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Original Article

Optimization of a method for refolding of bacterial recombinant proteins

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Abstract

Background: The over-expression of recombinant proteins in large amount is important for production of therapeutic proteins and structural study. There are several systems for expression of recombinant proteins. One of the most relevant expression systems is *Escherichia coli* (*E. coli*). Although this organism has many advantages, most of recombinant proteins expressed in *E. coli* hosts form inclusion bodies. For gaining biological activities, these structures should be refolded. Many techniques have been developed for in vitro protein refolding.

Methods: In this study, a method was designed for inclusion body solubilization and protein refolding. IBs were solubilized in the solution containing 2M urea. This is a mild solubilization method without creating random coil structures in the protein.

Results: Inclusion bodies undergo mild solubilization with maintain native-like secondary structures. Solubilized proteins were refolded on chromatography column by using native buffer conditions. The results showed the recombinant proteins were purified with high efficiency without aggregation.

Conclusions: The results suggest that this method is easy, efficient, cheap procedure and usable for obtaining refolded recombinant proteins. In addition, purified protein with the method can be used in diagnosis and/or treatment of diseases.

Keywords: Recombinant Protein, Refolding, Inclusion Body

Introduction

Genetic engineering has prepared various techniques for expression of desired genes in a foreign cell. It was shown that the host cell had significant effect on the quality and level of the recombinant protein. For example, mammalian cells produce recombinant active proteins with the desired posttranslational modifications. Disadvantages of the mammalian host cell include: high cost cultivation, complicated protein purification process, time consuming and particularly low level yield of the

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recombinant protein (Vallejo and Rinas, 2004). On the other hand, bacterial host cells lack these disadvantages. Due to its easy genetic manipulation, growth on cheap carbon sources, high protein expression, and etc, *Escherichia coli* (*E. coli*) has been widely employed for the recombinant protein production (Baneyx, 1999; Schmidt, 2004). However, recombinant proteins are not always folded in the correct conformation during translation. High recombinant protein expression in this organism mostly results in inclusion body (IB) formation, because of changing in kinetic competition between folding and aggregation caused by the higher rate of protein expression and insufficient level of chaperones to support proper protein refolding (Georgiou and Valax, 1996; Vonrhein et al., 1999). Many reasons have been mentioned for IB formation (Kiefhaber et al., 1991). Previous studies have shown that about 87% of human-derived proteins are expressed in *E. coli* in the IB form (Braun et al., 2002). Although formation of IB during recombinant protein expression is often considered undesirable, it has a set of advantages. The main advantages of IB formation include: 1) very high level of protein expression (in some cases more than 30% of the total protein), 2) easy extraction, 3) lesser degradation, 4) high resistance to proteolytic proteases, 5) lower contamination. Despite low biological activity, IBs formation facilitates purification of the interest protein (Singh and Panda, 2005).

So, in vitro refolding of these structures is critical to recover active form the protein. Although many in vitro refolding techniques have been developed, but refolding yields are not optimal. Development of rational methods for in vitro protein refolding with high yield is necessary (Basu et al., 2011).

Many studies have focused on developing new refolding methods or improving existing techniques by using new refolding materials can make the bacterial IBs as a promising alternative than other expression systems producing the recombinant proteins with proper conformation and activity. The main goal in improving refolding of bacterial IBs is to enhance the final refolding yield and protein concentration (Vallejo and Rinas, 2004).

In vitro refolding of IBs includes four steps: IB isolation, solubilization, refolding and purification. Because of high concentration, IB isolation is very easy. After this step, IB should be solubilized and refolded (Clark, 2001; Clark, 1998; Vallejo and Rinas, 2004).

In this study, IBs were solubilized in the solution containing 2M urea. This is a mild solubilization method without creating random coil structures in the protein. This situation results in storing native-like secondary structure of proteins in IBs (Singh and Panda, 2005). It can be a key element for recovery of

bioactive proteins with high efficiency. During protein purification, the solubilized proteins were refolded and purified on chromatography column by specific solutions.

Materials and methods

Bacterial strains, plasmids and media

E. coli strains BL21 (DE3) and DH5α were obtained from Pasteur Institute of Iran. These bacterial strains were grown at 37°C in LB broth or agar, supplemented when required with 100 µg/ml of ampicilin. Plasmid pET-32a (Novegen, USA) was used for gene expression.

