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Refolding of endostatin from inclusion bodies using high hydrostatic pressure

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ABSTRACT

High hydrostatic pressure was used for concomitant solubilization and refolding of insoluble endostatin (ES) aggregated as inclusion bodies (IBs). High hydrostatic pressure (200 MPa or 2 kbar) was applied in combination with nondenaturing concentrations of guanidine hydrochloride. High levels of correctly folded ES (90 mg/L culture) were obtained after optimization/standardization of the procedure by applying pressures of 200 MPa for 16 h in 1.5 M guanidine hydrochloride/0.5 mM oxidized glutathione and reduced glutathione. Refolded ES was purified by affinity chromatography on a heparin column and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting, size exclusion HPLC, circular dichroism, and intrinsic fluorescence. We demonstrated that high pressure can successfully convert insoluble IBs of ES expressed in *Escherichia coli* into an ES preparation with native tertiary structure and full biological activity.

Introduction

Escherichia coli is the most efficient and cost-effective host for transgenic protein production that does not require posttranslational modification. However, in some instances, *E. coli* is unable to fully refold the foreign protein during overexpression of recombinant proteins. Often, bacteria accumulate these misfolded intermediates in their cytoplasmic or even their periplasmic compartments as insoluble and misfolded aggregates known as inclusion bodies (IBs[1]) [1].

Chemical chaotropic agents in the presence of reducing agents are generally used to solubilize proteins from IBs. High concentrations of guanidine hydrochloride (GdnHCl) or urea affect both hydrophobic interactions and hydrogen bonds within the aggregates. They provide the chemical energy necessary to dissociate the aggregates with concomitant denaturation of the target proteins. Refolding of the soluble and denaturated protein preparation is then achieved by removing the chaotropic chemicals using dilution, dialysis, diafiltration, or solid-phase separation, for example, size exclusion and ion-exchange chromatography, in the presence of reducing and oxidizing agents [2].

Studies using Fourier transform infrared spectroscopy or Raman spectroscopy have demonstrated the presence of native-like secondary structures of proteins in a number of bacterial IBs [3–7]. Furthermore, recent data indicate the presence of some percentage of native-like proteins retaining tertiary structure in IBs concomitantly with misfolded protein intermediates [8,9].

High hydrostatic pressure modulates protein–protein and protein–solvent interactions through volume changes. Protein states with lower volumes, and thus minimal cavity space, with increased exposure of hydrophobic groups to solvent, and with more highly ionized states are favored by higher pressure. Aggregated and native structures possess water-excluded cavities, meaning lower hydration of the protein, and consequently have larger specific volumes than unfolded species. Volumes of protein intermediates decrease in the order aggregates > native > folding intermediates > unfolded [10]. Moderate pressures (100–300 MPa) have been reported to be effective in dissociating protein oligomers and aggregates [11], whereas relatively higher (>400 MPa) hydrostatic pressures are required for the denaturation of proteins [12]. Thus, under pressure, intermolecular hydrophobic interactions are disrupted, allowing the use of lower levels of chaotropic chemicals for aggregate dissolution. High-pressure disaggregation and refolding of IBs can occur under the same processing conditions; the protein preparation does not have to be fully unfolded for IB solubilization.

Several studies have demonstrated that moderate pressure (100–300 MPa) is effective for disaggregation and refolding of pure preparations of proteins, aggregated "in vitro" by dilution of denatured protein or by stirring [13–19]. The use of high pressure for disaggregation and refolding of proteins from IBs generally results in lower yields, probably because of the greater secondary structural perturbation and bacterial contamination of these types of aggregates [14,20,21].

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[1] *Abbreviations used:* IBs, inclusion bodies; GdnHCl, guanidine hydrochloride; ES, endostatin; LB, Luria broth; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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Endostatin (ES) is a monomeric 20-kDa C-terminal fragment of collagen XVIII that can specifically inhibit endothelial cell proliferation and, thus, potently inhibit angiogenesis and tumor growth [22]. ES is a globular protein with two pairs of disulfide bonds (Cys^{33}–Cys^{173} and Cys^{135}–Cys^{165}) in a nested pattern [23]. Refolding of ES from IBs has proven to be very difficult, with recovery less than 1% under physiological conditions [22]. We failed to obtain native protein using previously described protocols [24,25], which resulted mainly in insoluble and aggregated protein preparations.

In this article we describe a novel and robust method to disaggregate and refold murine ES from IBs expressed in *E. coli*, using nondenaturing concentrations of GdnHCl and high pressure (200 MPa) in the presence of reducing and oxidizing agents. We demonstrate that native, soluble, and bioactive ES is successfully produced using this optimized methodology.

