Short communication

Recombinant production of active microbial transglutaminase in *E. coli* using self-cleavable zymogen with mutated propeptide

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Abstract

Microbial transglutaminase (MTG) from *Streptomyces mobaraensis* has been used widely in the food industry, research, and medical applications. MTG can site-specifically modify proteins by the cross-linking reaction of a glutamine residue with a primary amino group. We have developed a recombinant expression system for MTG in *Escherichia coli* that readily and affordably yields high-purity MTG. The production of active and soluble MTG in *E. coli* was achieved using a chimeric protein of tobacco etch virus (TEV) protease and MTG zymogen. Experiments showed that using a chimera of TEV protease and MTG zymogen with native propeptide resulted in active MTG contaminated with cleaved propeptide. Contamination, likely due to strong interaction between the propeptide and catalytic domain of MTG; could be avoided by introducing mutations of K10R and Y12A to the propeptide. The mutations facilitated the dissociation of the cleaved propeptide and active MTG without any contamination was obtained. The specific activity of the active MTG was 22.7±2.6 U/mg.

Keywords: microbial transglutaminase; *Streptomyces mobaraensis*; zymogen; self-cleavable; chimeric protein.

Abbreviations

MBP, maltose binding protein; MTG, microbial transglutaminase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; TB, terrific broth; TEV, tobacco etch virus; TGase, transglutaminase; Tris, tris(hydroxymethyl)aminomethane.

Transglutaminase (TGase, protein-glutamine γ-glutamyltransferase, E.C. 2.3.2.13) catalyzes an acyl transfer reaction between the γ-carboxyamide group of a peptide-bound glutamine residue and the ε-amino group of a lysine residue within the same or another protein. Among many TGases from different sources (Griffin et al., 2002), a microbial transglutaminase (MTG) from *Streptomyces mobaraensis* is the most widely utilized (Strop, 2014). Although its main application is in the food industry (Kieliszek and Misiewicz, 2014; Motoki and Seguro, 1998), MTG has been used as a ligation tool for proteins in research (Milczek, 2018) and in pharmaceuticals (Schneider et al., 2020). MTG-mediated site-specific conjugations of proteins with small functional molecules (Kamiya et al., 2009), other proteins (Hirakawa et al., 2007), DNA (Kitaoka et al., 2011; Takahara et al., 2017), RNA (Kitaoka et al., 2012), synthetic polymers (Wakabayashi et al., 2017), and lipids (Takahara et al., 2019) have been reported. Moreover, MTG is currently utilized in the conjugation of anticancer agents with antibodies to generate antibody-drug conjugates for medicinal application (Dennler et al., 2014; Walker et al., 2019). Precise control of reaction conditions is necessary to achieve site-specific modification of proteins and correctly assess results, and thus highly purified MTG is required. Although commercially available MTG for the food industry is widely available and inexpensive, these products often contain additives and other proteinaceous substances, so they require further purification before use. Therefore, it was our aim to develop an *Escherichia coli* expression system for high purity and activity MTG for laboratory use.

In nature, MTG is produced as a zymogen, carrying a propeptide at the N-terminus of the mature MTG domain (Marx et al., 2007). Proteolysis of the propeptide is required for activation of MTG and post-proteolysis purification is necessary (Marx et al., 2008). Furthermore, since the propeptide has the function of intramolecular chaperone, expression of MTG without the propeptide in *E. coli* results in the formation of an inclusion body (Kawai et al., 1997). The active MTG can be

obtained by refolding from the inclusion body (Yokoyama et al., 2000). These strategies are not straightforward because they require the time-consuming steps of protease treatment followed by purification or the refolding process.

