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Investigation on solubilization protocols in the refolding of the thioredoxin TsnC from *Xylella fastidiosa* by high hydrostatic pressure approach

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ABSTRACT

The lack of efficient refolding methodologies must be overcome to take full advantage of the fact that bacteria express high levels of aggregated recombinant proteins. High hydrostatic pressure (HHP) impairs intermolecular hydrophobic and electrostatic interactions, dissociating aggregates, which makes HHP a useful tool to solubilize proteins for subsequent refolding. A process of refolding was set up by using as a model TsnC, a thioredoxin that catalyzes the disulfide reduction to a dithiol, a useful indication of biological activity. The inclusion bodies (IB) were dissociated at 2.4 kbar. The effect of incubation of IB suspensions at 1–800 bar, the guanidine hydrochloride concentration, the oxidized/reduced glutathione (GSH/GSSG) ratios, and the additives in the refolding buffer were analyzed. To assess the yields of fully biologically active protein obtained for each tested condition, it was crucial to analyze both the TsnC solubilization yield and its enzymatic activity. Application of 2.4 kbar to the IB suspension in the presence of 9 mM GSH, 1 mM GSSG, 0.75 M guanidine hydrochloride, and 0.5 M arginine with subsequent incubation at 1 bar furnished high refolding yield (81%). The experience gained in this study shall help to establish efficient HHP-based protein refolding processes for other proteins.

Introduction

To obtain biomedically relevant proteins that occur at low concentrations in their native sources, researchers have expressed recombinant proteins in *Escherichia coli*, the bacterial species that is most often employed to produce heterologous proteins for structural and functional studies and to obtain commercially valuable proteins.

Hydrophobic interactions among misfolded molecules determine aggregation and inclusion body (IB) formation. Soluble recombinant proteins are occasionally expressed, especially when the transformed bacteria are cultivated at low temperature. Expression of recombinant proteins in *E. coli* as a soluble state in the cytoplasm is preferred as a norm, because these compounds usually acquire a native state without the need for further refolding processing. Nevertheless, a number of recombinant proteins may aggregate even if the host is cultivated at low temperature. In this condition, IB with higher degree of intramolecular interactions emerges at the cost of intermolecular contacts and generates proteins with secondary structures that resemble the corresponding native state more closely than when the bacteria is grown at higher temperatures [1,2]. For subsequent refolding, it is interesting that the IB displays a network of intermolecular contacts that is easy to break, to facilitate protein solubilization at relatively mild conditions while preserving the IB native-like existing structures [3]. The expression of proteins as aggregates offers important advantages over their production in the soluble form. First, their expression level is generally high. Second, it is possible to physically remove the undesirable host contaminants from the insoluble aggregates, to increase the purity of the target protein. Unfortunately, establishing a simple and efficient protocol for protein refolding from the IB remains a difficult challenge [4].

High hydrostatic pressure (HHP)[1] has been widely used to study protein conformation. Application of 1–3 kbar disrupts the

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[1] *Abbreviations used:* HHP, high hydrostatic pressure; IB, inclusion bodies; GSH, reduced glutathione; GSSG, oxidized glutathione; TrxR, thioredoxin reductase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 2-nitro-5-thiobenzoic acid; NADPH, β-nicotinamide adenine dinucleotide 2′-phosphate reduced.

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intermolecular electrostatic and hydrophobic interactions, dissociating oligomeric and aggregated proteins. The same kind of interactions that maintain the aggregated states also preserve the secondary and tertiary protein structures in their native conformation [5–7]. However, more efficient protein hydration followed by disruption of intramolecular bonds and subsequent protein denaturation generally requires pressure higher than 4 and 5 kbar [8]. Therefore, the use of 2–3 kbar constitutes a non-denaturing technique that solubilizes aggregates and keeps the secondary and tertiary protein structure intact, being potentially applicable in protein refolding [9,10]. In this context, HHP has become a technique to obtain high-yield protein refolding [11–14]. Quantification of the target protein in the soluble fraction often aids determination of the refolding process efficiency [15,16]. Nevertheless, the fact that a protein is soluble does not necessarily imply that it is in the native conformation, with full biological activity. Therefore, it can be important to determine both the degree of solubilization as well as the biological activity of the target protein during optimization of the refolding process.

