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DnaK/DnaJ-assisted recombinant protein production in *Trichoplusia ni* larvae

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Abstract The DnaK/DnaJ *Escherichia coli* chaperone pair, co-produced along with recombinant proteins, has been widely used to assist protein folding in bacterial cells, although with poor consensus about the ultimate effect on protein quality and its general applicability. Here, we have evaluated for the first time these bacterial proteins as folding modulators in a highly promising recombinant protein platform based on insect larvae. Intriguingly, the bacterial chaperones enhanced the solubility of a reporter, misfolding-prone GFP, doubling the yield of recombinant protein that can be recovered from the larvae extracts in a production process. This occurs without negative effects on the yield of total protein (extractable plus insoluble), indicative of a proteolytic stability of the chaperone substrate. It is in contrast with what has been observed in bacteria for the same reporter protein, which is dramatically degraded in a DnaK-dependent manner. The reported data are discussed in the context of the biotechnological potential and applicability of prokaryotic chaperones in complex, eukaryotic factories for recombinant protein production.

Keywords Protein folding · Chaperones · Insect larvae · Protein quality · DnaK · Protein factories

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Introduction

Recombinant protein production is a routine practise in structural and functional genomics and the methodological source of many biotechnological and biomedical products, both at laboratory and industrial scales. The use of microbial cell systems (namely bacteria, yeast and filamentous fungi) encounters several obstacles during production processes, including conformational stress (Gasser et al. 2008), diverse metabolic responses that compromise productivity (Hoffmann and Rinas 2004; Mattanovich et al. 2004; Martinez-Alonso et al. 2008a), lack of post-translational modifications and poor solubility and stability of many heterologous proteins (Baneyx and Mujacic 2004; Rosano and Ceccarelli 2009) and the affectation of the whole cell integrity as response to protein production (Villa et al. 2009). Among the most critical points, many

recombinant proteins are produced as insoluble versions, and in particular, in bacterial systems, it is commonly observed the occurrence of inclusion bodies, namely large clusters of aggregated polypeptides that are not suitable for most of biomedical or biotechnological applications (Villaverde and Carrió 2003). These bottlenecks have pressed the exploration of new biological systems as alternative platforms to obtain higher quality proteins, especially under the urgent need of new protein pharmaceuticals (Leader et al. 2008) for which microbial cells might be inefficient as factories. In this context, mammalian and insect cell lines and transgenic plants and animals are being progressively incorporated to production activities (Ferrer-Miralles et al. 2009). Many recombinant therapeutics (that are subjected to highly restrictive quality controls) produced in mammalian cells have already been approved for use, competing in number to those obtained in microbial factories (Ferrer-Miralles et al. 2009). Among these alternative platforms for protein production, baculovirus-based expression systems show a highly promising potential, and insect larvae have several advantages compared with the insect cell line version, scaling-up of which is both methodologically complex and costly. While keeping the glycosylation abilities offered by the insect system, insect larvae do not require sterile conditions or growth media, importantly reducing production costs (Shi and Jarvis 2007). Also, larvae factories render biologically safe products either for diagnostic or vaccine purposes (Gomez-Sebastian et al. 2008; Ferrer et al. 2007; Lopez et al. 2005; Perez-Filgueira et al. 2007; Perez-Filgueira et al. 2006; Barderas et al. 2000). Furthermore, scaling up is straightforward once optimal larval infection conditions are defined. In contrast to protein production in mammals' milk, that requires the establishment of transgenic animal lines, the batch-based protein production in larvae skips complex cell and DNA manipulation procedures and clone selection.

