J Ind Microbiol Biotechnol (2015) 42:647–653
DOI 10.1007/s10295-014-1582-5

SHORT COMMUNICATION

SIMB
Society for Industrial Microbiology and Biotechnology

Expression and one-step purification of the antimicrobial peptide cathelicidin-BF using the intein system in *Bacillus subtilis*

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Received: 17 August 2014 / Accepted: 30 December 2014 / Published online: 13 January 2015

Abstract The intein expression system has been widely applied in *Escherichia coli* to express various proteins and peptides. However, the removal of endotoxin from the recombinant proteins expressed in *E. coli* is very difficult and therefore complicates the purification process. In this study, we constructed an intein-based expression vector for an antimicrobial peptide (cathelicidin from *Bungarus fasciatus*) and expressed the intein fusion peptide in a *Bacillus subtilis* expression system. The fusion peptide was secreted into the culture medium, identified by Western blot and purified by affinity chromatography and intein self-cleavage in just one step. Approximately, 0.5 mg peptide was obtained from 1 litre of culture medium. The purified peptide showed antimicrobial activity. Our results indicate that the intein expression system may be a safe and efficient method to produce soluble peptides and proteins in *B. subtilis*.

Keywords Intein · *Bacillus subtilis* · Cathelicidin · Protein purification

Introduction

Antimicrobial peptides (AMPs, also known as host defence peptides), endogenous polypeptides produced by multicellular organisms to protect the host against pathogenic microbes, play an important role in the innate immune response [3, 15]. AMPs are proposed to be a potential solution to the problem of antibiotic-resistant pathogens because they show broad spectrum antimicrobial activities against various micro organisms and possess low propensity for developing resistance [4, 9]. Cathelicidin-BF (CBF), the first identified cathelicidin antimicrobial peptide in reptiles, was isolated from the snake venom of *Bungarus fasciatus*. Despite possessing an atypical structure, it showed potent, broad spectrum, salt-independent antimicrobial activities against many pathogenic microbes, including both Gram-negative and Gram-positive bacteria and fungi, especially drug-resistant strains of clinical isolates [17, 18].

However, isolation of CBF or AMPs from natural sources is a labour-intensive and time-consuming process, and therefore is not an efficient method to obtain large amounts of peptides [6]. Fortunately, recombinant DNA technology provides an economical method to obtain large quantities of recombinant products [6, 24]. In general, recombinant proteins contain a fusion tag for purification through affinity chromatography, after which the tag is removed using a specific protease. Recently, engineered inteins have been used to create convenient self-cleaving tags for tag removal [19]. The intein fusion system avoids the use of exogenous proteases to remove the affinity purification tag [5]. This system utilises the inducible self-cleavage activity of intein to separate the recombinant protein from the purification tag and simplifies the purification of recombinant proteins. Previous research has shown that the intein fusion system can be applied for high-yield soluble expression and simple purification of AFPs with bioactivity [10, 22].

Among the systems available for recombinant protein expression, the *Escherichia coli* expression system is the most widely used platform [6]. With the advantages of

Electronic supplementary material The online version of this article (doi:10.1007/s10295-014-1582-5) contains supplementary material, which is available to authorized users.

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rapid growth, large availability of commercial expression vectors, well-established DNA manipulation protocols, and well-known genetics, biochemistry and physiology, *E. coli* has proven to be the most cost-effective method of recombinant protein production [13]. However, as a Gram-negative bacterium, *E. coli* has several disadvantages. First, the recombinants products are always contaminated by endotoxins (also known as lipopolysaccharides) liberated from Gram-negative bacteria during culture. Small amounts of endotoxin can cause severe side effects in host organisms, such as endotoxin shock, tissue injury, and even death [8]. Additionally, endotoxin is extremely difficult to remove from recombinant products due to its temperature and pH stability [14]. Second, recombinant proteins are not secreted into the supernatant but are retained in the periplasm in the *E. coli* expression system, which complicates the purification process [1].

