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ORIGINAL PAPER

Characterization and large-scale production of recombinant *Streptoverticillium platensis* transglutaminase

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Abstract Recombinant *Streptomyces platensis* transglutaminase (MtgA) produced by the *Streptomyces lividans* transformant 25-2 was purified by ammonium sulfate fractionation, followed by CM-Sepharose CL-6B fast flow, and blue-Sepharose fast flow chromatography. The purification factor was ~33.2-fold, and the yield was 65%. The molecular weight of the purified recombinant MtgA was 40.0 KDa as estimated by SDS-PAGE. The optimal pH and the temperature for the enzyme activity were 6.0 and 55 °C, respectively, and the enzyme was stable at pH 5.0–6.0 and at temperature 45–55 °C. Enzyme activity was not affected by Ca^{2+}, Li^{+}, Mn^{2+}, Na^{+}, Fe^{3+}, K^{+}, Mg^{2+}, Al^{3+}, Ba^{2+}, Co^{2+}, EDTA, or IAA but was inhibited by Fe^{2+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, PCMB, NEM, and PMSF. Optimization of the fermentation medium resulted in a twofold increase of recombinant MtgA activity in both flasks (5.78 U/ml) and 5-l fermenters (5.39 U/ml). Large-scale productions of the recombinant MtgA in a 30-l air-lift fermenter and a 250-l stirred-tank fermenter were fulfilled with maximal activities of 5.36 and 2.54 U/ml, respectively.

Keywords Large-scale production · Properties · Purification · Recombinant · *Streptomyces platensis* · Transglutaminase

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Abbreviations

TGase	Transglutaminase
MtgA	*Streptomyces platensis* transglutaminase
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
EDTA	Ethylenediamine tetraacetic acid
IAA	Iodoacetic acid
PCMB	*p*-Chloromercuribenzoate
PMSF	Phenyl methyl sulfonyl fluoride
NEM	*N*-ethylmaleimide

Introduction

Transglutaminase (TGase; protein-glutamine:amine γ-glutamyltransferase, EC 2.3.2.13) catalyzes an acyl transfer reaction using the peptide bond of a glutamine residue as the acyl donor and one of several primary amines as an acceptor. For example, it catalyzes the formation of cross-links between the γ-carboxyamide group of glutamine and the ε-amino group of lysine or other primary amine, resulting in the formation of an isopeptide bond either within or between polypeptide chains and the covalent incorporation of polyamine into proteins [1, 2].

TGases have been found in bacteria, plants, fish, and mammals, where they are widely distributed in various tissues and bodily fluids [3–6]. TGases play an important physiological role in hemostasis, wound healing, assembly and remodeling of the extracellular matrix, cell signaling and apoptosis [7]. Fibrin-stabilizing FXIII (factor XIII) is an extracellular TGase which is important in the process of blood coagulation. Pathologically, congenital FXIII deficiency has been described in a number of disorders including liver disease, ulcerative colitis, autoimmune IgG anti-FXIII, and some leukemias [8]; and patients with these

disorders experience improvements upon receiving intravenous injections of human FXIII concentrate to prevent hemorrhages [9].

While in general TGase activity in vertebrates and some invertebrates requires Ca^{+2} to expose a cysteine residue in the active site domain [10], TGase activity in bacteria is Ca^{+2} independent. Most microbial TGase (MTGase)-catalyzed reactions can be used to modify the functional properties of food proteins [11], such as texture, viscosity, foaming and emulsifying properties, and lead to improvements in their nutritional value [12, 13]. In addition, MTGases have extensive uses in biomedical research, human diagnostics and therapeutics, such as biomaterial cross-linking [14], protein immobilization [15], fabricating microfluidic devices for cell culture [16], MTGase-mediated gelatin matrices for tissue engineering [17], cross-linking enhancement in cell attachment [18], enzymatic biotinylation of antibodies [19], and formation of novel erythropoietin (EPO) conjugates [20].

It was recently shown that leaky expression of the MTGase gene cloned from *Streptoverticillium mobaraense* in *Lactococcus lactis* subsp. *cremoris* strain NZ9000 [21]. The enzymes from *Streptoverticillium* species [22–26] and *Bacillus* species [27, 28] have been purified and characterized. While MTGases are found extracellularly in *Streptoverticillium* and *Streptomyces* species, the enzyme is localized on the spores in *B. subtilis*. The genes for MTGases of *Streptoverticillium* sp. S-8112 [23], *Stv. cinnamoneum* CBS 683.68. [26], *Stv. mobaraense* DSMZ strain [24], *Stv. ladakanum* B1 [29], and *B. subtilis* [28] have been cloned and sequenced. Several expression systems including *S. lividans* [29, 30], *Escherichia coli* [31], and *Corynebacterium glutamicum* [32] have been used for the production of recombinant MTGases. However, with the exception of the *C. glutamicum* system, the expression levels of recombinant MTGases are very low [32]. A more efficient production system for MTGase is desirable for commercial applications.

Recently, we cloned the transglutaminase gene, *mtgA*, of *S. platensis* M5218 and expressed it in *S. lividans*, creating the *S. lividans* transformant, 25-2 [30]. In the current report, we describe the production, purification, and characterization of recombinant *S. platensis* MtgA produced by this transformant. We also describe the optimization of the growth medium and fermentation conditions which resulted in a significant increase in the production of the recombinant MtgA. To our knowledge, this report provides the first description of the procedures for expressing MTGase in an efficient *S. lividans* expressing system with a high yield and for a flexible and efficient MTGase purification system.

Materials and methods

Bacterial strain and culture conditions

Cultures of 25-2, an *S. lividans* JT46 transformant carrying the pAE053 plasmid [30] were grown on R2YE agar [33] or in liquid medium A [1% (w/v) glucose, 2% (w/v) polypeptone, 0.2% (w/v) KH_2PO_4, 0.2% (w/v) $MgSO_4$ $7H_2O$, 0.2% (w/v) yeast extract, 0.3% (w/v) glycine, and 5 μg/ml thiostrepton, pH 7.2]. A loop of fresh spore suspension was inoculated into 50 ml of medium and cultivated at 30 °C and 220 rev/min for 2–5 days.

