Intein-mediated fusion expression, high efficient refolding, and one-step purification of gelonin toxin

Chenyun Guo a, Zhuoyu Li a, Yawei Shi a, Mingqun Xu b, John G. Wise c, Wolfgang E. Trommer c, Jingming Yuan a, *

a Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Institute of Biotechnology, Shanxi University, Taiyuan 030006, PR China
b New England Biolabs, Beverly, MA 01915, USA
c Department of Chemistry, University of Kaiserslautern, Kaiserslautern 67653, Germany

Received 5 April 2004, and in revised form 14 June 2004
Available online 6 August 2004

Abstract

An open reading frame of gelonin (Gel), one of ribosome inactivating proteins, was inserted into the vector pBSL-C which contains the coding region of chitin binding domain (CBD)-intein, resulting in the fusion expression of CBD-intein–Gel in Escherichia coli BL21 (DE3) by the induction of IPTG. The fusion product formed an aggregate of the misfolded protein, commonly referred to as inclusion bodies (IBs). The IBs were denatured and then refolded by step-wise dialysis. About 69% fusion protein was in vitro refolded to native state in the presence of GSSG and GSH as monitored by size-exclusion HPLC. The refolded CBD-intein–Gel was loaded onto chitin beads column equilibrated with 10mM Tris buffer, 500mM NaCl, pH 8.5, and about 2.4mg Gel/L culture with 96% homogeneity was directly eluted from the captured column by incubation at 25°C under pH 6.5 for 48h based on intein C-terminal self-cleavage. Western blot, ELISA, and in vitro inhibition of protein synthesis demonstrated that the bioactivity of recombinant Gel was comparable to that of native Gel purified from seeds. This implied that the purified Gel by this method is biologically active and suitable for further studies.

© 2004 Elsevier Inc. All rights reserved.

Keywords: Gelonin; Intein; In vitro Refolding; Self-cleavage; One-step purification; Bioactivity

Protein self-splicing is a post-translational processing event in which an internal protein segment, the intein, can catalyze its own excision from a precursor protein and concomitantly ligate the flanking regions, the exteins, to form a mature protein [1,2]. Since the mechanism of protein splicing was elucidated, the research and application of splicing element, intein, have been developed in the field of protein engineering, purification of recombinant proteins in particular [3,4]. A conventional method for recombinant protein expression and purification is to make the target protein to be a fusion product harboring an affinity tag, such as polyhistidine (His-tag) [5], Escherichia coli maltose-binding protein (MBP) [6], Schistosoma glutathione S-transferase (GST) [7], Staphylococcus protein A [8], and so on. However, all of these methods suffer a drawback that a site-specific protease is necessary to cleave the target protein from its affinity tag, the high cost and uncompleted cleavage of these proteases have limited their application. In recent years, a rapid, simple protein expression, and purification system has been performed by using intein self-cleavage. The intein reactivity can be controlled to reach the cleavage reaction at either its C-terminus or its N-terminus. If C-terminal Asn of intein is substituted to Ala, the cleavage of fusion protein will

* Corresponding author. Fax: +86 351 7018268.
E-mail address: jmyuan@sxu.edu.cn (J. Yuan).

1046-5928/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2004.06.037
only occur at N-terminus of the intein. For example, human neurotrophin-3 (hNT-3) has been fused to N-terminus of intein from *Mycobacterium xenopi* gyrA (*Mxe* GyrA intein) and successfully purified on a chitin affinity column by one-step manipulation, based on DTT inducible peptide bond cleavage [9]. On the other hand, a target protein can also be fused to the C-terminus of an intein whose N-terminal CySH (Ser, Thr) was substituted to Ala, then the target protein can be purified by cyclization of the Asn residue at the C-terminus of intein with a pH or temperature shift [10].

