J Biosci Vol. 42, No. 4, December 2017, pp. 537–546
DOI: 10.1007/s12038-017-9707-4

Specific mutation of transglutaminase gene from *Streptomyces hygroscopicus* H197 and characterization of microbial transglutaminase

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MS received 7 May 2017; accepted 17 August 2017; published online 20 September 2017

Microbial transglutaminase (MTG) gene (*mtg*) from *Streptomyces hygroscopicus* H197 strain was cloned by PCR and mutated by deleting a specific 84 bp fragment using overlapping extension PCR. The mutant MTG and the wild MTG genes expressed by recombinant plasmid pET32a^{+}-mutant *mtg* and pET32a^{+}-mtg, respectively, and were harvested by alternating freeze–thaw steps and purified by Ni column. The purified mutant MTG and the wild MTG exhibited 0.22 U/mg and 0.16 U/mg activity, respectively, and 0.69 U/mg and 0.54 U/mg activity, respectively, after activated by trypsin. The molecular weight of mutant MTG was estimated as 67 kDa by SDS-PAGE. Both MTGs showed optimum activity at pH 6–8 for hydroxamate formation from N-CBZ-Gln-Gly and hydroxylamine, and exhibited higher stability at 40°C and 1–3% salinity. The two types of MTG were not stable in the presence of Zn(II), Cu(II), Hg(II), Pb(II), Fe(III), and Ag(I), suggesting that they could possess a thiol group. In addition, the mutant MTG and the wild MTG were strongly affected by ethanol. Furthermore, the mutant MTG was obviously ($P < 0.05$ or $P < 0.01$) more stable than the wild MTG at 50°C and 60°C, at pH 4, 5, and 9, at 7% and 9% salinity, 30% and 35% ethanol concentration, and in the presence of Li(I) and Ag(I). The polyhydroxy compounds as protein stabilizers could elevate MTG stability.

Keywords. Enzymatic properties; microbial transglutaminase; overlapping extension PCR; purification

Abbreviations: DTT, Dithiothreitol; EDTA, Ethylenediaminetetraacetic acid; MTG, Microbial transglutaminase; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TG, Transglutaminase; Trx, Thioredoxin

1. Introduction

Transglutaminases (TG; protein-glutamine γ-glutamyl-transferase, EC 2.3.2.13) catalyse acyl transfer reactions by introducing covalent cross-linking between proteins as well as peptides and various primary amines (Ohtsuka *et al*. 2000; Yokoyama *et al*. 2004). TGs are widely found in animals (Yasueda *et al*. 1995; Folk *et al*. 1966a; Schmid *et al*. 2011), plants (Carvajal *et al*. 2011), and microorganisms (Yokoyama *et al*. 2004). Microbial TG (MTG) was first obtained by the fermentation of a *Streptomyces mobaraensis* (Ando *et al*. 1989), and it was subsequently discovered in new microbial strains such as *Streptomyces* (Duran *et al*. 1998; Zhang *et al*. 2010) and *Bacillus* (Zhu *et al*. 1996; Kobayashi *et al*. 1998; De Barros *et al*. 2003). MTG produced by *Streptomyces mobaraenis* has been widely used in the food industry to improve the functional properties of food products (De Jong and Koppelman 2002; Yokoyama *et al*. 2004). Recent studies have suggested that TG-mediated cross-linking also has great potential in tissue engineering, leather and textiles processing, biotechnological tools, and other non-food applications (Zhu and Tramper 2008; Heck *et al*. 2013).

Several attempts have been made to obtain MTG with high yield, high activity, or high stability, such as by using optimizing expression system: MTG is produced in a secreted or soluble way (Marx *et al*. 2008a; Liu *et al*. 2011). Random mutagenesis of recombinant transglutaminase has been made to get new variants with heat-resistance and heat-sensitive properties (Marx *et al*. 2008b); saturation mutagenesis method has been carried on to delete N-terminal amino acid residues of microbial transglutaminase produced by *Streptomyces hygroscopicus*, and thus could enhance the enzyme activity and thermal stability (Chen *et al*. 2012). From the practical viewpoint, it is desirable to develop novel MTG with high activity or stability and seek soluble expression systems.

To determine whether the deletion of a particular sequence analysed by PyMOL software (Yuan *et al*. 2016) could enhance MTG activity or stability, in this study, a pair of primers with complementary sequence was designed to implement gene mutation by overlapping extension PCR technique. The mutant microbial

transglutaminase gene (mutant mtg) was ligated with pET32a$^+$ plasmid which can express a solubility promoting peptide thioredoxin (Trx) and was expressed in *E. coli* Rosetta. This research offers a reference to the study of the effect of specific peptide fragment on enzyme stability by using overlapping extension PCR technique and provide an alternative mutant MTG with high stability for industry applications.

2. Materials and methods

2.1 *Bacterial strains and plasmid*

Streptomyces hygroscopicus H197 containing the *mtg* gene (GenBank ID: KY111759), which secretes TG, was isolated in a previously study. *Escherichia coli* Rosetta (DE3) (Novogen, ON, Canada) and pET32a$^+$ (Novogen, ON, Canada) were used for the expression of pro-TG.

