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REFOLDING OF LYSOZYME ASSISTED BY MOLECULAR CHAPERONES IMMOBILIZED IN CELLULOSE: THE OPERATIONAL CONDITIONS THAT AFFECT REFOLDING YIELDS

REPLEGAMIENTO DE LIZOSIMA ASISTIDA POR CHAPERONAS MOLECULARES INMOVILIZADOS EN CELULOSA: LAS CONDICIONES OPERATIVAS QUE AFECTAN LOS RENDIMIENTOS DE REPLEGAMIENTO

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Abstract

Expression of recombinant proteins in *Escherichia coli* often leads to formation of inclusion bodies (IBs). To recover the protein activity, the IBs are isolated, solubilized and refolded. The protein refolding processes play a major role in the production of recombinant proteins; thus, various methodologies have been implemented, including dilution, dialysis and column chromatography with or without the assistance of molecular chaperones. Recently, it was demonstrated that the apical domain of GroEL (AD), DsbA and DsbC immobilized on cellulose improved the efficiency of chromatographic refolding of rhodanese and lysozyme. Although immobilized chaperones and foldases greatly improve refolding yields, their use has been limited. To improve their potential use at the bioprocess scale, it is essential to understand the effects of operational conditions and additives on refolding yields. Therefore, we investigated the lysozyme refolding kinetics assisted by the apical domain of GroEL (AD), DsbA and DsbC in either soluble or immobilized on cellulose with different lysozyme concentrations, different chaperone:lysozyme ratios, absence of redox pairing, presence of glycerol and presence of high concentrations of GdnHCl and β-mercaptoethanol (β-ME). Our results provide insight to improve the use of molecular chaperones in the refolding of recombinant proteins expressed as inclusion bodies.

Keywords: protein refolding, inclusion bodies, chaperone immobilization, cellulose, lysozyme.

Resumen

Las proteínas recombinantes expresadas en *Escherichia coli* en muchas ocasiones se acumulan en forma de cuerpos de inclusión (IBs) por lo que para recuperar la actividad biológica de éstas, es necesario solubilizarlas de los IBs y llevar a cabo su replegamiento, proceso que representa una etapa limitante en la producción de proteínas recombinantes. Metodologías como la diálisis, dilución, uso de chaperones moleculares y técnicas cromatográficas, se han implementado con éxito en el laboratorio. Recientemente, para facilitar el uso de chaperones, se demostró que el dominio apical de GroEL (AD), y las oxidoreductasas DsbA y DsbC inmovilizadas en celulosa, asistieron eficientemente el replegamiento de rodanasa y lisozima. Sin embargo, para mejorar su potencial uso a una escala de producción, se requiere conocer cómo afectan las condiciones de operación y aditivos en los rendimientos de plegamiento. En este trabajo, evaluamos la cinética de replegamiento de lisozima asistida por dominio apical de GroEL (AD), y las oxidoreductasas DsbA y DsbC, solubles o inmovilizadas en celulosa usando diferentes concentraciones de lisozima, glicerol, GdnHCl y β-mercaptoethanol (β-ME), así como diferentes relaciones molares de chaperón:lisozima y la ausencia de un par redox. Estos resultados reportados pueden contribuir al diseño de estrategias para mejorar el uso de los chaperones molecular en el replegamiento de proteínas expresadas como cuerpos de inclusión.

Palabras clave: replegamiento de proteínas, cuerpos de inclusión, chaperones inmovilizados, celulosa, lisozima.

