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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Heterologous signal peptides-directing secretion of *Streptomyces mobaraensis* transglutaminase by *Bacillus subtilis*

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Abstract

Microbial transglutaminase (MTG) from *Streptomyces mobaraensis* has been widely used for crosslinking proteins in order to acquire products with improved properties. To improve the yield and enable a facile and efficient purification process, recombinant vectors, harboring various heterologous signal peptide-encoding fragments fused to the *mtg* gene, were constructed in *Escherichia coli* and then expressed in *Bacillus subtilis*. Signal peptides of both WapA and AmyQ (SP_{wapA} and SP_{amyQ}) were able to direct the secretion of pre-pro-MTG into the medium. A constitutive promoter (P_{hpaII}) was used for the expression of SP_{wapA}-*mtg*, while an inducible promoter (P_{lac}) was used for SP_{amyQ}-*mtg*. After purification from the supernatant of the culture by immobilized metal affinity chromatography and proteolysis by trypsin, 63.0 ± 0.6 mg/L mature MTG was released, demonstrated to have 29.6 ± 0.9 U/mg enzymatic activity and shown to crosslink soy protein properly. This is the first report on secretion of *S. mobaraensis* MTG from *B. subtilis*, with similar enzymatic activities and yields to that produced from *Escherichia coli*, but enabling a much easier purification process.

Keywords Transglutaminase · *Bacillus subtilis* · Secretion · Signal peptides

Introduction

Transglutaminase (EC 2.3.2.13, protein-glutamine gamma-glutamyltransferase, TG) is an enzyme that catalyzes the acyl transfer reaction between γ-carboxyamide groups and primary amines within or between peptides or proteins (Gundersen et al. 2014). The crosslinked products display great stability, with high resistance to protease-degradation and chemical damage. Thus, they have been widely applied in various fields, including food and feed (Jaros et al. 2006; Yokoyama et al. 2004).

Eukaryotic TGs are widely distributed in animals (Chung et al. 1974; Folk and Cole 1966) and plants (Della et al. 2004), but are hard to be extracted from these organizations due to limited source availability and a relatively difficult purification process (Kieliszek and Misiewicz 2014). Furthermore, eukaryotic TGs ususally have a relatively narrow substrate specificity, for example, β-casein and several of its derivatives are excellent substrates for factor XIII, but cannot be catalyzed by the liver transglutaminase (Gorman and Folk 1980; Nielsen 1995). In prokaryotes, microbial transglutaminase (MTG) has been first detected from the culture medium of *Streptomyces* sp. (Ando et al. 1989), where it is initially expressed as a pre-pro-enzyme possessing 331 amino acids which becomes active after proteolysis (Kikuchi et al. 2003; Masayo et al. 2004; Yang et al. 2011). Compared to eukaryotic TGs, MTG has several advantages: (a) microbial fermentation costs less than feeding animals and growing plants, (b) the enzymatic activity of MTG is calcium-dependent only when measuring NH_3-release during crosslinking of caseinate (Macedo et al. 2011;

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Nielsen 1995). Therefore, MTG has been more widely used in the food industry, leading to improved texture and stability of foods with regard to temperature, syneresis, emulsifying properties, gelation, and increased water-binding capacity, without changing the pH, color, flavor, or nutritional quality of the food product. The process may even render food more nutritious thanks to the possibility of adding essential amino acids (Gaspar and de Góes-Favoni 2015).

To date, mainly a bacterial expression system with *Streptoverticillium mobaraensis* has been used to biosynthesize transglutaminases. However, this system has some drawbacks, involving, e.g., problems related to post-translational protein modification (Griffin et al. 2002). Thus, developing a cheaper and more efficient production system that will allow for a reduction of costs associated with the distribution, storage, extraction, and purification of TG recombinant proteins is worthy of attempting. *Escherichia coli* is the most commonly used one for the production of heterologous proteins. However, the formation of intracellular inclusion bodies of MTG in *E. coli* limited the purification process to an uneconomic level (Salis et al. 2015; Yokoyama et al. 2000). Some attempts to extracellularly express MTG from *E. coli* by fusing a PelB signal peptide in front of the *mtg* gene have failed and lead protein into the periplasm instead of the culture medium (Marx et al. 2007).

Bacillus subtilis is one of the most well-known host strains for efficient secretion of proteins of interest and is generally recognized as safe (Liu et al. 2013; Maarten and Michael 2013; Song et al. 2015). In this study, we constructed two separate MTG secretion systems in *B. subtilis*: one involves constitutive expression based on vector pMA5 (Zhang et al. 2006), and the other involves inducible expression based on vector pHT43 (Nguyen et al. 2007). MTG with a hexahistidine tag (MTG-6His) was secreted and purified successfully from both systems (constitutive system, 63.0 ± 0.6 mg/L; inducible system, 54.1 ± 0.3 mg/L) and proven to be highly active after proteolysis by trypsin with enzymatic activity of up to 29.6 ± 0.9 U/mg. These two newly established systems provide effective toolboxes for easy purification of MTG and for its future bioengineering.

Materials and methods

Vectors, strains, and growth conditions

Strains and vectors used in this work are listed in Table 1. *S. mobaraensis* (CGMCC 4.5591) was purchased from the China General Microbiological Culture Collection Center (CGMCC, Beijing, China) and was cultured in TSBY medium (30 g/L Oxoid tryptone soya broth, 340 g/L sucrose, 5 g/L Oxoid yeast extract) (Guan et al. 2015). *E. coli* DH5α (Novagen Company, Shanghai, China) was cultured in Luria-Bertani (LB) medium or on agar plates. *B. subtilis* 168 (ATCC 33712) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and was cultured in Luria-Bertani (LB) medium or on agar plates. For fermentation, fermentation medium (2.5 g of corn starch, 20 g of peptone, 0.8 g of urea, 3.26 g of $K_2HPO_4.3H_2O$, 2.54 g of KH_2PO_4, 0.92 g of $MgSO_4$, 3 g of NaCl, and 35 g of sucrose per liter of distilled water) was used for *B. subtilis* (Feng et al. 2017). Ampicillin was added to the growth medium of *E. coli* DH5α at a final concentration of 100 μg/ml whenever necessary. Kanamycin was added to the growth medium of *B. subtilis* at a final concentration of 25 μg/ml whenever necessary. Chloramphenicol was added to the growth medium of *B. subtilis* at a final concentration of 5 μg/ml whenever necessary.