Thioredoxin are enzymes that play key roles in cell biology, among them as hydrogen donors for the reduction of ribonucleotides to deoxyribonucleotides (building blocks of DNA) [17]; and as substrates for peroxiredoxin, the most relevant cellular pathway for removal of hydroperoxides [18]. Thioredoxins are thiol-disulfide oxido-reductases of about 12 kDa; they display a Trp-Cys-Gly-Pro-Cys motif that reversibly switches between the disulfide and dithiol states. Dithiol thioredoxin can reduce proteins such as ribonucleotide reductase and peroxiredoxin, to generate disulfide thioredoxin. NADPH then reduces the enzyme in its disulfide form in a reaction catalyzed by thioredoxin reductase, a flavo-enzyme [17,19].

This work employed TsnC, a thioredoxin from *Xylella fastidiosa*, in refolding studies. *X. fastidiosa* is an economically important gram-negative bacterium: it is the causative agent of variegated chlorosis disease (CVC) in Brazil and of Pierce's disease (grapes) and oleander leaf scorch in southern United States [20]. TsnC displays the characteristic features of thioredoxins such as low molecular weight and the Trp-Cys-Gly-Pro-Cys motif in the active site. Remarkably, TsnC can reduce peroxiredoxin PrxQ [21] but not Ohr [22], two Cys-based, thiol-dependent peroxidases from *X. fastidiosa*. Because the enzymatic assay for TsnC is well established, this protein is suitable for application in refolding studies that consider not only solubility, but also activity for optimization purposes. More specifically, the present study describes the refolding of IB aggregates of the thioredoxin TsnC from *X. fastidiosa* produced in *E. coli*. The aggregates were dissociated at 2.4 kbar. The refolding conditions were optimized with respect to the reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio, the guanidine hydrochloride concentration, and the presence of additives. TsnC quantification in the soluble fraction and activity determination by the thioredoxin activity assay aided analysis of the refolding process efficiency. The best TsnC refolding yield was 81%: each liter of bacterial culture (2.5 g wet cells) afforded 55 mg of biologically active TsnC.

Materials and methods

TsnC expression, growth conditions, cell fractionation, and IB isolation

To express recombinant TsnC, in this work a BL21(DE3) *E. coli* strain was transformed with the vector pET15b containing the TsnC gene cloned into the EcoRI/Hind III restriction sites [21,22]. A colony was randomly picked from transformants that were grown on LB plates (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl) containing 100 μg mL^{-1} ampicillin and inoculated in 0.5 L of 2-HKSII rich medium [23]. Cells were grown at 37 °C, and expression was induced by addition of isopropyl–D-thiogalactopyranoside (0.5 mM) when the absorbance at 600 nm reached approximately 3.0. After incubation with constant orbital agitation (150 rpm) for 16 h, the bacteria were collected by centrifugation at 5000*g* at 4 °C, for 10 min. The pellet containing approximately 1.2 g of wet cells was resuspended in 50 mL of 100 mM Tris–HCl, pH 8.0, and 5 mM EDTA. Lysozyme at a final concentration of 50 μg mL^{-1} was added to the suspension, which was followed by incubation at room temperature for 15 min. The suspension was sonicated in the presence of 0.1% sodium deoxycholate and centrifuged at 8600*g* for 10 min. The supernatant was discarded, and the pellet was suspended in 50 mL of 100 mM Tris–HCl, pH 8.0, with 5 mM EDTA and 0.1% sodium deoxycholate. The pellet was washed twice in 50 mL 50 mM Tris–HCl, pH 8.0; suspended in 10 mL of the same buffer; distributed in aliquots; and stored at −20 °C.