2.2 *Cloning of the MTG gene*

Streptomyces hygroscopicus H197 genomic DNA was extracted by using Tris-saturated phenol after H197 strain was ground under liquid nitrogen. For the amplification of *mtg* gene from *Streptomyces hygroscopicus* H197 genomic DNA, a pyrobest DNA polymerase (Takara, Dalian, China) was used. The amplification mix contained 2.5 μL 10× Ex Taq buffer, 1 μL dNTPs (2.5 mmol/L), 1 μL forward primer (10 ρmol/μL), 1 μL reverse primer (10 ρmol/μL), 30 ng of genomic DNA, 0.5 μL pyrobest DNA polymerase (5 U/μL) and water to 25 μL.

The forward primer named F_1 (5′-CGA GGA TCC ATG TAC AAA CGC CGG AGA TT-3′) contained a *Bam*HI recognition sequence, and the reverse primer named R_1 (5-CGC AAG CTT ACG GCC AGC CCT GCT T-3′) contained a *Hind*III recognition sequence.

The reaction was performed in a thermocycler (Bio-Rad, USA) for 5 min at 95°C, 34 cycles of 1 min at 95°C, 1 min at 56°C, 1 min at 72°C and a final elongation step of 10 min at 72°C. The amplified gene products were observed on 1% agarose under ultraviolet at 254 nm.

2.3 *Construction of recombinant plasmid pET-32a$^+$-mtg*

Ten microliters of pET-32a$^+$ (Novagen, about 20 ng/μL) were digested with 0.5 μL *Bam*HI (15 U/μL) and 0.5 μL *Hind*III (15 U/μL) in 5 μL 10× buffer (Takara) and water to 20 μL. The PCR product was digested accordingly. Incubation was carried out for 16 h at 37°C followed by a heat inactivation of the restriction enzymes for 20 min at 70°C.

The digested DNA was purified by gel electrophoresis, and the gel bands were purified with the AxyPrep^{TM} DNA Gel Extraction Kit (Axygen, USA) and eluted with 10 μL Tris-HCl (10 mmol/L, pH 8.5).

About 30 ng of PCR product were ligated with about 30 ng of digested plasmid (equivalent to a molar ratio of 4:1) with 1 μL of T4 DNA ligase (350 U/μL, Takara) in 2 μL of 10× T4 DNA ligase buffer and water to 20 μL. The sample was incubated at 16°C for 16 h followed by an inactivation of ligase at 70°C for 10 min.

2.4 *Overlapping extension PCR for mutant mtg gene*

Overlapping extension PCR technique containing two rounds of PCR reaction was used to achieve a specific fragment knockout of the *mtg* gene (figure 1). F_1 primer and reverse primer R_2 (5′-ACC CGC AGC CAG GGC CCT-3′) primer were used in the first round of PCR reaction for amplifying fragment A, and R_1 primer and forward primer F_2 (5′-AGG GCC CTG GCT GCG GGT CCC GCC GAG CCG CTC AAC CGG AT-3′) were used for amplifying fragment B. The sequence A and sequence B could be connected in the second round PCR reaction due to the complementary sequence provided by a pair of F_2 and R_2 primers.

The first round of PCR reaction amplification was conducted in a 25 μL system containing 2.5 μL 10× pyrobest buffer, 1 μL dNTPs (2.5 mmol/L), 1 μL forward primer (10 pmol/μL), 1 μL reverse primer (10 pmol/μL), about 25 ng of genomic DNA, 0.5 μL pyrobest DNA polymerase (5 U/μL) and water to 25 μL. The second round of PCR reaction amplification was carried out in a 25 μL system containing 2.5 μL 10× pyrobest buffer, 1 μL dNTPs (2.5 mmol/L), 1 μL F_1 (10 pmol/μL), 1 μL R_1 (10 pmol/μL), 1 μL sequence A, 1 μL sequence B, 0.5 μL pyrobest DNA polymerase (5 U/μL) and water to 25 μL.

The two rounds of PCR reaction were performed in a thermocycler (Bio-Rad, USA) for 5 min at 95°C, 25 cycles of 1 min at 95°C, 1 min at 56°C, 1 min at 72°C and a final elongation step of 10 min at 72°C. The amplified gene products were observed on 1% agarose under ultraviolet at 254 nm.

2.5 *Construction of recombinant plasmid pET-32a$^+$-mutant mtg*

The mutant *mtg* gene was cloned into pET-32a$^+$ employing the steps discussed above. The only difference here was that the ligation was carried out between pET-32a$^+$ and mutant *mtg* gene derived from a specific fragment deletion of *mtg* gene.

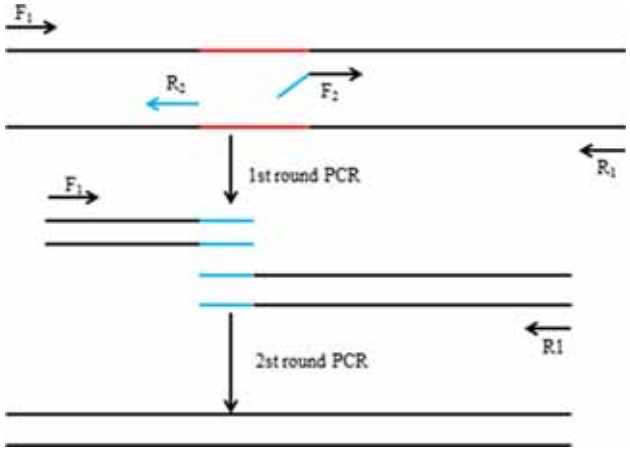

Figure 1. Procedures of overlapping extension PCR for deleting specific DNA fragments. In the first round PCR, primer F_1 and primer R_2, and primer F_2 and primer R_1 were used to amplify fragment A and fragment B, respectively. In the second round PCR, fragment A and fragment B were ligated due to the complementary sequence (blue) provided by primer R_2 and primer F_2, and thus the specific fragment (red) was deleted.

