Food Sci. Biotechnol. 23(5): 1507-1513 (2014)
DOI 10.1007/s10068-014-0206-1

RESEARCH ARTICLE

Heterologous Expression and Purification of *Zea mays* Transglutaminase in *Pichia pastoris*

Hongbo Li, Yanhua Cui, Lanwei Zhang, Huaxi Yi, Xue Han, Yuehua Jiao, Ming Du, Rongbo Fan, and Shuang Zhang

Received October 29, 2013; revised February 23, 2014; accepted March 29, 2014; published online October 31, 2014

Abstract Transglutaminases (TGases) are a family of enzymes that catalyze the cross-linking of proteins and are widely used in the food industry to improve the texture of dairy, meat, and bread products. *Zea mays* transglutaminase (TGZ) is a new type of TGase with a wide potential. TGZ was expressed in the yeast *Pichia pastoris* under an alcohol oxidase promoter. Maximal expression of recombinant TGZ was achieved by inducing recombinant GS115 (pPIC9K-*tgz*) in BMMY medium using 1.5% methanol for 96 h. Secreted TGZ was initially separated using Superdex 200 resin and further purified on cation exchange resin. The activity of TGZ following purification was 0.32 U/mg of protein. The polymerization effect of TGZ on casein catalyzed by recombinant TGZ was slightly lower than the effect of microbial transglutaminase (MTG). TGZ is a new potential additive for the food industry.

Keywords: *Zea mays* transglutaminase, *Pichia pastoris* GS115, protein expression, purification, polymerization effect

Introduction

Transglutaminases (protein-glutamine γ-glutamyltransferase, TGases; EC 2.3.2.13) are a family of enzymes that catalyze post-translational modifications of proteins by establishing ε-(γ-glutamyl) links and covalent conjugations between a primary amine group in a lysine or polyamine (amine donor), and a γ-carboxamide group of the glutamyl residue (amine acceptor) (1). Due to effects on the properties of proteins, TGases are widely used in the food processing, wool textiles, biopolymers, and cosmetics industries, and for clinical applications (2). The most prevalent application of TGase is in the food industry where this enzyme is used to improve the breaking strength and viscoelastic properties of foods (3).

The enzymatic activity of TGases have been widely identified in different organisms and enzymatic properties and functions have been extensively studied (4). A typical TGase from *Streptomyces mobaraensis* has been widely used in food processing as a protein cross-linking enzyme (5). However, TGases and their functions have been less studied in plants than in microorganisms and animals. Plant TGase was first observed in pea seedlings, then in both lower and higher plants (6). TGases from plants have different substrate specificities, protein sequence characteristics, Ca^{2+}-dependencies, and activities (7-12).

In previous studies, a maize plastidial TGase was first found mainly in the grana-appressed thylakoids of mesophyll light-exposed cell chloroplasts (11). The activity of this enzyme was significantly increased in enzymatic assays under light conditions (12). The TGZ genes, termed *tgz21*, *tgz15,* and *tgz4* according to the number of B-type repeats that are located in the non-catalytic domain of the enzyme, were cloned into *Escherichia coli* (13,14). However, recombinant TGZ was mainly present as inclusion bodies, and a complicated refolding process would have been necessary to obtain active TGZ for which the yield and the activity would be low (14).

The methylotrophic yeast *Pichia pastoris* is an excellent host for production of heterologous proteins. *P. pastoris*

Hongbo Li, Yanhua Cui, Lanwei Zhang (✉), Huaxi Yi, Xue Han, Yuehua Jiao, Ming Du, Rongbo Fan, Shuang Zhang
School of Food Science and Engineering, Harbin Institute of Technology, Harbin, Heilongjiang 150090, China
Tel, Fax: +86-451-86282901
E-mail: zhanglw@hit.edu.cn

Rongbo Fan
College of Food Science & Engineering, Qingdao Agricultural University, Qingdao, Shandong 266109, China

can use methanol as a sole carbon source, and offers the advantages of simple genetic manipulation and performance of eukaryotic post-translational modifications (15,16). Fermentation of *P. pastoris* is divided into the 2 phases of cell growth and expression of target proteins induced using methanol under a methanol-induced alcohol oxidase promoter (17). A variety of heterologous proteins have been successfully expressed in *P. pastoris* (18,19).

