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The solubility of recombinant proteins expressed in *Escherichia coli* is increased by *otsA* and *otsB* co-transformation

Tina Schultz [a], Jing Liu [a,b], Paola Capasso [c], Ario de Marco [a,c,*]

[a] *EMBL Scientific Core Facilities, Meyerhofstr. 1, D-69117 Heidelberg, Germany*
[b] *Department of Biological Engineering, School of Chemical Engineering & Technology, Tianjin University, Tianjin 300072, PR China*
[c] *IFOM-IEO Campus, Biochemistry Unit, via Adamello 16, I-20139 Milano, Italy*

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Abstract

The osmolyte trehalose strongly limits protein aggregation both *in vitro* and *in vivo*. The addition of trehalose to the culture medium reduced the aggregation of recombinant proteins expressed in *Escherichia coli* in a concentration-dependent manner. Comparable positive effects were obtained when the host bacteria were engineered to overexpress the gene products of *otsA* and *otsB*, the two enzymes involved in trehalose synthesis. Apparently, the osmolyte preserves protein monodispersion rather than directly facilitating protein folding. However, the stabilization of the protein folding intermediate(s) resulted in higher yields of native proteins and aggregates of lower complexity. Other osmolytes have been tested *in vitro* in comparison with trehalose. Di-myo-inositol1,1′-phosphate (DIP) seems to be a good candidate to test in *in vivo* applications, although the opportunity of using *otsA*/*B* overexpressing cells is simpler and less expensive.

Keywords: Recombinant expression; Protein aggregation probe; Aggregation index; Trehalose; Di-myo-inositol1,1′-phosphate; Hydroxyectoine

The expression of recombinant proteins remains a trial-and-error matter. Despite the success in improving the solubility of some proteins proved by several different protocols [1,2], no method universally successful can be envisaged, due to the protein heterogeneity. Therefore, a preferable approach is the comparison of many different expression conditions at a preliminary screening step to identify the most promising strategy to use for up-scaling [3–5].

Osmolytes are small molecules known to stabilize the cell environment *in vivo* during stress conditions [6,7] and the addition of osmolytes was beneficial to protein stability in experimental *in vivo* and *in vitro* conditions [8–11]. In particular, the biotechnological applications of the sugar trehalose have been described in relation to the possibility of conferring desiccation tolerance in plants and cells, inhibiting huntingtin protein aggregation, and protect protein denaturation during heat stress conditions [6,7,9–14]. These results stimulated our interest in using trehalose as a positive effector to reduce aggregation during the expression of recombinant proteins.

In the last years, the development of new methodologies allowed for the quantitative study of the protein aggregation and the achievement of a more detailed picture of the aggregate complexity [15–17]. Soluble and insoluble aggregates of different complexity have been separated and their biochemical features have been described, native activity has been found in large, insoluble precipitates, and the *in vivo* re-conversion of aggregates into soluble proteins has been exploited for biotechnological aims [4,15–19]. In this paper, we present the results describing the anti-aggregation effects of trehalose evaluated by fluorimetric assay, protein separation on sucrose-gradient [15–17], and using

* Corresponding author. Present address: IFOM-IEO Campus, Biochemistry Unit, via Adamello 16, I-20139 Milano, Italy. Fax: +39 02 574 303 231.
E-mail address: ario.demarco@ifom-ieo-campus.it (A.de Marco).

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a reporter-probe for monitoring protein aggregation *in vivo* [20]. Three model proteins with different solubility features (ClipA5, GST-GFP, and GST) were compared.