One-step expression of active MTG in *E. coli* has been demonstrated using polycistronic expression of the propeptide and the mature MTG domain (Javitt et al., 2017; Liu et al., 2011). The co-expressed propeptide facilitated the folding of mature MTG domain and active MTG was obtained in soluble form without additional processing. Another approach was to co-express MTG zymogens with a protease (Rickert et al., 2016). MTG zymogens with a 3C protease recognition sequence between the propeptide and mature MTG domain were successfully processed by the co-expressed 3C protease in *E. coli*; active MTG was obtained. For both strategies, the expression level of two genes would have an impact on the overall yield of active MTG. The simplest and the most precise way to control the expression ratio of two proteins is to genetically fuse and express them as a single polypeptide.

Herein, we report another strategy to make active MTG in soluble form by constructing a chimeric protein of a protease and MTG zymogen. Tobacco etch virus (TEV) protease was selected to cleave off the propeptide from MTG zymogen, since TEV protease has stringent substrate specificity and it can be expressed efficiently in *E. coli*. Two variants of protein chimera of TEV protease and MTG zymogen were produced using the pMAL-c5E vector (**Fig. 1A**). Both variants contained maltose-binding protein (MBP) at the N-termini to promote the correct folding of the chimera proteins and to increase their solubility.

MBP-TEV-Pro-MTG has the TEV protease recognition site between the propeptide and the mature MTG domain (**Fig. 1B**). Since the N-terminal amino group of glycine can be a substrate for MTG (Tanaka et al., 2005), we chose a TEV cleavage sequence of GSENLYFQ↓SGG to prevent self-cross-linking of MTG after propeptide cleavage by TEV protease.

Modulation of interaction between the propeptide and catalytic domain of MTG is key to increasing the productivity of active MTG (Rickert et al., 2016). Therefore, another chimeric protein was constructed with mutations in the propeptide. MBP-TEV-Pro(K10R/Y12A)-MTG has mutations of K10R and Y12A in the propeptide and a longer TEV protease recognition sequence (GGGSENLYFQ↓SGGGGS) than MBP-TEV-Pro-MTG, obtained by deleting 8 amino acid residues (AGPSFRAP) at the C-terminal of propeptide (**Fig. 1C**). We anticipated the mutation of Y12A in the propeptide would attenuate interaction with the catalytic domain of MTG and facilitate dissociation of the propeptide from active-MTG after self-cleavage (**Fig. 1D**) and the mutation of K10R would prevent cross-linking of MTG zymogen via K10. The complete amino acid sequences of both MTG variants are detailed in the Supplementary Information (**Table S1**).