In order to obtain a large amount of active TGZ for study of properties and applications, the *Zea mays* transglutaminase gene (*tgz*) was expressed in *P. pastoris* GS115, and the expression conditions for the recombinant strain were optimized in this study. TGZ was successfully expressed in the yeast *P. pastoris* with an activity of 0.2 U/mg of protein. Soluble TGZ was purified from fermentation supernatants using a 2-step method. The activity of TGZ following purification was increased up to 0.32 U/mg of protein. Purified TGZ was used to catalyze polymerization of casein in order to define the functions of recombinant TGZ, which could effectively catalyze the cross-linking effect of casein. Recombinant TGZ is a new additive for use in the food industry.

Materials and Methods

Strains, plasmids, and reagents *E. coli* DH5α, used in all DNA manipulations, was obtained from the School of Food Science and Engineering at Harbin Institute of Technology (Harbin, China). *P. pastoris,* the host strain for GS115 (his^- mut^+) and the pPIC9K vector were purchased from Invitrogen (Carlsbad, CA, USA). DNA polymerase KOD-Plus-neo was purchased from Toyobo (Shanghai, China). Restriction enzymes were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). All oligonucletides were synthesized by Sangon (Shanghai, China). A Hiload 16/60 Superdex 200 prepacked column and a SP Sepharose High Performance were purchased from GE Healthcare (Piscataway, NJ, USA). Microbial transglutaminase (MTG) derived from *Streptomyces mobaraensis* DSM40587 was obtained from the Harbin Institute of Technology (Harbin, China) (20). All other chemical reagents were of analytical grade.

Plasmid construction The *tgz* gene was amplified using PCR from the cloning plasmid pET28a-*tgz* (School of Food Science and Engineering, Harbin Institute of Technology, Harbin, China) that contained the full length *tgz* gene (21). The forward primer YS-F (5'-ATCGAATTCATGGCTCATCGTGGACATCTAGA-3', an *Eco*RI site underlined), and the reverse primer YS-R (5'-ATTAGCGGCCGCGATTTCACCATATTTGTCT-3', a *Not*I site underlined) were used to amplify this gene. Thermal cycling conditions were 94°C for 2 min, followed by 30 cycles of 94°C for 15 s, 62°C for 30 s, 68°C for 50 s, and a final extension step of 68°C for 5 min. The PCR product was cloned into the pTA2 vector (Toyobo) using a Target clone-plus kit (Toyobo) following the instructions provided by the manufacturer. Positive clones were selected based on resistance screening and colony PCR. Plasmid DNA was prepared and sequenced at least twice in both directions using an ABI PRISM 377 DNA sequencer (Perkin-Elmer Cetus Instruments, Norwalk, CT, USA). Then, the target gene was ligated into *Eco*RI-*Not*I-digested pPIC9K to create the recombinant vector pPIC9K-*tgz*.

Transformation of *P. pastoris* and selection of transformants The plasmid pPIC9K-*tgz* and the parent plasmid pPIC9K (Invitrogen) were linearized using *Sac*I (Takara Biotechnology Co., Ltd.) and transformed into *P. pastoris* GS115 cells at the *AOXI* locus (His^+Mut^+) using electroporation with an ECM399 Gene Pulser (BTX, Holliston, MA, USA). The recombinant clones grown on regeneration dextrose medium plates (1 mol/L of sorbital, 2% dextrose, 1.34% yeast nitrogen base without amino acids (YNB), 4×10^{-5}% biotin, 5×10^{-3}% amino acid, 2% agarose) were cultured on minimal dextrose agar plates (2% dextrose, 1.34% YNB, 4×10^{-5}% biotin, 1.5% agar) and minimal methanol (MM) agar plates (1.34% YNB, 0.5% methanol, 4×10^{-5}% biotin, 1.5% agar) at 28°C for 3-4 days. Transformants that grew normally on both plates were selected following the instructions for the *Pichia* Expression Kit (Invitrogen). Then, the His^+ Mut^+ transformants were spotted on yeast extract peptone dextrose agar plates (2% tryptone, 1% yeast extract, 2% glucose, and 1.5% agar) containing 0.25, 0.5, 0.75, 1.0, 2.0, and 3.0 mg/mL of geneticin (G418; Sigma, Shanghai, China). Colonies picked from the highest concentration of G418 (Sigma) were detected using PCR with the 5' AOX1 primer (5'-GACTGGTTCCAATTGACAAGC-3') and the 3' AOX1 primer (5'-GGCAAATGGCATTCTGACATCC-3').

