Reports of Biochemistry & Molecular Biology
Vol. 4, No. 1, Oct 2015

Original article

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Refolding Process of Cysteine-Rich Proteins: Chitinase as a Model

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Abstract

Background: Recombinant proteins overexpressed in *E. coli* are usually deposited in inclusion bodies. Cysteines in the protein contribute to this process. Inter- and intra- molecular disulfide bonds in chitinase, a cysteine-rich protein, cause aggregation when the recombinant protein is overexpressed in *E. coli*. Hence, aggregated proteins should be solubilized and allowed to refold to obtain native- or correctly- folded recombinant proteins.
Methods: Dilution method that allows refolding of recombinant proteins, especially at high protein concentrations, is to slowly add the soluble protein to refolding buffer. For this purpose: first, the inclusion bodies containing insoluble proteins were purified; second, the aggregated proteins were solubilized; finally, the soluble proteins were refolded using glutathione redox system, guanidinium chloride, dithiothreitol, sucrose, and glycerol, simultaneously.
Results: After protein solubilization and refolding, SDS-PAGE showed a 32 kDa band that was recognized by an anti-chitin antibody on western blots.
Conclusion: By this method, cysteine-rich proteins from *E. coli* inclusion bodies can be solubilized and correctly folded into active proteins.

Keywords: Chitinase, Cysteine-rich proteins, Protein refolding, Protein solubilization

Introduction

Expression of recombinant proteins in *E. coli* is a fast, cost-effective, and high-throughput approach; however, the recombinant proteins are usually deposited in insoluble aggregated particles called inclusion bodies (1). If the recombinant protein contains cysteine (Cys) residues, which are able to form inter- and intra- molecular disulfide bonds and constitute the molecules with stable structure, protein aggregation as inclusion bodies can be anticipated. If the native or correctly-folded structure of the recombinant protein is desired, the aggregated proteins can be solubilized and refolded (2-4). Developing a protein-refolding procedure has been a fundamental problem in recombinant protein synthesis. Current strategies include dilution, dialysis, and chromatography (5). Using these methods, inclusion bodies at concentrations of 1-3 mg/ml can be separated from lysed bacterial cell suspensions by centrifugation (6, 7). The aggregated recombinant proteins can be solubilized and refolded using chaotropic agents such as guanidinium chloride (GdnCl), urea, or thiocyanate salts at high concentrations (8, 9), in combination with reducing agents such as 2-mercaptoethanol (2-ME), dithiothreitol (DTT), or cysteine (7, 10). These techniques yield similar results in inclusion bodies

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Received: Jan 12, 2015; Accepted: 21 Feb, 2015

purification and protein solubilization; however protein refolding is limited in these systems. To obtain properly refolded Cys-rich proteins, redox buffers containing glutathione, L-cysteine, or cysteamine have been used. These compounds promote reshuffling of disulfide bonds and their use has resulted in high yields of naturally-folded proteins (7, 11).

Chitinases, as pathogenesis-related proteins (PR proteins), are one class of Cys-rich proteins that play crucial roles in allergies. Chitinases are produced in some plants, such as grape berry, which catalyze chitin as a homopolymer of N-acetyl-D-glucosamine as a defense against pathogenic microorganisms (12-15). The chitinase proteins contain chitin-binding domains with several conserved Cys residues that form disulfide bonds resulting in protein aggregation. Class IV chitinase, which is synthesized as grapes ripen and a major grape allergen (14, 16), is often produced as a recombinant protein for diagnosis and treatment of allergies (1, 16, 17). In this work, chitinase was investigated to develop an effective method of solubilization and refolding of Cys-rich recombinant proteins.

One method utilized to refold recombinant proteins slowly adds the unfolded soluble protein to refolding buffer. This method is applied in industry because of the broad applicability of the procedure in which a rotary shaker and peristaltic pumps are used (Fig. 1) (3). Here, we demonstrate that this dilution method of gradual addition of the Cys-rich recombinant protein into the refolding buffer improved the yield of the active form of the refolded protein over that of the un-refolded protein. This method could be useful in the solubilization and refolding of the other Cys-rich aggregated recombinant proteins from inclusion bodies.

