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An analysis of the factors that affect the dissociation of inclusion bodies and the refolding of endostatin under high pressure

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ABSTRACT

An optimization of the refolding of endostatin (ES), by a study of the conditions that can affect (i) dissociation of inclusion bodies (IBs) and (ii) renaturation under high hydrostatic pressure (HHP), is described. IBs produced by bacteria cultivated at 25 °C were shown to be more soluble than those produced at 37 °C and their dissociation by application of 2.4 kbar at 20 °C was shown to be further enhanced at −9 °C. A red shift in intrinsic fluorescence spectra and an increase in binding of the hydrophobic fluorescent probe bis-ANS show subtle changes in conformation of ES in the presence of 1.5 M GdnHCl at 2.4 kbar, while at 0.4 kbar the native conformational state is favored. The 25% refolding yield obtained via compression of IBs produced at 37 °C by application of 2.4 kbar, was increased to 78% when conditions based on the insights acquired were utilized: dissociation at 2.4 kbar and −9 °C of the IBs produced at 25 °C, followed by refolding at 0.4 kbar and 20 °C. Besides providing insights into the conformational transitions of ES structure under HHP, this work proposes innovative conditions that are likely to have wide applicability to the HHP-induced refolding of proteins in general.

1. Introduction

Many of the proteins with a biomedical relevance are found at low concentrations in their native sources. *Escherichia coli* is a microorganism that is extremely useful for producing proteins of commercial interest at large scale and for structural and functional studies. However, the production of recombinant polypeptides in *E. coli* frequently results in an incomplete folding process that usually leads to the accumulation of insoluble aggregates, known as inclusion bodies (IBs), in the cytoplasm or in the periplasmic space of the host cells. Therefore, refolding is often problematic and results in low yields of soluble and biologically active proteins.

The aggregation of heterologous proteins is caused by a high local concentration of nascent polypeptides on the ribosome [1], which are not protected from aggregation due to insufficient number of chaperones present during the overexpression of the recombinant protein or even to an absence of chaperones [2]. It has been shown that IBs contained ordered structural segments of cross-β structures with specific amino acid sequences, which were surrounded by folded domains or disordered segments. These β-strands do not necessary have a β-sheet conformation in the corresponding soluble folded form of the protein. The number of cross-β segments is variable, and their sizes typically range between 7 and 10 amino acid residues in length [3]. The proteins in the IBs were considered to be completely inactive. However, the current knowledge of IBs has evolved, and today, it is recognized that proteins within the IBs present a native-like structure [4]. An analysis by infrared spectroscopy (FTIR) showed that despite an increase in the intermolecular β-sheet structures in relation to the native states, proteins in the IBs presented native-like secondary structures [5,6]. In addition, a percentage of some of the proteins in the inclusion bodies were shown to have a native tertiary structure and biological activity [7,8]. The characteristics of the proteins within the IBs were shown to be affected by the cultivation temperature of the host bacteria. The bioactivity in the IBs had an inverse correlation with the temperature of the cultivation. During cultivation at high temperatures, intermolecular interactions are favored at the expense of native intramolecular contacts, and consequently, the IBs become more resistant to chemical denaturation. These results demonstrated that the dissociation of IBs produced at lower temperatures can be performed in milder conditions. Thus, the functional states of the IBs can be released using conditions that break the network of intermolecular contacts that maintains the stability of the IBs without denaturing the native-like proteins that are embedded in these structures [9].

Traditionally, the proteins in IBs are solubilized using high concentrations of chaotropic agents, such as guanidine hydrochloride (GdnHCl) or urea, and the denatured and soluble proteins are refolded to their native states after the removal of the denaturing reagents. However, the refolding step is difficult with these

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conventional methods because of the generation of new protein aggregates during the removal of the chaotropic agents [10].

Aggregated proteins display specific volumes that are larger than those of the native states due to the presence of intermolecular cavities, which are not exposed to water. The application of high pressure favors transitions of the protein structures that reduce the volume of the system. In vitro, pressures of 1–3 kbar impair intermolecular and electrostatic interactions, thereby promoting the dissociation of oligomeric protein states and aggregates. However, a stronger hydration with the rupture of intramolecular bonds and a consequential denaturation of the proteins generally occurs at higher pressure levels, above 4–5 kbar [11,12]. The effectiveness of refolding strategies that use mild solubilization conditions to enable the preservation of secondary and tertiary structures is increased in comparison to the solubilization of the aggregates using high concentrations of denaturing agents, where the protein loses its existing native-like tertiary and secondary structures [13,14]. The application of high pressure is a mild dissociation technique for aggregated proteins that does not require the use of high levels of denaturing reagents and enables the maintenance of secondary and tertiary structures, which is useful for protein refolding.

High hydrostatic pressures have been utilized for the dissociation of aggregates and protein refolding [15,16]. However, the pressure level at which the refolding occurs is controversial. While some articles state that the dissociation and refolding occur concomitantly at pressure levels of 2.0–2.5 kbar [17,18], other authors only utilize this range of pressure to dissociate the aggregates. In those cases, it was shown that the refolding of proteins with quaternary structures occurred at atmospheric pressure [15,19].

At high pressures, the freezing point of water is lowered, which allows for studies on the concomitant effects of high pressures with low temperatures in aqueous solutions. At low temperatures, the proteins are driven to a lower entropic state that favors the interaction of non-polar amino acids with water [20]. Thus, the effect of high pressures can be potentiated by the use of subzero temperatures, which enhances the exposure of hydrophobic side chains to the solvent and the dissociation of the aggregates.

Endostatin (ES) is a 20 kDa C-terminal fragment digested from collagen XVIII, an endogenous antiangiogenic protein that shows a potent inhibitory effect on endothelial cell migration, proliferation and tumor angiogenesis; [21–24]. This protein suppresses tumor growth without toxicity or an acquired drug resistance [24], exhibits a broad spectrum and is not toxic [25]. Studies have demonstrated the difficulty of generating native ES [23] because ES easily aggregates, resulting mainly in insoluble and aggregated ES preparations [26]. Therefore, its correct refolding is essential for the assurance of structural stability and biological functions [27].

In the present study, we have analyzed the factors that are involved the dissociation of IBs and the refolding of ES under high hydrostatic pressure. The impact of the growth temperature on the quality of aggregated ES was assessed. We also studied the effect of high hydrostatic pressure (2.4 kbar) with subzero temperatures (−9 °C) on the dissociation of ES. The effects of application of HHP on conformation transitions of ES were investigated in order to determinate their influence on the ES folding. Additionally, we studied the use of small molecule additives to increase the recovery yields of native ES in association with the action of the pressure. All of these factors were investigated with the aim of increasing the efficiency of ES refolding under pressure.

2. Materials and methods

2.1. Expression of ES, growth conditions, cell fractionation, and inclusion body isolation

BL-21(DE3)pLysS was transformed with the vector pET-28 containing the DNA sequence coding for the amino acids methionine, alanine, and six histidine residues at the N terminus, which was followed by the sequence for murine ES (construct cTB01#8, from pETKH-1, ATCC number 63404). For the expression of ES, a colony was randomly picked from the transformants that were grown on Kan + LB plates (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 50 mg/L kanamycin) and inoculated in 2-HKSII rich medium [28]. Cells were grown at 37 °C, and the expression of ES was induced with isopropyl-β-D-thiogalactopyranoside (0.5 mM) at the beginning of the exponential phase (approximately 3.0 at A_{600nm}). The culture was separated into 1 L erlenmeyer flasks containing 250 mL 2-HKSII medium, which were then incubated at different temperatures (25 °C, 30 °C and 37 °C). After incubation with a constant orbital agitation (150 rpm) for a 16 h period, bacteria were collected by centrifugation at 2500 × *g* for 10 min at 4 °C. The pellet was resuspended in 50 mL of 0.1 M Tris–HCl, pH 7.5 and 5 mM EDTA. Lysozyme, at a final concentration of 50 μg/mL, was added, and the suspension was incubated for 15 min at room temperature. The suspension was sonicated in the presence of 0.1% sodium deoxycholate and centrifuged at 8000 × *g* for 10 min. The supernatant was discarded, and the pellet was resuspended in 0.05 M Tris–HCl, pH 7.5 with 5 mM EDTA and sodium deoxycholate, washed twice in 0.05 M Tris–HCl, pH 7.5, and stored at −20 °C.