Expression of recombinant TGZ The selected recombinant strains were randomly picked and cultivated in 5 mL of Buffer Glycerol-complex (BMGY) medium (2% peptone, 1% yeast extract, 100 mM phosphate buffer, pH 6.0, 1% glycerol, 1.34% YNB and 4×10^{-5}% biotin) for 24 h at 30°C at 250 rpm as a growth phase. The cultures were induced using 2 mL of Buffer Methanol-complex (BMMY) medium (2% peptone, 1% yeast extract, 100 mM phosphate buffer, pH 6.0, 0.5% methanol, 1.34% YNB, and 4×10^{-5}% biotin) for 96 h at 28°C at 250 rpm. A concentration of 100% methanol (Kermel Co., Ltd., Tianjin, China) was added every 24 h to sustain the methanol level. Culture supernatants were collected and stored at −80°C for analysis of protein expression.

Optimization of the expression conditions for TGZ The selected recombinant strain GS115 (pPIC9K-*tgz*) was cultured in BMGY medium and subsequently induced using MM medium (0.5% methanol, 1.34% YNB, and 4×10^{-5}% biotin), MM with phosphate buffer (BMM) medium (100 mM phosphate buffer, pH 6.0, 0.5% methanol, 1.34% YNB, and 4×10^{-5}% biotin), BMMY medium, and modified BMMY (mBMMY) medium (0.1% yeast extract, 1% $(NH_4)_2SO_4$, 100 mM phosphate buffer, pH 6.0, 0.5% methanol, 1.34% YNB, and 4×10^{-5}% biotin) according to the method mentioned above (22). Then, the final methanol concentration and the induction time were optimized. Along with GS115 (pPIC9K-*tgz*) induced in the BMMY medium, the supernatants induced at different methanol concentrations (0, 0.5, 1, 1.5, 2, and 2.5%) were collected after 96 h. The supernatant of GS115 (pPIC9K-*tgz*) induced in BMMY medium using 1.5% methanol was collected every 24 h for 120 h. During induction, 100% methanol (Kermel Co., Ltd.) was added every 24 h to maintain the methanol concentration. The activity was calculated to determine the optimized expression conditions for TGZ.

Protein purification The 96 h BMMY medium used for recombinant *P. pastoris* GS115 (pPIC9K-*tgz*) was clarified by centrifugation for 5 min at 1,000×*g* at 4ºC, and filtered through a 0.22-μm filter membrane (Xinya Purification Co., Ltd., Shanghai, China). The filter liquid was concentrated using ultrafiltration with a Labscale TFF System (Millipore Corp, Bedford, MA, USA) over a 10 kDa Mw cut-off membrane prior to protein purification.

A Hiload 16/60 Superdex 200 prepacked column (GE Healthcare) was used for the first purification step. The column was equilibrated using 50 mM phosphate buffer (Kermel Co., Ltd.), pH 7.0, and 0.15 M NaCl (Kermel Co., Ltd.). Then, 2.5 mL of concentrated sample was loaded onto the equilibrated Hiload 16/60 Superdex 200 prepacked column (GE Healthcare) and eluted with the same buffer at 1.0 mL/min. Fractions containing the protein were pooled and concentrated using polyethylene glycol 20000 (Sigma). Concentrated samples were dialyzed using 1.4 kDa cut off dialysis tubing (Spectrum Laboratories Inc., Los Angeles, CA, USA) against deionized water, then assayed for TGase activity.

Strong cation exchange resin SP sepharose High Performance (HP) in a XK16/10 column (GE Healthcare) was used for the second purification step. The column was equilibrated using 50 mM phosphate buffer (Kermel Co., Ltd.) at pH 7.0. The dialyzed sample was loaded onto the column, followed by washing with the same buffer. The bound protein was eluted at different concentrations of sodium chloride (15, 23, 36, and 100%; Kermel Co., Ltd.) in 50 mM phosphate buffer (Kermel Co., Ltd.) at pH 7.0 at 3.0 mL/min. The purification fractions were analyzed using, 12% SDS-PAGE gel and assayed for TGZ activity.

TGZ activity assay The activity of recombinant TGZ was assayed according to the colorimetric method (23) with modification. The reaction mixture was performed in a 100 μL volume containing 0.04 M N-CBZ-Gln-Gly (Sigma), 0.04 M NaOH (Kermel Co., Ltd.), 0.08 M Tris-HCl (pH 8.0; Sigma), 0.02 M hydroxylamine (Kermel Co., Ltd.), 0.002 M GSH (Kermel Co., Ltd.) and 50 μL of the enzyme solution with incubation at 37ºC for 10 min. The reaction was stopped by adding 50 μL of a stop solution (3 M HCl, 0.7 M trichloroacetic acid, 0.2 M $FeCl_3\cdot6H_2O$). Then the absorbance at 525 nm was measured using a microplate reader (BioTek, Winooski, VT, USA). The mixture without N-CBZ-Gln-Gly was used as a blank control and all assays were done in triplicate. An amount of 1 unit of TGase activity was defined as the amount of enzyme that formed 1 μmol of L-glutamic acid-γ-monohydroxamate per minute at 37ºC. Protein concentrations were determined using a Bradford Protein Assay Kit (Beyotime, Shanghai, China) with bovine serum albumin (Beyotime) as a standard.