PCR amplification and cloning

Synthetic construct *urease/omp19/omp31* fusion protein gene (GenBank accession number: JQ965699) was used to design specific forward (5-ATAGAATTCATGGCC TCGTTAC-3) and reverse (5-GCTACAAGCTT TTACAAAATATCTTCAGCAGCG-3) primers for *urease* from *Brucella* spp. Additionally, specific forward (5-ACTAGAATTCGCCA CCATGGTTGTGGTCAGC-3) and reverse (5-GGTAAAGCTTATTAGTGATGGTGATGG TGATG-3) primers for gene encoded of 31-kDa outer membrane protein from *Brucella melitensis* (*omp31*) were designed. The underlined parts of the forward and reverse primer sequences above represent the restriction sites for *EcoRI* and *HindIII*, respectively. The open reading frame of omp31 and middle part of urease alpha subunit gene (Ala^{201} to Leu^{350}) were amplified by using Expand high fidelity PCR system (Roche, Germany). The PCR products were digested by *EcoRI* and *HindIII* and purified by High Pure PCR Cleanup micro kit (Roche Applied Science). Furthermore, pET32a vector was digested with same enzymes. The purified PCR products were inserted in the digested vector. For validation of recombinant colonies, a colony PCR was performed. Plasmids were extracted from positive colonies. The plasmids were subjected to double digestion by *EcoRI* and *HindIII* enzymes. After analysis on agarose gel, the purified plasmids were sequenced (Seq Lab, Germany). The recombinant pET32a-urease and pET32a-omp31 plasmids transformation into *E. coli* BL21 (DE3) and DH5α was performed by electroporation method. The hosts were grown overnight on the LB agar plate containing ampicilin.

Expression of recombinant protein

Expression of urease and Omp31 was achieved by the addition of 1 mM IPTG (Fermentas). 5 ml of bacterial cells were harvested by centrifugation at 10000 rpm/10 minutes and each pellet was resuspended in 0.1 ml of lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 0.2 mg/ml lysozyme). The samples were analyzed by 12% SDS-PAGE gel.

Isolation and solubilization of bacterial IBs from *E.coli* cells

After induction of *E.coli* cells by 1mM IPTG (isopropyl ß-D-thiogalactoside) and protein expression, the cells were harvested by centrifugation (8000 rpm, 4°C, 20 minutes). The harvested cells were dissolved in PBS and then were lysed by sonication. As a starting point use four 15 seconds cycles with 45 seconds cooling. After addition of wash buffer I (Tris 100mM, EDTA 5mM, DTT 5mM, Triton X100 0.6ml, 2M urea, pH 7), cells were precipitated by centrifugation (8000 rpm, 4 C, 30 min). The supernatant was removed and wash buffer II (Tris 100mM, EDTA 5mM, DTT 5mM, pH 7) was added to the pellet. After centrifugation (8000 rpm, 4°C, 30 minutes), the supernatant was removed. Extraction buffer (Tris 50mM, EDTA 5mM, DTT 5mM, 2M Urea, pH 7) was added to the pellet. The pellet was completely dissolved in extraction buffer after 60 minutes. Cell debris was precipitated by centrifugation (12000 rpm, 4°C, 30 minutes) and IBs were solubilized in supernatant containing extraction buffer.

Protein refolding and purification

The solution containing the solubilized recombinant protein were applied to Nickel–nitrilotriacetic acid (Ni–NTA) resin (Qiagen), washed with five column volumes of buffer A (50 mMTris-Cl, 150 mM NaCl, 1 mM 2-mercaptoethanol and 20 mM imidazole, pH 8), and the protein was eluted with 1.8 ml of buffer B or elution buffer (buffer A plus 250 mM imidazole, 10% Glycerol, pH 8.0). Addition of buffer B was repeated four times. All steps of IB isolation, solubilization, protein refolding and purification have been summarized in Figure 1.