2.2. Scanning electron microscopy

Scanning electron microscopy was performed by drying water-dialyzed insoluble aggregates onto clean polished Philips stubs and 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. The sizes of the IBs were analyzed using the software ImageTools.

2.3. Sample pressurization

Suspensions of ES IBs were diluted in refolding buffer (50 mM Tris–HCl, pH 7.5 with 1 mM EDTA) containing the final concentrations of 1.5 M GdnHCl and 0.5 mM oxidized (GSSG) and 0.5 mM reduced (GSH) glutathione. Samples of the suspension (2 mL) were placed into plastic bags, which were sealed and then placed into a larger plastic bag that was vacuum/heat-sealed. The bags were placed in a pressure vessel (R4-6-40, High-Pressure Equipment) with a oil for a pressure-transmitting fluid, and high pressure was applied (2.4 kbar). Samples were compressed to 2.4 kbar, incubated for 2 h and then decompressed to 1.2 kbar, 0.8 kbar or 0.4 kbar, which was maintained for 16 h, and was then followed by a decompression to atmospheric pressure. The samples were then centrifuged at 12,000 × *g* for 15 min. The supernatant was dialyzed against 50 mM Tris–HCl, pH 7.5 and centrifuged at 12,000 × *g* for 15 min to remove insoluble aggregates that formed during the dialysis process. To test the effects of additives on the HHP-induced refolding, 4,4'-dianilino-1,1'-binaphthyl-5, 5'-sulfonate (bis-ANS, 6.5 mM), heparin (2.5–20 mM), L-arginine (0.5 M), glycerol (2.5 M), sodium chloride (0.15 M), sucrose (1 M), Tween 20 (1 mM) or Triton X-100 (0.5 mM) were added to the refolding buffer.

2.4. Quantification of ES by SDS-PAGE

SDS-PAGE analysis was carried out on 15% SDS-polyacrylamide gels using the method described by Laemmli and stained with Coomassie Blue G-250. Suspensions of ES IBs were heated at 95 °C for 5 min in SDS-PAGE sample buffer (Tris–HCl 50 mM pH 8.5 containing 2% SDS and 1% dithiotreitol, 0.01% bromophenol blue and 10% glycerol) for complete ES solubilization and therefore the respective bands in the electrophoresis gels were used as a reference for the total amount of ES within IBs. The soluble fractions of the HHP-treated suspensions of IB were applied to SDS-PAGE gels under non-reducing conditions. Image J software (http://www.ncbi.nlm.nih.gov) was utilized for the analysis of the bands in the digital photography of the gels for the determination of the percentage of soluble ES in HHP-treated samples, in comparison to the total amount of ES in IBs. The total protein content was determined by a Bradford assay using pure bovine serum albumin as standard.

2.5. Determination of the solubilization of the IBs in the presence of GdnHCl

ES IBs, which were expressed at different temperatures (25 °C and 37 °C), were suspended in solutions containing 0–8 M guanidine hydrochloride (GdnHCl). After a 72 h incubation at 180° vertical rotation (35 rpm), the samples were centrifuged at 12,000 × *g* for 10 min and the absorbance of the supernatants were measured at 280 nm. The amount of ES in the insoluble fractions was quantified by SDS-PAGE analysis, as described above.

2.6. Fourier transform infrared spectroscopy (FTIR)

Attenuated total reflectance (ATR)-FTIR spectra were obtained from dry samples deposited directly onto the ATR crystal in a Nicolet 6700 IR spectrometer (Thermo Corp., USA). Spectra were collected with a 4 cm^{-1} resolution and are the result of the accumulation of 256 scans. Fourier self-deconvolution of the amide I band was performed with a 1.6 enhancement factor and a 20 cm^{-1} bandwidth. The peak positions and area assignments were done with the software OMNIC (Thermo Corp., USA) with a Voigt distribution for each peak.

2.7. Purification of ES

The HHP-treated supernatants of IBs suspensions were dialyzed and applied to a 1 mL HiTrap Heparin HP (GE Healthcare) that had been pre-equilibrated with 50 mM Tris–HCl, pH 7.5, at a flow rate of 1 mL/min. The column was washed with 10 mL of the equilibration buffer and then eluted using an increasing gradient of NaCl (0–1 M) in 40 mL.

For size-exclusion chromatography, a TosoHaas G2000 SW column (60 cm–7.5 mm i.d., particle size 10 μm, pore size 125 Å; Montgomeryville, PA, USA) coupled to a 7.5 cm–7.5 mm i.d. SW guard column was used. The mobile phase that was used was 0.05 M NaCl and 0.02 M sodium phosphate, pH 7.0 at a flow rate of 1.0 mL/min. The sample elution was detected by UV absorbance at a wavelength of 220 nm.

2.8. Circular dichroism

CD spectra were obtained using a Jasco-J810 spectropolarimeter equipped with a temperature-controlled liquid system and a 0.1 cm-light path cuvette. The reported curves of ellipticity are the averages of five measurements that were collected over a 3 min period. All measurements were carried out at 20 °C. The K2d program (http://www.embl-heidelberg.de/_andrade/k2d) was used for the analysis of the protein secondary structures.

2.9. Fluorescence and light-scattering (LS) measurements

The LS and fluorescence measurements were recorded on Cary Eclipse spectrofluorometer (Varian). Data were collected using a 1 cm-path length cuvette at atmospheric pressure. The LS measurements were performed at 320 nm, at an angle of 90° relative to the incident light, and the emission was recorded from 315 to 325 nm. Fluorescence measurements of Trp emissions were carried out with an excitation wavelength of 288 nm. The emission fluorescence spectra were collected between 300 and 400 nm by using a response time of 1 s and scan speed of 240 nm/min. In order to evaluate the binding of bis-ANS to ES, 5 μM bis-ANS was added to a protein sample (1 μM) that was then excited at 360 nm and fluorescence emission was measured between 400 and 600 nm.

For studies under pressure, a high-pressure cell equipped with optical sapphire windows (ISS), was connected to a pressure generator (High pressure equipment) and ethanol was utilized as pressure-transmitting fluid. Round quartz cuvettes filled with the samples and sealed with flexible polyethylene caps were placed into the high-pressure cell and subjected to HHP.

2.10. ES biological activity

The viability of HUV-EC-C endothelial cells (ATCC Number CRL-1730) was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega). In brief, 5×10^3 cells were plated in a 96-well tissue culture plate in DMEM supplemented with 10% FBS at a final volume of 0.1 mL, and the plates were incubated for 24 h at 37 °C in a humidified 5% CO_2 incubator. The medium was replaced with fresh medium containing 2% FBS, 5 ng/mL bFGF (RD systems) and different concentrations of ES and then incubated for 48 h. The cell viability was measured by the addition of 20 μL of an MTS/PMS (phenazine methosulphate) (20:1) solution and a 2 h incubation. The microplates were read in a spectrophotometer at 490 nm.

3. Results and discussion

3.1. Analysis of ES IBs

Recombinant bacteria expressing ES were grown at the temperatures of 25 °C and 37 °C, and the ES was always found in the insoluble fraction. The content of ES was relatively high, as it represents 39–45% of the total bacterial proteins. The variation of the level of ES concentration among bacteria grown in different temperatures was low (Fig. 1A). IBs of ES that were extracted from a culture grown at 25 °C presented a regular spherical shape with a rough surface and a diameter of 0.65 ± 0.07 μm, as depicted in Fig. 1B. The diameters of the IBs produced at 37 °C presented a slightly higher diameter of 0.74 ± 0.06 μm and a smoother surface (Fig. 1C).

The secondary structure of proteins in solid phase can be estimated by analysis of the vibration spectra of the amide I band, which is mainly due to the CO stretching vibration of the peptide bonds and occurs in the 1700–1600 cm^{-1} absorption region of the infrared spectra [29]. ATR-FTIR spectroscopy was used for the analysis of the secondary structure content of IBs in solid state.