Fermenter cultivation

Bench-scale

Fermentation experiments were carried out in a 5 l stirred-tank fermenter (BioFlo IV, NBS Edison, NJ, USA) filled with 3 l of fermentation media A and B [1% (w/v) glucose, 1% (w/v) glycerol, 1% (w/v) starch, 0.1% (w/v) sucrose, 1% (w/v) fructose, 0.5% (w/v) glycine, 0.05% (w/v) casein, 0.5% (w/v) yeast extract, 0.05% (w/v) tryptone peptone, 0.05% (w/v) $(NH_4)_2SO_4$, 0.05% (w/v) polypeptone, 0.2% (w/v) $MgSO_4$ $7H_2O$, 0.2% (w/v) K_2HPO_4, pH 7.2], respectively, and inoculated with 10% (v/v) of seed culture. Five μg/ml thiostrepton and the appropriate antiform (KM-72 deformer, Shin Etsu silicone, Japan) were added automatically, and cultures were grown at 30 °C in conditions of 2.0 vvm and 400 rpm for 50 h and uncontrolled pH. Samples of 10 ml were collected from fermenters and analyzed at intervals as specified in the text.

Laboratory scale

Fermentation was carried out in a 30 l air-lift fermenter (Biostat C, B. Braun, Melsungen, Germany) with 20 l medium B inoculated with 10% (v/v) of the seed culture, 5 μg/ml thiostrepton, and antiform. This culture was then grown at 30 °C, and aeration was maintained by injecting air at a rate of 1 vvm (1 standard liter of air per liter of broth per minute) for 65 h. The seed culture was prepared in a 5 l fermenter (BioFlo 3000, NBS) and was grown in medium A at 30 °C for 16 h.

Pilot plant scale

One hundred and thirty liter of autoclaved medium B in a 250 l stirred-tank fermenter (Model IF-250, NBS) were inoculated with 10% (v/v) of seed culture (13 l), 5 μg/ml thiostrepton, and antiform. The culture was then cultivated at 30 °C with 1.2 vvm and 150 rpm for 65 h. Seed cultures

were prepared in 20 l fermenters (BioFlo 3000, NBS) containing 13 l of medium A with 5 μg/ml thiostrepton at 30 °C with an aeration rate of 1.0 vvm, a rotation rate of 150 rpm, and the antiform controlled automatically. Samples of 10 ml were collected from the fermenters at various intervals, and the amounts of dry cell weight and activity of the recombinant MtgA were estimated.

Purification of the recombinant MtgA

A crude culture supernatant was obtained by centrifuging the culture broth at 5,000*g* for 10 min and concentrating the broth by filtering through a hollow fiber membrane (Koch membrane system, 10,000 MWCO, Wilmington, MA, USA) to about one-eleventh of the starting volume (200 ml). The concentrated broth was then precipitated with 20% saturated ammonium sulfate in an ice bath for 1 h. The obtained precipitate was separated by centrifugation at 12,000*g* for 20 min, and the resultant supernatant was further precipitated with solid ammonium sulfate powder at 50% saturation. The precipitate was then resuspended in a minimal amount of buffer A (0.05 M sodium acetate, pH 5.5) and dialyzed against 3 l of the buffer three times at 4 °C. The resultant solution was filtered through a 0.45 μm membrane filter (Millipore Co., Bedford, MA, USA). After filtration, the supernatant was loaded onto a CM-Sepharose CL-6B fast flow column (1.6 × 20 cm, bed volume 40 ml) which had been equilibrated with buffer A. After being washed with ten bed volumes of buffer A, the absorbed contaminants were eluted with 0.2 M NaCl in buffer A at a flow rate of 2 ml/min. Fractions of 5 ml were collected using a fraction collector (LKB Fract100, Pharmacia Biotech, Piscataway, NJ, USA), and fractions with MTGase activity were combined and dialyzed against buffer B (50 mM phosphate buffer, pH 7.0) at 4 °C overnight. Afterwards, the supernatant was loaded onto a blue-Sepharose fast flow column (1.6 × 10 cm, bed volume 20 ml) which had been equilibrated with buffer B. After washing with buffer B, the absorbed contaminants were eluted with 0.2 M NaCl in buffer B at a flow rate of 2 ml/min. Fractions were collected, and those with the recombinant MtgA activity were combined, dialyzed against buffer B at 4 °C overnight, and concentrated into 10 ml by ultrafiltration (Amicon model 8200, Amicon, Inc., MA, USA). The sample was stored at −80 °C until needed.

Enzyme assays

MTGase activity was determined by a colorimetric hydroxamate procedure using *N*-carbobenzoxy-L-glutaminyl-glycine as a substrate [29]. A calibration curve was prepared with L-glutamic acid-γ-monohydroxamic acid. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of hydroxamic acid per min at 37 °C. Samples were read by a colorimetric plate reader (Model DU-7400 Spectrophotometer, Beckman).

Determination of protein concentration

Protein concentration was determined by using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as the protein standard according to the manufacturer's instructions.

SDS-PAGE

The purified and concentrated protein preparation was electrophoresed by SDS-PAGE according to the method of Laemmli [34]. Samples were separated on 12% polyacrylamide gels on a vertical mini-gel apparatus (Model SE 250, Pharmacia Biotech) at 40 mA for 2 h. Gels were stained with Coomassie brilliant blue R 250 according to the manufacturer's instructions [35].

Effect of pH and temperature

MTGase enzyme activity was measured using purified recombinant MtgA (61.0 U/ml) with *N*-carbobenzoxy-L-glutaminyl-glycine as a substrate. pHs ranging from 3.0 to 9.0 were tested at 1.0 pH unit intervals. Results were expressed as a percentage of the activity obtained at the optimal pH. To determine the optimal pH, enzyme activity was measured in 50 mM citrate buffer with pH ranging from 3.0 to 6.0 and in 50 mM Tris–HCl buffer with pH ranging from 6.0 to 9.0. The effect of temperature was determined within the range of 25–65 °C at 5 °C intervals. Each experiment was performed in triplicate.

Effects of metal cations and inhibitors

Purified recombinant MtgA (61.0 U/ml) in 50 mM Tris–acetate buffer (pH 7.0) was preincubated with the indicated metal cations (1 mM) and various inhibitors (0.1–5.0 mM) at room temperature for 30 min, and enzyme activity was measured as described above. Each experiment was performed in triplicate.

