Intein-mediated intracellular production of active microbial transglutaminase in *Corynebacterium glutamicum*

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Abstract

Background The microbial transglutaminase (mTGase) from *Streptomyces mobaraense* has been widely used in the food industry. Recombinant production of mTGase is tricky because the mTGase is synthesized as an inactive zymogen, which needs to be activated by proteolytic processing. Self-cleaving inteins have been applied to activate the zymogen in a simple and highly specific manner as compared with proteolytic processing. However, self-cleaving inteins suffer from the inherent problem of premature cleavage. Moreover, self-cleaving inteins normally require an additional step of long time incubation to induce the cleavage. These two inherent problems limit self-cleaving inteins for their potential application in the production of mTGase.

Results In this study, the premature cleavage of intein *Ssp* DnaB was observed in *Corynebacterium glutamicum* when the *Ssp* DnaB was used to activate mTGase precursor protein. Rather than suppressing it, the premature cleavage was applied to produce active mTGase in *C. glutamicum*. The SDS-PAGE analysis and the mTGase activity assay indicated that the premature cleavage of intein *Ssp* DnaB was successfully applied to activate the mTGase intracellularly in *C. glutamicum*. The subsequent N-terminal amino acid sequencing and site-directed mutagenesis studies demonstrated that the premature cleavage activated the mTGase intracellularly in a highly specific manner. Finally, in a jar fermentor, the intracellular mTGase activity was up to 49 U/mL, which was the highest intracellular mTGase activity ever reported.

Conclusions An efficient and simple approach with great potential for large-scale industrial production of active mTGase was presented in this study. This approach employed premature cleavage of intein *Ssp* DnaB to activate mTGase in *C. glutamicum*, resulting in high-level intracellular production of active mTGase. Moreover, this approach did not require any further processing steps such as protease treatment or long time incubation, greatly simplifying the production of active mTGase.

Introduction

Transglutaminase (EC 2.3.2.13, TGase) catalyzes the acyl-transfer between glutamine residues and varieties of primary amines and results in the crosslinking of proteins [1, 2]. Owing to the crosslinking properties, TGase shows great potential for application in the food, pharmacological and biotechnological industries [3–5]. Initially, the relatively costly extraction of TGase from mammalian sources resulted in little industrial interests. Moreover, mammalian TGase requires Ca^{2+} for its activation. However, proteins in the food industry are easily precipitated by Ca^{2+}, which further impeded the use of mammalian TGase in the food industry [6–8].

The microbial transglutaminase (mTGase) is Ca^{2+} independent and can be produced cost-effectively by traditional fermentation technology. Therefore, the discovery of mTGase in *S. mobaraense* greatly prompted the industrial use of TGase [9–12]. The mTGase from *S. mobaraense* is synthesized in the form of zymogen (pro-mTGase) [2, 13]. The pro-peptide is essential for zymogen folding but must be

posttranslationally cleaved to activate the zymogen [14]. Therefore, zymogen consisting of the pro-peptide and mature part of mTGase was normally expressed first and the protease treatment was subsequently applied to remove the pro-peptide [15–25]. The protease treatment was either conducted *in vitro* [15–18, 20, 22, 23, 25] or accomplished through co-expression of the protease *in vivo* [19, 21, 24]. Either way, proteases treatment complicates the process of mTGase production and increases the cost of mTGase production. Moreover, proteases are known to be relatively nonspecific for their substrates and therefore often result in the uncertainty during pro-mTGase activation [26].

Inteins are self-splicing proteins and have been engineered to exhibit a highly specific self-cleaving activity [27, 28]. Despite self-cleaving inteins have been used to cleave the pro-peptide from the pro-mTGase, two major limitations were encountered with self-cleaving inteins [29, 30]. First, self-cleaving inteins normally require an additional process of long time incubation to induce their cleaving activities. This process is time-consuming and carries uncertainty for mTGase production [29, 30]. Second, self-cleaving inteins are subjected to the inherent problem of premature *in vivo* cleavage. The cleavage activity of intein was normally induced under a specific condition such as temperature or pH changes. Premaute cleavage denotes that the cleavage of intein occurs simultaneously before the induction. Therefore, premature cleavage resulted in the spontaneous cleavage of intein during the expression and decreased the production of the precursor proteins [30, 31]. Many efforts including low-temperature expression and protein engineering have been made to suppress the premature cleavage [31–34].

Here, for the first time, we employed rather than suppressed the premature cleavage of intein *Ssp* DnaB to activate pro-mTGase intracellularly in *C. glutamicum*. As a result, high-level production of active mTGase was obtained intracellularly in *C. glutamicum*.

Results

Development of T7 expression system for high-level expression of mTGase in *C. glutamicum*

To enhance the expression of mTGase, a T7 RNA polymerase (RNAP) -dependent expression system was developed for *C. glutamicum* [35]. The lac promoter in the plasmid pXMJ19 was replaced by the T7 promoter, yielding the plasmid pXMJ19T7. Correspondingly, the gene fragment containing the *T7 RNAP* and *lacI* gene was integrated into the chromosome of *C. glutamicum*, yielding the strain *C. glutamicum* ATCC 13032 (DE3). All the *C. glutamicum* strains used in the following experiment were *C. glutamicum* ATCC 13032 (DE3) unless otherwise specified. To test the T7 expression system, the gene encoding pro-mTGase was cloned on the pXMJ19T7 and expressed under the control of the T7 promoter (Fig. 1A). As shown in Fig. 2A, a strong band at approximate 43.3 kDa was observed in cell lysate, suggesting the high-level expression of pro-mTGase in *C. glutamicum*. Furthermore, to secret pro-mTGase, the cspA signal peptide was fused to pro-mTGase and the resulting fusion protein was expressed by using this T7 expression system (Fig. 1B). A strong band corresponding to the 43.3 kDa pro-mTGase was observed in the culture supernatant, suggesting that the high-level expression and secretion of pro-mTGase (Fig. 2B). Meanwhile, a clear band corresponding to 45.7 kDa was observed in the cell lysate, indicating that some

of the fusion protein was not secreted but resided intracellularly. All these results demonstrated that the T7 RNAP-dependent expression system was successfully established in *C. glutamicum* and can be used in the following study.

