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Fusion tags and chaperone co-expression modulate both the solubility and the inclusion body features of the recombinant CLIPB14 serine protease

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Abstract

Chaperone co-expression and the fusion to different tags were used to modify the aggregation pattern of the putative serine protease CLIPB14 precipitated in *Escherichia coli* inclusion bodies. A set of common tags used in expression vectors has been selected, as well as two bacterial strains over-expressing the chaperones GroELS and ibpA/B, respectively. The presence of the fused tags resulted in an improved solubility of CLIPB14 but also in a higher presence of contaminants in the inclusion bodies, while chaperone co-expression promoted the binding of all the chaperone machinery involved into the disaggregation to the CLIPB14.

Furthermore, each tag influenced in a specific manner the re-aggregation of the denatured CLIPB14 constructs during urea dilution and the preliminary trials indicated that the CLIPB14 fusions with higher homogeneity and lower re-aggregation rate were the optimal candidates for refolding assays.

In conclusion, it is possible to tune the quality of the inclusion bodies by choosing the suitable combination of tag and chaperone co-expression that minimize the non-productive side reactions during refolding.

Keywords: Fusion-tags; Chaperone co-expression; Inclusion bodies; Soluble aggregates; CLIPB14

1. Introduction

The major limitation of the recombinant expression of heterologous proteins in *Escherichia coli* is the high rate of protein aggregation. In the worst cases no soluble recombinant protein can be recovered, but it accumulates in precipitates generically identified as inclusion bodies (Rinas and Bailey, 1992). Recently, the heterogeneity of the inclusion body structure has been described (Carrió et al., 2000), as well as the dynamic interaction of the protein precipitated in inclusion bodies with its soluble fraction (Carrió and Villaverde, 2001). The existence of a cellular mechanism to recycle proteins aggregates in vivo

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has been exploited biotechnologically in combination with chaperone co-expression to improve the yields of the recombinant soluble protein (de Marco and De Marco, 2004). The intensity of the exchange between the soluble and the aggregate forms of a protein is probably protein-specific and, therefore, it is possible that for some proteins the inclusion bodies represent, in practice, a no-return trap.

Proteins with structures stabilized by disulfide bridges are among the most critical to express correctly folded in *E. coli*. In fact, the bacterial cytoplasm is too reducing and the cysteine oxidation in the periplasm is often non-specific. The fusion with the major enzymes involved in disulfide building and isomerisation (DsbA and DsbC) has improved the yield of functional recombinant proteins (Zhang et al., 1998; Jurado et al., 2002). Nevertheless, the rate of success is indirectly correlated with the number of cysteines present in the sequence. Other proteins, like GST or thioredoxin (Trx), have been often used as fusion partners and succeeded to improve the solubility of the passenger proteins in large-scale screenings (Dyson et al., 2004). Finally, we documented that in some cases fusions with NusA and Ftr stabilized the passenger proteins even better than GST (de Marco et al., 2004; De Marco et al., 2004).

An alternative approach for the production of recombinant proteins considers the purification of inclusion bodies and the recovery of native proteins after in vitro refolding (Middelberg, 2002). The quality of the inclusion bodies in terms of purity and homogeneity is apparently crucial to success because non-productive side reactions driven by contaminants can prevent the correct folding acting as re-aggregation seeds (Maachupalli-Reddy et al., 1997). The fact that the aggregates in the inclusion bodies can have different complexity and that the cell growth conditions can modify the rate of aggregation of the recombinant proteins in bacteria (Rinas and Bailey, 1992; Hunke and Betton, 2003) urges to identify protocols for selecting inclusion bodies with features suitable for refolding.

We used the CLIPB14 protein from *Anopheles gambiae* (Christophides et al., 2002), a putative protease, as a model because seven disulfide bridges are present in its native structure and this feature makes improbable its soluble accumulation in bacteria. Different fusion partners, chaperone co-expression and cell localisation have been considered to optimise both the soluble protein yield and the recovery of inclusion bodies with suitable features for in vitro refolding.

2. Material and methods

2.1. Growth of bacterial cultures and soluble protein purification

The cDNA corresponding to the putative protease domain of CLIPB14 (EAA05468) was cloned into the following expression vectors: pGEX6P (Amersham, between *Eco*RI and *Not*I), pETM11 (*Bsa*I-*Xho*I), pETM20 (*Bsa*I-*Not*I), pETM50 (*Bsa*I-*Xho*I), pETM60 (*Bsa*I-*Xho*I), pETM80 (*Bsa*I-*Not*I) (described at http://www.embl-heidelberg.de/ExternalInfo/protein_unit/draft_frames/index.html) and pETM90 (*Bsa*I-*Xho*I) (de Marco et al., 2004). The constructs expressed from pETM 50 and 80 were secreted into the periplasm while the others remained in the cytoplasm. All the vectors were used to transform BL21 (DE3) competent cells and pETM11 was further transformed in *E. coli* BL21 (DE3) cells co-expressing either GroELS or ibpA/B (Mogk et al., 2003a,b). Over-night pre-cultures were exploited to inoculate 500 mL of LB medium. Bacteria were grown at 37 °C until an OD_{600} of 0.4, and then the cultures were cooled down to 20 °C, induced with 0.1 mM IPTG and grown for further 18 h. The bacteria were pelleted by centrifugation, washed in 10 mL of PBS, and finally stored at −20 °C.