Thermal stability

Purified recombinant MtgA (61.0 U/ml) in 0.1 M Tris–HCl buffer (pH 7.0) was incubated at various temperatures (45–65 °C) for 2–30 min. At indicated time intervals, the enzyme solution was chilled immediately in ice for 5 min, and the residual activity was measured as described above.

Rate constants for thermal inactivation of the recombinant MtgA at various temperatures were calculated using the following equation: $K_D = (\ln A_0 - \ln A_t)/t$, where A_0 and A_t were enzyme activities before and after t min incubation, respectively [36]. The activation energy (E_a) was obtained from the slopes of the inactivation curve on an Arrhenius plot. The thermodynamic parameters, including ΔH^*, ΔG^*, and ΔS^* were calculated as follows:

$$\Delta H* = E_a - RT$$

$$\Delta G* = RT * (\ln K/h + \ln T - \ln K_D)$$

$$\Delta S* = (\Delta H * - \Delta G*)/T$$

where ΔH^*, ΔG^*, and ΔS^* are enthalpy, free energy and entropy changes, respectively; R, K, and h are the gas constants (1.98 cal/deg), Boltzmann constant (1.38×10^{-16} erg/deg), and Planck's constant (6.62×10^{-27} erg/deg), respectively; and T is the absolute temperature (K).

Medium optimization for MTGase production

A fractional factorial experimental design [37] was used for optimization of the medium for the recombinant MtgA production by *S. lividans* 25-2. The optimal amounts of glucose, glycerol, starch, sucrose, fructose, glycine, casein, yeast extract, tryptone peptone, $(NH_4)_2SO_4$, polypeptone, $MgSO_4$ $7H_2O$, and K_2HPO_4 were empirically determined.

Results and discussion

Production of the recombinant MtgA

Previously, we cloned the gene encoding transglutaminase, *mtgA*, in *S. platensis* M5218 and expressed it at high levels in *S. lividans* JT46 [30]. This transformant was also used in the present study for producing the recombinant MtgA. The expression system appeared stable in medium containing 5 μg/ml thiostrepton. Enzyme production, activity, sugar consumption, and dry cell weight in a 5.0 l stirred-tank fermenter culture in medium A were measured over time (Fig. 1). The production of the recombinant MtgA reached its highest level after 32 h incubation, while its maximal activity was achieved after 32 h of cultivation (2.61 U/ml). Enzyme production in the stirred-tank fermenter paralleled that in a flask-shaker. No apparent link was observed between cell growth and enzyme production. Production of the enzyme for the recombinant transformant (*S. lividans* 25-2) was about 3.3 times that from the wild-type strain (*S. platensis* M5218) [30], and both were much greater than that of *S. lividans* 3131-TS, in which MTGase was reported to be secreted at a level of not more than 0.1 mg/l [38]. Our results demonstrate a clear advantage of using recombinant techniques and indicate the potential for use in industrial-scale production of MTGase.

Purification of the recombinant MtgA

The purification of the recombinant MtgA from the culture supernatant was performed by liquid chromatography after ultrafiltration and pooling the 20–50% fractions from ammonium sulfate precipitation. About 88% of the recombinant MtgA activity was recovered after precipitation by this method (Table 1). The recombinant MtgA had high binding ability with CM-Sepharose and blue-Sepharose columns, especially the latter, and was eluted with 0.2 M NaCl in 20 mM phosphate buffer (pH 7.0). After CM-Sepharose fast flow chromatography and blue-Sepharose fast flow chromatography, the recombinant MtgA was purified to a specific activity of 61.0 U/mg. The final recovery was 65% with 33.2 purification fold (Table 1). In spite of one minor band in about 10.0 kDa,

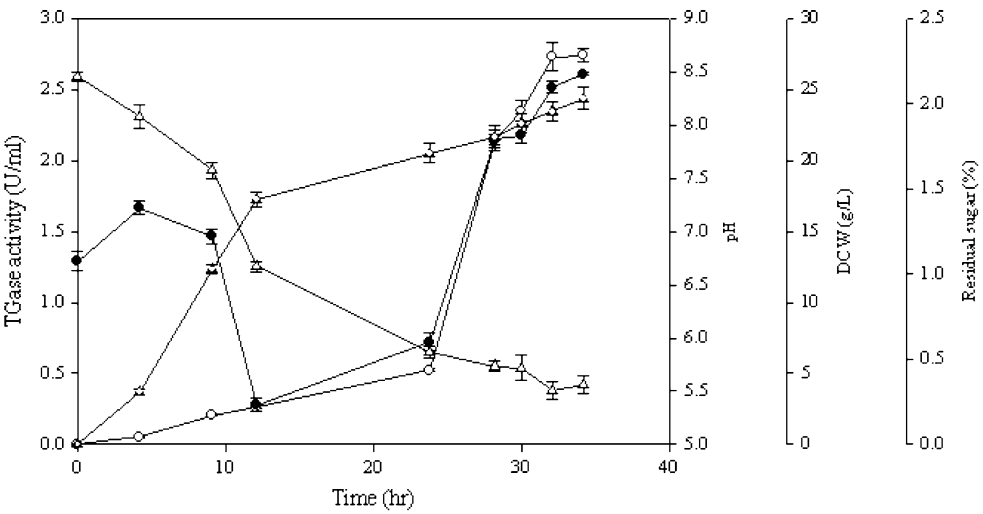

Fig. 1 The production of the recombinant MtgA using medium A in a 5 l stirred-tank fermenter. The fermenter was operated under the following conditions: medium A [1% (w/v) glucose, 2% (w/v) polypeptone, 0.2% (w/v) KH_2PO_4, 0.2% (w/v) $MgSO_4$ $7H_2O$, 0.2% (w/v) yeast extract, 0.3% (w/v) glycine, pH 7.2]; aeration rate 2.0 vvm; agitation speed 400 rpm; temperature at 30 °C. *Open circle* activity, *filled circle* pH, *filled triangle* biomass, *open triangle* sugar. The graph reflects data from experiments performed in triplicate

