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Extracellular production of *Streptomyces ladakanum* transglutaminase in a food-grade strain, *Bacillus subtilis*

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Research

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Lihong Fu[1,2,3], Jiansong Ju [3], Hamed M. El-Shora[4], Shuwen Zhang[1], Bo Yu[2], Limin Wang[1,2*], Jiaping Lv[1]

[1] *Key Laboratory of Agro-Products Processing，Ministry of Agriculture and Rural Affairs/ Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences, Beijing 100193, People's Republic of China*

[2] *CAS Key Laboratory of Microbial Physiological and Metabolic Engineering, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, PR China and State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100190, People's Republic of China*

[3] *College of Life Science, Hebei Normal University, Shijiazhuang 050024, PR China*

[4] *Department of Botany, Faculty of Science, Mansoura University, Dakahlia 35516, Egypt*

CORRESPONDENCE AUTHOR

L. Wang
E-mail: wanglimin@im.ac.cn
Phone/Fax: +86-10-64806132

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ABSTRACT

Background: Transglutaminase (TG) is an enzyme of the transferase family with cross-linking properties, which has been widely used in the food industry. Traditionally, TG is isolated from strains of *Streptomyces* sp. However, the development of a facile and efficient production of commercial TG is always desirable.

Purpose: In the current study, we described an efficient route for TG production in a food-grade strain of bacteria, *Bacillus subtilis.*

Method: Two strategies were employed for the extracellular production of *S. ladakanum* TG in *B. subtilis*. Sixteen signal peptides were optimized to secret TG into the extracellular medium. Site-directed mutageneses in pro-peptide were further utilized to improve the enzymatic activity. The enzymatic characteristics of *S. ladakanum* TG expressed in *B. subtilis* were analyzed.

Results: The N-terminal amino acids played important roles in enzymatic activity. Signal peptides of SacB (SP_{SacB}) and AbnA (SP_{AbnA}) showed good abilities to direct the secretion of TG into the medium. The enzyme was secreted into the medium and exhibited good Ca^{2+} stability and temperature stability, which were comparable to those produced by commercial strains. The enzymatic activity in the supernatant of culture reached 7.6 U/mg.

Conclusion: Our study demonstrated that *B. subtilis* may be a good candidate for the efficient and stable production of TG and has a much easier purification process.

Keywords: Transglutaminase (TG), *Streptomyces* TG, *Bacillus subtilis*, Food-grade strain.

1. INTRODUCTION

Transglutaminase (TG, EC 2.3.2.13) is an enzyme that catalyze the cross-linking between proteins, peptides and various primary amines [1]. It has a great commercial use in food industry. In recent years, it is also widely used in tissue engineering, textile and leather processing, biotechnology tools and other non -food fields, which increases the demand for TG [2]. TGs are distributed in various sources, including mammals, plants, microorganisms. It was firstly found in animal's tissues in 1973. But animal TG is rarely used in food industry, because of the red pigmentation and the property of calcium (Ca^{2+})-dependence [3, 4]. Microbial TG was discovered in bacteria belonging to the actinomycetes [5]. Microbial TG has low molecular weight (approximately 40 KDa) and is stable at a pH range of 4.5 to 8.0 [6]. It is Ca^{2+}-independent and could be obtained by conventional fermentation. Because some ingredients in food are easily precipitated by Ca^{2+}, Ca^{2+}-independent TG has a great potential in food industries [7]. Until now, many TG producing microorganisms have been identified, and some TGs were heterologously expressed in *Escherichia coli*, *Corynebacterium glutamicum* and *Streptomyces* species [6]. Although microbial sources of TGs allow for simplified extraction processes compared with those from animal sources, the development of an efficient and easy-to-use method for TG production, which requires less energy and provides economical savings, is highly desirable [8].

In *Streptomyces,* TG is synthesized as inactive zymogen containing the central body and an N-terminal pro-peptide and is then activated by the removal of pro-peptide. Apart from pro-peptide and central body, the N-terminal signal peptide plays an important role in targeting TG to the periplasm [9]. The obtain of soluble TG is one of the key problems for heterologous TG. It has been reported that expression of propeptide prior to that of TG was essential for the active TG production in *E. coli* [8]. *E. coli* is a popular host for heterologous protein expression. However, considering TG's application in food processing, *E. coli* is not a suitable host for TG production because of the formation of endotoxin [10]. Compared with *E. coli*, *Bacillus subtilis* is a commonly used host for secreted expression of heterologous proteins. It is recognized as GRAS (Generally Recognized as Safe) strain by FAD in the United States with the known genetic background [11]. Some wild-type *B. subtilis* strains can produce TG, but the yield is too low to be industrial used [12]. It has been reported that *S. mobaraensis* TG expressed in *B. subtilis* showed similar enzymatic activities to that produced from *E. coli* and catalyzed the cross-linking reactions [13]. In a previous report, we demonstrated that active-form *S. mobaraensis* TG was efficiently secreted by *B. subtilis* when a modified *Saccharomyces cerevisiae* vacuolar ATPase subunit (VMA) intein was introduced into TG zymogen [14]. In this study, two strategies were employed to express *S. ladakanum* TG in *B. subtilis*. Sixteen signal peptides were optimized to secret TG into the extracellular medium. Site-directed mutageneses in pro-peptide were further utilized to improve the enzymatic activity. Our study demonstrated that *B. subtilis* may be a good candidate for the efficient and facile production of TG.