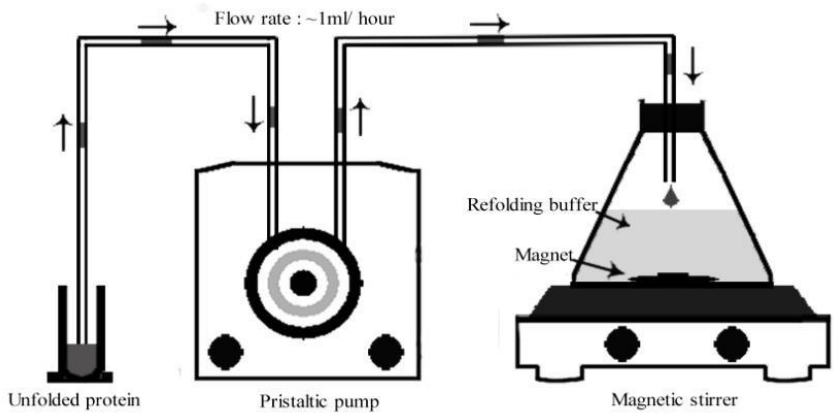

Fig. 1. A schematic diagram of Cys-rich protein refolding by the dilution method. The unfolded protein is slowly added to refolding buffer with ~1 ml/hour flow rate with stirring at 200 rpm at 4 °C.

Materials and Methods

Expression and evaluation of the recombinant protein

The previously cloned chitinase IV was expressed using plasmid pET21b+ in *E. coli* BL21-CodonPlus (18). Chitinase IV expression was induced by the addition of isopropyl β-d-thiogalactopyranoside (IPTG) to a final concentration of 0.1 µg/ml and the bacteria were cultured in 600 ml of Luria-Bertani (LB) broth for 12 h at 18 °C. The bacteria were harvested by centrifugation at 7000 x g for 5 min at 4 °C (Fig. 2, step 1).

Inclusion body isolation

The harvested bacterial pellet was resuspended in 3 ml of lysis buffer [3 ml lysis buffer/0.3 gram of *E. coli* (wet weight)] containing 50 mM Tris-HCl, pH 8.5, 100 mM KCl, and 2 M urea. To disrupt the bacteria thoroughly, the lysate was vortexed for 5 min and centrifuged at 9000 x g for 5 min at 4 °C. Following supernatant removal, the lysis step was repeated by adding 3 ml lysis buffer, vortexing for 1 min, and centrifuging at 9000 x g for 5 min at 4 °C (Fig. 2, step 2).

Protein solubilization

The pellet containing the inclusion bodies was resuspended in 5 ml of solubilization buffer consisting of 50 mM Tris-HCl, pH 8.5, 100 mM KCl, 5 M GdnCl, 1 mM EDTA, and 20 mM DTT. The mixture was vortexed for 1 min, incubated overnight at 4 °C, and incubated for 2 h at 37 °C. The solution was centrifuged at 9000 x g, for 10 min at 4 °C, the supernatant was collected for the protein refolding step (Fig. 2, step 3), and the pellet was saved for further analyses

Protein refolding

The 5 ml of solubilized recombinant protein was added to 300 ml of refolding buffer containing 50 mM Tris-HCl, pH 8.5, 0.4 M sucrose, 10% glycerol, 0.5% Triton X-100, 0.3 mM glutathione disulfide (GSSG), and 3 mM glutathione (GSH), at a rate of 1 ml/hour and stirred at 200 rpm at 4 °C. The mixture was then stirred for 24 h at 4 °C. The resulting solution was centrifuged at 9000 x g for 10 min at 4 °C and the supernatant was collected for protein purification (Fig. 2, step 4).