2.6 *Protein expression in E. coli*

E. coli Rosetta cells carrying pET-32a$^+$-mtg plasmid or pET-32a$^+$-mtg plasmid were inoculated to 10 mL of LB medium containing 100 μg/mL ampicillin (Sigma, USA) and incubated at 37°C with shaking at 200 rpm for 12 h. Five milliliters of this culture were added to 100 mL of LB medium containing 100 μg/mL ampicillin and incubated at 37°C. At an OD600 value of 0.5, cells were induced by adding 60 mmol/L of IPTG (Sigma, USA) stock solution (IPTG final concentration was 0.3 mmol/L). Growth was continued at 37°C or 25°C for 8 h.

MTG expressed by recombinant plasmid pET-32a$^+$-mtg and pET-32a$^+$-mutant *mtg* were designated as wild MTG and mutant MTG, respectively.

2.7 *Protein acquisition and localization*

Five milliliters of bacterial suspension samples were collected and centrifuged at 10,000*g* for 10 min and the pellets resuspended in 2 mL of phosphate buffer saline (1× PBS) (8.1 mmol/L Na_2HPO_4, 1.9 mmol/L KH_2PO_4, 153.8 mmol/L NaCl, pH 7.4) after bacteria were washed twice by 5 mL of 1× PBS (pH 7.4). Cells were lysed by five alternating freeze-thaw-steps (1 h at −70°C, 20 min at 20°C) and the whole cell extract was centrifuged at 10,000*g* for 10 min and the supernatant was retained.

For determining the distribution of MTG expressed by *E. coli* Rosetta, fermentation supernatant, lysate supernatant, and total cell proteins were analysed by SDS-PAGE.

2.8 *MTG purification and activation*

Recombinant Rosetta cells carrying pET-32a$^+$-mtg or pET-32a$^+$-mutant *mtg* were cultivated in 100 mL LB medium, and induced by IPTG at 25°C for 12 h. The cells were harvested by centrifuging at 10,000*g* for 10 min and crushed by five alternating freeze–thaw steps as described above. The lysed cells were centrifuged at 10,000*g* for 15 min. The crude extract was applied to a 'High Affinity Ni-Charged Resin' column (GenScript, USA) and washed with 8× bed volumes of Washing buffer (20 mmol/L Tris-HCl, 20–100 mmol/L imidazole, 0.5 mol/L NaCl), and then eluted by using 10× bed volumes of Elution buffer (20 mmol/L Tris-HCl, 500 mmol/L imidazole, 0.5 mol/L NaCl). The elution liquid was collected and dialysed in 1× PBS (pH 7.4) for 12 h. The purified protein was stored at −20°C for subsequent experiments.

The strategy of the expression of pro-TG (inactive zymogen) followed by processing *in vitro* is often used in TG production, the pro-MTG can be activated by adding exogenous proteases (Marx *et al*. 2008a). The activation of pro-MTG (mutant MTG and wild MTG) to a constant activity level was performed by adding 10 μL of 2.5% trypsin (Thermo) to the purified MTG samples and incubated at 30°C for 30 min, according to Pasternack *et al*. (1998).

2.9 *Determination of enzyme activity*

The activity of MTG was assayed according to the colorimetric hydroxamate procedure (Folk and Cole 1966b). A calibration curve was measured using commercially available L-glutamic acid γ-monohydroxamate in the range from 0 to 10 mmol/L. One unit of MTG is defined as the formation of 1 μmol L-glutamic acid γ-monohydroxamate per min at 37°C.

To 50 μL of enzyme solution, 90 μL of substrate solution (final concentrations: 0.2 mol/L Tris-HCl buffer, 100 mmol/L hydroxylamine, 10 mmol/L reduced glutathione, 30 mmol/L Z-Gln-Gly, pH 6.0) were added. After incubation in a thermomixer at 37°C for 10 min, the reaction was stopped with 160 μL reagent A (1 vol. 3 mol/L HCl, 1 vol. 12% trichloroacetic acid, 1 vol. 5% $FeCl_3 \cdot 6H_2O$ (in 0.1 mol/L HCl)). An aliquot of 200 μL supernatant was transferred to a transparent 96-well MTP (Boyang, China) after the mixture was centrifuged at 10,000*g* for 5 min, and the extinction was measured at 525 nm using a microplate reader (Thermo, USA).