Bacillus subtilis, a Gram-positive bacterium, is an alternative expression system for the production of recombinant proteins on a large scale [13, 16]. Compared to *E. coli*, *B. subtilis* shows a naturally high secretory capacity without endotoxin contamination. Recombinant proteins can be secreted directly into culture supernatant, which simplifies downstream purification [7, 20]. However, the limited availability of commercial expression vectors restricts its application. In this study, we developed an intein fusion CBF expression plasmid from two commercial vectors, one for *E. coli* and the other for *B. subtilis.* The chitin binding domain (CBD)–intein–CBF fusion protein was expressed in *B. subtilis.* Next, CBF was purified from the culture supernatant using a one-step purification procedure with a chitin affinity column using the self-cleavage characteristics of intein. Furthermore, the antimicrobial activity of purified CBF remained intact.

Materials and methods

Strains, vectors, and materials

E. coli DH5α (preserved in our laboratory) was used as the host for cloning and plasmid amplification. *B. subtilis* WB800 N (MoBiTec, Goettingen, Germany) was used as the host for protein expression. Gram-negative bacteria *E. coli* ATCC 25922 and *E. coli* K88, Gram-positive bacterium *Staphylococcus aureus* ATCC 2592, and *Pichia pastoris* (preserved in our laboratory) were used for antimicrobial activity detection. The shuttle vector pHT43 (MoBiTec) was used for secretion of target proteins. pTWIN1 (NEB, Beverly, MA, USA) was used for constructing fragments encoding CBD–intein–fused CBF. All enzymes were purchased from Takara (Shiga, Japan). Chitin beads were purchased form NEB. Positive control cathelicidin-BF was synthesised by GL Biochem (Shanghai, China). Primers were synthesised by Sangon Biotech (Shanghai, China). Chemical reagents were purchased from Sigma (St. Louis, MO, USA) and Amresco (Solon, OH, USA). All chemical reagents were analytical grade.

Construction of the recombinant pHT-CI-CBF plasmid

A partial gene of Ssp DnaB intein together with the mature CBF gene (part of the entire CBF gene, GenBank, EU753183.1) [17] were synthesised as two oligonucleotides with 86 nt (Supplementary Table 1, Forward and Reverse). One oligonucleotide contained an *Nru*I site, while the other contained an *Nco*I site. There was a 20 nt overlap between these two long primers. A fragment (152 bps) containing part of the intein gene together with the CBF gene was obtained by elongation of the two long primers. The complete sequence of the mature CBF gene is located from nucleotide number 39 to 131 in this 152-bp fragment. The elongation was performed in a total volume of 50 μL containing 1U *ExTaq* DNA polymerase (Takara, Shiga, Japan), 1 × PCR buffer (Mg^{2+} plus), 0.4 mM dNTP mixture and 0.4 mM of each primer with the following cycle: 94 °C for 1 min, 55 °C for 5 min and 72 °C for 2 min. The products were purified using a MicroElute Cycle Pure Kit (Omega, Norcross, GA, USA), digested with *Nru*I and *Nco*I, and then cloned into the *Nru*I/*Nco*I site of pTWIN1 to generate pTWIN1-CBF. A fragment (795 bps) containing CBD-intein-CBF was then amplified from pTWIN1-CBF using primers CBD-F and CBF-R (Supplementary Table 1), digested with *BamH*I and *Sma*I and then cloned into the *BamH*I/*Sma*I site of pHT43 to generate the recombinant expression vector pHT-CI-CBF (Fig. 1). The recombinant plasmid was confirmed by sequencing (Sangon).

Expression of the fusion protein in *B. subtilis*

Recombinant plasmid pHT-CI-CBF was transformed into *B. subtilis* strain WB800 N and the transformant was expressed as previously reported with modifications [23]. Briefly, the pHT-CI-CBF plasmid was first incubated with competent *B. subtilis* at 37 °C for 2 h. Next, the mixture was spread on Luria–Bertani (LB) plates with 20 μg/mL chloramphenicol and cultured overnight at 37 °C. Positive colonies were picked and identified. A positive clone was selected, inoculated into LB broth containing 20 μg/mL chloramphenicol and incubated for 12 h at 30 °C in a shaker with a rotation speed of 220 rpm. The culture was then inoculated into 100 mL fresh LB broth containing 20 μg/mL chloramphenicol in a 500 mL flask at a 1:50