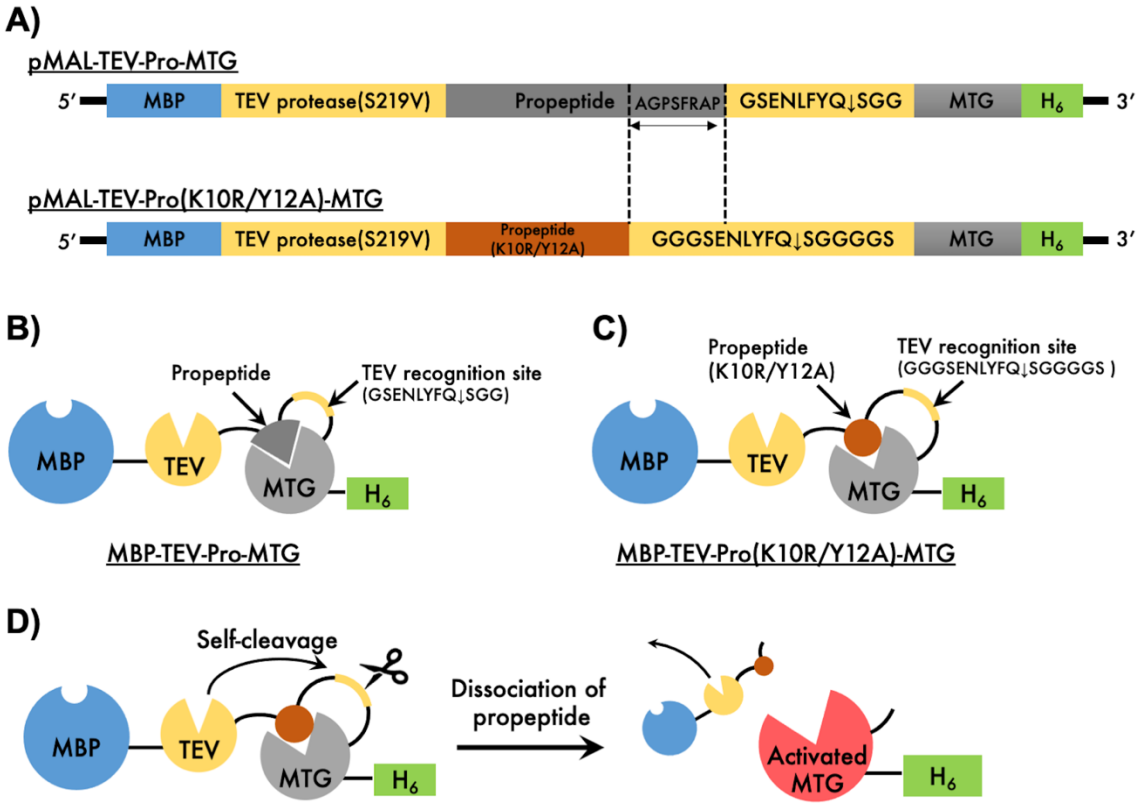

Fig. 1. Constructs of MTG variants evaluated in this study. A: DNA constructs of pMAL-TEV-Pro-MTG and pMAL-TEV-Pro(K10R/Y12A)-MTG. B and C: Schematic illustrations of MBP-TEV-Pro-MTG and MBP-TEV-Pro(K10R/Y12A)-MTG, respectively. D: Self-cleavage reaction of MBP-TEV-Pro(K10R/Y12A)-MTG to obtain active MTG. MBP: maltose binding protein; TEV: tobacco etch virus; MTG: microbial transglutaminase.

Both MTG variants were expressed in *E. coli* BL21(DE3) in 250 mL of Terrific broth (TB) medium and the wet weights of cell pellets obtained after expression were measured in triplicate. The average wet weights of cell pellets of MBP-TEV-Pro-MTG and MBP-TEV-Pro(K10R/Y12A)-MTG were 4.2 ±0.1 g and 1.0 ±0.1 g, respectively. Expression of MBP-TEV-Pro(K10R/Y12A)-MTG significantly reduced the weight of *E. coli* cells, suggesting that the MTG was catalytically active in the cells and resulted in toxicity. Both MTG variants were purified with HisTag affinity chromatography and size-exclusion chromatography (SEC) and the eluates were analyzed with SDS-PAGE (**Fig. 2**).

In case of MBP-TEV-Pro-MTG, the bands of the active MTG and cleaved MBP-TEV-Pro appeared around 38 kDa and 70 kDa, respectively (**Fig. 2A**). The cleaved MBP-TEV-Pro, which lacks HisTag, was highly retained by the nickel-nitrilotriacetic acid column because of attachment to active MTG. MBP-TEV-Pro(K10R/Y12A)-MTG showed mainly the band of active MTG at around 38 kDa (**Fig. 2B**) and the most of cleaved MBP-TEV-Pro(K10R/Y12A) could be removed from the active MTG. The remaining portion of MBP-TEV-Pro(K10R/Y12A) and highly cross-linked proteins were successfully removed from the active MTG by purification with SEC (**Fig. 2E and 2F**). In contrast, in the SEC purification of MBP-TEV-Pro-MTG, all active MTG co-eluted with the cleaved MBP-TEV-Pro and highly cross-linked proteins (**Fig. 2C and 2D**) and pure active MTG could not be isolated.