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1 Introduction

The *Escherichia coli* expression system is frequently used for the production of recombinant proteins that are not compromised by posttranslational modification because of its high expression yields and inexpensive culture media (Baneyx *et al*., 1999). Many strains of *E. coli*, as well as a variety of expression vectors and culture conditions, are available to express recombinant proteins in their soluble and correctly folded conformation (Koths, 1999; De Bernardez, 1998). However, these expression systems often fail, producing misfolded recombinant proteins that aggregate into inclusion bodies (IBs). To recover their biological activity, the recombinant proteins must be refolded, a step that has become the major limitation of the *E. coli* expression system. To obtain correctly folded proteins after solubilization of the IBs, excess denaturants and reducing thiol reagents have to be removed, with the reduced proteins transferred to oxidizing conditions (Middelberg, 2002; Misawa *et al*., 1999). There are numerous refolding methods available, including dilution, dialysis and column chromatography, designed to improve protein refolding (Jungbauer *et al*., 2004). The simplest method is reducing the concentration of denaturant by dilution with an appropriate refolding buffer. This strategy remains widely used at the production scale (Jungbauer *et al*., 2004; Chow, 2006; Jungbauer *et al*., 2007). The efficiency of protein refolding depends on the competition between correct folding and aggregation. This competition can be modulated by adjusting several operational conditions (Misawa *et al*., 1999; Jungbauer *et al*., 2004; Chow *et al*., 2006; Jungbauer *et al*., 2007; Altamirano *et al*., 1997; Basu *et al*., 2011). *In vivo*, these competing reactions are modulated by chaperones and foldases (Hartl *et al*., 2009). *In vitro*, refolding is assisted by immobilized chaperones and foldases, significantly improving refolding yields. However, the application of these *In vitro* technologies at the bioprocess scale has been limited by the costs associated with the use of molecular chaperones under operational conditions (Basu *et al*., 2011; Jhamb *et al*., 2008; Ramon-Luing *et al*., 2006). In previous studies, DsbA, DsbC, and the apical domain of GroEL (AD) were fused to the carbohydrate-binding module CBD_{Cex} of *Cellulomonas fimi* (CBM) to facilitate the use of molecular chaperones. Recombinant proteins were then purified and immobilized on cellulose to assist the refolding of denatured rhodanese and lysozyme either by dilution (Ramon-Luing *et al*., 2006; Antonio-Pérez *et al*., 2012) or oxidative chromatographic refolding (Antonio-Pérez, Ramón-Luing., *et al*., 2012). However, little is known about the operational conditions that may affect refolding yields. Understanding these operational conditions may improve the use of molecular chaperones at the bioprocess scale for refolding of recombinant proteins expressed as IBs. Here, we characterize the effects of the GSH/GSSG redox system, glycerol and denatured protein concentrations and the molar ratio of chaperone:lysozyme on the refolding yields of lysozyme. We also investigate the stability of chaperones (AD-CBM, DsbA-CBM and DsbC-CBM) immobilized on cellulose under high concentration of denaturants and reducing agents.

2 Materials and methods

Microcrystalline cellulose particles (Sigmacell Type 50), lysozyme and dried *Micrococcus lysodeikticus* cells were purchased from Sigma (St. Louis, MO, USA). Chromatographic columns, BioGel 6P fine, and MacroPrep Methyl were purchased from Bio-Rad (Hercules, CA, USA). Ni-Sepharose 6 Fast Flow was obtained from GE Healthcare (Piscataway, NJ, USA). Only analytical grade reagents and chemicals were used.

2.1 Preparation of denatured lysozyme

Native lysozyme was dissolved in a denaturing buffer (6 M GdnHCl and 120 mM β-ME) to a final concentration of 3.0 mg/mL and incubated at 4 °C for at least 4 h before the refolding experiments were performed.

2.2 Lysozyme activity assays

Lysozyme activity was measured at 25 °C by monitoring the decrease in the absorbance at 450 nm of 0.06 mg/mL *M. lysodeikticus* in 1 M phosphate buffer at pH 7.0. The refolded lysozyme sample (50 μL) was added to 1.45 mL of the substrate solution. The decrease in absorbance was then monitored every 5 s for 3 min using a Hewlett Packard 8453 diode array spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The specific enzyme activity was estimated from the initial rate per mg of protein (Dong *et al*., 2000). The refolding yield was determined by setting the specific activity of the native enzyme to 100%.

2.3 Purification and immobilization of AD-CBM, DsbA-CBM and DsbC-CBM

AD, DsbA, and DsbC fused to CBM from *C. fimi* (henceforth denoted AD-CBM, DsbA-CBM and DsbC-CBM) were expressed, purified and finally immobilized on cellulose particles (Sigmacell Type 50), as previously described (Ramon-Luing *et al*., 2006; Antonio-Pérez *et al*., 2012). A known amount of AD-CBM, DsbA-CBM or DsbC-CBM was then added to cellulose particles that were equilibrated ahead of time in the interaction buffer (20 mM Tris-HCl, pH 7.5, 20 mM NaCl). The mixture was incubated for 4 h at 4 °C in a minirotator shaker at 18 rpm. Afterwards, the microcrystalline cellulose particles were washed with 20 mM Tris-HCl containing 800 mM NaCl, pH 7.5. The immobilization coupling densities of AD-CBM, DsbA-CBM and DsbC-CBM were varied to achieve chaperone:lysozyme molar ratios of 1:1, 2:1 and 5:1.

