



Protein purification involving a unique auto-cleavage feature of a repeated EAAAK peptide

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ABSTRACT

Protein purification generally requires many steps of column chromatography that typically involve ion-exchange, hydrophobic-interaction and gel-filtration separations. More sophisticated purification of protein might be achieved through an application of affinity binding on a functionalized gel such as a nickel column, glutathione-modified column, maltose-modified gel column or others. Of several drawbacks existing in these methods, fusion proteins are commonly obtained, protease digestion might be necessary to remove the fusion moiety; a costly gel is employed for affinity binding, etc. Here we report that an expression vector derived from pREST was constructed to compose the gene of the chitin-binding protein (CBP) and the nucleotide sequence of the (EAAAK)₅ peptide linker following restriction sites for target gene insertion. Fusion proteins were expressed with *E. coli* and purified with a chitin column. The (EAAAK)₅ linker is shown to possess a pH-dependent auto-cleavage feature. In the range pH 6–7, the target protein becomes automatically released from the fusion protein without proteolytic treatment. Although the mechanism of this auto-cleavage property of an (EAAAK)₅ linker is unclear, this feature has been successfully employed for many cases of protein purification without the tag of a fusion protein.

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1. Introduction

An indispensable tool for the purification of a recombinant protein is an affinity tag, with which a target protein is obtainable from a crude cell extract via a one-step process [1]. The specificity of a target protein purification and the simplicity of its chromatographic manipulation are two attractive factors, but common drawbacks of many affinity purification systems are the costly affinity matrix and the post-proteolytic process.

Because of demands of research and industrial applications, several commercial kits have been developed using fusion proteins as tags for affinity purification, such as glutathione S-transferases (GST) (Novagen GST Bind kits), histidine tagging (Novagen His Bind Resin and Purification kit), a maltose-binding protein (pMAL system, New England BioLabs) and a chitin-binding domain (IMPACT system, New England BioLabs). The glutathione S-transferases (GST) are used to label proteins for expression and purification applications. GST-Mag agarose beads serve to bind the GST fusion protein, and gentle elution with reduced glutathione buffer. Enterokinase suffices to cleave GST and the target protein [2]. His-tag can be used in affinity chromatography with a column that has nickel bound to it; histidine is an effective ligand for nickel

so that the fusion protein with His-tag binds to the nickel column, and the protein can be eluted with a solution of imidazole. In Novagen products, the Ni-NTA His-Bind Resin is used to purify the His-tag fusion protein. To cleave the fusion protein, most Novagen vectors containing the His-Tag sequence on the N-terminus also encode protease cleavage sites such as that for thrombin, Factor Xa, or enterokinase [3,4]. Maltose-binding protein (MBP) has a high affinity for binding to maltose. In the pMAL system (New England BioLabs), MBP has been used as an N-terminal fusion tag to purify over-expressed proteins with affinity chromatography based on amylose resins [5,6]. Factor Xa protease is used to cleave MBP and a target protein. The expression system with chitin-binding domain fusion protein enables efficient coupling to chitin-agarose affinity resins and purification with single-step chromatography [7]. In the IMPACT system (New England BioLabs), the chitin beads serve to purify a fusion protein; IMPACT utilizes the inducible self-cleavage activity of inteins for its ability to separate a recombinant protein from the affinity tag without the use of a protease. Nevertheless, a chitin bead is still a costly material for purification.

Our objective in this work was to develop a system for affinity purification with a cheap solid support and also possibly to eliminate the treatment of protease to retain the target protein. As chitin, a linear polymer of β -(1–4)-linked *N*-acetylglucosamine, is an abundant natural material produced in the biosphere [8] and exhibits substantial mechanical and chemical strength, it is a perfect candidate for our purpose. Three crystalline forms of chitin

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have been described in terms of the arrangement of the chitin chains: α -chitin in which chains run anti-parallel, β -chitin in which chains run parallel, and γ -chitin with mixed parallel/anti-parallel chains. These natural polymers would provide an economical solid support for further protein purification if an appropriate binding apparatus were designed. In addition to a chitin-binding domain in chitinase, a chitin-binding protein (CBP) was found in the culture medium of *Serratia marcescens* when it was grown on chitin [9]. This protein belongs to the carbohydrate-binding module (CBM) family 33 and is known to bind to β -chitin [9,10]. Because the binding of CBP to chitin leads to non-hydrolytic disruption of the substrate structure [11], CBP can increase the substrate accessibility and strongly promote the hydrolysis of crystalline β -chitin by chitinase [12,13]. Previous work showed also that CBP can bind to β -chitin at a pH about 8.0 and be eluted in acidic conditions, pH < 7. This feature is prospectively useful for establishing a system for both protein purification and enzyme fixation.