Immunorecognition by Western blot

The expression of the recombinant proteins was verified by Western blotting by mouse anti-His antibodies. Accordingly, proteins were separated on a 12% SDS-PAGE gel and transferred onto PVDF membrane. Non-specific binding was blocked by incubating the membrane in blocking buffer of 5% skim milk in PBST (phosphate-buffered saline, pH 7.2, % 0.05 Tween 20) for 2 hours at room temperature. The membrane was washed three times with PBST at each step. After the treatment of blotted membrane with 1:6000 dilution of horseradish peroxidase (HRP)-conjugated anti-penta-His Antibody (Qiagen, Germany), the specific protein bands were discovered using their exposure to Dia-minobenzidine (DAB) substrate (Roche, Germany).

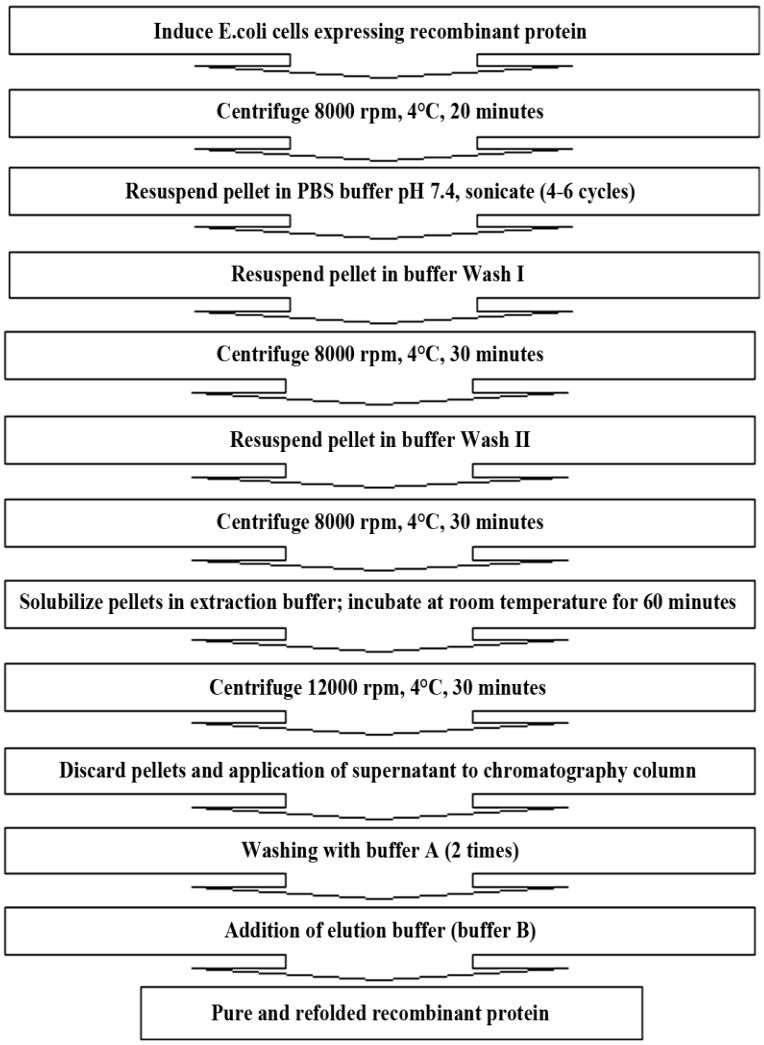

Figure 1 Scheme of IB isolation, solubilization, refolding and purification

Results

PCR amplification and cloning

Amplification of *urease* and *omp31* genes produced 482 bp and 708 bp DNA fragments, respectively (Figures 2a & 2b). The PCR products were cloned successfully in the pET32a (+) expression vector. The integrity of cloning process was confirmed by double digesting, colony PCR and sequencing of produced plasmids. Figure 2c displays the results of colony PCR from recombinant bacteria.

Expression of recombinant protein

Both *urease* and *omp31* genes were expressed in *E. coli* BL21 (DE3), with the N-terminal 6X-His-tag. The SDS-PAGE analysis showed the presence of 26 kDa and 32 kDa bands

related to urease and Omp31 proteins, respectively (Figure 3). The results showed that both urease and Omp31 are expressed in IBs form (Data not shown).