Soluble and bioactive TsnC was used as the positive control in the solubilization protocols and, as thioredoxin reductase (TrxR), was obtained as described below. ADA94(DE3) *E. coli* transformed with pET-15b/TsnC or pET-15b/TrxR were cultured overnight in 50 mL LB medium containing ampicillin (100 μg mL^{-1}). Each culture was then transferred to 1 L of fresh LB plus antibiotics and grown until the OD_{600nm} reached 0.6–0.8. Afterwards, IPTG (0.5 mM) was added to the culture, and the cells were grown overnight at 20 °C, followed by harvesting via centrifugation and stored at −20 °C. Frozen cells were suspended in 20 mL buffer (20 mM sodium phosphate buffer pH 7.4, 0.5 M sodium chloride, 20 mM imidazole) and disrupted by sonication. Cell extracts were kept on ice during 1% streptomycin sulfate treatment for 20 min. The suspension was centrifuged at 16,000*g* for 40 min to remove nucleic acid precipitates and cell debris. Finally, the soluble fraction was applied to a nickel affinity column (Hi-Trap from GE Healthcare) and the conditions of His-tagged protein purification were optimized according to the manufacturer's instructions. [21]. The recombinant proteins were quantified by A_{280} through *in silico* prediction of extinction coefficients (ExPASy; available on the World Wide Web).

Sample compression

The IB suspensions were diluted in 1 mL of refolding buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA) in the presence of the additives NaCl (0.15 M), L-arginine (0.5 M) or L-arginine HCl (0.5 M), glucose (1 M), sucrose (1 M), PEG 6000 (0.1%), glycerol (2.5 M), Tween 20 (1 mM), Triton X-100 (0.5 mM) or in the absence of additives, as indicated in the figures. The diluted IB suspensions were placed into plastic bags that were subsequently sealed and then placed in another larger vacuum/heat-sealed plastic bag. The bags were placed in a pressure vessel (R4-6-40, High-Pressure Equipment), and oil was used as a pressure-transmitting fluid. After decompression, the samples were centrifuged at 12,000*g* for 15 min. The supernatants were dialyzed to remove GdnHCl, arginine GSH/GSSG and other additives and centrifuged again. The pellets were discarded, and the soluble fractions were stored at −20 °C for subsequent analysis.

Determination of the percentage of solubilized TsnC

SDS–PAGE analysis was performed on 15% SDS–polyacrylamide gels. Equivalent volumes of untreated IB suspensions or of soluble fractions of the HHP-treated suspensions containing the same original amount of IB were heated in SDS–PAGE sample buffer (50 mM Tris–HCl, pH 8.5 containing 2% SDS, 1% dithiothreitol, 0.01% bromophenol blue, and 10% glycerol) at 95 °C for 5 min and were applied on SDS–PAGE. The gels were and stained with Coomassie Blue G-250. Image J software (http://www.ncbi.nlm.nih.gov) was used to analyze the bands that emerged in digital

photographs of the gels, to determine the percentage of soluble TsnC in HHP-treated samples as compared with the total amount of TsnC originally present in IB.

Circular dichroism

CD spectra were recorded on a Jasco-J810 spectropolarimeter equipped with a temperature-controlled liquid system, using an optical cuvette with a light path of 0.1 cm. TsnC was analyzed at a concentration of 18.2 μM. The reported ellipticity curves correspond to the average of five measurements collected over 5 min. The measurements were conducted at 20 °C.

Determination of the enzymatic activity of the thioredoxin TsnC

Thioredoxin activity was assayed on the basis of its ability to catalyze the reduction of the artificial disulfide 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to 2-nitro-5-thiobenzoic acid (TNB), as previously described [22]. Briefly, the protein TsnC refolded at HHP at concentrations of 1, 0.75, 0.5 or 0.25 μM were incubated with 200 μM β-nicotinamide adenine dinucleotide 2′-phosphate reduced (NADPH), 1 μM thioredoxin reductase (TrxR), and 100 μM DTPA in 20 mM Tris–HCl, pH 8.0, in a 96-well plate, to give a total reaction volume of 370 μL. The reaction was initiated by addition of DTNB (500 μM) at 37 °C; TNB production was monitored in a plate reader at a wavelength of 412 nm ($\varepsilon = 14150\ M^{-1}\ cm^{-1}$) [24]. A sample containing no TrxR was used as negative control.

