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A novel approach for improving the yield of *Bacillus subtilis* transglutaminase in heterologous strains

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Abstract The transglutaminase (BTG) from *Bacillus subtilis* is considered to be a new type of transglutaminase for the food industry. Given that the BTG gene only encodes a mature peptide, the expression of BTG in heterologous microbial hosts could affect their normal growth due to BTG's typical transglutaminase activity which can catalyze cross-linking of proteins in the cells. Therefore, we developed a novel approach to suppress BTG activity and reduce the toxicity on microbial hosts, thus improving BTG yield. Genes encoding the respective regions of transglutaminase propeptide from seven species of *Streptomyces* were fused to the N-terminal of the BTG gene to produce fusion proteins. We found that all the fused propeptides could suppress BTG activity. Importantly, BTG activity could be completely restored after the removal of the propeptides by proteolytic cleavage. Of the seven propeptides tested, the propeptide proD from *Streptomyces caniferus* had the strongest suppressive effect on BTG activity (70 % of the activity suppressed). Moreover, fusion protein proD-BTG (containing proD) also exhibited the highest yield which was more than twofold of the expression level of BTG in an active form in *Escherichia coli*. Secretion expression of BTG and proD-BTG in *Corynebacterium glutamicum* further showed that our novel approach was suitable for the efficient BTG expression, thus providing a valuable platform for further optimization of large-scale BTG production.

Keywords Transglutaminase · *Bacillus subtilis* · Propeptide · Fusion protein · Secretion expression

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Introduction

Transglutaminases (TGases, protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) are a family of enzymes that catalyze an acyl transfer reaction [3]. They catalyze the formation of the cross-linking between a γ-carboxyamide group of a glutamine residue and a γ-amino group of a lysine residue, resulting in the formation of an isopeptide bond either within or between polypeptide chains and the covalent incorporation of polyamine into proteins [13]. A microbial TGase (MTG), the first TGase obtained from a nonmammalian source, has been widely used in the food industry, such as dairy, meat and fish products, etc. [5, 14, 15, 26]. Moreover, it has huge potential utility in the textile, leather and pharmaceutical industries [4, 7, 29].

MTG was first isolated from the culture broth of *Streptoverticillium* S-8112 in 1989, later identified to be a variant of *Streptomyces mobaraense* [1, 24]. MTG as an active enzyme was lethal on microbial hosts, it was expressed as an inactive form with its propeptide [8, 17, 21, 27]. Currently, quite a number of studies for increasing MTG production have been reported. For instance, MTG was expressed in *Streptomyces lividans* (less than 0.1 mg/l) and *Escherichia coli* (about 5 mg/l) [21, 24]; MTG codons optimization for expression in *E. coli* resulted in a significant increase of MTG expression level, but MTG was

mainly produced in the form of inclusion bodies [28]. To overcome this hurdle, the extracellular overproduction of MTG was achieved in *Corynebacterium glutamicum* [2] and other coryneform bacteria [6]; MTG was achieved soluble expression in *E. coli* by reducing the temperature after induction [16].

The TGase (BTG) from *Bacillus subtilis* was first studied in 1996 [9]. BTG synthesis is controlled by σ^K in the mother cell and assembled onto the spore surface [30]. It is involved in catalyzing the covalent cross-linking of a fraction of the coat proteins to resist lytic enzymes, noxious chemicals and ensures normal germination [19, 30]. Compared with MTG, BTG can catalyze the formation of ε-(γ-Glu)Lys isopeptides as well [9], but it also has notable differences. The optimal temperature (60 °C) and pH (8.2) of BTG were clearly different from the optimal temperature (50 °C) and pH (6–7) of MTG [1, 20]; the N-terminal region and even the sequence around the active site of BTG had little similarity to MTG [10, 21]. Based on these properties, BTG is considered to be a new type of TGase with different industrial value for food processing, but its current yield is insufficient for its large-scale cost-effective production. A few studies have been conducted to increase its production. For example, the BTG gene was expressed in *E. coli*, but the yield was very low [10]; the yield of BTG was significantly improved by auto-induction, achieving a level of 50 mg/l, but was far below the production level of MTG [18]. Because BTG has the typical TGase activity and the BTG gene only encodes a mature peptide, unlike MTG which exists in the form of proenzyme, the expression of BTG will have a certain toxic effects on microbial hosts, affecting cell growth, leading to the relatively low yield of BTG. Thus, more efficient systems for increasing BTG production are needed.