Materials and methods

Preparation of the otsA–otsB expression vector. The bicistronic plasmid containing the *otsA* (trehalose-6-phosphate synthase) and *otsB* (trehalose-6-phosphate phosphatase) cDNAs was prepared starting from a CDFDuet-1 vector (Novagen). Five microliters of top 10 *Escherichia coli* cells was cultured at 37 °C until the OD_{600} of 0.4 and then incubated for 4 h at 16 °C to induce the *otsA–otsB* mRNA accumulation [21]. After pelleting, total RNA was isolated using the NucleoSpin RNAII Total RNA isolation kit (Macherey-Nagel), analyzed for its integrity and cDNA was amplified by reverse transcriptase PCR using the Smart™ Race cDNA amplification kit (BD Bioscience), according to the manufacturers' instructions. The resulting cDNA was used as a template in a PCR performed to generate the constructs corresponding to *otsA* and *otsB*. The following primers were used: (*otsA* fw) 5′-gtctgcagacatgagtcgtttggtcgta-3′; (*otsA* rev) 5′-gcgcggccgctcactacacaagctttggaaaggt-3′; (*otsB* fw) 5′-taca tatgcgatgacagaaccgttaacc-3′; and (*otsB* rev) 5′-ctctcgagctattagatgctacgacta aatga-3′. The two products were successively ligated into the CDFDuet-1 vector using the pairs of restriction sites *Not*1 and *Pst*1 (*otsA*), and *Nde*1 and *Xho*1 (*otsB*). Their correct insertion was tested by digestion and sequencing. The bicistronic plasmid was finally transformed and the resulting bacteria was made competent for being co-transformed with the vector harboring the sequence corresponding to the target proteins.

Cell culture, sucrose gradients, and protein purification. The estimation of the *in vivo* aggregation was performed using bacteria co-transformed with the plasmid pHK57 containing the *ibpB*-promoter β-galactosidase fusion [22] and one of the plasmids expressing ClipA5 (EAA00427), GST, or the GST-GFP construct [23]. Glycerol stocks (20%) were frozen and used to inoculate overnight cultures which grew at 30 °C in the presence of 1% glucose. The pre-culture was diluted 1:100 in flasks containing 10 mL of Lauria Bertani medium incubated at 37 °C in an orbital shaker. The temperature was lowered to 20 °C at a bacterial $OD_{600} = 0.2$ and trehalose (5 or 50 mM final concentration) and 0.4 M NaCl were added. The recombinant expression was induced by IPTG (0.1 mM) after further 45 min. The cells were pelleted 90 min after induction and used for β-galactosidase activity measurement.

Large scale purification of GST-GFP cultured overnight at 20 °C was performed starting from a 1 L culture medium pellet. The pellet was first lysated in 50 mM Tris–HCl, pH 8.0, 0.5 M NaCl, 5 mM $MgCl_2$, 1 mg/mL lysozyme, and 10 μg/mL DNase, sonicated in a water bath (Branson 200) for 5 min and the lysate was incubated for 30 min on a shaker at room temperature. The soluble fraction was recovered by ultracentrifugation of the total lysate (35 min at 115,000*g*) and the protein was purified by metal affinity chromatography [17] using a HiTrap column and a FPLC equipment (GE Healthcare). The purified protein was used for *in vitro* assays in the presence of trehalose (Sigma), betaine (Sigma), hydroxyectoine, or di-myo-inositol1,1′-phosphate (DIP, both kindly provided by bitop AG).

Total cell lysates (1 mL) was loaded onto 14 × 95 mm Ultra-Clear centrifuge tubes (Beckman) prepared with a step gradient formed by four layers of 20 mM Tris–HCl buffer, pH 8, containing 80%, 70%, 50%, and 30% sucrose, respectively. The tubes were centrifuged 15 h at 180,000*g* at 4 °C using a SW40Ti rotor and a L-70 Beckman ultracentrifuge.

Protein aggregation estimation in vivo and *in vitro*. The β-galactosidase activity was measured as explained previously in detail [20] and used to estimate the aggregation level *in vivo*.

The aggregation rate of the proteins was analyzed *in vitro* according to Nominé et al. [15] using an AB2 Luminescence Spectrometer (Aminco Bowman Series 2) equipped with SLM 4 software. The excitation was induced at 280 nm and the emission scan was recovered between 260 and 400 nm. The aggregation index is given by the ratio between the absorbance at 280 and 340 nm.