From these results, we concluded that introduction of mutations to the propeptide is essential to obtain active MTG without any contamination of the propeptide domain. Some portions of active MTG did co-elute with the cross-linked proteins in SEC purification of MBP-TEV-Pro(K10R/Y12A)-MTG. The productivity of the active MTG obtained via this recombinant expression system could be enhanced by reducing the formation of such highly

cross-linked proteins by eliminating the cross-linking sites of MBP-TEV-Pro(K10R/Y12A). Note that the purified active MTG in this study was stable and showed no self-cross-linking behavior (**Fig. S1**).

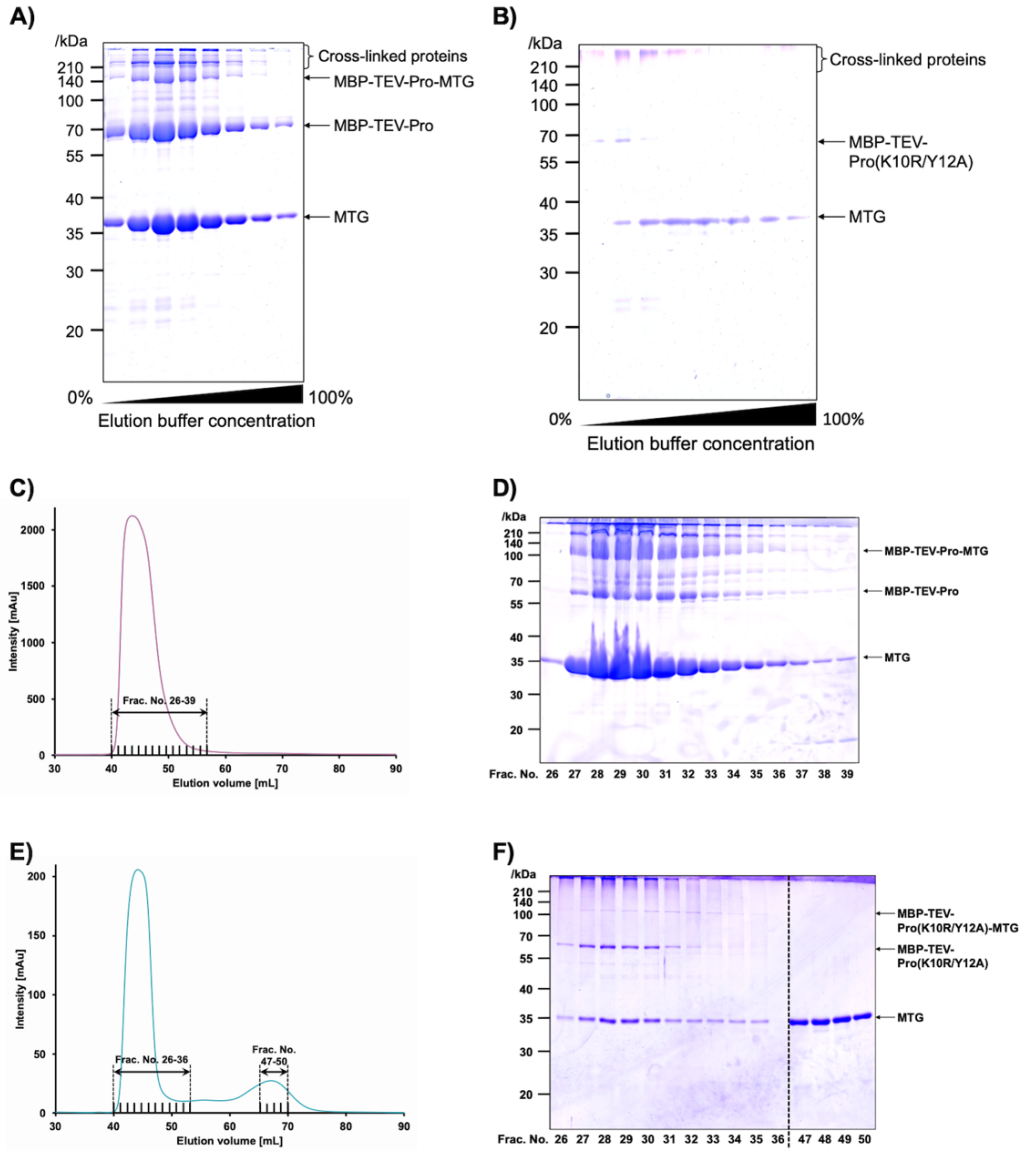

Fig. 2 Purification of active MTG obtained from MBP-TEV-Pro-MTG and MBP-TEV-Pro(K10R/Y12A)-MTG. A and B: SDS-PAGE analyses of fractions in the HisTag purification of MBP-TEV-Pro-MTG and MBP-TEV-Pro(K10R/Y12A)-MTG, respectively. The elution buffer used was 25 mM Tris-HCl, pH7.4, 500 mM NaCl, 500 mM imidazole. C and D: SEC chromatogram and the SDS-PAGE analysis of fractions indicated in the chromatogram of

MBP-TEV-Pro-MTG after HisTag purification (A). E and F: SEC chromatogram and the SDS-PAGE analysis of fractions indicated in the chromatogram of MBP-TEV-Pro(K10R/Y12A)-MTG after HisTag purification (B).

After purification, MTG activity measurement was conducted by hydroxamate assay (Folk and Cole, 1966). The specific activities of the active MTG prepared from MBP-TEV-Pro-MTG and MBP-TEV-Pro (K10R/Y12A)-MTG were 4.0 ±0.1 U/mg and 22.7 ±2.6 U/mg, respectively (**Fig. 3**). The low specific activity of the active MTG obtained