Molecular cloning

Molecular cloning techniques were performed as described by (Sambrook and Russell 2001). Preparation of competent cells and transformation of *E. coli* (Dower et al. 1988) and *B. subtilis* (Cao et al. 2011) were performed as described previously. Fast digest restriction enzymes and ligase were supplied by Fermentas (St. Leon-Rot, Germany) and used according to the manufacturer's instructions. The sequence of the *mtg* gene from *S.mobaraense* was deposited in the GenBank database under accession number DQ132977. The sequence of the *wapA* gene from *B. subtilis* was deposited in the GenBank database under accession number JQ302213. The sequence of the *amyQ* gene from *Bacillus amyloliquefasciens* was deposited in the GenBank database under accession number J01542.

Construction of recombinant vectors

Plasmid isolation and genomic DNA extraction were performed with the plasmid DNA extraction kit and genomic DNA extraction kit (TransGen Biotech, Beijing, China), respectively. Primers used in this work are listed in Table 2. Plasmid expressing the recombinant MTG gene consisting of the signal sequence of *B. subtilis wapA* was constructed by overlap PCR (Fig. 1a) (Heckman and Pease 2007). Primers p1 and p2 were designed to amplify the WapA signal peptide gene fragment (*Nde*I-SP_{wapA}-*overlapseq*) from genomic DNA of *B. subtilis* 168 (Harwood 1992). A *Nde*I site was added to the 5′ end of primer p1. The DNA fragment of *mtg* lacking its initial peptide sequence (*mtg-6his-Nhe*I) was amplified by primers p3 and p4. Codons of hexa histidine followed by a *Nhe*I site were added to the 5′ end of primer p4. Primers p2 and p3 were designed to be reversely complementary by overlapping the 5′ ends of each other. The fragment SP_{wapA}-*mtg-6his* was generated by spliced overlap extension PCR with primers p1 and p4 using the

Table 1 Strains and vectors used in this work

Strain or vector	Characteristic	Information	Reference
Strains	Used for cloning of *mtg* gene	CGMCC 4.5591, (CGMCC, Beijing, China)	(Ando et al. 1989)
S. mobaraensis			
B. subtilis 168	Expression host strain	ATCC 33712, (ATCC, Manassas, VA, USA)	(Harwood 1992)
B. subtilis WB600	Protease deficiency type, expression host strain	Novagen Company, Shanghai, China	(Wu et al. 1991)
E. coli DH5α	Intermediate host for the vector constructions	Novagen Company, Shanghai, China	(Sambrook and Russell 2001)
Vectors			
pNZ8048	Used for cloning of random sequence	Novagen Company, Shanghai, China	(de Ruyter et al. 1996)
pMA5	Shuttle vector; Amp^R(*E. coli*); Kan^R(*B. subtilis*)	Novagen Company, Shanghai, China	(Zhang et al. 2006)
pHT43	Shuttle vector; Amp^R(*E. coli*); Cm^R(*B. subtilis*)	Novagen Company, Shanghai, China	(Nguyen et al. 2007)
$pMA5_{mtg}$	Recombinant expression vector; Amp^R(*E. coli*); Kan^R (*B. subtilis*)	Carrys fused *mtg* gene containing *mtg* propeptide from *S. mobaraensis*	This work
$pHT43_{random}$	Intermediate vector; Amp^R(*E. coli*); Cm^R (*B. subtilis*)	The distance between *Bam*HI and *Xba*I on $pHT43_{random}$ was lengthened compared with pHT43	This work
$pHT43_{mtg}$	Recombinant expression vector; Amp^R(*E. coli*); Cm^R (*B. subtilis*)	Carrys fused *mtg* gene containing *mtg* propeptide from *S. mobaraensis*	This work

Amp^R ampicillin resistance, *Kan^R* kanamycin resistance, *Cm^R* chloramphenicol resistance

mixture of *Nde*I-SP_{wapA}-*overlapseq* and *mtg-6his-Nhe*I amplicons as the templates (Niwa et al. 1996). After the digestion by *Nde*I and *Nhe*I, SP_{wapA}-*mtg-6his* was cloned into pMA5 (Zhang et al. 2006), digested with the same enzymes to create the plasmid $pMA5_{mtg}$ in which the recombinant SP_{wapA}-MTG-6His will be expressed under control of the constitutive promoter P_{hpaII} (Fig. 1a).

In vector pHT43 (Nguyen et al. 2007), restriction sites *Bam*HI and *Xba*I following the *amyQ* signal sequence locate in close proximity to each other, which hampers insertion of an exogenous gene. In order to insert *mtg* into vector pHT43 between *Bam*HI and *Xba*I to form the fused SP_{amyQ}-*mtg* gene, a random region amplified from vector pNZ8048 (de Ruyter et al. 1996) by primers p5 and p6 was introduced between