2. MATERIALS AND METHODS

2.1. Strains and chemicals

Bacterial strains and plasmids used in the study are listed in Table 1. *S. ladakanum* (China Center of Industrial Culture Collection, CICC 11018) was a gift from Prof. Chunbo Lou, Institute of Microbiology CAS, China. The *B. subtilis* strain WB600 (Novagen Company, Shanghai, China) and *Bacillus subtilis* 168 were stock preserved and maintained in our laboratory. Competent *E. coli* TOP10 was purchased from Tiangen Co., China. Plasmid pWB980 was purchased from Novagen Company, Shanghai, China. Plasmid pMD19-T was purchased from TaKaRa Company, Beijing, China. Kanamycin (Km) and ampicillin (Amp) were purchased from Tiangen Co., China, and added to the medium at a final concentration of 20 μg/mL and 100 μg/mL, respectively, whenever necessary. The restriction enzymes and DNA modifying enzymes were all purchased from TaKaRa Co., Ltd, China. The Clone Express® MultiS One Step Cloning Kit was purchased from Vazyme Biotech Co., Ltd, China. Other chemicals and reagents were purchased from Sigma-Aldrich (Shanghai, China).

Table 1. Bacterial strains and plasmids used in this study.

Strains	Genotype/properties	Source
Escherichia coli top10	Cloned strain; *mcr*A Δ (*mrr-hsd* RMS-*mcr*BC) *φ*80 *lacZ* ΔM15 Δ*lac*X74 *rec*A1 *ara* Δ139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps* (*Strr*) *end*A1 *nup*G	Tiangen
Bacillus subtilis 168	Cloning of promoters (P_{abrB}, P_{spoVG}, P_{yabR}, P_{rsbW}, P_{gsiB}, P_{yqfD}, P_{sigW}, P_{ftsZ}, P_{yvyD}) encoding genes; wild-type	Our laboratory
Bacillus subtilis WB600	*Bacillus subtilis* 168 derivate,Δ*nprE* Δ*aprE* Δ*epr* Δ*bpr* Δ*mpr* Δ*nprB*; Protease deficiency type, expression host strain	Our laboratory
Streptomyces ladakanum	Cloning of *Lpro* gene； wild-type	A gift from Prof. Chunbo Lou
Plasmids		
pMD19-T	Efficient TA cloning vector constructed based on pUC19; Ap^r	TaKaRa
pMD19-T-*LTG*	pMD19-T carrying *TG* gene from *S. ladakanum*; Ap^r	This work
pMD19-T-$LTG_{DSD80\text{-}82AAA}$	pMD19-T carrying *TG* gene from *S. ladakanum* with DSD80-82AAA mutation; Ap^r	This work
pMD19-T-$LTG\Delta_{PS}$	pMD19-T carrying *TG* gene with PS deletion from *S. ladakanum*; Ap^r	This work
pMD19-T-$LTG_{SGS34\text{-}36AAA}$	pMD19-T carrying *TG* gene with SGS34-36AAA	This work
pMD19-T-$LTG_{PS74\text{-}75AA/DSD80\text{-}82AAA}$	pMD19-T carrying *TG* gene with PS74-75AA/	This work
pWB980	Expression plasmid with signal peptide *SacB* in *B. Subtilis*; Km^r	Novagen
pWB_{sacB}-*LTG*	pWB980 carrying *TG* gene from *S. ladakanum*; Km^r	This work
pWB_{sacB}-pro-*sacB-LTG*	pWB980 carrying the genes encoding SP_{sacB}, pro-peptide, SP_{sacB} and TG from *S. ladakanum*; Km^r	This work
pWB_{sacB}-$LTG_{DSD80\text{-}82AAA}$	pWB980 carrying *TG* gene from *S. ladakanum* with DSD80-82AAA mutation; Km^r	This work
pWB_{sacB}-$LTG\Delta_{PS}$	pWB980 carrying *TG* gene from *S. ladakanum* with PS deletion; Km^r	This work
pWB_{sacB}-$LTG_{PS74\text{-}75AA}$	pWB980 carrying *TG* gene from *S. ladakanum* with PS74-75AA mutation; Km^r	This work
pWB_{sacB}-$LTG_{SGS34\text{-}36AAA}$	pWB980 carrying *TG* gene from *S. ladakanum* with SGS34-36AAA mutation; Km^r	This work
pWB_{sacB}-$LTG_{PS74\text{-}75AA/\ DSD80\text{-}82AAA}$	pWB980 carrying *TG* gene from *S. ladakanum* with PS74-75AA/ DSD80-82AAA mutation; Km^r	This work
pWB_{n}-$LTG_{PS74\text{-}75AA/\ DSD80\text{-}82AAA}$	pWB_{sacB}-$LTG_{PS74\text{-}75AA/DSD80\text{-}82AAA}$ carrying SP_n (15 signal peptides) instead of SP_{sacB}; Km^r	This work

Ap^r, ampicillin resistance**;** Km^r, kanamycin resistance.