In this study, we first describe a novel approach to improve the yield of BTG in heterologous strains. Genes encoding the respective regions of transglutaminase propeptide from seven species of *Streptomyces* were fused to the N-terminal of the BTG gene to produce the forms of activity suppressed as fusion BTG (F-BTG). The optimal fusion BTG was screened in *E. coli* through measuring the suppressive effects of the propeptides on BTG activity. Then the effectiveness of the approach was further verified by the secretion expression of BTG and the optimal fusion BTG in *C. glutamicum*.

Materials and methods

Bacterial strains, plasmids, and culture medium

E. coli JM109, *E. coli* BL21 (DE3), *C. glutamicum* ATCC 13032 and *B. subtilis* 168 ATCC 23857 were stored in our lab. *E. coli* JM109 was used as an intermediate host for various plasmid constructions, *E. coli* BL21 (DE3) and *C. glutamicum* ATCC 13032 were used as the host for the expression of recombinant proteins. *E. coli* JM109 and *E. coli* BL21 (DE3) were grown in Luria–Bertani (LB) medium (yeast extract 5 g/l, tryptone 10 g/l, and NaCl 10 g/l) at 37 °C. MMTG medium (60 g of glucose, 1 g of $MgSO_4$, 30 g of $(NH_4)_2SO_4$, 1.5 g of KH_2PO_4, 0.01 g of $FeSO_4{\cdot}7H_2O$, 0.01 g of $MnSO_4{\cdot}4H_2O$, 450 μg of thiamine hydrochloride, 450 μg of biotin, 0.15 g of DL-methionine, 50 g of $CaCO_3$ per liter of distilled water, adjusted to pH 7.5) as a production medium for C. *glutamicum* was used at 30 °C. Kanamycin was added to final concentration of 50 μg/ml to the growth media of *E. coli* JM109 and BL21 (DE3). Chloramphenicol was added at a final concentration of 50 and 10 μg/ml to the growth media of *E. coli* JM109

Table 1 Plasmids used in this study

Plasmid	Characteristics	Source or reference
pET28a(+)	*E. coli* expressing vector; Kan^r	Novagen
pXMJ19	*E. coli-C. glutamicum* shuttle vector; Cm^r	Laboratory preserved
pEBTG1	pET28a(+) carrying BTG gene	This study
pECTG1	pET28a(+) carrying fused BTG gene containing TGase propeptide from *Streptomyces cinnamoneus*	This study
pEDTG1	pET28a(+) carrying fused BTG gene containing TGase propeptide from *Streptomyces caniferus*	This study
pEFTG1	pET28a(+) carrying fused BTG gene containing TGase propeptide from *Streptomyces fradiae*	This study
pEHTG1	pET28a(+) carrying fused BTG gene containing TGase propeptide from *Streptomyces hygroscopicus*	This study
pEMTG1	pET28a(+) carrying fused BTG gene containing TGase propeptide from *Streptomyces mobaraense*	This study
pENTG1	pET28a(+) carrying fused BTG gene containing TGase propeptide from *Streptomyces netropsis*	This study
pEPTG1	pET28a(+) carrying fused BTG gene containing TGase propeptide from *Streptomyces platensis*	This study
pXSBTG1	pXMJ19 carrying fused BTG gene containing signal peptide ΔS0949	This study
pXSDTG1	pXMJ19 carrying fused BTG gene containing signal peptide ΔS0949 and TGase propeptide from *S. caniferus*	This study

and C. *glutamicum*, respectively. The plasmids used in this study are listed in Table 1.

Oligonucleotide synthesis

The TGase propeptides from seven species of *Streptomyces* (*Streptomyces cinnamoneus*, *Streptomyces caniferus*, *Streptomyces fradiae*, *Streptomyces hygroscopicus*, *Streptomyces mobaraense*, *Streptomyces netropsis* and *Streptomyces platensis*) were represent as proC, proD, proF, proH, proM, proN and proP, respectively (Fig. 1a). Their gene sequences were deposited in GenBank: AB085698.1, AM746294.1, DQ432028.1, EU477523.1, AF531437.1, EF195356.1 and AM746296.1. The seven synthetic sequences (A1, A2, A3, A4, A5, A6 and A7) contain a region encoding proC, proD, proF, proH, proM, proN or proP, respectively, a cleavage sequence (Ile-Glu-Gly-Arg) of factor Xa, and the N-terminal 31 base pairs of the BTG gene (Fig. 1b).