Table 2 Primers used in this study

Gene	Primer	Sequence (5′–3′)	Characteristic/function
wapA	p1	GGAATTCCATATGAAAAAAAGAAAGAGGCGA	*Nde*I cleavage site
	p2	CTCTTCCCCCGCGCCATTGTCTGCTAGTACATCGGCTGGCAC	Overlap the 5′ end of p3
mtg	p3	GTGCCAGCCGATGTACTAGCAGACAATGGCGCGGGGGAAGAG	Overlap the 5′ end of p2
	p4	GCGGCCGCTAGCTCAGTGATGGTGATGGTGATGCGGCCAGCCCTGCTTTACCTTG	Codons of hexa histidine followed by a *Nhe*I cleavage site
random	p5	CGCGGATCCTCCTGACTCAATTCCTAATG	*Bam*HI cleavage site
	p6	TCCCCCCGGGTCTAGATAACTTGCTCTATATCCACACTG	*Xba*I-*Xma*I cleavage site
mtg	p7	CGCGGATCCGACAATGGCGCGGGGGAAGAG	*Bam*HI cleavage site
	p8	GTAGTCTAGATCAGTGATGGTGATGGTGATGCGGCCAGCCCTGCTTTACCTTG	Codons of hexa histidine followed by a *Xba*I cleavage site

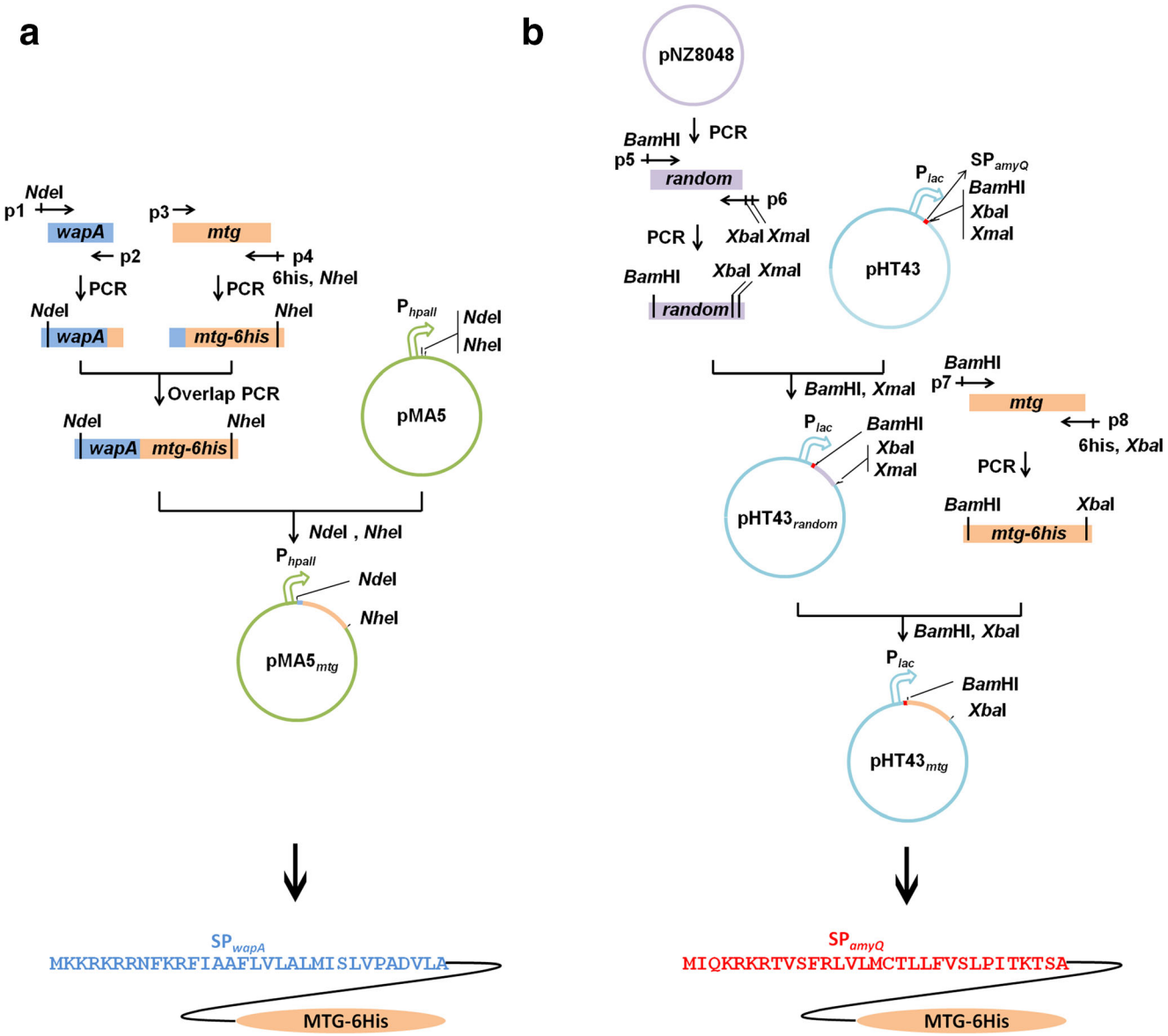

Fig. 1 Expression systems used in this study. **a**: Construction of pMA5$_{mtg}$, **b**: Construction of pHT43$_{mtg}$

*Bam*HI and *Xma*I in pHT43 by relative restriction enzyme digestion. The resulting vector was designated as pHT43$_{random}$. Because a *Xba*I site was added to the 5′ end of p6, the distance between *Bam*HI and *Xba*I on pHT43$_{random}$ was lengthened making inserting exogenous gene within *Bam*HI-*Xba*I possible. A *Bam*HI site was added to the 5′ end of primer p7, and codons of hexa histidine followed by a *Xba*I site were added to the 5′ end of primer p8. The DNA fragment of *mtg* lacking its initial peptide sequence (*BamHI-mtg-6his-XbaI*) was amplified by primers p7 and p8. After the digestion by *Bam*HI and *Xba*I, *mtg-6his* was fused after AmyQ signal peptide encoding gene resulting in pHT43$_{mtg}$ where the recombinant SP$_{amyQ}$-*mtg-6his* will be controlled by inducible promoter P$_{lac}$ (Fig. 1b).