Refolded protein purification and dialysis

Recombinant chitinase IV was purified by metal affinity chromatography (Amersham Pharmacia Biotech) using step-by-step imidazole concentration. The affinity chromatography column with 5 ml of packed gel was washed with 100 ml of distilled water, 50 ml of 0.1 M nickel chloride solution, and 100 ml of distilled water, and then equilibrated with 100 ml of start buffer containing 50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, and 5 mM imidazole. Sodium chloride and imidazole were added to the supernatant fraction of the refolded protein to final concentrations of 1.5 M and 5 mM, respectively, and this solution was applied to the chromatography column. The column was then washed with 200 ml of wash buffer [50 mM Tris-HCl, pH 8.0, 0.5 M NaCl] containing 5 mM imidazole and then with 200 ml of wash buffer containing 40 mM imidazole to remove contaminating bacterial proteins. Finally, the recombinant protein was eluted with 10 ml of elution buffer [50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, and 500 mM imidazole] and dialyzed two times; first in 250 ml of phosphate buffered saline (PBS) with, and then without, 50% glycerol overnight at 4 °C.

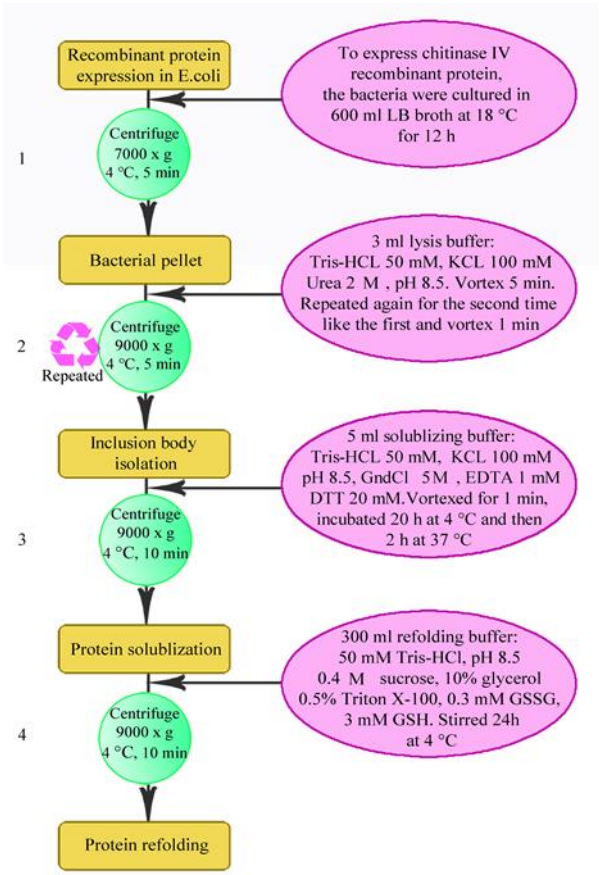

Fig. 2. A detailed schematic process of inclusion body isolation followed by protein solubilization and refolding. (Step 2 was repeated two times).

Refolded protein electrophoresis and western blotting

To evaluate the solubility of the solubilized recombinant protein, 10 µl of each fraction of the bacterial pellet, unfold-soluble protein before refolding process, and purified refolded protein of chitinase IV were separated by reducing 12.5% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (Mini-Protean II Cell, Bio-Rad). The purified refolded protein was compared with the bacterial pellet and supernatant of insoluble protein by western blotting (Mini-Protean II Cell, Bio-Rad). The membrane was incubated with a primary anti-chitinase IV antibody (12) diluted 1:500 in 2% bovine serum albumin (BSA) in PBS overnight at 4°C and then with a 1:5000 dilution of horseradish peroxidase (HRP) conjugated goat anti-mouse secondary antibody (Bio-Rad, CA, USA). The immunocomplexes were visualized using the enhanced chemiluminescence (ECL) detection system (GE Healthcare, Buckinghamshire, UK).