However, yields of heterologous proteins in larvae may be reduced due to protein aggregation (Ailor et al. 1999) that could be at least partially accounted for by the use of strong viral promoters, namely polyhedrin or p10 promoters in the expression systems. Compared with microbial cells, much fewer approaches to improve recombinant protein production and solubility have been described in insect larvae. In bacteria, one of the most common strategies to increase solubility is the co-production of homologous chaperones (usually as sets of cooperating chaperones or chaperone-co-chaperone pairs) along with the recombinant protein, as folding modulators are believed to be limiting in recombinant cells (Kolaj et al. 2009; de Marco et al. 2007; de Marco 2007). We have here explored for the first time the potential of the main *E. coli* chaperone DnaK and its co-chaperone DnaJ to modulate the quality of a reporter misfolding-prone GFP (mGFP) when produced in insect larvae. In *E. coli*, mGFP forms highly visible, fluorescent inclusion bodies rapidly after induction of gene expression (Garcia-Fruitos et al. 2007). The proposed approach has resulted in the doubling of the yield of extractable mGFP, proving that prokaryotic proteins act efficiently in complex eukaryotic systems for protein production. A moderate reduction in mGFP conformational quality (estimated through its specific fluorescence) is in agreement with a general concept proposed for bacterial systems under which protein yield and conformational quality cannot be gained simultaneously (Martinez-Alonso et al. 2008a). These results indicate the applicability of prokaryotic chaperones DnaK/DnaJ as folding modulators in complex, whole body protein factories, and opens possibilities of expanding the catalogue of platforms, in which largely characterized folding modulators (such as those from *E. coli*) can be successfully used.

Material and methods

Vectors and virus stocks

mGFP (Garcia-Fruitos et al. 2007) has been used as a reporter to study the effect of a set of *E. coli* chaperones when produced in a baculovirus expression system. This protein consists of the aggregation-prone VP1 capsid protein of foot-and-mouth disease virus fused to the amino terminus of the Green Fluorescent Protein (GFP). mGFP has been produced either alone or with the *E. coli* chaperone pair DnaK/J. For this purpose, two different transfer vectors based on pAcAB4 (Belyaev and Roy 1993) were constructed. The *mGFP* gene was cloned alone for single expression, and for chaperone co-expression a second vector containing both mGFP and the DnaK/J chaperone pair was constructed. Kozak sequences were added to each gene. The p10 promoter was used to drive the expression of *mGFP* and *dnaJ* genes, and *dnaK* expression was driven by the polyhedrin promoter.

Recombinant baculoviruses were obtained by co-transfection of each transfer vector with Bsu36I-linearized viral DNA BAC10:KO1629 (Zhao et al. 2003) into *Sf9* cells. Individual clones were plaque purified after 5 days, amplified once and screened for protein expression by Western blot analysis. Recombinant baculoviruses expressing the desired proteins were further amplified in Sf9 cells by infecting them at MOI=0.1 when cell density was 1×10^6 cells/ml. After 4 days, the culture supernatant was harvested, cleared by centrifugation at $9{,}500\times g$ for 10 min, and titered by standard plaque assay. This virus stock was amplified once more to produce a larger high titer stock. This time, *Sf9* cells were grown to a density of 2×10^6 cells/ml and infected at a MOI of 0.1. Culture

supernatants were harvested after 6 days, processed as described above and titered. These virus stocks were used directly to infect cells for protein expression.

Cells were maintained in Insect Xpress medium (Lonza, #12-730) and supplemented with 2% Foetal Calf Serum at the time of infection. Both cells and infected cultures were kept at 27°C and 110 rpm. Cell density was determined by cell counting using a haemocytometer, and Trypan Blue was used to assess viability. All virus stocks were stored in dark at 4°C.

Insect growth conditions and inoculation

Trichoplusia ni (Cabbage looper) larvae (*T. ni*) were reared under level-2 biosafety conditions following previously described methodology (Perez-Filgueira et al., 2006). For all experiments, fifth-instar larvae (last instar larvae before pupation) of about 300 mg weight, were injected with the recombinant baculoviruses (budded virus forms) in the body cavity near the proleg using different pfu/larva doses, as indicated on each experiment. Infected larvae were kept in growth chambers at 28°C and collected at indicated times. Larvae were then immediately frozen and kept at −20°C until processed.