2.10 *Biochemical characteristics of MTG*

To assess how pH, temperature, metal ion and inhibitor, salinity, polyhydroxy compound, and ethanol concentration affected the activity of mutant MTG and wild MTG, single

factor experiments were conducted to determine the effects of these factors. In the pH experiments, the pH of purified MTG samples were adjusted to 4, 5, 6, 7, 8, and 9 by adding the same volume of different buffers. These buffers were as follows: 50 mmol/L of acetic acid-sodium acetate buffer, pH 4 and 5; 50 mmol/L of Tris-HCl buffer, pH 6–9. The mixtures were pre-incubated at 10°C for 30 min. In the temperature experiments, the purified MTG samples were pre-incubated at different temperature ranging from 20 to 80°C for 30 min. In the salinity experiments, the purified MTG samples were adjusted to different salinity ranging from 1% to 11% (w/v) by adding NaCl, the mixtures were pre-incubated at 10°C for 30 min. In the metal ion and inhibitor experiments, the MTG samples supplemented with K(I), Na(I), Li(I), Ca(II), Zn(II), Cu(II), Co(II), Hg(II), Pb(II), Fe(III), Mn(II), Ni(II), Ag(I), EDTA (all metal ions final concentration was 5 mmol/L), and DTT (final concentration was 0.8 mmol/L), and the mixtures were pre-incubated in the same condition. In the ethanol concentration experiments, ethanol was separately added to the purified MTG samples and adjusted to the final concentrations ranging from 10% to 50% (v/v), the mixtures were pre-incubated at 10°C for 30 min. In the polyhydroxy compound experiments, 10% (v/v) of glycerol, sorbitol, mannitol, cellulose, and sucrose, and PEG (final concentration was 0.8 mol/L) were separately added to the purified MTG samples and pre-incubated at 60°C for 30 min. After pre-incubation, 50 µL of the treated enzyme solution was extracted for activity determination. The relative activity was calculated as the percentage of activity remaining after incubation in different conditions, and the relative activity of a sample without pretreatment was taken as 100%.

2.11 *Assessment of cross-linking performance*

To explore the activity of mutant MTG and wild MTG in practical cross-linking reaction, the enzymatic reactions catalysed by MTG were carried out at 25°C in 1× PBS (pH 7.4) containing 1% (w/v) casein. The reaction mixtures were incubated for various times ranging from 0 to 100 min, and then stopped by directly mixing the reagent A described above. The reaction level was determined by measuring the amount of ammonia release using Nessler's reagent spectrophotometry (APAH 1998).

3. Results

3.1 *Overlapping extension PCR for mutant mtg gene*

The spatial configuration of MTG was simulated by PyMOL software (figure 2a). The MTG had a triplet active center Cys151-Asp342-His361. The specific 84 bp DNA fragment was deleted by overlapping extension PCR as shown in figure 2b. The size of mutant mtg gene (1173 bp) was clearly smaller than that of mtg gene (1257 bp). The 84 bp DNA fragment could be translated to a hydrophobic sequence with 28 amino acid residues (QPG NSL AEL PPS VSA LFR APD AAD ERV T). The deletion of 28 amino acid residues fragment presented an external free state that

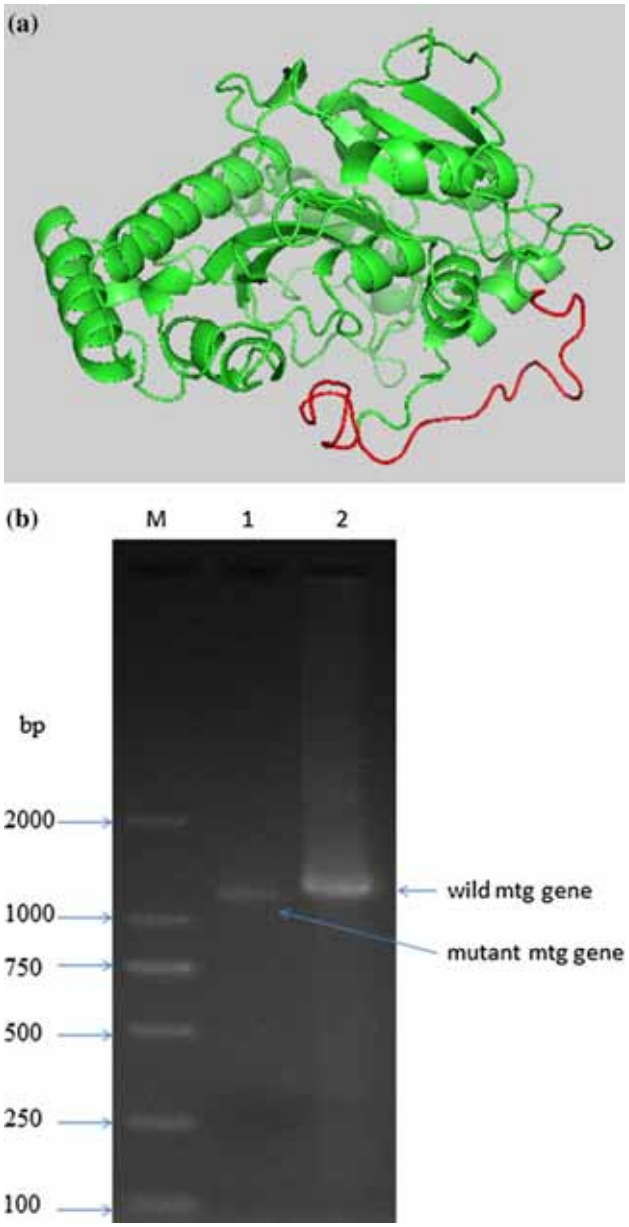

Figure 2. MTGase spatial configuration and *mtg* gene size. (**a**) MTGase tertiary structure according to the known configuration template of 3IU0, the specific 28 amino acid residues sequence (red) was removed to get mutant MTGase. (**b**) The specific 84 bp DNA fragment was deleted by overlapping extension PCR. (M: DL2000 marker; Lane 1: 1173 bp of purified mutant mtg gene; Lane 2: 1257 bp of purified *mtg* gene).

might result in the mutant MTG spatial configuration to be compressed and thus could enhance the stability or solubility of mutant MTG.