Gelonin (Gel) is one of the single chain plant ribosome inactivating proteins (RIPs). Due to lacking lectin subunit, single chain RIPs are generally not as highly toxic to intact cells as the double chain RIPs such as ricin. Therefore, the single chain RIPs have mostly been selected to construct the potent and specific immunoconjugates. Gel has been conjugated with some monoclonal antibodies by gene fusion or chemical cross to effectively kill the target cell, attempting to cure tumors and autoimmune diseases [11,12]. Herein, Gel gene was inserted into plasmid pBSL-C harboring Sp Sp DnaB mini-intein attached CBD as a chitin affinity tag and transformed into *E. coli* BL21(DE3). Then pure Gel can be directly eluted from chitin beads column by a pH shift and purified by single-step manipulation without chemical cleavage or protease digestion (Fig. 1). Thus, the intein purification system provides a facile method for preparing recombinant proteins.

### Materials and methods

**Bacterial strains, plasmids, reagents, and media**

*Escherichia coli* BL21(DE3) was stored in this laboratory at −70°C. Vector pBSL-C was generously provided

---

**Fig. 1.** A diagrammatic sketch for one-step purification of Gel by using intein C-terminal self-cleavage. The N-terminal cleavage of intein in vector pBSL-C was blocked due to the mutation of CySH1–Ala1. The expression product, CBD-intein-Gel, can bind to chitin beads and the unbound proteins are washed off the beads directly with washing buffer. When the captured beads are incubated at pH 6.5, nucleophilic attack will occur at the peptide bond between Asn at C-terminus of intein and CySH at N-terminus of target protein. Therefore, the Asn was transformed to an intermediate, succinic amide, and Gel was cleaved from the fusion product and eluted from the beads directly.
Then the IBs were dissolved in buffer B (10mM Tris–HCl, 500mM NaCl, pH 8.5) containing 8M urea to reach a protein concentration of about 0.3mg/mL. The dissolved precipitate was kept for 2–3h at room temperature to completely solubilize the aggregate. After centrifugation at 12,000rpm for 30min, about 10mL supernatant was step-wisely dialyzed against 1L buffer B containing 4, 2, and 1M urea sequentially at 4°C for about 17–18h each. Finally, the protein solution was dialyzed against 500mL buffer B containing 0.2mM GSH, 0.02mM GSSG, and 0.5mL-Arg for in vitro refolding at 4°C for 24h. After centrifugation at 12,000rpm for 30min, the supernatant was further purified in the next step. The in vitro refolding process was simultaneously monitored by size-exclusion HPLC equipped with a SW 300 gel column (7.8 × 300mm) with 10mM Tris buffer, pH 8.5, as the mobile phase at a flow rate of 1.0mL/min, and absorbance at 280nm was monitored with a UV detector.

Construction of plasmid pBSL-Gel and expression of recombinant in E. coli

To fuse the target gene to the C-terminus of CBD-intein reading frame, the Gel gene (753bp) was amplified from plasmid pUC-Gel by polymerase chain reaction (PCR) with the following primers which, respectively, contained unique restriction sites for AgeI (forward) and PstI (reverse) to facilitate cloning: forward primer: 5′-GGT GGT ACC GTG GAT ACC GTG AGC-3′, reverse primer: 5′-GGT CAG TTA TTT CGG ATC TTT ATC G AC-3′. The conditions used were: 95°C for 5min, 30 cycles of (94°C 30s, 52°C 30s, and 72°C 1min), and a final extension of 72°C for 10min. The PCR product was purified and digested with corresponding enzymes, subsequently inserted into the vector pBSL-C at AgeI and PstI sites of MCS to form the recombinant plasmid pBSL-Gel [14]. After restriction enzyme digestion and DNA sequencing verification, the pBSL-Gel was transformed into competent E. coli strain BL21(DE3) by calcium chloride method [13]. The resulting engineered strain E. coli BL21(DE3)/pBSL-Gel was grown in LB medium supplemented with 100g/mL ampicillin at 37°C until the optical density (OD) at 600nm reached 0.5–0.6, and then induced by 0.5mM IPTG at 12°C for 16h. The fusion expression product, CBD-intein–Gel was confirmed by SDS-PAGE and Western blot analysis.

