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Split intein facilitated tag affinity purification for recombinant proteins with controllable tag removal by inducible auto-cleavage

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ABSTRACT

Purification tags are robust tools that can be used to purify a variety of target proteins. However, tag removal remains an expensive and significant issue that must be resolved. Based on the affinity and the trans-splicing activity between the two domains of Ssp DnaB split-intein, a novel approach for tag affinity purification of recombinant proteins with controllable tag removal by inducible auto-cleavage has been developed. This system provides a new affinity method and avoids premature splicing of the intein fused proteins expressed in host cells. The affinity matrix can be reused. In addition, this method is compatible with his-tag affinity purification technique. Our methods provide the insights for establishing a novel recombinant protein preparation system.

1. Introduction

Protein splicing is a post-translational process that removes an internal protein segment, intein, from the precursor protein. The flanking protein segments, N- and C-terminal exteins, are then ligated with a peptidyl bond. This protein backbone rearrangement is catalyzed by intein itself as an autocatalytic and spontaneous reaction [1–3]. The majority of inteins contain the splicing domain and the homing endonuclease domain [4]. Some called mini-inteins have no endonuclease domain but remain splicing proficient [5–7]. Others termed split inteins even lost the sequence continuity [8,9]. Split inteins may occur naturally, such as *synechocystis* sp. Pcc6803 DnaE intein that has two fragments and is fully capable of protein trans-splicing [10]. The split dnaE gene encodes two precursor polypeptides that bind tightly and splice concomitantly [11–13]. Split intein may also be generated artificially from regular contiguous intein by splitting the coding sequence at several specific sites [14]. Both naturally occurring and artificial split inteins have been explored for a variety of protein engineering approaches [15–20]. Generally, the single fragment of a split intein has no splicing activity [3,21–23]. When fused with a target protein, it is stable to be expressed in different hosts and intactly purified. After added with another fragment, trans-splicing occurs at the fused protein to get rid of the intein fragment [21].

Ssp DnaB mini-intein has been wildly used in commercial plasmids, as the IMPACT^{TM} system. However, when the continuous intein was used as the fusion tag, the in vivo cleavage between the target proteins and intein could happen and thus lower down the final yields. Recently, it has been found that the purified fragments of artificially split Ssp DnaB mini-intein, which are expressed separately in *Escherichia coli*, are active in protein trans-splicing under native conditions [3]. The split DnaB mini-intein has 106 amino acids in its N-terminal domain (DnaB^{N}) and 48 amino acids in the C-terminal domain (DnaB^{C}) [8,24]. Its self-cleaving activity at the N-terminus or C-terminus is independent and requires for distinct amino acid residues. The mutation at Cys1 to Ala disables the cleavage at N-terminus but not C-terminus, while the mutation at the C-terminal Asn154 to Ala does oppositely [25].

We have developed a novel approach for tag affinity purification of recombinant proteins with controllable tag removal by inducible auto-cleavage (Fig. 1A), using DnaB^{N} and DnaB^{C} as the affinity ligand and tag based on their affinity and trans-cleavage activity. This method can solve the problem of unwanted in vivo auto-cleavage when the continuous intein was used as the fusion tag. The controllable tag removal and purification of the target protein could

Abbreviations: DnaB^{N}, N-terminal domain of Ssp DnaB intein; DnaB^{C}, C-terminal domain of Ssp DnaB intein; CBD, chitin binding domain; Trx, thioredoxin; MBP, maltose binding protein; ETI, Erythrina trypsin inhibitor; BNP, brain natriuretic peptide.

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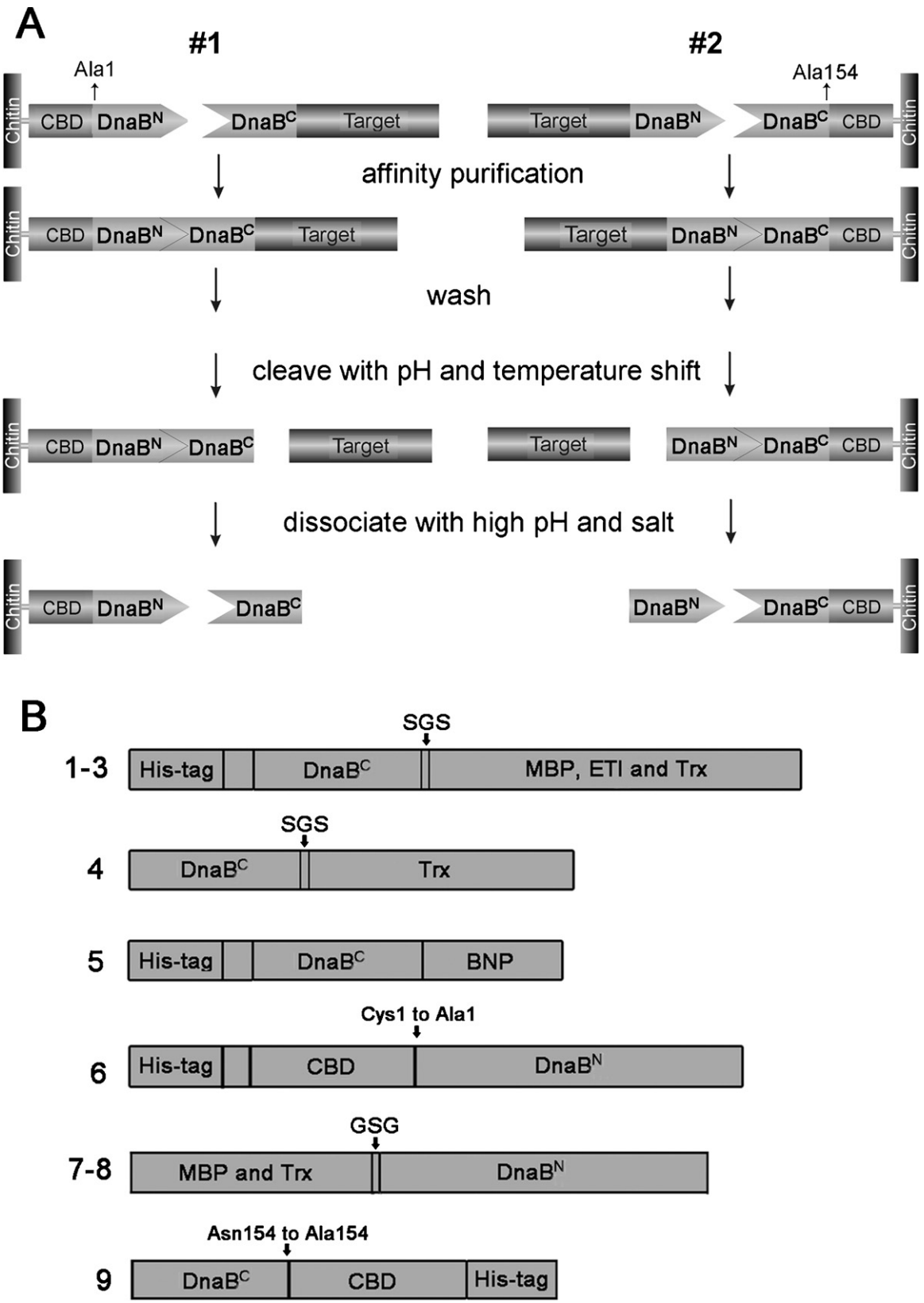