Preparation of protein extracts

Total protein extracts from *T. ni* larvae were obtained as previously described (Perez-Filgueira et al. 2006). Briefly, frozen insect material was homogenised using an extraction buffer containing phosphate buffered saline (PBS) pH7.2, 0.01% Triton X-100, 1% sodium dodecyl sulphate, 2.5 mM, dithiothreitol, 10 mM β-mercaptoethanol and a protease inhibitor cocktail (Complete, Roche, Germany). Such material was directly used for the analysis of the total recombinant protein, while for the evaluation of the soluble fraction the extract was clarified by centrifugation at $10{,}000\times g$ for 10 min and the pellet discarded. The supernatant was filtered through Whatman papers and centrifuged once again as described above. In both cases, total concentration of extracted protein was quantified by Bradford assay (Bradford 1976).

Protein analysis

To check for protein expression of the plaque-purified individual clones, *Sf9* cells were seeded on 6-well plates (1×10^6 cells/well) and infected with the individual clones. At least five clones were checked for either single expression of mGFP or expression of mGFP together with the DnaK/J pair. Cells were harvested 72 hpi, washed and resuspended in cold PBS. Three volumes of cells in PBS were mixed with one volume of sample buffer (50 mM Tris-HCl pH6.9, 10% sodium-dodecyl sulphate (SDS), 10% β-mercaptoethanol, 25% glycerol, 0.02% bromophenol blue) and loaded onto 12% acrylamide sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels for protein analysis. Proteins were transferred to a nitrocellulose membrane and probed with the appropriate antibodies. mGFP was detected using a commercial rabbit polyclonal antibody against GFP (Santa Cruz Biotechnology, Inc., # sc-8334). DnaK was detected using a hyperimmune rabbit serum, and for DnaJ a commercial rabbit polyclonal antibody (Stressgen, #SPA-410) was used. In all cases, goat anti-rabbit IgG (H+L)-HRP conjugate (Bio-Rad, #172-1019) was used as second antibody.

For the analysis of protein production in larvae, samples were prepared by mixing three volumes of extracts with one volume of sample buffer for protein analysis in 12% acrylamide SDS-PAGE gels followed by Western blotting. For that, proteins were transferred to a nitrocellulose membrane and GFP was detected as described above. Bands were scanned at high resolution and quantified by Quantity One Software (Bio-Rad). Experiments were carried out in duplicate.

Fluorescence determination

The emission of 40 μg of the larvae extracts (lysates or soluble extracts) was measured at 535 nm after being excited at 485 nm using a GENIOS fluorimeter. Each value was taken by measuring four times each extract. Fluorescence values were referred to mGFP amounts to obtain specific fluorescence of mGFP, defined as fluorescence units per microgram of mGFP.

Results

The amounts of mGFP released from larvae extracts (namely soluble mGFP species) were monitored by Western Blot upon viral infection at different pfu, when produced alone or with simultaneous production of *E. coli* DnaK/DnaJ chaperones. As observed (Fig. 1a), mGFP bands were hardly visible at 24 and 48 hpi in the absence of the bacterial proteins, but the co-production of DnaK/DnaJ dramatically increased the amount of soluble mGFP in all viral doses, making the product clearly immunodetectable already at 48 hpi.