Refrolding of CBD-intein–Gel aggregate

The cultured cells were harvested by centrifugation at 6000rpm for 10min and the cell pellets from 1L IPTG induced culture were suspended and sonicated in lysis buffer A (10mM Tris–HCl, 500mM NaCl, 1mM PMSF, pH 8.5, and 0.1% Triton X-100). The lysate was then centrifuged at 12,000rpm for 30min. Because the fusion protein almost completely existed in the form of IBs by 10% SDS–PAGE analysis, the precipitate was washed with buffer A containing 4M urea and centrifuged at 12,000rpm for 30min. This step was repeated twice to remove the cell debris and other impurities. Then the IBs were dissolved in buffer B (10mM Tris–HCl, 500mM NaCl, pH 8.5) containing 8M urea to reach a protein concentration of about 0.3mg/mL. The dissolved precipitate was kept for 2–3h at room temperature to completely solubilize the aggregate. After centrifugation at 12,000rpm for 30min, about 10mL supernatant was step-wisely dialyzed against 1L buffer B containing 4, 2, and 1M urea sequentially at 4°C for about 17–18h each. Finally, the protein solution was dialyzed against 500mL buffer B containing 0.2mM GSH, 0.02mM GSSG, and 0.5mL-Arg for in vitro refolding at 4°C for 24h. After centrifugation at 12,000rpm for 30min, the supernatant was further purified in the next step. The in vitro refolding process was simultaneously monitored by size-exclusion HPLC equipped with a SW 300 gel column (7.8 × 300mm) with 10mM Tris buffer, pH 8.5, as the mobile phase at a flow rate of 1.0mL/min, and absorbance at 280nm was monitored with a UV detector.

One-step purification of Gel by intein C-terminal self-cleavage

The refolded protein supernatant was applied to a 5mL chitin beads column pre-equilibrated with buffer B at 4°C. The captured column was washed with 20 column volumes of buffer B to remove unbound proteins and flushed with 15mL cleavage buffer (10mM Tris–HCl, 500mM NaCl, pH 6.5) before the outlet was closed. After incubating the captured column at 25°C for 48h, 5–10mL cleavage buffer was flowed through the column, the fractionation of Gel was collected according to the absorbance at 280nm in LKB protein purification chromatography system and then examined by 12% SDS–PAGE. The chitin resin could be regenerated with buffer C (10mM Tris–HCl, 500mM NaCl, and 1–2% SDS, pH 8.5).

 Determination of protein concentration and purity

Protein concentration was determined by Bradford method using bovine serum albumin as the standard [15]. Protein expression level and protein purity were estimated by comparing the intensity of Coomassie brilliant blue staining of samples run on SDS–PAGE. The stained gel was quantified by gel document scanning (GDS) with BIO-PROFIL Bio-ID V99.01 software.

Western blot and ELISA

Fusion protein CBD-intein–Gel and purified Gel were run on 12% SDS–PAGE and transferred electrophoretically to a nitrocellulose membrane using a mini trans-blot electrophoretic transfer cell (Bio-Rad) at 100V for 1h in transfer buffer (25mM Tris, 192mM glycine, and 0.025% SDS, pH 8.3). After blocking with 1% BSA, the membrane was incubated with mouse anti-Gel
coli (data not shown). The engineered strain pBSL-C and suitable for fusion expression in E. coli. Gel gene fragment was correctly inserted into expression and DNA sequence analysis for plasmid pBSL-C.

Results and discussion

Cloning and expression of Gel gene

The bioactivity of Gel on inhibition protein synthesis was assessed using a cell-free rabbit reticulocyte lysate protein translation system purchased from GIBCO (Grand Island, NY, USA). After diluted in PBS, 5μl Gel sample and 40μl cell-free rabbit reticulocyte lysate containing 10mM creatine phosphokinase, and 0.5mM KCl were added into 96 wells on the microtiter plate, incubating at 37°C for 5min, then 10μl master mixture containing 10mM creatine phosphate, 0.5mM MgCl₂, 79mM KCl, 5μCi/mL [³⁵S]valine, and amino acid mixtures were added and continuously incubated at 37°C for 10min. Five microliter reaction mixture was taken from the wells and added into 1mL ice chilled water containing 500μl valine (1mg/mL), incubating 10min at 37°C. The assay was repeated four times at each protein concentration. The reaction was stopped by adding 25% trichloroacetic acid. The protein obtained by the glass microfiber filter was dried and counted by liquid scintillator.