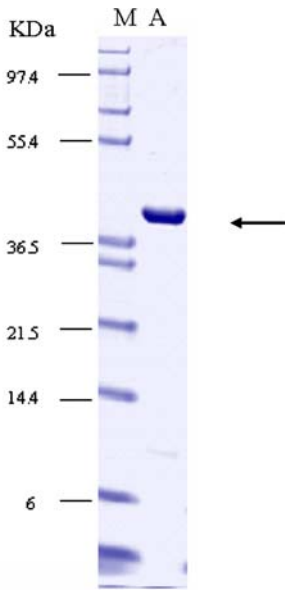

Fig. 2 SDS/PAGE of the recombinant MtgA. *Lane M* protein molecular weight marker: phosphorylase B (97.4 KDA); glutamic dehydrogenase (55.4 KDa); lactate dehydrogenase (36.5 KDa); trypsin inhibitor (21.5 KDa); lyzoyme (14.4 KDa); aprotinin (6.0 KDa). *Lane A* culture filtrate after ammonium sulfate precipitation, CM Sepharose fast flow, and blue Sepharose fast flow chromatography. The position of the recombinant MtgA is indicated by an *arrow*

SDS-PAGE demonstrated that the enzyme was purified to almost electrophoretic homogeneity with purity greater than 99% by densitometric analysis using the ImageQuant 7.0 software (Amersham Biosciences) (Fig. 2). The molecular size of the purified recombinant MtgA was about 40.0 kDa, slightly larger than that predicted by its amino acid sequence (37.7 kDa) [30].

Optimal pH and temperature

Enzyme assays were carried out at different pH and temperature values. Using *N*-carbobenzoxy-L-glutaminyl-glycine as a substrate, we determined that the optimal pH for the recombinant MtgA activity was 5.0–6.0 (Fig. 3a). The purified recombinant MtgA was stable at pH 4.0–8.0 after 30 min incubation at 37 °C, in which more than 80% activity was retained (Fig. 3b). When the pH of the enzyme solution was adjusted to lower than 3.0 or greater than 9.0, the enzyme activity decreased to less than 30 and 50% of its original activity, respectively. The pH stability profile for the enzyme in various pHs was similar with the results of *Stv. ladakanum* [25].

The optimal temperatures for MTGase activity are between 45 and 55 °C, depending on the species. *Stv. mobaraense* MTGase has an optimal temperature at 55 °C, whereas the optimal temperature for MTGases of *Stv. cinnamoneum* spp. *cinnamoneum* and *Stv. griseocarneum* is 45 °C. *Stv. baldaccii* MTGase, which was cloned by Negus et al. [39] and expressed in an *E. coli* system, has a lower optimal temperature, a desirable trait for use in the food industry which requires having high catalytic activity at low temperatures. As shown in Fig. 3c, the optimal temperature for the recombinant MtgA activity was 50 °C for the 30 min reaction, and the enzyme was stable at 45–55 °C. However, the enzyme retained less than 20% activity at temperatures greater than 65 °C. Enzyme activity was stable at 4 °C after 1 month (data not shown).

Thermal stability

According to the thermal inactivation curves (Fig. 4), the rate constants (K_D) for the recombinant MtgA activity were 1.52×10^{-4}, 3.03×10^{-4} and 3.42×10^{-4} s^{-1} at 45, 50 and 55 °C, respectively (Table 2), which were higher than those from pig plasma [40] but slightly lower than those from *Stv. ladakanum* [41]. The ΔH^* and E_a of the purified recombinant MtgA at 55 °C were 39.84 and 40.49 kcal/mol, respectively, higher than those from *Stv. ladakanum* (33.6 and 34.3 kcal/mol, respectively) [25] but lower than

Table 1 Stepwise purification of the recombinant MtgA from *S. lividans* 25-2

Procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (factor)
Culture filtrate	1,861.0	3,094	1.84	100	1.0
Ultrafiltration	881.0	2,934	3.33	95	1.8
Ammonium sulfate (20–50%)	604.0	2,733	4.52	88	2.5
CM-Sepharose	43.8	2,114	48.2	68	26.2
CL-6B fast flow					
Blue-Sepharose fast flow	32.7	1,996	61.0	65	33.2

The recombinant MtgA was purified by filtration through a hollow fiber membrane (Koch membrane system, 10,000 MWCO), and then the concentrated broth was precipitated with 20–50% saturated ammonium sulfate. After dialysis, the supernatant was loaded onto a CM-Sepharose CL-6B fast flow column (1.6 × 20 cm, bed volume 40 ml) at 2 ml/min, and then followed by loading onto a blue-Sepharose fast flow column (1.6 × 10 cm, bed volume 20 ml) at 2 ml/min