At 10^5 pfu, chosen as a model infection dose, quantification of Western blot analyses revealed a twofold increase of the releasable mGFP at both 48 and 72 hpi, when referred either to larvae extract volume (Fig. 1b) or total protein (Fig. 1c; $p=0.003$ at 72 hpi). Importantly, the bacterial chaperones had no significant effects on the total amount of produced mGFP (soluble+retained in the

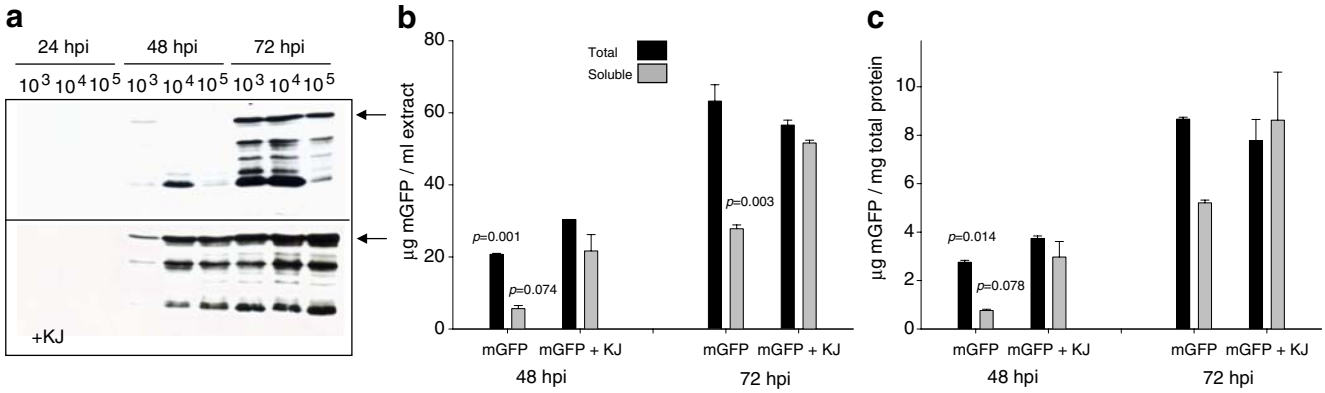

Fig. 1 (**a**) Western blot kinetic analysis of soluble mGFP production in larvae infected with different viral doses (pfu values per larva are indicated, in absence (*top*) and presence (*bottom*) of bacterial chaperones DnaK/DnaJ (*KJ*). *Arrows* indicate the position of the full-length protein. Quantitative analysis from several infection experiments with 10^5 pfu are shown in panels **b** and **c**, indicating the total and soluble mGFP referred to extract volume (**b**) or total protein (**c**). Significantly different values (always comparing data with and without chaperones for a given fraction) are indicated by the *p* parameter obtained in an ANOVA test. Only $p<0.1$ values are shown. For clarity, further analyses were done only on larvae infected with 10^5 pfu. *Bars* here and in the following figures indicate standard errors

insoluble extract fraction). In summary, the solubility of mGFP expressed as the percent of releasable mGFP over the total recombinant protein was significantly enhanced in co-expressing larvae up to around twofold, again at 48 and 72 hpi (Fig. 2; $p=0.001$ at 72 hpi).

We were interested in the analysis of protein quality, as we have recently shown that solubility of recombinant proteins is not necessarily linked to their conformational quality (Gonzalez-Montalban et al. 2007; Martinez-Alonso et al. 2009). Co-production of DnaK/DnaJ along with mGFP promoted a reduction of fluorescence in larvae extracts, particularly evident at 48 hpi ($p=0.005$ at 48 hpi and $p=0.041$ at 72 hpi), and a null effect on the fluorescence of the soluble fraction (Fig. 3a). As a consequence, the average specific fluorescence of mGFP in the whole cell extracts resulted negatively influenced (between around 40% and 60%) by the expression of bacterial genes ($p<0.001$ at 48 hpi and $p=0.026$ at 72 hpi) and this effect was also observed, although less robustly, in the soluble fraction ($p=0.017$ at 48 hpi and $p=0.107$ at 72 hpi; Fig. 3b). This is indicative of a moderate reduction in the conformational quality of mGFP irrespective of the protein fractioning. Such inactivation is in agreement with previous observations derived from systems level analysis of recombinant *E. coli,* indicating that up-modulation of protein productivity by genetic or process approaches is detrimental regarding conformational and functional protein quality (Garcia-Fruitos et al. 2007; Martinez-Alonso et al. 2009; Martinez-Alonso et al. 2008a).