The amount of protein bound to the cellulose was estimated from the difference in protein concentration in the supernatant before and after immobilization, measured using the BCA Protein Assay Kit, as previously reported (Antonio-Pérez, Ramón-Luing., *et al*., 2012). Briefly, 25 mg samples of cellulose from the refolding matrix were treated with Laemmli buffer and analyzed by 10% SDS-PAGE. The amount of protein was estimated by densitometric analysis of the protein bands in Coomassie brilliant blue-stained gels using Quantity One software (Bio- Rad, Hercules, CA, USA), with bands of known amounts of the same proteins used as standards.

2.4 Oxidative refolding

Lysozyme refolding was carried out using a 1:100 rapid dilution of the denatured, reduced lysozyme in refolding buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.4 mM GSSG, 2 mM GSH, 200 mM L-arginine, 1 M urea, 100 mM PMSF) to a final concentration of 25 mg/L in a reaction volume of 1.5 mL. The time required to recover lysozyme activity was determined over the course of 1 h incubation at 25 °C. Samples were collected every 5 min, and lysozyme enzymatic activities were immediately measured. In all cases, experiments were performed at least three times, giving similar results. The refolding yield was defined as the percentage of specific activity of the refolded lysozyme relative to the specific activity of the native lysozyme (set as 100%). To determine the effect of lysozyme concentration on refolding yields, final protein concentrations of 50, 75, 100, 150 and 200 mg/L in a reaction volume of 1.5 mL were tested. In another set of experiments, the refolding buffer was supplemented with 5%, 10%, 20% and 25% glycerol to determine the effect of glycerol concentration on refolding yield. In addition, oxidative refolding of lysozyme assisted by GroEL, DsbA and DsbC was evaluated with or without the addition of GSSG/GSH to the refolding buffer. In all assisted refolding assays, refolding buffer was supplemented beforehand with the appropriate amount of either soluble or immobilized AD-CBM, DsbA-CBM or DsbC-CBM or with an equimolar mixture of AD-CBM, DsbA-CBM and DsbC-CBM for a given chaperone:lysozyme molar ratio (e.g., 1:1).

2.5 Evaluation of the functional stability of immobilized foldases

DsbA-CBM, DsbC-CBM and AD-CBM immobilized on cellulose were incubated at 25 °C in 20 mM Tris-HCl, pH 8.0 containing GdnHCl and β-ME at concentrations of 1 M and 21 mM, 2 M and 42 mM, 3 M and 63 mM and 4 M and 80 mM, respectively. After 10, 30, 60 and 120 min of incubation, samples of immobilized chaperones were centrifuged for 5 min at 16,000 g, and supernatants were discarded. The immobilized chaperones were then resuspended in refolding buffer for the lysozyme oxidative refolding assay, as described above. The recovered lysozyme in the assisted refolding assay was used as an indicator of the functional stability of the immobilized chaperones.

3 Results

3.1 Lysozyme refolding yields

To diminish the mass transfer effect on lysozyme refolding assisted assays by the immobilized chaperones, the refolding assay for AD-CBM was performed at agitation speeds between 50 and 250 rpm (data not shown). The recovered lysozyme activity increased as agitation speed increased, plateauing at an agitation speed of 150 rpm. This result suggests that the effects of mass transfer on refolding can be diminished by working at agitation speeds greater than or equal to 150 rpm. Interestingly, for unassisted refolding, the refolding yields decreased at agitation speed greater than 150 rpm, suggesting that high agitation speeds favored unproductive protein-protein interactions that are prevented by the assistance of