Fig. 1. (A) Schematic diagram of the affinity purification and inducible auto-cleavage system. Strategy # 1: the $DnaB^C$ was used as the affinity tag fused with the target protein. Ala1-$DnaB^N$ was immobilized on chitin matrix as the affinity ligand. Strategy # 2: the $DnaB^N$ was used as the affinity tag fused with the target protein. Ala154-$DnaB^C$ was immobilized on chitin matrix as the affinity ligand. (B) Plasmids construction. In Strategy # 1, the $DnaB^C$ domain expressed in construct 1–5 was used as the affinity tag, and Ala1-$DnaB^N$ produced by construct 6 was used as the affinity ligand. Construct 1–3 contained an N-terminal his-tag and a linker sequence (SGS) incorporated between $DnaB^C$ and the target protein. Construct 4 lacking his-tag sequence was employed to evaluate the influence of his-tag on purification and cleavage. In construct 5, the sequence of BNP was inserted immediately after $DnaB^C$. Ala1-$DnaB^N$ was immobilized onto the chitin beads via chitin-binding domain (CBD), and was utilized as the affinity matrix to purify the $DnaB^C$ fusion protein. In Strategy # 2, $DnaB^N$ domain expressed in construct 7–8 was used as the affinity tag, while Ala154-$DnaB^C$ produced by construct 9 was used as the affinity ligand. In construct 7–8, the target proteins were fused at the N-terminus of $DnaB^N$ and a linker sequence (GSG) was incorporated between the target protein and $DnaB^N$. Ala154-$DnaB^C$ was immobilized on the chitin beads and utilized as the affinity matrix to purify the $DnaB^N$ fusion protein.

be combined into one step. This article also provided a new affinity purification method that can be a supplement of the existing methods.

There were two different approaches to carry out our work, as shown in Fig. 1A. Strategy # 1 (Fig. 1A) was using DnaB^{C} as the affinity tag to fuse with target proteins (Fig. 1B, 1–5), while the N-terminus cleavage disabled Ala1-DnaB^{N} (Fig. 1B, 6) was used as the affinity ligand immobilized on chitin-resin. Conversely, DnaB^{N} (Fig. 1B, 7–8) and Ala154-DnaB^{C} (Fig. 1B, 9) switched their roles in Strategy # 2 (Fig. 1A). After the fusion protein was purified on the affinity matrix, the target protein was released via trans-cleavage, which was induced with the changes in pH and temperature. The split intein affinity matrix was then recovered by extensively washing with the elution buffer.

According to our results, Strategy # 1 seems to be better than Strategy # 2 for the following reasons. First, when used as the fusion tag, DnaB^{C} only contains 48 amino acids, while DnaB^{N} has 154 amino acids. The smaller size of DnaB^{C} results in a higher stoichiometric ratio of target proteins in fusion proteins, and thereby generally increases the production yields of target proteins [26]. Second, DnaB^{C} has a strong hydrophilicity which may help to improve the solubility of DnaB^{C} fusion protein when expressed in *E. coli*. Therefore, our research has been focused more on Strategy # 1. In this study, we selected four different model proteins with different molecular weights to verify the effectiveness of the system introduced in this article. These four model proteins include maltose binding protein (MBP), *Erythrina* trypsin inhibitor (ETI), thioredoxin (Trx) and brain natriuretic peptide (BNP).

2. Experimental

2.1. Construction of the plasmids

When DnaB^{C} was used as the affinity tag and Ala1-DnaB^{N} was used as the affinity ligand, this system was referred as Strategy # 1. Otherwise, the DnaB^{N} was used as the affinity tag and Ala154-DnaB^{C} was used as the affinity ligand, the system was referred as Strategy # 2. We constructed 9 plasmids, named construct 1 to construct 9 (Fig. 1B). The construct 1–6 were used in Strategy # 1 and the construct 7–9 were used in Strategy # 2. All the constructs were inserted into the expression plasmids, pET30a or pET28a. The sequences of these constructs were all confirmed by DNA sequencing.