The pellets containing His-tagged proteins were resuspended in 10 mL of lysis-buffer (50 mM Tris–HCl buffer, pH 7.8, 0.5 M NaCl and 5 mM $MgCl_2$) and incubated in an ultrasonic water bath (Branson 200) for 5 min. Hundred-microlitre lysozyme (100 mg/mL) and 200 μL DNase I (500 μg/mL) were added and the lysates were incubated for 30 min on a shaker at room temperature. The supernatants were recovered after ultracentrifugation (35 min at 150,000 × *g*), loaded onto a HiTrap chelating affinity columns (Amersham Biosciences) pre-equilibrated with 20 mM Tris–HCl, pH 7.8, 500 mM NaCl, 15 mM imidazole and the His-tagged recombinant proteins were eluted in 20 mM Tris, pH 7.8, 125 mM NaCl and 250 mM imidazole.

The GST-fused protein was purified by affinity using a HiTrap GST column (Amersham Bioscience). The pellets were resuspended in PBS plus 5 mM $MgCl_2$, and treated as the His-tagged proteins. The elution was performed using 50 mM Tris–HCl, pH 8.0 and 10 mM reduced glutathione. The purified proteins were quantified detecting the absorbance at 280 nm and correcting the values according to the extinction factors (Gill and von Hippel, 1989). The quantification of the proteins separated by SDS-gel was performed applying the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

2.2. Inclusion body isolation and refolding test

Pelleted cells from 1 L culture were resuspended in 13 mL of solubilization buffer (50 mM Tris, pH 8.0, 25% sucrose and 10 mM DTT) and ultrasonicated in a water bath. Hundred-microlitre lysozyme (100 mg/mL), 500 μL DNaseI (0.5 mg/mL), 50 μL $MgCl_2$ (1 M) and 12.5 mL of lysis buffer (50 mM Tris, pH 8.0, 1% Triton X-100, 1% Na-desoxycholate, 100 mM NaCl and 10 mM DTT) were added and the cell suspension was incubated for 1 h while shaking at room temperature. The efficiency of the cell lysis was improved by a cycle of freezing (in liquid nitrogen) and thawing (30 min at 37 °C in a water bath). DNA digestion was optimised by a further incubation (1 h at room temperature) in the presence of 200 μL 1 M $MgCl_2$, 350 μL of 0.5 M EDTA at pH 8.0 were added before centrifugation (20 min at 11,000 × *g*) in a Sorvall SS34 rotor. The pellet was resuspended in 10 mL of Triton washing buffer (50 mM Tris, pH 8.0, 0.5% Triton X-100, 100 mM NaCl and 1 mM DTT), sonicated 5 min in water bath, centrifuged again at the same conditions, resuspended in 10 mL of washing buffer without Triton (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM DTT and 1 mM EDTA) sonicated, and finally centrifuged. Inclusion bodies were either recovered in PBS or dissolved in 4 or 8 M urea (pH 8) and 4 mM DTT. The samples denatured in 8 M urea were used for refolding tests. The FoldIt Screen kit (Hampton Research) was used according to the manufacturer's instructions exploiting the method of the direct dilution of the samples in the refolding buffers. The most promising buffers were also used for dialysis-dependent refolding.

2.3. Fluorimetric analysis and light scattering

An AB2 luminescence spectrometer (Aminco Bowman® Series 2) equipped with SLM 4 software was used to analyse the aggregation rate of the proteins according to Nominé et al. (2001). Fifty microgram of protein were sufficient to obtain a clear spectrum and three independent spectra were collected for each sample. The excitation was induced at 280 nm and the emission scan was recovered between 260 and 400 nm. The ratio between the values of the peaks at 280 and 340 nm is the aggregation index. Such an index is not an absolute value but is functional to follow the variations of the aggregate structures. Light scattering analysis was performed using a DynaPro 801 instrument (Protein Solution) equipped with Dynamics V software.