from MBP-TEV-Pro-MTG was due to the remaining cleaved propeptide, which has inhibitory effect. The specific activity of MTG from MBP-TEV-Pro (K10R/Y12A)-MTG in this study was comparable to previously reported active MTG purified from the original strain (Ando et al., 1989), prepared by in vitro activation of MTG zymogen (Chen et al., 2013) and commercially available MTG (Zedira, Germany) (25 U/mg). The total amount of active MTG obtained from MBP-TEV-Pro(K10R/Y12A)-MTG was 0.23 mg from 750 ml of TB medium. While the amount of active MTG obtained in this study is not sufficient for industrial application, it is acceptable for use in laboratories. Moreover, the purity and the specific activity of the MTG obtained are suitable for research applications requiring precise control of reaction conditions.

In conclusion, we have demonstrated the production of active and soluble MTG by constructing a chimeric protein of TEV protease and MTG zymogen. Introduction of mutations of K10R and Y12A to the propeptide was required to obtain active MTG free of contamination of cleaved propeptide, resulting in successful production of active MTG with a high specific activity. It is our expectation that research laboratories investigating MTG and its many applications will find this recombinant expression system in *E. coli* a useful method for the straightforward production of high purity and high activity MTG.

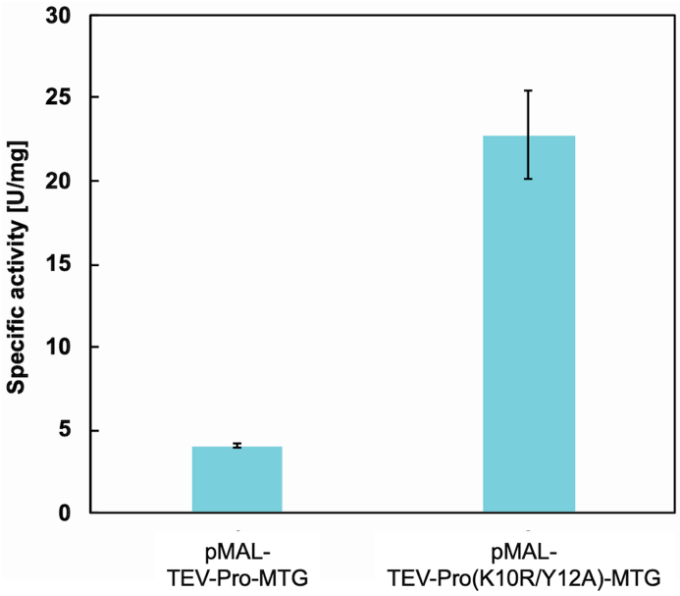

Fig. 3 Specific activities of the active MTG measured by hydroxamate assay.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found in the online version at