In Strategy # 1, four different target proteins with different molecular weights, including MBP, ETI, Trx and BNP, were genetically engineered to the C-terminus of DnaB^{C} to generate the target fusion proteins. The expression product of construct 6 was used as the affinity ligand. Since the C-terminal cleavage activity of Ssp DnaB intein is affected by the extein sequence and its first native extein residue is Ser, to ensure the cleavage activity, three amino acids "SGS" were incorporated between the target proteins and DnaB^{C} domain. We have previously shown that putting BNP directly to the C-terminus of Ssp DnaB min-intein could generate high cleavage efficiency and in this case the first three extein residues were "SPK" [27]. Therefore, "SGS" was inserted in construct 1–4 but not in construct 5, and the final products of MBP, ETI and Trx contained additional "SGS" at the N-terminus, while BNP was in its native amino acid sequence. To demonstrate the influence of the N-terminal his-tag on the system, the construct 4 that had no his-tag at its N-terminus was generated as a control.

In Strategy # 2, Trx and MBP were used as the target proteins. Since the first N-terminal extein residue is Gly, to ensure the cleavage, three amino acids "GSG" were inserted between the target protein and DnaB^{N} domain. The expression product of construct 9 was used as the affinity ligand.

2.2. Expression of the recombinant proteins

After the *E. coli* BL21(DE3) was transformed with the expression plasmid, a single-colony transformant was inoculated into the LB medium containing 50 μg/ml kanamycin and grown overnight at 37 °C. The culture was transferred to the fresh LB medium supplemented with kanamycin and allowed to grow at 37 °C until the optical density (OD600) reached about 0.8. IPTG was then added to a final concentration of 0.5 mM to induce the expression, and the cultivation was continued at 30 °C for 6 h. The cells were harvested by centrifugation at 5000 × *g* for 10 min.

2.3. Preparation of the affinity matrix

After the expression of construct 6, the recombinant protein Ala1-DnaB^{N} was purified with Ni-Sepharose. It was then loaded and immobilized onto the chitin column. The CBD tag was used for immobilization because the interaction between CBD tag and chitin is very strong and stable over a wide range of pH conditions and salt concentrations. The column was thoroughly washed with the wash buffer (20 mM Tris–HCl, 1 M NaCl, 5 mM EDTA, pH 8.0) and stored in PBS with 0.02% NaN_3. This was served as the affinity column for Strategy # 1. The preparation of the affinity column for Strategy # 2 was same as Strategy # 1, only replacing Ala1-DnaB^{N} with Ala154-DnaB^{C}, which was the expression product of construct 9.

2.4. Measuring the interaction between DnaB^{N} and DnaB^{C} by surface plasmon resonance

DnaB^{C} was immobilized on Biacore CM5 chip according to the manufacturer's instructions. The kinetic assay for Ala1-DnaB^{N} binding to immobilized DnaB^{C} in running buffer (10 mM HEPES, 0.15 M NaCl, 3 mM EDTA, pH 7.4) was performed on Biacore 2000.

2.5. Detecting the complex formation between DnaB^{N} and DnaB^{C} by native polyacrylamide gel electrophoresis

Ala1-DnaB^{N} (construct 6) was incubated with Ala154-DnaB^{C} (construct 9) in phosphate buffer (50 mM phosphate, 0.2 M NaCl, 5 mM EDTA, pH 6.5) for 30 min at room temperature. Ala1-DnaB^{N} or Ala154-DnaB^{C} alone was incubated in the same buffer and used as a control. The samples were then analyzed by native polyacrylamide gel electrophoresis.

2.6. Purification of the DnaB^{C} fusion proteins by the affinity matrix of Strategy # 1

DnaB^{C} fusion proteins were produced in *E. coli* transformed with construct 1–5. The fusionproteins of Trx and MBP were soluble. While the fusion proteins of ETI and BNP, which tend to form inclusion bodies when fused with other partners, were expressed mainly as inclusion bodies. For DnaB^{C}-MBP (construct 1) and DnaB^{C}-Trx (construct 3 and 4) purification, bacterial pellets were lysed by sonication in the binding buffer (50 mM NaAc, 0.5 M NaCl, pH 5.0) at 4 °C and the supernatants were recovered by centrifugation. The supernatants were then applied onto the affinity column of Strategy # 1, and DnaB^{C} fusion proteins were purified after the column was extensively washed with the binding buffer. If DnaB^{C}, the affinity tag, was not required to be removed, the fusion proteins were eluted directly with the elution buffer (0.6 M NaCl, 50 mM Tris–HCl, pH 9.0). The affinity matrix was regenerated by thoroughly washing with the elution buffer.

DnaB^{C}-ETI (construct 2) or DnaB^{C}-BNP (construct 5) inclusion bodies were dissolved in denaturing buffer (6 M guanidine hydrochloride, 5 mM EDTA, 50 mM DTT, 50 mM Tris–HCl, pH 8.5),