2.4. Western blots

After SDS-PAGE, proteins were transferred by wet blotting onto a PVDF membrane using a Bio-Rad chamber and the efficiency of the transfer was proved by Ponceau Red staining (Serva). The primary rabbit antibodies were a gift of Dr. Bukau and were purified from sera using Protein G Plus/Protein A Agarose (Oncogene) to minimize the background of the western immunoreaction due to unspecific interactions. Peroxidase-conjugated secondary antibodies for chemioluminescent detection were purchased from Dianova and the detection performed using the SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce), following the supplier's instructions. Blots were used repeatedly by effectively removing the antigen–antibody interaction using the Western Blot Recycling kit (Alpha Diagnostic Int.).

2.5. Protease activity test

Two independent standard tests were used to assay the putative protease activity of the purified CLIPB14 and trypsin activity was used as a control. BSA digestion was performed in PBS 1 h at room temperature using 1 μg of the substrate and 0.5 μg of the enzyme in the presence of 5 mM $CaCl_2$. For the endopeptidase assay 2 μg of bovine haemoglobin were incubated in the presence of 1 μg of CLIPB14 at room temperature for 30 min. The reaction was stopped by the addition of 5% trichloroacetic acid and the proteolitic processing

of the substrates was finally analysed by 10% SDS-PAGE followed by Coomassie staining.

The reactivity of the serine residue necessary for the protease function was tested by inducing its reaction with the conformation-specific inhibitor ^{3}H-diisopropylfluorophosphate (DFP) followed by direct fluorographic detection of radioactivity in the gels according to Skinner and Griswold (1983).

2.6. Sample preparation for electron microscopy

Samples were fixed by using the "single-droplet" parafilm protocol. Fifty microlitre of 1% fresh prepared uranyl acetate were pipetted on a clean parafilm surface. Five microlitre of protein sample were pipetted on each grid (carbon-coated formvar 400 mesh-grids, Agar Scientific) and incubated 1 min while keeping the grid blocked with forceps. The excess of fluid was removed using filter paper, the unbound protein was washed and the grids were placed on the drop of 1% uranyl acetate with the section side downwards. Finally, the grids were dried, placed in the grid-chamber and stored in desiccators before their observation in a CM120 BioTwin electron microscope (Philips).

3. Results and discussion

The effect of fusion tags and chaperone co-expression on the recombinant protein solubility has been widely documented (Zhang et al., 1998; Jurado et al., 2002; Nygren et al., 1994; LaVallie et al., 1993; De Marco et al., 2004; de Marco and De Marco, 2004; Dyson et al., 2004). The improved solubility does not prevent that part or even most of the expressed protein still precipitates into insoluble aggregates.

The complexity of the recombinant protein aggregates has been recognised only recently, thanks to the fine analysis of inclusion bodies (Carrió and Villaverde, 2001) and the identification of several classes of precipitates (Stegemann et al., 2005). Nevertheless, despite the large biotechnological interest in inclusion bodies as a starting material for refolding protocols (Middelberg, 2002), only sporadic information (Sachdev and Chirgwin, 1998) is available about how different constructs and the expression conditions can influence the features of the aggregates. In this paper, we report the data concerning the effect of expressing the aggregation-prone putative serine protease CLIPB14 fused to different fusion tags or in combination with different chaperones.

Table 1
Effect of fusion tags and chaperone co-expression on the solubility of the CLIPB14 serine protease

Vector	Fusions and chaperones	Precipitated protein (%)
pETM11	His-tag	98 ± 2
pETM11	His-tag, GroELS	80 ± 8
pETM11	His-tag, ibpA/B	95 ± 3
pGEX6P	GST-His-tag	80 ± 4
pETM20	Trx-His-tag	95 ± 4
pETM90	Ftr-His-tag	90 ± 3
pETM50	DsbA-His-tag	50 ± 11
pETM60	NusA-His-tag	1 ± 0.4
pETM80	DsbC-His-tag	10 ± 2

BL21 (DE3) bacterial strains were transformed with the listed expression vectors and the produced recombinant proteins were isolated from the insoluble fraction. The vector pETM11 was also transformed in strains co-expressing the chaperones GroELS or ibpA/B. The data are the average of three independent experiments

3.1. Effect of the tags on the solubility of CLIPB14

The structural model for the putative protease CLIPB14 indicates that the protein could be stabilized by as much as seven disulfide bonds. The correct folding of such a demanding structure in bacteria is very improbable, even when the construct is secreted into the oxidizing periplasm. Therefore, it was expected that almost no soluble His-tagged CLIPB14 was recovered by affinity purification when the protein was expressed alone in the reducing cytoplasm (Table 1 and Fig. 1A). The co-expression of the same construct together with the folding-helper GroELS chaperones resulted in 20% of soluble CLIPB14 while the effect of the co-expression with the aggregate-binding ibpA/B chaperones on the solubility of the protease was negligible (Table 1). No yield improvement of the soluble CLIPB14 was observed when it was co-expressed together with a control protein without chaperone activity (CAI58668, data not shown), indicating the specificity of the solubilizing effect induced by GroELS.