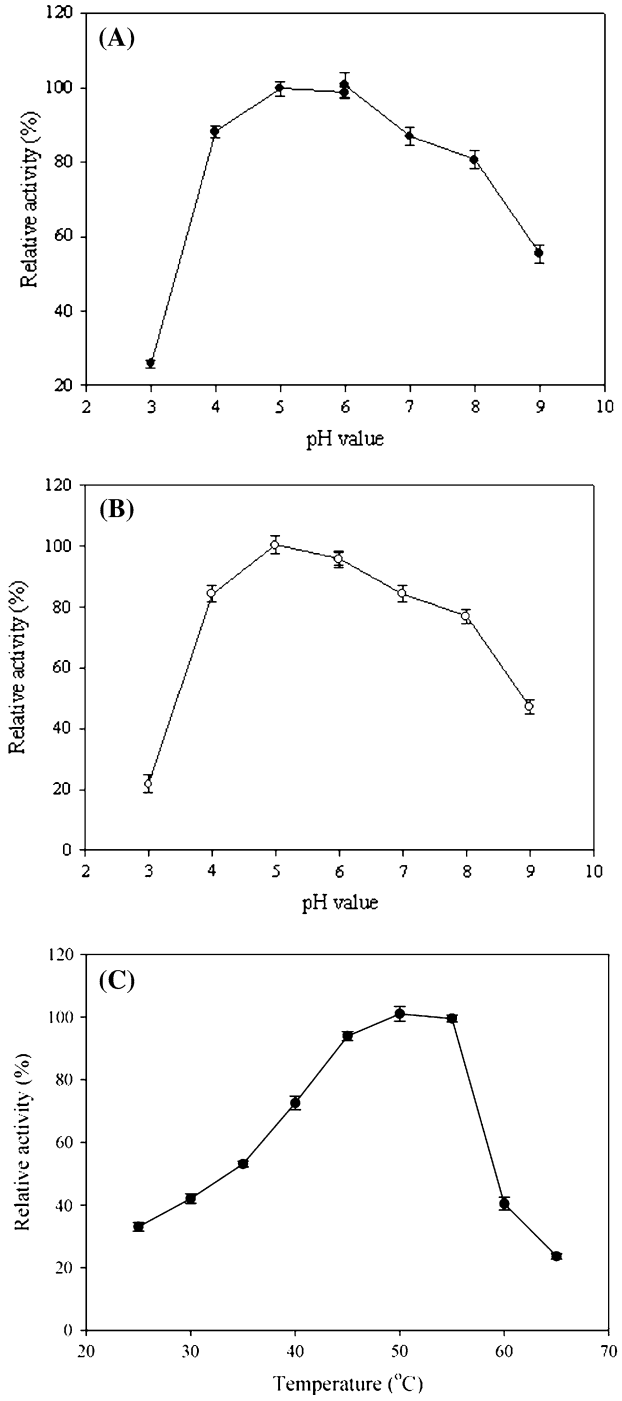

Fig. 3 Effect of pH and temperature on the purified recombinant MtgA. **a** Effect of pH on the activity. The pH of the reaction mixture was adjusted with 50 mM citrate buffer (pH 4.0–6.0) and with 50 mM Tris–HCl buffer (pH 6.0–9.0), **b** pH stability of the purified recombinant MtgA. The pH of the reaction mixture was adjusted with 50 mM citrate buffer (pH 4.0–6.0) and with 50 mM Tris–HCl buffer (pH 6.0–9.0). The enzyme was preincubated at 37 °C for 30 min and then assayed for enzyme activity, **c** profile of temperature dependent activity in 50 mM Tris–HCl buffer, pH 7.0. The graph reflects data from experiments performed in triplicate

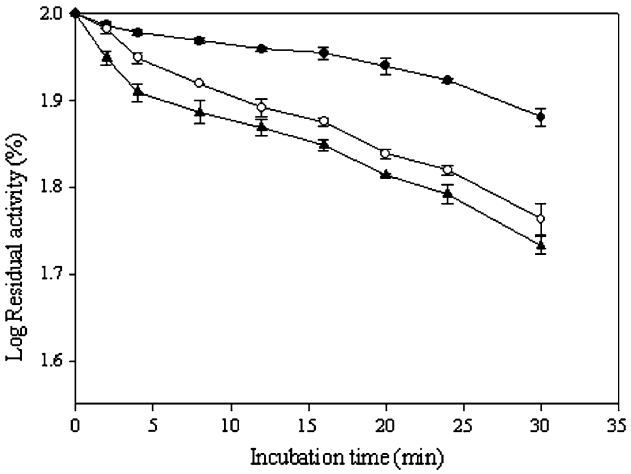

Fig. 4 Thermal stability of the purified recombinant MtgA. The purified recombinant MtgA in 50 mM Tris–HCl buffer, pH 7.0 was incubated at 45, 50, and 55 °C, respectively, for 2–30 min. At indicated time intervals, residual activity of the enzyme was determined. *Filled circle* 45 °C, *open circle* 50 °C, *filled triangle* 55 °C. The graph reflects data from experiments performed in triplicate

those from pig plasma (60.3 and 47.2 kcal/mol, respectively) [40]. These results demonstrate that the recombinant MtgA are less thermally stable than Factor VIIIa from pig plasma, but more thermostable than that purified from *Stv. ladakanum* [25, 41].

Effects of metal cations and inhibitors

The relative activity of the purified recombinant MtgA was determined in the presence of metal ions and inhibitors (Tables 3, 4). The purified recombinant MtgA was not affected by Ca^{+2} or EDTA, suggesting that it is calcium-independent, similar to those purified from *Streptoverticillium* species [26, 29] and *B. subtilis* [28]. The enzymatic reaction catalyzed by TGases of vertebrates and some invertebrates require Ca^{+2} to expose a cysteine residue in the active site [10]. The enzyme was strongly inhibited by Zn^{2+}, Cu^{2+}, Hg^{2+} and PCMB, and moderately inhibited by Pb^{2+}, NEM, and PMSF, suggesting the presence of a thiol group [42], a characteristic of the MTGase active site. Unlike the MTGases from *B. circulans* [27] and *Stv. mobarense* [22], the purified recombinant MtgA was completely inhibited by Hg^{2+}. The enzyme was not inhibited by IAA, suggesting that the involvement of sulfhydryl groups in the catalytic reaction of the purified recombinant MtgA might be greater than that in *Stv. mobarense*.

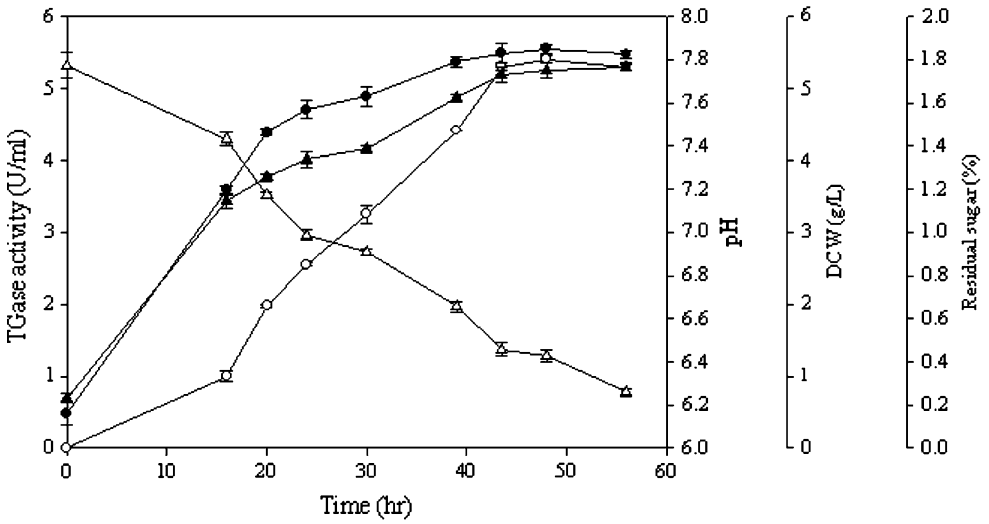

Fig. 5 The production of the recombinant MtgA using the optimized medium B in a 5 l stirred-tank fermenter. The fermenter was operated under the following conditions: in medium B [1% (w/v) glucose, 1% (w/v) glycerol, 1% (w/v) starch, 0.1% (w/v) sucrose, 1% (w/v) fructose, 0.5% (w/v) glycine, 0.05% (w/v) casein, 0.5% (w/v) yeast extract, 0.05% (w/v) tryptone peptone, 0.05% (w/v) $(NH_4)_2SO_4$, 0.05% (w/v) polypeptone, 0.2% (w/v) $MgSO_4$ $7H_2O$, 0.2% (w/v) K_2HPO_4, pH 7.2]; aeration rate 2.0 vvm; agitation speed 400 rpm; temperature at 30 °C. *Open circle* activity, *filled circle* pH, *filled triangle* biomass, *open triangle* sugar. The graph reflects data from experiments performed in triplicate