Intein *Ssp* DnaB was subjected to the premature cleavage in *C. glutamicum*

Despite pro-mTGase was expressed intracellularly and extracellularly at a high level by using the T7 expression system, no obvious enzyme activity was detected for pro-mTGase, which needs to be activated. To activate pro-mTGase, intein *Ssp* DnaB was fused with the pro-mTGase. The *Ssp DnaB* gene was inserted between gene fragments encoding pro-peptide and mature part of mTGase. The chimeric gene was cloned on pXMJ19T7 and expressed under the control of the T7 promoter, yielding the plasmid pXMT7-csp-pro-ssp-mTG (Fig. 1C). The expression of the fusion protein was then analyzed by SDS-PAGE. No clear band corresponding to fusion protein (60.7 kDa) or mature mTGase (38.8 kDa) was observed in the culture supernatant. Meanwhile, no mTGase activity was detected in the culture supernatant. Moreover, no mTGase activity was detected after the culture supernatant was treated under pH 6.5 for 24 hours, which was supposed to induce the cleavage. These results indicated that the precursor fusion protein was not secreted to the culture. However, in the cell lysate supernatant, a specific band that exactly matched the molecular weight of the mature part of mTGase (38.8 kDa) was observed (Fig. 3A). Meanwhile, the mTGase activity (0.2 U/mL/OD) was also detected in the cell lysate, further indicating the intracellular production of active mTGase. These results suggested that the intein *Ssp* DnaB was subjected to the premature *in vivo* cleavage when it was applied to activate the pro-mTGase. Consequently, the premature cleavage activated the pro-mTGase intracellularly and prevented the pro-mTGase secretion.

Premature cleavage was applied to active mTGase intracellularly

Observing the production of active mTGase through premature cleavage, we employed rather than suppressed premature cleavage to produce active mTGase intracellularly in *C. glutamicum*. To express the mTGase precursor intracellularly, the gene fragment encoding cspA signal peptide was removed from the plasmid pXMT7-csp-pro-ssp-mTG and the resulting plasmid was named pXMT7-pro-ssp-mTG (Fig. 1D). As shown in Fig. 3A, the active mTGase was successfully produced intracellularly in *C. glutamicum*. The removal of the cspA signal peptide increased the intracellular expression level of the active mTGase. Meanwhile, the mTGase activity of the cell lysate supernatant reached to 0.7 U/mL/OD, which is increased by 2.5 fold. To observe the process of premature cleavage, the expression of the fusion protein pro-ssp-mTG was analyzed at different time intervals. The protein bands for fusion protein continued to increase in 8 hours and began to decrease after 8 hours. Meanwhile, the protein bands for mature mTGase was observed in 4 hours and increased constantly thereafter (Fig. 3B). This result indicated that the premature cleavage of intein *Ssp* DnaB efficiently activated the pro-mTGase intracellularly in *C.*

glutamicum. Furthermore, the substitution of the first C-extein residue with proline was presumed to inhibit the self-cleaving activity of *Ssp* DnaB [36]. As we expected, the proline substitution resulted in the accumulation of precursor fusion protein. Meanwhile, the production of mature mTGase was significantly decreased (Fig. 3C). This result further indicated the premature cleavage of *Ssp* DnaB mediated the cleavage of the precursor fusion protein, resulting in the intracellular production of active mTGase.

Besides, the recombinant mTGase with the His_6 tag at the C-terminus was purified from the supernatant of cell lysate and the N-terminal amino acid sequencing was conducted. Five residues M-D-S-D-D, which were exactly matched with the mature mTGase N-terminus, were identified by the N-terminal sequencing. This result strongly suggested that the premature cleavage of *Ssp* DnaB activated the mTGase in a highly specific manner.

The first C-extein residue modulated the premature *in vivo* cleavage

The first C-extein residue is of vital importance to the modulation of C-terminal cleavage [36]. Thus, the Met in the +1 position of C-extein was substituted with the other 19 naturally occurring amino acids. The premature cleavage efficiency of each variant of mTGase was compared by analyzing the intracellular mTGase activities. As shown in Fig. 4, the mTGase variant with methionine in the +1 position exhibited the highest mTGase activity. Compared to the variant with Met at the +1 position, the variant with Leu at +1 position exhibited 78% activity, suggesting the Leu was the next favored residue for C-terminal cleavage. Fifteen variants with substitutions (Val, Phe, Ser, Ile, Trp, Arg, Asn, Gly, Ala, Pro, Glu, Tyr, Gln, Cys and Thr) yielded various extent of C-terminal cleavage (10–65%), while three substitutions (Asp, His and Lys) essentially decreased the cleavage efficiency to less than 10%. These results demonstrated that the substitution of the first C-extein residue modulated the premature cleavage and the resulting intracellular production of mature mTGase. The variant with methionine residue at the +1 position of the C-extein exhibited the highest intracellular mTGase activity and hence was used in the following experiment.

Characterization of recombinant mTGase

As shown in Fig. 5A and Fig. 5B, the optimal temperature and pH of the recombinant mTGase was 55 °C and 7.0, respectively. Furthermore, the enzyme was stable at 40 °C, and 30% of the activity was retained at 50 °C for 100 minutes. However, at 60 °C, the recombinant mTGase lost all its activity within 20 minutes (Fig. 5C). In addition, the purified recombinant mTGase was stable at pH 5.0–9.0 after 1 h incubation at room temperature, during which more than 70% activity was retained (Fig. 5D). The effects of inhibitors and various metal ions on the activity of recombinant mTGase were also detected. It was found that the activity of purified mTGase was not inhibited by Ethylene Diamine Tetraacetie Acid (EDTA), phenyl methyl sulfonyl fluoride (PMSF) and metal ions including Na^+, K^+, Mg^{2+} and Ca^{2+}. The enzyme activity was mildly inhibited by metal ions, such as Cu^{2+}, Mn^{2+}, Fe^{2+}. With the addition of Zn^{2+}, the

activity was almost totally inhibited (Table 1). These results demonstrated that the major properties of the recombinant mTGase produced in this study were not noticeably altered [20].

High-level production of active mTGase in a jar fermentor

The recombinant mTGase production was scaled up by using a 1 L fed culture. Cells were grown at 30 °C and then changed to 25 °C after IPTG was added. Samples were taken at different time intervals and the enzyme activity was determined. As shown in Fig. 6, the mTGase activity was constantly detected after IPTG induction owing to the premature cleavage. At 42 h, the mTGase activity was up to 49 U/mL, which was the highest intracellular mTGase activity ever reported. It is highly likely that a higher level production of mTGase would be available once the fermentation process is further optimized.

Discussion

C. glutamicum has been widely used as a platform chassis for the production of small biological molecules especially amino acids [37]. More recently, *C. glutamicum* has attracted considerable industrial interest as a recombinant protein expression host [38, 39]. Native promoters have been the most commonly used for *C. glutamicum* in synthetic biology and metabolic engineering experiments [40, 41]. However, for the recombinant protein expression in *C. glutamicum*, a strong promoter was highly desirable. Therefore, a strong T7 promoter-specific, inducible expression system was developed in *C. glutamicum* to obtain the high-level production of recombinant mTGase [35]. The T7 RNAP dependent expression system in *C. glutamicum* enabled the high-level expression of mTGase and should be easily adapted to express other recombinant proteins in *C. glutamicum*.