The construction of a fusion protein involves the linkage of two proteins or domains of proteins with a peptide linker. The selection of the linker sequence is particularly important for the construction of functional fusion proteins. Rigid α -helix peptide linkers (EAAAK)_n ($n \leq 6$) are generally used for this purpose [14–18]. These linkers are expected to form a monomeric hydrophilic α -helix, presumably stabilized by Glu⁻–Lys⁺ salt bridges. In this work, we introduced a helix-forming peptide linker (EAAAK)₅ between CBP and a target protein to discriminate effectively the bifunctional domains of a fusion protein. Interestingly, an unexpected auto-cleavage property of the repeated EAAAK linker was discovered and investigated.

2. Material and methods

S. marcescens (ATCC 990) (Food Industry Research and Development Institute, Hsinchu, Taiwan), buffers (Sigma–Aldrich, St. Louis, MO, USA), *Vent* DNA polymerase (NEB) and *Pfu* DNA polymerase (Merck) were obtained from the indicated sources.

2.1. Cloning of chitin-binding protein

The *cbp* was PCR-amplified using the genomic DNA of *S. marcescens* as template. Primers 1 and 2 (all sequences are summarized in Table 1) were designed on the basis of the *cbp* of *S. marcescens* (accession number AAU88202) with NdeI and XhoI sites (underlined) in the sequences, respectively. PCR amplification was performed using *Vent* DNA polymerase (NEB) for 25 cycles with the conditions set for each cycle as 94 °C, 30 s for denaturation, 58 °C, 30 s for annealing, and 72 °C, 4 min for extension. The PCR fragment

was inserted into NdeI and XhoI sites of pRSET A to form the vector of pRSET/CBP.

2.2. Construction of the expression vector of CBP fusion protein

Several steps were involved in the construction of the expression vector of the CBP fusion protein with a linker and a genenase I recognition site. In the first step, the NdeI site in pRSET/CBP was removed by site-directed mutagenesis using primers 3 and 4 for PCR amplification; the resulting vector is designated de-NdeI-pRSET/CBP. In the second step, a genenase I cutting site and a NdeI cutting site were inserted right after the CBP gene, with which the stop codon in *cbp* was deleted simultaneously; primers 5 and 6 were designed for this purpose. Both primers were phosphorylated with T4 polynucleotide kinase before use for PCR amplification. The amplified fragments were subject to self-ligation to form pRSET/CBP-G. Various peptide linkers can be introduced in the third step. For the case of adding a (EAAAK)₅ linker in pRSET/CBP-G, primers 7 and 8 were employed for backward PCR amplification. Similar to the second step, both primers were phosphorylated in advance so that the amplified DNA fragments were subject to self-ligation to give pRSET/CBP-V5G. Vectors with distinct repeated EAAAK linkers, such as (EAAAK)₂, (EAAAK)₃ and (EAAAK)₄, were constructed with a similar strategy using primers 9 and 10, primers 9 and 11, primers 9 and 12, respectively. A vector without the genenase I proteolytic site was also constructed using primers 13 and 14; the resulting vector was named pRSET/CBP-V5. Primers 15 and 16 were employed for vector construction without CBP. For functional evaluation of this system, the gene of laminaripentaose-producing- β -1,3-glucanase (LPHase) from *Streptomyces matensis* was inserted into NdeI/EcoRI sites; the gene of chitinase from *Bacillus cereus* [19] was inserted into NdeI/NcoI sites, and the gene of chitosanase (CNS) from *Aspergillus fumigatus* [20] was inserted into NdeI/HindIII sites of pRSET/CBP-V5G vector, separately, and expressed in *E. coli* BL21 (DE3). The three fusion proteins CBP-V5G-LPHase, CBP-V5G-chitinase and CBP-V5G-CNS were further purified on a β -chitin column (described in a following section).

2.3. β -Chitin matrix preparation

Squid pens (cartilages) were used to prepare a β -chitin matrix and column. Cartilages (50 g) were soaked in NaOH (3%, 1l) and heated at 100 °C for 3 h. The cartilages were then thoroughly washed with water to eliminate NaOH. The cartilages were further suspended in HCl (6M, 1l) and kept near 25 °C for 12 h. After washing with distilled water several times to increase the pH > 6, the cartilages were homogenized and sieved to diameter

Table 1
Sequences of primers.