Materials and methods

Expression of ES, cell fractionation, and inclusion body purification

BL-21(DE3)pLysS was transformed with vector pET-28 containing the DNA sequence coding for the amino acids methionine, alanine, and six histidine residues at the N terminus followed by the sequence of murine ES. For ES expression, a colony was randomly picked from the transformants grown in Kan + LB plates (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 50 mg/L kanamycin) and inoculated into 15 mL of LB medium containing kanamycin. The 15 mL LB overnight culture (37 °C, 280 rpm) was diluted to a final volume of 500 mL of rich culture medium (2 × HKSII) [26], in 1000 mL shaker flasks. Expression of ES was induced with isopropyl-β-D-thiogalactopyranoside (0.5 mM) when the $A_{600\ nm}$ reached 3.0. After incubating in the shaker for a 16-h period (37 °C, 280 rpm), 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 at room temperature. The suspension was sonicated in the presence of 0.1% sodium deoxycholate, then centrifuged at 8000*g* for 10 min. The supernatant was discarded; the pellet was resuspended in 0.05 M Tris–HCl, pH 7.5, 5 mM EDTA containing sodium deoxycholate. The centrifugation/resuspension step was repeated twice, and the resultant pellet was dissolved in 15 mL of 0.05 M Tris–HCl, pH 7.5, 100 mM NaCl, 3 M urea and centrifuged at 8000*g* for 10 min at 4 °C. The urea washing procedure was repeated four times. and the urea-washed pellet was then washed twice in 0.05 M Tris–HCl, pH 7.5, and stored at –20 °C in the same buffer for further assays.

Refolding buffer and sample pressurization

The IBs were diluted in refolding buffer (50 mM Tris–HCl pH 7.5, 1 mM EDTA) containing the final concentrations of GdnHCl and oxidized (GSSG) and reduced (GSH) glutathione as indicated in the figures. Samples of 0.5 ml of the suspension were placed into plastic bags, which were vacuum/heat-sealed. The bags were placed in a pressure vessel, with a mixture of water and oil as pressure-transmitting fluid, and high pressure was applied (200 MPa, 2000 bar, or 29,000 psi) for 16 h. Samples were then centrifuged at 12000*g* for 15 min to remove remaining insoluble aggregates. The supernatant was dialyzed against 50 mM Tris–HCl, pH 7.5, buffer and centrifuged at 12,000*g* for 15 min to remove insoluble aggregates eventually formed during the dialysis process.

ES purification

Dialyzed, refolded ES was applied to a 1 mL HiTrap Heparin HP (GE Healthcare) that had been preequilibrated 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. ES-containing tubes were pooled, diluted four times, and used again in the same pre-equilibrated column, under the same conditions.

Gel electrophoresis, Western blot analysis, and protein quantification

SDS–PAGE analysis was carried out on 15% SDS–polyacrylamide gels. The gels were stained with Coomassie Blue G-250 or the proteins were transferred by electrophoresis to a nitrocellulose membrane for immunoblotting. For Western blot, the membrane was probed for 10 h with rabbit anti-mouse ES polyclonal antibody (1:400, Chemicon, Temecula, CA, USA). Reactions were detected with secondary antibody conjugated to horseradish peroxidase using enhanced chemiluminescence (GE Healthcare). Total protein content was determined by Bradford assay, using pure bovine serum albumin as a standard. Stained gels were photographed, and the densitometry of the ES bands was analyzed using Image J software for determination of the percentage of ES.

Size-exclusion high-performance liquid chromatography

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

Circular dichroism

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

Fluorescence spectroscopy

Fluorescence spectra were colleted on a K2 fluorometer (ISS Inc, Champaign, IL, USA) with a 1 cm-path-length cuvette. Tryptophan emission fluorescence measurements were carried out with the excitation wavelength at 288 nm. This wavelength was chosen because ES contains four tryptophan residues, and excitation at 288 ensures that the fluorescence emission detected is due mainly to tryptophan residues. The emission fluorescence spectra were collected between 300 and 400 nm, using a response time of 1 s and a scan speed of 240 nm/min. For the urea-induced unfolding procedure, samples of purified ES were diluted (using the same dilution factor) into solutions of urea at different concentrations for 2 h, and Trp emission fluorescence measurements were carried out. Values for fluorescence obtained at different concentrations of urea in 50 mM Tris were used as blanks for the samples in urea buffer.

Activity of ES on endothelial cell proliferation

The bioactivity of refolded ES in inhibiting the proliferation of human endothelial cells was tested by determination of their [^{3}H]thymidine incorporation. Pure recombinant ES produced by Chinese hamster ovary cells [27] was used as a standard for biological activity of the native ES. HUVE-EC-C cells obtained from ATCC (CRL-1730) were maintained in minimal essential medium (MEM)

supplemented with 10% fetal bovine serum, 50 units/mL penicillin, 50 μg/mL streptomycin, and 2 mM L-glutamine. The cells were then incubated in a humidified environment at 37 °C, in the presence of 5% CO_2. Incubations were performed in 96-well plates in a final volume of 100 μL/well MEM containing 2% fetal bovine serum, with an initial cell density of 5×10^3 cells/well. After a 24-h incubation, the medium was replaced with fresh MEM containing 2% fetal bovine serum and 10 ng/mL bFGF with or without ES. The cells were pulsed with 1 μCi [^{3}H]thymidine for 24 h, and then the cells were harvested. Cell-associated radioactivity was determined using a liquid scintillation counter.