Construction of *E. coli* expression plasmids

All PCR primers used in this study are listed in Table 2. Primers F1 and R1 based on the sequence of *B. subtilis* TGase gene (GenBank accession no. E13095) were used for amplifying the BTG gene, included the mature peptide (244-amino acid residues), was inserted into the

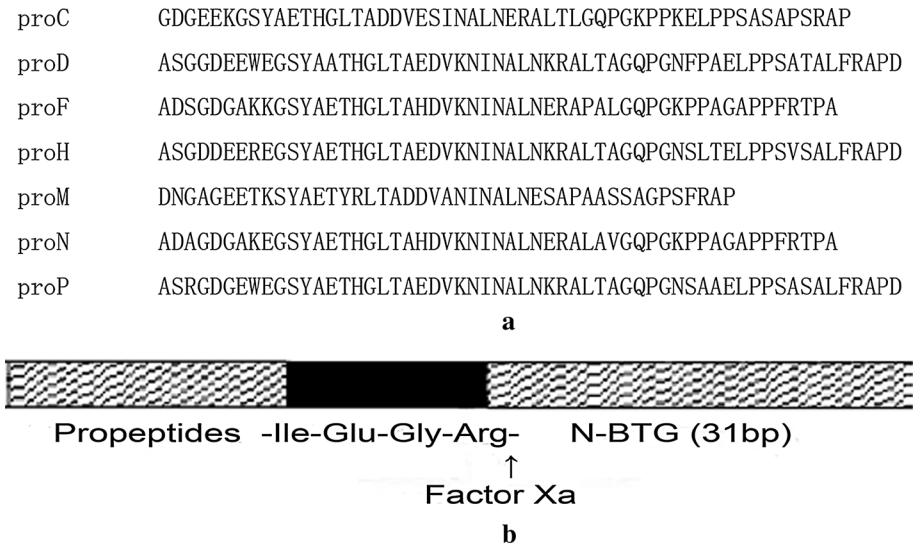

Fig. 1 **a** The respective amino acid sequences of transglutaminase propeptide from seven species of *Streptomyces*. **b** Schematic representation of the synthetic genes. Propeptides, genes encoding the respective regions of transglutaminase propeptide from seven species of *Streptomyces*; -Ile-Glu-Gly-Arg-, gene encoding the recognition sequence of factor Xa; N-BTG (31 bp), N-terminal 31 base pairs of the BTG gene; the cleavage site of the propeptides is indicated by an *arrow*

Table 2 Sequence of the primer used in the study

Primer	Sequence[a]	Restriction site
F1	5′-CGCCATATGATGATTATTGTATCAGGACAATTGC-3′	*Nde*I
F2	5′-ATGATTATTGTATCAGGACAATTGCTCCG-3′	
F3	5′-CGCCATATGGGCGATGGGGAAGAGA-3′	*Nde*I
F4	5′-CGCCATATGGCCAGCGGCGG-3′	*Nde*I
F5	5′-CGCCATATGGCCGACAGCGGC-3′	*Nde*I
F6	5′-CGCCATATGGCTAGCGGTGACGACG-3′	*Nde*I
F7	5′-CGCCATATGGACAATGGCGCGGG-3′	*Nde*I
F8	5′-CGCCATATGGCCGACGCCGG-3′	*Nde*I
F9	5′-CGCCATATGGCCAGCCGCGG-3′	*Nde*I
P1	5′-TATCGGCGCTGCCAGCATGTTTATGCCAAAGGCCAACGCCCAAGGAGCACTCGAGATGATTATTG TATCAGGACAATTGC -3′	
P2	5′-TATCGGCGCTGCCAGCATGTTTATGCCAAAGGCCAACGCCCAAGGAGCACTCGAGGCCAGCGGCGGC-3′	
P3	5′-CCCAAGCTTAAAGGAGGACACGCATGCAAATAAACCGCCGAGGCTTCTTAAAAGCCACCGCAGGACTT-GCCACTATCGGCGCTGCCAGCATG-3′	*Hin*dIII
R1	5′-CCCAAGCTTTTAGCGGACGATGCGG-3′	*Hin*dIII
R2	5′-CGCGGATCCTTAGCGGACGATGCGG-3′	*Bam*HI

