2, L-arginine (0.5 M) in the absence of GdnHCl; lane 3, L-arginine (0.5 M); lane 4, glucose (1 M); lane 5, sucrose (1 M); lane 6, PEG 6000 (0.1%); lane 7, glycerol (2.5 M); lane 8, Tween 20 (1 mM); lane 9, Triton X-100 (0.5 mM); lane 10, bis-ANS (6.7 mM); lane 11, absence of additives; lane 12: sample of suspension of IBs in reducing conditions. (B) Western blotting of the sample of the lane 8 of the (A). The results shown are representative of three experiments.

monomeric and pentameric CTB were observed in the absence of GdnHCl (data not shown).

3.3. Dissociation of the IB aggregates at HHP and low temperature

High hydrostatic pressures of up to 3–4 kbar, in general does not cause substantial irreversible changes in secondary or tertiary structure, but is an efficient method to dissociate macromolecular complexes (Mozhaev et al., 1996). The freezing point of water is lowered under high pressure, which allows studies of the effects of application of high pressures at temperatures below 0 °C in aqueous solutions. At temperatures below the freezing point of water, the hydrophobic effect, which is considered the main driving force for protein folding and also for intermolecular protein interactions that leads to aggregation is weakened because at low temperatures the interaction of non polar amino acids with water is favored (Tsai et al., 2002). To determine the effect of high pressure and of the association of high pressure and low temperature on the solubilization of aggregates, a suspension of CTB IB was subjected to increasing pressure levels and the dissociation of the aggregates was monitored by determination of the light scattering. Fig. 3 shows that the value of light scattering decreases at the higher pressures, indicating dissociation of the IB, an effect that is further improved at negative temperature, down to −9 °C.

The analysis of the suspension subjected to 2.4 kbar at 20 °C for 16 h shows that 64% of CTB was solubilized, while 36% of CTB was present in the insoluble fraction (data not shown), either because a fraction of the insoluble aggregates were not dissociated at this pressure level, or because of the reaggregation that can possibly occur after decompression. The value of light scattering was reduced at −9 °C and 2.4 kbar (Fig. 3) compared to the value obtained at 20 °C, indicating that the aggregates are further dissociated. Despite of this fact, the yield of CTB refolding at 2.4 kbar was not improved by dissociation at −9 °C, 0 °C or 10 °C (data not shown), possibly because the reduction of temperature induces the formation of misfolded states with lower capacity to achieve the correct folding.

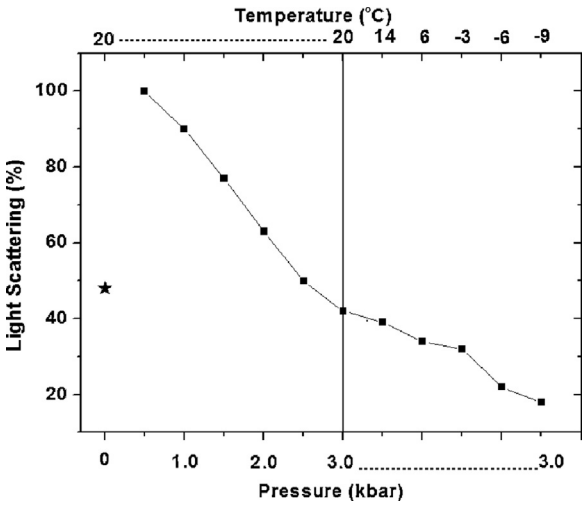

Fig. 3. Dissociation of CTB IB under HHP and low temperatures. The light scattering (LS) of the suspension of IB of CTB in buffer 50 mM Tris–HCl, 1 mM EDTA and 1 mM Tween 20, pH 8.5 (■) were followed during pressure and temperature variation. The values were collected after pressure and temperature stabilization. Values of LS after return to 20 °C and 1 bar (⋆).