All recombinant vectors were constructed in *E. coli* DH5α (Sambrook and Russell 2001), then transformed into *B. subtilis* and extracted to check by DNA sequencing.

Growth curve of recombinant MTG-His expression strains

B. subtilis strains with/without vector were inoculated into 5 ml of fresh LB medium with/without relevant antibiotics and cultured overnight at 37 °C. One milliliter of the overnight culture was inoculated into 100 ml of fresh LB; the growth curve was drawn by measuring OD_{600} absorbance value during 0–24-h cultivations and fresh LB medium was used as control.

Constitutive expression and purification of recombinant SP_{wapA}-MTG-6His

The overnight grown culture of *B. subtilis* strains harboring $pMA5_{mtg}$ were inoculated at 1:100 ratio into fresh fermention medium and grew for 12, 24, and 48 h. Supernatant was obtained after centrifugation at 8000 rpm for 10 min, and proteins were purified and analyzed by SDS-PAGE (Mu et al. 2018). Purification from *B. subtilis* 168 (pMA5) with fermentation time of 48 h was treated as control.

Inducible expression and purification of recombinant SP_{amyQ}-MTG-6His

B. subtilis 168 harboring $pHT43_{mtg}$ was grown overnight at LB plates containing a final concentration of 5 μg/ml chloramphenicol at 37 °C, then a single clone was picked into 5 ml of fresh LB medium containing 5 μg/ml chloramphenicol and cultured overnight at 37 °C in a shaker at 200 rpm. The seed culture of *B. subtilis* 168 harboring $pHT43_{mtg}$ was inoculated at 1:100 ratio into fresh fermention medium. When the OD_{600} reached 0.5, the culture was induced by 10 μM IPTG (final concentration) and grew for another 2, 12, 24, and 48 h. Supernatant was obtained after centrifugation at 8000 rpm for 10 min, and proteins were purified and analyzed by SDS-PAGE (Mu et al. 2018).

Optimization of the inducer concentration

The overnight grown culture of *B. subtilis* 168 harboring $pHT43_{mtg}$ was inoculated at 1:100 ratio into fresh fermention medium. When the OD_{600} reached 0.5, the culture was splitted into five parallel samples, which were induced by 10, 20, 40, 80, and 120 μM of IPTG, respectively, and grew for another 12 h (since the induction time is optimized at 12 h in the above process). Supernatant was obtained after centrifugation at 8000 rpm for 10 min, and proteins were purified and analyzed by SDS-PAGE (Mu et al. 2018).

Protein purification and digestion

Following centrifugation at 8000 rpm for 10 min, 20 ml of supernatant was taken from the culture of each sample and used for immobilized metal affinity chromatography (IMAC). The obtained supernatants were directly applied to a nickel-nitrilotriacetic acid (Ni-NTA) column. The nickel-nitrilotriacetic acid (Ni-NTA) column resin was equilibrated twice with 38.5 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8), and then 20 ml of supernatants were allowed to bind to 2 ml of the column resin on a rotor at room temperature for 2 h. Subsequently, the column was washed twice with 35 ml of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8). Purified proteins were collected by elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8) in the same volume of column resin, and analyzed by SDS-PAGE (Mu et al. 2018).

Twenty milliliters of supernatants taken from either 48-h cultivation of *B. subtilis* 168 harboring $pMA5_{mtg}$ or 12-h cultivation of 80 μM IPTG-induced *B. subtilis* 168 harboring $pHT43_{mtg}$ were digested with 200 μg/ml (final concentration) trypsin, respectively, for 1 h at 37 °C. Mature MTG-6His were purified by IMAC (see above), analyzed by SDS-PAGE (Mu et al. 2018) and stored at − 80 °C for further use.

Enzymatic activity assay of MTG-6His

In order to measure the enzymatic activity of mature MTG-6His, the concentration of MTG-6His was determined using a Bradford Protein Assay Kit (Bradford 1976), a colorimetric hydroxamate procedure using N-benzyloxycarbonyl-L-glutaminylglycine (Z-Gln-Gly, Sigma-Aldrich Co., St. Louis, MO, USA) as a substrate was then carried out (Grossowicz et al. 1950). Fifty microliters of enzyme solution was mixed with 90 μl substrate solution (final concentrations: 200 mM Tris/HCl-buffer, 100 mM hydroxylamine, 10 mM reduced glutathione, 30 mM Z-Gln-Gly, pH 6.0). After incubation at 37 °C for 10 min, the reaction was stopped with 160 μl stopping reagent (1 vol. 3 M HCl, 1 vol. 12% trichloroacetic acid, 1 vol. 5% $FeCl_3.6H_2O$ (in 0.1 M HCl)). The extinction of the reaction mixture was measured at 525 nm using a microtiter plate reader. One unit of MTG activity was defined as the amount of enzyme required for the formation of 1 μmol L-glutamic acid γ-monohydroxamate/min at 37 °C and pH 6.0.

Crosslinking of soy protein isolate (SPI)

One percent (*w*/*v*) SPI solution was prepared with distilled water and stirred for 12 h at room temperature. The solution was centrifuged at 13,300 rpm for 5 min to filter soluble proteins existing in the supernatant; the supernatant was then mixed with MTG at a ratio of 50:5 (*v*/*v*). The mixture was incubated at 37 °C in a shaker at 200 rpm, samples were taken from the reactions at 30, 60, and 120 min. Two controls were incubated in the same conditions consisting of mature MTG-6His in water and SPI in water. Finally, all the samples and controls were mixed with sample buffer and analyzed by SDS-PAGE gels after staining with 0.25% Coomassie brilliant blue R250.

Statistical analysis

All tests were repeated at least three times, and the data were expressed as mean ± standard deviation (SD). All analyses were performed using the SPSS software (v.13.0, SPSS Inc., Chicago, Ill., U.S.A.).