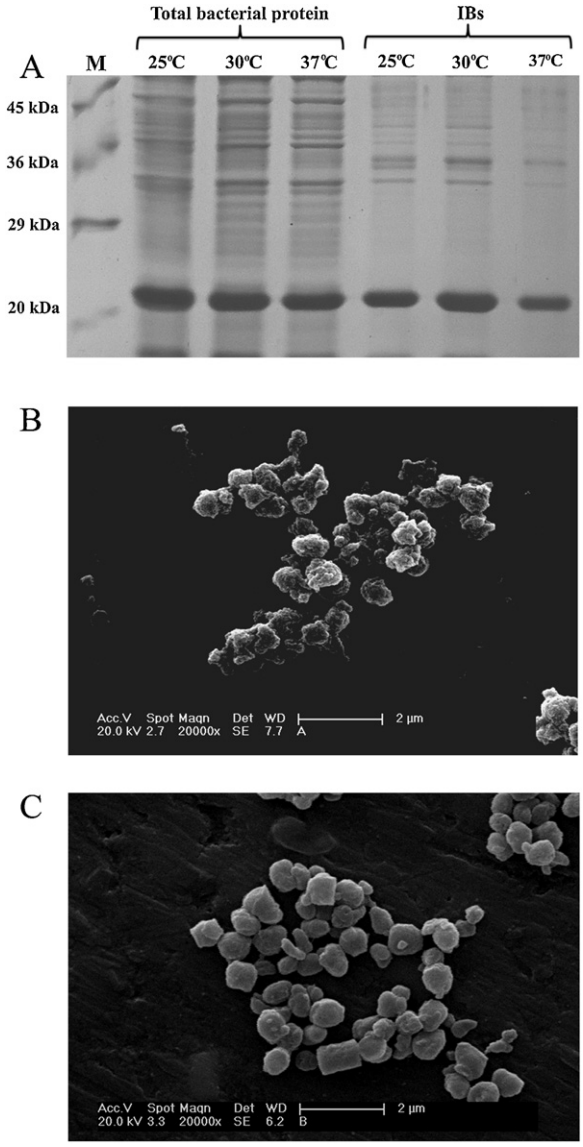

Fig. 1. Expression of ES. (A), SDS-PAGE analysis of bacterial cultures that were grown at different temperatures in the rich culture medium 2HK2 and isolated IBs. (B) Scanning electron micrograph of ES IBs produced at 37 °C; (C) scanning electron micrograph of ES IBs produced at 25 °C. Magnification: 20,000×. The bars represent 2 μm. Samples were normalized by volume of culture (30 μL).

The single globular domain of endostatin has a compact fold. The secondary structure contains a large fraction of irregular loop structures and β-sheets, a small fraction of α-helices and two pairs of disulfide bonds in a nested pattern [30]. The spectra of lyophilized ES obtained by HHP refolding process [31] was examined by ATR FTIR spectroscopy, as shown in Fig. 2A, and was similar to the spectra described for native ES in solution [32], which indicated that the process of lyophilization did not provoke effect on the secondary structure of the dry protein. The determination of the secondary structure percentages were calculated by the decomposition of the FTIR spectra (Fig. 2A and B and Table 1). The spectra obtained for the native ES show the presence of non-native β-sheets (bands at 1684 and 1613 cm^{-1}) at 10%, but the main β-sheet peak obtained after deconvolution was found at 1631 cm^{-1} (Table 1), which is characteristic of native β-sheets [33]. Notably, the appearance of a peak at ~1620 cm^{-1} (position also assigned as β-sheet) in the spectra of the IBs can be assigned as non-native β-sheets because it is not found in the native ES IR spectra (Fig. 2A and Table 1).

Table 1
FTIR analysis of the secondary structure content of native ES and of IBs.

	Native		IBs		IBs	
			25 °C		37 °C	
Structural assignment	Wavenumber (cm^{-1})	Area (%)	Wavenumber (cm^{-1})	Area (%)	Wavenumber (cm^{-1})	Area (%)
β-sheets	1684, 1613	10	1691	3	1691	3
β-sheets	1631	26	1636, 1621	52	1637, 1622	54
Turns	1663	29	1675	10	1675	10
Disordered	1646	32	–	–	–	–
α-helix/random	1653	2	1656	35	1656	33

The estimated error in the wavenumbers is ± 4 cm^{-1}, and ± 2% for the peak areas.
The data are representative of 3 experiments.

Another peak at 1656 cm^{-1} is also present in the spectra of the IBs produced at 25 °C and 37 °C, suggesting an increase of α-helices [29,34], at the expense of the peak at 1646 nm assigned as disordered structures in the spectrum of the native protein, which is likely due to the high intrinsic α-helical propensity of the unfolded polypeptide backbone of ES [32,35]. The determination of the secondary structure percentages (Fig. 2A and B and Table 1), show that besides subtle differences among the IB spectra, the conformational statuses of IBs produced at both 25 °C and 37 °C presented similar secondary structure profiles (Fig. 2A and Table 1), which may be due to the high tendency of ES to aggregate even at low cultivation temperatures.

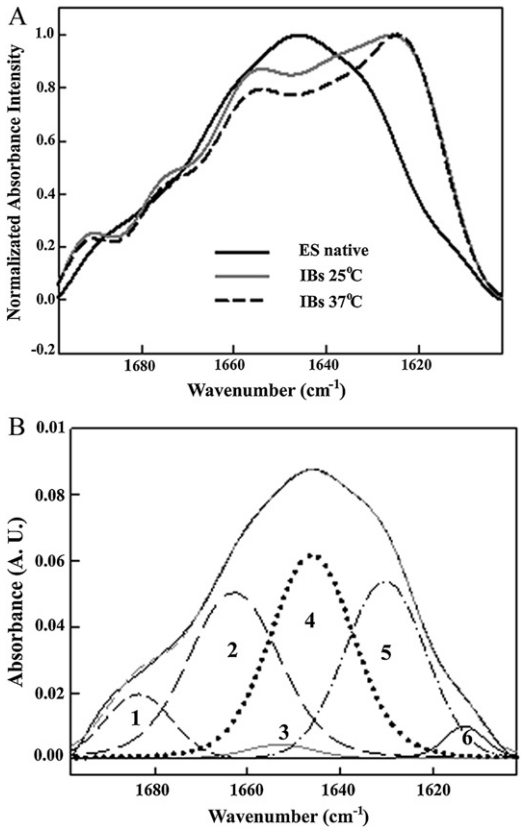

Fig. 2. IBs produced at different temperatures present similar secondary structure. (A) Fourier self-deconvoluted (FSD) spectra of lyophilized native endostatin (black solid line) and IBs obtained at 25 °C (gray line) or at 37 °C (dashed line). Only the amide I region is shown. (B) Curve fitting and peak assignment of the deconvoluted IBs produced at 25 and 37 °C IBs amide I band yielded five Voigt functions: (1 and 6) 1684 and 1613 cm^{-1}, β-sheets; (2) 1663 cm^{-1}, turns; (3) 1653 cm^{-1}, α-helix; (4) 1646 cm^{-1}, disordered; and (5) 1631 cm^{-1}, β-sheets. The data are representative of 3 experiments.

3.2. The temperature for bacterial growth influences the solubility of IBs

During recombinant protein production, aggregation is generally favored at higher temperatures due to the strong temperature dependence of hydrophobic interactions [36].

The effect of growth temperatures on the quality of IBs was studied. To investigate the solubility of ES IBs, we explored whether the cultivation temperature influences the resistance of IBs to the breakage of intermolecular hydrogen bonds and hydrophobic interactions, which sustain IBs architecture, by the chaotropic agent GndHCl. Thus, suspensions of IBs, which were produced at different temperatures, were tested for a resistance to dissociation by GdnHCl. As illustrated in Fig. 3A, the solubility of the proteins from the IBs produced at 25 °C was slightly higher than the protein solubility in the IBs expressed at 37 °C. However, when we specifically analyzed the solubility of ES by the determination of the respective bands of insoluble fractions of the GdnHCl curve, we verified that it was greatly improved in IBs formed at 25 °C in comparison to IBs produced at the higher temperature (Fig. 3B). For example, the concentration of GdnHCl necessary to solubilize 50% of the ES within IBs formed at 25 °C (0.7 M), doubled for IBs produced at 37 °C (1.5 M). Further, incubation for 72 h in the absence of GdnHCl reduced the amount of ES in the insoluble fraction to 62% and 81% in IBs formed at 25 °C and 37 °C, respectively.