3.4. Effect of schemes of compression/decompression on the refolding of CTB

In order to improve the yield of refolded pentameric CTB from suspensions of CTB IB, the compression/decompression schemes 1–6 presented in the legend of Fig. 4 were analyzed (Fig. 4A and B). The incubation for 1.5 h at 2.4 kbar (schemes 1, 3–6), was effective to disaggregate IB (Fig. 3) however incubation at this pressure for a longer time, 16 h (scheme 2), increased the yield of soluble monomeric CTB. Thus, in order to generate higher concentration of soluble monomeric forms, likely able to interact for the oligomerization to the native pentamer, the compression for 16 h at 2.4 kbar was utilized for the subsequent assays. Incubation at the intermediary pressures of 0.8 kbar (scheme 4), 0.4 kbar (scheme 5) or 0.2 kbar (scheme 6) was not useful for increasing the concentration of neither CTB monomers nor pentamers. The application of 2.4 kbar for 1.5 h and incubation at 1 bar for 16 h (scheme 1) was the most effective scheme to obtain pentameric CTB, which suggests that the formation of oligomers occurs preferentially at atmospheric pressure. Therefore, incubation at atmospheric pressure during longer periods of time was subsequently analyzed.

3.5. Effect of incubation at atmospheric pressure after compression of CTB IB

To determine the effect of incubation at low pressure in the formation of the CTB pentamer, a suspension of IB was subjected to 2.4 kbar for 16 h and incubated for periods of time ranging from 0 h to 168 h at atmospheric pressure.

Incubation for 96 h and higher periods of time at atmospheric pressure is necessary for achievement of pentameric CTB. The suspension of IB that was not incubated at 1 bar and the one that was incubated for 24 h at this condition (Fig. 5, lanes 1 and 2 respectively) only present monomers and other forms of intermediate molecular mass between the CTB monomer and pentamer. In contrast, the presence of a band corresponding to the CTB pentamer is present in the supernatant of the suspensions that were incubated for longer periods at atmospheric pressure (lanes 3–6). Our results are in agreement with those described for the refolding of another oligomeric protein, the trimeric protein tailspike, which was described to occur during incubation at atmospheric pressure