Primer	Sequence	Usage
1	5'-GGAATTCATATGAACAAAACCTCCCGTACC-3'	CBP (+)
2	5'-CCGCTCGAGCTCTTATTGCTCAGGTTGAC-3'	CBP (-)
3	5'-GGAGATATAGGGATGAACAAA AC-3'	CBP-de-NdeI (+)
4	5'-GTTTGTTCATCCCTATATCTCC-3'	CBP-de-NdeI (-)
5	5'-CCGGTGC GG CACTACCATATGGAGCTCGAGATCTGCAGTGGTAC-3'	Genenase I site (+)
6	5'-GATCAACACGTTTAGCTTTGCTCAGGTTGACGTCGATC-3'	Genenase I site (-)
7	5'-CTGCTAAGAAGCTGCTGCTAAAGAAGCTGCTGCTAAACCGGGTGC GG CACTAC-3'	Linker V5 (+)
8	5'-CAGCTCTTTAGCAGCAGCTTCTTTAGCAGCAGCTTCTTTGCTCAGGTTGACGTC-3'	Linker V5 (-)
9	5'-GAAGCTGCTGCTAAAGAAGCTGCTGCTAAACCGGGTGC GG CACTACCATATG-3'	Linker V2-4 (+)
10	5'-TTTGCTCAGGTTGACGTCACATCGC-3'	Linker V2 (-)
11	5'-TTTAGCAGCAGCTTCTTTGCTCAGGTTGACGTC-3'	Linker V3 (-)
12	5'-TTTAGCAGCAGCTTCTTTAGCAGCAGCTTCTTTGCTCAGGTTGACGTC-3'	Linker V4 (-)
13	5'-GTAGTGTGCCGACCCCGCATATGTTTAGCAGCAGCTTC-3'	V5-de-genenase I site (-)
14	5'-GGAATTCATATG TACAATTTGCCAAAC-3'	V5-de-genenase I site (+)
15	5'-GCGATCGACGTCAACCTGCTCGAGGAAGCTGCTGCT-3'	deCBP (+)
16	5'-CGATTCGACATACGAGAGCATTCGCTGTTGCGA-3'	deCBP (-)

0.3–0.6 mm of a β -chitin matrix, corresponding to 30–50 mesh particle size. The β -chitin matrix was kept at 4 °C for further applications.

2.4. Cultural conditions and purification of CBP and fusion proteins

E. coli BL21 (DE3) served as the host strain for protein expression. A single colony bearing pRSET/CBP was inoculated into LB medium (5 ml) containing ampicillin (0.1 mg/ml) and cultured at 37 °C on a rotary shaker for 12 h. The overnight culture was then transferred into a conical flask (2 l) containing LB medium (1 l) with ampicillin (0.1 mg/ml) and IPTG at 37 °C for 15 h. The culture broth was centrifuged at 4 °C for 10 min at 7000 \times g. The cell pellet was resuspended in sodium phosphate buffer (10 ml, 20 mM, pH 7.0) and then subjected to cell disruption with ultrasonication. After removal of the cell debris with centrifugation, the supernatant (~10 ml) was mixed with β -chitin matrix (100 ml, Tris 50 mM, $(\text{NH}_4)_2\text{SO}_4$ 1 M, pH 8.0, 5% (v/w) 0.3–0.6 mm diameter of β -chitin) and incubated at 4 °C overnight. The resulting mixture was then packed onto a 2.5 cm \times 60 cm I.D. column and washed with 250 ml of the buffer solution (Tris 50 mM, $(\text{NH}_4)_2\text{SO}_4$ 1 M, pH 8.0) as a 10-fold volume of the solid chitin matrix to remove the non-binding protein. CBP was eluted from the β -chitin matrix using 50 ml of acetate buffer (20 mM, pH 3.6) with flow rate at 2 ml/min. Each fraction of 5 ml eluent was collected. All other CBP fusion proteins, including CBP-V5G-LPHase, CBP-V5G-chitinase and CBP-V5G-CNS, were purified based on the general protocol described above. The activity of the purified enzyme was assayed by using the corresponding polysaccharide as substrate (described in the following section) and the purity was analyzed by SDS-PAGE. The protein recovery yield and the purity enhancement were calculated based on the enzymatic activity and the amount of the protein retained. The recombinant chitinase fusion protein without CBP (i.e. V5G-CNS) was purified according to the literature [20].

2.5. Protein determination

The protein content of the enzyme preparation was determined by the BCA method as described in the manufacturer's protocol (BCA-1 kit for protein determination; Sigma–Aldrich, St. Louis, MO).

2.6. Enzyme activity assay

The enzymatic assay of LPHase, chitinase, and CNS were performed by using β -1,3-glucan, colloidal chitin, and chitosan as the substrate, respectively. The activities were analyzed by estimating the amount of the reducing ends of sugars using the dinitrosali-

cyclic acid method [21]. The standard assay was prepared by mixing 0.3 ml of polysaccharide substrate (1%) and 0.3 ml of enzyme (at a suitable dilution) and incubated for 4 h at 37 °C; 0.6 ml of dinitrosalicylic acid reagent was then added, and the resulting mixture was boiled for 15 min, chilled, and centrifuged to remove the precipitants. The resulting adducts of reducing sugars were analyzed and measured spectrophotometrically at 540 nm.