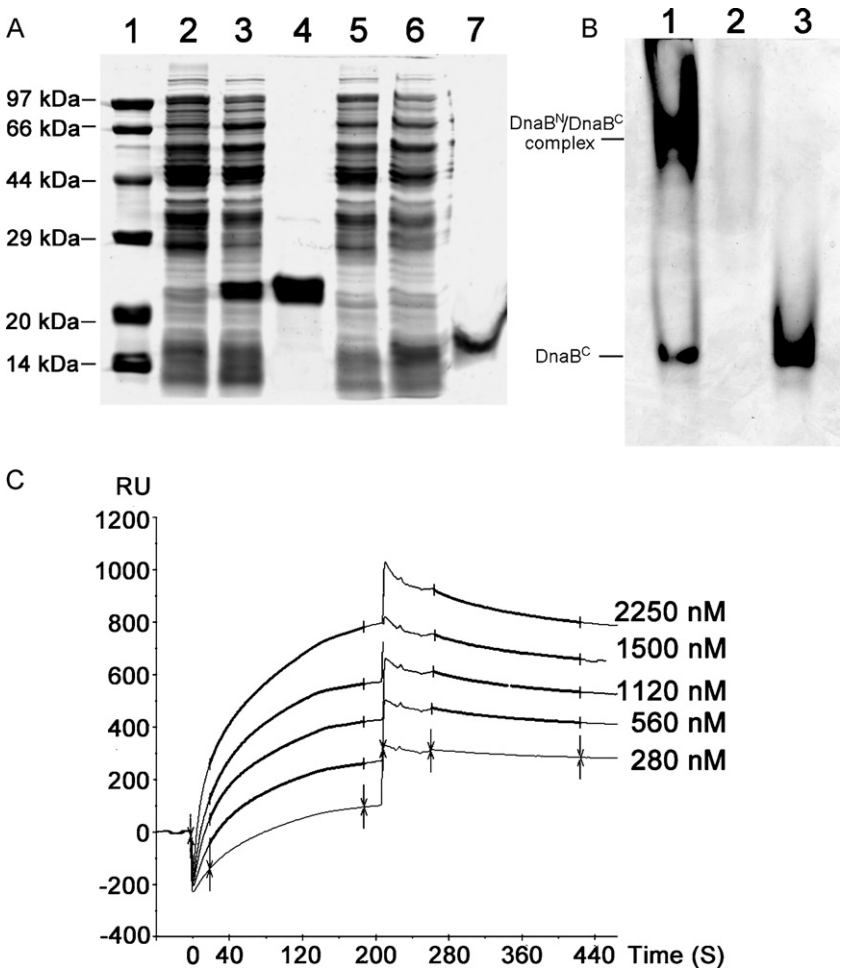

Fig. 2. (A) Expression and purification of Ala1-DnaB^N and Ala154-DnaB^C. Lane 1: molecular weight marker; lane 2: non-induced bacterial lysate of construct 6 (Ala1-DnaB^N); lane 3: IPTG induced bacterial lysate of construct 6; lane 4: purified Ala1-DnaB^N; lane 5: non-induced bacterial lysate of construct 9 (Ala154-DnaB^C); lane 6: IPTG induced bacterial lysate of construct 9; and lane 7: purified Ala154-DnaB^C. (B) Detecting the complex formation between DnaB^N and DnaB^C by native polyacrylamide gel electrophoresis (Tris–Glycine). Lane 1: DnaB^N and DnaB^C mixture; lane 2: DnaB^N control; lane 3: DnaB^C control. (C) Measuring the interaction between DnaB^N and DnaB^C by surface plasmon resonance.

and the fusion protein was then re-natured by diluting with refolding buffer (2.5 M urea, 2 mM EDTA, 50 mM Tris–HCl, pH 8.8) and incubated overnight at 10 °C. After concentrated by ultrafiltration and dialyzed against the binding buffer (50 mM NaAc, 0.5 M NaCl, pH 5.0), the refolding sample was purified with affinity column using the same procedure as that for DnaB^C-MBP and DnaB^C-Trx. All the collected samples were analyzed by SDS–PAGE.

2.7. The induced cleavage of DnaB^C fusion proteins and further purification by the affinity matrix of Strategy # 1

After the cell lysate (DnaB^C-MBP and DnaB^C-Trx) or refolding sample (DnaB^C-ETI and DnaB^C-BNP) was loaded onto the affinity column, the column was extensively washed with binding buffer. Then the affinity matrix carrying fusion protein was flushed with the cleavage buffer (0.3 M L-arginine, 5 mM EDTA, 50 mM phosphate buffer, pH 6.5) for 3 bed volumes. The cleavage was completed by incubating the column at room temperature for 20 h. The column was then washed with cleavage buffer, and the target protein was recovered in the pass-through fraction. After extensively dialyzed against 5 mM NH_4HCO_3, the target protein was lyophilized. The purified target proteins were then characterized with mass spectrometry, reverse phase HPLC and biological activity assay.

The affinity tag (DnaB^C fragment) released from the fusion protein was bound to the affinity matrix and could be removed from the affinity column by washing with the elution buffer (0.6 M NaCl, 50 mM Tris–HCl, pH 9.0). The efficiency of on-column cleavage was evaluated by SDS–PAGE.

2.8. The controllable cleavage

To minimize the premature cleavage during sample loading and column washing, we investigated the effects of pH and temperature on the cleavage of DnaB^C fusion protein. Using DnaB^C-MBP as the model, on-column cleavage of the fusion protein was studied under pH 5–7 at 4 °C or room temperature.