These results indicate that the cultivation temperature influences the stability of the intermolecular interactions among the polypeptide chains embedded within the IB aggregates.

Low levels of GndHCl are frequently used for high hydrostatic pressure-refolding protocols because its presence is necessary to disrupt hydrogen bonds that are insensitive to the high pressure application [17].

We had previously demonstrated that ES can be obtained from a suspension of IBs by an application of 2.0 kbar for 16 h. The application of this process to suspensions of IBs produced at 37 °C rendered ES refolding yields of up to 35.6% [31]. One of the benefits of refolding with high pressure is the dissociation of aggregates under mild conditions, which is induced by the high pressure, and the minimum levels of chaotropic reagents required that do not hamper the obtainment of the refolded protein from intermediary states, such as molten globules. However, in order to supplant the difficulty of disaggregating all of the protein states that are embedded within the aggregates under non-denaturing conditions, it is convenient to obtain IBs with increased solubility. The higher solubility of the IBs formed at 25 °C suggests that higher yields of dissociation would likely be obtained by application of high pressure. In fact, the solubilization of ES using HHP rendered a higher yield of soluble ES from the IBs produced at 25 °C (32.4%) than from that produced at 37 °C (25.3%) allowing for a greater proportion of the protein to refold.

Therefore, despite the similarities between the secondary structures of the IBs formed at different temperatures, as shown by FTIR

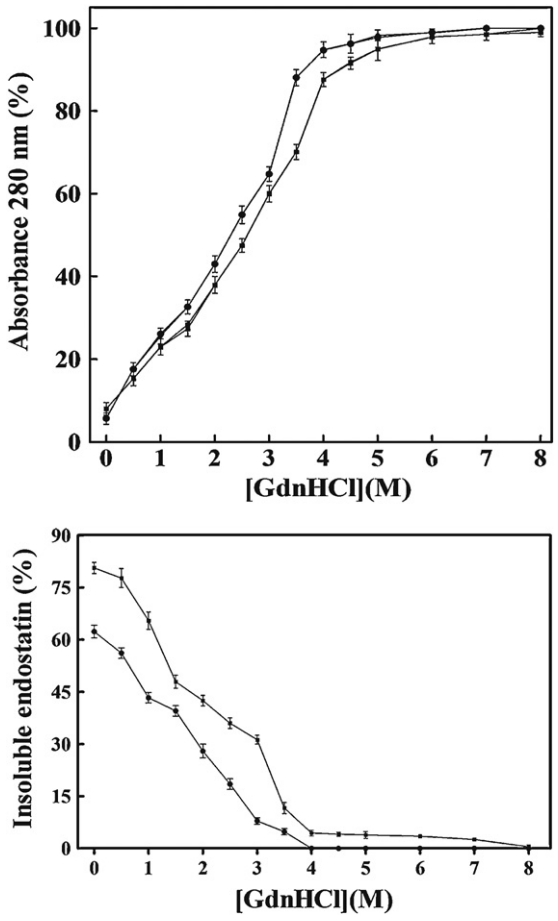

Fig. 3. The temperature of production affects the solubility of IBs. The suspensions of IBs were incubated with different concentrations of GdnHCl for 72 h with 35 rpm of 180° vertical rotations. (A) The solubilization behavior of IBs produced at different growth temperatures was monitored by estimating the amount of total protein at 280 nm in the soluble fraction; (B) The percentage of ES found in the insoluble fraction of IBs produced at different growth temperatures was determined by SDS-PAGE analysis. Chart symbols: IBs produced at 25 °C (●) and IBs produced at 37 °C (■). The data points represent the average of two sets of experiments.

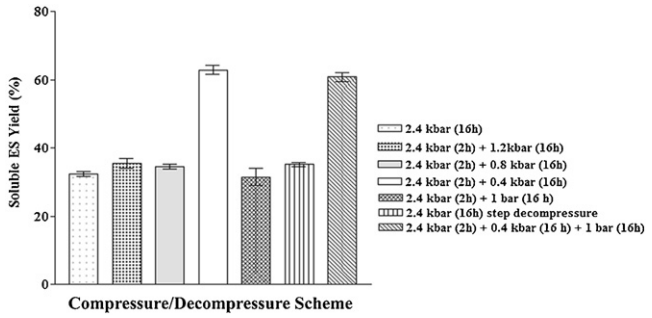

Fig. 4. The refolding yield of ES depends on the pressure level at which the protein was incubated after dissociation at 2.4 kbar. Suspensions of ES IBs in refolding buffer (50 mM Tris–HCl, pH 7.5 with 1.5 M GdnHCl, 0.5 mM GSSG and 0.5 mM GSH) were compressed at 2.4 kbar for 2 h and then decompressed to 0.4, 0.8 or 1.2 kbar and incubated for 16 h before the complete release of pressure. The scheme of decompression was straight for all of the samples, except for the one indicated in the figure as a "step decompression", in which 0.4 kbar of pressure was released every 30 min. The data are representative of 4 experiments.

(Fig. 2A), the yield of soluble ES obtained from the IBs formed at lower temperatures were improved.

3.3. Incubation at 0.4 kbar improves the yield of soluble ES

Tertiary and quaternary protein structures are stabilized by similar types of interactions [37], and high pressure is effective at destabilizing both structures, promoting the dissociation of aggregates and partially or totally unfolding polypeptides, depending on the pressure level. The application of relatively low pressures (1–2 kbar) has been shown to induce structural alterations in the native tertiary structure of monomeric proteins [38,39]. Hence, pressure levels that can cause dissociation of protein aggregates likely can also affect the interactions involved in the maintenance of the tertiary structure.

We and other authors have previously performed dissociations of protein aggregates and then refolding the proteins under similar pressure levels (2.0–2.5 kbar) [16,17,31,40]. However, recent studies performed with IBs of green fluorescent protein (GFP) demonstrated that incubation at pressure levels higher than 1 kbar hinders the refolding of GFP to the native state, likely by hampering the connections involved in the generation of the protein's native structure. Thus, high yields of biologically active GFP were only obtained by an application of 2.4 kbar for 30 min to dissociate the aggregates with an incubation at lower pressure levels (0.35–0.70 kbar), which were shown to favor GFP refolding, for 16 h [41] Hence, we investigated if the effect of incubations at pressure levels lower than those applied to dissociate the aggregates of ES were beneficial. A suspension of IBs, which were produced at 25 °C in the presence of 1.5 M GdnHCl, 0.5 mM GSSG and 0.5 mM GSH, was subjected to different schemes of compression/decompression. The first scheme was performed following the conditions of pressure that were previously described by our group. This scheme consists of a compression at 2.4 kbar for 16 h followed by a direct decompression to atmospheric pressure. In the second scheme, IBs were compressed at 2.4 kbar for 2 h and then decompressed to 1 bar, 0.4 kbar, 0.8 kbar or 1.2 kbar, which was maintained for 16 h before a full decompression to atmospheric pressure. Other authors reported that a slow decompression can improve the yield of refolded protein after an application of high hydrostatic pressure, which suggests that rapid depressurization rates can trap aggregation-prone species [42]. To assess the effect of a gradual decompression on the refolding of ES, IBs samples were compressed at 2.4 kbar for 2 h that was followed by a stepwise decompression by 0.4 kbar reductions every 30 min. Each sample was dialyzed to withdraw the GdnHCl and glutathione reagents, and the percentage of recovery of ES in the soluble fraction was assessed by SDS-PAGE analysis. A higher solubilization yield (63%) was obtained by the incubation of the IB suspension at 2.4 kbar for 2 h and at 0.4 kbar for 16 h, as compared to the sample that was compressed at 2.4 kbar for 16 h (32%) (Fig. 4), which suggests that 0.4 kbar is an effective pressure level to foster the refolding of this protein. These results indicate that the incubation at 0.4 kbar after dissolution of ES IBs at higher pressure (2.4 kbar) was efficient to avoid the reaggregation of non-native states, associated to the formation of the contacts involved in the folding of the native-like ES, which thereby doubled the solubilization yield.

