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

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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.
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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

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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

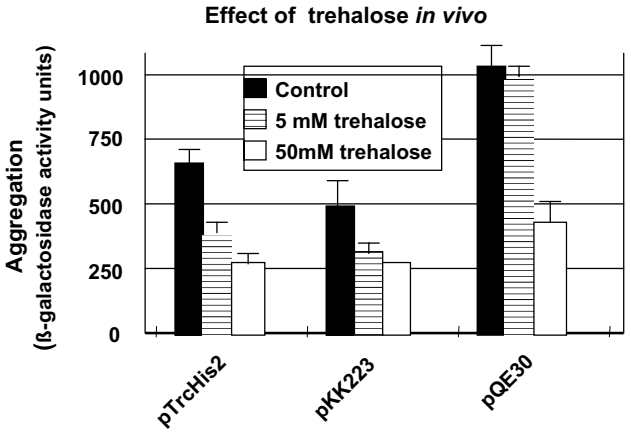

Fig. 1. Trehalose addition to the culture medium reduces the *in vivo* accumulation of protein aggregates. Three plasmids with different expression rates were compared. ClipA5 was expressed in bacteria co-transformed with an *ibpB*-promoter-β-galactosidase aggregation reporter plasmid and 5 or 50 mM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

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