2.9. The biological activity assays for ETI and BNP

The activity of BNP was analyzed by the relaxing effect to the isolated rabbit aortic strips in vitro [28]. Rabbit aortic strips, which were approximately 3.0 cm long, were prepared and suspended in an organ bath containing 20 ml Krebs solution (37 °C, pH 7.4). The solution was continuously gassed with air. Contraction of the strip was recorded with a 1.0 g load on the tissues. After an equilibration period of 1 h, stable baseline tone was reached. Precontractions were elicited by adding norepinephrine hydrotartrate to the organ bath. When a stable contraction plateau was reached, the peptide

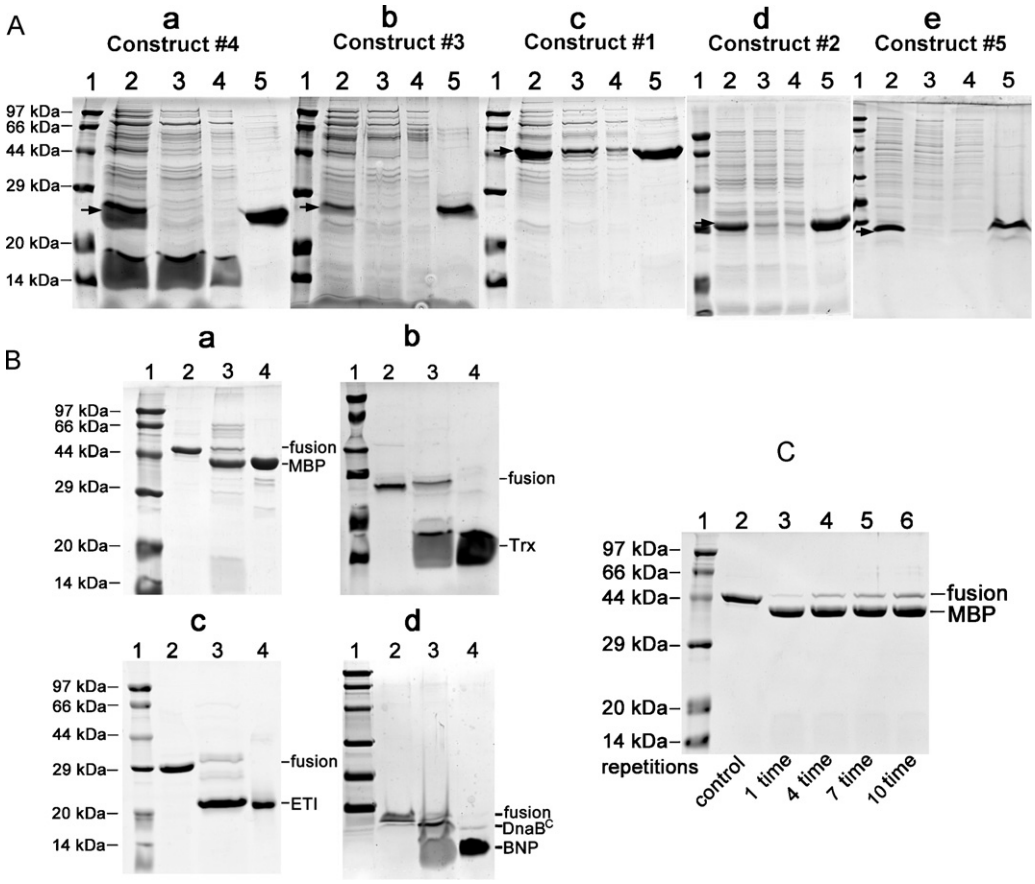

Fig. 3. (A) Purification of DnaB^C fusion proteins by affinity column immobilized with Ala1-DnaB^N. (a) Without N-terminal His-tag; (b–e) all contain N-terminal his-tag. Lane 1: marker; lane 2: samples before purification; lane 3: fraction of pass-through; lane 4: the fraction of column wash; and lane 5: the fraction of elution (0.6 M NaCl, 50 mM Tris–HCl, pH 9.0). (a) DnaB^C-Trx fusion protein. (b) DnaB^C-Trx fusion protein. (c) DnaB^C-MBP fusion protein. (d) DnaB^C-ETI fusion protein. (e) DnaB^C-BNP fusion protein. (B) Induced cleavage of DnaB^C fusion protein and purification of target protein on Ala1-DnaB^N affinity column. Lane 1: marker; lane 2: fusion protein control; lane 3: sample after on-column cleavage; lane 4: purified target protein. (a) Trx, (b) MBP, (c) ETI, and (d) BNP. (C) Reusability of the Ala1-DnaB^N affinity matrix. The induced cleavage procedure was performed repeatedly for 10 times using DnaB^C-MBP fusion protein as the model. Each time the affinity matrix was regenerated by extensive washing with the elution buffer. The cleavage efficiency of each run was determined by SDS–PAGE.

samples were added to the bath with various concentrations. The relaxing effects were measured and the EC_{50} results were calculated.

The biological activity of ETI was analyzed by its inhibition against trypsin [29]. The ETI purified from *Erythrina caffra* seeds was used as the standard control. An appropriate amount of trypsin was mixed with increasing amounts of ETI samples. Then the residual enzyme activities were assayed by the amidolytic release of p-nitroanilide from the chromogenic substrates. The mixture was incubated in 50 mM Tris–HCl, pH 8.0, 0.1 M NaCl, 10 mM $CaCl_2$, 0.005% Triton-X 100 for 5 min at 25 °C. Assay was started by the addition of substrate and the amidolytic activity was calculated from the increase in absorbance at 410 nm. The inhibitory activity (Ki) was calculated and compared with the control.

2.10. Strategy # 2: purification using N-terminal cleavage promoted by a modified DnaB split intein

DnaB^N-MBP and DnaB^N-Trx were produced in *E. coli* transformed with construct 7 and 8, respectively. DnaB^N-Trx was expressed as inclusion body and we did not get enough soluble protein after refolding. The DnaB^N-MBP fusion protein was selected as the model for the feasibility study of Strategy # 2, which uses the same procedures as Strategy # 1.