2.7. Electrospray mass-spectrometric (ESI-MS) analysis

Mass spectra were recorded with a quadrupole time-of-flight mass filter (Q-TOF, Micromass, UK). This analyzer was scanned over a range 500–3000 μm of mass-to-charge ratio (m/z) for protein analysis, with a scan step 2 s and an interscan 0.1 s/step.

3. Results and discussion

3.1. Cloning, expression and affinity-column purification of CBP from *S. marcescens*

Based on the gene (*cbp*) of *S. marcescens* chitin-binding protein [9–11], two oligonucleotides were designed and used as primers for PCR cloning. With the chromosomal DNA of *S. marcescens* as template, a DNA fragment (0.6 kb) was amplified, and the CBP was confirmed with DNA sequencing. Sequence analysis revealed that the amplified CBP gene contained an open reading frame of 594 bp encoding 197 amino-acid residues with the first 27 amino acids as a signal peptide. The full CBP gene was further subcloned into pRSET A. The resulting plasmid was transformed into the *E. coli* BL21 (DE3) strain for protein expression. The recombinant enzyme, expressed as a soluble form, was further purified to high homogeneity with a β -chitin matrix. To investigate the binding affinity of CBP toward chitin, the crude recombinant protein was mixed with suitable amount of chitin matrix and further loaded onto a column and extensively washed with NaCl (50 mM) before elution with various buffers under varied pH conditions (pH 4–8), that included NaOAc (50 mM, pH 4–5), phosphate (50 mM, pH 6–7) and Tris–HCl (50 mM, pH 7–8). The eluted samples were further analyzed with SDS-PAGE. The results showed that CBP was effectively eluted from the β -chitin column when the pH was less than 7. SDS-PAGE analysis revealed that the recombinant CBP was obtained with a high homogeneity (>90%) (Fig. 1). The estimated molecular mass is ~20 kDa. ESI-MS analysis gave a more precise measurement, a molecular mass 18,785 Da (Fig. 1), consistent with a theoretical calculation of the deduced protein without the signal peptide.

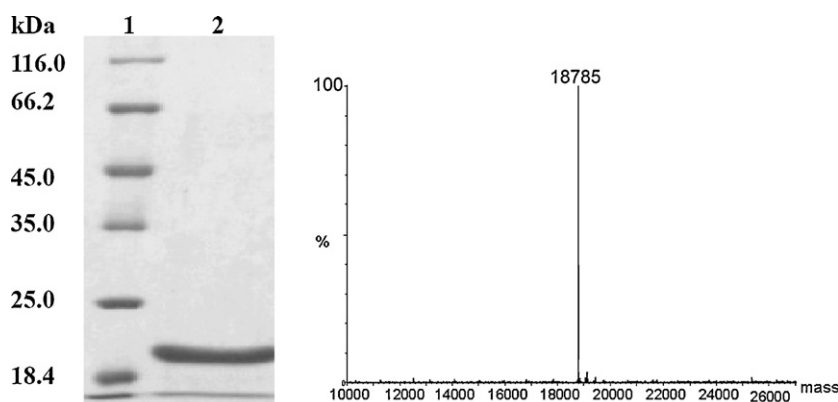


Fig. 1. SDS-PAGE and MS analysis of CBP. (A) Lane 1: protein marker; lane 2: purified CBP; (B) mass spectrum of recombinant CBP.