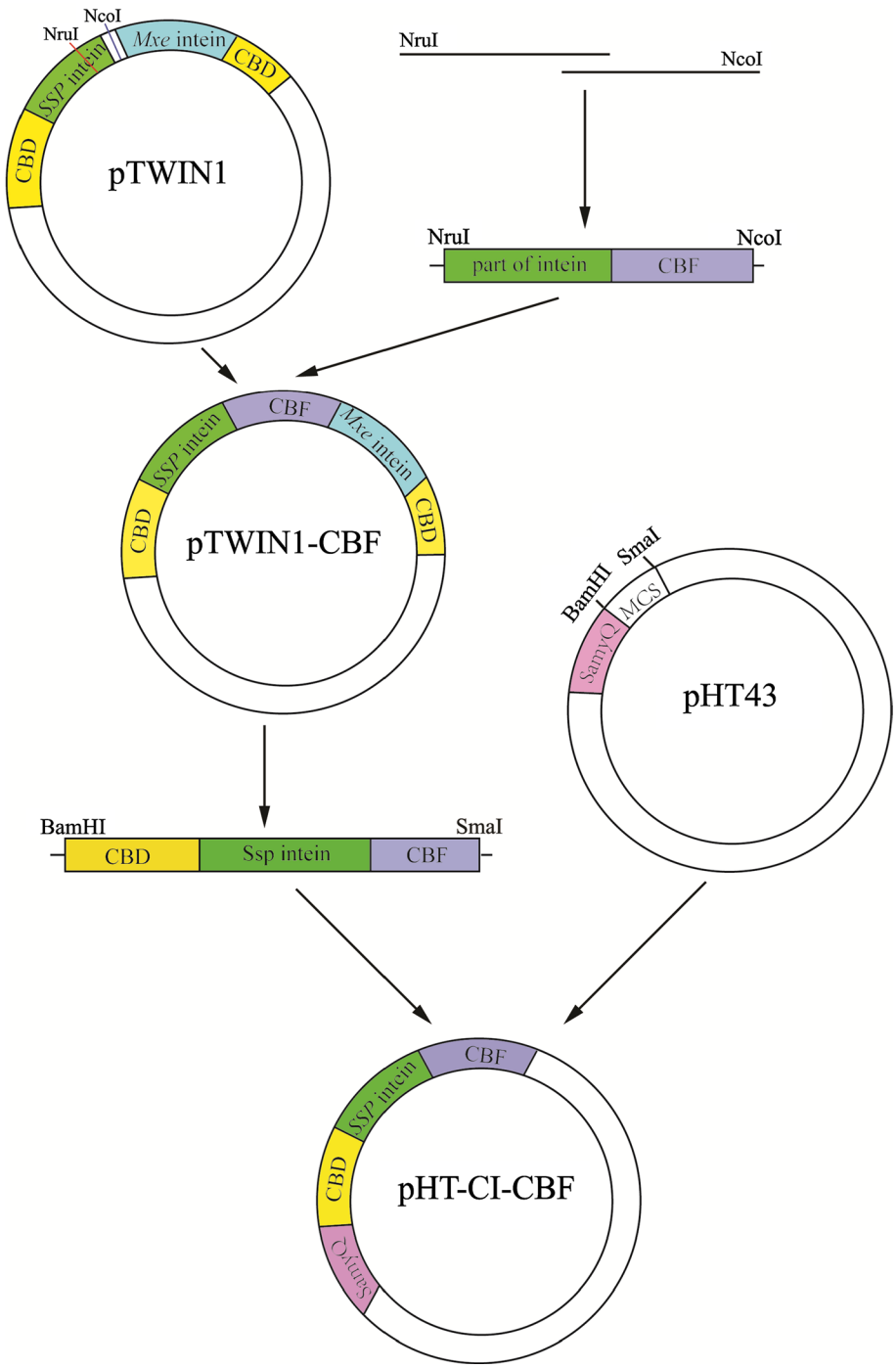

Fig. 1 A flowchart representing the construction of the recombinant expression plasmid. The fragment containing part of the intein gene together with the mature CBF gene was obtained by annealing and elongating the two long primers. One primer contains part of the intein sequence and a second primer contains part of the intein sequence and the mature CBF sequence. There is a 20 nt overlap between the two primers. *CBD* chitin binding protein, *Ssp intein* Ssp DnaB intein, *Mxe intein* Mxe GyrA intein, *CBF* cathelicidin-BF, *MCS* multiple cloning site, *SamyQ* signal peptide for amyQ