3. Results

3.1. Detecting the complex formation between DnaB^N and DnaB^C

DnaB^N and DnaB^C proteins were expressed and purified from *E. coli* (Fig. 2A). Based on surface plasmon resonance data, the resultant complex between DnaB^N and DnaB^C was stable as illustrated by the slow dissociation rate (Fig. 2C). Five concentrations of DnaB^N were selected and the Kd was determined to be 59 ± 8 nM by the Bio-evolution software.

As shown in the native polyacrylamide gel electrophoresis (Fig. 2B), there was no band for DnaB^N control on the native gel (Fig. 2B, lane 2), which was due to its net positive charge at pH 8.8 (its isoelectric point is pH 9.7). DnaB^N could be shown as a single band in the native gel at pH > 10 (data not shown). Compared with DnaB^C alone (Fig. 2B, lane 3), the mixture of DnaB^C and DnaB^N displayed an additional band with a much slower migration rate (Fig. 2B, lane 1), indicating that a complex was formed between DnaB^C and DnaB^N.

3.2. Purification of the DnaB^C fusion proteins by the affinity matrix of Strategy # 1

As the first step of the purification, the samples containing the DnaB^C fusion proteins (construct 1–5, Fig. 1B) were loaded onto the affinity matrix of Ala1-DnaB^N (construct 6, Fig. 1B) at pH 5.0 and

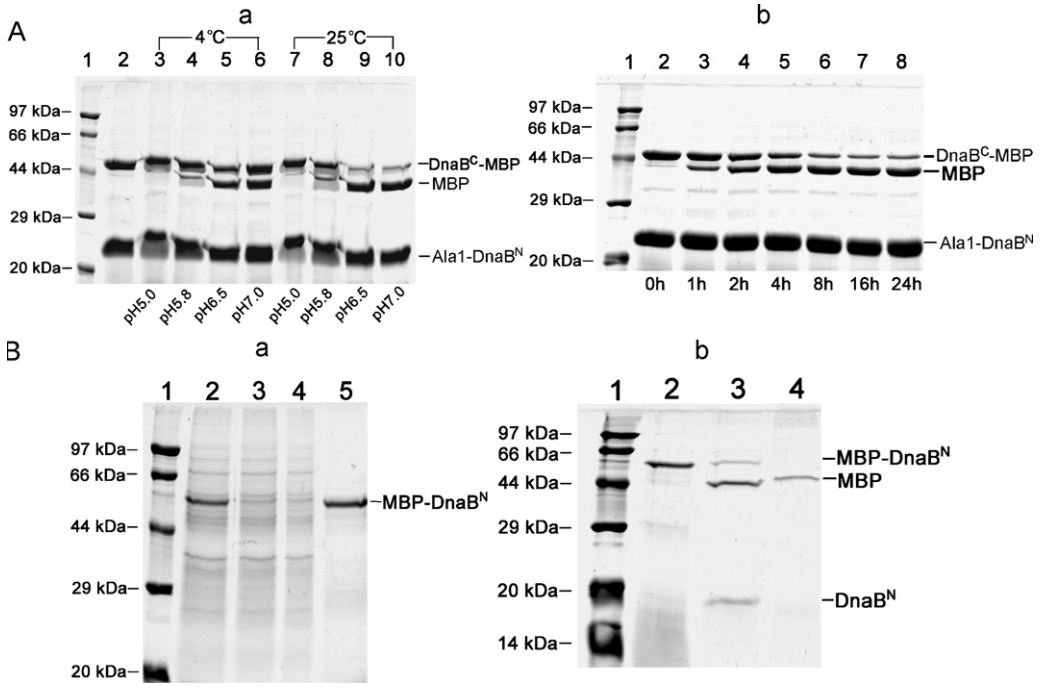

Fig. 4. (A) The controllable cleavage. (a) The effects of pH and temperature on the efficiency of on-column cleavage for 16 h. Lanes 3–6 and lanes 7–10 were cleaved at 4 °C and room temperature, respectively. Lane 1: marker; lane 2: control (before cleavage); lanes 3 and 7: pH 5.0; lanes 4 and 8: pH 5.8; lanes 5 and 9: pH 6.5; lanes 6 and 10: pH 7.0. (b) The efficiency of DnaB^C-MBP cleavage on N-chitin column after incubation at pH 6.5 and room temperature for different time. Lane 1: marker; lane 2: 0 h; lane 3: 1 h; lane 4: 2 h; lane 5: 4 h; lane 6: 8 h; lane 7: 16 h; lane 8: 24 h. (B) Strategy # 2: purification using N-terminal cleavage promoted by a modified DnaB split intein. (a) Purification of the DnaB^N-MBP fusion protein on C-chitin column of Strategy # 2. Lane 1: marker; lane 2: total bacterial lysate; lane 3: the fraction of pass through; lane 4: the fraction of column wash; lane 5: the fraction of elution. (b) Induced cleavage of DnaB^N-MBP fusion protein on C-chitin column. Lane 1: marker; lane 2: DnaB^N-MBP control; lane 3: on-column cleavage product; lane 4: pass through fraction.

4 °C. Since the complex of Ala1-DnaB^N and DnaB^C was stable at pH 4.0–7.5 in the presence of 1 M NaCl (data not shown), the contaminant proteins could be removed from the column by washing with a buffer containing 0.5 M NaCl at pH 5.0.

As the second step of the purification, in case there was no need to remove the fusion tag DnaB^C, the fusion protein could be eluted directly. The elution was done by raising pH to 9.0 with 0.6 M NaCl. The target fusion proteins were highly purified (Fig. 3A), suggesting that the binding between Ala1-DnaB^N and DnaB^C was specific.