The fusion to the tags strongly influenced the solubility of CLIPB14, while Trx, Ftr and GST, showed a positive but limited effect, the fusion with NusA was almost completely soluble (Table 1 and Fig. 1A). The addition of 2% glucose to the growth medium in order

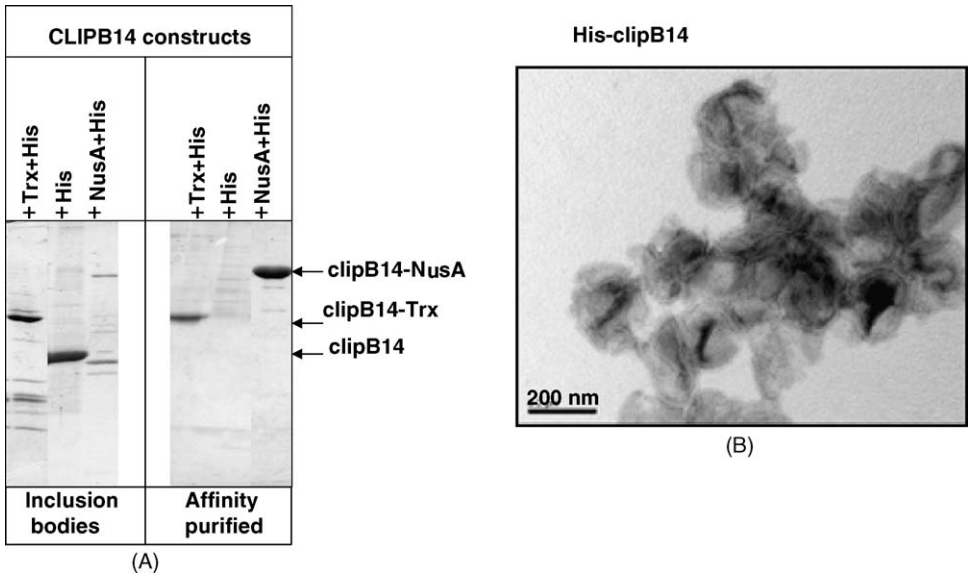

Fig. 1. Effect of the fusion tags on the solubility and homogeneity of the recombinant CLIPB14. (A) Protein fractions recovered from purified inclusion bodies and affinity-purified CLIPB14 constructs were analysed by SDS-PAGE. (B) Electron microscopy picture of His-tagged CLIPB14 inclusion bodies.

to prevent the expression leakage had negligible effects on the solubility of the CLIPB14-fusions.

The fusion with both the secreted proteins DsbA and DsbC resulted in high rates of soluble CLIPB14 and the significant better performance of DsbC with respect to DsbA could be related to the further isomerase activity of such a fusion tag. It would result in a more correct pairing of the cysteines involved into the disulfide bridges and, consequently, in a more stable structure. The light scattering and fluorimetric analyses indicated that the soluble CLIPB14 was monodispersed. Nevertheless, in contrast to the positive control trypsin, ^{3}H-DFP binding to CLIPB14 samples could not be demonstrated, implying that the protease did not have the expected folding. Furthermore, the protease activity tests failed to show proteolytic processing of the substrates.

3.2. The tags can affect the composition of the inclusion bodies

The inclusion bodies formed by His-tagged CLIPB14 had a diameter of around 200 nm and were almost homogeneous (Fig. 1A and B). In contrast, the inclusion bodies isolated from bacteria expressing fusions of CLIPB14 with larger partners were often contaminated by other proteins (Fig. 1A). This observation suggests that the presence of large fusion partners often resulted in the co-aggregation of bacterial proteins. The identity of such contaminants is crucial. In fact, the presence of unspecific contaminants is an important limiting factor during in vitro refolding because they can act as re-aggregation seeds (Maachupalli-Reddy et al., 1997). Differently, the binding of the recombinant fusions to chaperones could improve the refolding efficiency because their presence could prevent non-productive side reactions and facilitate the achievement of the native structure (Goloubinoff et al., 1999; Altamirano et al., 1999; Mogk et al., 2003b).

We induced a mild protein unfolding resuspending the inclusion bodies in 4 M urea and, after centrifugation, the soluble and insoluble fractions were analysed by coomassie staining of SDS-gels and by western blot using specific antibodies against the chaperones ClpB, DnaK, GroEL and ibpB. The same experiments were repeated after urea dilution to 2, 1, 0.5 and 0.1 M.