Polymerization of casein with recombinant TGZ To estimate the polymerization of casein with recombinant TGZ, MTG was used as a control. An amount of 10 μL of different concentrations (5 and 10 mg/mL) of TGZ and MTG were incubated with 40 μL of 1.0% casein (Sigma) for 4 h at 37ºC. Then, polymerization of casein was examined using SDS-PAGE. The sample loading volume and the concentrations of the separating gel and the stacking gel were 10 μL, 12, and 5%, respectively. Images were scanned using the Gel Doc XR system (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Results and Discussion

Plasmid construction The *tgz* gene was amplified using PCR with pET28a-*tgz* (Harbin Institute of Technology) as a template, and YS-F and YS-R as primers. The amplified PCR product was cloned into the pTA2 vector (Toyobo) to obtain plasmid pTA2-*tgz*. DNA sequencing showed that the sequence was identical to the *tgz* gene of maize (GenBank: AJ421525.2). Plasmid pTA2-*tgz* was digested using *Eco*RI (Takara Biotechnology Co., Ltd.) and *Not*I (Takara Biotechnology Co., Ltd.) and small fragments were ligated into the same sites of the pPIC9K yeast-shuttle vector (Invitrogen) to obtain recombinant plasmid pPIC9K-*tgz*. Restriction analysis and PCR analysis were performed and approximately 1.6 kb was observed in both analyses. Due to the α-factor signal sequence for target protein secretion expression in the N-terminal of the multiple

cloning site of the pPIC9K plasmid, the initiation codon ATG of *tgz* was removed from the PCR product (24).

Expression of recombinant TGZ *P. pastoris* has become an important eukaryotic expression system for production of target proteins (25). In order to express active TGZ, the expression plasmid pPIC9K-*tgz* was transformed into *P. pastoris* GS115 (Invitrogen) and the selected positive clone was expressed. Compared with the negative control of the induction product of *P. pastoris* GS115 (pPIC9K), a band of 70 kDa from the supernatant of GS115 (pPIC9K-*tgz*) was detected using SDS-PAGE (Fig. 1). TGZ was successfully expressed in the yeast *P. pastoris* GS115 (his^-mut^+) as a soluble form.

Prior to expression of TGZ in *P. pastoris*, TGZ was expressed in *E. coli*, however, the recombinant protein was mainly present in inclusion bodies (data not published) as reported previously (13,14). A temperature shift strategy was unsuccessfully applied with growth at 37°C and a post-induction temperature of 15°C to achieve soluble expression of TGZ. In order to improve the level of TGZ expression, some factors that influence expression of TGZ, such as the IPTG concentration, induction time, and initial OD_{600} value before IPTG induction, were optimized. However, the level of TGZ expression was low (data not shown) and a complicated refolding process was necessary. Therefore, the eukaryotic expression system using *P. pastoris* was applied for soluble expression of recombinant TGZ.

Optimization of the expression conditions for TGZ To produce a large amount of soluble recombinant TGZ, the induction medium was optimized. TGZ production of the positive strain GS115 (pPIC9K-*tgz*) induced in different medium is shown in Fig. 2A. When induced in either MM or BMM for 96 h with 0.5% methanol, the activity of TGZ was low. In contrast, TGZ activity reached 529 mU/mL when cells were induced using BMMY medium. When the mBMMY medium was modified from the BMMY medium by removing peptone, the yeast extract concentration was reduced from 1 to 0.1% (22) and cell growth barely

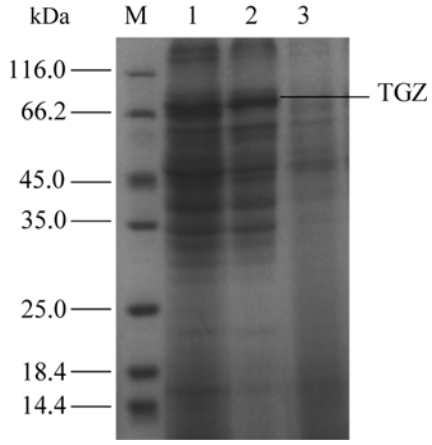

Fig. 1. Expression of TGZ in *Pichia pastoris* detected using 12% SDS-PAGE. Lane M, protein marker; lanes 1-2, supernatants from GS115 (pPIC9K-*tgz*); lane 3, supernatants from GS115 (pPIC9K), a negative control. The position of recombinant TGZ is indicated by an arrow.