Table 2 Thermodynamic parameters for thermal inactivation of the purified recombinant MtgA

Temp (Δ)	K_D (s^{-1})	ΔG^* (kcal/mol)	E_a (kcal/mol)	ΔS^* (cal/K mol)	ΔH^* (kcal/mol)
45	1.52×10^{-4}	24.13	40.49	49.42	39.86
50	3.03×10^{-4}	24.08	40.49	48.79	39.85
55	3.42×10^{-4}	24.39	40.49	47.09	39.84

K_D rate constant of thermal inactivation, ΔG^* free energy change, E_a activation energy, ΔS^* entropy change, ΔH^* enthalpy change

Table 3 Effect of metal ions on the recombinant MtgA activity

Metal ion (1 mM)	Relative activity (%)
None	100 ± 2.12
Ca^{2+}	101.9 ± 4.23
Li^{+}	98.7 ± 3.35
Mn^{2+}	98.5 ± 4.56
Na^{+}	98.4 ± 2.34
Ni^{+}	98.3 ± 5.12
Fe^{3+}	98.2 ± 1.87
K^{+}	95.6 ± 3.72
Mg^{2+}	94.2 ± 5.21
Al^{3+}	93.9 ± 2.56
Ba^{2+}	93.8 ± 4.34
Co^{2+}	93.0 ± 5.86
Fe^{2+}	73.2 ± 3.63
Pb^{2+}	59.8 ± 2.06
Zn^{2+}	32.6 ± 3.48
Cu^{2+}	1.4 ± 0.23
Hg^{2+}	0.8 ± 0.18

Results are mean ± SD for triplicate experiments

Medium optimization for the production of the recombinant MtgA

A fractional factorial experimental design [37] was used for the optimization of the medium for the recombinant MtgA production by the *S. lividans* 25-2 based on components of medium A. The optimal medium, designated medium B (see "Materials and methods" for composition) was obtained. Comparisons of the recombinant MtgA production in media A and B were carried out in both flask and jar experiments. In shaking-flask experiments, the production of the recombinant MtgA cultured in medium B reached a maximal activity of 5.78 U/ml, a twofold increase of that produced in medium A (data not shown). In 5 l stirred-tank fermenter experiments, a maximal activity of 5.39 U/ml was obtained in medium B after 56 h fermentation (Fig. 5), also a twofold increase of that produced in medium A (2.61 U/ml) after 32 h fermentation (Fig. 1), even though the dry cell weight of the former was far lower than that of the latter. The initial lag for the recombinant MtgA production in medium A was shorter than that in medium B. Taken together, the results showed that

Table 4 Effect of protein inhibitors on the recombinant MtgA activity

Concentration (mM)	EDTA	IAA	PMSF	NEM	PCMB
0	100	100	100	100	100
0.1	–	–	–	–	0.5 ± 0.03
0.5	–	–	98.2 ± 3.51	74.4 ± 4.41	0.2 ± 0.08
1.0	98.7 ± 3.26	101.2 ± 2.41	86.7 ± 3.76	46.3 ± 2.67	–
2.0	97.6 ± 4.23	88.3 ± 4.31	52.1 ± 4.87	31.7 ± 3.47	–
5.0	96.3 ± 2.31	84.2 ± 5.12	34.4 ± 1.83	6.1 ± 0.57	–

Results are mean ± SD for triplicate experiments

EDTA ethylenediamine tetraacetic acid, *IAA* iodoacetic acid, *PMSF* phenylmethyl sulfonyl fluoride, *NEM* *N*-ethylmaleimide, *PCMB* *p*-chloromercuribenzoate

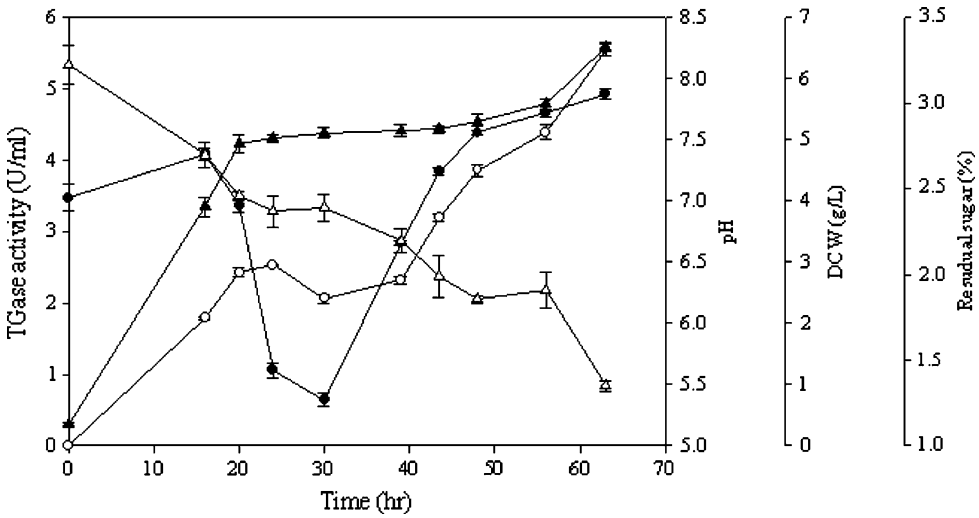

Fig. 6 The time profiles of mycelial biomass and the recombinant MtgA in a 30 l air-lift fermenter. The fermenter was operated under the following conditions: in medium B; aeration rate 1.0 vvm; temperature at 30 °C. *Open circle* activity, *filled circle* pH, *filled triangle* biomass, *open triangle* sugar. The graph reflects data from experiments performed in duplicate

although medium A appears to be better for growth of *S. lividans* 25-2, medium B is better for the recombinant MtgA production in both shaking-flask and jar fermentation scales of experiments.