The mentioned reduction in the fluorescence emission observed in whole cell extracts (Fig. 3a) was confirmed by a direct observation of infected, mGFP-producing larvae (Fig. 4), which were less fluorescent when bacterial proteins were also produced.

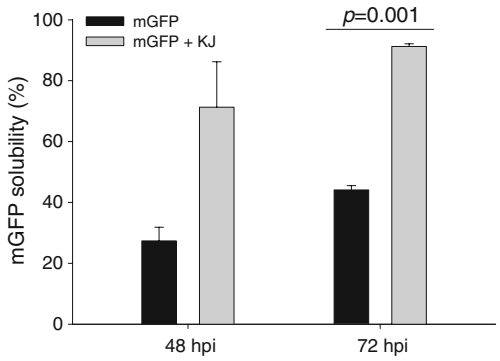

Fig. 2 mGFP solubility as influenced by DnaK/DnaJ (*KJ*) co-production, determined at two times post-infection. Significantly different values (always comparing data with and without chaperones for a given fraction) are indicated by the *p* parameter obtained in an ANOVA test. Only $p<0.1$ values are shown

Discussion

Protein production in recombinant hosts is severely compromised by protein misfolding and poor solubility (Baneyx and Mujacic 2004), host stress responses (Gasser et al. 2008) and different host-specific bottlenecks (Ferrer-Miralles et al. 2009), including the absence of many post-translational modifications in prokaryotic cells (de Marco 2009). The urgent need of new recombinant products, including protein drugs for molecular therapies, has strongly pushed the incorporation of new cell types and organisms into the protein production arena, including mould, filamentous fungi, methilotrophic yeasts and insect larvae, among others.

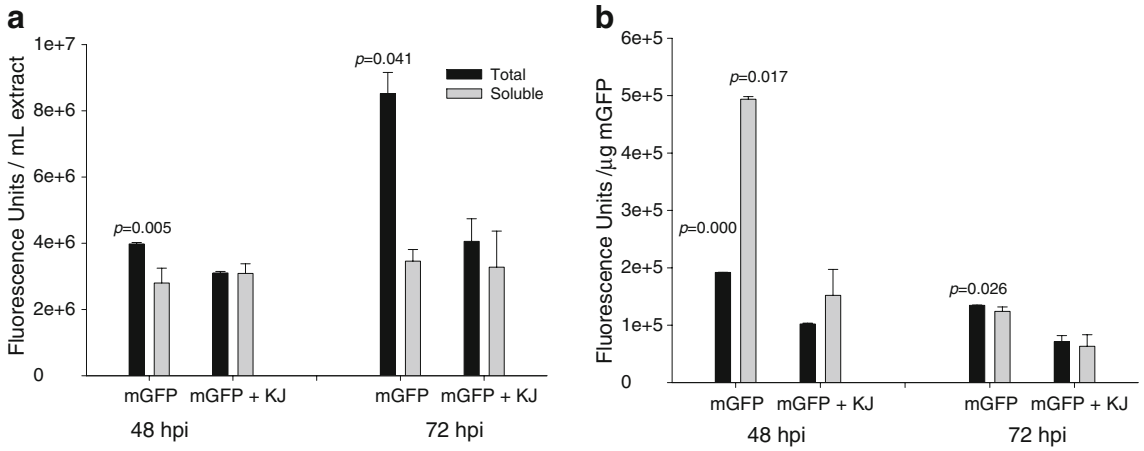

Fig. 3 Fluorescence emission of mGFP, produced in absence or presence of bacterial chaperones DnaK/DnaJ (*KJ*), referred to extract volume (**a**) or given as mGFP specific fluorescence (**b**). Significantly different values (always comparing data with and without chaperones for a given fraction) are indicated by the *p* parameter obtained in an ANOVA test. Only $p<0.1$ values are shown