2.2. Construction of TG expression plasmids

The primers used in the study are listed in Table S1. The sequence of TG gene from *S. ladakanum* was deposited in the GenBank database under accession number AAO48277.1. Two separate TG secretion systems were constructed in *B. subtilis*. The first one involved constitutive expression of the *TG* gene encoding the pro-peptide domain and central body of *S. ladakanum* TG with signal peptide SacB (SP_{sacB}) (Fig. 1A). *TG* gene was amplified from *S. ladakanum* genomic DNA by polymerase chain reaction (PCR) using primers TG-F/TG-R. The amplified DNA fragment and the pWB980 expression vector containing SP_{sacB} were each double-digested with *Xma*I and *Xba*I and ligated to generate plasmid pWB_{sacB}-*LTG* .

For the second system, gene encoding the pro-peptide domain of TG was PCR amplified using primers ProTG-F/ProTG-R and the gene encoding the central body was PCR amplified using primers CD-F/CD-R. Each PCR product was paired with a preceding *sacB* secretory sequence. The *sacB* gene was PCR amplified from plasmid pWB980 using primers SacB-F/ SacB-R. The PCR products of the three genes were ligated by overlap extension PCR using primers ProTG-F/CD-R. The DNA fragment was double-

digested with *Xma*I and *Nhe*I and ligated into the *sacB* signal peptide-containing pWB980 expression vector that had been treated with the same enzymes, generating plasmid pWB$_{sacB}$-pro-*sacB-LTG* (Fig. 1B).

Plasmids pWB$_{sacB}$-*LTG* and pWB$_{sacB}$-pro-*sacB-LTG* were transformed into *B. subtilis* WB600, respectively. Competent cells of *B. subtilis* were prepared and transformed as described [14]. The recombinant *B. subtilis* strains were grown at 37 °C for 12 h in Luria-Bertani (LB) medium with 20 μg/mL Km. The positive clones were selected for colony PCR amplification and DNA sequencing. The molecular weight of the TG was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. Site-directed mutagenesis construction

The pro-peptide region folded into an L-shape to cover the active site. So, N-terminal residues played important roles in enzymatic activity. The candidate mutation sites of Ser^{34}–Ser^{36} and Pro^{74}–Ser^{75} in the pro-peptide region and mutation sites of Asp^{80}–Glu^{83} in the junction between pro-peptide and corn domain were selected in this study (Fig. 2). Site-directed mutations were carried out by PCR-based overlap extension mutagenesis [15]. Briefly, the $Asp^{80}Ser^{81}Asp^{82}$→$Ala^{80}Ala^{81}Ala^{82}$ substitution (DSD80–82AAA) was completed using primers DSD-F/DSD-R. The deletion of Pro^{74}–Ser^{75} (ΔPS) was completed using primers ΔPS-F/ΔPS-R. The $Pro^{74}Ser^{75}$→$Ala^{74}Ala^{75}$ substitution (PS74–75AA) was completed using primers PS-F/PS-R. The $Ser^{34}Gly^{35}Ser^{36}$→$Ala^{34}Ala^{35}Ala^{36}$ substitution (SGS34–36AAA) was completed using primers SGS-F/SGS-R. Plasmid pMD19-T-*LTG* was used as a template. The fragments obtained by overlap PCR were ligated into vector pMD19-T, yielding plasmid pMD19-T-*LTG*$_{DSD80-82AAA}$, pMD19-T-*LTG*Δ$_{PS}$, pMD19-T-*LTG*$_{SGS34-36AAA}$, and pMD19-T-*LTG*$_{PS74-75AA}$, respectively. The mutations were verified by DNA sequencing. The correct fragments were digested by *Xma*I/*Xba*I and inserted into *Xma*I/*Xba*I-digested pWB980, yielding pWB$_{sacB}$-*LTG*$_{DSD80-82AAA}$, pWB$_{sacB}$-*LTG*Δ$_{PS}$, pWB$_{sacB}$-*LTG*$_{PS74-75AA}$, and pWB$_{sacB}$-*LTG*$_{SGS34-36AAA}$, respectively.

Multi-mutation at positions $Pro^{74}Ser^{75}$ and $Asp^{80}Ser^{81}Asp^{82}$ (PS74–75AA/DSD80–82AAA) was completed using primers PD-F/PD-R with plasmid pMD19-T-*LTG*$_{PS74-75AA}$ as template. The mutations were verified by DNA sequencing, and the correct DNA product was transformed into *E. coli* TOP10 to obtain plasmid pMD19-T-*LTG*$_{PS74-75AA/DSD80-82AAA}$. Plasmid pMD19-T-*LTG*$_{PS74-75AA/DSD80-82AAA}$ were digested by *Xma*I/*Xba*I and inserted into *Xma*I/*Xba*I-digested pWB980, yielding pWB$_{sacB}$-*LTG*$_{PS74-75AA/DSD80-82AAA}$. The recombinant plasmid was transformed into *B. subtilis* WB600 as described [14]. The positive clones were selected for colony PCR amplification and DNA sequencing.