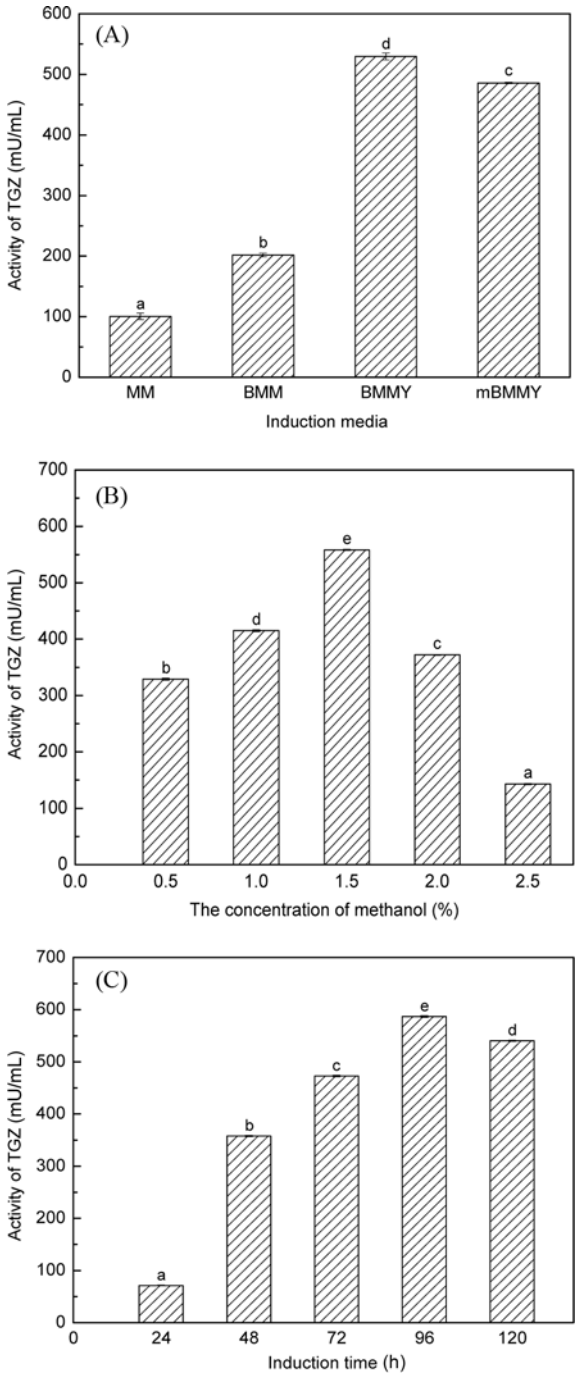

Fig. 2. Effect of different induction media (A), methanol concentration (B) and induction time (C) on TGZ activity. Data are given as mean±standard deviation (SD), n=3. Values followed by different letters in the same row are significantly different (p<0.05).

changed with use of either BMMY or mBMMY media while the activity of TGZ was slightly lower than for induction in BMMY medium (485 mU/mL).

For *P. pastoris* induction, protein expression strongly depends on the methanol concentration (26). A total of 6 methanol concentrations (0, 0.5, 1, 1.5, 2, and 2.5%) were maintained for induction. The effect of the methanol concentration on TGZ expression is shown in Fig. 2B. The activities of TGZ were 329, 414, 558, 372 and 142 mU/mL upon induction using 0.5, 1, 1.5, 2, and 2.5% methanol after 96 h. Percentage improvements of 69.6 and 34.7% for TGZ activity were observed at the 1.5% methanol levels compared to that at 0.5 and 1.0% methanol levels, respectively, perhaps due to a shortage of carbon compensated for by residual methanol (27). The activity of TGZ was decreased at the 2 and 2.5% methanol levels, perhaps as a result of methanol toxic effects on cell growth and inhibition of the TGZ expression (17).

Recombinant strain GS115 (pPIC9K-*tgz*) was induced in BMMY medium using 1.5% methanol (Kermel Co., Ltd.) and the supernatant was collected every 24 h. The activity of recombinant TGZ was increased with time and TGZ activity reached 586 mU/mL at 96 h (Fig. 2C). However, the TGZ activity decreased after 96 h, perhaps due to an increase in the concentration of the metabolite increasing with the induction time, leading to a toxic effect on the cells. Partial recombinant TGZ was hydrolyzed by protease secreted by *P. pastoris*, and the yield of TGZ was reduced.

