Cross-system excision of chaperone-mediated proteolysis in chaperone-assisted recombinant protein production

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Main *Escherichia coli* cytosolic chaperones such as DnaK are key components of the control quality network designed to minimize the prevalence of polypeptides with aberrant conformations. This is achieved by both favoring refolding activities but also stimulating proteolytic degradation of folding reluctant species. This last activity is responsible for the decrease of the proteolytic stability of recombinant proteins when co-produced along with DnaK, where an increase in solubility might be associated to a decrease in protein yield. However, when DnaK and its co-chaperone DnaJ are co-produced in cultured insect cells or whole insect larvae (and expectedly, in other heterologous hosts), only positive, folding-related effects of these chaperones are observed, in absence of proteolysis-mediated reduction of recombinant protein yield.

In living cells, the protein quality control system surveys the folding of nascent polypeptides and full-length proteins in a kinetically driven process. The folding machinery acts in a set of ATP-dependent, coordinated activities intended to avoid the accumulation and aggregation of misfolded protein species, as protein deposits might be unusable reservoirs of amino acids¹ and the amyloid-like stretches contained in them might be cytotoxic. Chaperones, included in the set of heat shock proteins, have high affinity for hydrophobic surfaces displayed in partially folded polypeptides leading them to either a proteolytic pathway, or a refolding pathway by modifying misfolded structures to convert them

into a closer to native-like conformation. In fact, conformational deficiencies are associated to degenerative conditions and aging,^{2,3} being chaperones proposed as therapeutic agents against accumulation of these protein aggregates.^{4,5}

In biotechnology, the production of soluble, biologically active recombinant proteins in prokaryotic gene expression systems such as *Escherichia coli* is limited by several factors, including saturation of the folding machinery, differential post-translational modifications or the absolute incapability to perform some of them.⁶ In many cases, overexpression of recombinant genes leads to the accumulation of misfolded, aggregated protein forms known as inclusion bodies,⁷ a fact that drastically reduces the yield of soluble protein. However, in prokaryotic systems, protein solubility can be improved by modulating cell growth conditions (i.e., lowering growth temperature⁸), fusing highly soluble protein partners (i.e., GST and MBP⁹), creating a more oxidative environment (i.e., Origami strains,¹⁰ fusion to DsbC,¹¹ periplasmic secretion¹²), optimizing codon usage,¹³ introducing eukaryotic glycosylated moieties in produced recombinant proteins¹⁴ or co-expressing protein factors involved in the protein folding control system (i.e., DnaK/J, GroEL/S and others).¹⁵⁻¹⁷

The genetic supplementation of folding modulators in recombinant protein production driven by *E. coli* can alleviate the saturation of the folding system caused by the input of newly synthesized polypeptides in the overproducing stressed cell. However, in some cases increasing

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Figure 1. Model of DnaK and DnaJ differential action in bacterial and eukaryotic systems. By rehosting the chaperones to a system lacking orthologs of the bacterial proteases Lon and ClpP, proteolysis can be avoided while keeping the conserved foldase activity of the DnaK/J pair.

the amount of folding modulators in over-expressing cells to improve solubility results in decreased or constant protein yields or an increase only in aggregated soluble conformers.^{15,18,19} This dual and contradictory effect reflects the complex equilibrium that drives misfolded polypeptides to either folding attempts or to proteolytic degradation. In that sense, it has been demonstrated that defined sets of several folding modulators might increase the success rate of recombinant protein production and global solubility,¹⁷ although, as discussed later, gaining solubility is not always accompanied by a real increase in the soluble protein yield and quality.