2.4. Signal peptide optimization

The construction of plasmids containing different signal peptides (SP) were shown in Fig. S1. Signal peptides used in the study are listed in Table S2. The encoding genes of SP$_{wapA}$, SP$_{epR}$, SP$_{nucB}$, SP$_{yncM}$, SP$_{yhcR}$, SP$_{wprA}$, SP$_{amyE}$, SP$_{lytD}$, SP$_{penP}$, SP$_{abnA}$, SP$_{nprB}$, SP$_{bglS}$, SP$_{motB}$, SP$_{lipB}$ and SP$_{lipA}$ were PCR-amplified from the genome of *B. subtilis* W168 using primers WapA-F/WapA-R, EpR-F/EpR-R, NucB-F/NucB-R, YncM-F/YncM-R, YhcR-F/YhcR-R, WprA-F/WprA-R, AmyE-F/AmyE-R, LytD-F/LytD-R, PenP-F/PenP-R, AbnA-F/AbnA-R, NprB-F/NprB-R, BglS-F/BglS-R, MotB-F/MotB-R, LipB-F/LipB-R, and LipA-F/LipA-R, respectively. Then, the PCR products were digested and inserted into *Bsa*BI-*Xma*I-cleaved pWB$_{sacB}$-*LTG*$_{PS74-75AA/DSD80-82AAA}$, yielding pWB$_{n}$-*LTG*$_{PS74-75AA/ DSD80-82AAA}$ (n denotes 15 signal peptides).

2.5. Expression of TG in *B. subtilis*

B. subtilis strains carrying the recombinant plasmids were inoculated into 5 mL LB medium containing 20 μg/mL Km and cultured overnight at 37 °C with shaking at 200 rpm. The seed cultures were inoculated at 2% (*v*/*v*) into the fresh LB medium containing 20 μg/mL Km and cultured at 37 °C, 200 rpm. Samples were taken at 48 h, and supernatants were collected following centrifugation at 4000 × *g* for 10 min. The clarified supernatants were evaluated for enzymatic activity.

2.6. Activity assay of TG

TG activity was measured by a modification method of calorimetric hydroxamate [16]. Enzyme solution (50 μL) was mixed with 50 μg/mL dispase, and incubated at 37 °C for 20 min. Then, the mixture was added with 90 μL of reagent A (0.2 M Tris-HCl (pH 6.0), 30 mM CBZ-Gln-Gly, 100 mM hydroxylamine, and 10 mM glutathione), and incubated at 37°C for 10 min. The reaction was stopped by adding 160 μL of reagent B (a, 3 M HCl; b, 12% TCA; c, 5% ferric chloride–trichloroacetic acid; a:b:c=1:1:1). The supernatant was collected by centrifugation at 4000 × *g* for 10 min, and the absorbance at 525 nm was measured to determine the TG activity. One unit of microbial TG activity was defined as the amount of enzyme needed to produce 1 μmol of hydroxamic acid per min. Protein concentration was determined using a Bradford protein assay kit (Bio-Rad, 17).

2.7. Optimal pH and temperature

The optimal pH was assayed using the following various buffers (0.2 M): sodium phosphate-citrate buffer (pH 3, pH 4, and pH 5) and Tris-HCl (pH 6, pH 7, pH 8, and pH 9) at 37 °C. The optimal temperature was assayed in Tris-HCl, pH 6 at 20 °C, 30 °C, 40 °C, 50 °C, 60 °C, and 70 °C. The relative activities were expressed as percentages of the activity at the optimal temperature or pH [18]. All tests were repeated at least three times and the data were expressed as mean ± standard deviation (SD). The highest activity was defined as 100%.

2.8. The effects of metal ions on TG activity

Various metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}) with a final concentration of 5 mM were added to the enzyme solution. The mixtures were incubated for 30 min on ice prior to performing the enzyme assay. The enzymatic activity with no metal ion was set to 100%. To determine the effect of Ca^{2+} concentration on the activity of TG, different concentrations of calcium chloride ($CaCl_2$) was added to the enzyme solution (0 mM, 5 mM, 10 mM, 15 mM, 20 mM, and 25 mM). The mixtures were incubated for 30 min on ice prior to performing the enzyme assay [18]. All tests were repeated at least three times and the data were expressed as mean ± SD.

2.9. TG production in a fermentor

High-level production of TG was carried out in a 5-L bioreactor (NBS BioFlo 310, Eppendorf, Shanghai, China) with a working volume of 2 L. The pH was adjusted to 7.0. The dissolved oxygen was related to speed, and it was higher than 15% during fermentation. Seed culture (1%, *v/v*) was inoculated into fresh LB medium containing 20 μg/mL Km. Samples were collected periodically to determine cell density (OD_{600}). The clarified supernatants were measured for enzymatic activity.

3. RESULTS AND DISCUSSION

3.1.Synthesis of TG in *B. subtilis* without its native signal peptide

S. ladakanum is a TG producer . *S. ladakanum* TG is Ca^{2+}-independent and is stable at pH 5.0~7.0 [19]. Crystal structure showed that *Streptomyces* TG contained signal peptide, pro-peptide, and central body (Fig. 2A). To produce secreted TG in *B. subtilis, B. subtilis* WB600, a strain deficient in six extracellular proteases [20], was used as host, and two separate secretion systems were constructed. As shown in Fig. 1A, the original signal peptide in *S. ladakanum* TG was replaced with the *sacB* signal peptide in the first strategy. The gene (1172 bp) encoding the pro-peptide domain and central body of *S. ladakanum* TG was fused with *sacB,* and the recombinant plasmid was transformed into *B. subtilis* WB600 (Fig. S1A). A protein band was detected in the supernatants of *B. subtilis* WB600 carrying plasmid pWB_{sacB}-*LTG* in SDS-PAGE (Fig. S1B), which suggested that TG was successfully expressed extracellularly. Extracellular enzyme activity was determined to be as high as 1.62 U/mg (Table 2).