Purification of soluble recombinant TGZ Recombinant TGZ was expressed under optimal conditions. After ultrafiltration, the supernatant was directly loaded onto an equilibrated Hiload 16/60 Superdex 200 prepacked column (GE Healthcare) to exclude brown pigment. The elution profile is shown in Fig. 3A. Eluted fractions were pooled, concentrated, and dialyzed against deionized water to remove excess salt, and approximately 58.3% of TGZ activity was retained with a 2.4× increase in the specific activity (Table 1). Based on the anticipated isoelectric point (10.8) of TGZ, strong cation exchange resin SP Sepharose High Performance (GE Healthcare) was used to carry out the second purification step, during which TGZ activity was detected as a symmetrical peak (Fig. 3B). At the end of this step, TGZ activity was purified 4.6× and the specific activity was 0.32 U/mg of protein, which was 0.6x higher than the activity before optimization.

Purified TGZ was obtained after SP sepharose HP purification (Fig. 4). The Mw of purified TGZ was 70 kDa, therefore, the size of the recombinant TGZ was larger than the calculated Mw of maize TGZ (approximately 61 kDa) (12). Since the N-glycosylation site (N-K-S-L) was located in a glutamic acid-rich region (12), the site might be have been glycosylated when TGZ was secreted from *P. pastoris*, leading to an increase in the size of recombinant TGZ.

Polymerization of casein with recombinant TGZ SDS-PAGE was carried out in order to determine the polymerization of casein catalyzed by recombinant TGZ, with MTG used as a control. The bands corresponding to casein decreased as the concentration of TGZ increased (Fig. 5). There was a slight decrease of casein bands in samples treated with 5 mg/mL of TGZ. With an increase in the enzyme level (10 mg/mL), a greater intensity for the high Mw protein band was observed. In contrast, the intensity of the band representing casein decreased after incubation with different concentrations of MTG, and the

Table 1. Purification of TGZ from *P. pastoris*

Step	TGZ activity (U)	Protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Ultrafiltration	21.8	298.1	0.07	1	100
Superdex 200	12.7	74.6	0.17	2.4	58.3
SP sepharose HP	2.3	7.2	0.32	4.6	10.6

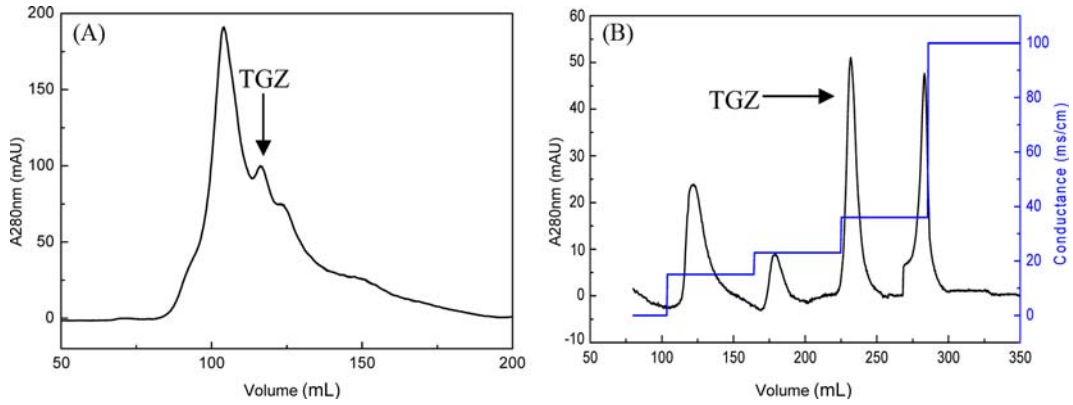

Fig. 3. Chromatography of crude TGZ expressed in *P. pastoris* on Superdex 200 prepacked and SP sepharose HP columns. Chromatography of TGZ purified using a (A) Superdex 200 prepacked column, (B) and the SP sepharose HP column. The arrow indicates the fraction containing TGZ activity.