Reticulocyte lysate activity

It was shown from the results of double-enzymatic digestion and DNA sequence analysis for plasmid pBSL-Gel that Gel gene fragment was correctly inserted into vector pBSL-C and suitable for fusion expression in E. coli (data not shown). The engineered strain E. coli BL21(DE3)/pBSL-Gel can express the target product, CBD-intein–Gel in the form of IBs only as estimated by SDS-PAGE (Fig. 2) or Western blot analysis (data not shown). To explore its soluble expression, various factors were examined, including the optical density of culture at induction (OD₆₀₀ₙ₉ 0.4–0.8), the concentration of inducer IPTG (0.1–1.0 mM), induction temperature (37, 25, 16, and 12°C) [16–18] as well as co-expression with molecular chaperone GroESL [19]. Unfortunately, no significant amount of the soluble target product was observed under any of the above conditions. Over-expression of a recombinant harboring the gene of eukaryotic cells in host strain E. coli often results in the formation of biologically inactive aggregates. Fortunately, it is possible to make the aggregates soluble by some refolding methods [20].

It is well known that the IBs occurred during recombinant expression in bacteria as a random protein aggregate in an unfolded, partially folded or inactive conformational state, which can be in vitro refolded to partially recover its active and native state under the defined conditions [20]. Due to the retention time of linear and globular macromolecules being quite different on size-exclusion chromatography, the whole process of in vitro refolding was accurately monitored by size-exclusion HPLC while the urea concentration was reduced in step-wise dialysis (Fig. 3). The IBs were initially washed with 4M urea to remove the cell debris and other impurities before completely solubilized in 8M urea. As can be seen in Fig. 3, the protein was in linear or random state under strong chaotropic solvent condition (purple trace, 2). There was little difference for the chromatograms (purple and green traces, 2 and 3) in the presence of 8 or 4M urea, which indicated that the complete unfolded polypeptide was still dominative with 4M urea [21]. However, when the characteristics of solvent surrounded denatured protein were changed with decreasing urea concentration, nucleation of protein conformation seemed to be appearing after dialyzed against 2M urea, as evidenced by the appearance of a new peak at the retention time of 7.48min (turquoise trace, 4). The unsymmetrical chromatogram profile indicated that the protein could partially be folded to an intermediate, and there existed a competition between the first-order (correct) folding reaction and the higher-order aggregation reaction. After dialyzed against 1M urea, the majority of the target protein was refolded as indicated by the main peak around 11min (pink trace, 5), and also a significant amount of protein seem to be in a misfolded or aggregate state as indicated by the peak around 5min (pink trace, 5). The misfolding or aggregation of the protein could be from the association of
hydrophobic surfaces that were exposed in folding intermediate or improper disulfide bridge formation [22]. The competition of misfolding may kinetically limit the protein to be folded into its native state [23]. In our study, the majority of the protein was shifted to the correct conformation after urea was completely removed by dialysis in the refolding buffer (10 mM Tris–HCl, 500 mM NaCl, pH 8.5, 0.2 mM GSH, 0.02 mM GSSG, and 0.5 M LL-Arg) (brown trace, 6). Reduced and oxidized glutathione (GSH and GSSG) are commonly used as oxidido-shuffling reagents, because thiol-disulfide exchange reactions are rapidly reversible. The oxidido-shuffling reagents can increase both the rate and the yield of correct disulfide bond formation by rapid reshuffling of improper disulfide bonds [23]. LL-Arg contains a guanidino group and it may play a role in suppressing aggregation of the protein during refolding [24]. In conclusion, the results from size-exclusion HPLC analysis figuratively demonstrated in vitro refolding process during the step-wise dialysis, which was also confirmed by following bioactivity assay.