Scanning electron microscopy

Scanning electron microscopy was performed by drying the water-dialyzed insoluble IB onto clean polished Philips stubs, followed by sputter coating in a SCD-040 sputter coater (Balzer) at 38 mA, for 120 s. The samples were then viewed and photographed using a Philips XL-200 scanning electron microscope.

Results and discussion

TsnC expression

In the present study, we expressed the *X. fastidiosa* TsnC gene, which generated a product containing an N-terminal Histidine tag with a molecular weight of 14.7 kDa. At 20 °C, we were only able to express TsnC at low levels, mainly in the soluble fraction (Fig. 1). At higher temperature, this protein chiefly emerged as higher levels of insoluble aggregates (Fig. 1). Important advantages of using the TsnC produced at 37 °C and in the insoluble fraction is the lower level of contamination by bacterial proteins and higher expression levels. To produce the protein for the subsequent assays, we decided to induce bacterial expression of TsnC at the temperature that furnished the highest level of insoluble TsnC (37 °C).

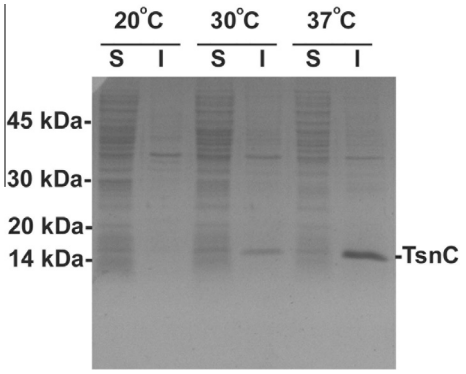

Fig. 1. TsnC (14.7 kDa) production in *E. coli* cultivated in rich medium (2HK2) at different temperatures, after activation. S: soluble fraction of bacteria; I: insoluble fraction of bacteria.

Effect of disulfide-shuffling reagents on TsnC refolding

HHP cannot cleave the disulfide intermolecular covalent cross-links of protein aggregates. In the case of polypeptides containing disulfide bonds, it can be necessary to add an oxidant-reducer pair to the refolding solution. This should disrupt the intermolecular disulfide bonds and trigger a cycle of reduction and oxidation steps, generating the disulfide bonds of the native conformation. Low free energy should stabilize these bonds [16]. Because TsnC contains two Cys residues (C33–C36), we initially optimized the redox conditions of the refolding buffer by compressing the TsnC IB suspensions at 2.4 kbar for 16 h in the presence of different GSH/GSSG ratios. After decompression, a dialyzation was performed to withdraw the reagents GSH, GSSH and GdnHCl before performing the quantification of TsnC in the soluble fraction and of its enzymatic activity. Compression of the IB suspension afforded soluble TsnC in 60–80% yield (Fig. 2A). However, TsnC presented up to 6.6-fold higher activity upon refolding at GSH/GSSG ratios equal to or higher than 1 (Fig. 2B), suggesting that refolding of TsnC was more efficient in the reduced rather than in the oxidized state. The results also showed that determining the biological activity of the soluble recombinant protein could be critical when establishing the optimal refolding conditions. This approach allow the identification of a protocol that eliminates the chance to select a