[a] Underlined nucleotides represent restriction sites

*Nde*I-*Hin*dIII sites of pET28a(+), giving rise to recombinant plasmid pEBTG1 to express His_6-BTG. To engineer the synthetic propeptides before BTG, respectively, crossover PCR were performed. The first round of PCR to amplify the BTG gene was performed using primers F2 (as the forward primer), R1 (as the reverse primer) and template pEBTG1. The second round of PCR was performed with the amplified DNA fragments from the first PCR as one of the two templates, then used primers F3/R1 and template A1, primers F4/R1 and template A2, primers F5/R1 and template A3, primers F6/R1 and template A4, primers F7/R1 and template A5, primers F8/R1 and template A6, primers F9/R1 and template A7 to amplify the F-BTG genes. Each amplified gene was inserted into the *Nde*I-*Hin*dIII sites of pET28a(+), giving rise to recombinant plasmids pECTG1, pEDTG1, pEFTG1, pEHTG1, pEMTG1, pENTG1 and pEPTG1 to express His_6-proC-BTG, His_6-proD-BTG, His_6-proF-BTG, His_6-proH-BTG, His_6-proM-BTG, His_6-proN-BTG and His_6-proP-BTG. All the recombinant plasmids and parental plasmid pET28a(+) were transformed into *E. coli* BL21 (DE3), respectively. The sequences were confirmed by DNA sequencing.

Expression of His_6-BTG and His_6-F-BTG in *E. coli*

The recombinant *E. coli* strains that expressed His_6-BTG and His_6-F-BTG were grown at 37 °C for 12 h in LB medium with kanamycin (50 μg/ml). Subsequently, these seed cultures were, respectively, transferred to 800 ml of LB medium supplemented with 50 μg/ml kanamycin and incubated at 37 °C until the optical density at 600 nm (OD_{600}) reached about 0.8, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, followed by an additional 20 h cultivation at 20 °C. Taking 20 ml culture in 50-ml centrifuge tube, cells were centrifuged at 4,000×g for 10 min and washed two times with deionized water, incubated at 60 °C until the weight of cells became constant. All assays were carried out in triplicate. The rest of cells were harvested by centrifugation at 4,000×g for 15 min at 4 °C and resuspended in 25 ml buffer A (20 mM CAPS, pH 10.0, 0.5 M NaCl, 10 mM imidazole and 10 % glycerol). Then cells were disrupted by the Low Temperature Ultra-high Pressure Continuous Flow Cell Disrupter (JNBIO, China).

Purification and enzymatic cleavage of His_6-BTG and His_6-F-BTG

To facilitate the purification of products, a single affinity chromatographic step was used [18]. The disrupted cells were centrifuged at 12,000×*g* for 30 min at 4 °C and the supernatants were loaded on a His-Trap, Ni^{2+}-nitrilo-triacetic acid affinity column (GE Health Sciences) pre-equilibrated with buffer A. After the affinity column was washed with buffer A containing 30 mM imidazole, elution was performed using buffer A containing 150 mM imidazole. Then, the elutions were dialyzed against ice-cold cleavage buffer (20 mM Tris–HCl, 100 mM NaCl, 2 mM $CaCl_2$, pH 8.0) at 4 °C overnight and concentrated to about 2 ml using the Amicon ultra-15 cell (Millipore Corporation), with a molecular weight cut-off of 10 kDa. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce). The purified and concentrated samples were stored at −80 °C until use.

Six hundred microliter of the sample of His_6-BTG was divided into two equal portions, one portion was added a certain amount of thrombin (Sigma, USA), the other portion was added the equal volume of the cleavage buffer, both the two portions were incubated for 4 h at 20 °C and examined by 12 % SDS-PAGE and the gel was stained by Coomassie Brilliant Blue R-250. To 600 μl of the sample of His_6-F-BTG was added a certain amount of thrombin and incubated for 4 h at 20 °C. Then the sample was divided into two equal portions, to one portion was added a certain amount of factor Xa (Sigma, USA), the other portion was added the equal volume of the cleavage buffer; both the portions were incubated for 4 h at 23 °C and examined by 12 % SDS-PAGE and the gel was stained by Coomassie Brilliant Blue R-250.

Activity assay of TGase

Forty microliter of the reaction system of BSA cross-linking containing 5 μl of BSA (10 mg/ml in 0.1 M Tris–HCl, pH 8.0), 2 mM dithiothreitol (DTT) was incubated at 50 °C for 2 h with or without 10 μl (1 mg/ml) of the recombinant enzyme [12, 18]. The reaction products were analyzed by 12 % SDS-PAGE and the gel was stained by Coomassie Brilliant Blue R-250.