3.2 *Expression and localization of MTG*

In order to overexpress MTG, the expression strain was cultivated in a 100 mL shaking flask containing LB medium and ampicillin at 37°C. Protein expression was induced by IPTG either at 37°C, i.e. maintaining cultivation temperature or after cooling to 25°C. Bacterial suspensions were harvested after induction and MTG activity was determined (table 1). The activity of the mutant MTG and the wild MTG were improved after activated by adding trypsin.

To investigate the distribution of mutant MTG, bacterial suspensions were treated as described above to obtain fermentation supernatant, lysate supernatant, lysate precipitation, and total cell proteins. According to the result of SDS-PAGE shown in figure 3, the mutant MTG did not exist in fermentation supernatant (see lane 5), and mostly existed in lysate supernatant (see lanes 1 and 2, 3 and 4). The molecular weight of mutant MTG was close to 70 kDa. The results indicated that the mutant MTG was intracellular expression protein and mainly expressed in soluble form.

3.3 *Effects of environmental factors on enzyme activity*

The effect of pH on MTG activity was determined by testing enzyme activity as described previously at pH 4–9. The mutant MTG and wild MTG exhibited optimum activity for the catalytic reaction of N-CBZ-Gln-Gly and hydroxylamine in a range of pH 6–8, and showed acceptable activity at pH 4, 5, and 9 (figure 4a). The mutant MTG could keep 57%, 78%, 72% activity at pH 4, 5, and 9, respectively. Similarly, the wild MTG could maintain 50%, 66%, and 50% activity at pH 4, 5, and 9, respectively. Date analysis showed that the mutant MTG was significantly more stable than the wild MTG at pH 4, 5, and 9 ($P < 0.05$ or $P < 0.01$). The mutant MTG and wild MTG shared a same optimum pH range of 6–8, which was nearly the same as that from *Streptoverticillium mobaraense* (Ando *et al*. 1989) and *Streptomyces hygroscopicus* (Cui *et al*. 2007). The mutant MTG and the wild MTG activities decreased gradually at alkaline condition and reduced rapidly at acidic condition. It was unlike

Table 1. Summary of MTGase enzyme activity.

	Mutant MTGase (U/mg)	Wild MTGase (U/mg)
Crude enzyme	0.34	0.28
Purified	0.22	0.16
Activated	0.69	0.54

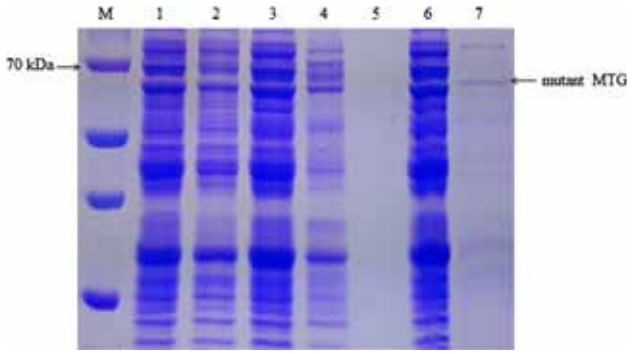

Figure 3. Localization and purification of mutant Trx-pro-MTG-His_6 (Lane M, standard marker; Lane 1, total cell proteins at 37°C; Lane 2, lysate supernatant at 37°C; Lane 3, total cell proteins at 25°C; Lane 4, lysate supernatant at 25°C; Lane 5, fermentation supernatant at 25°C; Lane 6, lysate precipitation at 25°C; Lane 7, purified MTGase). Ten microliters of sample were applied in each lane; the feeble bands were useless protein expressed by host cell gene.

transglutaminase produced by *Bacillus subtilis* having an optimal pH value of 8.2 (Suzuki *et al*. 2000). The TG from mammals and fishes had an optimum pH of 8.0 and soybean TG had an optimal pH value of 7.6 (Gerber *et al*. 1994; Zhu *et al*. 1996).

The effects of temperature on MTG activity were studied by determining the activity between 20°C and 70°C after enzymes were incubated at pH 7 for 30 min, with the reaction mixtures as indicated previously. The mutant MTG and wild MTG exhibited optimum activity at 20–40°C, and acceptable activity at 50°C for the catalytic reaction of N-CBZ-Gln-Gly and hydroxylamine (figure 4b). 43% activity of the MTG and 33% activity of the wild MTG were retained at 60°C. Besides, less than 15% activity and nearly no enzyme activity were detected at 70°C and 80°C, respectively. Statistical analysis demonstrated that mutant MTG was significantly more stable than wild MTG at 50°C and 60°C ($P < 0.05$). Comparing with other MTG from different sources, the optimal temperature of MTG almost was the same as that from *Streptoverticillium ladakanum* and diverging from *Streptoverticillium mobaraense* and *Bacillus subtilis* MTG which had the optimum temperature of 55°C and 60°C, respectively with the same substrate and incubation time (Ando *et al*. 1989; Ho *et al*. 2000; Suzuki *et al*. 2000).