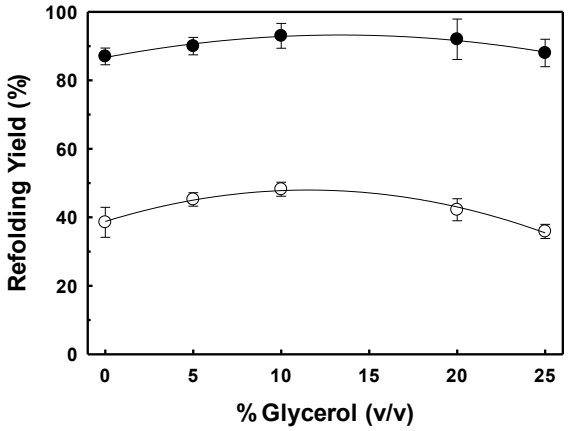

Fig. 1. **Effect of glycerol on the lysozyme refolding**. Lysozyme refolding was carried out using a 1:100 rapid dilution of the denatured, reduced lysozyme in refolding buffer to give a final lysozyme concentration of 0.025 mg/mL in a 1.5 mL reaction volume. The time course for recovery of lysozyme activity was determined over the course of 1 h of incubation at 25ºC. The enzyme activity was immediately determined after incubation. The symbols represent the refolding yield after 1 h for assisted (•) and unassisted (○) refolding experiments. For the assisted refolding assays, buffer was supplemented beforehand with the appropriate amount of free AD-CBM at a 1:1 molar ratio to lysozyme. The refolding yield was defined as the percentage of specific activity of the refolded lysozyme relative to the specific activity of the native lysozyme (set as 100%). The vertical bars indicate standard error calculated from at least three assays.

chaperones. Therefore, experiments were performed at 150 rpm, unless other agitation conditions were specified.

3.2 Effect of glycerol on lysozyme refolding yields

The stability of stored AD-CBM, DsbA-CBM and DsbC-CBM greatly increased with the addition of 20-50% glycerol (Antonio-Pérez, Ramón-Luing, *et al*., 2012). To determine the effect of glycerol on recovered lysozyme activity, lysozyme refolding assays were performed at different glycerol concentrations (Fig. 1). Glycerol concentrations < 20% with (filled circles) or without (open circles) the assistance of AD-CBM concentrations improved the refolding yields of lysozyme; however, at glycerol concentrations greater than or equal to 20%, a decrease in the refolding yields was noted, especially with unassisted refolding. It is known that glycerol favors formation of secondary structures and inhibits aggregation of many proteins by hindering contact between hydrophobic regions and preventing intermolecular hydrophobic interactions in a concentration-dependent manner (De Bernardez, 1998; Tsumoto *et al*., 2003; Gekko *et al*., 1981). However, high concentrations of glycerol increased the viscosity of the solution. This increase most likely affected lysozyme refolding kinetics, favoring unproductive protein-protein interactions that were partially prevented by the presence of chaperones. Therefore, glycerol concentrations below 20% could be used in the refolding assisted by immobilized chaperones, but the increase in the viscosity should be considered specially for chromatographic refolding.

3.3 Effect of redox system addition

The influence of the redox environment on refolding yield has been extensively studied (Tsumoto *et al*., 2003; Zapun *et al*., 1995; Lyles *et al*., 1991; Ruoppolo *et al*., 1996; Langenhof *et al*., 2005; Lu *et al*., 2008; Maskos *et al*., 2003). It was previously demonstrated that a dynamic redox environment, promoted by the addition of GSSG/GSH, improved folding yields (Ruoppolo *et al*., 1996; Langenhof *et al*., 2005). However, the addition of GSSG/GSH represents an economic burden at the process scale. To determine the contribution of GSSG/GSH to refolding assisted by AD-CBM, DsbA-CBM and DsbC-CBM, lysozyme refolding yield experiments with AD-CBM, DsbA-CBM and DsbC-CBM in solution (Panel A) or immobilized on cellulose (Panel B) with (filled bars) and without (open bars) the redox pairs were performed (Fig. 2). As expected, the absence of GSSG/GSH negatively affected the refolding of lysozyme. This decrease was even more drastic for unassisted refolding. For unassisted refolding, the recovered lysozyme activity was less than 10%. For assisted refolding without GSSG/GSH, 25% to 70% of the lysozyme activity was recovered. In fact, 70% or 35% of lysozyme activity was recovered for assisted refolding without the par redox by free or immobilized foldases (AD, DsbA and DsbC), respectively, compared with 90% or 70%, respectively with the redox pair (Figs. 2A, 2B). Moreover, the refolding yields for lysozyme assisted by the three foldases without GSSG/GSH were greater than unassisted refolding with the par redox, suggesting that in the absence of GSSG/GSH,