Results

In this study, the previously cloned chitinase IV as a Cys-rich protein model was induced in *E. coli*. After centrifugation of the bacterial lysate, chitinase production was evaluated by SDS-PAGE of the bacterial pellet. Despite the low induction temperature of 18 °C, the SDS-PAGE showed an abundant 32 kDa band in the pellet fraction as aggregated protein (Fig. 3, lane A). There was no 32 kDa detectable band of chitinase in supernatant fraction of protein before solubilization and refolding procedures (Fig. 3, lane B). This result indicates that much of the induced protein was aggregated in inclusion bodies. After purification a single 32 kDa protein band of chitinase IV was present in the refolded protein fraction (Fig. 3, lane C).

The purified refolded protein was analyzed by western blotting. The anti-chitinase IV antibody bound to a 32 kDa protein in all the fractions; however, the band was more intense in the purified re-folded fraction (Fig. 4, lane C) than in unfold-soluble protein before refolding process (Fig. 4, lane B). Western blotting results, which were belonging to protein fraction before refolding procedure, showed 66 kDa band that are likely dimmers of chitinase (Fig. 4 A and B).

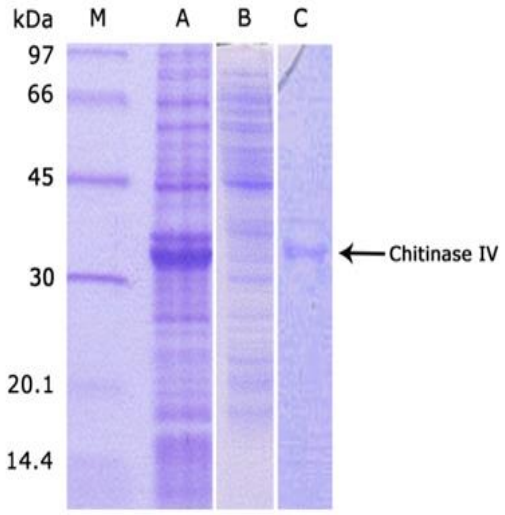

Fig. 3. SDS-PAGE analysis of chitinase IV expression in *E. coli* Bl21-CodonPlus. Lane M, protein molecular weight standards; lane A, bacterial pellet of induced *E. coli*; Lane B, supernatant fraction from induced *E. coli* before refolding procedure; Lane C, Purified refolded protein.

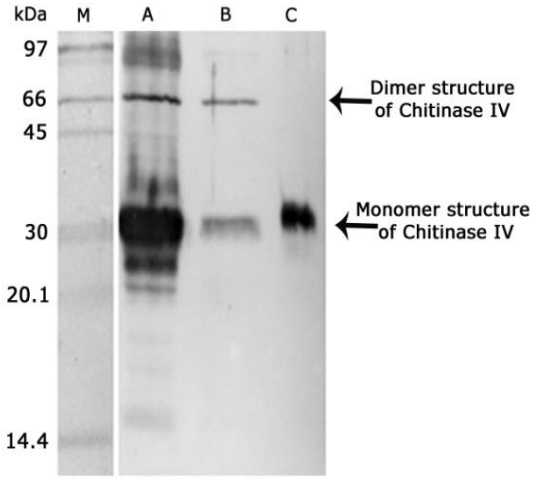

Fig. 4. Western blot analysis of chitinase IV expression in *E. coli* Bl21-CodonPlus. Lane M, protein molecular weight standards; lane A, bacterial pellet of induced *E. coli*; Lane B, supernatant fraction from induced *E. coli* before refolding procedure; Lane C, Purified refolded protein.

Discussion

Recombinant proteins, especially Cys-rich ones, are usually expressed in inclusion bodies of *E. coli* as insoluble or aggregated proteins. Insoluble proteins must be solubilized and then refolded to achieve their soluble and active forms. Protein solubilization and refolding methods include dilution, dialysis, and chromatography (5), which are similar in inclusion bodies purification and protein solubilization efficiency, but differ in protein refolding efficiency (7, 11). In this study, we evaluated a dilution procedure to refold Cys-rich proteins using refolding buffer containing glutathione as a redox system, guanidinium chloride, dithiothreitol, sucrose, and glycerol, simultaneously.