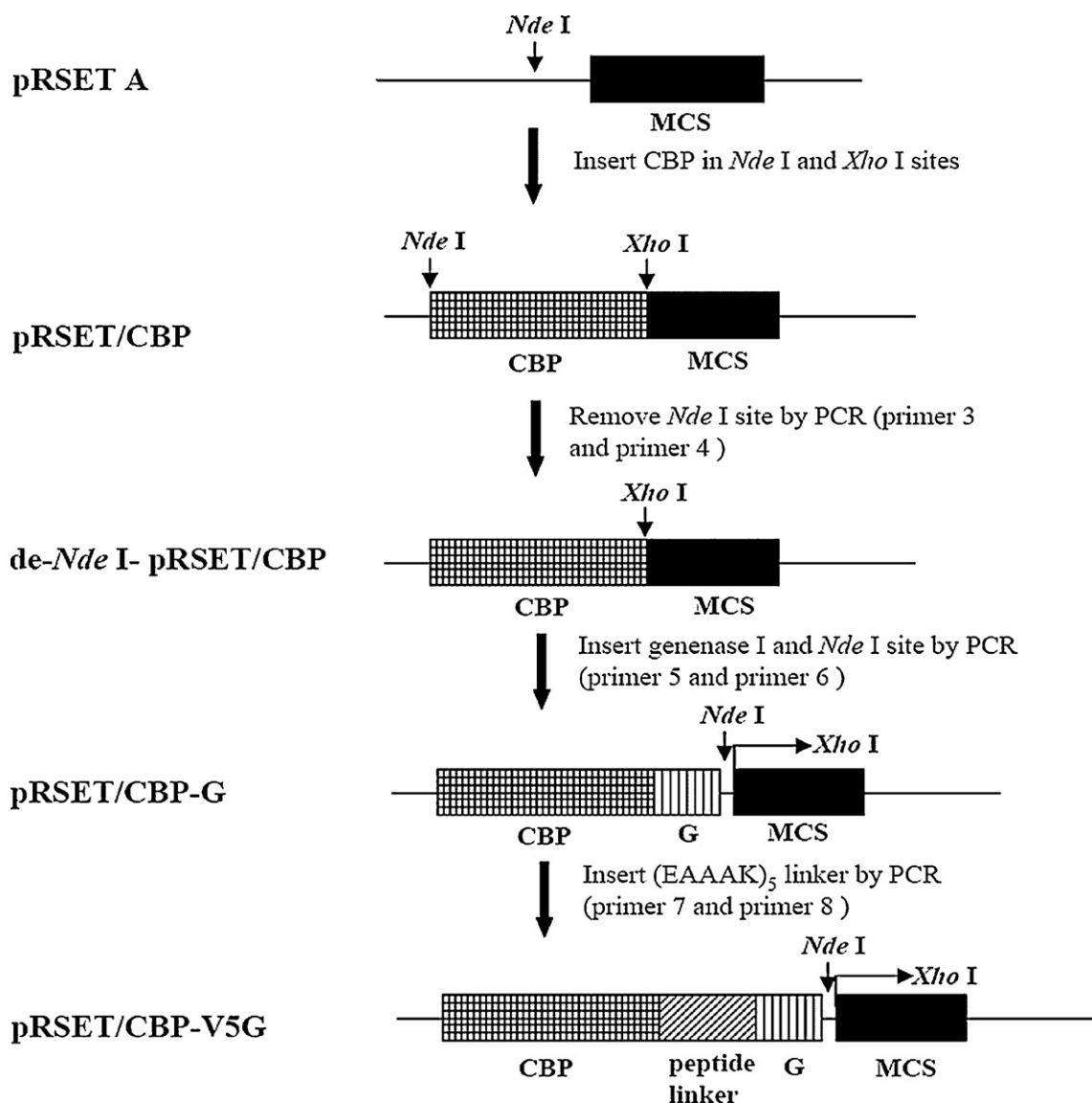


Fig. 2. Procedure of pRSET/CBP-V5G vector construction.

3.2. Construction and evaluation of the system of CBP fusion protein with peptide linkers

The expression vector was constructed based on pRSET A. The process of plasmid construction is illustrated in Fig. 2. First, the amplified *cbp* was inserted in Nde I and Xho I sites. The Nde I cutting site in the resulting vector was further removed to give a plasmid designated de-Nde I-pRSET/CBP, in which the sequences of various peptide linkers and a genease I cutting site (PGA AHY) were inserted after *cbp*. For further incorporation of a target gene, the Nde I site was reinserted after the genease I site. The resulting plasmid was ready for various insertions of genes. To evaluate the feasibility of this expression vector, a tested target protein, such as chitinase (CNS), was inserted. The proteolytic accessibility of the genease I and the chitinase activity of the fusion protein served as criteria to evaluate the feasibility of a linker; more than seven peptide linkers, as shown in Table 2, were examined. Among the tested linkers [15], fusion proteins with (EAAAK)₅, GTGGEGPGGGPGEGGTGGTGEGPGG, or (GGGGS)₅ linker showed significant chitinase activity. Also, all three fusion proteins could be purified with the chitin column, and further released chitinase (~25 kDa) after being treated with

genease I (data not shown), indicating that the peptide linkers did not perturb the structures of both proteins and that the specific proteolytic site was accessible in the fusion protein. As the (EAAAK)₅ peptide was known to form a helical structure and gave a yield and purity better than the other two fusion proteins after chitin-column chromatography, we selected it as the linker for our further work; the vector is designated pRSET/CBP-V5G.

To verify the function of this vector, we tested another two proteins, chitinase from *Bacillus cereus*, and LPHase from *S. matensis* for over-expression in *E. coli*. The fusion proteins, expressed in soluble form, were further purified to high homogeneity through the

Table 2
Peptides tested as linker of the CBP fusion protein.