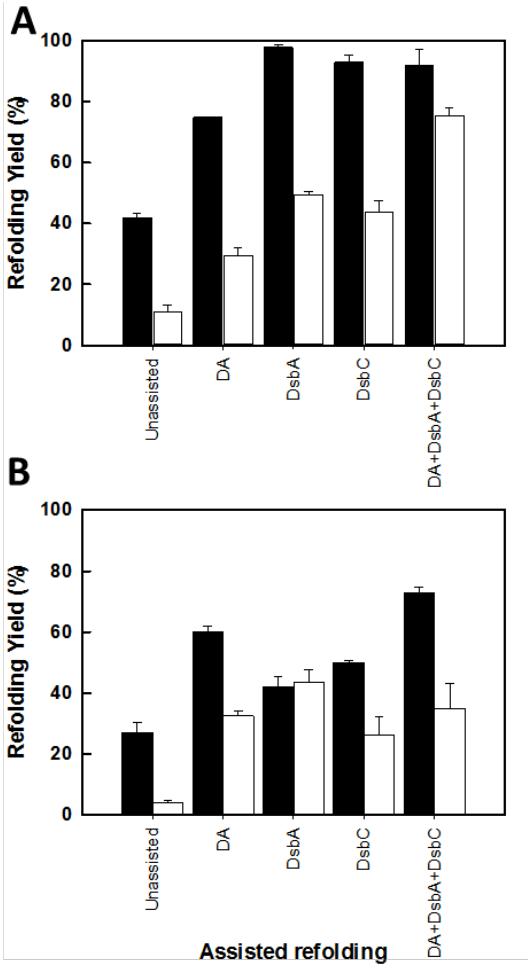

Fig. 2. **Effect of GSH/GSSG on the lysozyme refolding yield**. Lysozyme refolding was carried out using a 1:100 rapid dilution of the denatured and reduced lysozyme as previously described. The time course for recovery of lysozyme activity was determined over 1 h of incubation at 25ºC. The activity was immediately determined after incubation. For assisted refolding experiments, the refolding buffer was supplemented beforehand with the appropriated amounts of free (A) or immobilized (B) AD-CBM, DsbA-CBM, and DsbC-CBM or equimolar mixtures of two or three foldases at a 1:1 molar ratio of each of the folding biocatalysts to lysozyme. Experiments were performed in refolding buffer with (filled bars) or without (empty bars) the helper redox system (GSSG/GSH). The refolding yield was defined as the percentage of the specific activity of the refolded lysozyme relative to the specific activity of the native lysozyme (set as 100%). Bars represent standard error.

lysozyme disulfide bond formation is catalyzed by DsbA, most likely due to dithiol-disulfide exchange with DsbC. However, the DsbA-DsbC interaction is impaired by immobilization, decreasing the lysozyme disulfide bond formation rate when immobilized on cellulose, suggesting that GSSG/GSH or other redox couple is necessary to obtain acceptable column refolding yields. Further experiments will be needed to test such a hypothesis.

3.4 Effect of lysozyme concentration on refolding yields

To investigate the effect of unfolded protein concentration on the assisted refolding yield, denatured and reduced lysozyme was added into the refolding assay at concentrations ranging from 25 to 200 mg/L (Fig. 3). Although, the refolding yield for assisted refolding (filled circles) decreased as the lysozyme concentration increased, no aggregation was observed. In addition, the recovered lysozyme activity for assisted refolding was always higher compared with unassisted refolding (open circles). This observation is consistent with results reported for chromatographic refolding of lysozyme assisted by immobilized foldases, where 100% of lysozyme activity was recovered when samples of 1 mg/ml unfolded protein were loaded into the column and only 60% of lysozyme activity was recovered at a protein concentration of 3 mg/ml. Remarkably, a 100% refolding yield was obtained after six consecutive refolding batches, indicating that protein aggregation was prevented by chaperone-protein interactions (Antonio-Pérez, Ramón-Luing., *et al*., 2012). It is known that at high protein concentrations, unfolded polypeptides are in close physical proximity to each other, favoring intermolecular contact among partially folded intermediates, thus triggering aggregation (Gupta *et al*., 1998). Our data are consistent with the hypothesis that presence of chaperones in the refolding reaction prevents protein aggregation rather than modifying the rate of protein refolding (Antonio-Pérez *et al*., 2012).