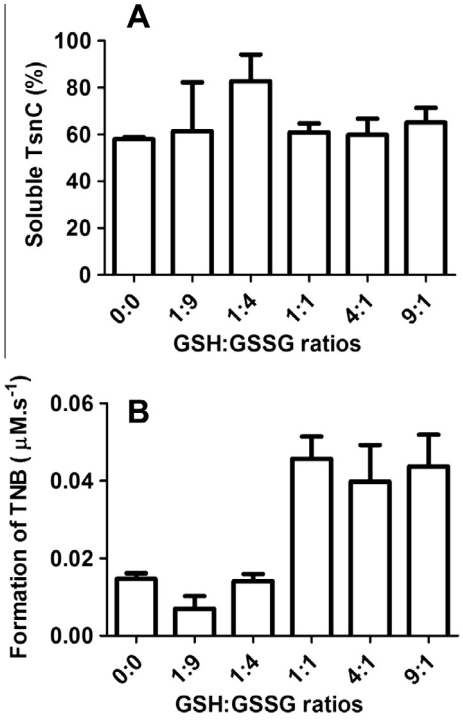

Fig. 2. The GSH/GSSG ratio affects TsnC solubilization and enzymatic activity of the product obtained by HHP treatment of IB suspensions. (A) Percentage of soluble TsnC as determined by SDS–PAGE in relation to the bands corresponding to TsnC in IB suspensions used as a 100% TsnC; (B) thioredoxin activity of 1 μM TsnC. Refolding buffer: 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, and 1.5 M GdnHCl. The samples were pressurized in buffer containing a final concentration of 10 mM glutathione (GSH + GSSG) at the proportions depicted in the *X*-axis, at a 2.4-kbar compression for 16 h. The supernatants were subjected to dialysis. Control (0:0) was performed in the absence of GSH/GSSG.

condition that favors the formation of a soluble but without enzymatic activity, therefore probably in a misfolded state. Therefore, we conducted subsequent assays at 9 GSH:1 GSSG.

Effect of GdnHCl on TsnC refolding

The establishment of non-native intermolecular hydrogen bonds in the aggregates interferes with pressure-induced dissolution, because these bonds are insensitive to pressure. On the other hand, chaotropic agents such as urea or GdnHCl can help to break these bonds, dissolving the insoluble aggregates [25]. Due to the fact that HHP favor the dissociation of aggregates, lower concentration of GdnHCl than that usually used for solubilization of IB at atmospheric pressure is usually required. Concentrations of GdnHCl from 0.5 to 6 M promoted 27–47% IB dissociation (Fig. 3A). GdnHCl at 0.75 M provided the maximum enzymatic activity (Fig. 3B). The lower yield of soluble TsnC at high GdnHCl concentration (6 M) probably stemmed from larger exposure of the TsnC hydrophobic domains, which re-aggregated during dialysis. On the basis of the data described in Fig. 3A and B, we used 0.75 M GdnHCl for the subsequent assays.

Effect of incubation at different pressure levels on TsnC refolding

The literature suggests that compression of aggregated proteins at high pressure (2–3 kbar) induces dissociation and refolding [10,26]. However, there are also articles in the literature that indicate that protein folding occurs during incubation at lower pressure levels (0.3–0.7 kbar) [13,27] or at atmospheric pressure [9]. To determine the conditions that favor TsnC refolding, IB suspensions were subjected to incubation at 2.4 kbar for 16 h. As alternative conditions, the IB suspensions were compressed at 2.4 kbar for 90 min for dissociation of the aggregates, which was followed by decompression to 0.8, 0.4, 0.2 kbar or 1 bar for refolding, condition that was maintained for 16 h before complete decompression and dialysis. Refolding of TsnC occurs preferentially at low pressure, as shown by the highest level of soluble protein with enzymatic activity that was found when the protein was incubated for 16 h at atmospheric pressure. Therefore, we chose the following conditions for refolding: incubation at 2.4 kbar for 90 min, followed by incubation at 1 bar for 16 h.