63% activity at 7% salinity, 35% activity at 9% salinity, and 16% activity at 13% salinity of mutant MTG could be maintained (figure 4c). Similarly, 51% activity, 21% activity, and 10% activity of the wild MTG could be kept at 7%, 9%, and 11% salinity, respectively. The mutant MTG was remarkable more stable than wild MTG at 7% and 9% salinity ($P < 0.05$). The result was similar to that of crude tilapia TG (Worratao and Yongsawatdigul 2003, 2005).

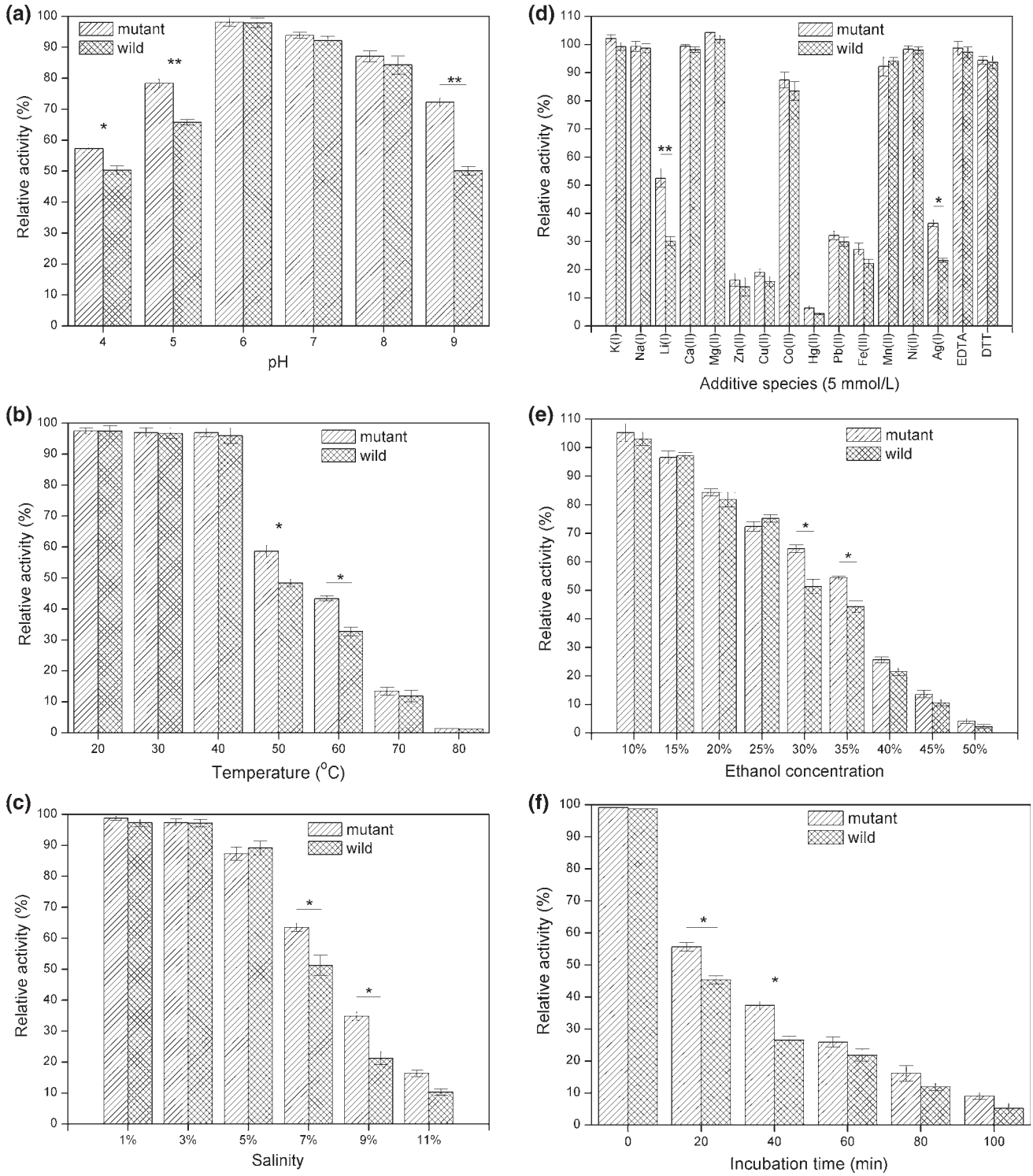

Figure 4. Effect of different environmental factors on the activity of purified MTGase: (**a**) pHs; (**b**) temperatures; (**c**) salinities; (**d**) metal ions and inhibitors; (**e**) ethanol concentrations; (**f**) incubation times. The results are the mean values of three experiments; error bars represent standard error (*$P < 0.05$, **$P < 0.01$).

Sensitivity of TG to NaCl was also reported in other TG, including those from carp dorsal muscle (Kishi *et al*. 1991) and from Japanese oyster (Kumazawa *et al*. 1997).