ratio and incubated at 30 °C in a shaker for approximately, 6–8 h until OD_{600} = 1.2. To prevent pre-cleavage of the fusion protein, fusion protein expression was induced at 16 °C. The culture was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (Amresco, Solon, OH, USA) and incubated at 16 °C with a rotation speed of 220 rpm for 48 h. The supernatant was collected by centrifugation (10,000×*g*, 10 min) at 4 °C.

Western blot

Fifty microlitre of the collected supernatant was mixed with 10 μL 6 × SDS loading buffer and boiled for 10 min. Ten-microlitre samples were loaded and separated by 10 % SDS-PAGE, then transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membranes were blocked with TBS (20 mM Tris–HCl [pH 7.4],

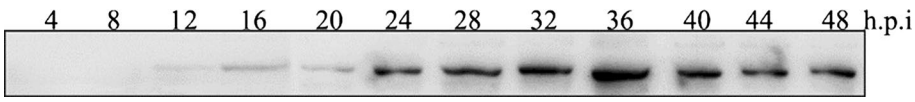

Fig. 2 Expression of fusion CBD–intein–CBF. The culture medium (50 μL) was collected every 4 h post-IPTG induction in recombinant plasmid transformed *B. subtilis*, mixed with 10 μL 6× SDS loading buffer and boiled for 10 min. Ten microliter sample from each time point was subjected to Western blot using CBD antibody. *h.p.i.* hours post induction

150 mM NaCl) containing 5 % (w/v) milk (Nestle, Geneva, Switzerland) for 1 h at room temperature (RT). The membranes were incubated with monoclonal antibodies against CBD (NEB) for 1 h at 37 °C, followed by three washes with TBS-T (TBS containing 0.1 % Tween-20) buffer. The membranes were then incubated with HRP-conjugated goat anti-mouse IgG (Pierce, Rockford, IL, USA) at a dilution of 1:5,000 in TBS for 1 h at RT, followed by three washes with TBS-T. Proteins were visualised using the Supersignal® West Pico chemiluminescent substrate (Pierce) on an AlphaEase® FC Imaging System (Alpha Innotech Corporation, San Leandro, CA, USA).

Purification of CBF and intein-mediated self-cleavage

All purification steps were performed at 4 °C. Due to the large volume of the supernatant, the fusion protein in the supernatant was first concentrated with ammonium sulphate and then purified using chitin beads according to the manufacturer's protocol (NEB, N6951S). One litre of culture supernatant was precipitated with ammonium sulphate to 60 % saturation. The ammonium sulphate was added very slowly in solid form. The precipitate was collected by centrifugation at 12,000×*g* for 15 min, resuspended in 20 mL binding buffer (20 mM Tris–HCl, 500 mM NaCl, pH 8.0) and dialysed against the same buffer. After dialysis, the dialysed mixture was passed through a 0.2 μm filter (Millipore) and then applied to a 15 mL chitin beads column (NEB) equilibrated with binding buffer. The column was washed with 800 ml binding buffer and then washed with 45 ml cleavage buffer (20 mM Tris–HCl, 500 mM NaCl, pH 6.0). The column was kept in the cleavage buffer overnight at 25 °C and then eluted with 30 ml binding buffer. The pass-through elution fraction (approximately 25 mL) was collected and dialysed against low-salt buffer (10 mM Tris–HCl, pH 8.0) at 4 °C and lyophilised.

The sample prior to chitin loading (after ammonium sulphate precipitation and dialysis), chitin beads (50 μL, 1/300 of the total beads) after loading, chitin beads (50 μL, 1/300 of the total beads) after extensive washing, and chitin beads (50 μL, 1/300 of the total beads) after intein self-cleavage were collected and subjected to SDS-PAGE. The samples from chitin beads were resuspended in 30 μL binding buffer, mixed with 80 μL 2× SDS loading buffer and boiled for 10 min. Then, the supernatants (approximately 100 μL) was collected and 10 μL of the samples (approximately 1/3,000 of the total sample) were used for SDS-PAGE.