The pro-peptide of zymogen was presumed to assist the zymogen folding but also to inhibit the toxicity of mature peptide [1, 42]. Without the coexpression of pro-peptide, mTGase was constantly expressed as inclusion bodies in *E. coli* [43, 44] and *C. glutamicum* (Additional file 1: Figure S1), suggesting that the pro-peptide of mTGase was essential for mTGase precursor folding. However, regarding the toxicity of mature peptide, the high-level expression of active mTGase intracellularly in *C. glutamicum* suggested that mTGase was not as toxic as previously presumed. Moreover, we did not observe any growth deficiency for the *C. glutamicum* strain in which active mTGase was produced (Additional file 1: Figure S2). Therefore, the pro-peptide of mTGase is essential for zymogen folding while it does not seem to be involved in the toxicity inhibition. In previous studies where mTGase was produced intracellularly in *E. coli*, the authors also suggested that mTGase was not completely toxic to *E. coli* [45, 46]. However, yet we could not rule out the possibility that the pro-peptide released by the intein-mediated premature cleavage might be involved in toxicity inhibition.

The coexpression of pro-peptide and mature part of mTGase is essential to obtain active mTGase. Two different strategies were employed for the coexpression of pro-peptide with mature part mTGase. In previous efforts to obtain active mTGase intracellularly in *E. coli*, the co-expression of pro-peptide and mature part of mTGase was accomplished by the polycistronic expression [45, 46]. The polycistronic expression resulted in the separate expression of pro-peptide and the mature part of mTGase as two proteins. In our study, however, the pro-peptide is coexpressed with the intein and the mature part of mTGase as a fusion protein, which is more similar to the native form of pro-mTGase. The fusion protein guaranteed the equivalent molecular ratio between pro-peptide and the mature part of mTGase. Moreover, the intramolecular interaction between the pro-peptide part and the mature part of mTGase precursor for fusion protein is more efficient than intermolecular interaction for two individual proteins. The difference in coexpression strategy might explain why a much higher level of mTGase was produced in *C. glutamicum* than that in *E. coli*. It is also possible that *C. glutamicum* is a better heterologous host than *E. coli* for the intracellular expression of mTGase. A much lower expression of mTGase in *E. coli* was observed when the exact plasmid expressing mTGase in *C. glutamicum* was transformed into *E. coli* BL21 (DE3) (Date not shown).

Inteins are protein introns and has been engineered to remove unwanted peptide sequences in a highly specific way. Normally, premature *in vivo* cleavage is an inherent but undesirable feature for the self-cleaving inteins. Self-cleaving inteins were initially applied to remove the purification tags. The premature cleavage resulted in the loss of the purification tags before the purification and greatly decreased the final production proteins of interest [31, 47]. More recently, self-cleaving inteins were used for zymogen activation [29, 30]. Du *et. al* encountered the problem of premature cleavage when using intein *Ssp* DnaB during the production of active mTGase in *E. coli*. In their study, the precursor fusion protein containing the pro-peptide, the self-cleaving intein, and the mature part of mTGase was expressed in *E. coli*. The premature cleavage persisted throughout the precursor fusion protein expression, resulting in a decrease in the precursor fusion protein and consequently a decline in mature mTGase production [30]. We also observed the occurrence of premature cleavage by intein *Ssp* DnaB in *C. glutamicum*. Moreover, premature cleavage occurred intracellularly in *C. glutamicum* in a fast and complete way. Therefore, the premature cleavage of *Ssp* DnaB was employed to establish a new approach to produce mTGase in *C. glutamicum*. This approach enabled the high-level intracellular production of mTGase in an active form. It did not require any further processing steps such as renaturing from inclusion bodies or protease treatment and hence greatly simplified the production of active mTGase. This approach should be adaptable in the expression of other zymogens. Besides, it is interesting to test other self-cleaving inteins besides *Ssp* DnaB for their potential use in the production of mTGase and other recombinant proteins in *C. glutamicum*.

Conclusions

Recombinant production of mTGase is tricky because the mTGase is synthesized as an inactive zymogen. The self-cleaving inteins have been applied to activate mTGase zymogen owing to its high specificity and simplicity. However, self-cleaving inteins suffer from the inherent problem of premature

cleavage. The intein *Ssp* DnaB was also subjected to the premature cleavage when it was used to activate the zymogen of mTGase in *C. glutamicum*. Instead of suppressing the premature cleavage, we used it to activate the mTGase intracellularly in *C. glutamicum*. Series of results demonstrated that the premature cleavage activated the mTGase in an efficient and highly specific manner, resulting in the intracellular production of active mTGase at a high level. Moreover, the premature *in vivo* cleavage occurred simultaneously, avoiding the additional step of long time incubation. Therefore, this work presented an efficient and simple approach to the high-level production of active mTGase. This approach shows great potential for the large-scale industrial production of active mTGase.

Materials And Methods

Bacterial strains and culture medium

The bacterial strains used in this study are listed in Table 2*. E. coli* Trans1-T1 was grown in Luria broth at 37 °C. *S. mobaraense* CGMCC4.1719 was grown in GYM streptomyces medium (per liter: glucose 4.0 g, yeast extract 4.0 g, malt extract 10.0g) at 30 °C [13]. *C. glutamicum* was grown at 30 °C in BHI medium (per liter: 37g brain heart infusion) or CGX medium (per liter: 40 g of glucose, 21 g of MOPS, 5 g of $(NH_4)_2SO_4$, 1 g of KH_2PO_4, 1 g of K_2HPO_4, 5 g of urea, 2 g of yeast extract, 0.25 g of $MgSO_4$, 0.002 g of biotin, 0.01 g of $CaCl_2$, 1mL of trace element buffer, pH7.0). Trace element buffer (per liter: 16.4 g of $FeSO_4.7H_2O$, 100 mg of $MnSO_4.H_2O$, 200 mg of $CuSO_4$, 1 g of $ZnSO_4.7H_2O$, 20 mg of $NiCl_2.6H_2O$). *C. glutamicum* was transformed by electroporation as described previously [35]. Chloramphenicol was used at a final concentration of 17 μg/mL for *E. coli* and *C. glutamicum*.