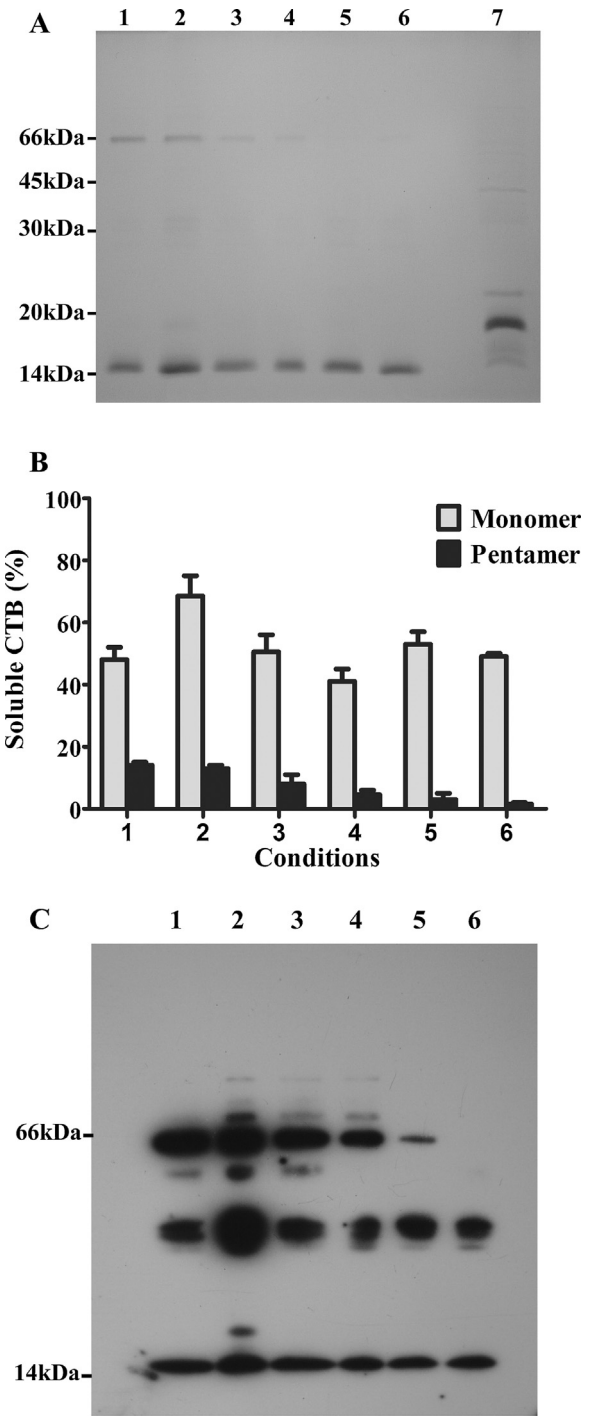

Fig. 4. Effect of different schemes of compression and decompression on the solubilization of CTB from IB suspensions. The buffer used was 50 mM Tris–HCl, 1 mM EDTA and 1 mM Tween 20, pH 8.5. (A) The samples were subjected to HHP on the following schemes: (1) 2.4 kbar for 1.5 h, direct decompression and incubation at 1 bar for 16 h; (2) 2.4 kbar for 16 h and direct decompression; (3) 2.4 kbar for 1.5 h, step decompression and incubation at 1 bar for 16 h; (4) 2.4 kbar for 1.5 h, decompression to 0.8 kbar and incubation for 16 h and direct decompression; (5) 2.4 kbar for 1.5 h, decompression to 0.4 kbar and incubation for 16 h and direct decompression (6) 2.4 kbar for 1.5 h, decompression to 0.2 kbar and incubation for 16 h and direct decompression; (7) suspension of IB. (B) Quantitative analysis of the samples shown in A in comparison to the amount of the insoluble CTB (lane 7 of the (A). Error bars: mean ± SEM. Statistical significance was determined using two way anova with Bonferroni post test. The percentage of monomer solubilized in condition 2 is statistically different from the all other conditions ($P < 0.01$). (C) Western blot analysis of the schemes of compression/decompression shown as in (A). The results shown are representative of three experiments.

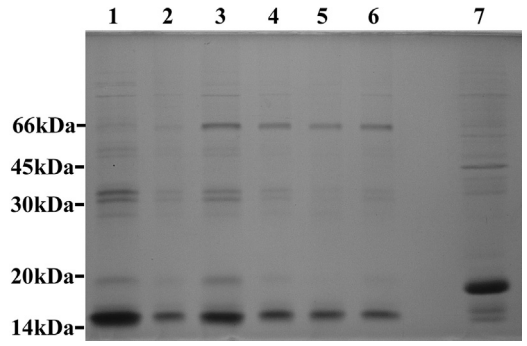

Fig. 5. Effect of incubation for different times at 1 bar at 20 °C after compression of the CTB IB at 2.4 kbar for 16 h. After incubation at atmospheric pressure, a fraction of the suspension was centrifuged and the supernatant was frozen for subsequent analysis. Lane 1, suspension not incubated at 1 bar; lane 2, suspension incubated at 1 bar for 24 h; lane 3, suspension incubated at 1 bar for 96 h; lane 4, suspension incubated at 1 bar for 120 h; lane 5, suspension incubated at 1 bar for 144 h; lane 6, suspension incubated at 1 bar for 168 h; lane 7, suspension of IB (reducing condition). The results shown are representative of two experiments.

after dissociation of the aggregates at high pressure (Foguel et al., 1999; Lefebvre et al., 2004).

3.6. Effect of protein concentration in the refolding of CTB

To determine whether the concentration of the dissociated monomers affects CTB refolding yield, suspensions of IB containing 30 μg/ml to 1920 μg/ml of CTB were subjected to high-pressure refolding (Fig. 6). The raise in the CTB concentration to 960 μg/ml increased 2.6-fold the refolding yield, in comparison with the concentrations used in the previous tests (120 μg/ml), rendering the higher percentage of pentameric CTB: 45.6%, indicating that the oligomerization of CTB can be optimized at higher concentration of monomers, likely induced by a higher number of productive contacts among them.