Effect of the presence of additives on TsnC refolding

A frequent problem encountered during protein refolding is re-aggregation due to intermolecular interaction between the exposed hydrophobic domains. To raise the refolding yields, it is common to use additives – they create an environment where the refolding rate increases, the aggregation rate decreases, and the native folded protein becomes stable instead of undergoing a transition to intermediate states. The presence of low-molecular-weight compounds (sugars, polyols, and hydrophilic polymers) enhances protein stability during refolding: they slow down the unfolding step or reduce the concentration of aggregation-prone species, improving the refolding yields. Unfavorable interactions between these additives and protein surfaces underlie such stabilizing effects. These interactions preferentially exclude the additives and reduce the protein surface exposed to the solvent [28–30]. However, because such stabilizers can enhance aggregation, they have been used in combination with aggregation inhibitors that, in opposition, are preferentially bound to the protein and augment the protein surface exposed to the solvent [31]. The mechanism through which arginine inhibits aggregation has not

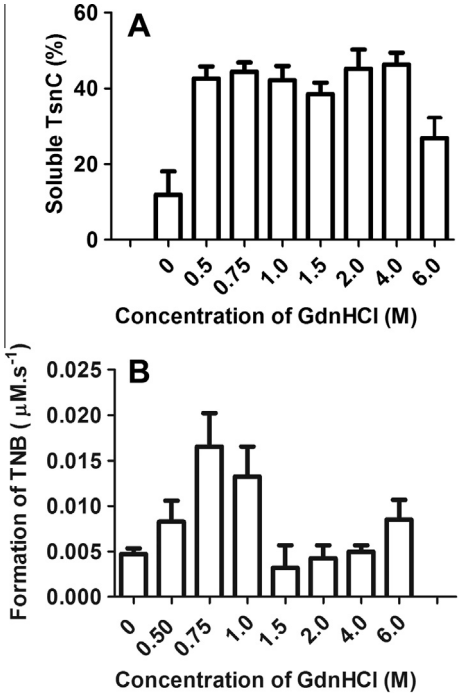

Fig. 3. GdnHCl concentration affects TsnC solubilization and enzymatic activity of the product obtained by HHP treatment of IB suspensions. (A) percentages of soluble TsnC as determined by SDS–PAGE in relation to the bands of the IB suspensions used as a 100% TsnC; (B) thioredoxin activity of 1 µM TsnC. Refolding buffer: 50 mM Tris–HCl, pH 8.0, 1 mM EDTA. The samples were pressurized in buffer containing a final concentration of 10 mM redox pair (9 mM GSH and 1 mM GSSG) at 2.4 kbar for 16 h. The supernatants were subjected to dialysis.

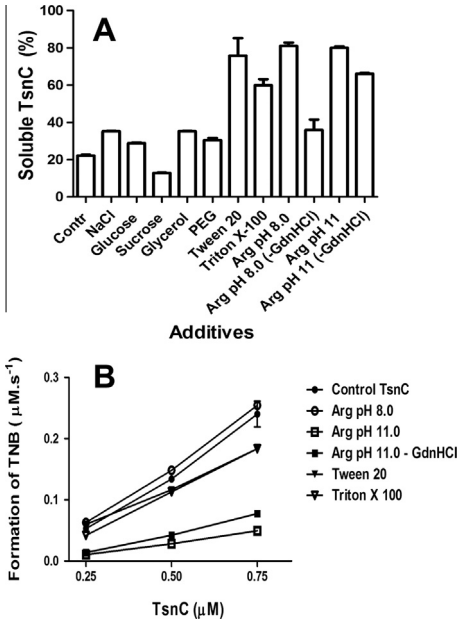

Fig. 4. Additives affect TsnC solubilization and enzymatic activity of the product obtained by HHP treatment of IB suspensions. (A) Percentages of soluble TsnC as determined by SDS–PAGE in relation to the bands of the IB suspensions used as a 100% TsnC. Control was performed in the absence of additives; (B) thioredoxin activity of TsnC refolded in the presence of the five additives that gave the highest yields of soluble protein, in relation to TsnC obtained by purification of the soluble fraction of bacteria, used as a control of thioredoxin activity. Refolding buffer: 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, and 0.75 M GdnHCl. Bars labeled "–GdnHCl" indicate that guanidine was not added to the reaction mixture. The samples were pressurized in buffer containing a final concentration of 10 mM redox pair (9 mM GSH and 1 mM GSSG) at 2.4 kbar for 90 min and incubated for 16 h at 1 bar. The supernatants were subjected to dialysis.