3.5 Effect of chaperone:lysozyme ratio on refolding yields

The molar ratios of chaperones, as well as the inhibitory effect of residual chemical denaturants, should be taken into account in the design of any assisted refolding process. To investigate the influence of the foldase:unfolded protein molar ratio,

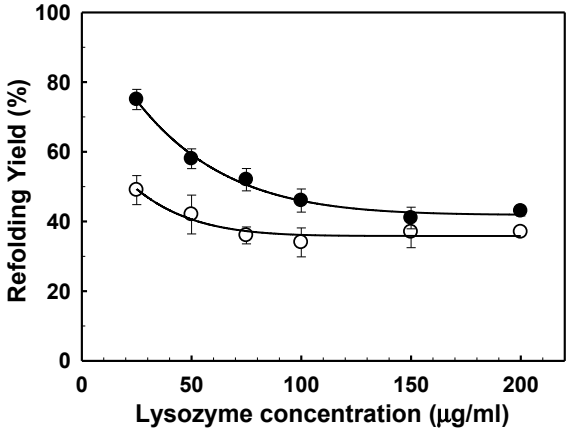

Fig. 3. **Effect of the unfolded protein concentration on the lysozyme refolding yield**. Lysozyme refolding was carried out using a 1:100 rapid dilution of the denatured and reduced lysozyme in the refolding buffer to give a final lysozyme concentration from 25 μg/mL to 200 μg/mL in a 1.5 mL reaction volume. The time course for recovery of lysozyme activity was determined over 1 h of incubation at 25ºC. The final percentage of refolded lysozyme was monitored for the assisted (●) and unassisted systems (○). The activity of the native lysozyme was set at 100%. Bars represent standard error.

the refolding yields for lysozyme oxidative refolding were determined for various concentrations of AD-CBM, DsbA-CBM and DsbC-CBM. Figure 4 shows the refolding yields assisted by AD-CBM (Panel A) and the oxidoreductases DsbA and DsbC (Panel B) at 1:1, 2:1, 3:1, 5:1 and 10:1 molar ratios of chaperone:lysozyme. The refolding rates and yields decreased with excess AD-CBM (from 90% to only 43% at molar ratios of 1:1 and 10:1) (Fig. 4A). Although excess DsbA and DsbC caused a decrease in refolding rate, after 55 min incubation time, the refolding yields were similar for all tested conditions (Fig. 4B).

A reasonable explanation is that AD-CBM specifically interacts with hydrophobic surfaces that are highly exposed on unfolded or partially folded polypeptides, while DsbA and DsbC only interact with disulfide bonds, a consequence of AD-CBM exhibiting stronger protein-protein interactions. This explanation is supported by previous reports on refolding of LDH with the assistance of holochaperonins. In these reports, the GroEL-LDH complex was stable over a period of at least 6 h, and the refolding of LDH was completely suppressed under GroEL saturation concentrations (5:1 molar