Because the order of expression of the pro-peptide and central body plays an important role in the reconstitution of TG and a successful example has been reported in *E. coli* [8], in the second strategy we paired each part of the pro-peptide region and central body region with a preceding *sacB* signal peptide (Fig. 1B). However, this expression system failed to produce active TG. No protein band was detectable by SDS-PAGE nor was any enzymatic activity detected in the cell supernatants (data not shown). The reason maybe that pro-peptide functions in an intermolecular manner to mediate TG folding. For the first strategy, pro-peptide and core domain secreted simultaneously. However, for the second strategy, pro-peptide and core domain were secreted outside cells separately. Wrong folding may occur, which resulted in no enzymatic activity detection. Based on our findings, the first expression system encoding the *sacB* signal peptide, pro-peptide, and central body was used in the subsequent studies. These findings also indicated that the *sacB* signal peptide could direct TG to be secreted into the periplasm.

Currently, TG has been identified from various microorganisms, and has been heterologously expressed in different strains. However, only *Streptomyces* TG has commercial value [10]. Microbial TG has received increasing interest for food treatment and has been shown to improve food flavor, appearance and texture [21]. The complexity of the current procedures prompted scientists to develop an efficient and easy-to-use system for the "green" production of TG. It has been reported that in *Streptomyces* sp., TG is secreted as an inactive zymogen in cultures and is activated during culture [22]. Obtaining soluble TG is a key problem for the recombinant expression. Although *Streptomyces* TG has been successfully expressed in *E. coli* by sequence expressing pro-peptide and central body [8], the pre-expression of pro-peptide does not result in soluble TG expression in this study. The reason may be the wrong folding of soluble TG [22]. *B. subtilis* is classified as a GRAS organism and is used as a secretion host for large amounts of commercial enzymes. The secretion of recombinant proteins into the culture medium greatly simplified downstream processing [23, 24]. In our previous study, active *S. mobaraense* TG was secreted by *B. subtilis* through the introduction of VMM into zymogen [14]. *S. mobaraense* TG was also secreted by *B. subtilis* through the Tat pathway [13]. These studies indicated that *B. subtilis* was a promising host for TG production.

The signal peptide was responsible for TG transport across the cytoplasmic membrane. However, it has been reported that heterologous *Streptomyces* TG using *E. coli* as a host was expressed as inclusion bodies with its native signal peptide [25]. In *B. subtilis*, the Sec pathway is the major route of protein transport [23]. To produce extracellular *S. ladakanum* TG in *B. subtilis*, the *sacB* signal peptide (SP_{sacB}) was used to replace its native signal peptide (Fig. 1). With the direction of SP_{sacB}, TG was released into extracellular medium of *B. subtilis*. A single band was detected in SDS-PAGE (Fig. S1).

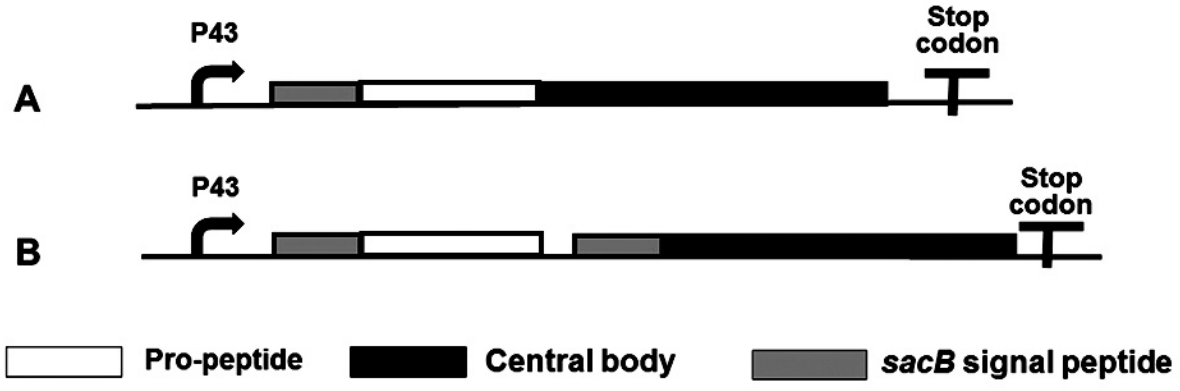

Fig. 1. Genetic organization for the construction of two separate TG secretion systems. Construction of plasmid pWB_{sacB}-*LTG* (A). **C**onstruction of plasmid pWB_{sacB}-pro-*sacB-LTG* (B).