Results

Effect of GSSG/GSH and pressurization on the solubilization of ES from IBs

ES was expressed as insoluble particles in *E. coli*. Following pressurization of the suspensions of IBs at 200 MPa for 16 h in refolding buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA) containing 1.5 M GdnHCl and different ratios of GSH/GSSG, the final solutions were centrifuged and the supernatants dialyzed. The proteins present in the supernatants following centrifugation were analyzed for protein concentration. The bands of approximately 20 kDa correspond to monomers of ES solubilized by pressurization in buffers containing 1.5 M GdnHCl and different ratios of the redox pair GSSG/GSH totaling 1 mM (Fig. 1A). In the absence of the GSSG/GSH mixture, there was a weak band of monomeric ES at the soluble fraction. ES solubilization was maximal at GSSG/GSH ratios of 2:1, 1:1, and 1:2, whereas 4:1 and 1:4 GSSG/GSH yielded less native properly folded protein. Controls incubated at atmospheric pressure (lanes 8–13) showed lower recovery of soluble monomeric ES, as seen in Fig. 1A.

When the total molar concentrations of the redox mixture, at a 1:1 ratio, were varied for pressure-induced refolding (Fig. 1B), good recovery of soluble ES was obtained at 0.5, 1, and 2.5 mM total concentrations of the redox pair. Lower yields of soluble ES were obtained when a total molar concentration of 5 mM was used. No