The relatively activity of MTG was investigated in the presence of several metal ions and two inhibitors (figure 4d). The purified mutant MTG and wild MTG was evidently ($P < 0.01$) inhibited by Li(I), Zn(II), Cu(II), Hg(II), Pb(II), Fe(III), and Ag(I), slightly ($P < 0.05$) restrained by Co(II), Mn(II), and DTT, and not depressed by K(I), Na(II), Ca(II), Mg(II), Ni(II), and EDTA ($P > 0.05$). In addition, the mutant MTG was significantly more stable than the wild MTG in the presence of Li(I) ($P < 0.01$) and Ag(I) ($P < 0.05$). These results were consistent with those of *Streptoverticillium mobaraense* was strongly inhibited by Zn(II), moderately by Pb(II), and unaffected by Cu(II) (Ando *et al*. 1989), and *Streptomyces hygroscopicus* was strongly by Zn(II), Cu(II), Fe(III), Hg(II), and Pb(II) (Cui *et al*. 2007). The purified mutant MTG and wild MTG were not inhibited by Ca(II) and EDTA, suggesting that it was Ca(II)-independent, which was completely different from those of Ca(II) dependent TG from animal tissues or organs (Icekson and Apelbaum 1987; Worratao and Yongsawatdigul 2005) and was similar to those from microorganisms (Ando *et al*. 1989; Ho *et al*. 2000; De Barros *et al*. 2003). This property was very useful in modifying food proteins, such as milk caseins, soybean globulins, and myosins were sensitive to and easily precipitated by Ca(II) (Yokoyama *et al*. 2004).

The mutant MTG activity and the wild MTG activity slightly elevated with a lower concentration of ethanol after incubated at 25°C for 30 min (figure 4e). With increasing ethanol concentration, the enzyme activity decreased, and almost had no activity in the presence of 50% ethanol. The mutant MTG was obviously more stable than the wild MTG in the presence of 30% and 35% ethanol ($P < 0.05$). The result was consistent with that of MTG produced by *Streptomyces hygroscopicus* (Cui *et al*. 2007) which showed high concentration ethanol inhibited MTG activity.

In order to know whether increasing incubation time could improve MTG activity, the mutant MTG and the wild MTG were incubated at 60°C for different times. The activity of both MTG descended sharply in 20 min and reduced gradually from 20 to 100 min (figure 4f). 9% activity of the mutant MTG and 5% activity of the wild MTG were left at the incubation end (100 min). However, the mutant MTG was also significantly ($P < 0.05$) more stable than the wild MTG when these two types of MTG incubated for 20 min or 40 min. The phenomenon might be due to that the ordered structure of MTG was gradually lost during heat treatment at 60°C, resulting in the decrease of MTG activity. The result was consistent that of MTG (Cui *et al*. 2008) which presented enzyme activity lost during heat treatment at 60°C.

3.4 *Enhancement of MTG activity*

To examine the protective effect of some protein stabilizers on thermal inactivation of MTG, the residual activity was measured after the purified MTG was incubated at 60°C for 30 min in the presence of glycerol, sorbitol, mannitol, sucrose, cellulose, and PEG, respectively. As shown in figure 5, about 60% activity of the mutant MTG and about 70% activity of the wild MTG were lost. At the given concentrations of different stabilizers, glycerol and sorbitol exhibited a higher stabilizing effect on the mutant MTG and more than 60% activity of the mutant MTG was preserved, while sorbitol and mannitol presented a higher stabilizing effect on the wild MTG and about 60% activity of the wild MTG was retained. Besides, polyhydroxy compounds of sucrose, cellulose, and PEG also showed a higher stabilizing effect on the mutant MTG and the wild MTG, and more than 50% activity of these two MTG was kept. Data analysis demonstrated that glycerol obviously stabilized the mutant MTG than the wild MTG ($P < 0.05$).

3.5 *Determination of cross-linking reaction*

The effects of ethanol on the mutant MTG cross-linking reaction represented by the amount of released ammonia were shown in figure 6. The presence of 20% ethanol did not evidently influence the cross-linking reaction of casein catalysed by the mutant MTG, and the amount of ammonia produced by cross-linking casein with the mutant MTG in

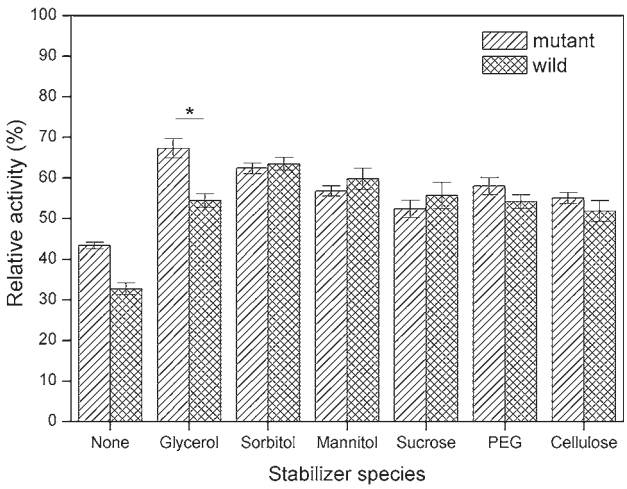

Figure 5. Effects of different stabilizers on the residual activity of MTGase after 30 min of incubation at 60°C with PEG at a concentration of 0.8 mol/L and with 10% (v/v) of glycerol, sorbitol, mannitol, sucrose and cellulose, respectively. The results are the mean values of three experiments; error bars represent standard error (*$P < 0.05$).