Construction of plasmids

The plasmids used in this study are listed in Table 2. *C. glutamicum* ATCC 13032 (DE3) was generated via the suicide integration vector pK18*mobsacB* [49]. The upstream and downstream homologous flanking sequences were PCR-amplified from *C. glutamicum* ATCC 13032 chromosomes with primer pairs UF/UR and DF/DR, respectively. The fragment containing *T7 RNA polymerase* and *lacI* gene was amplified with primer pairs T7PF/T7PR using *E. coli* BL21 (DE3) as the template. The three fragments were subsequently ligated into pK18*mobsacB* digested with *Bam*HI to generate vector pK18*mobsacB*-T7. *C. glutamicum* ATCC 13032 (DE3) was obtained by double homologous recombination via transformation of pK18*mobsacB*-T7. Plasmid pET28a (+) was used as the template to amplify the T7 promoter with primer pairs T7F/T7R. The resulting fragment was ligated into plasmid pXMJ19 amplified with P19-F/P19-R to construct pXMJ19T7. The extracellular expression plasmids were constructed as follows: *mTGase* gene containing pro-peptide and mature part was amplified by PCR using chromosomal DNA of *S. mobaraensis* CGMCC4.1719 as the template with primer pairs P3/P4. The signal peptide cspA [19] from *Corynabacterium ammoniagenes* was amplified with cspA-F/cspA-R using preserved plasmid as template. The two DNA fragments were ligated into the vector pXMJ19T7 amplified with P1/P2 to

construct pXMT7-csp-pro-mTG. The intein *Ssp* DnaB was amplified by PCR using primers P7/P_M employed synthesized DNA as the template (Qtsingke Biotech, Beijing, China). The resulting *Ssp* DnaB fragment with the insertion of methionine at position +1 of C-extein contained in PCR was introduced into the pro-peptide region and mature part of pXMT7-csp-pro-mTG amplified with primers P5/P6 yielding vector pXMT7-csp-pro-ssp-mTG. Then the intracellular expression plasmids were constructed: the sequence of pro-ssp-mTG without signal peptide was amplified with primers P3/P4 from pXMT7-csp-pro-ssp-mTG and ligated into vector pXMJ19T7 amplified with P1/P9 yielding pXMT7-pro-ssp-mTG. Using pXMT7-csp-pro-mTG as template, pro-mTG fragment which was amplified with primers P3/P4 ligated into vector pXMJ19T7 amplified with P1/P9 resulting in pXMT7-pro-mTG. To test the C-terminal self-cleavage efficiency, the other nineteen variants of methionine at position +1 of *Ssp* DnaB C-extein underwent PCR, the mutation sites contained in the primers were underlined in Table 3. The DNA fragments ligation in this study was conducted using ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech, Nanjing, China). The C-terminal His_6 tag was used to simplify the purification procedure. The plasmids constructed were verified by DNA sequencing. The primers used in the study are listed in Table 3.

Expression and purification of recombinant mTGase

glutamicum harboring mTGase expression vectors were cultivated in 50 mL CGX medium at 30 °C to the OD_{600} reached 0.8. The expression of mTGase was induced by 0.5 mM IPTG at 25 °C for 48 h. After cultivation, extracellular proteins were prepared by centrifugation at 10,000×g for 20 min at 4 °C. To extract intracellular proteins, cells harvested by centrifugation with the same optical density at 600 nm were resuspended by Tris-buffer (pH 8.0) and lysed via ultrasonic-method [50]. After centrifugation (10,000×g, 4 °C; 20 min), the supernatant of cell lysate was used as the intracellular protein samples. All extracellular and intracellular protein samples were subsequently analyzed by SDS-PAGE [45] and enzyme activity assay. The purification of the recombinant enzyme was conducted using nickel affinity chromatography (HisTrap™ FF crude, GE Healthcare) [30].

Activity assay of recombinant mTGase

The mTGase activity was determined by the colorimetric hydroxamate procedure as previously described with some modifications [29, 46]. One unit of transglutaminase was defined as the amount of enzyme needed for the formation of 1 μmol hydroxamic acid per minute at 37 °C. The buffer A for reaction contained 0.2 M Tris-HCl buffer (pH 6.0), 30 mM CBZ-Gln-Gly, 100 mM hydroxylamine, and 10 mM glutathione. 0.5 mL buffer A was mixed with 0.2 mL of properly diluted enzyme. After incubation for 10 min at 37 °C, 0.2 mL buffer B (1 M HCl, 4% trichloroacetic acid, 5% $FeCl_3$) was added to stop the reaction. The supernatant was collected by centrifugation at 4000×g for 5 min and the absorbance at 525 nm was measured to determine the mTGase activity.

N-terminal sequencing of mTGase

To determine the N-terminal amino acid sequence, the purified protein samples were transferred from the SDS–PAGE gel to polyvinylidene difluoride (PVDF) membrane in transfer buffer [51]. The transferred protein band on the membrane was visualized by staining with ponceau and subjected to Edman degradation-based N-terminal peptide sequencing (Peking University, Beijing, China).

Characteristics of the recombinant enzyme

The optimal temperature for the enzymatic activity of the recombinant mTGase was determined by subjecting mTGase under the temperature range from 30 to 60 °C for 10 min at pH 8.0. The optimal pH was detected by subjecting mTGase to pH range from 4.0 to 9.0 at 55 °C for 10 min. Thermal and pH stability were determined by measuring the residual activity after preincubating the enzyme at various temperatures and pH values. The effects of various metal ions and possible inhibitors on the recombinant mTGase were measured after incubation at room temperature for 30 min. The reagents were added at a final concentration of 1 mM. All measurements were repeated at least in triplicate.

Fed-batch cultivation

100 mL of *C. glutamicum* bearing pXMT7-intein-mTGase was inoculated into 1 L CGX medium in a 2 L jar fermenter. Before the addition of IPTG, the culture was cultivated at 30 °C until OD_{600} reached 10. Following induction, the culture was incubated at 25 °C for 65 hours in the condition of 1.0 vvm and 700 rpm. Glucose solution (50%) was added to the culture as the carbon source. The pH during cultivation was maintained at 7.0 by adding the ammonia solution. Cell growth was monitored by measuring the OD_{600} [51].

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Contributions

ZYD, SZ and NZ conceived the project and designed the study. NZ, YZH, XC and YFZ performed the experiments. SZ and NZ analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 The effects of various metal ions and inhibitors on the activity of purified recombinant mTGase

Regent (1mM)	Activity (%)
Cu^{2+}	76.19±2.27
Mn^{2+}	77.19±6.85
Na^{+}	113.46±3.64
K^{+}	113.67±3.90
Ca^{2+}	108.20±3.46
Zn^{2+}	1.18±0.17
Fe^{2+}	87.23±1.20
Mg^{2+}	111.07±2.40
EDTA	116.33±6.11
PMSF	113.73±4.96
Control	100

Metal ions and inhibitors were added at the concentration of 1 mM. After the mixture incubated at room temperature for 30 min, the activity was measured. The values are means standard deviations from quadruplicate independent experiments.