In bacteria, chaperone DnaK and its co-chaperone DnaJ have been observed as promising tools to improve solubility (de Marco et al. 2007; de Marco 2007). They act in a sequential network of folding assistant proteins that involves trigger factor and GroEL/GroES proteins among others (Deuerling et al. 1999; Langer et al. 1992; Krueger and Walker 1984; Gragerov et al. 1992) and have a wide spectrum of protein substrates that expose hydrophobic

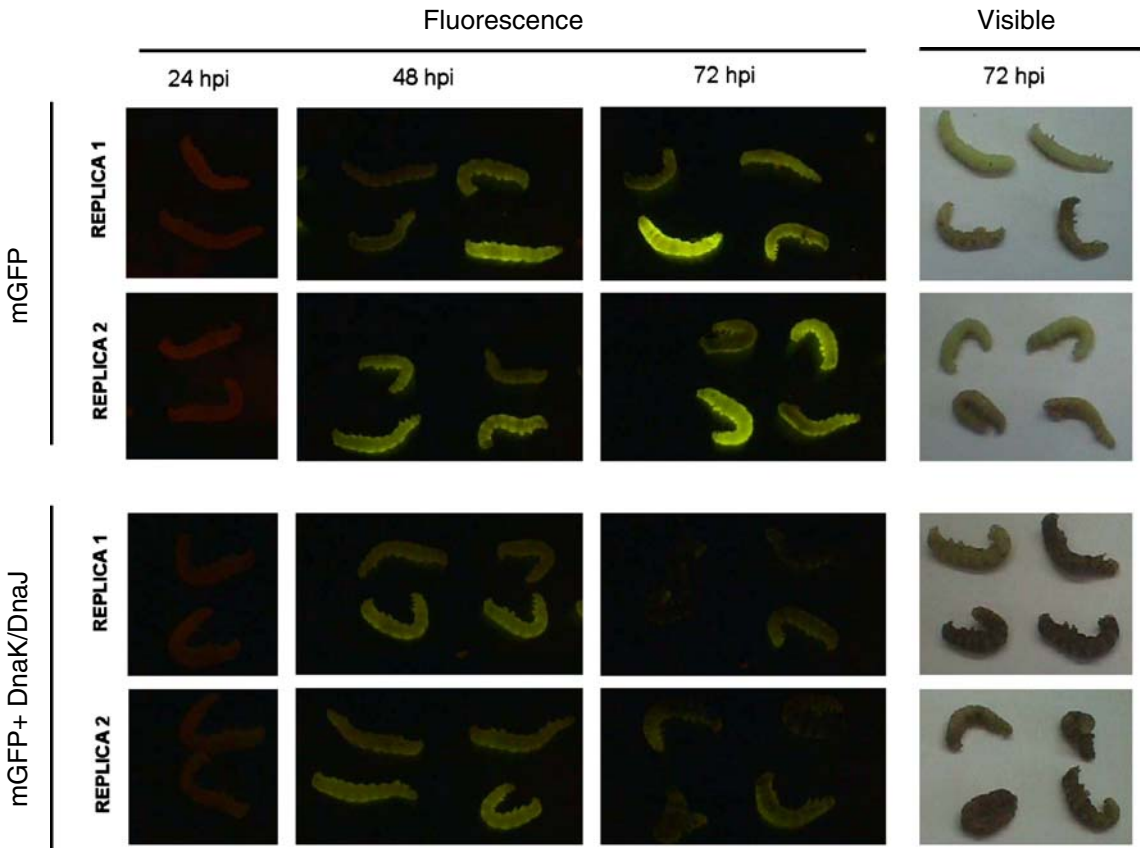

Fig. 4 Fluorescence and visible imaging of infected larvae (two replicas) producing mGFP in absence or presence of bacterial chaperones DnaK/DnaJ (*KJ*), at different post-infection times