TGase activity was measured by the incorporation of the fluorescent amine, dansyl cadaverine [*N*-(5-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide, MDC] into *N,N*-dimethylcasein by the method of fluorescence [22], the fluorescence intensity of MDC will be increased about 13-fold upon the incorporation into casein. The assay system of TGase activity consisted of 90 μl (5 mg/ml) of the recombinant enzyme and 1.98 ml of 50 mM Tris–HCl buffer, pH 7.5, containing 12.5 μM MDC, 0.2 % *N,N*-dimethylcasein and 4.5 mM DTT. The reaction was conducted at 37 °C for 30 min and stopped by the final concentration of 42 mM $(NH_4)_2SO_4$. The fluorescence intensity was measured with a Fluorescence spectrophotometer-F-7000 (Hitachi, Japan) using an excitation and emission wavelength of 350 and 500 nm, respectively. The experiments were performed in triplicate. One unit of TGase was defined as the amount of enzyme which 1.0 nM MDC

incorporated into casein per minute at 37 °C. TGase activity could be analyzed as the following formula:

$$[\mathrm{MDC}]_{\mathrm{incorporated}} = \frac{I - I_0}{13 \times I_0} \times [\mathrm{MDC}]_{\mathrm{total}}$$

I_0 denotes the fluorescence intensity of the reaction mixture without TGase, I denotes the fluorescence intensity of the reaction mixture with TGase.

Construction and expression of *C. glutamicum* extracellular expression plasmids

To engineer a newly identified signal peptide named ΔS0949 from *C. glutamicum* R [25] before the BTG and proD-BTG genes, two rounds of PCR were performed: primers P1/R2 and template pEBTG1, primers P2/R2 and template pEDTG1 were used for the first round PCR amplification; the resultant PCR products were purified and, respectively, used as the templates for the second round PCR reaction with the primers P3 (as the forward primer) and R2 (as the reverse primer). Each amplified fragment was inserted into the *Hind*III-*Bam*HI sites of pXMJ19, giving rise to recombinant plasmids pXSBTG1 and pXSDTG1, which were subsequently transformed into *C. glutamicum* by electrotransformation [23]. Recombinant *C. glutamicum* cells were, respectively, cultivated in MMTG at 30 °C for 12 h, then 1 mM IPTG inducer was added, followed by additional cultivation for 40 h. The supernatants were harvested by centrifugation at 5,000×g for 10 min. Factor Xa was added in the supernatant of the recombinant *C. glutamicum* carrying plasmid pXSDTG1 to cleave F-BTG. Subsequently, the activity of both the supernatants was determined by the fluorescence assay.

Results

Construction of expression vectors

First, a recombinant plasmid pEBTG1 for the expression of BTG in an active form in *E. coli* was constructed. Then, to test whether the synthetic propeptides could suppress BTG activity, seven recombinant plasmids, pECTG1, pEDTG1, pEFTG1, pEHTG1, pEMTG1, pENTG1 and pEPTG1 for expression of F-BTG was constructed. The above recombinant plasmids and parental plasmid pET28a(+) were, respectively, transformed into *E. coli* BL21 (DE3), recombinant strains pEBTG1/BL21(DE3), pECTG1/BL21(DE3), pEDTG1/BL21(DE3), pEFTG1/BL21(DE3), pEHTG1/BL21(DE3), pEMTG1/BL21(DE3), pENTG1/BL21(DE3), pEPTG1/BL21(DE3) and pET28/BL21(DE3) were selected on LB agar plates containing kanamycin. Finally, to achieve the secretion expression of BTG and F-BTG in

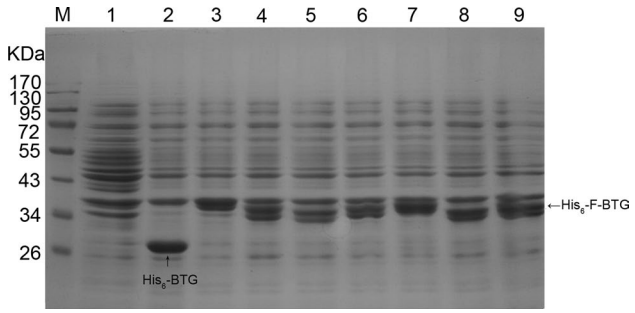

Fig. 2 SDS-PAGE analysis of expression of recombinant proteins in *E. coli* BL21 (DE3). The positions of His_6-BTG and His_6-F-BTG are shown by *arrowheads*. *Lane M* protein MW marker, *lanes 1–9* whole cell lysates of induced pET28/BL21(DE3), pEBTG1/BL21(DE3), pECTG1/BL21(DE3), pEDTG1/BL21(DE3), pEFTG1/BL21(DE3), pEHTG1/BL21(DE3), pEMTG1/BL21(DE3), pENTG1/BL21(DE3) and pEPTG1/BL21(DE3)

C. glutamicum, we constructed two plasmids pXSBTG1 and pXSDTG1, respectively. Subsequently, the expression plasmids were, respectively, transformed into *C. glutamicum* ATCC 13032 by electroporation. Transformants pXSBTG1/13032 and pXSDTG1/13032 were selected on LB agar plates containing chloramphenicol.