3.4. Conformational analysis of ES at HHP as determined by extrinsic and intrinsic fluorescence

The intrinsic fluorescence of the ES was previously shown to present a maximal emission at 319 nm [43]. This blue-shifted structured emission reflects the fact that the four tryptophan residues distributed within the structure present their side chains into the molecule [30], which makes tryptophan fluorescence a useful marker for evaluating the conformation of the ES structure. Fig. 5A depicts the intrinsic fluorescence spectra of ES that were

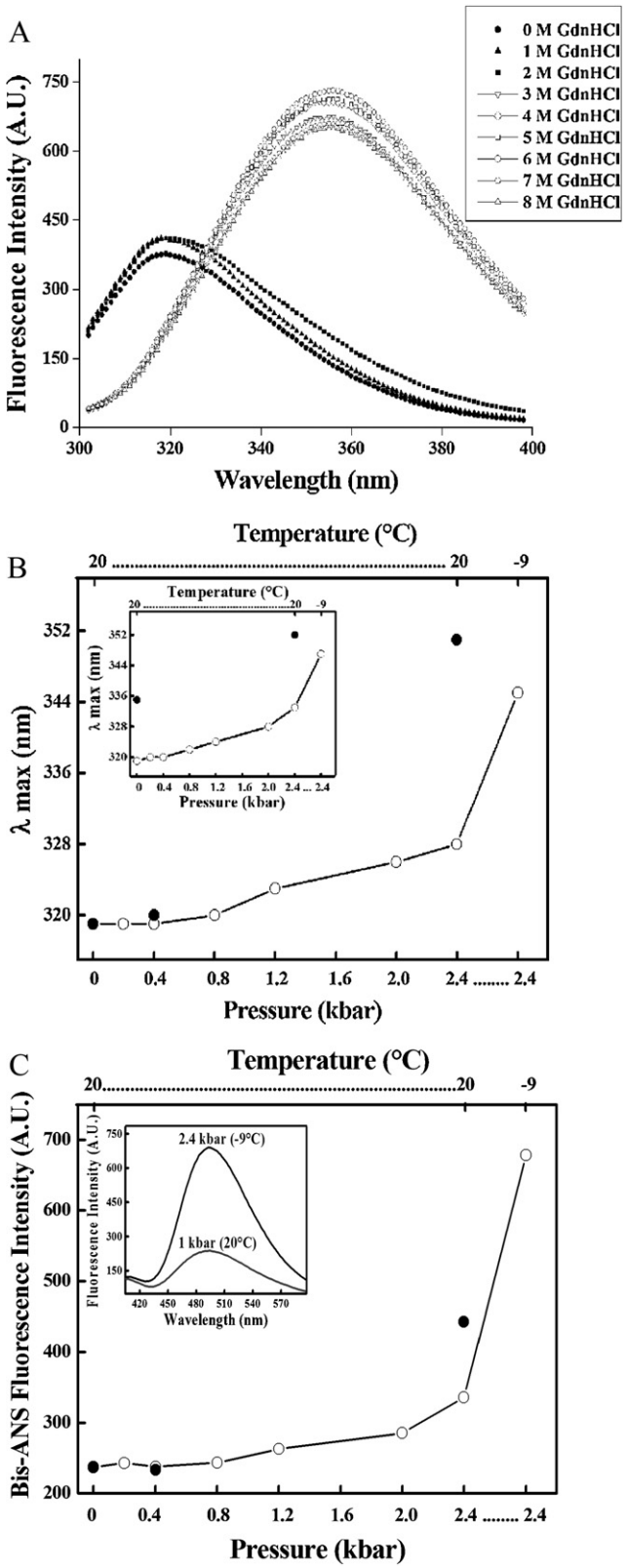

Fig. 5. HHP induced reversible changes in the intrinsic and extrinsic fluorescence emission of ES. (A) Effect of GdnHCl on the intrinsic fluorescence of native ES; (B) effect of the compression and the decrease of the temperature on maximal intrinsic fluorescence of ES. Maximal intrinsic fluorescence obtained by compression and decrease of the temperature (○) and maximal intrinsic fluorescence obtained at the return of the temperature from −9 to 20 °C and of the pressure level from 2.4 kbar to 0.4 kbar or directly to 1 bar (●) (as indicated in the graph B and in the inset). The inset shows the maximal fluorescence of the ES that was straightly decompressed from 2.4 kbar to 1 bar; (C) effect of the compression and the decrease of the temperature on the intensity of bis-ANS fluorescence. Fluorescence obtained by compression and decrease of the temperature (○) and fluorescence obtained at the return of the temperature from −9 to 20 °C and of the pressure level from 2.4 kbar, 0.4 kbar and 1 bar (●) (as indicated in the graph C). Experiments were performed in refolding buffer (50 mM Tris–HCl, pH 7.5, 0.5 mM GSSG and 0.5 mM GSH containing 1.5 M GdnHCl in graphs B and C) and 1 μM ES. For intrinsic fluorescence determination samples were excited at 288 nm and the emission was measured between 300 and 400 nm. For bis-ANS fluorescence determination, samples were excited at 360 nm and emission was measured between 400 and 600 nm; a.u., arbitrary units. The data are representative of 2 experiments.

displaced from the value obtained for the native state, reaching a maximum at 356 nm, which indicates protein structural changes and full exposure of Trp residues to the aqueous environment, as the concentration of GdnHCl is raised to 3 M and higher.

In order to gain insight into the structural stability of ES under HHP and low temperature, the determination of intrinsic fluorescence was performed. ES in the presence of 1.5 M GdnHCl presents a fluorescence emission maximum at 319 nm. Compression of ES in the presence of 1.5 M GdnHCl induces a progressive red-shift displacement of maximum intrinsic fluorescence, indicating partial exposure of the Trp residues to the aqueous environment (Fig. 5B). The decrease in temperature to −9 °C induced a further red-shift displacement of maximum intrinsic fluorescence to 351 nm, consistent with Trp residues almost fully exposed to the aqueous environment, which is in agreement with the expectations because it is well known that low temperature destabilizes hydrophobic interactions [44]. The red-shift in maximum fluorescence is accompanied by a decrease in fluorescence quantum yield (not shown). Decompression to 0.4 kbar induced the return of the maximum intrinsic fluorescence to a value very similar to that obtained for the native state (320 nm), indicating absence of hysteresis effect and the reversibility of ES denaturation after complete decompression. In opposition, the maximal intrinsic fluorescence obtained for the ES that was straightly decompressed did not return to the initial value, even after 16 h incubation at atmospheric pressure (inset Fig. 5B). These results show the importance of the incubation at the intermediary pressure level for the reversibility of the ES unfolding, in agreement with the results depicted in Fig. 4.

Bis-ANS is a hydrophobic probe that has been amongst the most frequently used dyes for protein characterization due to its ability to fluoresce mainly upon binding to hydrophobic regions of partially unfolded proteins, and also to positively charged amino acids by electrostatic interactions [45]. Fig. 5C shows a progressive enhancement in the fluorescence emission of this probe with the raise of pressure level, indicating an increase of bis-ANS binding to ES. This result suggests that HHP converts the protein into intermediate species that exposes hydrophobic tertiary contacts able to bind bis-ANS more effectively than the native state. The fluorescence of bis-ANS is abrogated in case of unfolding of ES by incubation of with 6 M GdnHCl (not shown). Thus, the 3-fold raise of the bis-ANS fluorescence at 2.4 kbar and −9 °C indicate a further improvement of exposure of ES hydrophobic patches, and suggests that ES is not completely unfolded. The reversibility at 0.4 kbar was also shown by bis-ANS fluorescence (Fig. 5C), in accordance with the data obtained by determination of instrinsic fluorescence (Fig. 5B).

Altogether these data show that incubation at 2.4 kbar induces alterations in ES structure that likely impairs the folding of this protein. The fact that the alterations that occur at 2.4 kbar were shown to be reversible by incubation at 0.4 kbar, explains the higher ES solubilization yield obtained by incubation of the IBs dissociated states at this pressure level (black bar, Fig. 4).