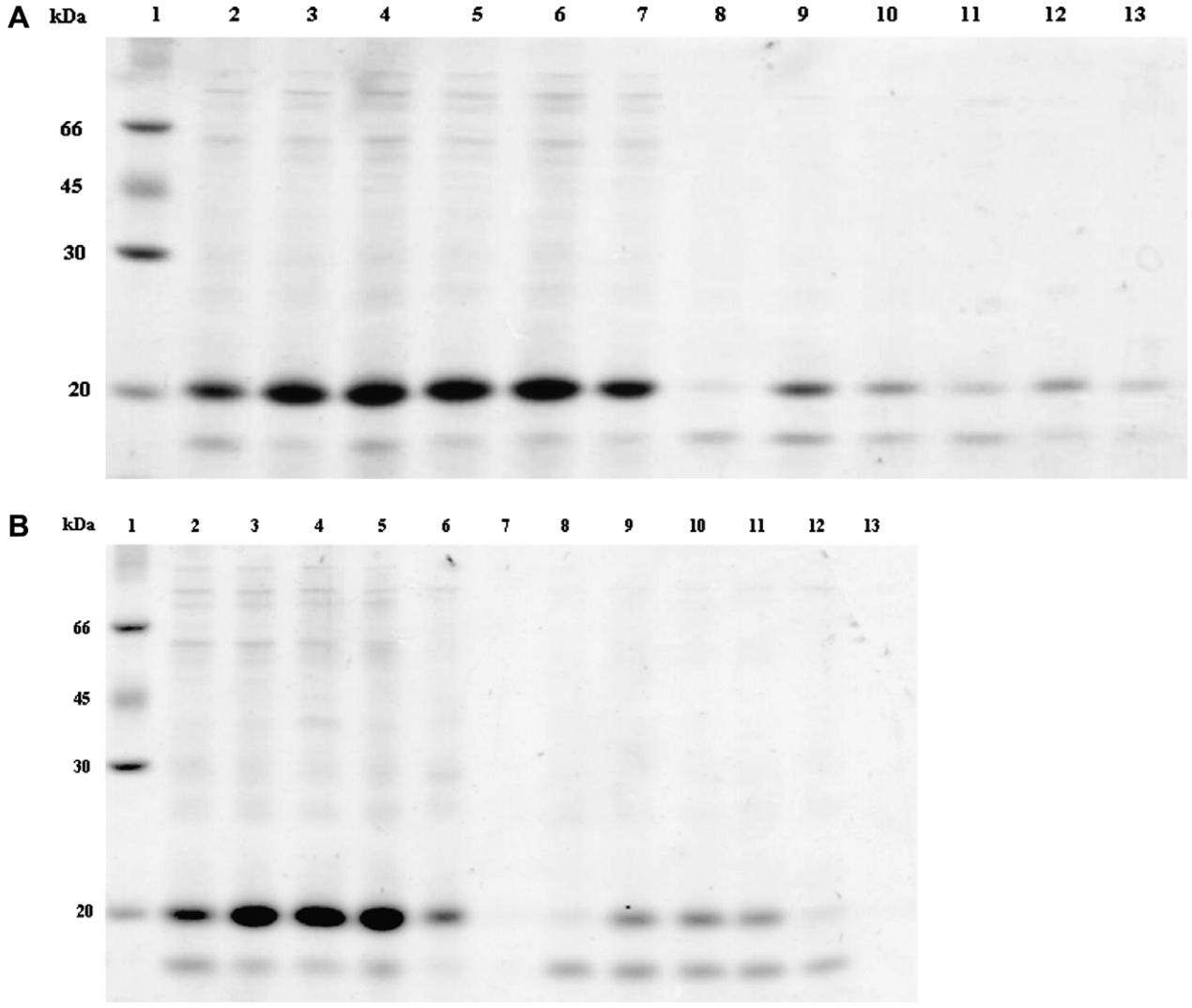

Fig. 1. Effect of the ratios of GSH:GSSG on the solubilization of ES from IBs. SDS–PAGE analysis of the soluble ES obtained in buffer containing 1.5 M GdnHCl and different proportions of GSSG and GSH. Lanes 2–7: samples subjected to high hydrostatic pressures (200 MPa) for 16 h. Lanes 8–13: samples subjected to atmospheric pressure. (A) Ratios of the redox pair GSH and GSSG with a total molar concentration of 1 mM: Lane 1, molecular weight standard. Lanes 2 and 8: samples without GSH/GSSG. Lanes 3 and 9: GSH:GSSG = 4:1. Lanes 4 and 10: GSH:GSSG = 2:1. Lanes 5 and 11: GSH:GSSG = 1:1. Lanes 6 and 12: GSG:GSSG = 1:2. Lanes 7 and 13: GSH:GSSG = 1:4. (B) Variation in concentrations of the redox pair maintaining the 1:1 ratio of GSH:GSSG. Lane 1: molecular weight standard. Lanes 2 and 8: samples without GSH/GSSG. Lanes 3 and 9: GSH/GSSG = 0.5 mM. Lanes 4 and 10: GSH/GSSG = 1 mM. Lanes 5 and 11: GSH/GSSG = 2.5 mM. Lanes 6 and 12: GSH/GSSG = 5 mM. Lanes 7 and 13: GSH/GSSG = 10 mM. MWS, molecular weight standard.

bands corresponding to ES could be seen in the SDS–PAGE-stained gel at 10 mM GSH and GSSG in the refolding buffer.

Effect of GdnHCl and pressurization on solubilization of ES

Previous studies had shown that pressure-induced disaggregation and refolding of human growth hormone and lysozyme can be optimized with the addition of nondenaturing levels of GdnHCl [15–17]. To achieve the best conditions to obtain a higher yield of native ES, we incubated the suspension of IBs at 200 MPa for 16 h in refolding buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA) and 1:1 GSSG:GSH, with varying concentrations of GdnHCl. ES did not solubilize in the absence of GdnHCl, as can be seen in the SDS–gel (Fig. 2), and at increasing concentrations of GdnHCl up to 2 M, there was an increase in intensity of the bands corresponding to native soluble monomeric ES. At 6 M GdnHCl, the intensity of the ES band diminished, and the amount of pellet formed after dialysis of the pressurized sample increased (data not shown). The higher precipitation of protein during dialysis at higher concentrations of GdnHCl indicates that the protein was solubilized during pressurization but did not fold correctly, thus being more prone to aggregation during dialysis. At atmospheric pressure, lower solubilization of aggregates of proteins from IBs was observed at all concentrations of GdnHCl, and also, lower yields of native monomeric ES were visualized. On the other hand, under high pressure (200 MPa), the aggregated protein preparation from IBs was dissolved in lower, nondenaturing concentrations of GdnHCl. Recovery of soluble ES was not altered by pressurizing the IB suspensions for 62 h compared with 16 h (data not shown).

ES characterization by SDS–PAGE and Western blot

Refolded ES showed a 20-kDa main band at the expected position of monomeric ES under nonreducing conditions, in SDS–PAGE analysis (Fig. 3A). The band corresponding to ES was displaced in the reduced sample, indicating a reduction in the disulfide bonds. Densitometry of the Coomassie-stained gel indicated that ES constituted up to 100% of the soluble protein obtained after pressurization and dialysis of the IBs. Western blot confirmed the immunological authenticity of both reduced and unreduced bands (Fig. 3B).

Monomeric ES characterization by size exclusion HPLC

Refolded and soluble ES present in the supernatant following pressurization and dialysis, when applied to a G200SW size-exclusion column, exhibited a single symmetrical peak with a retention time (t_r) of 17.8 min, in accordance with what is expected for a 20-kDa (ES) monomer (Fig. 4).The retention time of a protein (human growth hormone) with a slightly higher mass, 22.4 kDa, run under