Table 2 Strains and plasmids used in the study

Strains and plasmids	Relevant characteristics	Source
Strains		
E. coli Trans1-T1	F-*φ80(lacZ)ΔM15ΔlacX74hsdR (rk -, mk +) ΔrecA1398endA1tonA*	Transgene
S. mobaraense CGMCC4.1719	wild type	Our laboratory
C. glutamicum 13032	wild type	Our laboratory
C. glutamicum 13032 (DE3)	13032 with chromosomally encoded T7 RNA polymerase	This study
Plasmids		
pK18*mobsacB*	pMB1 ori, kan, sacB, lac (pro)	Our laboratory
pK18*mobsacB*-T7	pK18*mobsacB* with T7polymerase, upstream and downstream region	This work
pXMJ19	ori pUC, ori pBL1, cat, *lacIq*, tac (pro), rrnB (ter)	Our laboratory
pXMJ19T7	Cm^r; ori pUC, ori pBL1, cat, *lacIq*, T7 (pro),	This work
pXMT7-csp-pro-mTG	Cm^r; pXMJ19T7 with *pro-mTG* gene from *S. mobaraense* and cspA signal peptide from *C. ammoniagenes*,	This work
pXMT7-csp-pro-ssp-mTG	Cm^r; pXMT7-csp-pro-mTG inserted with *Ssp DnaB*	This work
pXMT7-pro-mTG	Cm^r; pXMT7-csp-pro-mTG without signal peptide	This work
pXMT7-pro-ssp-mTG	Cm^r; pXMT7-csp-pro-ssp-mTG without signal peptide	This work

Table 3 Primers used in the study

Name	Sequence (5′-3′)
T7PF	GCAACTCGTGAAAGGTAGGC
T7PR	GCGTTACGCGAACGCGAAGT
UF	GAGCTCGGTACCCGGGACAACTCTTTCACGTAAGTTC
UR	CCTTTCACGAGTTGCGGAAGTAGGGGCCTTACGC
DF	GCGTTCGCGTAACGCGGAAATAGGGGCCTTTTGTTG
DR	GCAGGTCGACTCTAGACTTCCAAGATGGCATGGGGC
T7F	GCACCAATGCTTCTGGCGTCCGCTCATGAGCCCGAAGTGG
T7R	AATTCGAGCTCGGTACCCGGGGGGAATTGTTATCCGCTCAC
19-F	CCGGGTACCGAGCTCGAATTC
19-R	GACGCCAGAAGCATTGGTGC
cspA-F	AAGAAGGAGATATACATATGAAACGCATGAAATCGCTGGCT
cspA-R	TCTTCCCCCGCGCCATTGTCTGCCGTTGCCACAGGTGCGG
P1	AGGTAAAGCAGGGCTGGCCGCACCACCACCACCACCACTGA
P2	CATATGTATATCTCCTTCTTAAAGTTAAAC
P3	GACAATGGCGCGGGGGAAGA
P4	CGGCCAGCCCTGCTTTACCT
P5	GACTCCGACGACAGGGTCACCC
P6	GGGGGCCCGGAACGACGGGC
P7	GCCCGTCGTTCCGGGCCCCCGCTATCTCTGGCGATAGTCTGATCAG
P_M	GTGACCCTGTCGTCGGAGTC<u>CAT</u>GTTGTGTACAATGATGTCATTCGCGA
P9	TCTTCCCCCGCGCCATTGTCCATATGTATATCTCCTTCTT

The mutation sites produced by PCR at position +1 of *Ssp* DnaB C-extein are underlined.

Figures

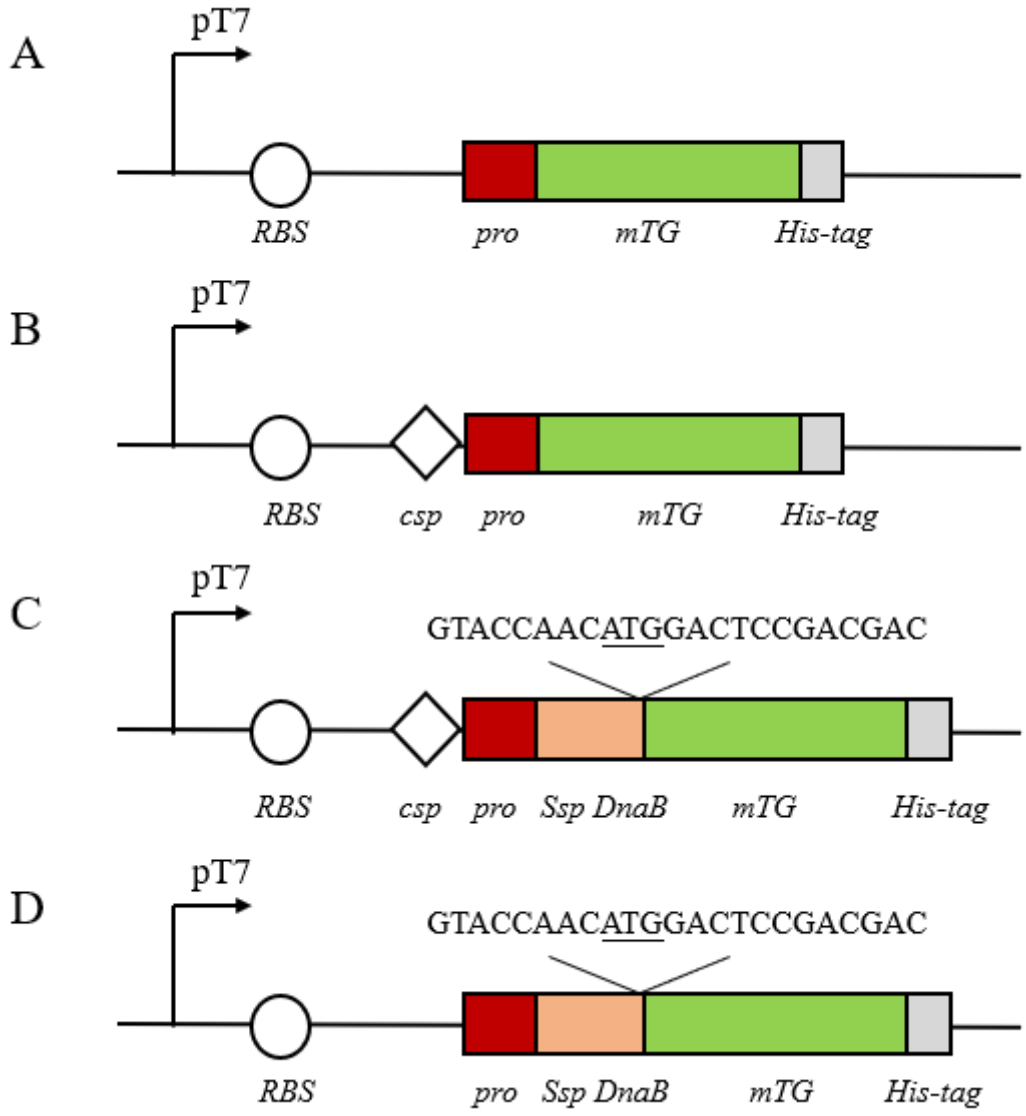

Figure 1

Schematic representation of plasmids for mTGase expression used in this study. (A) pXMT7-pro-mTG. (B) pXMT7-csp-pro-mTG. (C) pXMT7-csp-pro-ssp-mTG. (D) pXMT7-pro-ssp-mTG. RBS is short for ribosome binding site; csp is short for cspA signal peptide; pro is short for pro peptide of mTGase; mTG is short for mature peptide of mTGase. The insertion of methionine at position +1 of Ssp DnaB C-extein are underlined. All plasmids contain a C-terminal His-tag (18 bp).

