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Purification and characterization of transglutaminase from a newly isolated *Streptomyces hygroscopicus*

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Abstract

Transglutaminase (TGase, EC 2.3.2.13) from a *Streptomyces hygroscopicus* strain isolated from soil was purified from culture broth by ethanol precipitation, followed by successive chromatographies on CM-cellulose and Sephadex G-75 columns with a yield and purification-fold of 21.1% and 30%, respectively. The enzyme's molecular weight was estimated as 38,000 Da by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The purified microbial transglutaminase (MTG) exhibited optimum activity at 37–45 °C and in a range of pH 6.0–7.0 for hydroxamate formation from *N*-carboxybenzoyl-L-glutaminyl-glycine and hydroxylamine. The enzyme was not stable above 50 °C and was stable within a pH range of 5.0–8.0 at lower temperature. The MTG was not inhibited by Ca^{2+} and ethylenediaminetetraacetic acid, suggesting it was calcium-independent. Purified MTG was strongly inactivated by 5,5′-dithiobis (2-nitrobenzoic acid), Cu^{2+}, Zn^{2+}, Pb^{2+}, and Hg^{2+}, suggesting that this enzyme could possess a thiol group at the active site. The MTG stability was strongly affected by ethanol concentration. The enzyme activity was slightly elevated at a lower concentration of ethanol at 25 °C.

Keywords: Microbial transglutaminase; *Streptomyces hygroscopicus*; Purification; Enzyme characterization; Ethanol

1. Introduction

Transglutaminase (TGase; protein-glutamine-glutamyltransferase, EC 2.3.2.13) is an enzyme capable of catalyzing acyl transfer reactions by introducing covalent cross-links between proteins as well as peptides and various primary amines. Transglutaminases present in most animal tissues and body fluids are involved in several biological processes, including blood clotting, wound healing, epidermal keratinization, and stiffening of the erythrocyte membrane (Aeschlimann & Paulsson, 1994). Recently, TGase has captured peoples' interest due to its attractive potential application in food industries (Motoki & Seguro, 1994; Zhu, Rinzema, Tramper, & Bol, 1995), immobilization of enzymes (Josten, Meuse, Spener, & Haalck, 1999; Yoshiro, Erika, & Masao, 1992) and textile industries (Cortez, Bonner, & Griffin, 2004). The relatively small quantity obtained and the complex separation and purification procedure required for these enzymes from tissues led to the search for microbial sources. Ando et al first (Ando et al., 1989) reported that strains from the genus *Streptoverticillium* screened from several thousands microorganisms had the ability to produce transglutaminase using the hydroxamate assay. These microorganisms excreted the enzyme, and one of them classified as a variant of *Streptoverticillium mobaraensis* (Washizu et al., 1994) produced a high activity. The enzyme from microorganisms was named microbial transglutaminase (MTG). Subsequently Ajinomoto Co., Inc., produced the enzyme at an industrial scale in collaboration with Amano Enzyme Inc. Since then, efforts have been made to obtain TGase not only from *Streptoverticillium* species but also from other genus such as *Bacillus* (Barros, Assmann, & Zachia, 2003; Zhu, Rinzema, Tramper, & Bol, 1996).

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To achieve commercialization of MTG, studies have been continually performed in our laboratory including the screening of microorganisms, scaled-up cultivation, downstream process optimization and its application (Cui et al., 2006; Lu, Zhou, Tian, & Chen, 2003; Yan, Du, Li, & Chen, 2005; Zheng, Du, & Chen, 2002). Recently, we have isolated a new strain producing a high activity TGase classified as *Streptomyces hygroscopicus WSH03-13* from soil and optimal conditions for production of MTG in an agitating fermenter has been investigated in our laboratory. The objectives of this study were to purify and characterize TGase from the newly isolated strain. The properties of the enzyme were also to be compared to TGases from other sources including microorganisms or tissues. Furthermore, the effect of ethanol on MTG activity and casein cross-linking due to the activity of transglutaminase in the presence of low concentration of ethanol was examined. This investigation will help to provide an alternative strain to mass-produce TGase by traditional fermentation technology and determine the suitability of this enzyme for food industry applications.

2. Materials and methods

2.1. Materials

All the chemicals used were of analytical grade and mainly purchased from Sinopharm Chemical Reagent Co., Ltd, China, unless otherwise mentioned. Low molecular mass protein calibration kit was from Sino-American Biotechnology Co., China. *N*-carboxybenzoyl-L-glutaminyl-glycine (N-CBZ-Gln-Gly), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., Ltd.

2.2. Microorganism and crude enzyme preparation

Streptomyces hygroscopicus WSH03-13 collected in our laboratory was used throughout this study. The strain was cultivated on malt-meat agar (pH 7.0) at 30 °C for 2 weeks. The seed culture medium was composed of (g/l): starch (20), peptone (20), yeast extract (5), $MgSO_4$ (2), K_2HPO_4 (2) and KH_2PO_4 (2) at pH 6.80. The fermentation medium consisted of (g/l): starch (5), glucose (5), glycerin (10), peptone (15), soybean powder (20), yeast extract (5), $MgSO_4$ (2), K_2HPO_4 (2), KH_2PO_4 (2) and $CaCO_3$ (5) at pH 6.5.

For seed preparation, the microorganism from a fresh slant tube was inoculated into 100 ml fresh seed medium in 500 ml flasks and cultivated on a rotary shaker at 200 rpm and 32 ± 1 °C for 24 h. Seed culture (8%, v/v) was then inoculated into the fermentation medium (initial pH value of 6.5) for production of transglutaminase in a 7 l fermenter (KF-7L, Korea Fermenter Co., Inchon, Korea) with a working volume of 4.5 l. The cultivation was carried out at 32 ± 1 °C and at an agitation speed of 200 rpm. After 42 h, the culture broth was centrifuged at 8000*g* for 20 min at 4 °C to remove microorganisms and the resulting supernatant solution was used as the crude enzyme preparation.