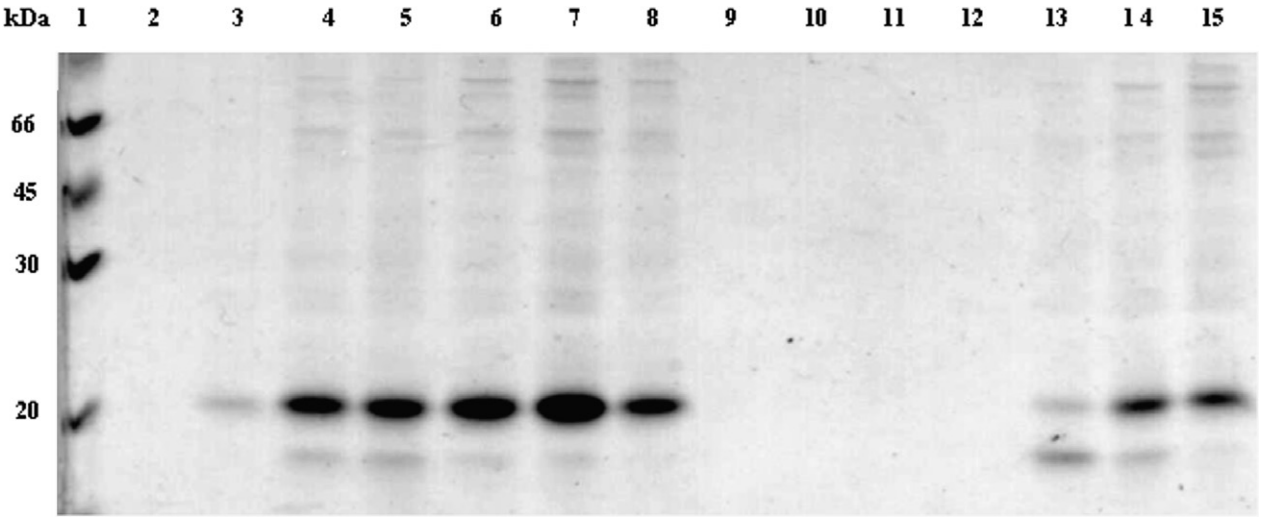

Fig. 2. Effect of molarity of GdnHCl and pressurization on the solubilization of ES. SDS–PAGE analysis of the soluble ES obtained in buffer containing 0.5 mM concentrations of both GSH and GSSG. Lane 1: molecular weigh standard. Lanes 2–8: Samples subjected to high hydrostatic pressures (200 MPa) for 16 h. Lanes 9–15: samples subjected to atmospheric pressure. Lanes 2 and 9: samples without GdnHCl. Lanes 3 and 10: 0.5 M GdnHCl. Lanes 4 and 11: 0.75 M GdnHCl. Lanes 5 and 12: 1 M GdnHCl. Lanes 6 and 13: 1.5 M GdnHCl. Lanes 7 and 14: 2 M GdnHCl. Lanes 8 and 15: 6 M GdnHCl.

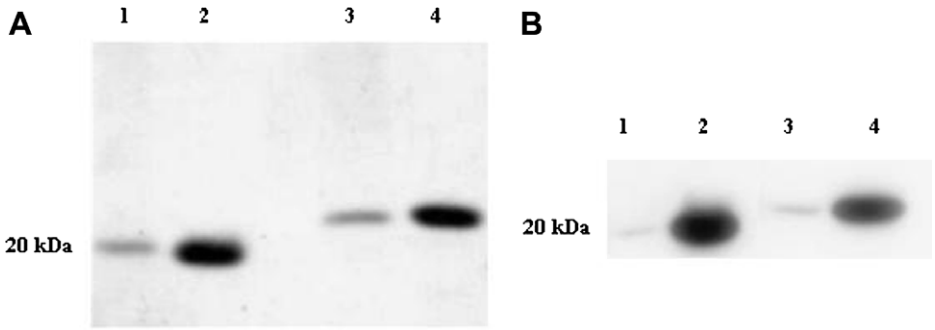

Fig. 3. Representative assays of reducing and nonreducing SDS–PAGE (A) and Western blot analysis (B) of ES. Lane 1: ES nonreduced, produced in CHO cells. Lane 2: ES nonreduced, refolded by high pressure. Lane 3: ES reduced, produced in CHO cells. Lane 4: ES reduced, refolded by high pressure.

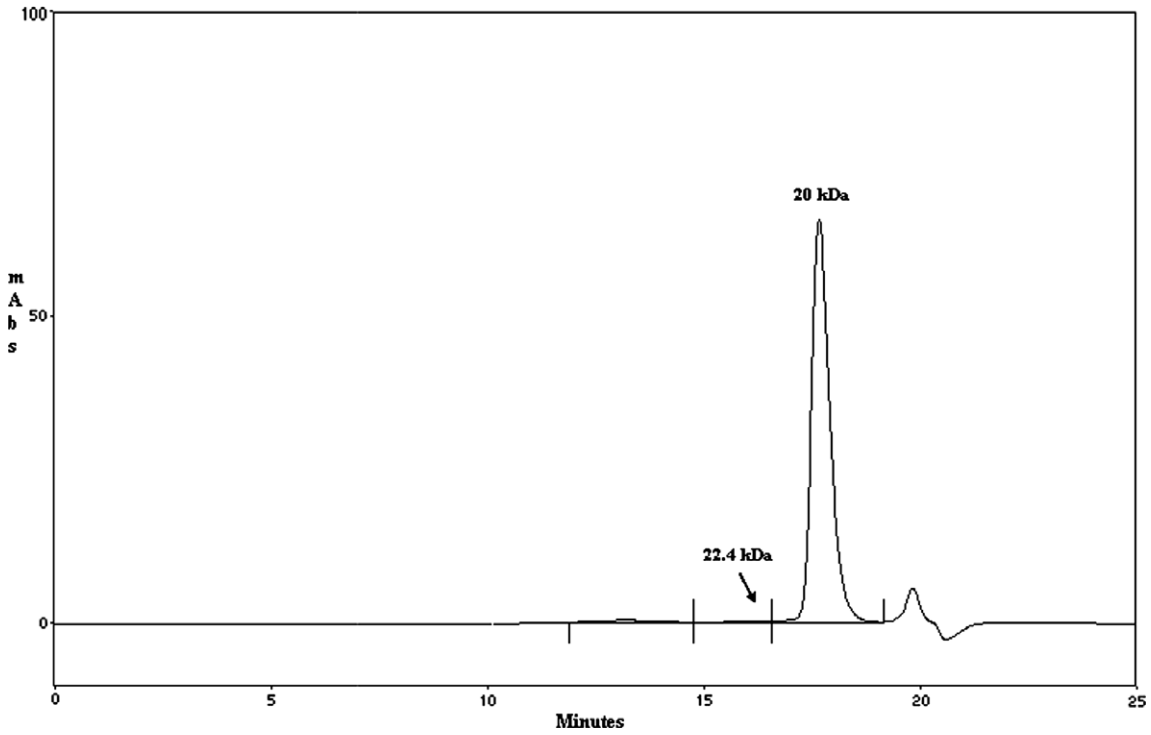

Fig. 4. HPSEC retention times of the soluble ES, without purification, showing a single symmetrical peak with a retention time of 17.8 min. The arrow indicates the retention time of human growth hormone with a slightly higher mass as a standard (22.4 kDa).