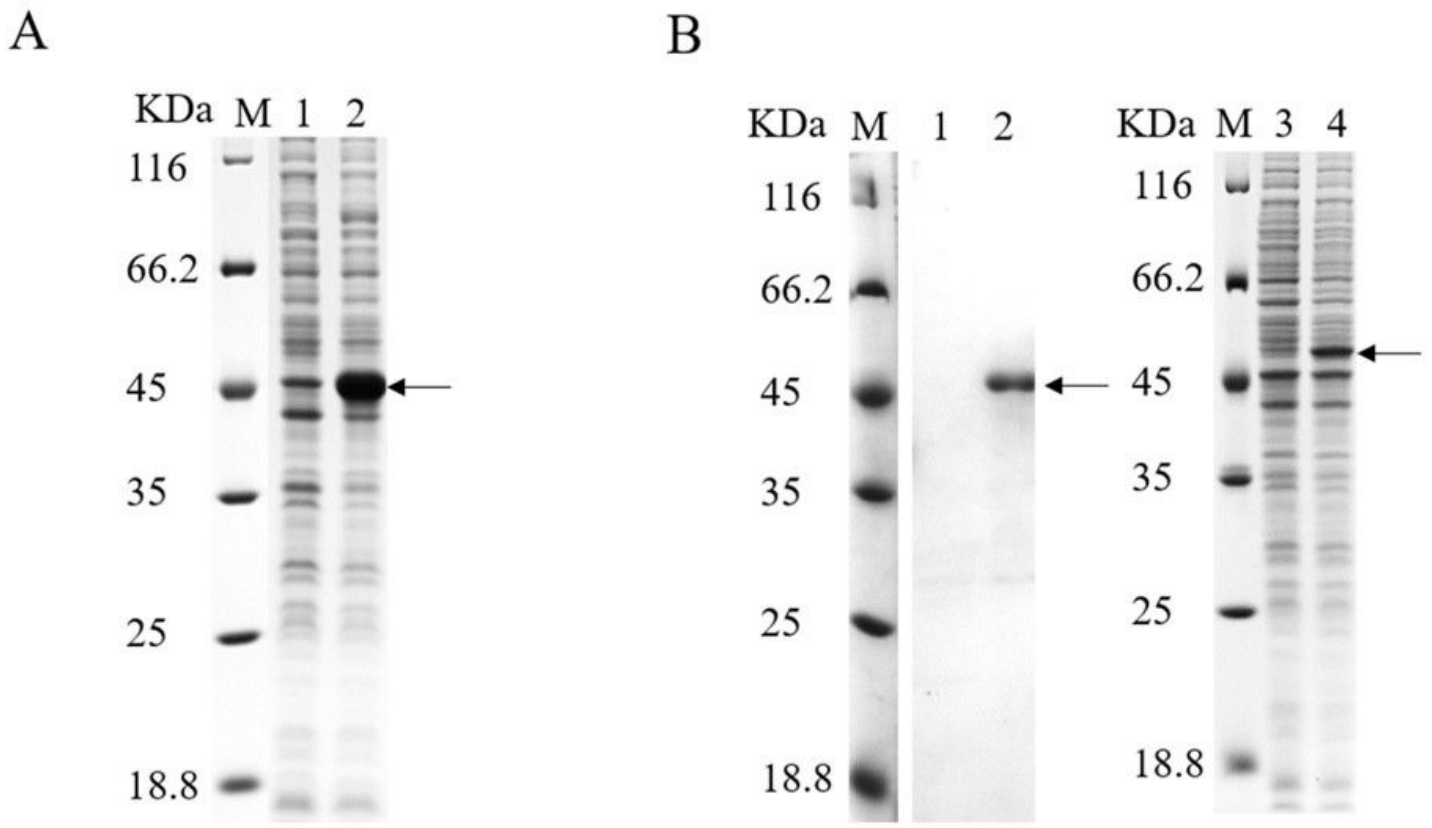

Figure 2

The expression of pro-mTGase in C. glutamicum by using the T7 expression system. (A) SDS-PAGE analysis of the intracellular expression of pro-mTGase. M: protein molecular mass markers. Lane 1: soluble lysate from cells harboring pXMT7-pro-mTG without IPTG induction. Lane 2: soluble lysate from cells harboring pXMT7-pro-mTG with IPTG induction. (B) SDS-PAGE analysis of the secretion of pro-mTGase. Lane 1: culture supernatant from cells harboring pXMT7-csp-pro-mTG without IPTG induction. Lane 2: culture supernatant from cells harboring pXMT7-csp-pro-mTG with IPTG induction. Lane 3: soluble cell lysate from cells harboring pXMT7-csp-pro-mTG without IPTG induction. Lane 4: soluble lysate from cells harboring pXMT7-csp-pro-mTG with IPTG induction.