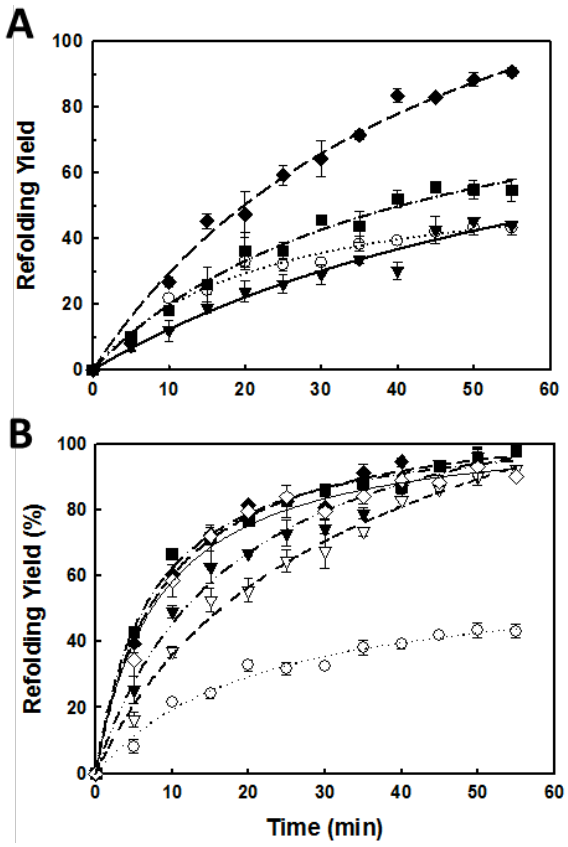

Fig. 4. **Effect of lysozyme:chaperon molar ratio on the lysozyme refolding yield**. Lysozyme refolding was carried out using a 1:100 rapid dilution of the denatured and reduced lysozyme in the refolding buffer to give a final lysozyme concentration of 0.025 mg/mL in a 1.5 mL reaction volume. The time course for recovery of lysozyme activity was determined over 1 h of incubation at 25ºC. The activity was immediately determined after incubation. For assisted refolding experiments, the refolding buffer was supplemented beforehand with the appropriate amount of free AD-CBM (A), oxidoreductases DsbA (B) or DsbC (C) at 1:1 (◆), 2:1 (■), 3:1 (▲), and 5:1 (▼) molar ratios of chaperone:lysozyme. For unassisted refolding, no chaperone was added (○). Refolding yield was defined as the percentage of the specific activity of the refolded lysozyme relative to the specific activity of the native lysozyme (set as 100%). Vertical bars denote the standard error of at least three assays.

ratio). This complex was also dissociated with the addition of 2 mM ATP. After 6 hours of reaction, the enzyme activity was recovered between 50% and 55%

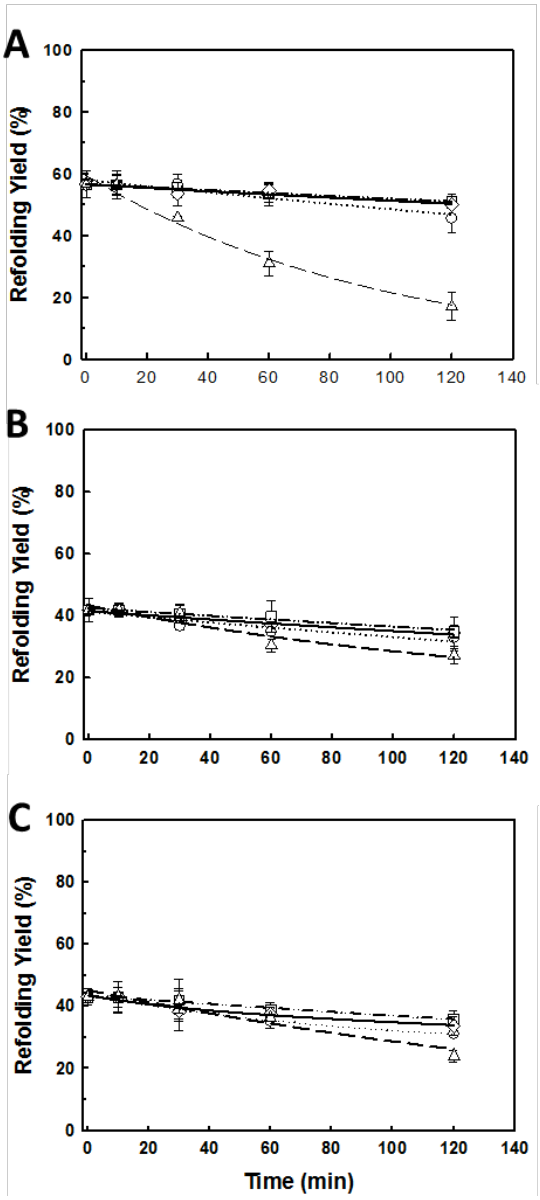

Fig. 5. Operational stability of cellulose-immobilized AD-CBM, DsbA-CBM, and DsbC-CBM. AD-CBM (A), DsbA-CBM (B), and DsbC-CBM (C) immobilized on cellulose were mixed with BioGel P-6 fine (Bio-Rad) and added into the refolding buffer supplemented with GdnHCl/β-ME at 1 M/20 mM (□), 2 M/40 mM(⋄), 3 M/60 mM(○), and 4 M/80 mM (△) for 2 h. After this denaturing incubation, the cellulose was washed three times with 20 mM Tris-HCl, pH 7.5, and 100 mM NaCl and used to assist lysozyme refolding by dilution, as previously described. The foldase activities were then determined. The initial values for refolding yield (without denaturing incubation) were set at 100%.

due to refolding (Teshima *et al*., 2000; Mizobata *et al*., 2000). According to these results, protein refolding should be carried out at an equimolar ratio between unfolded polypeptide and the molecular chaperone (AD-CBM, DsbA-CBM or DsbC-CBM).