Linker	Amino-acid sequence	Activity
1	AKRGWI	No
2	GTGGEGPGG	No
3	GTGGEGPGGGPGEGGTG	No
4	TTLGSNLGTLGLK	No
5	(EAAAK) _n , n = 2, 3, 4, 5	Yes
6	GTGGEGPGGGPGEGGTGGTGEGPGG	Yes
7	(GGGGS) ₅	Yes

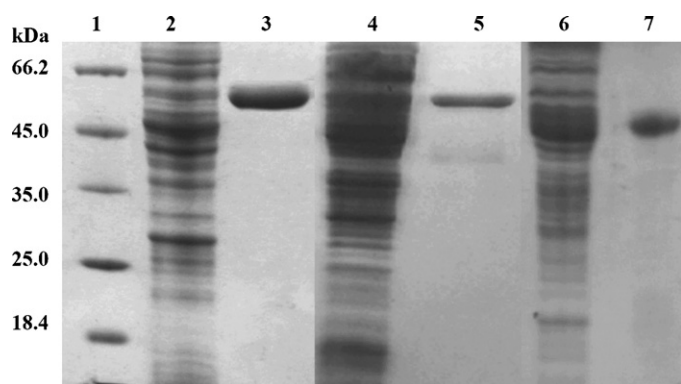


Fig. 3. SDS-PAGE analyses of purified fusion proteins. Lane 1: protein marker; lane 2: CBP-V5G-LPHase crude enzyme; lane 3: CBP-V5G-LPHase pure enzyme (MM 65 kDa); lane 4: CBP-V5G-chitinase crude enzyme; lane 5: CBP-V5G-chitinase pure enzyme (MM 58 kDa); lane 6: CBP-V5G-CNS crude enzyme; lane 7: CBP-V5G-CNS pure enzyme (MM 45 kDa).

application of a chitin column as described previously. The unique characteristic of the interaction between the chitin-binding protein and the chitin matrix allows us to construct a versatile system for affinity purification of a recombinant protein. The system provides a simple and rapid way to purify a large quantity of fusion protein. The yield of purification and purity enhancement were 27% and 53-fold, 29% and 30-fold, and 58% and 137-fold for the fusion proteins of LPHase, chitinase, and chitosanase, respectively. SDS-PAGE analysis revealed that highly homogenous proteins (>90%) were obtained (Fig. 3). The fusion proteins were then subject to proteolytic cleavage with genenase I to release the recombinant enzymes (data not shown). Because of the presence of the NdeI site, the recombinant protein obtained after genenase I treatment is expected to contain an extra histidine residue at the N-terminus.

3.3. Control of the acid-dependent auto-cleavage of (EAAAK)₅ linker

The striking finding of the auto-cleavage of (EAAAK)₅ linker was derived from a study of the optimization conditions for fusion protein elution. The binding affinity of CBP toward β -chitin depends on pH; at pH < 7, CBP loses its binding affinity on chitin [2–4]. In our preliminary work we discovered that, when CBP-V5G-CNS fusion protein, derived from the expression of vector pRSET/CBP-V5G-CNS, was incubated with phosphate buffer (pH 6–7, 25 °C) for 12 h, a substantial amount of CNS was released from the fusion protein spontaneously without genenase I treatment. This unexpected cleavage might be due to contamination with a trace proportion of protease; to test this possibility, we purified the CBP-V5G-CNS protein with a chitin column with the eluent condition at pH 3.6, such that the recombinant protein remained as the original fused form of protein. This fusion protein was then heated at 100 °C for 10 min under pH 3.6. The resulting sample was subjected to exchanging buffers with pH 4.2–8.0 and then kept at 25 °C for 12 h. SDS-PAGE analysis (Fig. 4) showed that the feature of auto-cleavage of fusion protein was retained, and that the auto-cleavage occurred exclusively within pH 6.0–7.0. The best effect was observed at pH 6. Although the mechanism of this auto-cleavage is unclear, this fascinating finding might provide a new method to obtain a target protein from a fusion protein without protease treatment.

3.4. Analysis of auto-cleavage of fusion CNS with various repeated EAAAK linkers

To understand the detailed auto-cleavage feature, we incorporated varied repeat units, (EAAAK)_n with $n=2, 3, 4, 5$, in the

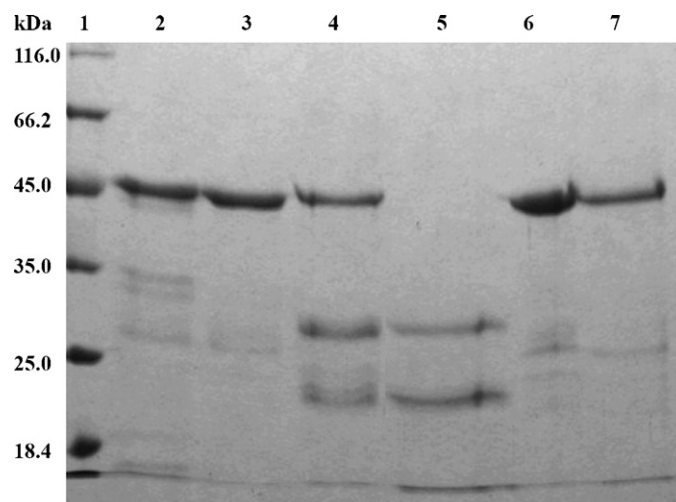


Fig. 4. SDS-PAGE analyses of CBP-V5G-CNS fusion protein at varied pH to test auto-cleavage. Lane 1: protein marker; lane 2: Tris, pH 8.0; lane 3: Tris, pH 7.5; lane 4: phosphate, pH 7.0; lane 5: phosphate, pH 6.0; lane 6: NaOAc, pH 5.1; lane 7: NaOAc, pH 4.2. Note that, in lane 5, the upper band and the lower band are presumable to be the CNS and CBP, respectively. The band at 45 kDa is the intact fusion protein.