Large-scale production of the recombinant MtgA

The recombinant MtgA production by *S. lividans* 25-2 was scaled up by using a 30 l air-lift batch culture under an aeration rate of 1.0 vvm (Fig. 6). High recombinant MtgA production (5.36 U/ml) was achieved after 64 h, comparable to that produced in the 5 l fermenter after 56 h (5.39 U/ml) (Fig. 5). The activity was considerably higher than those reported to date for most other actinomyces that produce MTGase [29, 30]. Oxygen supply was effective for cell growth and the recombinant MtgA production in the air-lift fermentation, and an increase in the aeration rate increased both these parameters. These results are in good agreement with those of Techapun et al. [43]. Our results demonstrate that in addition to genetic manipulation, optimization of cultivation conditions can lead to significant improvements in the production of heterologous MTGase by *S. lividans*.

To scale up the production of the recombinant MtgA further, a pilot plant scale of batch culture in a jar fermenter (250 l stirred-tank) was carried out with an aeration rate of 1.0 vvm and a rotation rate of 200 rpm. The fermentation took a period of 48 h with a maximal activity of 2.54 U/ml (data not shown). Limitations in DO after the cell growth became maximal or inadequate aeration may result in the low activity of the recombinant MtgA in the 250 l stirred-tank fermenter. Feed-batch fermentation with continuous feeding of both glucose and tryptone may lead to a further increase in production of the enzyme [44], and we are continuing to investigate this possibility.

Conclusions

In summary, we have purified, characterized and made large-scale production of the recombinant *Stv. platensis* transglutaminase, MtgA, from *S. lividans*. The enzyme was purified to near homogenecity by a stepwise method with high recovery. The enzyme had an optimal pH and temperature of 6.0 and 55 °C, respectively, and it was stable at

pH 5.0–6.0 and at 45–55 °C. It had a rate constant (K_D) of 3.42×10^{-4} s^{-1} at 55 °C. In our large-scale production of the enzyme in a 30 l air-lift fermenter, the fermentation took a period of 64 h with maximal productivity of 5.36 U/ml. In a pilot plant scale of MtgA production in 250 l stirred-tank fermenter, the process took a period of 48 h with maximum productivity of 2.54 U/ml. Furthermore, the recombinant MtgA is of great potential for industrial use based on its broad optimal activity range, substrate specificity, and stability. Further improvement of the large-scale production of the recombinant MtgA is now in progress. With the help of recombinant approach, the recombinant MtgA may have potential to provide new alternatives particularly suitable for therapeutic and diagnostic applications.

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References

1. Folk JE, Chung SI (1985) Transglutaminases. Methods Enzymol 113:358–375. doi:10.1016/S0076-6879(85)13049-1
2. Aeschlimann D, Paulsson M (1994) Transglutaminases: protein cross-linking enzymes in tissues and body fluids. Thromb Haemost 71:402–415
3. Folk JE, Finlayson JS (1997) The ε-γ-(glutamyl) lysine crosslink and the catalytic role of transglutaminases. Adv Protein Chem 31:1–133. doi:10.1016/S0065-3233(08)60217-X
4. Muszbek L, Yee VC, Hevessy Z (1999) Blood coagulation factor XIII: structure and function. Thromb Res 94:271–305. doi:10.1016/S0049-3848(99)00023-7
5. Yasueda H, Nakanishi K, Kawakawa Y, Nagase K, Motoki M, Mastsui H (1995) Tissue-type transglutaminase from red sea bream (Paris major): sequence analysis of the cDNA and functional expression in *Escherichia coli*. Eur J Biochem 232:411–419. doi:10.1111/j.1432-1033.1995.tb20826.x
6. Kang H, Cho YD (1996) Purification and properties of transglutaminase from soybean (*Glycine max*) leaves. Biochem Biophys Res Commun 223:288–292. doi:10.1006/bbrc.1996.0886
7. Wodzinska JM (2005) Transglutaminases as targets for pharmacological inhibition. Mini Rev Med Chem 5:279–292
8. Anwar R, Miloszewski KJ (1999) Factor XIII deficiency. Br J Haematol 107:468–484. doi:10.1046/j.1365-2141.1999.01648.x
9. Tosetto A, Castaman G, Rodeghiero F (1993) Acquired plasma factor XIII deficiencies. Haematologica 78:5–10
10. Casadio R, Polverini E, Mariani P, Spinozzi F, Carsughi F, Fontana A, Polverino de Laureto P, Matteucci G, Bergamini CM (1999) The structural basis for the regulation of tissue transglutaminase by calcium ions. Eur J Biochem 262:672–679. doi:10.1046/j.1432-1327.1999.00437.x
11. Nielsen PM (1995) Reactions and potential industrial applications of transglutaminase. Food Biotechnol 9:119–156
12. Zhu Y, Rinzema A, Tramper J, Bol J (1995) Microbial transglutaminase-a review of its production and application in food processing. Appl Microbiol Biotechnol 44:277–282. doi:10.1007/BF00169916
13. Yokoyama K, Nio N, Kikuchi Y (2004) Properties and applications of microbial transglutaminase. Appl Microbiol Biotechnol 64:447–454. doi:10.1007/s00253-003-1539-5
14. Chen RN, Ho HO, Sheu MT (2005) Characterization of collagen matrices crosslinked using microbial transglutaminase. Biomaterials 26:4229–4235. doi:10.1016/j.biomaterials.2004.11.012
15. Kamiya N, Doi S, Tominaga J, Ichinose H, Goto M (2005) Transglutaminase-mediated protein immobilization to casein nanolayers created on a plastic surface. Biomacromolecules 6:35–38. doi:10.1021/bm0494895
16. Paguirigan A, Beebe DJ (2006) Gelatin based microfluidic devices for cell culture. Lab Chip 6:407–413. doi:10.1039/b517524k
17. Ito A, Mase A, Takizawa Y, Shinkai M, Honda H, Haata K-I, Kobayashi T (2003) Transglutaminase-mediated gelatin matrices incorporating cell adhesion factors as a biomaterial for tissue engineering. J Biosci Bioeng 93:196–199
18. Chau DY, Collighan RJ, Verderio EA, Addy VL, Griffin M (2005) The use of transglutaminase as a novel biocatalyst in the modification of collagen for the development of new biomaterials. Biomaterials 26:6518–6529. doi:10.1016/j.biomaterials.2005.04.017
19. Josten A, Haalck L, Spener F, Meusel M (2000) Application of microbial transglutaminase for the enzymatic biotinylation of antibodies. J Immunol Methods 240:47–54. doi:10.1016/S0022-1759(00)00172-1
20. Chadler P (2006) Formation of novel erythropoietin conjugates using transglutaminase. United States Patent 6995245
21. Fu R-Y, Chen J, Li Y (2005) Heterologous leaky production of transglutaminase in *Lactococcus lactis* significantly enhances the growth. Appl Environ Microbiol 71:8911–8919. doi:10.1128/AEM.71.12.8911-8919.2005
22. Ando H, Adachi M, Umeda K, Matsuura A, Nonaka M, Uchio R (1989) Purification and characteristics of a novel transglutaminase derived from microorganisms. Agric Biol Chem 53:2613–2617
23. Kanaji T, Ozaki H, Takao T, Kawajiri H, Ide H, Motoki M, Shimonishi Y (1993) Primary structure of microbial transglutaminase from *Streptoverticillium* sp. strain s-8112. J Biol Chem 268:11565–11572
24. Gerber U, Jucknischke U, Putzien S, Fuchsbauer HL (1994) A rapid and simple method for the purification of transglutaminase from *Streptoverticillium mobaraense*. Biochem J 299:825–829
25. Tsai GJ, Lin SM, Jiang ST (1996) Transglutaminase from *Streptoverticillium ladakanum* and application to minced fish product. J Food Sci 61:1234–1238. doi:10.1111/j.1365-2621.1996.tb10968.x
26. Duran R, Junqua M, Schmitter JM, Gancet C, Goulas P (1998) Purification, characterisation, and gene cloning of transglutaminase from *S. cinnamoneum* CBS 683.68. Biochim 80:313–319. doi:10.1016/S0300-9084(98)80073-4
27. de Barros Soares LHB, Assmann F, Zachia Ayub MA (2003) Purification and properties of transglutaminase produced by *Bacillus circulans* strain isolated from the amazon environment. Biotechnol Appl Biochem 37:295–299. doi:10.1042/BA20020110
28. Kobayashi K, Hashiguchi KI, Yokozeki K, Yamanaka S (1998) Molecular cloning of the transglutaminase gene from *Bacillus subtilis* and its expression in *Escherichia coli*. Biosci Biotechnol Biochem 62:1109–1114. doi:10.1271/bbb.62.1109
29. Lin YS, Chao ML, Liu CH, Chu WS (2004) Cloning and expression of the transglutaminase gene from *Streptoverticillium ladakanum* in *Streptomyces lividans*. Process Biochem 39:591–598. doi:10.1016/S0032-9592(03)00134-1
30. Lin YS, Chao ML, Liu CH, Tseng M, Chu WS (2006) Cloning of the gene coding for transglutaminase from *Streptomyces platensis* and its expression in *Streptomyces lividans*. Process Biochem 41:519–524. doi:10.1016/j.procbio.2005.09.009
31. Yokoyama K, Nakamura N, Saguaro K, Kubota K (2000) Overproduction of microbial transglutaminase in *Escherichia coli*: In vitro refolding and characterization of the refolded form.