3.7. CTB IB

Scanning electron microscopy of the IB indicate a high concentration of IB aggregates (Fig. 7A). The HHP treatment of the suspension of the IB was very effective to reduce the amount of IB,

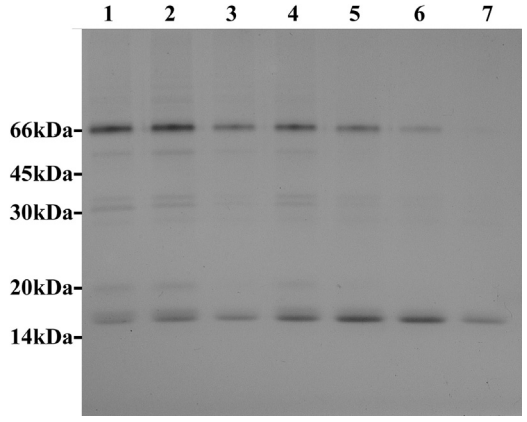

Fig. 6. Effect of the protein concentration in the yield of CTB refolding. The suspensions were subjected to 2.4 kbar for 16 h and 96 h at 1 bar. The buffer for refolding was 50 mM Tris–HCl, 1 mM EDTA and 1 mM Tween 20, pH 8.5. The volumes applied to the SDS-PAGE were normalized. Lane 1, suspension refolded at 1920 μg CTB/ml, lane 2, suspension refolded at 960 μg CTB/ml; lane 3, suspension refolded at 480 μg CTB/ml; lane 4, suspension refolded at 240 μg CTB/ml; lane 5, suspension refolded at 120 μg CTB/ml; lane 6, suspension refolded at 60 μg CTB/ml, lane 7, suspension refolded at 30 μg CTB/ml.

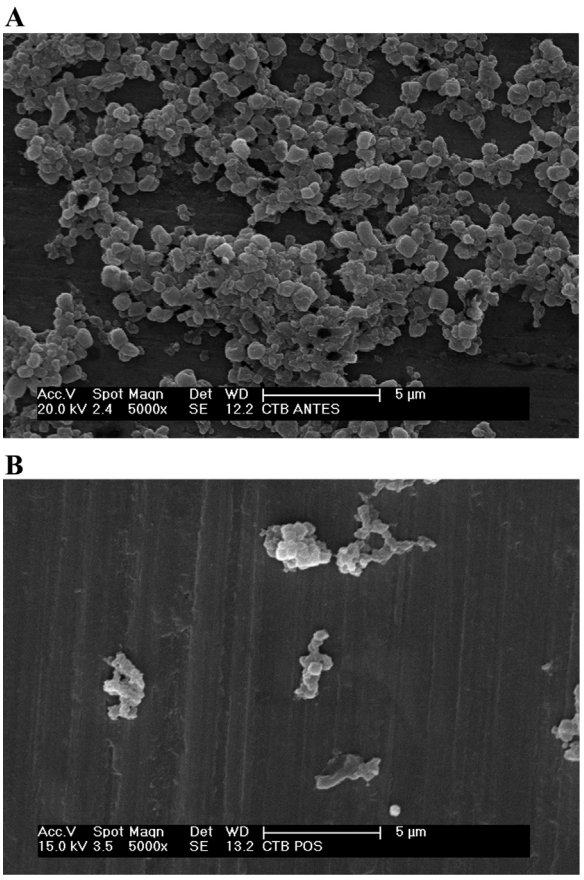

Fig. 7. Scanning electron microscopy of the IB. (A) Scanning electronic microscopy of the dried CTB IB; (B) scanning electronic microscopy of the structures that remain insoluble after HHP treatment. The insoluble material was dialyzed against water to withdraw the Tris HCl buffer, in order to avoid the formation of Tris crystals that interfere with the visualization of the IB. Scale: 5 µm.