fusion protein. Sufficient fusion protein was purified and subjected to auto-cleavage assessment. All these fusion proteins were incubated at 16 °C in phosphate buffer (100 mM, pH 7.0) containing NaCl (300 mM) for 12 h. Under these conditions, the auto-cleavage of fusion protein was incomplete. As shown in Fig. 5, the SDS-PAGE analysis revealed a systematic pattern of protein bands for all fusion proteins. The protein ladder near 20 kDa might result from a separate degree of cleavage of the repeated EAAAK linker. For instance, after partial auto-cleavage, the fusion protein CBP-V2G-CNS (lane 2 in Fig. 5) exhibited four clear bands, showing with the estimated molecular masses 45, 26, 20 and 19 kDa, which presumably correspond to the intact fusion protein, the PGAAHY-CNS (CNS fusion protein with genenase I recognition site), CBP-(EAAAK)₂, and CBP-EAAAK, respectively. Similarly, for the case of CBP-V3G-CNS, the five protein bands shown from large to small molecular mass were expected to be the intact fusion protein, CNS linked with PGAAHY, CBP-(EAAAK)₃, CBP-(EAAAK)₂, and CBP-EAAAK, respectively. Other fusion proteins with longer linkers demonstrated the same auto-cleavage pattern. We further removed either the peptide sequence of the genenase I cutting site or the CBP from the fusion protein and tested the auto-cleavage feature of the proteins. The results showed that, without the existence of the CBP portion or the recognition peptide fragment of genenase I in the fusion protein, the (EAAAK)₅ linker was still susceptible to auto-cleavage at pH 6.

The suggested auto-cleavage pattern of the repeated EAAAK linkers in the fusion proteins was further confirmed with ESI/MS analyses (Fig. 6). The calculated molecular masses of CBP-(EAAAK)₅, CBP-(EAAAK)₄, CBP-(EAAAK)₃, CBP-(EAAAK)₂, CBP-(EAAAK)₁ are 20,996, 20,526, 20,056, 19,586 and 19,115 Da, respectively, whereas PGAAHY-CNS has 24,430 Da. As shown in Fig. 6, the m/z signals in each spectrum correlate well with the expected CBP-(EAAAK)_n portion and the PGAAHY-CNS. For the CBP/CNS fusion protein, to cleave at the EAAAK unit adjacent to CNS seems more efficient because there was no EAAAK-linked CNS observed with ESI-MS analysis throughout the reaction. The linker continued to be cleaved from the CBP-(EAAAK)_n portion, releasing a unit of (EAAAK) peptide (molecular mass 470 Da) at a time. Although the mechanism of the auto-cleavage is unclear, the auto-cleavage is likely chemically controlled without an involvement of protease digestion. More extensive work is necessary to solve the enigma of the intrinsic property of auto-cleavage.

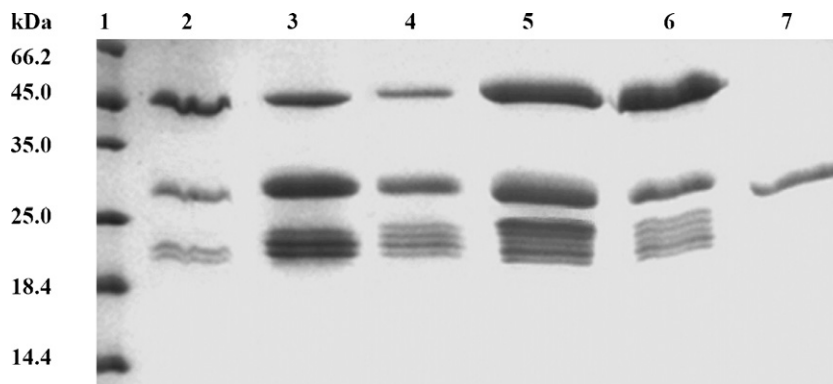


Fig. 5. SDS-PAGE analyses of linkers auto-cleavage. The fusion proteins were incubated in phosphate buffer (pH 6.0 at 16 °C) so that partial auto-cleavage was obtained. Lane 1: protein marker; lane 2: CBP-V2G-CNS; lane 3: CBP-V3G-CNS; lane 4: CBP-V4G-CNS; lane 5: CBP-V5G-CNS; lane 6: CBP-V5-CNS (protein expressed without the peptide sequence of the genase I cutting site); lane 7: V5G-CNS (protein expressed without CBP).