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Supplementary Information

Recombinant production of active microbial transglutaminase in *E. coli* using self-cleavable zymogen with mutated propeptide.

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Materials

PrimeSTAR® MAX DNA Polymerase and In-Fusion® HD Cloning Kit were purchased from Takara Bio Inc. (Shiga, Japan). Luria-Bertani (LB) broth (Miller) was purchased from Merck Millipore Corp (Billerica, MA, USA). Tryptone, dried yeast extract, tris (hydroxymethyl) aminomethane (Tris), acrylamide/bis mixed solution (29:1), and Rapid Stain CBB kit were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Hydrochloric acid, sodium chloride, potassium chloride, sodium phosphate dibasic dodecahydrate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, glutathione (reduced form), iron (III) chloride hexahydrate, and trichloroacetic acid were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). *N,N,N′,N′*-Tetramethylethylenediamine, ammonium persulfate, and hydroxylamine hydrochloride were purchased from Kishida Chemical Co., Ltd (Osaka, Japan). Z-QG was purchased from Peptide Institute, Inc. (Osaka, Japan). L-glutamic acid γ-monohydroxamate was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO). Ultrapure water supplied by Milli-Q® Reference Water Purification System (Merck Millipore Corp., Billerica, MA, USA) was used for buffer preparation.

Construction of expression vectors and preparation of recombinant proteins

The gene encoding tobacco etch virus (TEV) protease mutant S219V (Kapust et al., 2001) was inserted at the 3′ end of gene encoding maltose binding protein (MBP) in pMAL-c5E vector (New England Biolabs, MA, USA) to construct pMAL-TEV vector. A synthetic gene encoding pro-MTG from *Streptomyces mobaraensis* that has a C-terminal HisTag sequence (H_6), TEV protease recognition sequence, and linkers (GSENLYFQSGG) between the propeptide and catalytic domain of MTG was then fused to the 3′ end of the TEV protease gene in the pMAL-TEV vector to construct pMAL-TEV-Pro-MTG. Two mutations at K10R and Y12A in the propeptide of MTG were then introduced to pMAL-TEV-Pro-MTG. Also the linker sequence at TEV protease recognition site

was extended (GGGSENLYFQSGGGGS) by deleting 8 amino acid residues (AGPSFRAP) at the C-terminal of propeptide to construct pMAL-TEV-Pro(K10R/Y12A)-MTG. (**Figure 1**) The complete amino acid sequences of the constructed MTG variants are provided in **Table S1**.

The expression of MTG variants was conducted on an *Escherichia coli* BL21 Star (DE3) strain. One nanogram of the expression vectors of the MTG variants was transferred into the cells by a heat shock method and cultured on LB agar medium containing 100 μg/ml of ampicillin sodium. A single colony was inoculated to 5.0 ml of LB medium containing the same amount of ampicillin sodium and shaken at 220 rpm at 37°C for 6 hours. One milliliter of the precultured medium was added into 250 ml of Terrific broth (TB) medium containing the same antibiotics and cultured with shaking at 220 rpm at 37°C until the optical density at 600 nm reached around 0.5. Expression of each protein was induced by addition of isopropyl β-d-1-thiogalactopyranoside to a final concentration of 0.1 mM, and the cells were cultured at 17°C for 16 hours. The cells were harvested by centrifugation at 5,000 × g for 20 min and supernatant was removed. The cell pellet was resuspended in phosphate-buffered saline (PBS) (-) (pH 7.4) and the wet weight of each pellet was measured after removing PBS (-). The expression in TB medium was conducted in triplicate for each MTG variant.