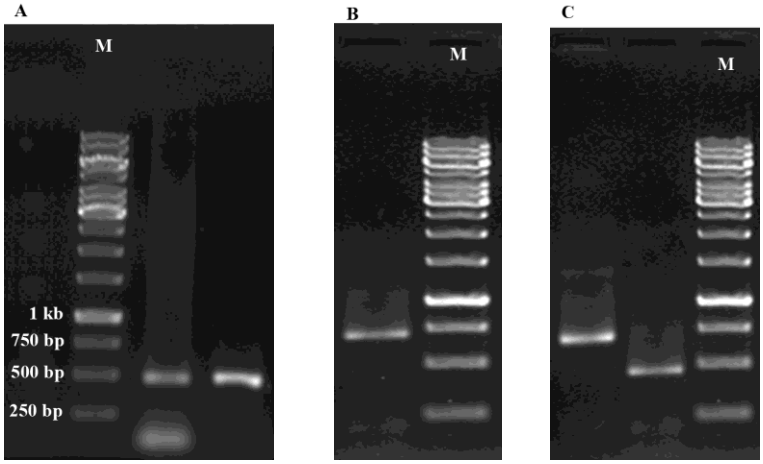

Figure 2 PCR product of middle fragment of *urease* (a) and *omp31* (b) genes. Results of colony PCR from recombinant bacteria with pET32a-urease and pET32a-omp31 (c). Lane M) 1 kb DNA ladder (Fermentas)

Protein refolding and purification

Purification of the protein was performed under native condition and SDS-PAGE analysis showed presence of the recombinant proteins (urease and Omp31) in the eluted fraction (Figure 4). No aggregation was observed after protein elution. Concentration of the purified proteins was measured by the Bradford protein assay method. The average yield of urease and Omp31 were 1.34 mg/ml and 0.51 mg/ml, respectively.

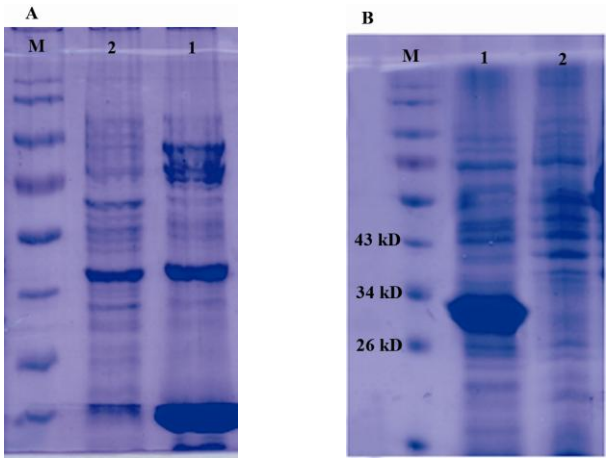

Figure 3 Expected 26 kDa urease (a) and 32 kDa Omp31 (b) proteins were determined by SDS-PAGE. M) PageRuler prestained protein ladder (Fermentas, SM0671). Lane 1) Induced cell lysate, Lane 2) Uninduced cell lysate of urease and Omp31 expressing *E. coli* cells

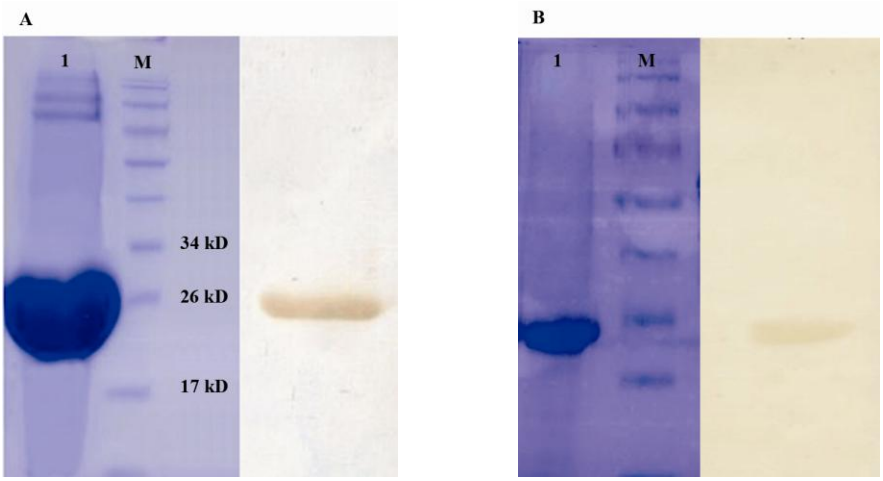

Figure 4 Purification and Western blot of recombinant urease (a) and Omp31 proteins (b). Western blotting was done using an anti-His antibody dilution ratio of 1:6000. M) 2 µl protein weight marker of PageRuler prestained protein ladder (Fermentas, SM0671). Lane 1, 20 µl purified protein after elution with 250 mM imidazole