When the His-tagged CLIPB14 construct grown in wild type BL21 (DE3) was used, the recombinant protein recovered from inclusion bodies remained soluble

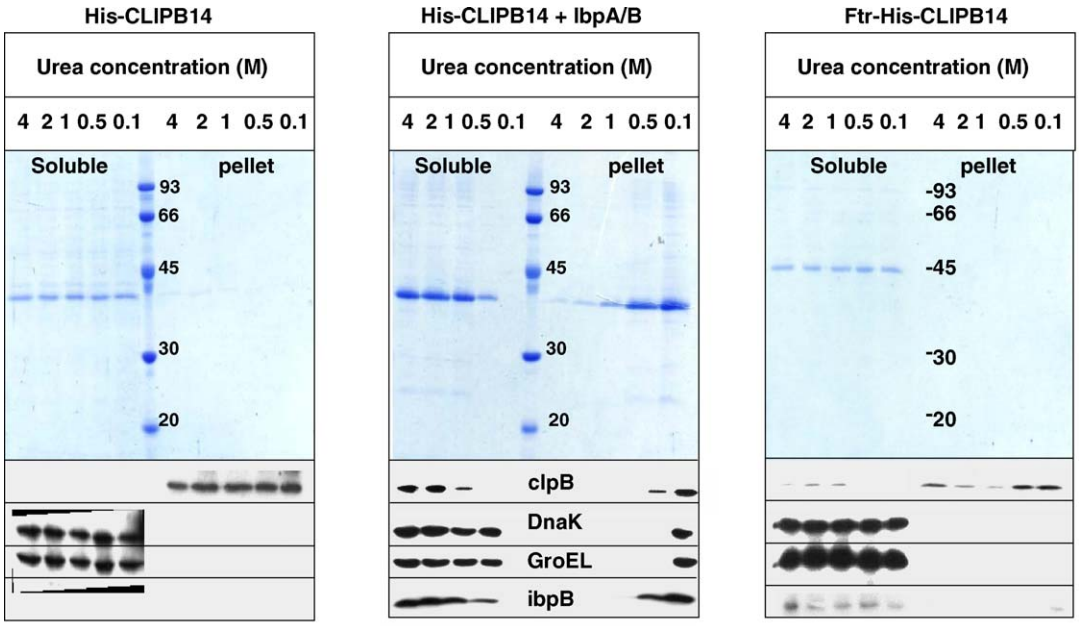

Fig. 2. Specificity of the chaperone binding to the different aggregated CLIPB14 constructs. Inclusion bodies recovered from the strain expressing His-tagged CLIPB14 (left), from the same construct co-expressed with ibpA/B (middle) and from Ftr-His-CLIPB14 transformed cells (right) were partially denaturated in 4 M urea plus 4 mM DTT and the denaturant was step-wise diluted to 2, 1, 0.5 and 0.1 M. For each concentration the soluble and the precipitated fractions were separated by centrifugation and the same amount of protein was loaded on SDS-PAGEs for coomassie staining and western blot analysis using specific antibodies against ClpB, DnaK, GroEL and ibpB. Each picture shows the data of a single experiment representative of three repetitions.

in all the urea concentrations and the western blot analysis indicated that DnaK and GroEL, even though not detectable by Coomassie staining, were present in the fractions and had the same pattern of distribution of CLIPB14 (Fig. 2, left). Differently, ClpB was always recovered in the insoluble fractions and ibpB was not detected at all (Fig. 2, left). Therefore, these data suggest that the His-tagged CLIPB14 strongly bound to DnaK and GroEL during precipitation and that mild denaturing conditions are not sufficient to remove the chaperones. In the inclusion bodies fraction it was possible to identify some ClpB but its affinity for the CLIPB14 was negligible (Fig. 2, left).

The pattern of solubility changed completely when the sample isolated from His-tagged CLIPB14 co-expressed with ibpA/B was used for the same experiments. The recombinant CLIPB14 became progressively insoluble at lower urea concentrations and all the four chaperones co-migrate with it (Fig. 2, middle), suggesting their binding to CLIPB14. Now, while at least ClpB did not bind the His-tagged CLIPB14 expressed alone, the over-expression of ibpA/B seems to lead to an aggregate conformation capable of recruiting the other chaperones. Such a mechanism could represent the starting point of the in vivo disaggregation route (Carrió and Villaverde, 2001) in which ibpA/Band ClpB play a key role in the preliminary steps leading to the refolding (Mogk et al., 1999, 2003a,b).

Also the co-expression of GroELS together with CLIPB14 induced an apparent co-migration of all the four chaperones analysed by western blot but, in this case, chaperones and CLIPB14 remained always soluble (data not shown).