Since his-tag is specific and widely used for protein purification, we investigated the combined use of his-tag with DnaB^C by inserting his-tag into the N-terminus of the DnaB^C domain (Fig. 1B, construct 1–3, and 5). The insertion of his-tag had no effect on both purification and cleavage (compare Fig. 3Aa and Ab). Therefore, our DnaB^C affinity purification method is compatible with his-tag affinity technique.

3.3. The induced cleavage of DnaB^C fusion proteins and further purification by the affinity matrix of Strategy # 1

As the third step of the purification, when the fusion tag was to be removed from the target protein, the cleavage at the fusion protein could be induced by increasing pH to 6.5–7.0 and incubating overnight at 4 °C or room temperature. The target protein was released from the affinity matrix and recovered from the pass-through fraction (Fig. 3B), while the affinity tag was still binding with the column and could be removed by washing with elution buffer.

Importantly, the affinity matrix of our system had good reusability. After 10 times of repetitive use, the affinity matrix retained high activity to perform the C-terminal cleavage at fusion proteins (Fig. 3C). The cleavage efficiency was determined by monitoring the eluted fraction after each cleavage. With the repetitive usage of the matrix, decreased efficacy of purification was observed, which probably was due to the denaturation of the immobilized ligand protein with elution buffer. Mutants of split intein with better stability and cleavage efficiency should be more useful in our purification system.

3.4. The controllable cleavage

It was found that on-column cleavage was enhanced with the increase of pH and temperature. The fusion protein was most effectively cleaved when the pH is greater than 6.5, while very little cleavage occurred at pH 5.0 (Fig. 4Aa). These data suggested that low pH and temperature could be applied to prevent unwanted auto-cleavage. It is important to keep the pH of washing buffer below 5.8, in order to avoid premature cleavage of the fusion protein on the affinity column. The on-column cleavage of the fusion protein reached a plateau after incubation under pH 6.5 for 16 h at room temperature (Fig. 4Ab).

3.5. Characterization of the recombinant target proteins

All the target proteins, MBP, ETI, Trx and BNP, were successfully produced and purified (Fig. 3A and B). Their molecular weights were determined by mass spectrometry (Fig. 5A), which were consistent with their theoretical values (40685.20, 19464.76, 12971.76 and 3466.08), indicating that the fusion proteins were specifically cleaved between intein Asn154 and extein1 by the C-terminal cleavage. Using the analytic C18 HPLC, BNP was found to have the same retention time as the synthetic standard BNP (Fig. 5B). The biological activity of BNP was measured by detecting its vasodilation effect on rabbit aortic strips pre-contracted with norepinephrine [28]. It was found that recombinant BNP vaso-relaxed aortic strips with an EC_{50} of $(1.55 \pm 0.42) \times 10^{-6}$ mg/ml, which was similar to $(1.67 \pm 0.48) \times 10^{-6}$ mg/ml of the BNP stan-