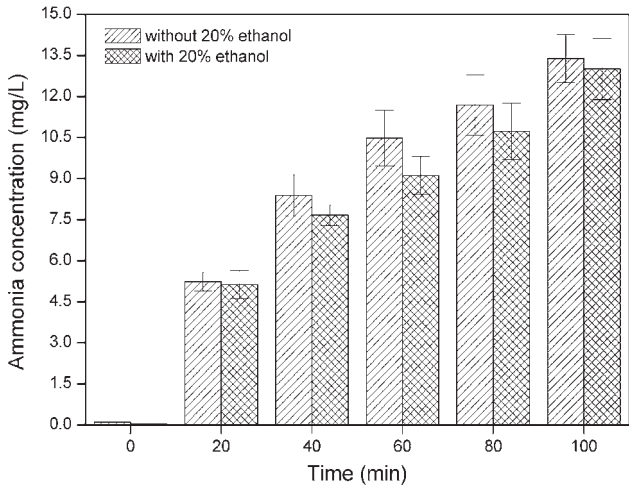

Figure 6. Effect of ethanol on the cross-linking reaction of casein catalysed by the mutant MTGase by determining the amount of ammonia produced. The results are the mean values of three experiments; error bars represent standard error.

the presence of 20% ethanol was less than the control. The amount of ammonia gradually increased and got to the highest value of 13.4 mg/L and 13.0 mg/L after incubation for 100 min in the presence of without 20% ethanol reaction system and with 20% ethanol reaction system, respectively. The result indicated that the mutant MTG could exhibit good activity in terms of cross-linking reaction and was not affected by 20% ethanol.

4. Discussion

Today, MTG has captured researcher's interest due to its attractive potential application for its special catalytic character. So, it is very necessary to obtain the enzyme with high thermal stability and activity for its preparation and application in industrial purposes. In this study, in order to gain high stability or high activity of MTG, a specific 84 bp fragment was deleted from *mtg* gene by overlapping extension PCR and the mutant *mtg* gene carried by pET32a^{+} plasmid was expressed in *E. coli* Rosetta cell.

The mutant MTG and the wild MTG expressed in a soluble way at 25°C or 37°C might be mainly due to the function of Trx peptide which could promote MTG dissolution. It has been reported that the deletion pelB signal peptide of TG also could achieve soluble expression (Marx *et al*. 2007). After the MTGase was activated, the activity of the mutant MTGase and the wild MTGase were 0.69 U/mg and 0.54 U/mg, respectively, which were evidently higher than the purified MTGase. Besides, the supernatant crude enzyme of the mutant MTGase was higher than that of MTGase produced by *Streptomyces hygroscopicus* (0.25 U/mg) (Cui *et al*. 2007).

The specific sequence deletion performed in this study preliminarily suggested that the 28 amino acid residues could affect MTG stability, and thus the mutant MTG presented different activity in different conditions. It has been reported that pH and temperature could influence MTG stability (Cui *et al*. 2007, 2008). Besides, high concentration of NaCl could induce conformational changes of TG, resulting in a decrease of TG activity (Kishi *et al*. 1991; Kumazawa *et al*. 1997). However, NaCl significantly increased TG activity from marine species, such as scallop, botan shrimp (*Pandalus nipponensis*), and squid (Nozawa *et al*. 1997). Some metal ions, like Zn(II), Cu(II), Hg(II), or Pb(II), was preferable to react with thiol groups, which responded to the mutant MTG and wild MTG carrying a triplet Cys-Asp-His active center were inhibited in the presence of these four metal ions. De Barros *et al*. (2003) reported that MTG produced by *Bacillus circulans* possessed a thiol group and inhibited by Zn(II), Pb(II), and Cu(II). Furthermore, it has been found that ethanol remarkably affected MTG activity might be due to the fact that ethanol could modify the water holding capacity, compete with water during the formation of chemical bonds and alter the water activity (Lerici and Manzocco 2000). Single factor experiments demonstrated that the mutant MTG were obviously more stable than the wild MTG at 50 and 60, at pH 4.0, 5.0, and 9.0, at 7% and 9% salinity, and in the presence of Li(I) and Ag(I), 30% and 35% ethanol. These results might be due to the spatial configuration of the mutant MTG was more compact than that of the wild MTG.

The stability of the mutant MTG and the wild MTG were improved in high temperature by adding some polyhydroxy compounds. The improvement of thermal stability by adding polyhydroxy compounds was probably due to the reinforcement of the hydrophobic interactions among nonpolar amino acids inside the MTG molecules, and thus increased their resistance to inactivation. Back *et al*. (1979) reported that polyhydroxy compounds could modify the structure of water and strengthen hydrophobic interactions among nonpolar amino acids inside the protein molecules. Furthermore, PEG could evidently increase the viscosity of the enzyme solution, which might cause reduction of chemical or biological reaction rate (Lee and Choo 1989).

In addition, the mutant MTG could exhibit acceptable activity in practical cross-linking reaction. Therefore, the mutant MTG has a broad application potential.

Acknowledgements

This study was funded by the Nature Science Foundation of Hubei Province (BZY10033).

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Corresponding editor: María Luz Cárdenas