Expression of His_6-BTG and His_6-F-BTG in *E. coli*

The above recombinant *E. coli* strains were cultured in LB medium, after induction, the whole cell lysates were analyzed by SDS-PAGE (Fig. 2). The whole cell lysates of pEBTG1/BL21(DE3) showed an obvious protein band at about 30 kDa (lane 2), consistent with the expected size of BTG plus a HIS-tag, indicating that His_6-BTG was successfully expressed in *E. coli.* SDS-PAGE analysis detected apparent protein bands (lanes 3–9) of about 38 kDa in the whole cell lysates of other seven recombinant strains, consistent with the expected size of BTG plus a HIS-tag and a propeptide, indicating that BTG was also successfully expressed in the form of His_6-F-BTG in *E. coli*.

Purification and enzymatic cleavage of His_6-BTG and His_6-F-BTG

In this work, His_6-BTG and His_6-F-BTG were purified by the method of affinity chromatography, the elutions were dialyzed against the cleavage buffer and concentrated. The N-terminal of His_6-BTG and His_6-F-BTG contained a HIS-tag. To exclude potentially suppressive effects of the HIS-tag on the activity of BTG and avoid interference with the analysis of the suppressive effects of the propeptides on BTG activity, it was removed by the cleavage of thrombin. As shown in Fig. 3a, after the cleavage by thrombin, the relative molecular mass of His_6-BTG was decreased slightly

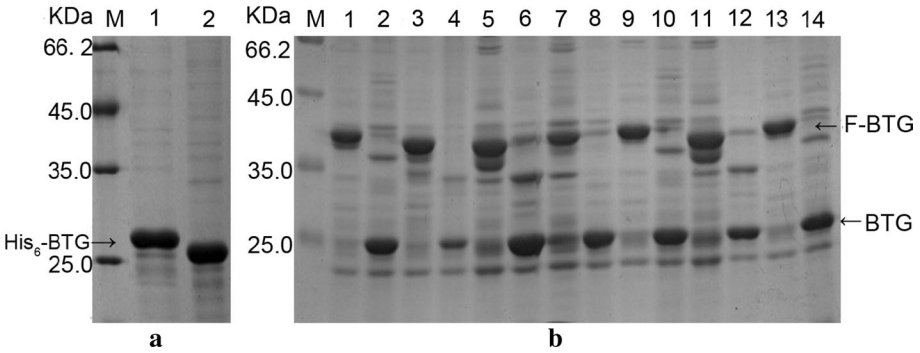

Fig. 3 SDS-PAGE analysis of purified recombinant proteins by proteolytic cleavage. The positions of the recombinant proteins before and after proteolytic cleavage are indicated by *arrowheads*. **a** *Lane M* protein MW marker, *lane 1* concentrated His_6-BTG, *lane 2* concentrated His_6-BTG after the cleavage by thrombin. **b** *Lane M* protein MW marker, *lanes 3*, *5*, *7*, *9*, *11*, *13*, *15* concentrated proC-BTG, proD-BTG, proF-BTG, proH-BTG, proM-BTG, proN-BTG and proP-BTG, *lanes 2*, *4*, *6*, *8*, *10*, *12*, *14* concentrated proC-BTG, proD-BTG, proF-BTG, proH-BTG, proM-BTG, proN-BTG and proP-BTG after the cleavage by factor Xa