3.2. Involvement of the N-terminal region of TG in increasing enzyme activity

To further increase the enzyme activity in this study, site -directed mutagenesis was used. *S. mobarensis* TG was used as model since it is currently the type of TG used commercially and its structure has been extensively investigated [26]. Candidate mutation sites were selected based on sequence homology and structural analysis of *S. mobaraensis* TG (PDB: 3IU0), an enzyme highly homologous to *S. ladakanum* TG sharing 88% identity at the amino acid level. Studies have shown that the pro-peptide in the N-terminal region of *S. mobarensis* TG are the most important for achieving active and soluble enzyme [27]. It folded into an L-shape and covered the active-site cleft in a complementary manner [26]. It is assumed to act as an intermolecular chaperone to stimulate TG folding, enzymatic activity, or secretion [16]. Therefore, the site-mutations in the current study were all within the pro-peptide region (Ser^{34}–Ser^{36} and $Pro^{74}Ser^{75}$) and at the junction between the pro-peptide and central body (Asp^{80}–Asp^{82} and Glu^{83}) (Fig. 2B). Ala has propensity to form alpha helices but can also occur in beta sheets. It is generally an accepted single residue first choice for mutations because it may not cause changes in polar and differently charged. So, it was chosen for site-directed mutations in this study. The results in Table 2 demonstrated that the changes in the N-terminal residues indeed affect the activity of TG. Although the contents of extracellular proteins of different mutations maintained at 0.24~0.27 mg/mL, there was a difference in enzymatic activities of mutations. The mutation SGS34–36AAA in the N-terminal of the pro-peptide showed little decrease in enzymatic activity in the supernatant with 97% of the activity remaining. However, the mutation DSD80–82AAA at the junction of the pro-peptide and central body resulted increase in enzymatic activity with 98% higher than that of the wild type enzyme. Similarly, the Pro^{74}–Ser^{75} deletion (ΔPS) showed improved activity with a 125% increase compared to that of the wild type. In the same way, the pro-peptide mutant PS74–75AA also displayed improved TG activity. Multi-mutations at positions Pro^{74}, Ser^{75}, and $Asp^{80}Ser^{81}Asp^{82}$ (PS74–75AA/DSD80–82AAA) resulted in the highest enzymatic activity of 5.84 U/mg. This was 261% higher than that of the wild type enzyme. The *B. subtilis* strain transformed with the gene encoding the mutated $TG^{PS74–75AA/DSD80–82AAA}$ was used in further study, and its enzymatic characteristics were systematically evaluated to explore its potential utilization.

The deletion or substitution at positions Asp^{80}–Asp^{82} and $Pro^{74}Ser^{75}$ resulted in good performance in our study. Enzymatic activity was efficiently improved, especially in the case of multi-mutations, which exhibited activity levels 361% compared to that of the wild type enzyme (Table 2). The results obtained in this study provide clear evidence that mutations in the pro-domain affect TG activity. Several proteases, including subtilisins and TG, require the N-terminal pro-peptide to produce mature enzymes [28]. Although pro-peptide is essential only during the late stages of the folding process, a mutated pro-peptide may result in an altered conformation of protease and affect its bioactivity. This phenomenon is known as “protein memory” [29]. Therefore, mutations in the pro-peptide region may make changes in the structure of the mature TG resulting in high enzymatic activities being obtained in these mutants. In the current study, substitutions in Asp^{80}–Asp^{82} (DSD80–82AAA), which were located at the junction between the pro-peptide and central body, may have affected the association of the central TG region with the pro-peptide resulting in the enhancement of the enzyme activity. Our results clearly demonstrated that engineering of the pro-peptide region is an efficient method for modifying enzymatic characteristics.

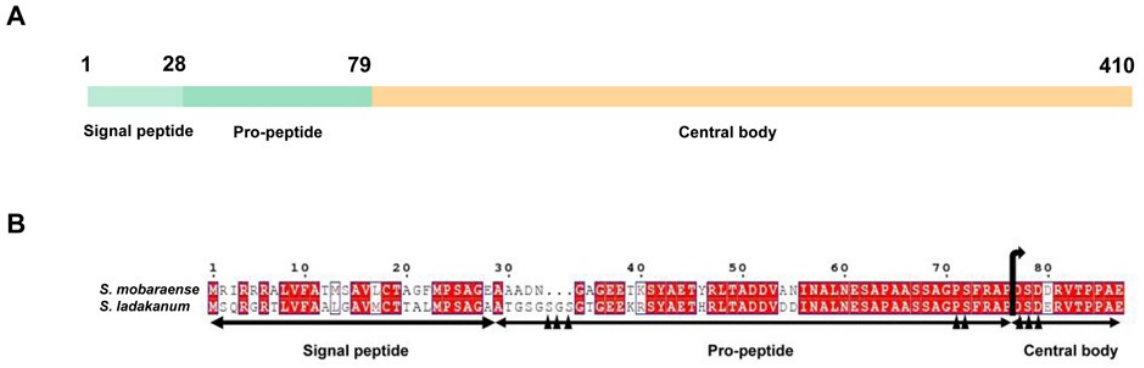

Fig. 2. Schematic representation of *S. ladakanum* TG (A). Homology of the N-terminal region of TG from *S. ladakanum* and *S. mobaraense* (B). Triangle indicates the mutation sites.

Table 2. Relative amounts of TG accumulation in the culture supernatant of *B. subtilis*[a].

Type	Feature	Enzyme activity (U/mg)	Extracellular protein content (mg/mL)	Relative amount[b] (%)	Region in TG
WT[b]	Wild type	1.62 ± 0.08	0.23 ± 0.02	100	—
DSD80-82AAA	$Asp^{80}Ser^{81}Asp^{82}$→Ala substitution	3.21 ± 0.03	0.26 ± 0.05	198	Conjunction
ΔPS	Pro^{74} to Ser^{75} deletion	3.65 ± 0.02	0.27 ± 0.03	225	Pro-peptide
PS74-75AA	Pro^{74} Ser^{75}→Ala substitution	4.32 ± 0.04	0.25 ± 0.01	267	Pro-peptide
SGS34-36AAA	Ser^{34} $Gly^{35}Ser^{36}$→Ala substitution	1.57 ± 0.04	0.24 ± 0.03	97	Pro-peptide
PS74-75AA/ DSD80-82AAA	Substitutions of Pro^{74} Ser^{75}→Ala and $Asp^{80}Ser^{81}Asp^{82}$→Ala	5.84 ± 0.06	0.25 ± 0.04	361	Multi-mutations

[a] Results are the average of duplicates.
[b] The enzyme without mutation sites was named as WT.
[c] The enzyme activity of mutation (U/mg) /that of mild type. The enzymatic activity produced by wild type was set as 100%.