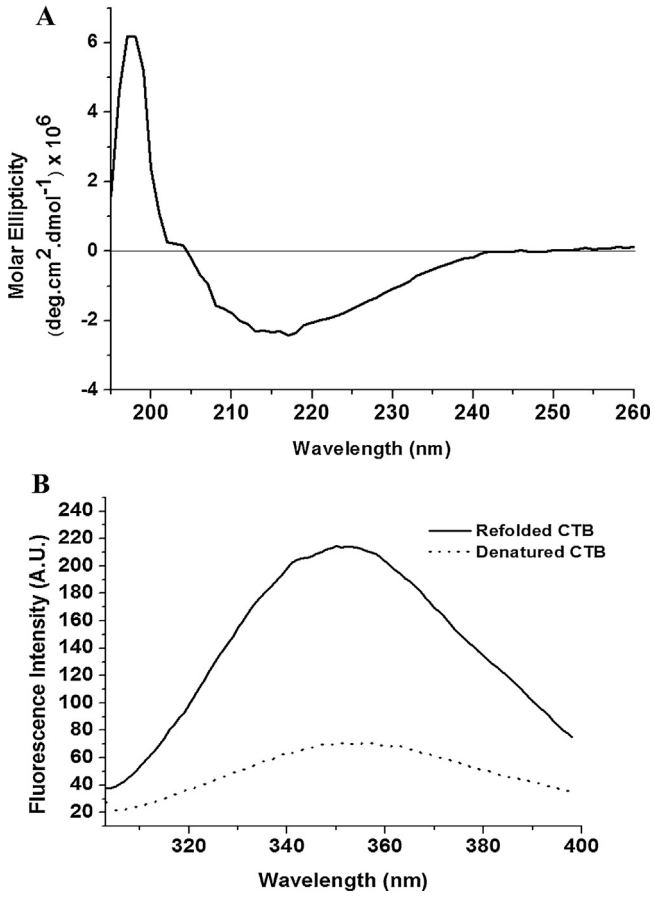

Fig. 8. Spectra of refolded and purified CTB. (A) Spectrum of circular dichroism; (B) spectra of intrinsic fluorescence of refolded CTB (continuous line) and CTB denatured by incubation in the presence of 8 M urea for 2 h (dotted line). Excitation at 288 nm. CTB refolded at HHP was purified in IMAC column and the pentamer was separated from the monomeric form by size-exclusion chromatography.

as verified by the microscopy of the insoluble fraction of the suspensions that had been subjected to high pressure (Fig. 7B), showing again that this treatment was effective in the dissociation of the aggregates. The re-pressurization of the remaining aggregates that were previously subjected to high pressure rendered low yields (4.5%) of pentameric CTB (data not shown).

3.8. Characterization of the refolded CTB

CTB refolded using high hydrostatic pressure was purified by immobilized $Ni2^+$ affinity chromatography and by size exclusion chromatography (Superdex 75 10/300 GL) to separate the pentamer from the monomer and other forms. The characterization of the secondary structure of the purified pentameric form of CTB was achieved by circular dichroism (Fig. 8A). The refolded protein showed a profile of a structured protein with a profile very similar to the previously published, with a negative peak at 215 nm (Dertzbaugh et al., 1990).

Intrinsic fluorescence emission of Trp was used for the analysis of the tertiary structure of refolded and purified pentameric form of CTB (Fig. 8B). Each monomer of CTB has one residue (W88), totaling five Trp in the pentameric structure. The fluorescence spectrum of the refolded CTB showed an emission maximum at 350 nm, in agreement with spectra for native CTB reported (Mertz et al., 1996). The sample denatured in the presence of 8 M urea showed a red shift in the maximum emission of fluorescence to 354 nm, by increased exposure of Trp to the aqueous solvent that indicates protein denaturation. These data confirm the efficacy of the refolding process.

3.9. Biological activity of CTB

The analysis of the functionality of refolded CTB, in comparison with a denatured sample, was performed by an ELISA assay that determines the binding of the protein to the GM1 receptor. The ability of the refolded CTB to bind to the GM1 receptor was shown

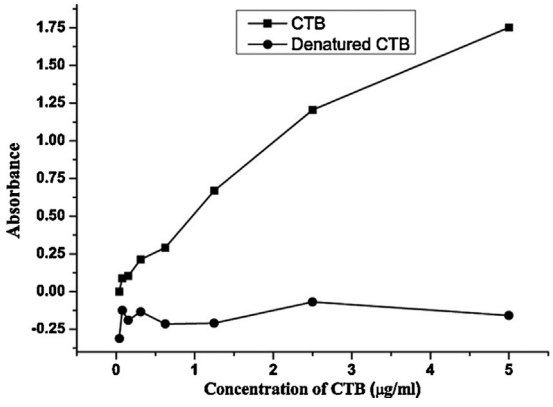

Fig. 9. Functional analysis of CTB. CTB refolded at HHP was purified as described. The ability of the CTB pentamers to bind to the GM1 receptor coated on plates was assessed by ELISA. The results shown are representative of five experiments.

to be proportional to the protein concentration (Fig. 9), while the denatured protein did not bind to the immobilized GM1 receptor. Therefore, the refolded CTB was shown to present biological activity.