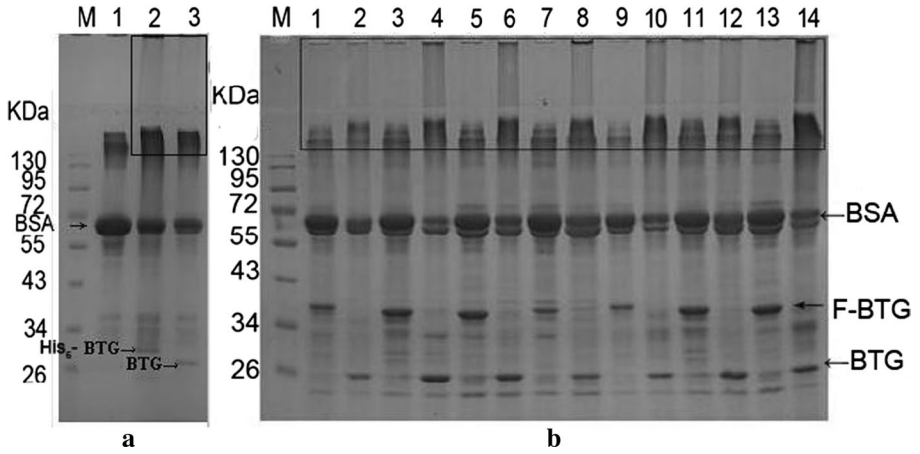

Fig. 4 Cross-linking of BSA by the recombinant proteins. Both the recombinant proteins with and without cleavage by thrombin or factor Xa were tested for cross-linking of BSA in 0.1 M Tris–HCl, 2 mM DTT, pH 8.0 at 50 °C for 2 h. The positions of the BSA preparation and the recombinant proteins before and after proteolytic cleavage are indicated by *arrowheads*. The area of the gel delimited by a *solid line* indicated cross-linked polymers of BSA**. a** *Lane M* protein MW marker, *lane 1* BSA control, *lane 2* BSA polymerization by His_6-BTG, *lane 3* BSA polymerization by cleaved His_6-BTG. **b** *Lane M* protein MW marker, *lanes 1*, *3*, *5*, *7*, *9*, *11*, *13* BSA polymerization by proC-BTG, proD-BTG, proF-BTG, proH-BTG, proM-BTG, proN-BTG and proP-BTG, *lanes 2*, *4*, *6*, *8*, *10*, *12*, *14* BSA polymerization by activated proC-BTG, proD-BTG, proF-BTG, proH-BTG, proM-BTG, proN-BTG and proP-BTG

and appeared only one new band at about 28 kDa (lane 2), indicating that His_6-BTG was cleaved at the specific site to remove the HIS-tag and convert BTG. His_6-F-BTG was also cleaved by thrombin to remove the HIS-tag and convert F-BTG (data not shown). Then, to test the suppressive effects of the propeptides on BTG activity, F-BTG activity both in the fractions with and without the removal of the propeptides would be measured and compared. As shown in Fig. 3b, after the cleavage by factor Xa, one new band appeared at the same position as BTG about 28 kDa (lanes 2, 4, 6, 8, 10, 12, 14) instead of the original bands about 38 kDa (lanes 1, 3, 5, 7, 9, 11, 13), indicating that F-BTG was cleaved to remove the propeptides and convert BTG.

Suppressive effects of the propeptides on BTG activity

To test the suppressive effects of the propeptides and the HIS-tag on BTG activity, BSA cross-linking assay was used. As shown in Fig. 4a, whether the removal of the HIS-tag or not, BTG exhibited the same activity for BSA cross-linking as reflected by the same concentration of polymerized BSA, indicating that the N-terminal HIS-tag of BTG showed no apparent suppressive effects on BTG activity. As shown in Fig. 4b, F-BTG showed very low activity for BSA cross-linking as reflected by the slight decrease of BSA concentration and the generation of small amounts of polymers. However, after the removal of the propeptides

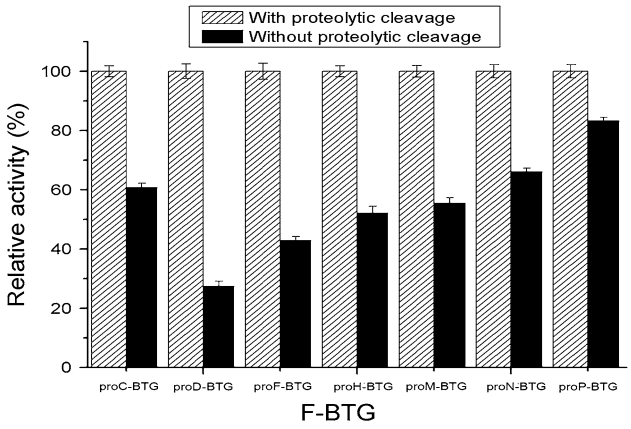

Fig. 5 Suppressive effects of the propeptides on the activity of BTG. F-BTG activity with and without cleavage by factor Xa was detected to analyze the inhibitory effects of the propeptides on the activity of BTG. F-BTG activity with proteolytic cleavage was taken as 100 %. Results were determined from triplicates

by factor Xa, BTG was shown to catalyze the formation of BSA polymers and consequent decrease of BSA concentrations, some of the polymerized BSA could not penetrate the stacking gels. The above observation strongly suggests that BTG activity for BSA cross-linking was increased significantly after the removal of the propeptides and the propeptides were capable of suppressing BTG activity.