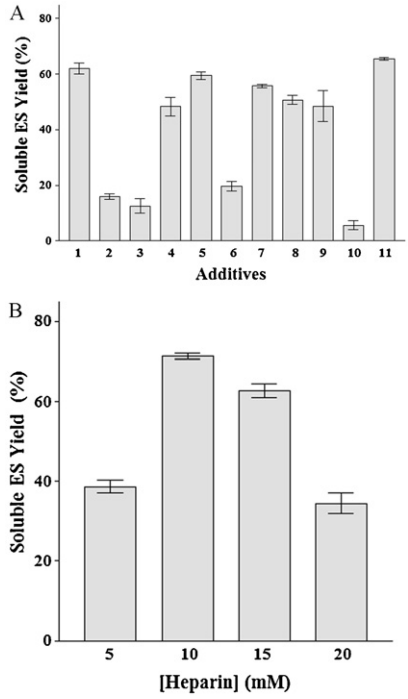

Fig. 6. The effect of the presence of different additives on ES solubilization. Suspensions of ES IBs in refolding buffer (50 mM Tris–HCl, pH 7.5 with 1.5 M GdnHCl, 0.5 mM GSSG and 0.5 mM GSH) were subject to 2.4 kbar for 2 h and then decompressed at 0.4 kbar for 16 h before complete pressure release. (A) Yields of ES solubilization in buffer containing: (1) Absence of additive; (2), 0.5 M L-arginine without GdnHCl; (3), 0.5 M L-arginine; (4), 0.15 M NaCl; (5), 1 M Glucose; (6), 1 M sucrose; (7), 0.1% PEG; (8), 1 mM Tween 20; (9), 0.5 mM Triton X-100; (10), 6.5 mM bis-ANS; and (11), 15 mM heparin. (B) Yields of ES solubilization in buffer containing increasing concentrations of heparin (5–30 mM). The data are representative of 4 experiments.

3.5. Effect of the presence of additives on the solubilization of ES

It has been known that additives, especially low molecular weight compounds, may significantly enhance the yield of the refolding process [46].

To assess if the solubilization and refolding of ES could be enhanced by the presence of some additives commonly used for improving the refolding yields at atmospheric pressure, they were added to the refolding buffer and IBs suspensions were pressurized at the optimized conditions (2.4 kbar for 2 h, decompression to 0.4 kbar and incubation for 16 h). The majority of the additives did not have a positive effect on augmenting the yield of soluble ES (Fig. 6A). Bis-ANS was described as blocking solvent-exposed hydrophobic patches in nonnative proteins and preventing them from forming insoluble aggregates. Bis-ANS was shown to be an effective inhibitor of a thermal- or chemical-induced aggregation of proteins [47,48]. Surprisingly, the presence of bis-ANS induced massive reaggregation, as shown by the reduction from 60% to 6% of ES in the soluble fraction. This result suggest that the binding of bis-ANS to the ES hydrophobic domains causes a steric hindrance of intramolecular contacts and prevents the coiling of misfolded states to the native one, thus leading to reaggregation.

The utilization of heparin as an artificial chaperone to control aggregation and promote refolding has rarely been reported [49,50]. Heparin consists of mixtures of polysaccharide chains with multiple acidic groups, polyanions that give it a highly negative charge. Heparin has been shown to bind to ES by an extensive patch of basic amino acids that is formed by 11 arginine residues present on its surface [30]. The presence of 15 mM heparin increased the refolding yields from 60% to 66%. On the basis of these results, we analyzed the effect of different concentrations of heparin on the refolding of ES. The presence of 10 mM heparin increased the ES refolding yield to 70% (Fig. 6B). This result suggests that the binding of heparin can stabilize ES intermediaries at a conformation that has a higher tendency to enter a productive folding pathway and/or a lower tendency for reaggregation.

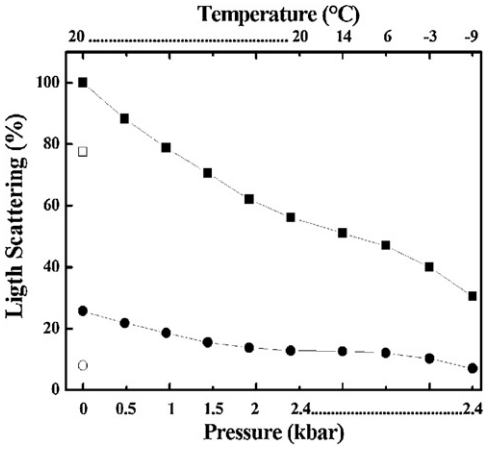

Fig. 7. Sub-zero temperatures in association with high hydrostatic pressure elevated the dissociation of IBs. Light scattering of the samples in the presence (●) or in the absence of 1.5 M GdnHCl (■) were followed as a function of pressure and temperature. Each spectroscopic data-point was collected 5 min after the pressure or temperature value had stabilized. The isolated open symbols on the left side of the panel represent the light-scattering value after the return to atmospheric pressure.

3.6. Sub-zero temperatures in association with high pressure improve the dissociation and enhance the yield of soluble ES

High hydrostatic pressure was utilized to reach subzero temperatures without freezing the water because it lowers the freezing point of water. The effect of the application of high pressure and low temperature on the dissociation of ES IBs, which was monitored by the scattering of visible light (350 nm), is shown in Fig. 7. At a high pressure (2.4 kbar) and 20 °C, a suspension in the absence of GdnHCl (squares) exhibited a pronounced decrease in the LS to 56% of the initial value. Lowering the temperature to −9 °C further enhanced the decrease of the LS to 30%. However, a return of pressure to 1 bar following the return of the temperature to 20 °C induced a recovery of 77% of the initial LS intensity, a decay of only 23%, a result that suggests that the dissociation of the aggregates by application of high pressure and low temperature is reversible resulting in the reaggregation of ES. The presence of 1.5 M GdnHCl induced a decrease in the intensity of the LS by the IB suspension to 26% of the LS value obtained for the sample in the absence of the chaotropic reagent. The application of high pressure (2.4 kbar) at 20 °C (circles) to this sample induced a further decrease of the LS to 13%. A decrease in the temperature to −9 °C induced a decrease of the LS to 7%, indicating enhancement of the dissociation of the aggregates. The return of the sample to 20 °C and atmospheric pressure did not provoke significant reaggregation of this sample (LS of 8%). The results indicate that the presence of GdnHCl, is essential for enhancing and maintaining the ES dissociation after the return to atmospheric pressure.

With the aim to verify if the increase in the dissociation of aggregates due to the application of a negative temperature in association with high pressure can improve the yield of soluble ES, we performed a comparison of the process under high pressure with IBs dissociation at −9 °C (2.4 kbar) that was followed by incubation at 20 °C (0.4 kbar) to compare it to the former process that was performed entirely at 20 °C. As expected, the application of a low temperature increased the yield of soluble ES from 69% to 78%.

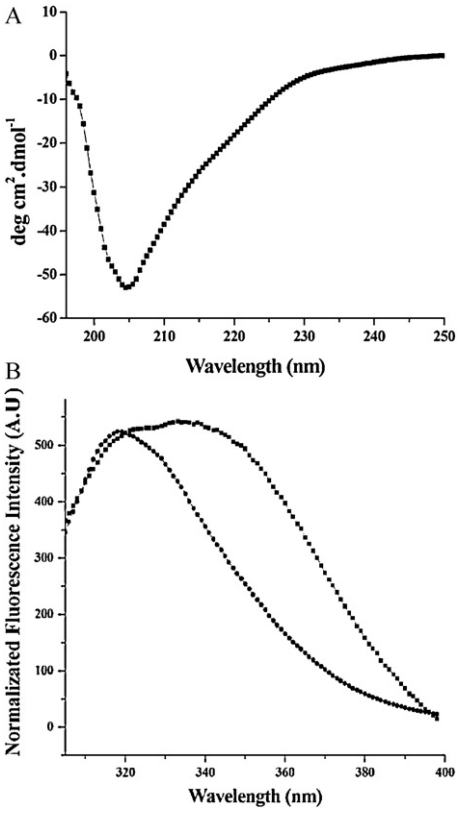

Fig. 8. HHP-treated ES present native secondary and tertiary structure. (A) Circular dichroism spectra of purified ES that was refolded under pressure; (B) normalized emission spectra for tryptophan fluorescence of the soluble fraction of ES IBs that were refolded at 0.4 kbar (▲) and the same sample that was purified in a size-exclusion TosoHaas G2000 SW column on an HPLC system (●). The fluorescence was measured at an excitation wavelength of 288 nm, and the emission spectra were collected between 300 and 400 nm.