regions to the solvent (Mayer et al. 2000; Rodriguez et al. 2008). Although this fact enables these proteins to be used as folding tools in recombinant processes, co-production of DnaK/DnaJ might eventually occur at the expense of productivity since in the bacterial quality control network, DnaK can reduce the proteolytic stability of recombinant proteins (Martinez-Alonso et al. 2007; Garcia-Fruitos et al. 2007). In an attempt to evaluate how the well-studied bacterial chaperone DnaK, could promote protein folding in a complex eukaryotic platform we have expressed the encoding *dnaK* and *dnaJ* genes in insect larvae producing an aggregation prone GFP.

While the total yield of mGFP was not significantly affected by these folding assistant proteins (Fig. 1b), both the yield of soluble mGFP (the fraction that can be recovered from larvae extracts) and the solubility ratio were significantly improved (Fig. 1b, c and 2). Contrarily to what occurs in *E. coli* cells, in which the inactivation of the *dnaK* gene or its plasmid-driven overexpression increases or decreases the recombinant protein yield, respectively (Carrio and Villaverde 2003; Martinez-Alonso et al. 2007; Garcia-Fruitos et al. 2007), the results presented here indicate that in larvae, DnaK has quality-restricted, yield-independent effects on protein production.

On the other hand, the folding assistant proteins moderately reduced the specific fluorescence of the soluble mGFP (Fig. 3b). This fact could be accounted for by a recently proposed concept for bacterial platforms (in particular *E. coli*) in which protein yield and quality are observed as antagonistic parameters that evolve divergently when modifying the production conditions or tuning the cell's genetic background (Garcia-Fruitos et al. 2007; Martinez-Alonso et al. 2008a). The results obtained here in a completely different biological system prompt to consider such principle as not restricted to a specific production platform and probably as a general rule in the recombinant protein production arena. Importantly, the soluble versions of aggregation-prone proteins consist of molecular isoforms that can adopt a wide conformational spectrum (de Marco and Schroedel 2005; Martinez-Alonso et al. 2008b; de Marco 2008) and exhibit a gradation in their biological activity (Gonzalez-Montalban et al. 2007). The relative proportion of such soluble, but biologically diverse protein species seems to be strongly influenced by the in vivo protein concentration (Martinez-Alonso et al. 2007), what in the production process context results in an incapability to simultaneously favour soluble yield and protein quality (Martinez-Alonso et al. 2008a).

In few previous studies, several human chaperones have been individually tested on insect larvae producing recombinant proteins (Nakajima et al. 2009), being the human chaperone Bip the only that had positively affected protein solubility and functionality in a significant way (Nakajima et al. 2009; Hsu and Betenbaugh 1997). Here we have proved, for the first time, the utility of DnaK/DnaJ as folding modulators in complex eukaryotic platforms, increasing the extractable amounts of a model protein. The functional performance of bacterial chaperones in insect larvae largely expands the catalogue of folding modulators, beyond chaperones of eukaryotic origin, which can be used in this platform, by the incorporation of deeply characterized proteins and protein teams of bacterial origin. In particular, the cooperative mode of action of DnaK/DnaJ, through their simultaneous binding to distinct target sites within the same substrate protein, has been recently determined using the σ^{32} transcription factor as a model (Rodriguez et al. 2008). Furthermore, DnaK/DnaJ, IbpA/IbpB and ClpB cooperate in the physiological removal of misfolded species from protein aggregates (Mogk et al. 2003; Schlieker et al. 2004; Weibezahn et al. 2005). The positive results presented here with DnaK/DnaJ, and the absence of undesired side effects on the stability of the target protein prompt to explore, in insect larvae, appropriate combinations of bacterial proteins that have been reported as highly successful in *E. coli* (de Marco et al. 2007; de Marco 2007).

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