Circular dichroism. The modifications of the protein secondary structure were monitored by means of circular dichroism (CD) spectra recorded between 250 and 190 nm using suprasil precision cells (Hellma) and a Jasco J-710 instrument.

Results

ClipA5 is a secreted protein that needs an oxidative environment to correctly fold. However, the degree of its aggregation is dependent on the expression rate and can be influenced by culture factors [20]. The amount of ClipA5 aggregates *in vivo* can be estimated by using a β-galactosidase reporter under the control of an *ibpB* promoter. Protein aggregates specifically activate the promoter [22] and the β-galactosidase construct down-stream is expressed. The aggregate relative concentration in cell transformed with the reporter is estimated by measuring the β-galactosidase activity. The system enabled to evaluate the effect of culture modifications on recombinant protein solubility [20].

ClipA5 was expressed using three vectors that differ for expression rate [20]. As expected, the β-galactosidase reporter indicated variable aggregation levels inside the cells. Cells transformed with pTrcHis2-ClipA5 and pKK223-ClipA5 accumulated moderate amounts of aggregates and the addition of 5 mM trehalose to the culture medium was sufficient to further reduce them (Fig. 1). Increasing the trehalose concentration to 50 mM resulted in minimal solubility gain. In contrast, cells transformed with the pQE30 vector accumulated high amounts of ClipA5 aggregates and 5 mM trehalose was ineffective in reducing aggregation (Fig. 1), but 50 mM trehalose was very beneficial (Fig. 1).

This first experiment showed that the addition of trehalose to the culture medium was effective in increasing the monodispersity of an aggregation-prone protein. Next, we wished to follow the trehalose effect on the complexity of protein aggregates using the fluorescent construct

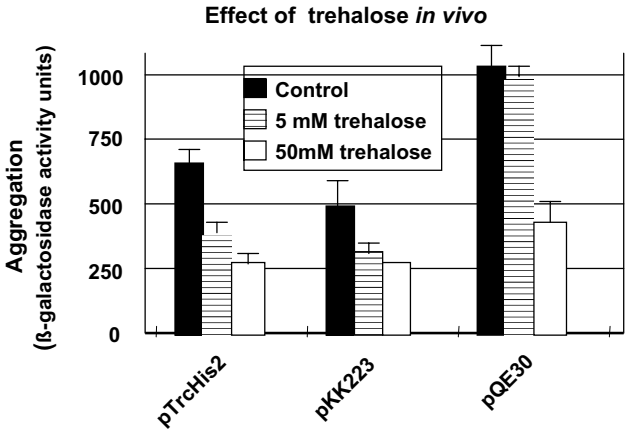

Fig. 1. Trehalose addition to the culture medium reduces the *in vivo* accumulation of protein aggregates. Three plasmids with different expression rates were compared. ClipA5 was expressed in bacteria co-transformed with an *ibpB*-promoter-β-galactosidase aggregation reporter plasmid and 5 or 50 mM trehalose, respectively, were added to the medium 45 min before expression induction of the recombinant protein.