3.7. ES treated by the HHP process present native structure and biological activity

With the aim of confirm that the structure of the ES that was solubilized utilizing the process of HHP was properly refolded, a purified sample of ES was analyzed. The CD spectrum exhibited a negative peak at 205 nm (Fig. 8A), and the analysis show a content of 7% α helices, 51% β sheets, and 42% of random coils. This result is in agreement with the literature concerning the ES secondary structure [27] and thus confirms the efficiency of the refolding process to obtain ES with a native secondary structure.

The emission spectra of the soluble fraction of a suspension of IBs treated under pressure presented a broad peak (Fig. 8B) with a maximal emission at 337 nm. To verify if this red shift in the maximal emission was due to an alteration in the conformation of the ES state or, alternatively, to the presence of bacterial contaminant proteins in the soluble fraction of the treated suspension of IBs, the intrinsic fluorescence of ES that was purified by size-exclusion HPLC was analyzed. The fluorescence of the refolded and purified ES presents a sharper peak with a maximum intensity at 319 nm, indicating that the protein has a native tertiary structure. Furthermore, it was reported that the maximal intrinsic fluorescence emission wavelength of ES mutants, which lack the two disulfide bonds due to exchange of the Cys residues for Ala (C33A/C173A and C135A/C165A), was at 340 nm, which is close to the denatured state [27]. The peak of fluorescence with a maximum emission at 319 nm was found for the refolded and purified ES, which corroborates our presumption that the ES refolded under a high pressure has a native tertiary structure retained by the disulfide bonds.

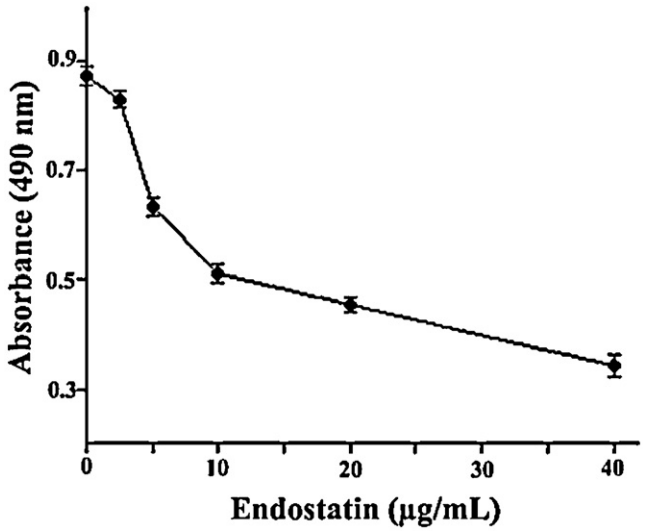

Fig. 9. Purified ES refolded under HHP reduces the viability of endothelial cells. The viability of HUV-EC-C endothelial cells was monitored by the MTS assay after 48 h of treatment with ES. The absorbance at 490 nm indicates the conversion of a tetrazolium salt into a colored, aqueous soluble formazan product by the mitochondrial activity of viable cells and is directly proportional to the number of living cells. Each value is a mean of triplicate cultures from a representative experiment and the error bars represent the standard deviation.

The ES that was refolded under HHP presented a dose-dependent effect on the viability of endothelial cells, as depicted in Fig. 9, which shows that the molecule presents a biological activity, in addition to a native structure.

3.8. The refolding yields were improved by application of the new HHP process

The improvement in the refolding yields of native ES, at a concentration of 180 µg ES/mL suspension of IBs, by applications of high pressure can be seen in Table 2. The factor that induced the most substantial improvement was the incubation at an intermediary pressure (0.4 kbar), which doubled the yield of soluble and native ES. The optimization of the process rendered an improvement of the refolding yields of more than 3-fold, from 25.3% to

Table 2
Yields of ES refolding under HHP.

Temperature of IBs production	Pressure of refolding	Heparin in refolding buffer	Temperature of dissociation	Initial ES content (mg/L)	Refolding yield (%)	Refolded ES per liter of bacterial culture	Purity (%)
37 °C	2.4 kbar	–	20 °C	237	25.3 ± 1.5	60 mg/L	76,8
25 °C[a]	2.4 kbar	–	20 °C	238	32.4 ± 1.2	77 mg/L	80,8
25 °C	0.4 kbar[a]	–	20 °C	238	62.9 ± 1.8	150 mg/L	82,7
25 °C	0.4 kbar	10 mM[a]	20 °C	238	70.2 ± 1.0	167 mg/L	83,2
25 °C	0.4 kbar	10 mM	−9 °C[a]	238	78.0 ± 1.4	185 mg/L	82,9

[a] Condition changed.

78.0%, with an obtainment of 185 mg ES with a native conformation in 1 L of bacterial culture. Notably, in comparison to the method using HHP, the refolding protocol performed at atmospheric pressure rendered a refolding yield of only 4.7%.

4. Concluding remarks

The protein refolding from aggregates that was recently described for some proteins utilized the same pressure levels (2.0–2.5 kbar) and temperatures (20–25 °C) for both the dissociation of the aggregates and the protein refolding [40,51,52]. In the present study, the optimization of the method for ES refolding was based on a separation in to a HHP-based two-step process: (1) the dissociation of the IBs (at 2.4 kbar) and (2) the refolding of ES to the native state (at 0.4 kbar). The dissociation step utilizing IBs that were produced at a lower temperature (25 °C), which despite containing similar secondary structures, were more soluble than those produced at higher temperatures, was optimized and resulted in an increase in the refolding yields. The utilization of a negative temperature (−9 °C) was effective in amplifying the effect exerted by the high pressure on the dissociation of the aggregates, which also increased the ES refolding yields.

However, the condition that produced the greatest effect in doubling the yield of ES refolding was the incubation at 0.4 kbar for 16 h after the dissociation of the IBs. This is likely due to the fact that this pressure level enables the formation of native intramolecular contacts in the ES, which does not occur at higher pressures, while preventing reaggregation of the unfolded states that occur at atmospheric pressure. The presence of heparin, polysaccharide chains with an affinity for ES, also increased the yield of HHP-induced ES refolding possibly by increasing the stability of the ES molecules.

Thus, we have shown that by using this high pressure-induced refolding method, the pressure level, temperature and presence of additives can be chosen to disfavor the nonnative intermolecular connections that form aggregates, and subsequently the conditions can be selected to favor the intramolecular connections involved in shaping the native protein state. Further studies on additional proteins will demonstrate whether this method is widely applicable.