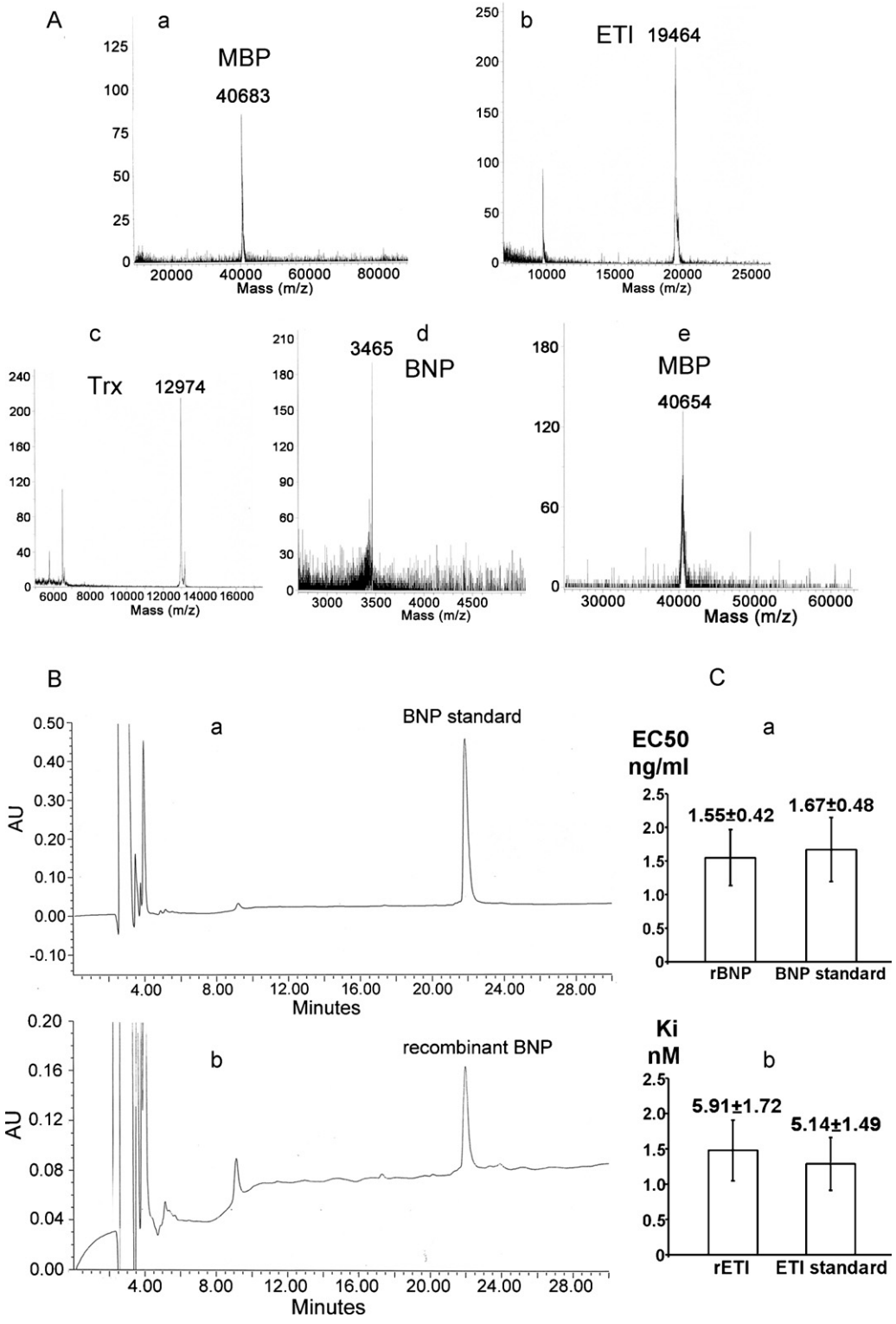

Fig. 5. (A) Mass spectroscopy spectra of purified target proteins. (a) MBP (expected mass 40685.20), (b) ETI (expected mass 19464.76), (c) Trx (expected mass 12971.76), (d) BNP (expected mass 3466.08), and (e) MBP produced by Strategy # 2 (expected mass 40655.17). (B) The retention time of BNP samples on C18 reverse phase HPLC. (a) The synthetic BNP standard, and (b) purified recombinant BNP. (C) The biological activities of purified proteins. (a) The vasodilation activities (EC50) of purified recombinant BNP (rBNP) and BNP standard, and (b) The inhibitory activities (Ki) of purified recombinant ETI (rETI) and ETI standard to trypsin.

dard, indicating that recombinant BNP prepared by the current system was identical to the native BNP (Fig. 5C). The activity assay of recombinant ETI was performed based on its inhibition against trypsin [29]. The inhibitive constant (Ki) of recombinant ETI was (5.91 ± 1.72 nM), which was similar to (5.14 ± 1.49 nM) of ETI purified from *E. caffra* seeds (Fig. 5C).

3.6. Strategy # 2: purification using N-terminal cleavage promoted by a modified DnaB split intein

DnaB^{N}-MBP fusion protein in the supernatant of the bacterial lysate was purified with affinity matrix of Ala154-DnaB^{C} (construct 9, Fig. 1B), and induced cleavage of the fusion protein was achieved

after flushing the column with cleavage buffer (0.3 M L-arginine, 5 mM EDTA, 50 mM phosphate buffer, pH 6.5) and incubated at room temperature for 24 h (Fig. 4B). The molecular weight of purified MBP was determined by mass spectrometer (Fig. 5Ae), which was consistent with its theoretical value (40,655.17), indicating the specificity of the on-column cleavage of the fusion protein.

4. Discussion

To the best of our knowledge, our method using the split intein for protein preparation has not been reported. Utilizing immobilized C-terminal domain of Ssp DnaE split-intein, Chen et al. demonstrated the interaction between intein fragments [12], however their work did not refer to the protein preparation. Additionally, the pH of the buffer they applied to dissociate the N-terminal and C-terminal domain of the intein was 2.7, which was too harsh for many proteins. Other intein mediated protein production systems, such as the IMPACT^{TM} system, use the continuous intein as the fusion partner [17,30]. The unwanted auto-splicing occurs when the fusion protein is expressed in host cells [21]. In some cases, more than 90% of the fusion proteins are cleaved due to in vivo auto-cleavage, which leads to much low purification yield of the target proteins [31]. In contrast, as described in our method, the two domains of Ssp DnaB mini-intein were artificially split, stably expressed and separately purified. The cleavage activity was regained only after two intein fragments were mixed together [21]. However, the intein fragment is unable to increase the solubility of target protein which contains disulfide bonds and is easy to form inclusion body by itself, such as ETI and BNP. In order to prevent the premature cleavage, the purification was performed at pH below 5.8. For the proteins not stable under this pH, other split inteins with different splicing conditions could be chosen. Our method should be especially useful for the soluble proteins and peptides that are stable at a pH range of 5–9.

Since the C-terminal cleavage of Ssp DnaB intein is efficiently conducted with various extein1 residues [26,27], it is suitable to be fused with the N-terminus of most target proteins. When the extein1 residues are unsuitable for cleavage, extra amino acids could be inserted between Asn154 and extein1 to preserve the cleavage efficiency. For example, since the native extein1 of Ssp DnaB intein is Ser, we chose to insert Ser–Gly–Ser between intein Asn154 and extein1 (Fig. 1B). Compared with a previous report that added a negative charged Asp residue at the C-terminus of target proteins [32], attachment of Ser–Gly–Ser to the N-terminus might be less likely to affect the functions of target proteins. Additionally, we have also successfully immobilized Ala154-DnaB^{C} domain as the ligand to purify the DnaB^{N} fusion protein (Figs. 1 and 4B).

In conclusion, we have developed a novel affinity purification and controllable automatic tag removal system, in which the affinity column can be used for the initial purification of fusion proteins or for a temperature and pH inducible on-column cleavage to release target proteins. It does not require for any extra enzymes or chemical reagents to remove affinity tags [33]. The system has solved the problem that intein fused proteins are auto-spliced in host cells. The new affinity matrix can be repeatedly used without significant loss of the activity. And our affinity purification method is compatible with his-tag affinity techniques. Moreover, Ssp DnaB mini-intein is capable of trans-splicing at multiple split sites [14,21]. With more split-inteins being discovered [34,35], our research may reveal insights to using other split sites or even other split-inteins for recombinant protein preparation.

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