the same conditions, is indicated by an arrow as a standard for the retention time. The presence of ES as the main peak is in agreement with the data obtained with SDS–PAGE (Fig. 1A), where we can see the refolded ES as the main band. The evidence obtained by size-exclusion chromatography indicates that the refolding procedure yielded an ES preparation of high purity (up to 98.5%), even before additional purification steps.

Purification of refolded ES

The affinity of ES for heparin [22] seems to be related to the presence of an extensive patch of basic amino acids formed by 11 arginine residues [23]. ES in 50 mM Tris–HCl, pH 7.5, buffer was completely adsorbed to a heparin resin column (data not shown). ES was eluted in the range 0.4–0.5 M NaCl, indicating a relatively high affinity of ES for heparin (Fig. 5A and B). These results suggest that ES obtained by pressure refolding has the arginine-containing epitope in its correct conformation. Refolded ES (90.0 mg/L culture) yielded up to 61.6 mg of pure protein in the heparin purification procedure. The yields of soluble correctly folded ES obtained with the refolding procedure and after purification were 35.6 and 24.3%, respectively, relative to the starting amount of ES expressed on bacteria as IBs (Table 1).

It is noteworthy that the protein obtained is stable in solution and does not aggregate during purification, dialysis, or freezing/thawing procedures, underscoring the novelty and importance of this methodology as a robust technique to produce ES in its native and monomeric form from *E. coli.*

Circular dichroism of native ES

The secondary structure of ES contains a large fraction of irregular loop structures and β sheets and a small fraction of α helices in a compact fold. ES contains two pairs of disulfide bonds that form a nested pattern (C133–C173 and C135–C165). Such a unique nested pattern of disulfide bonds makes the structure of ES very tight in a symmetrical sphere conformation [23]. CD experiments were employed to detect the secondary structure of ES and its changes under unfolding by heating procedures. The CD spectrum at 20 °C (Fig. 6) exhibits a negative peak at 205 nm, which is in good agreement with the literature reports for ES secondary structure [28,29], with a content of 7% α helices, 51% β sheets, and 42% random coil. The CD spectra of ES preparations start to display a different pattern at 50 °C. At higher temperatures, the spectra change, with an increase in α helices (15%), a decrease in β sheets (32%), and an increase in random coil content (54%). Even at a temperature as high as 90 °C, the spectra recorded still show ES with a secondary structure. The α -helix content of ES was previously reported to increase under unfolding conditions [30], which may explain the increase in α -helix content at high temperatures.

Fluorescence of native ES

ES contains four tryptophan residues (W83, W114, W120, and W138) evenly distributed within the structure. All their side chains stretch inside the molecule [23], which makes intrinsic tryptophan fluorescence a good marker of modifications in tertiary structure. The tryptophan fluorescence emission spectrum of native monomeric ES exhibits maximal emission wavelength at 318 nm when excited at 288 nm (Fig. 7). The fluorescence emission spectra are red-shifted in ES urea-induced unfolding. Their fluorescence intensity increases significantly, with maximal emission at 355 nm. ES starts to unfold at 4 M urea and reaches the largest unfolded state at around 6 M urea. These data are in agreement with fluorescence emission spectra for native ES described in previous studies [31].

Biological activity of native ES

The presence of refolded ES led to dose-dependent growth inhibition of the endothelial cells, as determined by a decrease in thymidine incorporation on basic fibroblast growth factor-stimulated cell proliferation (Fig. 8). We observed that refolded ES produced