4. Conclusions

This article describes the dissociation of CTB from IB aggregates using high hydrostatic pressure and refolding of this pentameric protein at atmospheric pressure. High hydrostatic pressure was effective in the dissociation of the IB CTB. The fact that the oligomerization of CTB is optimized in the presence of the surfactants Triton X100 and specially Tween 20, indicates that other concentrations of Tween or the presence of other surfactants can have a positive effect on the oligomerization of CTB. The incubation at 2.4 kbar for 16 h and direct decompression to 1 bar yielded the highest level of monomeric protein and incubation for longer periods at atmospheric pressure was essential to increase the yield of pentameric CTB. Another important factor to obtain high yields of the pentameric form of CTB was the incubation at high (960 μg/ml) protein concentration. Purification of CTB and separation of the forms generated after the high pressure treatment has proved troublesome due to the presence of monomeric forms together with the pentamers of interest. Therefore, two purification steps were necessary to obtain pentameric CTB with high purity.

Circular dichroism and intrinsic tryptophan fluorescence have demonstrated that CTB refolded at high hydrostatic pressure has regular secondary and tertiary structure. In addition, CTB refolded at high hydrostatic pressure showed biological activity.

The final yield of refolded CTB from the aggregated protein was very high: 213 mg pentameric CTB/liter of culture (up to 59%). The experience gained in this study can be advantageous for refolding of proteins with quaternary structure, a difficult task to achieve by other methods.

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References

Arakawa, T., Chong, D.K.X., Langridge, W.H.R., 1998. Efficacy of a food plant-based oral cholera toxin B subunit vaccine. Nat. Biotechnol. 16, 292–297.

Areas, A.P.D., de Oliveira, M.L.S., Ramos, C.R.R., Sbrogio-Almeida, M.E., Raw, I., Ho, P.L., 2002. Synthesis of cholera toxin B subunit gene: cloning and expression of a functional 6XHis-tagged protein in *Escherichia coli*. Protein Expr. Purif. 25, 481–487.

Areas, A.P.M., Oliveira, M.L.S., Miyaji, E.N., Leite, L.C.C., Aires, K.A., Dias, W.O., Ho, P.L., 2004. Expression and characterization of cholera toxin B – pneumococcal surface adhesin A fusion protein in *Escherichia coli*: ability of CTB-PsaA to induce humoral immune response in mice. Biochem. Biophys. Res. Commun. 321, 192–196.

Arimitsu, H., Tsukamoto, K., Ochi, S., Sasaki, K., Kato, M., Taniguchi, K., Oguma, K., Tsuji, T., 2009. Lincomycin-induced over-expression of mature recombinant cholera toxin B subunit and the holotoxin in *Escherichia coli*. Protein Expr. Purif. 67, 96–103.

Bam, N.B., Cleland, J.L., Randolph, T.W., 1996. Molten globule intermediate of recombinant human growth hormone: stabilization with surfactants. Biotechnol. Prog. 12, 801–809.

Bernardi, K.M., Forster, M.L., Lencer, W.I., Tsai, B., 2008. Derlin-1 facilitates the retrotranslocation of cholera toxin. Mol. Biol. Cell 19, 877–884.

Chura-Chambi, R.M., Cordeiro, Y., Malavasi, N.V., Lemke, L.S., Rodrigues, D., Morganti, L., 2013. An analysis of the factors that affect the dissociation of inclusion bodies and the refolding of endostatin under high pressure. Process Biochem. 48, 250–259.

Chura-Chambi, R.M., Genova, L.A., Affonso, R., Morganti, L., 2008. Refolding of endostatin from inclusion bodies using high hydrostatic pressure. Anal Biochem. 379, 32–39.

de Groot, N.S., Ventura, S., 2006. Effect of temperature on protein quality in bacterial inclusion bodies. FEBS Lett. 580, 6471–6476.

Dertzbaugh, M.T., Peterson, D.L., Macrina, F.L., 1990. Cholera-toxin B-subunit gene fusion – structural and functional-analysis of the chimeric protein. Infect. Immun. 58, 70–79.