The identification of purified recombinant protein was analysed by Tricine-PAGE. A 100 μL elution fraction was taken out of 25 mL, mixed with 20 μL 6× SDS loading buffer and boiled for 10 min. Thirty microlitres (approximately 1/1,000 of the total sample) of the boiled sample was used for Tricine-PAGE.

The concentration of the protein was estimated by comparing the intensity of the specific protein band to one marker band with a known concentration. The comparison of band intensity was performed using image-pro-plus software (Media Cybernetics, Rockville, MD, US).

Bioactivity of CBF

The bioactivity of CBF was determined using an antimicrobial assay. The minimum inhibitory concentration (MIC) of recombinant CBF and synthetic CBF was determined by incubation with bacteria in LB broth or with yeast in yeast nitrogen base broth as previously reported [17]. Briefly, serial two-fold dilutions of the peptides were added to sterile 96-well plates (Costar, Cambridge, MA, USA) in a volume of 10 μL, followed by addition of 90 μL bacteria or yeast (6×10^5 colony-forming units/mL). Each bacterium or yeast was incubated with one concentration of peptide in quintuplicate. The MIC was determined as the lowest concentration at which no visible growth occurred. Triplicate assays were carried out and data are presented as the mean of three independent assays.

Results

Purification of CBF

To assess the optimal induction time for expression, the supernatants were collected every 4 h from 0 to 48 h post-induction and subjected to Western blot. A band of approximately 26 kDa was detected at 12 h post-induction, reached the highest expression level at 36 h post-induction and showed little decrease thereafter (Fig. 2). Therefore, the supernatant was collected at 36 h post-induction when the fusion protein was expressed on a large-scale fermentation.