For the purification of the MTG variants, the cells were resuspended in PBS (-) and disrupted by sonication on ice for 12.5 min. The cell debris was removed by centrifugation at 18,000 × g for 20 min at 4°C. The supernatant was applied to a HisTrap FF crude 5 mL column (GE Healthcare UK Ltd.), which had been pre-equilibrated with the HisTrap binding buffer (20 mM Tris-HCl, 500 mM NaCl, 35 mM imidazole, pH 7.4). The column was washed with the same buffer until all the unbound proteinaceous substances eluted. The MTG variants were eluted by a gradient of HisTrap elution buffer (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 7.4) up to 100% with 13 column volumes. The fractions containing MTG variants were concentrated to a volume of approximately 5 mL using ultrafiltration membrane of 10 kDa MWCO (Vivaspin Turbo 15, Sartorius). MTG variants were then purified with size exclusion chromatography using HiLoad 16/600 Superdex 75 pg (GE Healthcare UK Ltd.) with PBS (pH 7.4) containing 0.1 mM of dithiothreitol as a running buffer. The MTG variants were concentrated using the 10 kDa MWCO ultrafiltration membrane. The protein concentrations of purified MTG variants were determined by measuring absorbance at 280 nm using an ND-1000 Nanodrop and calculated by using the extinction coefficient of MTG predicted by ProtParam at ExPASy website at https://web.expasy.org/protparam/.

Activity measurement of the active MTG by hydroxamate assay

The activities of MTG variants were assessed by quantifying the amount of hydroxamate produced by the MTG reaction using a complex formation of hydroxamate and Fe (III) ions (Folk and Cole, 1966). The substrate solution containing 30 mM Z-QG, 100 mM hydroxylamine hydrochloride and 10 mM glutathione in 0.2 M Tris-Acetate (pH 6.0) was added to MTG variants. After 10 min of incubation at 37°C, the stop solution (200 mM trichloroacetic acid in 50 mM HCl) containing 50 mM of iron (III) chloride hexahydrate was added to the reaction mixture and the absorbance at 525 nm was measured using Synergy HTX Multi-Mode Reader (BioTek, Winooski, USA). A standard curve was constructed using a L-glutamic acid γ-monohydroxamate reference solution with known concentration. One unit of MTG was defined as the amount of MTG that catalyzes the formation of 1 μmol of hydroxamic acid in 1 minute.