The repartition of the material recovered from the inclusion bodies formed by the other CLIPB14-tag combinations is exemplified by the case of Ftr-CLIPB14 (Fig. 2, right). The CLIPB14 constructs were soluble in all the urea dilutions used and DnaK, GroEL and ibpB co-migrated with them. Nevertheless, the amount of bound ibpB was very limited and ClpB was mostly identified in the precipitates. It could be speculated that a limiting concentration of ibpB bound to the

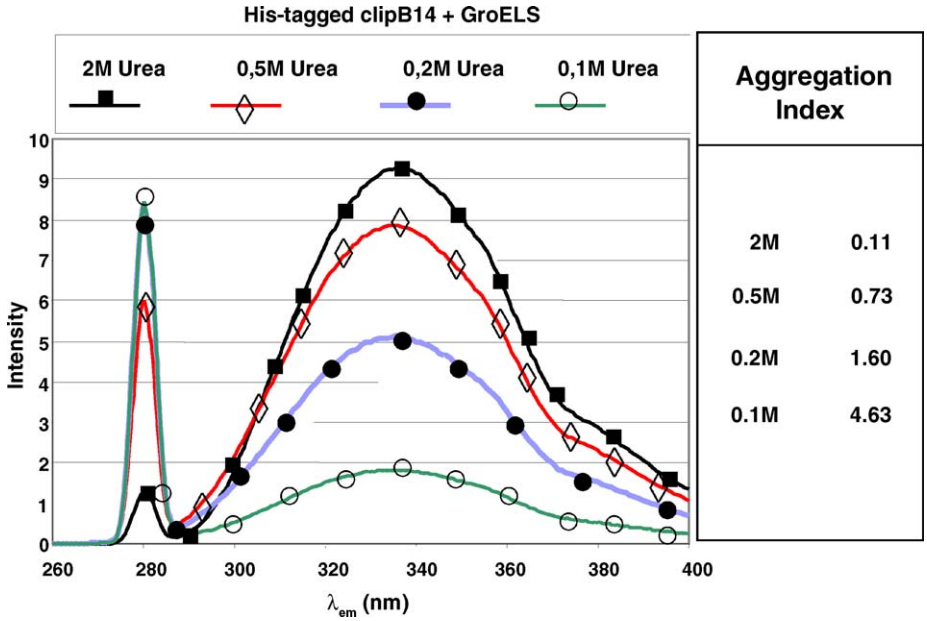

Fig. 3. Urea dilution-dependent re-aggregation of His-tagged CLIPB14. The emission spectra of the protein were measured at different urea concentrations and the ratio between the values at 280 and 340 nm (aggregation index) was calculated. The data are representative of four independent experiments.

substrate must be reached to allow the ClpB recruitment to the aggregates.

3.3. Re-aggregation pattern of the denatured CLIPB14 constructs

The solubility of a protein is not a measure of its monodispersity. A recent publication (Nominé et al., 2001) showed the presence of soluble aggregates and introduced a rapid fluorimetric method to analyse the aggregation state of the proteins that can be performed using even diluted proteins. The proposed aggregation index considers the ratio of the emissions at 280 and 340 nm. We have reported above that the His-tagged CLIPB14 obtained from inclusion bodies recovered from cells over-expressing GroELS was soluble even at concentrations of 0.1 M urea. Nevertheless, the protein was monodispersed, probably unfolded, at 2 M urea but progressively aggregated at lower urea concentrations, even though it did not precipitate after centrifugation (Fig. 3). We analysed more in detail the effect of the fusion tags on the re-aggregation pattern of the unfolded CLIPB14 fusions.

The inclusion bodies were solubilized in 8 M urea plus 4 mM DTT and progressively diluted in PBS buffer. The re-aggregation pattern was followed by the fluorimetric analysis and seems being specific for each protein construct and chaperone combination (Fig. 4). The purified His-tagged CLIPB14 from wild type BL21 (DE3) cells remained monodispersed until the concentration of 1 M urea but strongly aggregated at higher dilutions. Surprisingly, the same construct recovered from inclusion bodies generated in bacteria co-expressing GroELS showed a reduced level of aggregation at low urea concentrations. A direct solubilizing effect of GroEL(S) during the process of urea dilution seems improbable, since rather DnaK and ClpB are crucial in the first disaggregation steps (Mogk et al., 1999, 2003a,b; Goloubinoff et al., 1999). We can only argue that the interaction with the chaperones during the formation of the inclusion bodies let a sort of “structure memory” in the target protein, something like the effect of the proline isomerisation that can facilitate the folding of linearized peptides already processed into the folding-compatible proline conformation (Schmid et al., 1993).