Table 1
Specific activity of TsnC refolded in the presence of additives as compared with Control TsnC.

Additive	Specific activity (μM TNB.s^{-1}.μM TsnC^{-1})
Control TsnC	0.374 ± 0.0334
Arg pH 8.0	0.383 ± 0.0119
Arg pH 11.0	0.078 ± 0.0054
Arg pH 11.0 – GdnHCl	0.127 ± 0.0040
Tween 20	0.247 ± 0.0094
Triton X-100	0.283 ± 0.0002

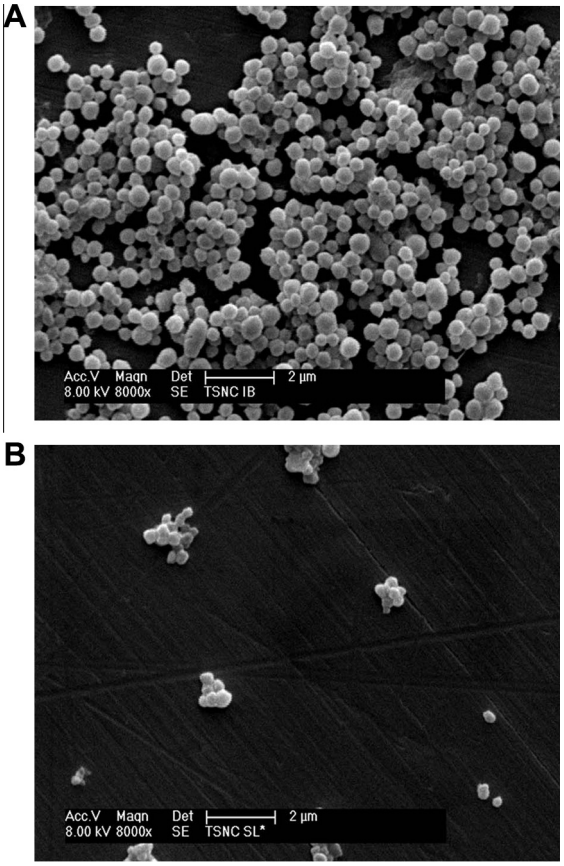

Fig. 5. Scanning electron microscopy of the insoluble aggregates. (A) TsnC IB; (B) insoluble fraction of the IB suspension that remained after HHP. Gain of 8000×. The bar indicates a size of 2 μm.

been completely elucidated. However, it has been proposed that this amino acid inhibits non-native hydrophobic interactions and stabilizes denatured and partially folded proteins by interacting with non-native exposed hydrophobic stretches, thereby diminishing protein–protein interactions and aggregation [32–34].

Indeed, arginine is a very useful reagent for protein refolding at atmospheric pressure or even at high pressure [35]. To test how useful these types of reagents are, we compressed an IB suspension in the presence of additives. The addition of 0.5 M arginine in buffer which pH was not adjusted (11.0) in the presence or in the absence of GdnHCl, buffer containing arg and adjusted to pH 8.0 in the presence of 0.75 M GdnHCl, 1 mM Triton X-100 and 0.5 mM Tween 20 were the conditions that best enhanced the TsnC solubility as compared with the sample compressed in the absence of additives (Fig. 4A). We assessed the TsnC enzymatic activity of the protein that was subjected to compression and incubated in buffers containing the five additives that enhanced the refolding yields to the highest levels (Fig. 4B). TsnC obtained in the soluble