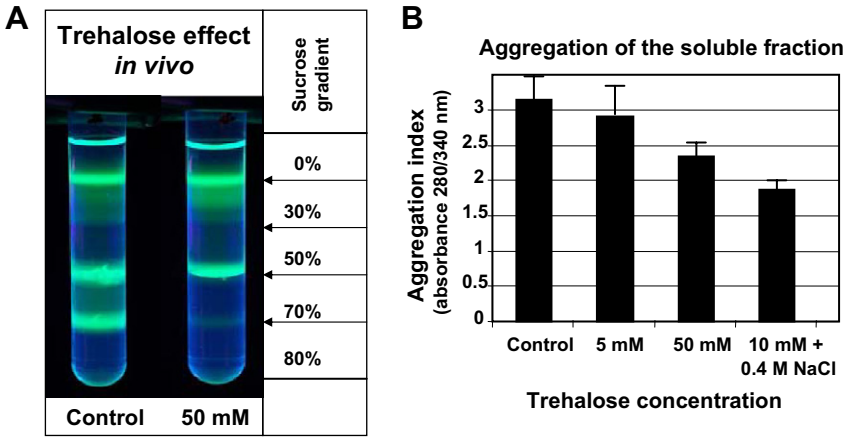

Fig. 2. Trehalose reduces the mass of GST-GFP insoluble aggregates and increases the monodispersity of GST-GFP soluble fraction. (A) The GST-GFP aggregates resulting from recombinant expression in bacteria cultured in LB medium or in the same medium supplemented with 50 mM trehalose were separated onto a sucrose gradient. (B) GST-GFP purified from the soluble fractions of bacteria cultured in media supplemented with different concentrations of trehalose and salt were analyzed at the fluorimeter for determining their aggregation index.

GST-GFP. This fusion protein is expressed in both soluble and insoluble form when transformed bacteria were grown at temperatures between 20 and 30 °C [17].

The addition of trehalose to cell culture increased the solubility measured *in vivo* by the β-galactosidase reporter (Fig. 4). The trehalose addition effect on the GST-GFP aggregate mass was further investigated by loading the total lysate of GST-GFP expressing cells onto a sucrose gradient (Fig. 2A). The protein accumulated at the interfaces 50–70% and 70–80% have been previously characterized as insoluble, with increasing aggregation complexity [17]. The addition of trehalose to the culture medium strongly reduced the accumulation of aggregates with higher density, namely with larger mass (Fig. 2A).

The highest visible band of the sucrose gradient corresponds to the soluble fraction after cell lysate ultra-centrifugation. In this fraction, there are both native protein and soluble aggregates and their ratio can be estimated by a fluorimetric assay [15,17]. Cells transformed with GST-GFP were cultured at 25 °C and the soluble GST-GFP was purified from the supernatant and analyzed for its content in soluble aggregates. The addition of 50 mM trehalose to the culture medium, or of a combination of trehalose (10 mM) and 0.4 M NaCl to improve the osmolyte uptake inside the cell (Fig. 2B), decreased the complexity of soluble aggregates.

These results showed that trehalose can contribute to stabilize the structure of recombinant proteins *in vivo*. Next, purified GST-GFP was heated either for 30 min at 42 °C or 10 min at 50 °C in the presence of increasing amounts of trehalose. The fluorimetric aggregation index clearly showed that trehalose prevented the protein to aggregate at 50 °C (Fig. 3A). The result was confirmed by circular dichroism using the GST-GFP fraction heated for 10 min at 50 °C in the presence of 900 mM trehalose (Fig. 3B). No alteration of the secondary structure was detectable in comparison with the control.