3.6 Effect of GdnHCl and β-ME on the stability of AD-CBM, DsbA-CBM and DsbC-CBM immobilized on cellulose

To determine the effect of denaturing and reducing conditions on the functional stability of AD-CBM, DsbA-CBM and DsbC-CBM immobilized on cellulose, each foldase was incubated in varying concentrations of GdnHCl and β-ME. Their refolding assistance capacity was then determined at different incubation times (Fig. 5). At concentrations of urea and β-ME lower than or equal to 3 M and 60 mM, respectively, all of the foldases lost only 20% of their activity after 120 min of incubation. Remarkably, DsbA and DsbC incubated in 4 M urea and 80 mM β-ME retained more than 70% of their activity after 60 min, while AD-CBM only retained 50% and 25% of its chaperone activity in the refolding assay after 60 and 120 min of incubation, respectively. The refolding assistance capacities of the foldases immobilized on cellulose depend on the stability of the immobilization, as well as on the specific chemical stability of each foldase. The former is correlated with the protein lost from the cellulose. The latter is correlated with the loss of functional conformation during incubation in denaturing conditions. Because all foldases are bound to cellulose through CBM, the fact that in 4 M urea, AD-CBM (Fig. 5 A) lost its refolding ability faster than DsbA or DsbC (Fig. 5B and 5C) suggests that AD has a lower chemical stability than DsbA and DsbC. The presence of low concentrations of denaturant (up to 3 M urea and 60 mM β-ME) did not affect binding of the foldases to cellulose. The conformational changes occurring under these concentrations were recovered during the refolding assay. However, incubation in 4 M urea and 80 mM β-ME caused greater conformational changes for AD than for DsbA and DsbC, affecting the recovery of its chaperone activity. One probable explanation is that AD has lower chemical stability than DsbA or DsbC because AD is one of three domains found in each of the 14 monomers that form the chaperonin GroEL, while DsbA and DsbC are whole enzymes. Previous observations indicate that immobilized AD-CBM, DsbA-CBM and DsbC-CBM on packed cellulose in a refolding column exhibited

greater operational stability after several consecutive refolding batches for protein samples solubilized in 8 M urea or 6 M GdnHCl and 120 mM β-ME (Antonio-Pérez, Ramón-Luing., *et al*., 2012), concentrations of denaturant that were much higher than those tested in our study. However, in the column, the time that immobilized chaperones are exposed to harsh denaturing and reducing conditions is short, allowing them to recover their functional conformation after being perturbed by the short pulse of urea or GdnHCl. This is consistent with the fact that less than 8% of the protein was removed from the refolding column and more than 85% of its refolding capability remained after eight consecutive chromatographic refolding batches (Antonio-Pérez, Ramón-Luing., *et al*., 2012). Further studies are needed to correlate our data with the reusable refolding columns containing foldases immobilized on cellulose.

Conclusions

Immobilized chaperones are an efficient alternative strategy for renaturing recombinant proteins from IBs. Despite the fact that their use has been limited by several operational drawbacks, in this paper, we provided evidence for their potential use at the bioprocess scale. According to our results, AD-CBM, DsbA-CBM and DsbC-CBM immobilized on cellulose displayed high operational stability for long periods of time in the presence of extreme denaturant conditions. Such a result opens the possibility for greater reuse potential. We used an immobilized chaperone to denatured peptide molar ratio of 1:1; however, this does not necessarily imply that equimolar amounts are required for consecutive chromatographic refolding batches. AD-CBM, DsbA-CBM and DsbC-CBM assisted the oxidative refolding of lysozyme even without the helper redox system. In conclusion, the use of molecular chaperones for refolding of recombinant proteins is possible at the bioprocess scale by optimizing the handling of additives and the operational conditions.

Acknowledgments

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