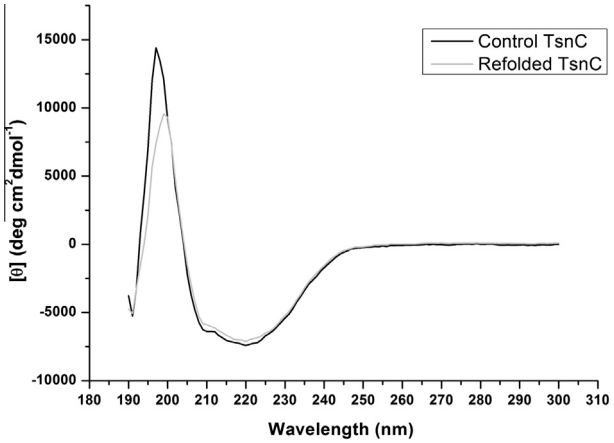

Fig. 6. Analysis of the secondary structure of thioredoxin TsnC by circular dichroism in comparison to TsnC used as control: soluble and bioactive TsnC produced by transformed bacteria cultivated at 20 °C, as described. The spectra were recorded in 20 mM Tris–HCl, pH 8.0 in the range of 200–300 nm, and expressed in terms of molar ellipticity [θ]. Three measurements were taken to obtain the average values.

fraction of *E. coli* cultivated at 20 °C (labeled as "Control TsnC") served as control of TsnC with full biological activity. The protein refolded in buffer containing 0.5 M arginine adjusted to pH 8.0 presented specific activity similar to the native protein, which indicate that the presence of arginine enhanced the yield of obtainment of soluble TsnC (Fig. 4A) with biological activity (Fig. 4B and Table 1). In contrast, the sample refolded in buffer containing arginine at high pH (11.0) exhibited markedly lower TsnC enzymatic activity, in both the presence and absence of GdnHCl (Fig. 4B). The presence of Tween 20 and Triton X-100 prompted smaller decrease in the TsnC specific enzymatic activity.

TsnC IB

Purified recombinant IBs appear as spherical, ellipsoidal, or cylindrical particles measuring between 0.5 and 1.8 mm, characterized by a smooth and porous surface [36,37]. TsnC IBs consisted of spherical particles (Fig. 5A). The particles present in the portion of the TsnC suspension that remained in the insoluble fraction after HHP treatment (Fig. 5B) emerged in lower quantity and were smaller, probably as a result of partial solubilization upon HHP or re-aggregation upon decompression.

Analysis of the secondary structure of refolded TsnC

The structure of *E. coli* thioredoxin presents five β-sheets flanked by four α-helices [38]. The circular dichroism spectrum of the refolded *X. fastidiosa* TsnC in buffer containing 0.5 M arginine adjusted to pH 8.0 and subsequently subjected to purification was typical of a regular secondary structure (Fig. 6). Remarkably, the spectra of the refolded and Control TsnC were highly similar, an additional indication that they present a resemblance of structure.

Conclusions

We optimized the GSH/GSSG ratio, GdnHCl concentration, type of additives, and incubation at different pressure levels for refolding of TsnC from IB dissociated at HHP (2.4 kbar). The solubilization yields of HHP-treated IB reached a maximum around 2 M GdnHCl, but enhanced thioredoxin activity occurred at 0.75 M GdnHCl. Solubilization yields varied little upon changing the GSH/GSSG ratio from 9:1 to 1:9, but thioredoxin activity was strikingly higher in

more reducing environments. The results showed that it is advantageous to select the refolding conditions on the basis of both the protein solubility and the biological activity. The following conditions afforded high TsnC yield: application of HHP (2.4 kbar) for 90 min to a suspension of IB containing non-denaturing levels of GdnHCl (0.75 M), 0.5 M arginine at pH 8.0, and 10 mM redox pair at a 9:1 GSH:GSSG ratio followed by incubation at 1 bar for 16 h. In the optimized conditions, the TsnC refolding process furnished 81% yield of soluble and biologically active protein from HHP-incubated IB. In the same buffer conditions, the yield of refolding of TsnC from IB suspensions maintained at atmospheric pressure was only 4.0%. The advantage of using the HHP-refolding protocol is evidenced by the facts that the production of the TsnC as insoluble IB by *E. coli* cultivated at 37 °C is higher than the soluble protein by bacteria cultivated at 20 °C, summed to the high refolding yield and the fact that the refolded protein presents a strikingly lower contamination by *E. coli* proteins than the soluble protein expressed at 20 °C.

Acknowledgments

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