3.3. Effects of signal peptide on TG activity in *B. subtilis*

In order to improve the production of extracellular TG in *B. subtilis,* 16 signal peptide from *B. subtilis* 168, including SP_{sacB}, SP_{wapA}, SP_{epR}, SP_{nucB}, SP_{yncM}, SP_{yhcR}, SP_{wprA}, SP_{amyE}, SP_{lytD}, SP_{penP}, SP_{abnA}, SP_{nprB}, SP_{bglS}, SP_{motB}, SP_{lipB} and SP_{lipA}, were used to replace the original signal peptide of *S. ladakanum* TG. The genes encoding the pro-peptide and core domain of *S. mobaraens* TG were fused with SP_n (n denotes 16 signal peptides) into plasmid pWB980, under the control of the constitutive promoter P_{43} (Fig. S1). As shown in Fig. 3, different signal peptides showed different secretion efficiency. SP_{SacB} and SP_{AbnA} showed better abilities to direct the secretion of TG into the medium than other SPs. The enzymatic activities in the supernatants of strains with SP_{wapA}, SP_{epR}, SP_{nucB}, SP_{wprA}, SP_{amyE}, SP_{lytD}, SP_{penP}, SP_{motB}, SP_{lipB} and SP_{lipA} were only 0.8% to 2% compared to those with SP_{SacB} and SP_{AbnA}. Our results indicated that SP_{wapA}, SP_{epR}, SP_{nucB}, SP_{wprA}, SP_{amyE}, SP_{lytD}, SP_{penP}, SP_{motB}, SP_{lipB} and SP_{lipA} could not complete the secretion of TG in *B. subtilis*. Although SP_{yncM}, SP_{yhcR}, SP_{nprB}, and SP_{bglS} could secrete TG outside the cell, the TG activity of the culture supernatant with SP_{yncM}, SP_{yhcR}, SP_{nprB}, and SP_{bglS} were only approximately 30% compared to those with SP_{SacB}. Although SP_{SacB} and SP_{AbnA} showed better abilities than other SPs. Considering SP_{sacB} is a commonly used levansucrase SP in *B. subtilis* [14], it was selected for further study. After shake-flask fermentation for 48 h, the TG activity of the culture supernatant was 5.98 U/mg .

In general, there is a hydrophobic signal peptide (SP) at the N-terminal of the secreted protein, which leads the protein to be secreted into the cell periplasm. SP is composed of a positively charged N-terminal domain, a long hydrophobic H-domain and a C-terminal domain, which is the signal peptidase recognition site [30]. The optimal match between SP and the target protein is very important. It is critical to the optimization of SP for the heterologous protein. *S. mobaraensis* TG was expressed in *B. subtilis* using SP_{wapA} and SP_{amyQ} [13]. Our study demonstrated that for *S. ladakanum* TG, the SP_{sacB} and SP_{abnA} were more suitable than SP_{wapA} (Fig. 3). Furthermore, SP_{amyQ} was also been detected for TG secretion and our results showed that SP_{sacB} and SP_{abnA} were better than SP_{amyQ} (data not shown).

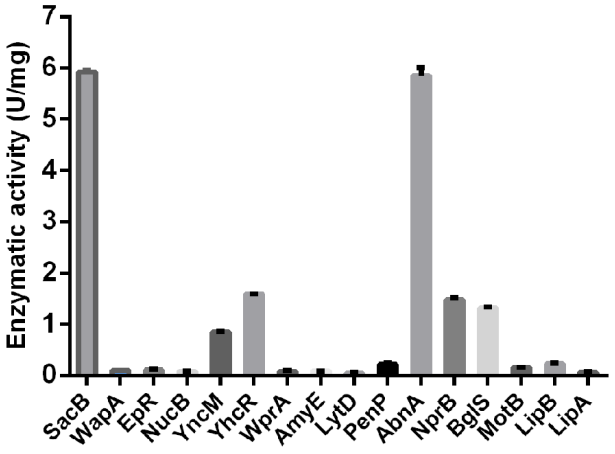

Fig. 3. Effects of signal peptides on TG secretion.Error bars represent SD calculated from three independent determinations.

3.4. Enzymatic characterization of TG expressed in *B. subtilis*

The extracellular TG expressed in *B. subtilis* exhibited optimum activity at pH 6.0. The activity gradually decreased at pH 9.0 and rapidly decreased at pH 4.0, indicating that this enzyme was not tolerant under acidic conditions. The extracellular TG expressed in *B. subtilis* was relatively stable at pH 6.0–8.0 (Fig. 4A). It exhibited high activity at 50 °C. The enzyme activity decreased rapidly when temperature was higher than 50 °C. Only approximately 30% activity remained when temperature was increased to 60 °C (Fig. 4B). Ca^{2+}, Na^{+} and Mg^{2+} had little influence on the activity of TG. The presence of K^{+} and Mn^{2+} could maintain the activity of TG at approximately 90%, but Cu^{2+} and Zn^{2+} significantly inhibited TG activity, especially Zn^{2+}. The relative activity was only 9.8% (Fig. 4C). The TG expressed in *B. subtilis* required no Ca^{2+} for activity. It was highly stable in the presence of Ca^{2+} and exhibited 113% activity in 25 mM Ca^{2+} (Fig. 4D).