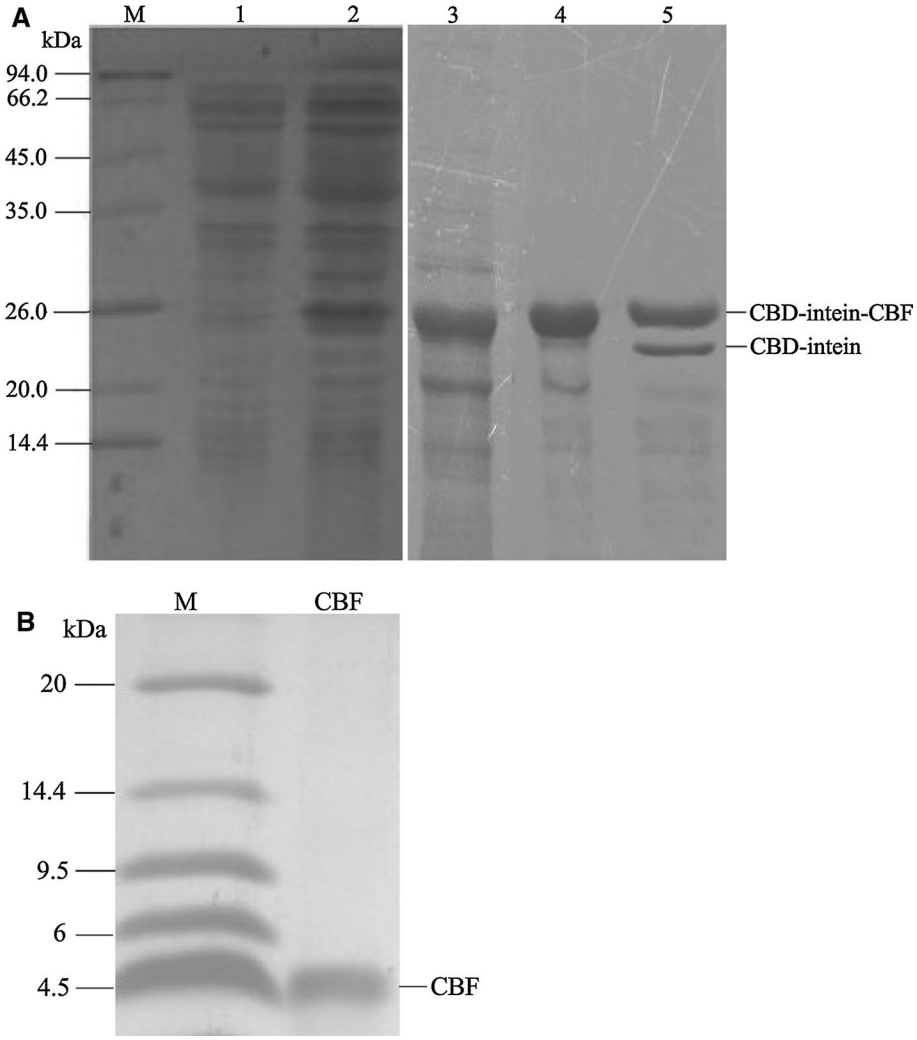

Fig. 3 Purification of recombinant CBF. **a** The culture medium was collected 36 h post induction, precipitated with ammonium sulphate, dialysed, and loaded into a chitin bead affinity column. Recombinant CBF was purified using intein self-cleavage through PH and temperature changes. M, Marker; lane 1, concentrated and dialysed supernatant from pHT43 transformed *B. subtilis*; lane 2, concentrated and dialysed supernatant from recombinant vector pHT-CI-CBF transformed *B. subtilis*; lane 3, proteins binding with chitin beads; lane 4, proteins binding with chitin beads after extensive washing; lane 5, proteins binding with chitin beads after intein self-cleavage. **b** Identification of recombinant CBF using Tricine-PAGE

In the large-scale fermentation, the supernatant was collected at 36 h post-induction by centrifugation and the proteins in the supernatant were concentrated by ammonium sulphate precipitation and applied to a chitin bead column for purification. There was approximately, 15 mg of fusion protein per litre of bacterial culture (Fig. 3a, lane 3). The concentration of the fusion protein was determined by comparison of its band intensity to a protein marker with a known concentration. The 26 kDa band of the marker was approximately 0.5 μg, and the fusion protein band intensity was approximately, 10 times of that band, and thus the concentration was approximately, 5 μg. As described in the Materials and methods, samples for SDS-PAGE represented 1/3,000 of the total sample, so the total amount of the fusion protein was approximately, 15 mg (0.5 μg × 3,000). Binding of the fusion protein was induced by self-cleavage through pH and temperature changes. The cleavage efficiency was approximately, 30 % as the uncleaved band intensity was about twice of the cleaved band (Fig. 3a, lane 5). Tricine-PAGE showed that there was approximately, 0.5 mg recombinant CBF obtained per litre of bacterial culture (Fig. 3b). The concentration of CBF was determined as described above. The 6 kDa band and 4.5 kDa band of the marker were approximately, 0.5 and 1 μg, respectively. The CBF band intensity was about the same as the 6 kDa band and the concentration of the CBF was therefore considered to be 0.5 μg. As described in the Materials and methods, samples used for Tricine-PAGE represented 1/1,000 of the total CBF, so the total amount of the purified CBF was approximately, 0.5 mg.

Antimicrobial activity of CBF

The antimicrobial activity of recombinant CBF was compared to that of synthetic CBF against two Gram-negative bacteria (*E. coli* ATCC 25922 and *E. coli* K88), one Gram-positive bacterium (*Staphylococcus aureus* ATCC 2592) and one yeast (*Pichia pastoris*). As shown in Table 1, recombinant CBF showed activity similar to that of synthetic CBF, with an MIC of 2 μg/mL for *E. coli* ATCC

Table 1 Antimicrobial activity of CBF

Microorganism strains	MIC [median (lower limit, upper limit), μg/mL]	
	Recombinant CBF	Synthetic CBF
E. coli ATCC 25922	2 (1, 4)	2 (1, 4)
E. coli K88	4 (2, 8)	4 (2, 8)
Staphylococcus aureus ATCC 2592	8 (4, 8)	4 (4, 8)
Pichia pastoris	2 (1, 2)	2 (1, 2)

25922, 4 μg/mL for *E. coli* K88, 8 μg/mL for *S. aureus* ATCC 2592, and 1 μg/mL for *Pichia pastoris*.