In eukaryotic cells, several families of chaperones have been identified.²⁰ Among them, the eukaryotic Hsp70 chaperone family and its co-factors Hsp40 and HsdJ (DnaJ family) are the orthologs of the prokaryotic DnaK and its co-chaperone DnaJ. In the use of eukaryotic cells (namely insect cells, mammalian cells and yeast) as protein factories, co-expression of eukaryotic chaperones also increases solubility.²¹⁻²³ Unfortunately, an assessment of the effect of such strategy on the conformational quality of the target proteins has not been performed side by side, making it difficult to completely evaluate the

convenience of using such folding modulators as a general approach. In *E. coli*, DnaK (the homologous counterpart of the eukaryotic HSP70) is the main cytosolic chaperone exhibiting foldase activity in cooperation with its DnaJ and GrpE co-chaperones. However, in IB-forming cells, DnaK species are found associated to the IB surfaces²⁴ and involved in the disaggregation of proteins from IBs, in cooperation with DnaJ, GrpE, ClpB, IbpA and IbpB.²⁵⁻²⁷ On the other hand, DnaK is a negative modulator of the heat-shock response through the proteolytic inactivation of the stress-activated RNA polymerase subunit $\sigma 32$. In a similar way, it has been shown that DnaK promotes ClpP- and probably also Lon-mediated proteolysis of recombinant proteins.²⁸ When producing recombinant enzymes and fluorescent proteins in DnaK-deficient *E. coli* cells, there is an increase in protein aggregation and yield compared to a wild type genetic background, in concordance with the loss of both chaperone and proteolytic enhancer activities.^{18,29} Surprisingly, an increase in specific activity is observed in the produced recombinant protein mainly in the insoluble cell fraction.⁸ This observation seems to be also related to the absence of DnaK-induced proteolytic activity since ClpP⁻ cells with a wild type DnaK also

produce protein conformers with higher specific activity associated to protein deposited in inclusion bodies.⁸ Therefore, in DnaK⁻ cells, protein yield and quality increase at expenses of protein solubility. In agreement, in cells overexpressing DnaK and its immediate co-chaperone DnaJ along with the target recombinant protein, solubility increases with a concomitant loss of conformational quality and protein yield, being these parameters mutually exclusive.¹⁸

In the context of the antagonistic activities of the DnaK and DnaJ pair observed in bacteria, the introduction of these prokaryotic folding modulators in heterologous expression systems such as cultured insect cells,³⁰ insect larvae³¹ or others can benefit from their conserved foldase activities while avoiding the undesirable adverse proteolytic effect mediated by ClpP and Lon proteases, which is not expected to be conserved in eukaryotes. This principle (Fig. 1) has been proved by the production of a modified aggregation-prone GFP (mGFP) along with DnaK and DnaJ in insect cells, where the chaperones compensate for the conformational stress during recombinant protein production in absence of proteolysis.³⁰ In fact, the recombinant GFP is proteolytically stabilized in the presence of DnaK and DnaJ,³⁰ contrarily to what had been observed in *E. coli*, where the half life of this protein is dramatically reduced under co-expression conditions.¹⁸ Moreover, when analyzing protein fractioning in cultured cells, protein yield is significantly favoured in the soluble fraction and also in the insoluble fraction. When extending the chaperone rehosting concept to the production of other recombinant proteins in insect cell culture, these are better produced under co-production conditions than in absence of bacterial DnaK and DnaJ,³⁰ showing an increase in the yield of extractable protein and a reduction of oligomer formation depending on the model protein.³⁰ When rehosting the chaperones to a whole insect larvae system, total protein levels were not significantly affected by chaperone co-production, but soluble protein amounts represented almost the total of the recombinant protein and solubility values higher than 90% were obtained.³¹ However, in both cultured insect cells and larvae, the

recombinant GFP exhibits slightly lower functional quality (measured through specific fluorescence) in presence of bacterial chaperones,^{30,31} which can probably be accounted for by the increased yield. This is in agreement with previous observations in bacteria indicating that increasing protein yield is accompanied by a reduction of the conformational quality,⁸ probably associated to the formation of soluble aggregates in the soluble cell fraction.¹⁹

All these observations add further evidences to the fact that, taken independently, solubility is a poor indicator of protein quality in protein production processes,³² and show that a more accurate case by case analysis is an inexcusable need for a proper control of conformational and functional protein quality, as recently claimed.^{33,34}

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