Immunorecognition by Western blot

Western blotting analysis showed a single band in the induced cell lysate of recombinant *E.coli* cells, corresponding to the expected bands of urease and Omp31 (Figure 4).

Discussion

The main goal of protein refolding from *E.coli* IBs is to recover high level of protein at low cost (Alibolandi and Mirzahoseini, 2011). Major problem during protein refolding process is protein aggregation. So, it is critical to decrease protein aggregation at each step of refolding process from protein isolation to final purification. If the protein is completely unfolded during solubilization, it will be more prone to aggregation than partially folded proteins (Singh and Panda, 2005). Previous studies have shown that misfolded secondary protein structures in the IBs formed in *E.coli* results in protein aggregation during the refolding process (Carrio and Villaverde, 2002; Carrio and Villaverde, 2001).

It has been known that the IBs expressed in various compartments of *E. coli* maintain most of their secondary structures (Bowden et al., 1991; Przybycien et al., 1994). Furthermore, the presence of considerable native-like secondary structures of proteins from IBs has been approved (Oberg et al., 1994; Przybycien et al., 1994; Umetsu et al., 2004). Hence, these findings show the protein in bacterial IBs has an extensive extent of secondary structures. Application of a method without disturbing existing native-like secondary structure of IBs results in low protein aggregation and high protein recovery. Maintaining the native-like secondary structure of protein during mild solubilization significantly reduces the propensity of protein aggregation (Singh and

Panda, 2005). This is followed by easy purification and refolding with high efficiency. Our results showed that both urease and Omp31 are expressed in IB form. The IBs were extracted and solubilized in a solution with storing the secondary structure of protein. Since ionic and hydrophobic interactions can facilitate the protein aggregation in IBs, presence of a low amount of urea results in mild solubilization that decreases the propensity of protein aggregation. Furthermore, mild solubilization conditions (2M urea) can result in higher final refolding products compared to solubilization by high concentrations of solubilizing agent such as urea (6-8 M) (Burgess, 1996; Khan et al., 1998).

Low molecular weight additives are commonly added to refolding buffer to improve the refolding process. Hence, the additives have key role in protein refolding process so that they can significantly affect the product quality and the refolding yield and also that the effects may not always be predictive from one protein to another. These materials are divided to two groups: folding enhancers that increase protein-protein interaction and aggregation suppressors that decrease side chain interactions. The folding enhancers promote interaction between proteins and thus enhance the stability of proteins whereas aggregation suppressors decrease side chain interaction of folding intermediates without interfering with refolding process (Tsumoto et al., 2003; Wang and Engel, 2009; Yamaguchi et al., 2013). It has been indicated that Triton X-100 (in wash buffer) can transiently bind to unfolded protein in the presence of denaturants (Nath and Rao, 2001). B-mercaptoethanol (in buffers A and B) helps to conserve cystein residues in a reduced state and thus prevents formation of non-native intra- or inter disulfide bond in extremely concentrated protein solutions at alkaline pH (De Bernardez Clark et al., 1999). The disulfide bonds are more effectively formed when a mixture of low molecular weight thiols in their reduced and oxidized state is added to the refolding buffer. Naturally disulfide-bonded proteins in their reduced conditions are mostly very unstable and display a high tendency towards aggregation, particularly during the early steps of refolding (Clark, 2001; Rudolph and Lilie, 1996). Usage of the oxidized form of a thiol reagent such as dithiothreitol can cause numerous charged residues into the protein, which prevent the intermolecular interactions responsible for aggregation. When the reduced form is available in excess and the pH of solution is slightly alkaline, disulfide-bonded proteins can be efficiently refolded. These situations let fast disulfide exchange reactions until the protein reaches the most stable disulfide-bonded form, in general the native state of the protein (Wetlaufer et al., 1987). The presence of dithiothreitol (DTT) is usually

necessitated during protein refolding. The DTT decreases unwanted inter- and intramolecular disulfide bond formation during protein extraction (Clark, 2001; De Bernardez Clark et al., 1999). EDTA (in extraction buffer and wash buffers) is a chelating agent that is mostly applied in the soublization buffer to prevent metal-catalyzed air oxidation of cysteins (Alibolandi and Mirzahoseini, 2011; Mayer and Buchner, 2004).