Discussion

In this study, for the first time we introduce an intein-based expression system into *B. subtilis*. Recombinant CBF with antimicrobial activity was successfully expressed and purified using a one-step protocol.

Bacillus subtilis is a Gram-positive bacterium that has been widely used in recombinant protein production for several reasons: it is nonpathogenic and classified as "generally recognised as safe" by the United States Food and Drug Administration; it contains no significant bias in codon usage; and it is capable of secreting exogenous proteins directly into the culture medium [2, 7]. Inteins are mobile genetic elements capable of self-splicing post-translationally [12]. The ability of inteins to cleave specific peptide bonds in a variety of contexts makes it a powerful tool in recombinant protein purification [21]. Intein fusion systems have proved to be efficient for soluble expression and simple purification of AFPs with bioactivity [10]. Fusion of the peptide with the C-terminus of intein enabled inactivation of the antimicrobial activities of the peptide, permitting expression of the fusion protein in *E. coli* [10]. In the present study, CBF was fused to the C-terminus of intein.

To date, commercial intein-based expression vectors such as IMPACT-TWIN can only be used with *E. coli*. To construct an intein-based CBF expression vector in the *B. subtilis* system, the CBF gene was first ligated into pTWIN downstream of Ssp intein to form pTWIN-CBF. Next, the CBD–intein–CBF fragment was obtained by PCR and subcloned into the *E. coli–B. subtilis* shuttle vector pHT43 to generate the recombinant expression vector pHT-CI-CBF for *B. subtilis*.

The cleavage activity of intein is strongly affected by temperature, with the most rapid cleavage observed at 37 °C [11]. To avoid unnecessary self-cleavage during expression, a low temperature condition (16 °C) was applied for the fusion protein. The cleavage activity of intein is also strongly influenced by the amino acids adjacent to intein. The preferred amino acids of Ssp intein are CRA or GRA (NEB website, https://www.neb.com/products/N6951-pTWIN1-Vector), while the first three amino acids of CBF are KFF, so we expected the cleavage efficiency to be very low. The results confirmed this hypothesis, because the data showed that the cleavage efficiency was approximately, 30 %. This is a big limitation of this expression system. In the future, other inteins that preserve high cleavage activity with KFF adjacent to intein should be tested to achieve high cleavage efficiency.

Recombinant CBF showed the same bio-activity as synthetic CBF for most tested bacteria or yeast, with the exception of one Gram-positive bacterium (*S. aureus* ATCC 2592). This discrepancy may be due to differences in cell wall penetration capacity of the two CBFs. A similar phenomenon was observed with recombinant CBF for another Gram-positive bacterium (*S. aureus* ATCC 25923) [7]. The bio-activity of CBF in the present study is comparable to previous reports [7, 17]. Our results indicated that this expression system can produce peptides with high bioactivities in *B. subtilis*.

Very recently, Luan et al. [7] expressed CBF in *B. subtilis* using SUMO technology and obtained 3 mg/L recombinant CBF, which is much higher than the product we obtained. Although the product in the present study was estimated using an imprecise method which may lead to either an over- or an under-estimated value, the actual total amount of the recombinant CBF should still be much less than 3 mg/L. In addition to the lower cleavage efficiency, the chaperon capacity and stability of SUMO contribute to the higher yield. The use of other inteins that preserve high cleavage activity when KFF is adjacent to intein or another chaperon in the intein-based expression system may aid in obtaining higher yields of fusion proteins. Compared with the Luan et al. study using the SUMO system the purification process with our intein system is much simpler because the intein-fusion protein can be purified in one step while the SUMO-fusion protein is purified in two steps. The fewer steps in the procedure, the easier the method is for application. Therefore, our expression system might be easier for large-scale use.

In conclusion, for the first time we expressed an antimicrobial peptide with bio-activity using the intein system in *B. subtilis*. This expression system allowed exogenous proteins to be secreted directly into the culture medium and therefore be purified in one step, which simplified the expression and purification of recombinant products. We believe that this expression system could be applied to the production of many valuable peptides or proteins in the future.

Acknowledgments This work was supported by grants from Natural Science Foundation of China (No. 21171031).

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