Results

SP_{wapA} is able to direct secretion of MTG-6His

To select the host strain with highest productivity, $pMA5_{mtg}$ (Fig. 1a) was transformed into *B. subtilis* WB600 (strain deficient in six extracellular proteases; Wu et al. 1991) and *B. subtilis* 168 (Harwood 1992) respectively, obtaining *B. subtilis* WB600 ($pMA5_{mtg}$) and *B. subtilis* 168 ($pMA5_{mtg}$). SP_{wapA}-MTG-6His purified from both *B. subtilis* WB600 ($pMA5_{mtg}$) and *B. subtilis* 168 ($pMA5_{mtg}$) with fermentation times of 12, 24, and 48 h were analyzed by SDS-PAGE and software of BANDSCAN and *B. subtilis* 168 (pMA5) was treated as control. As shown in Fig. 2a, a protein band of with a molecular weight equivalent to the calculated mass (46.9 kDa) of SP_{wapA}-MTG-6His was detected in all six samples except the control indicating the successful secretion of SP_{wapA}-MTG-6His in *B. subtilis*. Moreover, when fermentation time was prolonged, productivity of SP_{wapA}-MTG-6His increased (Fig. 2b) with 48 h fermented *B. subtilis* producing the largest quantity of SP_{wapA}-MTG-6His (*B. subtilis* WB600 3.7/2.6 times of that of 12 h/24 h fermented sample ($P < 0.01$); *B. subtilis* 168: 4.1/1.5 times of that of 12 h/24 h fermented sample) ($P < 0.01$). The amount of SP_{wapA}-MTG-6His obtained from 24 h/48 h fermented *B. subtilis* 168 is 1.9/1.1 times of that of *B. subtilis* WB600 ($P < 0.01$) implying deleting six proteases did not contribute to more accumulation of SP_{wapA}-MTG-6His in *B. subtilis.* Based on this result, *B. subtilis* 168 was selected as host strain for the rest of the experiments.

SP_{amyQ} is able to direct secretion of MTG-6His

The vector $pHT43_{mtg}$ (Fig. 1b) was transformed into *B. subtilis* 168, obtaining *B. subtilis* 168 ($pHT43_{mtg}$). Supernatants from 10 μM IPTG-induced culture of *B. subtilis* 168 ($pHT43_{mtg}$) with different inducing times (2, 12, 24, and 48 h) were collected and purified. As shown in Fig. 2c, a protein band of with a molecular weight equivalent to the calculated mass (47.1 kDa) of SP_{amyQ}-MTG-6His was detected in three of four samples indicating the successful secretion of SP_{amyQ}-MTG-6His in *B. subtilis*. Compared to samples with 2, 24, and 48 h of inducing time, the sample with 12 h inducing time produce most SP_{amyQ}-MTG-6His (1.5/1.1 times of that of 24 h/48 h samples) ($P < 0.01$) while no protein was detected after 2 h of induction (Fig. 2d). The inducing concentration of IPTG was also optimized. Figure 2e showed that after 12 h of induction, the secretion of soluble SP_{amyQ}-MTG-6His was improved efficiently, with concentrations of inducer increasing from 10 to 80 μM, while the sample induced by 120 μM IPTG did not continue this tendency only producing around 65% of that of sample induced by 80 μM IPTG (Fig. 2f).

Expression of MTG-6His slightly affects growth of *B. subtilis*

To explore the impact of expression of SP-MTG-6His imposed on the growth of *B. subtilis*, growth curves of *B. subtilis* 168 strains harboring no vector or four different vectors (pMA5, $pMA5_{mtg}$, pHT43, and $pHT43_{mtg}$) were investigated. As shown in Fig. 3, growth curves of *B. subtilis* 168 (pMA5) and *B. subtilis* 168 (pHT43) almost coincided with that of *B. subtilis* 168 indicating that either pMA5 or pHT43 does not affect the growth of *B. subtilis* 168. Unlike strains harboring other vectors, *B. subtilis* 168 ($pMA5_{mtg}$) had a lower growth profile during the exponential phase. After reaching stationary phase, the values of OD_{600} of all samples stabilized around 2.4 (Fig. 3).

SP_{wapA}-MTG-6His and SP_{amyQ}-MTG-6His produced by *B. subtilis* keep good activity after proteolysis

Two hundred microgram per milliliter of trypsin was used to digest both SP_{wapA}-MTG-6His and SP_{amyQ}-MTG-6His. Figure 4 showed that there is one band corresponding to the theoretical molecular weight (38.9 kDa) of MTG-6His appearing in the lanes of digested, indicating both SP_{wapA}-MTG-6His and SP_{amyQ}-MTG-6His were fully digested by trypsin to generate mature MTG-6His. The concentration of *B. subtilis*-produced MTG-6His was tested up to 63.0 ± 0.6 mg/L from SP_{wapA}-MTG-6His and 54.1 ± 0.3 mg/L from SP_{amyQ}-MTG-6His (Fig. 4c). The specific activities of *B. subtilis*-produced MTG-6His were tested with Z-Gln-Gly as a substrate, and the results were shown in Fig. 4c, after proteolysis by trypsin, mature MTG was tested to have the enzymatic ability of 27.0 ± 0.4 U/mg from SP_{wapA}-MTG-6His and 29.6 ± 0.9 U/mg from SP_{amyQ}-MTG-6His. The measured activities of MTG-6His were relatively equal to what was reported previously (Salis et al. 2015).