Biosci Biotechnol Biochem 64:1263–1270. doi:10.1271/bbb.64.1263
32. Date M, Yokoyama KI, Umezawa Y, Matsui H, Kikuchi Y (2003) Production of native-type *Streptoverticillium mobaraense* transglutaminase in *Corynebacterium glutamicum*. Appl Envion Microbiol 69:3011–3014. doi:10.1128/AEM.69.5.3011-3014.2003
33. Chater KF, Hopwood DA, Kieser T, Thompson CJ (1982) Gene cloning in *Streptomyces*. Curr Top Microbiol Immunol 96:69–95
34. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of. bacteriophage T4. Nature 227:680–685. doi:10.1038/227680a0
35. Neuhoff V, Arold N, Taube D, Ehrhardt W (1989) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie brilliant blue G-250 and R-250. Electrophoresis 9:255–262. doi:10.1002/elps.1150090603
36. Hashimoto A, Kobayashi A, Arai K (1982) Thermostability of fish myofibrillar Ca-ATPase and adaptation to environment temperature. Bull Jap Soc Sci Fish 48:671–684
37. DeMeo M, Larget M, Phan-Tan-Luu R, Mathieu D, Dumenil G (1985) Application of experimental designs for optimization of medium and culture conditions in fermentation. Bioscience 4:99–102
38. Washizu K, Ando K, Koikeda S, Hirose S, Matsuura A, Takagi H, Motoki M, Takeuchi K (1994) Molecular cloning of the gene for microbial transglutaminase from *Streptoverticillium* and its expression in *Streptomyces lividans*. Biosci Biotechnol Biochem 58:82–87
39. Negus SS (2002) A novel microbial transglutaminase derived from *Strptoverticillium baldaccii*. A Thesis of the degree of Doctor of Philosophy of Griffith University, Nathan Campus, Queensland
40. Jiang ST, Lee JJ (1992) Purification, characterization, and utilization of pig plasma factor XIIIa. Agric Food Chem 40:1101–1107. doi:10.1021/jf00019a002
41. Ho ML, Leu SZ, Hsieh JE, Jiang ST (2000) Technical approach to simplify the purification method and characterization of microbial transglutaminase produced from *Streptoverticillium ladakanum*. J Food Sci 65:76–80. doi:10.1111/j.1365-2621.2000.tb15959.x
42. Motoki M, Seguro K (1998) Transglutaminase and its use for food processing. Trends food Sci Technol 9:204–210. doi:10.1016/S0924-2244(98)00038-7
43. Techapun C, Poosaran N, Watanabe M, Sasaki K (2003) Improvement of cellulase-free xylanase production by *Streptomyces* sp. Ab 106 with optimized stirred type fermenter and repeated fed-batch cultivation using agricultural waste. J Biosci Bioeng 95:298–301
44. Payne GF, DelaCruz N, Coppella SJ (1990) Improved production of heterologous protein from *Streptomyces lividans*. Appl Microbiol Biotechnol 33:395–400. doi:10.1007/BF00176653