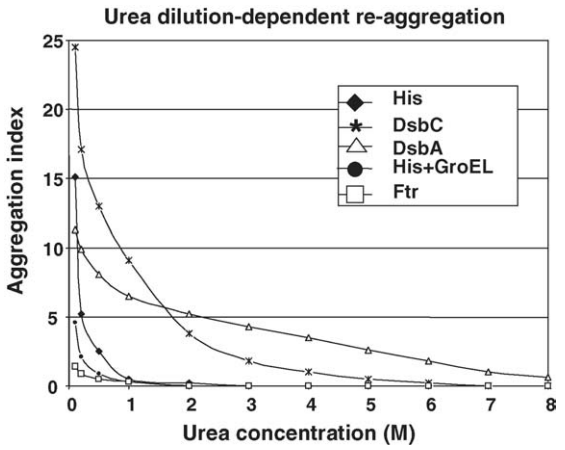

Fig. 4. Re-aggregation curves of different unfolded CLIPB14 constructs. CLIPB14 constructs initially denatured into 8 M urea and 4 mM DTT were step-wise diluted and their aggregation index calculated. The data are the average of three independent experiments.

The fusion with Ftr never re-aggregated significantly while the DsbC fusion was sensitive to any dilution of the urea concentration. DsbA was not completely unfolded even at 8 M urea but its re-aggregation was linear rather than progressive. It is interesting to observe that the effect of the fusion partners is different, even opposite, when we consider the soluble and the precipitated fractions. In fact, DsbA and DsbC fusions gave high rates of soluble proteins (Table 1) but their precipitated fractions have high tendency to (re)-aggregate after denaturation. In contrast, His and Ftr fusions are almost completely insoluble but their aggregates remain soluble and relatively monodispersed after denaturation, even at very low concentrations of urea.

The re-aggregation pattern of a denatured protein is an important factor to know for selecting the most promising material and for choosing the suitable refolding protocol. First, we performed a standard refolding screening using a commercial kit (FoldIt Screen) and a direct dilution protocol. The failure of the DFP labelling, however, revealed that the refolding of the protease was not complete and, therefore, only the aggregation rate of the protein sample was considered. The DsbC fusion precipitated in all the buffers while the Ftr fusion remained monodispersed in most of them and the other combinations were soluble in at least one buffer (data not shown). Similar results were obtained when a progressive dilution by dialysis was performed but in this case also DsbA always precipitated (data not shown). Therefore, the results confirmed that the constructs that re-aggregated at higher level during the urea dilution were also the most prone to precipitate during the refolding test.

4. Conclusions

The results underline the importance to screen different constructs and co-expression conditions to select the most suitable material for the further applications. In fact, the collected data demonstrate that it is possible to identify fusion tags and combinations of chaperones that can both improve the yield of the soluble target protein and modulate the homogeneity of the inclusion bodies. This last feature was crucial to optimise the refolding trials. Specifically, we showed the positive effect of GroELS co-expression and Ftr fusions to obtain CLIPB14 stable under mild denaturing conditions compatible with refolding protocols. Finally, we show the advantages of using an analytical method to evaluate the actual aggregation development of the denatured proteins.

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References

Altamirano, M.M., Garcia, C., Possani, L.D., Fersht, A.R., 1999. Oxidative refolding chromatography: folding of the scorpion toxin Cn5. Nat. Biotechnol. 17, 187–191.

Carrió, M.M., Cubarsi, R., Villaverde, A., 2000. Fine architecture of bacterial inclusion bodies. FEBS Lett. 471, 7–11.

Carrió, M.M., Villaverde, A., 2001. Protein aggregation as bacterial inclusion bodies is reversible. FEBS Lett. 489, 29–33.

Christophides, G.K., Zdobnov, E., Barillas-Mury, C., Birney, E., Blandin, S., Blass, C., Brey, P.T., Collins, F.H., Danielli, A., Dimopoulos, G., Hetru, C., Hoa, N.T., Hoffmann, J.A., Kanzok, S.M., Letunic, I., Levashina, E.A., Loukeris, T.G., Lycett, G., Meister, S., Michel, K., Moit, L.F., Muller, H.M., Osta, M.A., Paskewitz, S.M., Reichhart, J.M., Rzhetsky, A., Troxler, L., Vernick, K.D., Vlachou, D., Volz, J., von Mering, C., Xu, J., Zheng,

L., Bork, P., Kafatos, F.C., 2002. Immunity-related genes and gene families in *Anopheles gambiae*. Science 298, 159–165.