Crosslinking of SPI

To further test the enzymatic activity of *B. subtilis*-produced MTG-6His, an SPI crosslinking test was performed. MTG-6His digested from both SP_{wapA}-MTG-6His and SP_{amyQ}-MTG-6His were incubated with 1% (*w*/*v*) SPI solution at 5:50 (*v*/*v*) for different time intervals (30, 60, and 120 min) at 37 °C. The crosslinking was verified by production of high molecular weight products at the top of both the stacking gel and the separating gel, in addition to the disappearance of the β-conglycinin and acidic subunit glycinin protein bands in the middle of the separating gel (Fig. 5). SPI could not be crosslinked linked when signal peptide (SP_{wapA}/SP_{amyQ}) and pro-region were not removed (lane II, Fig. 5a) confirming that the crosslinking was catalyzed by the mature MTG-6His. Compared to commercial MTG (lane VI, Fig. 5a), MTG-6His produced by *B. subtilis* crosslinked the SPI with even more intensive extent.

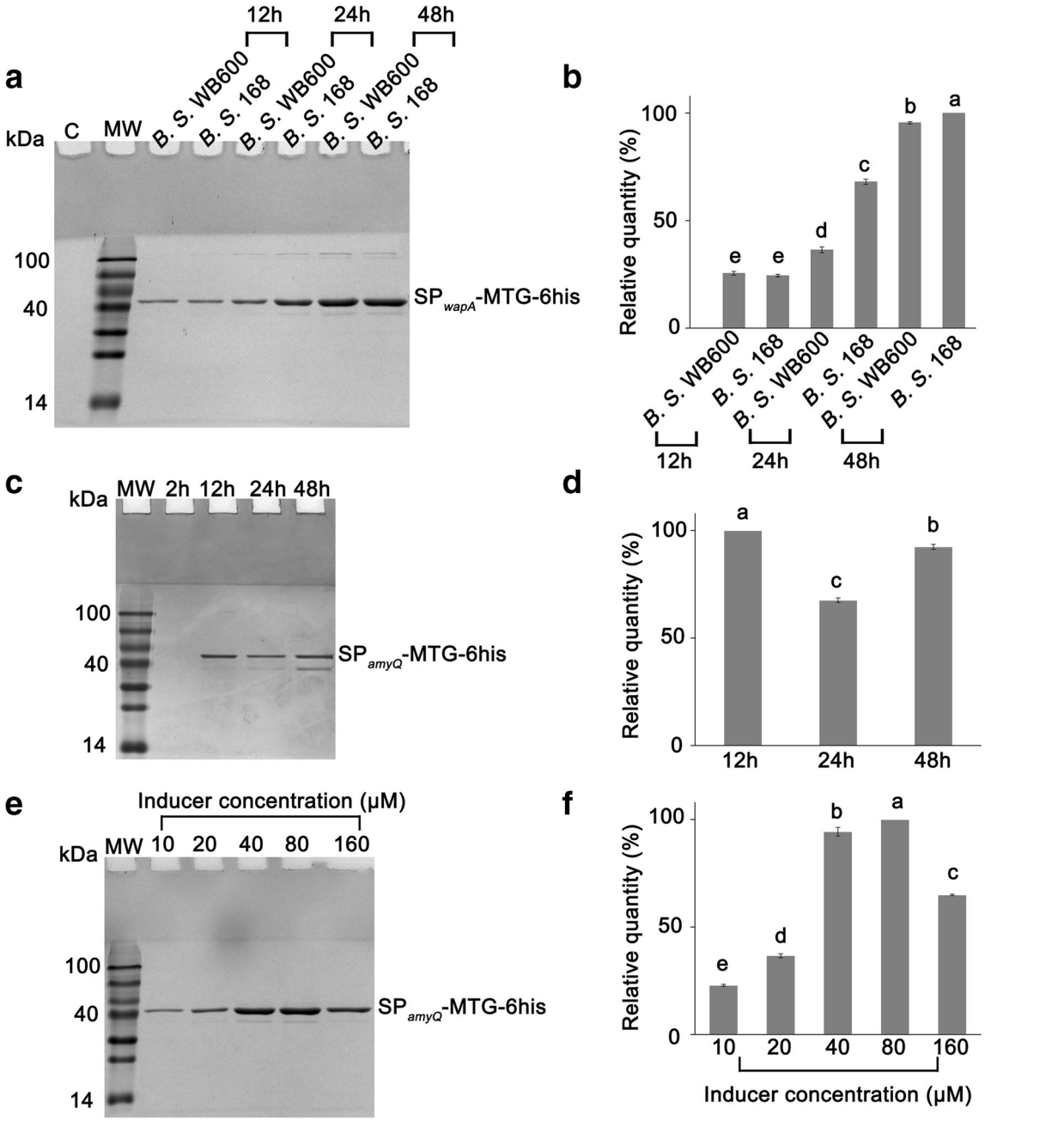

Fig. 2 SDS-PAGE analysis of SP-MTG-6His from *B. subtilis* strains. B. S. *B. subtilis*, MW molecular weight. **a** Purified SP$_{wapA}$-MTG-6His from *B. subtilis* strains (pMA5$_{mtg}$) with fermentation time of 12, 24, and 48 h; Purification from *B. subtilis* 168 (pMA5) with fermentation time of 48 h was treated as control. **b**. The relative quantities of SP$_{wapA}$-MTG-6His from *B. subtilis* strains (pMA5$_{mtg}$) with different fermentation times were estimated with Bandscan software. **c** Purified SP$_{amyQ}$-MTG-6His from 10 μM IPTG-induced culture of *B. subtilis* 168 (pHT43$_{mtg}$) with different inducing times (2, 12, 24, and 48 h). **d** The relative quantities of SP$_{amyQ}$-MTG-6His from 10 μM-IPTG-induced culture of *B. subtilis* 168 (pHT43$_{mtg}$) with different inducing times (12, 24, and 48 h) were estimated with Bandscan software. **e.** Purified SP$_{amyQ}$-MTG-6His from the culture of *B. subtilis* 168 (pHT43$_{mtg}$) with 12-h cultivation and different concentrations of IPTG as inducer. **f** The relative quantities of SP$_{amyQ}$-MTG-6His from the culture of *B. subtilis* 168 (pHT43$_{mtg}$) with 12-h cultivation and different concentrations of IPTG as inducer were estimated with Bandscan software. Values with different letters above the error bars are significantly different at $P < 0.01$ in the ANOVA test