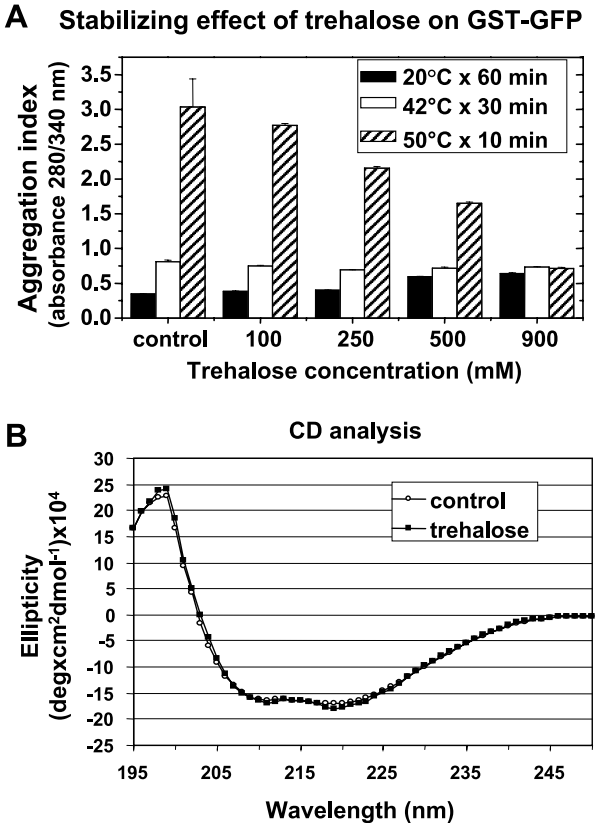

Fig. 3. Trehalose-dependent GST-GFP stabilization during *in vitro* heat treatments. (A) Concentration dependent effect of trehalose on the structural stabilization of heat-treated GST-GFP. The protein was first incubated in the presence of trehalose and then subdue to heating treatment. (B) CD analysis. The secondary structures of control GST-GFP and the same protein incubated in the presence of 900 mM trehalose and heated 10 min at 50 °C were compared.

Osmolytes are a large class of compounds and it was interesting to know whether the protective effect of trehalose was specific of this sugar or other osmolytes could

be even more efficient in preventing protein aggregation. Hydroxyectoine has been already proposed as a structure stabilizer and DIP has potentially useful features [8,9]. However, at least in our conditions, hydroxyectoine was inefficient (Table 1). In contrast, DIP scored even better than trehalose. The difference in protective potential between DIP and hydroxyectoine was confirmed when GST-GFP was destabilized in the presence of 5 mM betaine (Table 1). These results made DIP a good candidate for further analysis but, so far, its elevated cost of production impairs its *in vivo* application. Therefore, our efforts were focused on optimizing the trehalose alternative.

The efficiency of the trehalose uptake in the cells seems to be critical for its *in vivo* stabilizing activity (Fig. 2B). Instead of the external addition of the sugar to the culture medium, we thought that cytoplasm trehalose accumulation in the recombinant expressing cells could also be induced by the overexpression of the gene products of *otsA* and *otsB*, the two enzymes involved in the trehalose synthesis pathway [10]. Control bacteria, bacteria transformed with a bicistronic construct for OtsA and OtsB expression, and bacteria grown in the presence of 50 mM trehalose were compared for their capability of impairing the aggregation of three model proteins having different solubility. The cells co-transformed with *OtsA* and *OtsB* produced the lowest amount of aggregates with all the three different samples (Fig. 4). The positive results are an indication that an optimal cytoplasm concentration of trehalose was obtained. Therefore, the co-expression strategy resulted not only useful to control the aggregation of recombinant expressed proteins, but it was even more effective than the supplementation of the culture medium with trehalose.

Table 1
Stabilizing effect of DIP on GST-GFP during heat treatments

	Control	DIP		Hydroxyectoine	
		10 mM	100 mM	10 mM	100 mM
20 °C	0.2	0.1	0.1	0.2	0.1
42 °C	1.2	0.8	0.1	1.1	1.1
50 °C	3.2	2.4	1.3	3.0	2.5
20 °C + betaine	13.1	6.6	2.8	13.2	12.0

The GST-GFP construct was incubated in the presence of the osmolytes DIP and hydroxyectoine before being incubated at different combinations of time and temperature. Five millimolar betaine were added to increase the GST-GFP aggregation. The protein aggregation was estimated using the fluorimetric aggregation index (Abs 280/340 nm) and the values are the average of three independent measurements.

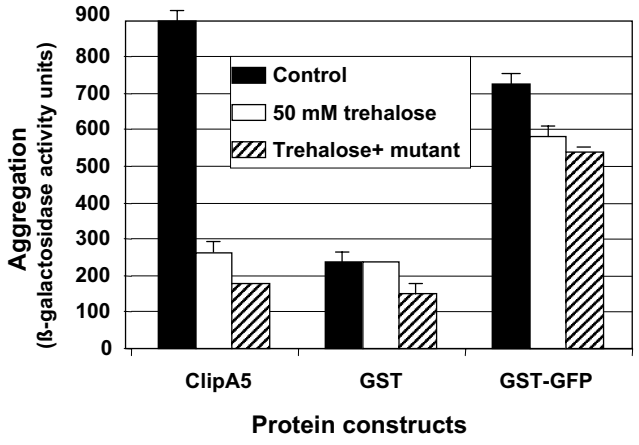

Fig. 4. Increased solubility of recombinant proteins expressed in bacteria overexpressing *otsA* and *otsB*. ClipA5, GST, and GST-GFP were overexpressed in control bacteria and bacteria either grown in media supplemented with 50 mM trehalose or co-expressing the genes *otsA* and *otsB* for boosting the accumulation of trehalose inside the cells.