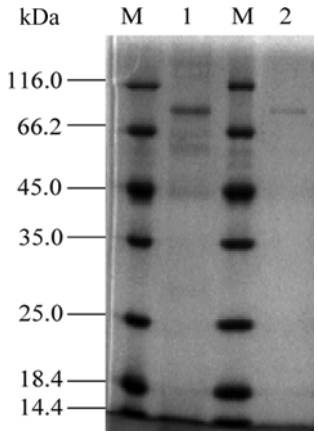

Fig. 4. Fractions containing TGZ activity were analyzed using SDS-PAGE. Lane M, protein marker; lane 1, TGZ after Superdex 200; lane 2, TGZ after SP sepharose HP

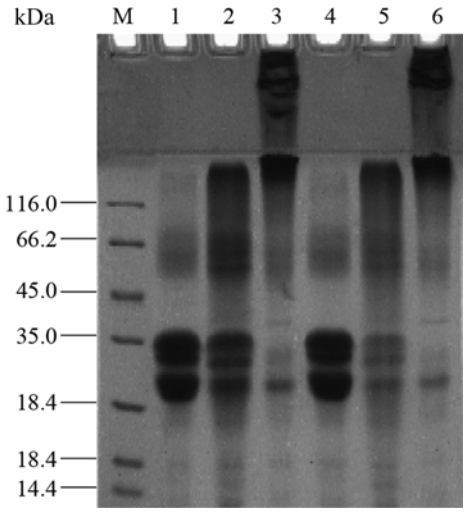

Fig. 5. Electropherograms of casein cross-linking using different concentrations of purified TGZ and MTG. Lane M, protein marker; lane1 and lane 4, 1.0% casein incubated without TGZ, negative control; lane 2 and lane 5, the cross-linking effect of 5 and 10 mg/mL of TGZ on 1.0% casein; lane 3 and lane 6, the cross-linking effect of 5 and 10 mg/mL of MTG on 1.0% casein

effect was more obvious than for samples treated with TGZ. In fact, polymerization catalyzed by MTG showed a decrease in the amount of casein, and resulted in formation of high Mw protein polymers that did not enter the stacking gel. This result indicated that, similar to MTG (28,29), casein was also a good substrate for TGZ.

In this work, TGZ was solubly expressed in *P. pastoris* GS115. After induction in BMMY medium using 1.5% methanol for 96 h and a 2-step purification process, the activity of purified TGZ was 0.32 U/mg of protein. The polymerization effect of recombinant TGZ on casein showed that polymerization of casein was effectively catalyzed by TGZ, further indicating that TGZ can be used as an additive in the food industry.

Acknowledgments This work was supported by the National Nature Science Foundation of China (Grant No.30972041/C110602), the Foundation of Harbin City (2010RFXXN039), and the Foundation of Heilongjiang Province (ZJN0605-02).

Disclosure The authors declare no conflict of interest.

References

1. Griffin M, Casadio R, Bergamini CM. Transglutaminases: Nature's biological glues. Biochem. J. 368: 377-396 (2002)
2. Yokoyama K, Nio N, Kikuchi Y. Properties and applications of microbial transglutaminase. Appl. Microbiol. Biotechnol. 64: 447-454 (2004)
3. DeJong G, Koppelman S. Transglutaminase catalyzed reactions: Impact on food applications. J. Food Sci. 67: 2798-2806 (2002)
4. Marx CK, Hertel TC, Pietzsch M. Purification and activation of a recombinant histidine-tagged pro-transglutaminase after soluble expression in *Escherichia coli* and partial characterization of the active enzyme. Enzyme Microb. Technol. 42: 568-575 (2008)
5. Santos M, Torne J. Recent patents on transglutaminase production and applications: A brief review. Recent Patents Biotechnol. 3: 166-174 (2009)
6. Icekson I, Apelbaum A. Evidence for transglutaminase activity in plant tissue. Plant Physiol. 84: 972-974 (1987)
7. Margosiak SA, Dharma A, Bruce-Carver MR, Gonzales AP, Louie D, Kuehn GD. Identification of the large subunit of ribulose 1, 5-bisphosphate carboxylase/oxygenase as a substrate for transglutaminase in *Medicago sativa* L. (Alfalfa). Plant Physiol. 92: 88-96 (1990)
8. Duca S, Tidu V, Bassi R, Esposito C, Serafmi-Fracassini D. Identification of chlorophyll-a/b proteins as substrates of transglutaminase activity in isolated chloroplasts of *Helianthus tuberosus* L. Planta 193: 283-289 (1994)
9. Kang H, Cho YD. Purification and properties of transglutaminase from soybean (*Glycine max*) leaves. Biochem. Bioph. Res. Co. 223: 288-292 (1996)
10. Bernet E, Claparols I, Dondini L, Asunción Santos M, Serafini-Fracassini D, Torné JM. Changes in polyamine content, arginine and ornithine decarboxylases and transglutaminase activities during light/dark phases (of initial differentiation) in maize calluses and their chloroplasts. Plant Physiol. Bioch. 37: 899-909 (1999)
11. Villalobos E, Torné J, Rigau J, Ollés I, Claparols I, Santos M. Immunogold localization of a transglutaminase related to grana development in different maize cell types. Protoplasma. 216: 155-163 (2001)
12. Villalobos E, Santos M, Talavera D, Rodrıguez-Falcón M, Torné J. Molecular cloning and characterization of a maize transglutaminase complementary DNA. Gene 336: 93-104 (2004)
13. Carvajal P, Villalobos E, Campos A, Torné JM, Barberà E, Santos M. *Zea mays* L. transglutaminase expression in *Escherichia coli*. Microb. Cell. Fact. 5: P8 (2006)
14. Carvajal P, Gibert J, Campos N, Lopera O, Barberà E, Torné JM, Santos M. Activity of maize transglutaminase overexpressed in *Escherichia coli* inclusion bodies: An alternative to protein refolding. Biotechnol. Prog. 27: 232-240 (2011)
15. Daly R, Hearn MTW. Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. J. Mol. Recognit. 18: 119-138 (2005)
16. Cereghino JL, Cregg JM. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol. Rev. 24: 45-66 (2000)
17. Zhang J, Wang X, Su E, Fang G, Ren Y, Wei D. A new fermentation strategy for S-adenosylmethionine production in recombinant *Pichia pastoris*. Biochem. Eng. J. 41: 74-78 (2008)