Discussion

Previously, MTG of *S. mobaraensis* had been secreted by *Corynebacterium glutamicum* through the Sec machinery with the signal peptide of CspB of *C. glutamicum* integrated in front of MTG (Kikuchi et al. 2003). In our study, SP$_{wapA}$, a native *B. subtilis* twin-arginine signal peptide, was proven to direct the transportation of MTG of *S. mobaraensis* out from *B. subtilis* implying MTG could be secreted through the Tat pathway as well (Ling

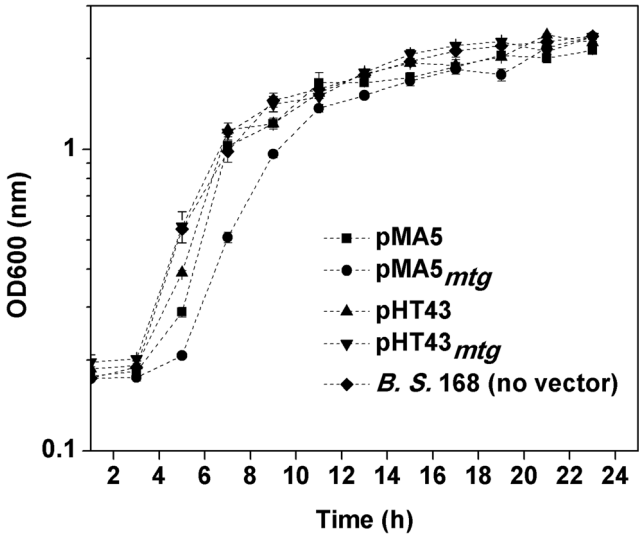

Fig. 3 Growth curves of different kinds of transformed *B. subtilis* 168 strains at 37 °C in a continuously shanking flask. B. S. *B. subtilis*

et al. 2007; Zhang et al. 2013). There are several publications demonstrating the effective transport capacity of the signal peptide of AmyQ from *Bacillus amyloliquefaciens* in *B. subtilis* (Guo et al. 2014; Phan et al. 2006). Similarly, in this study, SP_{amyQ} is shown to secret MTG up to 54.1 ± 0.3 mg/L from *B. subtilis*. Compared to SP_{wapA}, SP_{amyQ} directed slightly lower secretion of MTG of *S. mobaraensis* out from *B. subtilis*, which might be explained by that a native signal peptide has a better transporting capability in *B. subtilis* and/or because this will direct secretion via the Sec pathway, which might be less effective than the Tat pathway for this protein.

B. subtilis WB600 deficient in six extracellular proteases (neutral protease A/subtilisin/extracellular protease/metalloprotease/bacillopeptidase F/neutral protease B) was developed to improve the quality and quantity of the secreted foreign proteins (Wu et al. 1991). However, in our study, no significant difference in MTG production was observed between *B. subtilis* 168 and *B. subtilis* WB600 indicating that MTG is most probably not digested by the six endogenous extracellular proteases/peptidases in *B. subtilis,* although many foreign proteins were reported to be susceptible to them. According to (Kikuchi et al. 2003), subtilisin-like protease SAM-P45 was able to hydrolyze MTG at Ser41. Subtilisin shared high homology with SAM-P45 particularly in regions around the active sites meaning residues outside of this area might play