De Marco, V., Stier, G., Blandin, S., de Marco, A., 2004. The solubility and stability of recombinant proteins are increased by their fusion to NusA. Biochem. Biophys. Res. Commun. 322, 766–771.

de Marco, A., De Marco, 2004. Bacteria co-transformed with recombinant proteins and chaperones cloned in independent plasmids are suitable for expression tuning. J. Biotechnol. 109, 45–52.

de Marco, A., Casatta, E., Savaresi, S., Geerlof, A., 2004. Recombinant proteins fused to thermostable partners can be purified by heat incubation. J. Biotechnol. 107, 125–133.

Dyson, M.R., Shadbolt, S.P., Vincent, K.J., Perera, R.L., McCafferty, J., 2004. Production of soluble mammalian proteins in *Escherichia coli*: identification of protein features that correlate with successful expression. BMC Biotechnol. 4, 32.

Gill, S.C., von Hippel, P.H., 1989. Calculation of protein coefficients from amino acid sequence data. Anal. Biochem. 182, 319–326.

Goloubinoff, P., Mogk, A., Zvi, A.P., Tomoyasu, T., Bukau, B., 1999. Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. Proc. Natl. Acad. Sci. U.S.A. 96, 13732–13737.

Hunke, S., Betton, J.M., 2003. Temperature effect on inclusion body formation and stress response in the periplasm of *Escherichia coli*. Mol. Microbiol. 50, 1579–1589.

Jurado, P., Ritz, D., Beckwith, J., de Lorenzo, V., Fernandez, L.A., 2002. Production of functional single-chain Fv antibodies in the cytoplasm of *Escherichia coli*. J. Mol. Biol. 320, 1–10.

LaVallie, E.R., DiBlasio, E.A., Kovacic, S., Grant, K.L., Schendel, P.F., McCoy, J.M., 1993. A thioredoxin gene fusion expression system that circumvents inclusion body formation in *E. coli* cytoplasm. Biotechnology 11, 187–193.

Maachupalli-Reddy, J., Kelley, B.D., De Bernandez Clark, E., 1997. Effect of inclusion body contaminants on the oxidative renaturation of hen egg white lysozyme. Biotechnol. Prog. 13, 144–150.

Middelberg, A.P.J., 2002. Preparative protein refolding. Trends Biotechnol. 20, 437–443.

Mogk, A., Tomoyasu, T., Goloubinoff, P., Rüdiger, S., Röder, D., Langen, H., Bukau, B., 1999. Identification of thermolabile *Escherichia coli* proteins: prevention and reversion of aggregation by DnaK and ClpB. EMBO J. 18, 6934–6949.

Mogk, A., Deuerling, E., Vorderwulbecke, S., Vierling, E., Bukau, B., 2003a. Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation. Mol. Microbiol. 50, 585–595.

Mogk, A., Schlieker, C., Friedrich, K.L., Schonfeld, H.J., Vierling, E., Bukau, B., 2003b. Refolding of substrates bound to small Hsps relies on a disaggregation reaction mediated most efficiently by ClpB/DnaK. J. Biol. Chem. 278, 31033–31042.

Nominé, Y., Ristriani, T., Laurent, C., Lefevre, J.F., Weiss, E., Travé, G., 2001. A strategy for optimising the modispersity of fusion proteins: Application to the purification of HPV E6 oncoprotein. Protein Eng. 14, 297–305.

Nygren, P.A., Stahl, S., Uhlen, M., 1994. Engineering proteins to facilitate bioprocessing. Trends Biotechnol. 12, 184–188.

Rinas, U., Bailey, J.E., 1992. Protein compositional analysis of inclusion bodies produced in recombinant *Escherichia coli*. Appl. Microbiol. Biotechnol. 37, 609–614.

Sachdev, D., Chirgwin, J.M., 1998. Solubility of protein isolated from inclusion bodies is enhanced by fusion to maltose-binding protein or thioredoxin. Protein Expr. Purif. 12, 122–132.

Schmid, F.X., Mayr, L.M., Mücke, M., Schönbrunner, E.R., 1993. Prolyl isomerases: role in protein folding. Adv. Protein Chem. 44, 25–66.

Skinner, M.K., Griswold, M.D., 1983. Fluorographic detection of radioactivity in polyacrilamide gels with 2,5-diphenyloxazole in acetic acid and its comparison with existing procedures. Biochem. J. 209, 281–284.

Stegemann, J., Ventzki, R., Schrödel, A., de Marco, A., 2005. Comparative analysis of protein aggregates by blue native electrophoresis and subsequent SDS-PAGE in a three dimensional geometry gel. Proteomics, in press.

Zhang, Y., Olsen, D.R., Nguyen, K.B., Olson, P.S., Rhodes, E.T., Mascarenhas, D., 1998. Expression of eukaryotic proteins in soluble form in *Escherichia coli*. Protein Expr. Purif. 12, 159–165.