Similar to that of the wild-type *Streptomyces* TG, *S. ladakanum* TG expressed in *B. subtilis* is Ca^{2+}-independent, and its activation requires no special cofactors, which is highly beneficial in industrial applications. For instance, TG could be used to modify some Ca^{2+} sensitive proteins, such as soybean globulins. Because these proteins are easily precipitated by Ca^{2+}, Ca^{2+} cannot be used in this food [1, 31]. The enzyme obtained in our study exhibited stable activity in the absence of Ca^{2+} and therefore can be used to modify proteins that are sensitive to Ca^{2+}. Furthermore, the presence of Ca^{2+} did not inhibit the activity of *S. ladakanum* TG expressed in *B. subtilis* (Fig. 4), suggesting *S. ladakanum* TG expressed in *B. subtilis* may also be used to modify proteins containing Ca^{2+}. Taken together, it appears that *S. ladakanum* TG expressed in *B. subtilis* is likely to have a broad range of applications.

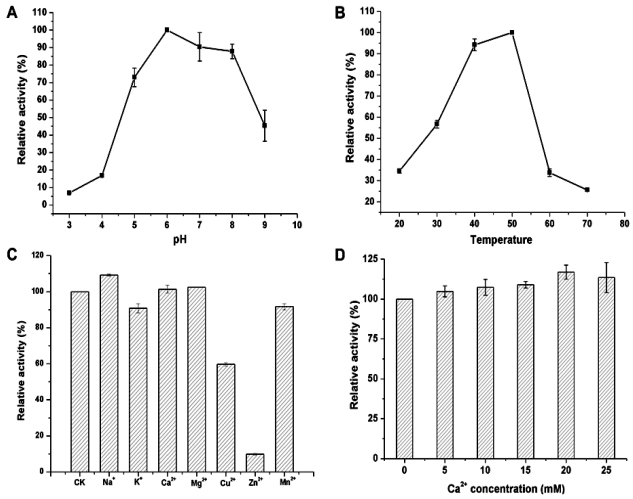

Fig. 4. Analysis of enzymatic characteristics. Optical pH of TG expressed in *B. subtilis* (A). Optical temperature of TG expressed in *B. subtilis* (B). Effects of different metal ions on enzyme activity (C). Effects of Ca^{2+} on enzyme activity (D). The highest activity was defined as 100%. Error bars represent SD calculated from three independent determinations.

3.5. Production of extracellular TG in *B. subtilis*

The *B. subtilis* transformant containing the optimal gene encoding $TG^{PS74–75AA/DSD80–82AAA}$ with SP_{sacB} was evaluated for small-scale TG production in a 5-L fermentor. As shown in Fig. 5, during the first 16 h, *B. subtilis* was in logarithmic growth stage. The OD_{600} value increased significantly and reached 64 at 21 h. Extracellular activity of TG in the culture broth steadily increased in a linear fashion during the first 48 h and reached a maximum of 7.60 U/mg at 48 h. Interestingly, the TG expressed in *B. subtilis* demonstrated good stability during fermentation compared with that of the *Streptomyces* TG. The maximum enzymatic activity at 48 h was set as 100% and it retained a large percentage of its initial activity (60%) at 60 h. Importantly, the 60% activity remained with increased incubation time (data not shown).

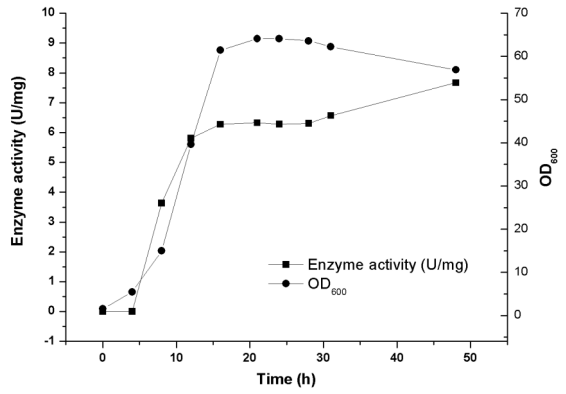

Fig. 5. TG production in *B. subtilis*. Error bars represent SD calculated from three independent determinations.

4. CONCLUSION

This study presents the secretion of *S. ladakanum* TG in *B. subtilis*. After replacing the native signal peptide with SP_{sacB} and site-directed mutagenesis, an activity of 7.60 U/mg was obtained in the supernatant of culture at 48 h. The enzyme from our current study is Ca^{2+}-independent and is stable at a wide range of pH, which are comparable to that of currently used commercial TG. This system could secret TG into the culture medium, which simplified downstream processing and may be useful for producing easily purified TG with widespread application.

Competing interests

The author hereby declares that there were no competing interests in this research.

Authors contributions

LF and LW performed the experiment. JJ, HE, SZ, BY and JL performed data analysis. LW wrote the paper. All authors read and approved the final manuscript.

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Supplementary Data

https://www.siftdesk.org/articles/images/10678/supplementary-files.pdf

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