Acknowledgments

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References

[1] Ventura S. Sequence determinants of protein aggregation: tools to increase protein solubility. Microb Cell Fact 2005;4:11.
[2] Rinas U, Bailey JE. Protein compositional analysis of inclusion bodies produced in recombinant *Escherichia coli*. Appl Microbiol Biotechnol 1992;37:609–14.
[3] Wang L, Maji SK, Sawaya MR, Eisenberg D, Riek R. Bacterial inclusion bodies contain amyloid-like structure. PLoS Biol 2008;6:e195.
[4] Przybycien TM, Dunn JP, Valax P, Georgiou G. Secondary structure characterization of beta-lactamase inclusion bodies. Protein Eng 1994;7:131–6.
[5] Ami D, Natalello A, Gatti-Lafranconi P, Lotti M, Doglia SM. Kinetics of inclusion body formation studied in intact cells by FT-IR spectroscopy. FEBS Lett 2005;579:3433–6.
[6] Ami D, Natalello A, Taylor G, Tonon G, Maria Doglia S. Structural analysis of protein inclusion bodies by Fourier transform infrared microspectroscopy. Biochim Biophys Acta 2006;1764:793–9.
[7] Garcia-Fruitos E, Aris A, Villaverde A. Localization of functional polypeptides in bacterial inclusion bodies. Appl Environ Microbiol 2007;73:289–94.
[8] Carvajal P, Gibert J, Campos N, Lopera O, Barbera E, Torne JM, et al. Activity of maize transglutaminase overexpressed in *Escherichia coli* inclusion bodies: an alternative to protein refolding. Biotechnol Prog 2011;27:232–40.
[9] de Groot NS, Ventura S. Effect of temperature on protein quality in bacterial inclusion bodies. FEBS Lett 2006;580:6471–6.
[10] Tsumoto K, Ejima D, Kumagai I, Arakawa T. Practical considerations in refolding proteins from inclusion bodies. Protein Expr Purif 2003;28:1–8.
[11] Silva JL, Weber G. Pressure stability of proteins. Annu Rev Phys Chem 1993;44:89–113.
[12] Silva JL, Foguel D, Royer CA. Pressure provides new insights into protein folding, dynamics and structure. Trends Biochem Sci 2001;26:612–8.
[13] Singh SM, Panda AK. Solubilization and refolding of bacterial inclusion body proteins. J Biosci Bioeng 2005;99:303–10.
[14] Patra AK, Mukhopadhyay R, Mukhija R, Krishnan A, Garg LC, Panda AK. Optimization of inclusion body solubilization and renaturation of recombinant human growth hormone from *Escherichia coli*. Protein Expr Purif 2000;18:182–92.
[15] Foguel D, Robinson CR, de Sousa Jr PC, Silva JL, Robinson AS. Hydrostatic pressure rescues native protein from aggregates. Biotechnol Bioeng 1999;63:552–8.
[16] St John RJ, Carpenter JF, Randolph TW. High pressure fosters protein refolding from aggregates at high concentrations. Proc Natl Acad Sci USA 1999;96:13029–33.
[17] St John RJ, Carpenter JF, Balny C, Randolph TW. High pressure refolding of recombinant human growth hormone from insoluble aggregates. Structural transformations, kinetic barriers, and energetics. J Biol Chem 2001;276:46856–63.
[18] St John RJ, Carpenter JF, Randolph TW. High-pressure refolding of disulfide-cross-linked lysozyme aggregates: thermodynamics and optimization. Biotechnol Prog 2002;18:565–71.
[19] Lefebvre BG, Gage MJ, Robinson AS. Maximizing recovery of native protein from aggregates by optimizing pressure treatment. Biotechnol Prog 2004;20:623–9.
[20] Tsai CJ, Maizel Jr JV, Nussinov R. The hydrophobic effect: a new insight from cold denaturation and a two-state water structure. Crit Rev Biochem Mol Biol 2002;37:55–69.
[21] Dhanabal M, Ramchandran R, Volk R, Stillman IE, Lombardo M, Iruela-Arispe ML, et al. Endostatin: yeast production, mutants, and antitumor effect in renal cell carcinoma. Cancer Res 1999;59:189–97.
[22] Yamaguchi N, Anand-Apte B, Lee M, Sasaki T, Fukai N, Shapiro R, et al. Endostatin inhibits VEGF-induced endothelial cell migration and tumor growth independently of zinc binding. EMBO J 1999;18:4414–23.
[23] O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 1997;88:277–85.
[24] Boehm T, Folkman J, Browder T, O'Reilly MS. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. Nature 1997;390:404–7.
[25] Folkman J. Antiangiogenesis in cancer therapy–endostatin and its mechanisms of action. Exp Cell Res 2006;312:594–607.
[26] Zheng MJ. Endostatin derivative angiogenesis inhibitors. Chin Med J (Engl) 2009;122:1947–51.
[27] Zhou H, Wang W, Luo Y. Contributions of disulfide bonds in a nested pattern to the structure, stability, and biological functions of endostatin. J Biol Chem 2005;280:11303–12.
[28] Jensen EB, Carlsen S. Production of recombinant human growth hormone in *Escherichia coli*: expression of different precursors and physiological effects of glucose, acetate, and salts. Biotechnol Bioeng 1990;36:1–11.
[29] Byler DM, Susi H. Examination of the secondary structure of proteins by deconvolved FTIR spectra. Biopolymers 1986;25:469–87.
[30] Hohenester E, Sasaki T, Olsen BR, Timpl R. Crystal structure of the angiogenesis inhibitor endostatin at 1.5 A resolution. EMBO J 1998;17:1656–64.
[31] Chura-Chambi RM, Genova LA, Affonso R, Morganti L. Refolding of endostatin from inclusion bodies using high hydrostatic pressure. Anal Biochem 2008;379:32–9.
[32] He Y, Zhou H, Tang H, Luo Y. Deficiency of disulfide bonds facilitating fibrillogenesis of endostatin. J Biol Chem 2006;281:1048–57.
[33] Cordeiro Y, Kraineva J, Suarez MC, Tempesta AG, Kelly JW, Silva JL, et al. Fourier transform infrared spectroscopy provides a fingerprint for the tetramer and for the aggregates of transthyretin. Biophys J 2006;91:957–67.
[34] Barth A, Zscherp C. What vibrations tell us about proteins. Q Rev Biophys 2002;35:369–430.
[35] Li B, Wu X, Zhou H, Chen Q, Luo Y. Acid-induced unfolding mechanism of recombinant human endostatin. Biochemistry 2004;43:2550–7.
[36] Schellman JA. Temperature, stability, and the hydrophobic interaction. Biophys J 1997;73:2960–4.
[37] Dill KA. Dominant forces in protein folding. Biochemistry 1990;29:7133–55.
[38] Cioni P, Strambini GB. Pressure effects on protein flexibility monomeric proteins. J Mol Biol 1994;242:291–301.
[39] Cioni P, Strambini GB. Tryptophan phosphorescence and pressure effects on protein structure. Biochim Biophys Acta Protein Struct Mol Enzymol 2002;1595:116–30.
[40] Cothran A, St John RJ, Schmelzer CH, Pizarro SA. High-pressure refolding of human vascular endothelial growth factor (VEGF) recombinantly expressed in bacterial inclusion bodies: refolding optimization, and feasibility assessment. Biotechnol Prog 2011;27:1273–81.
[41] Malavasi NV, Foguel D, Bonafe CFS, Braga CACA, Chura-Chambi RM, Vieira JM, et al. Protein refolding at high pressure: optimization using eGFP as a model. Process Biochem 2011;46:512–8.

[42] Kim YS, Randolph TW, Seefeldt MB, Carpenter JF. High-pressure studies on protein aggregates and amyloid fibrils. Amyloid, prions, and other protein aggregates. Pt C 2006;413:237–53.
[43] Fu Y, Wu X, Han Q, Liang Y, He Y, Luo Y. Sulfate stabilizes the folding intermediate more than the native structure of endostatin. Arch Biochem Biophys 2008;471:232–9.
[44] Foguel D, Weber G. Pressure-induced dissociation and denaturation of allophycocyanin at subzero temperatures. J Biol Chem 1995;270:28759–66.
[45] Hawe A, Friess W, Sutter M, Jiskoot W. Online fluorescent dye detection method for the characterization of immunoglobulin G aggregation by size exclusion chromatography and asymmetrical flow field flow fractionation. Anal Biochem 2008;378:115–22.
[46] Clark ED. Protein refolding for industrial processes. Curr Opin Biotechnol 2001;12:202–7.
[47] Fu X, Zhang X, Chang Z. 4,4'-Dianilino-1,1'-binaphthyl-5,5'-sulfonate, a novel molecule having chaperone-like activity. Biochem Biophys Res Commun 2005;329:1087–93.
[48] Cordeiro Y, Lima LM, Gomes MP, Foguel D, Silva JL. Modulation of prion protein oligomerization, aggregation, and beta-sheet conversion by 4,4'-dianilino-1,1'-binaphthyl-5,5'-sulfonate (bis-ANS). J Biol Chem 2004;279:5346–52.
[49] Meng F, Park Y, Zhou H. Role of proline, glycerol, and heparin as protein folding aids during refolding of rabbit muscle creatine kinase. Int J Biochem Cell Biol 2001;33:701–9.
[50] Lira CB, Gui KE, Perez AM, da Silveira RC, Gava LM, Ramos CH, et al. DNA and heparin chaperone the refolding of purified recombinant replication protein A subunit 1 from *Leishmania amazonensis*. Biochim Biophys Acta 2009;1790:119–25.
[51] Lee SH, Carpenter JF, Chang BS, Randolph TW, Kim YS. Effects of solutes on solubilization and refolding of proteins from inclusion bodies with high hydrostatic pressure. Protein Sci 2006;15:304–13.
[52] Arana ME, Powell GK, Edwards LL, Kunkel TA, Petrovich RM. Refolding active human DNA polymerase nu from inclusion bodies. Protein Expr Purif 2010;70:163–71.