Foguel, D., Robinson, C.R., de Sousa Jr., P.C., Silva, J.L., Robinson, A.S., 1999. Hydrostatic pressure rescues native protein from aggregates. Biotechnol. Bioeng. 63, 552–558.

Fraga, T.R., Chura-Chambi, R.M., Goncales, A.P., Morais, Z.M., Vasconcellos, S.A., Morganti, L., Martins, E.A.L., 2010. Refolding of the recombinant protein OmpA70 from *Leptospira interrogans* from inclusion bodies using high hydrostatic pressure and partial characterization of its immunological properties. J. Biotechnol. 148, 156–162.

Fujinaga, Y., Wolf, A.A., Rodighiero, C., Wheeler, H., Tsai, B., Allen, L., Jobling, M.G., Rapoport, T., Holmes, R.K., Lencer, W.I., 2003. Gangliosides that associate with lipid rafts mediate transport of cholera and related toxins from the plasma membrane to endoplasmic reticulm. Mol. Biol. Cell 14, 4783–4793.

Hamorsky, K.T., Kouokam, J.C., Bennett, L.J., Baldauf, K.J., Kajiura, H., Fujiyama, K., Matoba, N., 2013. Rapid and scalable plant-based production of a cholera toxin B subunit variant to aid in mass vaccination against cholera outbreaks. PLoS Negl. Trop. Dis. 7, 1–15.

Holmgren, J., 1981. Actions of cholera-toxin and the prevention and treatment of cholera. Nature 292, 413–417.

Jaenicke, R., Lilie, H., 2000. Folding and association of oligomeric and multimeric proteins. Adv. Protein Chem. 53, 329–401.

Jensen, E.B., Carlsen, S., 1990. Production of recombinant human growth hormone in *Escherichia coli*: expression of different precursors and physiological effects of glucose, acetate, and salts. Biotechnol. Bioeng. 36, 1–11.

Lefebvre, B.G., Gage, M.J., Robinson, A.S., 2004. Maximizing recovery of native protein from aggregates by optimizing pressure treatment. Biotechnol. Prog. 20, 623–629.

Lencer, W.I., Tsai, B., 2003. The intracellular voyage of cholera toxin: going retro. Trends Biochem. Sci. 28, 639–645.

Lhoir, C., Renard, A., Martial, J.A., 1990. Expression in *Escherichia coli* of 2 mutated genes encoding the cholera toxin-B subunit. Gene 89, 47–52.

Malavasi, N.V., Foguel, D., Bonafe, C.F.S., Braga, C.A.C.A., Chura-Chambi, R.M., Vieira, J.M., Morganti, L., 2011. Protein refolding at high pressure: optimization using eGFP as a model. Process Biochem. 46, 512–518.

Malley, R., Morse, S.C., Leite, L.C.C., Areas, A.P.M., Ho, P.L., Kubrusly, F.S., Almeida, I.C., Anderson, P., 2004. Multiserotype protection of mice against pneumococcal colonization of the nasopharynx and middle ear by killed nonencapsulated cells given intranasally with a nontoxic adjuvant. Infect. Immun. 72, 4290–4292.

Mekalanos, J.J., Swartz, D.J., Pearson, G.D.N., Harford, N., Groyne, F., Dewilde, M., 1983. Cholera-toxin genes – nucleotide-sequence, deletion analysis and vaccine development. Nature 306, 551–557.

Merritt, E.A., Sarfaty, S., Vandenakker, F., Lhoir, C., Martial, J.A., Hol, W.G.J., 1994. Crystal-structure of cholera-toxin B-pentamer bound to receptor G(M1) pentasaccharide. Protein Sci. 3, 166–175.

Mertz, J.A., McCann, J.A., Picking, W.D., 1996. Fluorescence analysis of galactose, lactose, and fucose interaction with the cholera toxin B subunit. Biochem. Biophys. Res. Commun. 226, 140–144.

Miyata, T., Harakuni, T., Taira, T., Matsuzaki, G., Arakawa, T., 2012. Merozoite surface protein-1 of *Plasmodium yoelii* fused via an oligosaccharide moiety of cholera toxin B subunit glycoprotein expressed in yeast induced protective immunity against lethal malaria infection in mice. Vaccine 30, 948–958.

Mozhaev, V.V., Heremans, K., Frank, J., Masson, P., Balny, C., 1996. High pressure effects on protein structure and function. Proteins 24, 81–91.