In the solubilization procedure, GdnCl as a denaturant at high concentration is able to solubilized aggregated protein in inclusion bodies through decreased oxidation of -SH groups and isomerization of disulfide bonds (19). Although GdnCl at high concentration is necessary for solubilization, it can interfere with correct protein folding; therefore, it must be removed prior to the protein refolding (19). Because the soluble protein was precipitated in the absence of GdnCl, the refolding step was performed at a low concentration of guanidine hydrochloride (3, 19). As mentioned above, EDTA, as a chelating agent, can be used in the solubilization to chelate metal ions and inhibit unwanted oxidation reactions. Dithiothreitol is the other agent in solubilization buffer that can keep

cysteine in its reduced form and prevent disulfide bonding during solubilization (7).

In Cys-rich proteins, constitution of disulfide bonds resulting in formation of dimmer structures of proteins. In the refolding procedure oxidation of disulfide bonds can be triggered by the thiol group of glutathione, as a redox system (oxido-shuffling reagents), that exists in both the reduced and oxidized forms (GSH and GSSG). (20-22). In consistent with this idea, there was a 66 kDa band in our western blotting results of protein fraction before refolding procedure, which are likely dimmers of chitinase. While after refolding procedure by reduction in the solubilization/refolding process, the 66 kDa protein band was disappeared. Hence, disappearing of 66 kDa band of chitinase in the western blotting results of refolded protein indicated that chitinase was in normal condition without dimmer structures. Tsuji and colleagues applied glutathione to refold reduced IL-2 in oxidative fashion successfully. The high cost of glutathione as an oxido-shuffling reagent is a disadvantage to its use in the protein refolding process (21).

Sucrose, as low molecular weight additive, and both ionic (sodium dodecyl sulfate (SDS)) and non-ionic (Triton X-100, Tween 20, and Tween 80) detergents can enhance protein refolding yields through the suppression of protein aggregation in refolding buffers. Jason and colleagues showed that the yield of protein refolding can be increased by the use of additives such as 500 mM sucrose, 0.1% Tween 20, and 500 mM Tris, in the refolding process (23). On the other hand, glycerol as a protein stabilizer can prevent protein aggregation in the refolding process, which reduces the refolding buffer turbidity (24). Tris concentrations greater than 0.4 M can enhance renaturation yields via inhibition of protein aggregation (25-27).

Because proteins, especially Cys-rich ones, are prone to aggregate at high concentrations, the dilution procedure may be the method of choice for refolding of highly-concentrated proteins. For this purpose, the soluble protein is slowly added to the refolding buffer drop-by-drop with stirring, which leads to intramolecular rather than intermolecular interactions. To achieve high refolding yields with low protein aggregation, the temperature, the rate of protein addition, and the volume ratio of the refolding buffer to the unfolded protein must be carefully maintained. The dilution procedure has been successfully applied for the refolding of *Pseudomonas* exotoxin recombinant proteins (24). Dialysis is recommended after protein refolding to remove any reducing and/or oxidizing agents from the protein mixture. Further manipulation of the current procedure for protein refolding to improve refolded protein yields has been performed with other additives such as acetone, acetoamide, dimethylsulfoxide, and polyethyleneglycol (28, 29).

In conclusion, this study has demonstrated that Cys-rich recombinant proteins can be refolded using dilution method as a simple and efficient procedure and refolding buffer containing glutathione, guanidinium chloride, dithiothreitol, sucrose, and glycerol, simultaneously. The SDS-PAGE and western blotting results indicated that the protein was soluble and refolded in compare with supernatant fraction of protein before refolding procedure. In the future, it will be necessary to determine the quantitative yield of protein refolding by the dilution method with different additives one at a time to achieve protein refolding with optimum yields.

Acknowledgements

This research was supported and funding by Immunology Research Center, Medical School, Mashhad University of Medical Sciences, Mashhad, Iran.

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