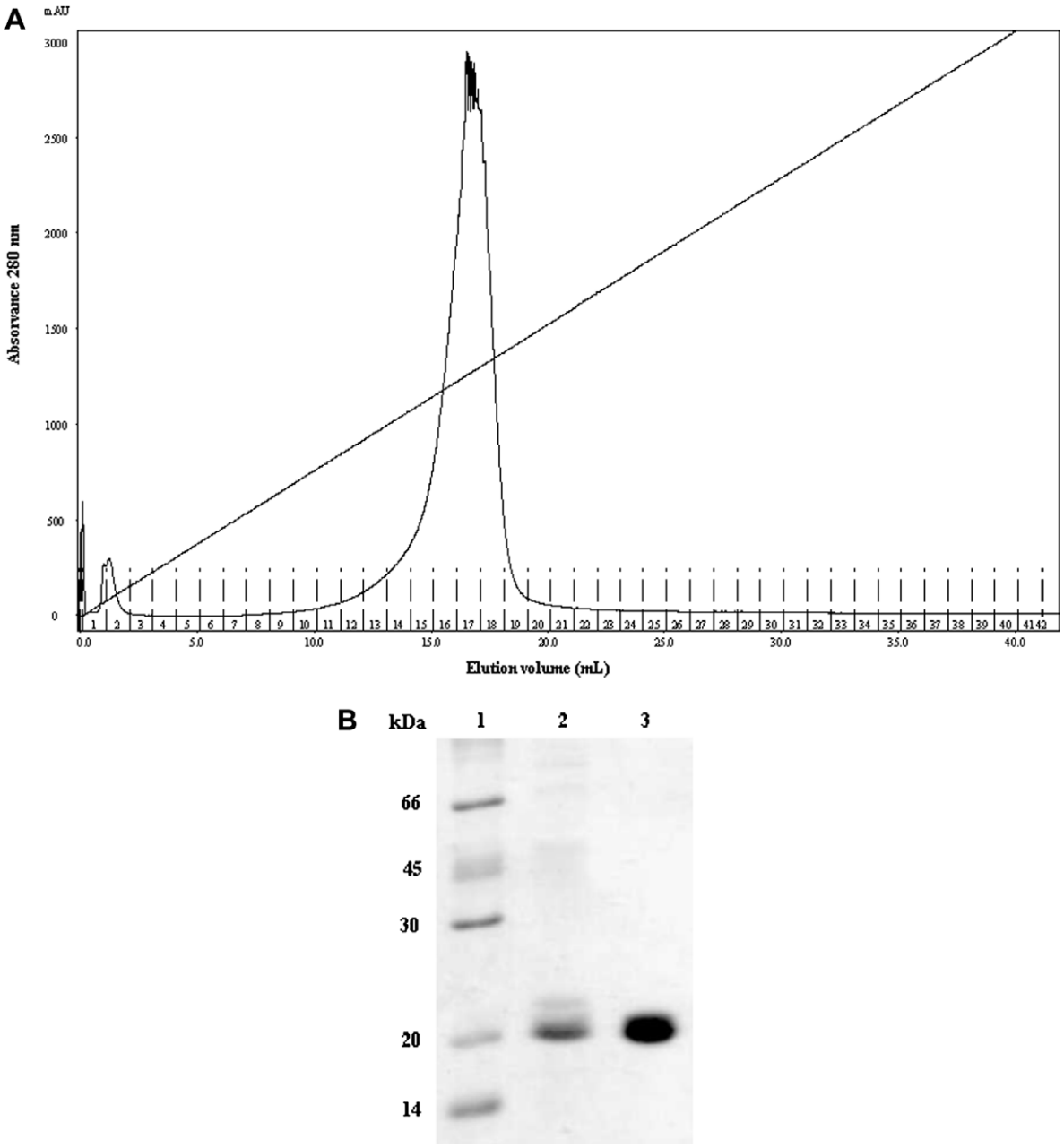

Fig. 5. Purification of soluble ES using a heparin column. (A) An increasing gradient of NaCl from 0 to 1 M was used to elute absorbed endostatin. (B) Electrophoretic analysis of purified ES from a heparin column with 15% SDS–PAGE. Lane 1: molecular weight standard. Lane 2: crude refolded protein. Lane 3: purified ES.

by high pressure inhibited the proliferation of endothelial cells (47.3 ± 4.5%) and had an inhibitory effect similar to that of a pure preparation of recombinant ES produced by CHO mammalian cells (50.8 ± 7.5%), suggesting not only correct folding but also the biological activity of this molecule.

Discussion

Recombinant heterologous proteins solubilized by high levels of chaotropic reagents generally reaggregate at the step when the denaturant is removed[2]. In our previous attempts to obtain native ES following described protocols [24,25], we failed to obtain soluble and native ES (data not shown) because of reaggregation during dialysis, purification, or freezing/thawing.

It has been shown that the process of dissolution and refolding of human growth hormone aggregates under high pressure (200 MPa) is impaired by intermolecular nonnative hydrogen bonds. This occurs because the hydrogen bonds are insensitive to pressure, as negligible volume change is associated with the breaking of these bonds [16]. Low nondenaturing levels of GdnHCl or urea disrupt hydrogen bonds, allowing dissolution of growth hormone and refolding to occur. The chaotropic agents also weaken hydrophobic interactions, further augmenting the effect of pressure, allowing a greater proportion of the protein preparation to dissociate and refold. Thus, as also shown here (Fig. 2), nondenaturing concentrations of GdnHCl (1.5–2 M) under high pressure increase dissociation of ES aggregates in IBs. In this study, we also manipulated glutathione redox concentrations and ratios to optimize high-pressure refolding of ES aggregates, obtaining maximum yields of refolded active protein of 35.6%.

It is noteworthy that high yields of refolded protein were obtained when 30 μg ES/mL refolding solution was used for pres-

Table 1
Yields of ES obtained by refolding ES under high hydrostatic pressure from IBs produced by *Escherichia coli* (1 L of culture)

Procedure	Total protein content (mg)[a]	ES content[b] (mg)	Specific activity (mg ES/mg protein)	Yield (%)
E. coli	468.0	252.7	0.54	100
Washed IBs	200.0	152.0	0.76	60.1
Refolding by HHP	90.0	90.0	1	35.6
Purification	61.6	61.6	1	24.3

[a] The protein concentration was determined with the Bradford assay.
[b] The concentration of ES was estimated by densitometry analysis.