Nochi, T., Takagi, H., Yuki, Y., Yang, L.J., Masumura, T., Mejima, M., Nakanishi, U., Matsumura, A., Uozumi, A., Hiroi, T., Morita, S., Tanaka, K., Takaiwa, F., Kiyono, H., 2007. Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. Proc. Natl. Acad. Sci. U.S.A. 104, 10986–10991.

Peltola, H., Siitonen, A., Kyronseppa, H., Simula, I., Mattila, L., Oksanen, P., Kataja, M.J., Cadoz, M., 1991. Prevention of travelers diarrhea by oral B-subunit whole-cell cholera vaccine. Lancet 338, 1285–1289.

Qadri, F., Wenneras, C., Ahmed, F., Asaduzzaman, M., Saha, D., Albert, M.J., Sack, R.B., Svennerholm, A.M., 2000. Safety and immunogenicity of an oral, inactivated enterotoxigenic *Escherichia coli* plus cholera toxin B subunit vaccine in Bangladeshi adults and children. Vaccine 18, 2704–2712.

Qoronfleh, M.W., Hesterberg, L.K., Seefeldt, M.B., 2007. Confronting high-throughput protein refolding using high pressure and solution screens. Protein Expr. Purif. 55, 209–224.

Qu, D., Zheng, B.J., Yao, X., Guan, Y., Yuan, Z.H., Zhong, N.S., Lu, L.W., Xie, J.P., Wen, Y.M., 2005. Intranasal immunization with inactivated SARS-CoV (SARS-associated coronavirus) induced local and serum antibodies in mice. Vaccine 23, 924–931.

Quiding, M., Nordstrom, I., Kilander, A., Andersson, G., Hanson, L.A., Holmgren, J., Czerkinsky, C., 1991. Intestinal immune-responses in humans –oral cholera vaccination induces strong intestinal antibody-responses and interferon-gamma production and evokes local immunological memory. J. Clin. Invest. 88, 143–148.

Sanchez, J., Holmgren, J., 2011. Cholera toxin – a foe & a friend. Indian. J. Med. Res. 133, 153–163.

Shukla, D., Schneider, C.P., Trout, B.L., 2011. Molecular level insight into intra-solvent interaction effects on protein stability and aggregation. Adv. Drug. Deliv. Rev. 63, 1074–1085.

Silva, J.L., Cordeiro, Y., Foguel, D., 2006. Protein folding and aggregation: two sides of the same coin in the condensation of proteins revealed by pressure studies. Biochim. Biophys. Acta 1764, 443–451.

Slos, P., Dutot, P., Reymund, J., Kleinpeter, P., Prozzi, D., Kieny, M.P., Delcour, J., Mercenier, A., Hols, P., 1998. Production of cholera toxin B subunit in *Lactobacillus*. FEMS Microbiol. Lett. 169, 29–36.

Spangler, B.D., 1992. Structure and function of cholera-toxin and the related *Escherichia coli* heat-labile enterotoxin. Microbiol. Rev. 56, 622–647.

St John, R.J., Carpenter, J.F., Randolph, T.W., 1999. High pressure fosters protein refolding from aggregates at high concentrations. Proc. Natl. Acad. Sci. U.S.A. 96, 13029–13033.

Tsai, C.J., Maizel Jr., J.V., Nussinov, R., 2002. The hydrophobic effect: a new insight from cold denaturation and a two-state water structure. Crit. Rev. Biochem. Mol. Biol. 37, 55–69.

Vera, A., Gonzalez-Montalban, N., Aris, A., Villaverde, A., 2007. The conformational quality of insoluble recombinant proteins is enhanced at low growth temperatures. Biotechnol. Bioeng. 96, 1101–1106.

Visudtiphole, V., Thomas, M.B., Chalton, D.A., Lakey, J.H., 2005. Refolding of *Escherichia coli* outer membrane protein F in detergent creates LPS-free trimers and asymmetric dimers. Biochem. J. 392, 375–381.

Wang, H., Andersen, K.K., Vad, B.S., Otzen, D.E., 2013. OmpA can form folded and unfolded oligomers. Biochim. Biophys. Acta Proteins Proteomics 1834, 127–136.