Table S1. Amino acid sequence of MTG variants prepared in this study

Proteins	Sequences
MBP-TEV-Pro-MTG	MKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFW AHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNP PKTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGA KAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFK GQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEELAKD PRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTNSSSNNNNNNN NNNLGAMESLFKGPRDYNPISSTICHLTNESDGHTTSLYGIGFGPFIITNKHLFRRNNGTLLV QSLHGVFKVKNTTTLQQHLIDGRDMIIIRMPKDFPPFPQKLKFREPQREERICLVTTNFQTKS MSSMVSDTSCTFPSSDGIFWKHWIQTKDGQCGSPLVSTRDGFIVGIHSASNFTNTNNYFTSVP KNFMELLTNQEAQQWVSGWRLNADSVLWGGHKVFMVKPEEPFQPVKEATQLMNRRRRRGGDNG AGEETKSYAETYRLTADDVANINALNESAPAASSAGPSFRAPGSENLYFQ↓SGGDSDDRVTPP AEPLDRMPDPYRPSYGRAETVVNNYIRKWQQVYSHRDGRKQQMTEEQREWLSYGCVGVTWVNS GQYPTNRLAFASFDEDRFKNELKNGRPRSGETRAEFEGRVAKESFDEEKGFQRAREVASVMNR ALENAHDESAYLDNLKKELANGNDALRNEDARSPFYSALRNTPSFKERNGGNHDPSRMKAVIY SKHFWSGQDRSSSADKRKYGDPDAFRPAPGTGLVDMSRDRNIPRSPTSPGEGFVNFDYGWFGA QTEADADKTVWTHGNHYHAPNGSLGAMHVYESKFRNWSEGYSDFDRGAYVITFIPKSWNTAPD KVKQGWPGSHHHHHH
MBP-TEV-Pro(K10R/Y12A)-MTG	MKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFW AHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNP PKTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGA KAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFK GQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEELAKD PRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTNSSSNNNNNNN NNNLGAMESLFKGPRDYNPISSTICHLTNESDGHTTSLYGIGFGPFIITNKHLFRRNNGTLLV QSLHGVFKVKNTTTLQQHLIDGRDMIIIRMPKDFPPFPQKLKFREPQREERICLVTTNFQTKS MSSMVSDTSCTFPSSDGIFWKHWIQTKDGQCGSPLVSTRDGFIVGIHSASNFTNTNNYFTSVP KNFMELLTNQEAQQWVSGWRLNADSVLWGGHKVFMVKPEEPFQPVKEATQLMNRRRRRGGDNG AGEETRSAAETYRLTADDVANINALNESAPAASSGGGSENLYFQ↓SGGGGSDSDDRVTPPAEP LDRMPDPYRPSYGRAETVVNNYIRKWQQVYSHRDGRKQQMTEEQREWLSYGCVGVTWVNSGQY PTNRLAFASFDEDRFKNELKNGRPRSGETRAEFEGRVAKESFDEEKGFQRAREVASVMNRALE NAHDESAYLDNLKKELANGNDALRNEDARSPFYSALRNTPSFKERNGGNHDPSRMKAVIYSKH FWSGQDRSSSADKRKYGDPDAFRPAPGTGLVDMSRDRNIPRSPTSPGEGFVNFDYGWFGAQTE ADADKTVWTHGNHYHAPNGSLGAMHVYESKFRNWSEGYSDFDRGAYVITFIPKSWNTAPDKVK QGWPGSHHHHHH

Light blue:maltose-binding protein with a linker sequence at the C-terminus.
Purple: Tobacco etch virus protease
Blue: oligo arginine tag
Orange: Propeptide of microbial transglutaminase
Green: Tobacco etch virus protease cleavage sequence(ENLYFQ↓S) with linkers
Red: mutations in the propeptide (K10R and Y12A)
Brown: mature microbial transglutaminase domain with C-terminal HisTag.

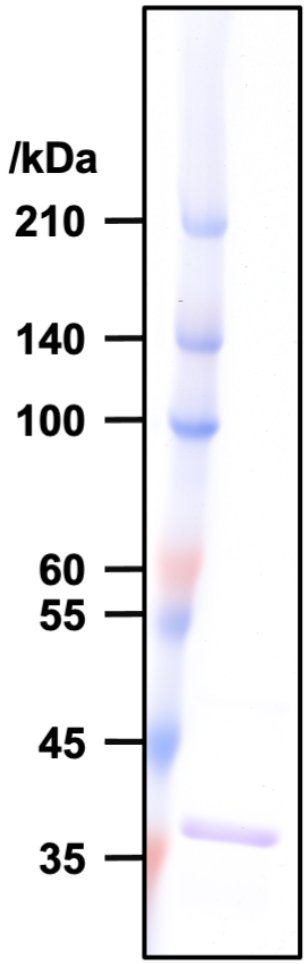

Fig.S1. SDS-PAGE analysis of active MTG from MBP-TEV-Pro(K10R/Y12A)-MTG. MTG sample was incubated at 37°C for 1 hour and analyzed with SDS-PAGE after heat treatment of 98°C for 3 min in SDS-PAGE sample buffer.

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