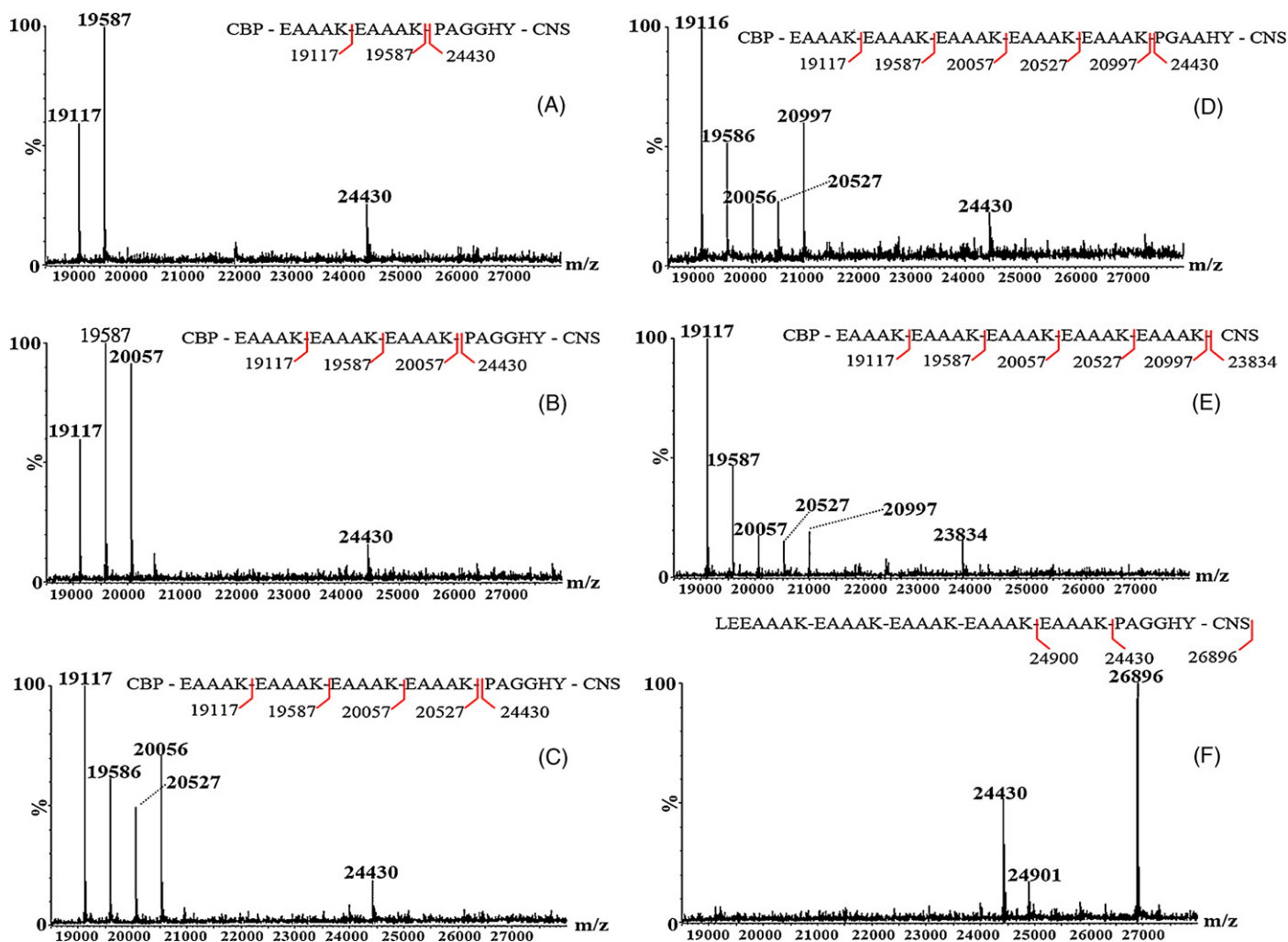


Fig. 6. ESI-MS analyses of fusion proteins after auto-cleavage. The samples used for mass analyses were those for SDS-PAGE analyses shown in Fig. 4. (A) CBP-V2G-CNS, (B) CBP-V3G-CNS, (C) CBP-V4G-CNS, (D) CBP-V5G-CNS, (E) CBP-V5-CNS, and (F) V5G-CNS. The molecular mass (MM) of the EAAAK peptide is 470 Da.

4. Conclusion

In summary, this study demonstrated a convenient system for protein purification with a re-usable and low cost chitin matrix. The repeated EAAAK linker provides a unique auto-cleavage property that commits an effective way to obtain protein of interest from fusion protein without protease treatment. It is potential useful for cost effective protein purification in large scale and the

chitin matrix can be reused for at least 6 times without losing the function.

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