18. Kang HK, Yun SI, Lim TY, Xia YM, Kim D. Cloning of levansucrase from *Leuconostoc mesenteroides* and its expression in *Pichia pastoris*. Food Sci. Biotechnol. 20: 277-281 (2011)
19. Ryu HJ, Seo ES, Kang HK, Kim YM, Kim D. Expression, purification, and characterization of human intestinal maltase secreted from *Pichia pastoris*. Food Sci. Biotechnol. 20: 561-565 (2011)
20. Zhang L, Zhang L, Yi H, Du M, Ma C, Han X, Feng Z, Jiao Y, Zhang Y. Enzymatic characterization of transglutaminase from *Streptomyces mobaraensis* DSM 40587 in high salt and effect of enzymatic cross-linking of yak milk proteins on functional properties of stirred yogurt. J. Dairy Sci. 95: 3559-3568 (2012)
21. Qin LX, Wang L, Zhang LW. Cloning and expression of maize transglutaminase in *Escherichia coli*. Food Sci. 31: 177-181 (2010)
22. Chen CC, Wu PH, Huang CT, Cheng KJ. A *Pichia pastoris* fermentation strategy for enhancing the heterologous expression of an *Escherichia coli* phytase. Enzyme Microb. Technol. 35: 315-320 (2004)
23. Grossowicz N, Wainfan E, Borek E, Waelsch H. The enzymatic formation of hydroxamic acids from glutamine and asparagine. J. Biol. Chem. 187: 111-125 (1950)
24. Wang H, Wang Q, Zhang F, Huang Y, Ji Y, Hou Y. Protein expression and purification of human Zbtb7A in *Pichia pastoris* via gene codon optimization and synthesis. Protein Expr. Purif. 60: 97-102 (2008)
25. Hermanrud CE, Lucas CL, Sykes M, Huang CA, Wang Z. Expression and purification of soluble murine CD40L monomers and polymers in yeast *Pichia pastoris*. Protein Expr. Purif. 76: 115-120 (2011)
26. Kupcsulik B, Sevella B. Effect of methanol concentration on the recombinant *Pichia pastoris* Mut^{s} fermentation. Chem. Eng. 48: 73-87 (2004)
27. Hu XQ, Chu J, Zhang SL, Zhuang YP, Wang YH, Zhu S, Zhu ZG, Yuan ZY. A novel feeding strategy during the production phase for enhancing the enzymatic synthesis of S-adenosyl-l-methionine by methylotrophic *Pichia pastoris*. Enzyme Microb. Technol. 40: 669-674 (2007)
28. Smiddy M, Martin JEGH, Kelly A, De Kruif C, Huppertz T. Stability of casein micelles cross-linked by transglutaminase. J. Dairy Sci. 89: 1906-1914 (2006)
29. Moon JH, Hong YH, Huppertz T, Fox PF, Kelly AL. Properties of casein micelles cross-linked by transglutaminase. Int. J. Dairy Technol. 62: 27-32 (2009)