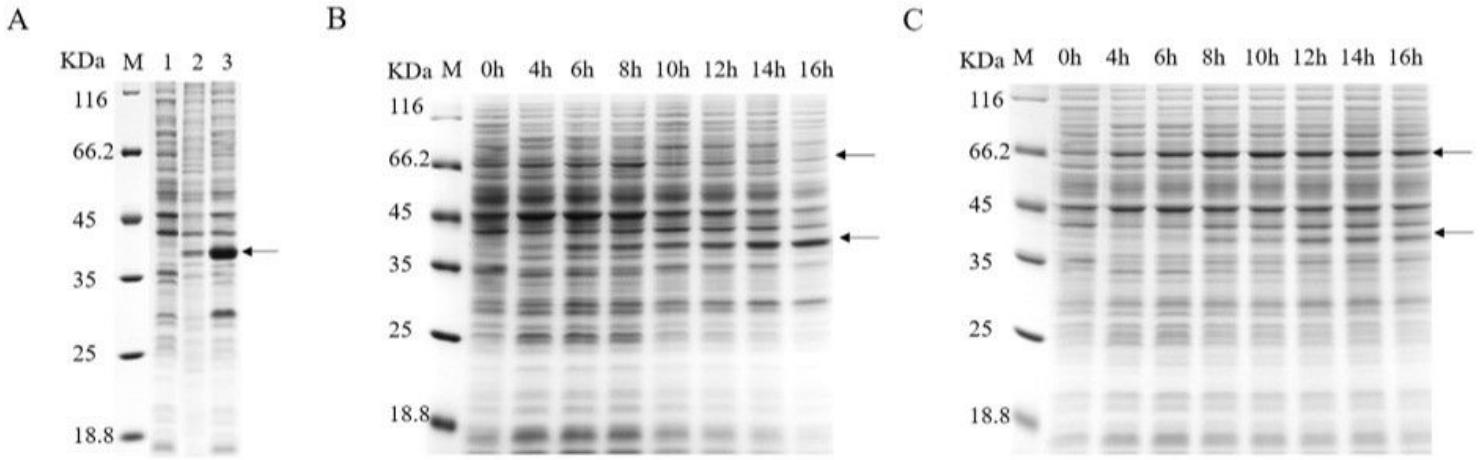

Figure 3

Premature cleavage of intein Ssp DnaB mediated the intracellular cleavage of fusion mTGase. (A) SDS-PAGE analysis of soluble lysate from cells harboring plasmid pXMT7-csp-pro-ssp-mTG (lane 2) and pXMT7-pro-ssp-mTG (lane 3). Lane 1: soluble lysate from cells harboring pXMT7-pro-ssp-mTG without IPTG induction. M: protein molecular mass markers. (B) Time-course analysis of soluble lysate from cells harboring plasmid pXMT7-pro-ssp-mTG. (C) Time-course analysis of soluble lysate from cells harboring variant plasmid in which the first C-extein residue of Ssp DnaB in pXMT7-pro-ssp-mTG was replaced by proline.

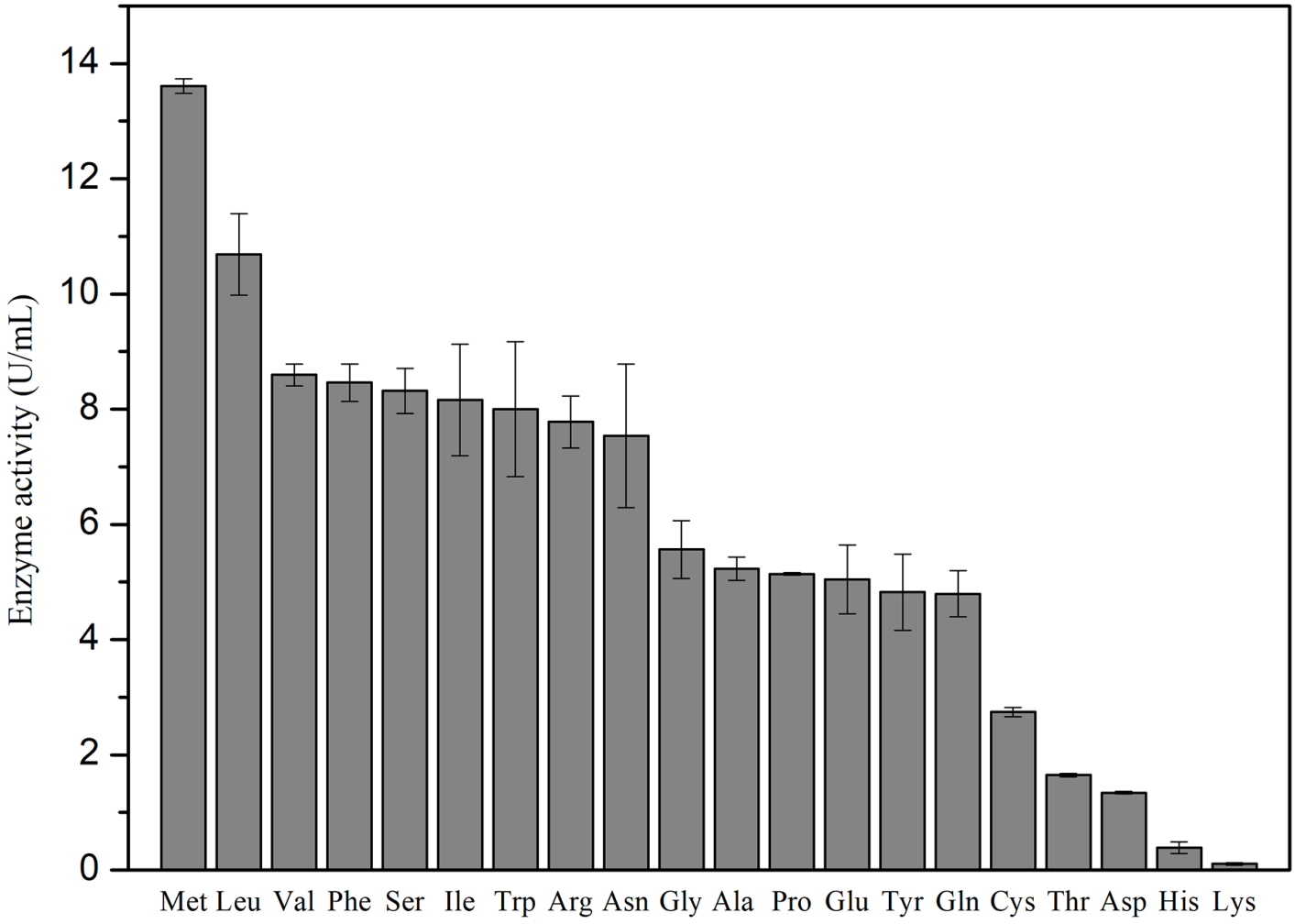

Figure 4

Enzyme activity comparison of mTGase variants. The amino acids at position +1 of Ssp DnaB C-extein are indicated under each bar. Error bars represent SD calculated from three independent determinations.