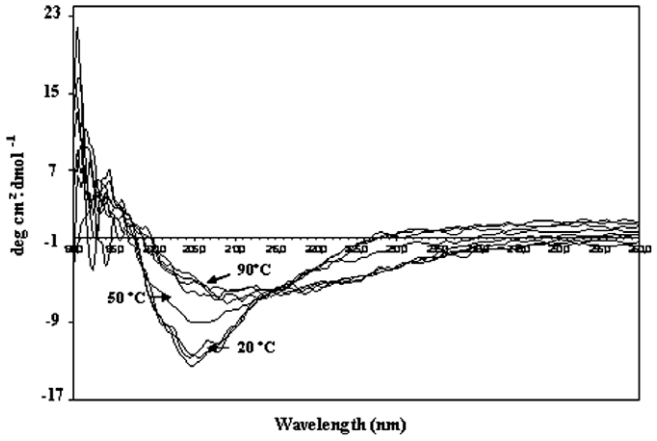

Fig. 6. Circular dichroism spectra of native ES at several different temperatures, from 20 to 90 °C, with increases in 10 °C steps. Changes in the protein secondary structure started at 50 °C; at the highest temperature (90 °C), ES maintained its secondary structure.

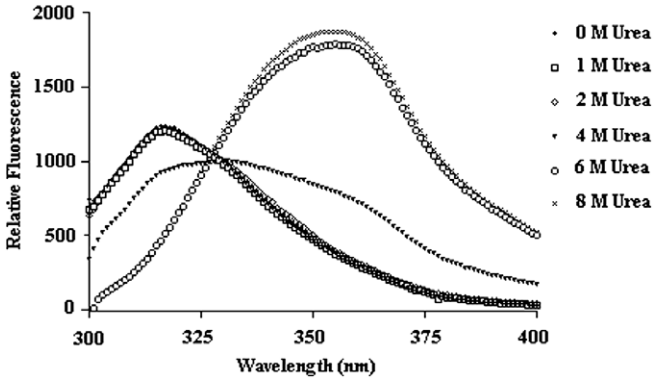

Fig. 7. Tryptophan fluorescence emission spectra of urea-induced unfolding of native ES. The fluorescence was measured at an excitation wavelength of 288 nm and emission different emission wavelengths as indicated.

surization, a concentration that is similar to those used for protein refolding in traditional methods, which typically use protein concentrations in the range 10–50 μg/mL [32].

ES is a globular protein with two pairs of disulfide bonds (Cys^{33}–Cys^{173} and Cys^{135}–Cys^{165}) in a nested pattern [23]. Zhou and colleagues [31] reported that the maximal tryptophan fluorescence emission wavelength of native ES was 320 nm, and that, of those mutants of ES lacking one of the two disulfide bonds, in which cysteine residues were changed for alanine residues, C33A/C173A and C135A/C165A were reported to present maximal tryptophan fluorescence emission at 320 and 335 nm, respectively. The mutant C33A/C173A/C135A/C165A lacking the two disulfide bonds has a maximal emission (340 nm) close to

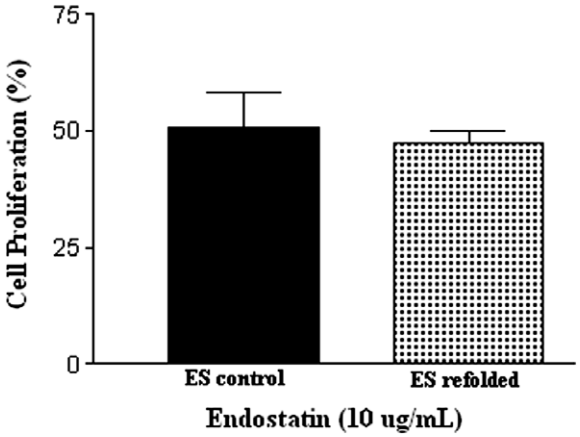

Fig. 8. Effect of refolded ES on endothelial cell proliferation assay. ES (10 μg/mL) was tested for its ability to inhibit [^{3}H]thymidine incorporation in human endothelial cells. Purified native ES was used as a control of biological activity.

that of a completely denatured state [31]. In addition, the mutant C33A/C173A, which has a native-like structure and a maximal fluorescence identical to that of wild-type ES (320 nm), was described as being more sensitive to unfolding by urea than the wild-type ES, exhibiting a red shift of maximal fluorescence at low urea concentration (1 M). Comparison of the data reported by Zhou [31] with the data obtained for ES refolded by high pressure, tryptophan maximal fluorescence (318 nm), and stability of ES structure at urea concentrations up to 2M (Fig. 7) corroborate our hypothesis that ES refolded at high pressure has the native tertiary structure retained by disulfide bonds.

In conclusion, the refolding of ES under the conditions described here resulted in a pure protein, as demonstrated by reducing and nonreducing SDS–PAGE (Table 1). The preparation also had a native tertiary structure of ES confirmed by CD, fluorescence spectra, and biological activity as shown by inhibition of proliferation of endothelial cells.

For years, refolding of ES from IBs has been a challenging task. In this study, we describe a novel, high-yield methodology that successfully produces ES with its correct tertiary structure, fully bioactive and in its monomeric form.

Acknowledgments

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