Next, we adopted a fluorescence-based method to determine the activity of TGase quantitatively. The activities of His_6-BTG and F-BTG with and without proteolytic cleavage were measured and compared to calculate the inhibitory effects of the propeptides and the HIS-tag on BTG activity. Results indicated that the N-terminal HIS-tag of BTG had no apparent suppressive effects on BTG activity (data not shown). However, as shown in Fig. 5, all the propeptides examined exhibited suppressive effects toward BTG, and the propeptide proD showed the strongest suppressive effect on BTG activity, achieving a level of 70 % inhibition. We also found that the specific activity of BTG released from F-BTG was the same as the BTG expressed in an active form (220 mU/mg), demonstrating that BTG activity can be completely restored after the removal of the propeptides by factor Xa.

Analyses of dry cell weight and yield

Through the analyses of cell dry weight and BTG yield, we routinely found that the recombinant *E. coli* strain that expressed His_6-proD-BTG had the maximum dry cell weight (about 4.16 g/l), and the yield of the fusion protein was also the highest (about 18 mg/l). However, because of the lower dry cell weight attained (about 2.89 g/l) by the expression of BTG in an active form, the yield of BTG was lower, routinely about 8 mg/l. The above results indicate that compared with the expression of BTG in an active form, BTG expression in the form of proD-BTG can enhance BTG production in *E. coli.*

Secretion expression of BTG and proD-BTG in *C. glutamicum*

In order to verify whether the expression of BTG in the form of proD-BTG could improve BTG yield, we explored extracellular expression of BTG and proD-BTG in *C. glutamicum* by utilizing the signal peptide ΔS0949. We found that compared with recombinant strain pXSBTG1/13032 (20 mU/ml), strain pXSDTG1/13032 produced higher level of BTG expression (55 mU/ml), demonstrating that the extracellular expression of BTG in the form of proD-BTG also can improve BTG yield in *C. glutamicum.*

Discussion

BTG is considered to be a new type of TGase with different industrial value for food processing, but few studies have been conducted to increase its production, BTG was expressed in an active form in *E. coli* [10, 18]. However, the expression of active BTG will cause toxic effects on microbial hosts, leading to the relatively low yield of BTG. To reduce the toxicity of BTG on microbial hosts, improving BTG yield, we fused genes encoding the respective regions of transglutaminase propeptide from seven species of *Streptomyces* to the N-terminal of the BTG gene to produce F-BTG to suppress BTG activity. To quantitatively analyze the suppressive effects of the propeptides on BTG activity and screen the optimal propeptide, we adopted a fluorescence-based method to measure BTG activity for the first time, which was more simple, quick and secure than the previous isotope method [9, 20]. The analysis of the 3D-crystal structure of MTG has suggested that its propeptide covers the active site cleft and prevents the substrates from accessing the active site [11]. We demonstrated that all the fused propeptides could suppress BTG activity in varying degrees (Fig. 5), the propeptide proD showed the strongest suppressive effect on BTG activity. In addition, full BTG activity could be restored through the cleavage by factor Xa. The observed protective effects of propeptides on BTG activity may be ascribed to similar steric hindrance, the fusion of the propeptides and BTG may result in its folding into such a three-dimensional structure that it hinders the interaction of the substrates and the active center. Through the cleavage by factor Xa, the effect of the steric hindrance is removed with the propeptides separated from BTG and the propeptides have no effects on the correct

fold of BTG in *E. coli*, and thus the activity of BTG can be completely restored.

The fact that the cell density of the recombinant strain expressing proD-BTG was higher than that of the recombinant strain expressing active BTG, and the yield of proD-BTG (about 18 mg/l) was more than twofold of the yield of active BTG (about 8 mg/l). It supports our premise that the heterologous propeptide proD can be used to inhibit the toxic effects of BTG on microbial hosts and alleviate the pressure of BTG activity on their normal growth, resulting in the increase of cell density, thus improving BTG yields.

However, even the optimal propeptide proD could only suppress 70 % of BTG activity, the residual activity might still have a certain toxicity to the host cell. Thus, further improvement of BTG production level could be achieved by rational enzyme engineering through the analysis on three-dimensional structure of F-BTG to improve the suppressive effects of the protective propeptides on BTG activity, which was beneficial to attain higher cell density.

Utilizing the expression system of *E. coli*, a novel approach to increase the yield of BTG was developed. Then the approach was further verified by the extracellular expression of BTG and proD-BTG in *C. glutamicum*. We found that the expression of proD-BTG produced higher level of BTG (55 mU/ml) than that of BTG in an active form (20 mU/ml), proving the effectiveness of this approach, and thus the approach may be generally applicable to other heterologous strains for the expression of BTG.

In conclusion, this study develops a novel approach to increase BTG production in heterologous strains, thus providing a valuable platform for further optimization of large-scale BTG production.

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Conflict of interest We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled 'A novel approach for improving the yield of *Bacillus subtilis* transglutaminase in heterologous strains'.

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