In vitro pH inducible cleavage of fusion protein

The target protein fused with intein can be cleaved at the C-terminus of intein by pH shift based on the background of the recombinant plasmid. To investigate the optimal pH for the cleavage reaction, the refolded CBD-intein–Gel was incubated in vitro at five different pH values, ranging from pH 6.0 to 8.0 at 25°C. It was shown from the results of SDS–PAGE in Fig. 4 that the cleavage reaction was completely inhibited at pH ≥7.5 and was gradually increased at either pH 6.0 or 7.0, while the optimal value for the yield and purity of Gel seemed to be at pH 6.5. During the cleavage process, three bands corresponding to fusion protein (55 kDa), CBD-intein (27 kDa), and Gel (28 kDa) should occurred by SDS–PAGE analysis. However, the molecular weights of Gel and its fusion partner are too close to be separated on the gel plate. For confirming the target protein Gel, a positive product at 28 kDa band was occurred by Western blot analysis (data not shown). It has been speculated that pH sensitivity of the intein aroused from protonation of the highly conserved penultimate histidine residue (pKₐ, approximately 6.5) of the intein C-terminus [25]. Mutation of the penultimate histidine inhibited intein C-terminal cleavage, which also indicated the importance of the conserved histidine residue for intein C-terminal cleavage [26–28].

One-step purification of gelonin

The refolded CBD-intein–Gel was loaded onto a chitin beads column at pH 8.5. After washing the captured column with the equilibrated buffer, about 90% of fusion protein was bound to the chitin beads (Fig. 5, lane 3). Pure Gel with 96% homogeneity was directly eluted from the column after incubation at pH 6.5,
25°C for 48 h (Fig. 5, lane 4). It is indicated from Table 1 that the refolding percentage of CBD-intein–Gel reached about 69%, but Gel yield is only 5%, compared with the quantity of inclusion bodies. The cleavage reaction as described above was so incomplete that about 20% fusion product still stuck in the affinity column as occurred by SDS–PAGE analysis with a small portion of affinity beads. There was not much improvement in the yield of Gel even if the cleavage reaction on-column was extended to longer time. However, this system requires no protease or chemical cleavage to obtain the target protein from its fusion product, compared with other affinity tag expression systems [29,30].

Bioactivity of recombinant Gel

It was demonstrated from ELISA (Table 2) and Western blot analysis (data not shown) that the recombinant fusion product or Gel only reveals the positive immunoreactivity. The bioactivity of recombinant Gel was further confirmed with the functional analysis by using inhibition assay of cell-free protein synthesis in rabbit reticulocyte lysates. As shown in Fig. 6, native Gel inhibits cell protein synthesis by 50% at 15 pM, whilst the recombinant Gel is at 20 pM with 50% inhibition. The result indicated that the bioactivity of the recombinant Gel was comparable to that of native Gel.

Conclusions

In this report, a fusion protein, CBD-intein–Gel was over-expressed in the form of inclusion bodies in E. coli and was successfully in vitro refolded through 8 M urea denaturation and step-wise dialysis. The refolding process along the renaturation was concomitantly monitored by size-exclusion HPLC, and the refolding recovery of the fusion product reaches about 69%. About 2.4 mg Gel/L culture with 96% homogeneity was obtained by single-step purification on chitin beads column, without any chemicals or protease treatment. Moreover, immunoreactivity and functional assay also demonstrated that the recombinant Gel possessed the same properties as the natural Gel purified from seeds. This intein-mediated purification scheme would provide

Table 1

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total proteins (mg)</th>
<th>Target protein (mg)</th>
<th>Purity (%)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inclusion bodies (in 8 mol urea)</td>
<td>76</td>
<td>46 (CBD–intein–Gel)</td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>Refolding supernatant</td>
<td>51</td>
<td>32 (CBD–intein–Gel)</td>
<td>63</td>
<td>69</td>
</tr>
<tr>
<td>Chitin column and intein-mediated cleavage</td>
<td>2.4</td>
<td>2.3 (Gel)</td>
<td>96</td>
<td>5</td>
</tr>
</tbody>
</table>

*Step and overall yields were calculated starting from the pure inclusion body preparation and protein concentration was determined by Bradford method.

*b Purity is defined as the percentage of target protein in the purified protein preparation.
a convenient and economic method to prepare other recombinant proteins.

Acknowledgments

This work was supported by the Natural Science Foundation of China (NSFC: 30270292). We also thank Dr. Tao Yuan in Aventis Pasteur, Canada, for his helpful suggestion.

References