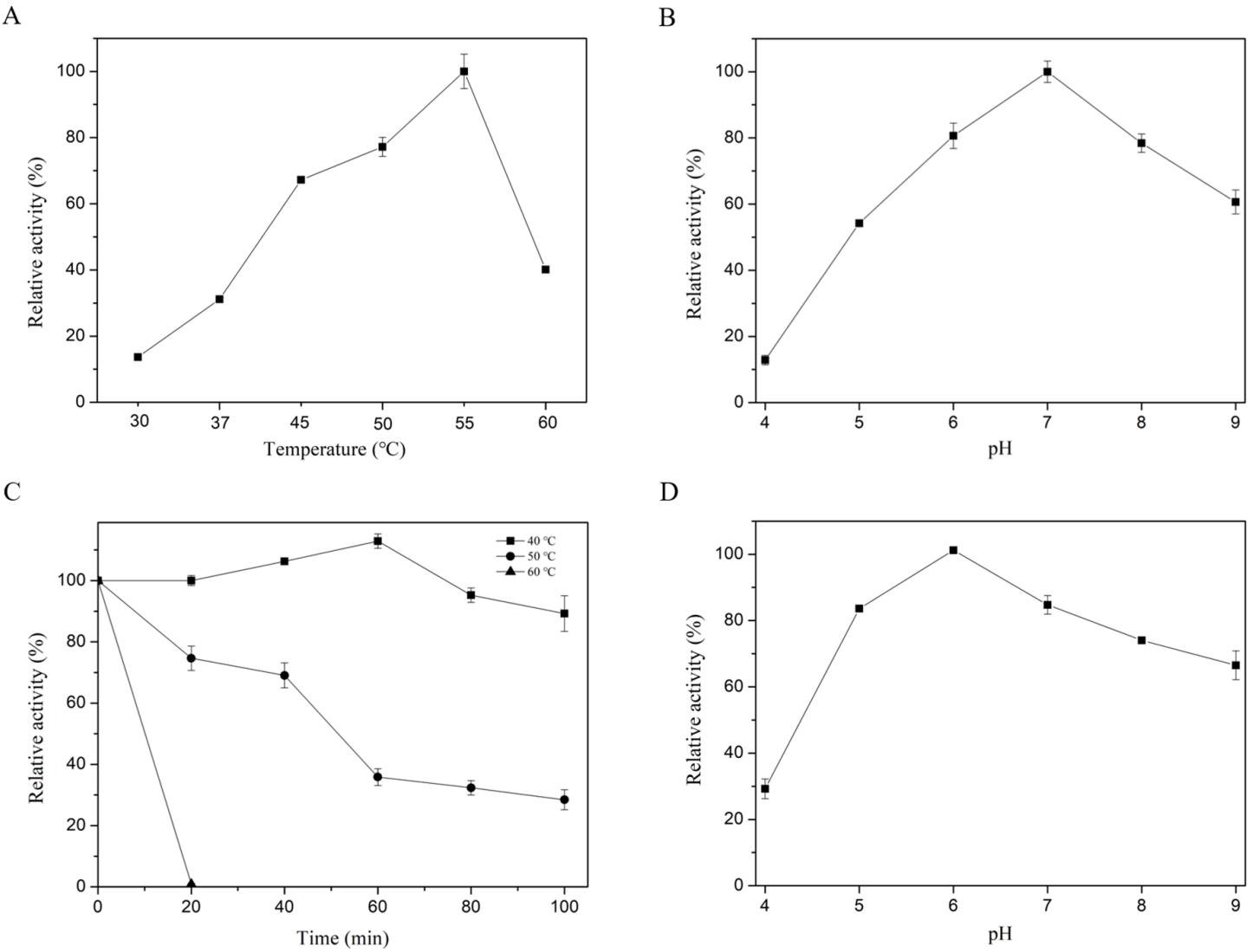

Figure 5

Characteristics of the purified recombinant mTGase. (A) The optimal temperature of the recombinant mTGase. (B) The optimal pH of the recombinant mTGase. (C) Effects of temperature on the thermostability of recombinant mTGase. The enzyme was incubated at 40 °C (■), 50 °C (●), 60 °C (▲) in 0.05 M Tris-HCl buffer (pH 8.0) for 1 h. Samples were harvested at different time intervals for residual enzyme activity assay under standard conditions. (D) Effects of pH on stability of the recombinant mTGase. The enzyme was incubated under different pH (4.0–9.0) at room temperature for 1 h before the residual enzyme activity assay under standard conditions. Error bars represent SD calculated from three independent determinations.

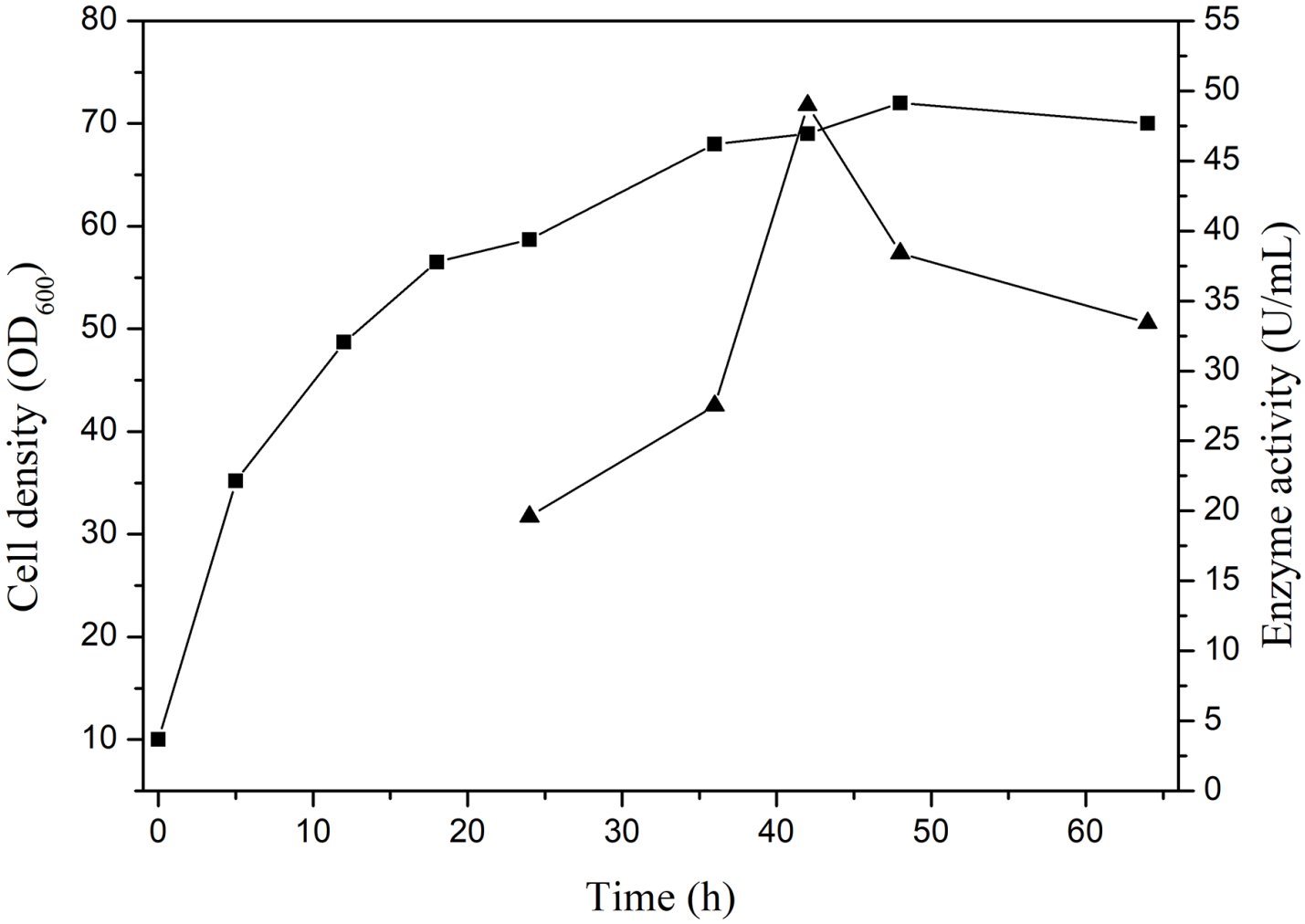

Figure 6

Fed- batch cultivation of C. glutamicum harboring pXMT7-pro-ssp-mTG (■: cell density; ▲: mTGase activity).

Supplementary Files

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