Another strategy for avoiding the undesired intermolecular interaction between aggregation–prone folding intermediates is binding of the unfolded and solubilized protein to the solid support such as chromatography column before exchanging from denaturing to native buffer conditions (Machold et al., 2005). Application of refolding methods based on chromatographic processes is advantageous as they combine refolding with an at least partial purification of the target protein (Cho et al., 2001; Rehm et al., 2001). Protein aggregates formed during the refolding process can also be removed during on column refolding as they have a diverse retention time compared to the correctly folded protein (Gu et al., 2001; Li et al., 2002; Rathore et al., 2013). Furthermore, on column refolding can be performed constantly with the feasibility to recycle aggregates formed during the refolding process thus resulting in processes with refolding yields up to 100% (Lanckriet and Middelberg, 2004; Schlegl et al., 2003). Protein immobilization on chromatographic matrices aids spatially isolation of proteins from each other. This decreases intermolecular interactions of the protein folding intermediates (Batas et al., 1997). To prevent protein aggregation during refolding by nickel chelating chromatography, the resin binding is done in batch mode, usually at concentrations of 1 mg of protein per ml of resin. Different types of binding motives and matrices have been used for the solubilized protein to the matrix (Glynou et al., 2003; Li et al., 2003; Suttnar et al., 1994; Werner et al., 1994). In this study, urease and Omp31 containing the hexahistidine tag bound to the immobilized nickel ions.

Addition of polyhistidine-tag to N- or C-terminal provides refolding on a solid support based on immobilized metal affinity chromatography (IMAC). In presence of chaotropic agents such as urea, immobilized divalent metal ions (such as Ni^{2+} or Co^{2+}) bind to the polyhistidine-tag with high affinity (Glynou et al., 2003; Liu et al., 2003; Zouhar et al., 1999). This method allows that solubilized IBs containing polyhistidine-tag first purified and then refolded. After changing from denaturing to native buffer conditions, the dissociation of the refolded protein from the solid support should easily be performed. Both urease and Omp31 were dissociated from the matrix by using elution buffer containing imidazole 250 mM and 10% glycerol.

The removal of the denaturant from denatured proteins is a critical stage in the effective recovery of the recombinant proteins. Therefore, various methods have been presented to refold an inactive protein into an active protein. As fast decreases in denaturant concentration results in misfolding and/or aggregation, a gradual decrease in denaturant concentration within a short period of time may result in impressive protein refolding (Gu et al., 2003; Upadhyay et al., 2014). For gaining high refolding yields, one of key elements that should be optimized is components of final buffer (Gekko and Timasheff, 1981; Timasheff, 2002). Presence of glycerol in elution buffer increases the stability of proteins at all concentrations. Glycerol acts as a protein stabilizer by increasing the hydrophobic interactions as a consequence of an increase in the solvent ordering around the proteins. Increasing the glycerol concentration raises the protein stability even at high protein concentrations. Hence, the glycerol can increase the refolding yield. Glycerol has uncommon features that diminishes the surface tension of water but enhances its viscosity (Timasheff, 2002). Enhancing glycerol concentrations are identified to enhance the stability of proteins at all concentrations although it diminishes refolding at high concentrations because of its ability to slow down the kinetics of refolding, which could cause off-pathway aggregation (Cao et al., 2002).

The results showed the recombinant proteins were purified with high efficiency without aggregation. Furthermore purified protein by using this method can be employed in diagnosis and treatment of diseases. Further studies are needed to determine the function of refolded proteins with this method.

Conclusions

This method can be easy and cheap procedure with high efficiency for in vitro protein refolding and purification.

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Conflict of interest

We declare that we have no conflicts of interest.

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