Discussion

The results clearly indicate that trehalose can have a positive effect in preventing recombinant protein aggregation in *E. coli* and that its concentration appears critical. In the case of ClipA5, for instance, the use of different expression vectors allowed the modulation of the *in vivo* aggregate amount (Fig. 1). In the bacteria transformed with the vectors pTrcHis2 and pKK223 that accumulated lower level of aggregates, the addition of 5 mM of trehalose in the culture medium was sufficient to obtain a significant reduction of the aggregates. In contrast, in the presence of the higher aggregate concentration induced by pQE30 expression, only the supplement of 50 mM trehalose to the culture medium resulted effective.

The biophysical characterization of the specific trehalose–ClipA5 interaction is beyond the scope of this communication but the theories concerning the macromolecule stabilization by osmolytes consider that these molecules prevent aggregation by favoring the protein hydration and generating a pressure that compact the protein structure [24–26].

ClipA5 presents several disulfide bonds in its native structure and cannot be expressed folded in bacteria cytoplasm but trehalose appears capable to maintain it monodisperse *in vivo*. The trehalose function in impairing unfolded proteins to aggregate by preventing non-productive interactions was previously reported [14]. *In vivo*, the prolonged time in which proteins remain monodispersed increases the chances that they will find their native conformation, probably through collaboration between stabilizing osmolytes and folding chaperones [12,14]. Such a function is of evident biotechnological interest and seems to be confirmed in the case of the GST-GFP construct. This construct can fold but its folding efficiency is low and several parameters influence it [17,20]. Trehalose addition *in vivo* increased the solubility of the recombinant GST-GFP and even the complexity of the aggregate fractions was reduced in an osmolyte concentration-dependent manner. Both the largest insoluble aggregates disappeared and the monodispersity of the soluble fraction increased (Fig. 2).

Trehalose was also effective in maintaining the protein structure *in vitro*, showing that it can oppose to potential aggregation factors like heating (Fig. 3). The possibility

to use trehalose for increasing the thermo-tolerance of relevant enzymes would be technologically beneficial, as in the case of lipases [27]. However, our main interest remained the increment of the soluble recombinant protein yields from bacterial culture. The observation that the trehalose concentration was critical urged us to identify solutions to increase the osmolyte accumulation in the bacteria cytoplasm. The addition of NaCl to the culture medium was efficient in boosting other osmolyte uptake [28,30] and in combination with trehalose improved its anti-aggregation effect. As an alternative, bacteria were transformed for obtaining an overexpression of the *otsA* and *otsB* products, namely the enzymes necessary for the synthesis of trehalose. Analogous transformations were successfully used in plants and human cells [9,10,13]. Bacteria co-transformed with a bicistronic vector for the cytoplasm expression of OtsA and OtsB and with a vector expressing a target protein were more efficient in reducing the aggregation level of the target proteins than to control bacteria cultured in the presence of 50 mM trehalose (Fig. 4).

In conclusion, the supplement of trehalose seems to be another opportunity to add to the list of conditions that can reduce protein aggregation. Our results indicate that the effect is dependent on the target protein features (Fig. 4) and a case by case approach is suggested. Other osmolytes have been proved efficient in reducing the aggregation of some proteins in the past [6]. We have showed promising *in vitro* data using DIP (Table 1), an osmolyte that has a physiological stabilizing role in Archaea [29]. Parameters like product costs, solubility, possible engineering of the synthesis pathways and synergic effect between osmolytes and molecular chaperones [12,14,28,30] should be considered for choosing among the different osmolytes.

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