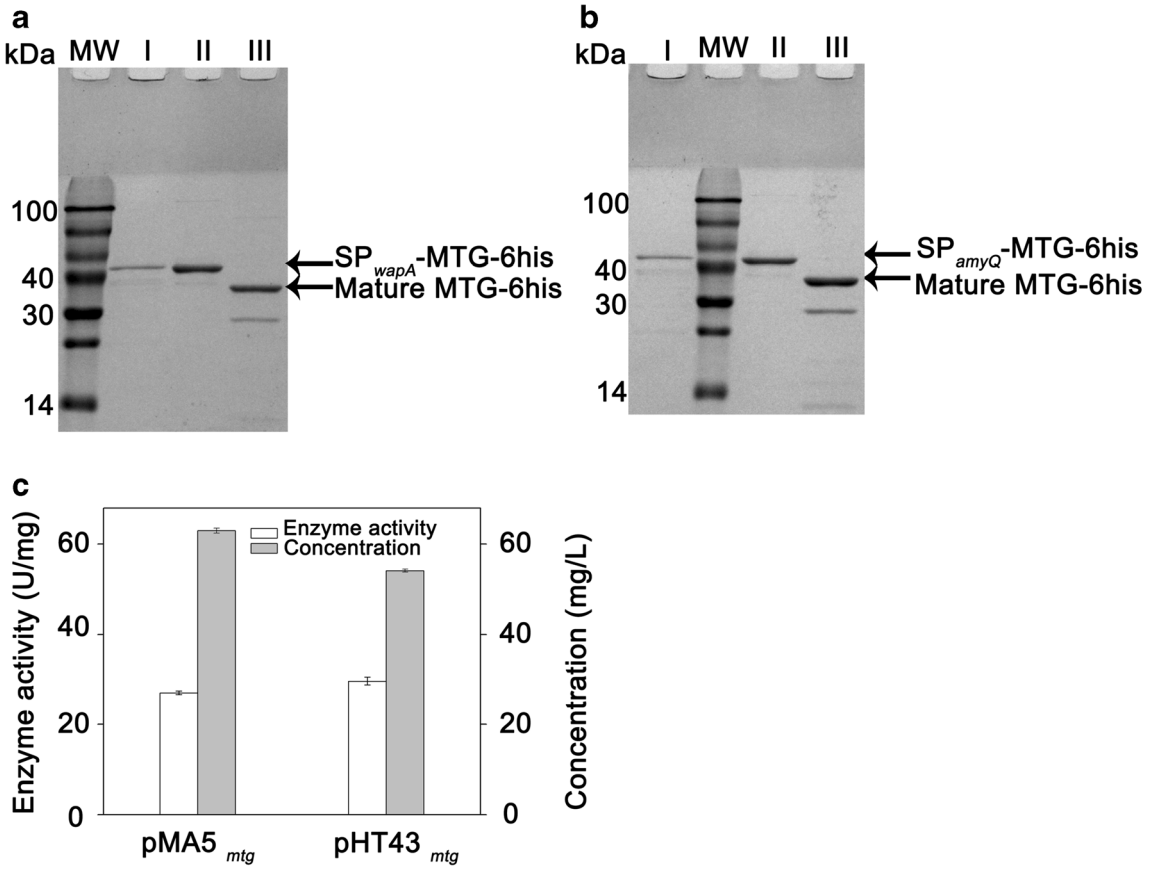

Fig. 4 Analysis of digested SP-MTG-6His. MW molecular weight. **a** Samples from *B. subtilis* 168 ($pMA5_{mtg}$). **b** Samples from *B. subtilis* 168 ($pHT43_{mtg}$). **c** Concentration and enzyme activity of *B. subtilis*-produced MTG-6His. I. protein from the supernatant, II. protein without trypsin digestion, and III. protein activated with terminal concentration of 200 μg/ml trypsin

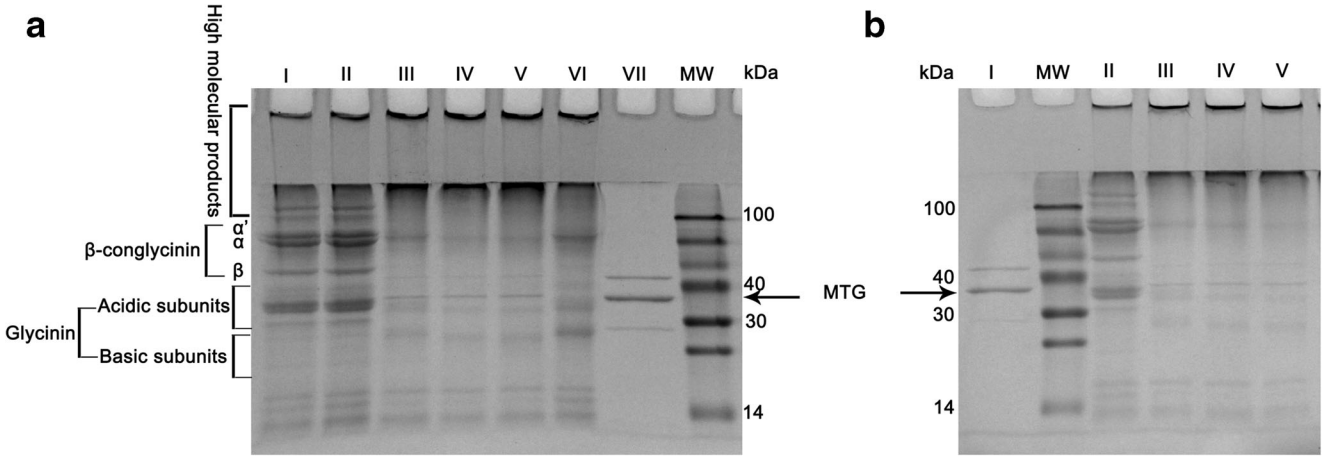

Fig. 5 SPI crosslinking by mature MTG-6His digested from both SP_{wapA}-MTG-6His (**a**) and SP_{amyQ}-MTG-6His (**b**). MW molecular weight. **a** I. SPI in water, II. SPI with SP_{wapA}-MTG-6His at 120 min, III. SPI with mature MTG-6His at 30 min, IV. SPI with mature MTG-6His at 60 min, V. SPI with mature MTG-6His at 120 min, VI. SPI with commercial MTG at 120 min, and VII. Commercial MTG in water. **b** I. Commercial MTG in water, II. SPI in water, III. SPI with mature MTG-6His at 30 min, IV. SPI with mature MTG-6His at 60 min, and V. SPI with mature MTG-6His at 120 min

critical role in digesting MTG (Suzuki et al. 1997; Taguchi et al. 2002).

Expression of SP_{wapA}-MTG-6His by constitutive promoter P_{hpaII} affected the growth of the host strain negatively. This might be because much energy which should be supplied for regular cell metabolism is used for production of SP_{wapA}-MTG-6His while in the case of SP_{amyQ}-MTG-6His, this process was buffered by using the inducible promoter P_{lac}, which was initiated at the middle of exponential phase. Similar results have been observed before. When GFP was under the control of inducible promoter P_{nisA} /P_{czcD}, the induction of GFP did not affect the growth profile of host strains (Mu et al. 2013).

The SPI crosslinking experiment has proven the usefull activity of MTG produced by *B. subtilis* for application. Although mature MTG derived from SP_{wapA}-MTG-6His and SP_{amyQ}-MTG-6His share 100% homology (not considering 6His tail) with commercial MTG, more intensive bands remained in the sample (lane VI, Fig. 5a) treated by commercial MTG. This might be caused by the incomplete proteolysis of pro-region for commercial MTG as observed in lane VII of Fig. 5a.

In this work, *S. mobaraensis* MTG has been for the first time secreted successfully from two systems by using *B. subtilis* as a host strain. The productivity of active MTG reached 63.0 ± 0.6 mg/L. Considering *B. subtilis* as the most widely used Gram-positive platform for protein engineering, our work provides the potential toolbox to engineer designed MTGs in the future research with an easy purification process. We demonstrate that *B. subtilis* has great potential as a host for the industrial production of MTG heterologous proteins.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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