2.3. Analytical methods

2.3.1. Determination of enzyme activity

MTG activity was determined by hydroxamate formation with the specific substrate, N-CBZ-Gln-Gly described by Grossowicz et al (Grossowicz, Wainfan, Borek, & Waelsch, 1950). A 1.0 ml substrate solution, containing 0.4 ml of 0.2 M Tris–HCl buffer (pH 6.0), 0.2 ml of 0.1 M hydroxylamine, 0.2 ml of 0.01 M reduced glutathione and 0.2 ml of 0.15 M N-CBZ-Gln-Gly, was mixed with 0.4 ml of appropriately diluted enzyme solution. The reaction mixture was incubated at 37 °C for 10 min and then stopped by adding 0.4 ml of ferric chloridetrichloracetic acid reagent (consisting of 1 volume 12% HCl, 1 volume 12% trichloracetic acid (TCA) and 1 volume 5% ferric trichloride solution in 0.1 M HCl). After 5 min centrifugation at 10,000*g*, the absorbance at 525 nm of the supernatant was measured. The calibration was performed using L-glutamic acid γ-monohydroxamate as standard. One unit of transglutaminase was defined as the amount of enzyme which causes the formation of 1.0 μmol L-glutamic acid γ-monohydroxamate per minute at 37 °C.

2.3.2. Protein determination

The amount of protein was determined by the Lowry method (Lowry, Rosenberg, Farr, & Randell, 1951) with bovine serum albumin as the standard.

2.4. Purification of MTG

All steps were performed at 4 °C, unless otherwise stated.

2.4.1. Ethanol precipitation

Chilled ethanol was added drop-wise to the crude enzyme solution with an initial total protein concentration of approximately 2% (w/v) while stirring to give a final ethanol concentration of 70% (v/v). The whole broth was left overnight at 4 °C. The precipitate formed was collected by centrifugation at 10,000*g* for 20 min, dissolved in 20 mM phosphate buffer, pH 6.0 and dialyzed against the same buffer overnight.

2.4.2. CM-cellulose chromatography

The dialyzed enzyme solution was applied to a CM-cellulose chromatography column (2.6 × 20 cm) previously equilibrated with 20 mM phosphate buffer, pH 6.0. The column was washed extensively with the same buffer and eluted with a linear gradient of 0.0–0.8 M NaCl in the same buffer at a flow rate of 1.0 ml/min. Fractions with MTG activity were pooled and dialyzed overnight against 20 mM phosphate buffer, pH 6.5.

2.4.3. Sephadex G-75 chromatography

The MTG was further purified by gel filtration on a column of Sephadex G-75 (1.6 × 80 cm) pre-equilibrated with 20 mM phosphate buffer, pH 6.5. The dialysed enzyme solution from CM-cellulose chromatography column was concentrated to about 2 ml with Polyoxyalkylene 20,000 and passed through the column. The column was washed with 0.1 M NaCl in the same buffer at a flow rate of 1.0 ml/min and the active fractions were collected.

2.4.4. Sodium dodecylsulfate polyacrylamide gel electrophoresis

The purified enzyme was analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli (Laemmli, 1970). A 12.5% separating gel was used. The proteins were stained with a 0.1% solution of Coomasiie brilliant blue R-250.

2.5. Biochemical characteristics of MTG

The effect of pH on MTG activity was determined using the reaction mixtures as described previously except for with various buffers: pH 3.7–6.0, using 50 mM acetate buffer; pH 6.0–9.0, using 50 mM Tris–HCl buffer. The enzyme activity was measured after incubation at 37 °C for 10 min. To check the pH stability enzyme was pre-incubated with the above respective buffer at 10 °C and 37 °C, respectively, for 30 min and enzyme activity was carried out using the substrate solution described above without adjusting the pH. The relative activities were determined by using the maximal activity of the enzyme at a specific pH as 100%.

The effect of temperature on MTG activity was tested by assaying the activity at different temperatures in the range 20–70 °C and pH 6.0 using the reaction mixtures as indicated above. The relative activities were determined by using the maximal activity of the enzyme at a specific temperature as 100%. Thermal stability was determined by pre-incubating the enzyme at various temperatures for 30 min and the samples were placed in ice immediately. The relative activities were then determined using the standard method described above, taking the activity of a sample without incubation as 100%.

To study the effect of various additives on MTG activity, the enzyme was pre-incubated with various metals and other reagents at 37 °C for 30 min and the samples were placed in ice immediately. The relative activity was monitored by the standard method. The relative activity assayed in the absence of additives and without incubation was taken as 100%.

Kinetic parameters were determined in the reaction mixtures indicated above containing variable amounts of the specific substrate N-CBZ-Gln-Gly (0–30 mM). The values of Michaelis constant (K_m) and maximum velocity (V_{max}) were determined from Lineweaver–Burk plots.

The effect of different ethanol concentrations on MTG activity was performed at pH 6.0 in 50 mM Tris–HCl buffer with an enzyme concentration of 1.0 U/ml at 25 °C. The samples were incubated for appropriate periods of time, aliquots were then withdrawn, and the relative activities of the enzyme were determined by using the standard method described above but the substrate solution containing the same concentrations of ethanol, taking the activity of a sample without ethanol and without incubation as 100%.

2.6. Cross-linking experiments

The enzymatic reactions were carried out at 25 °C in 20 mM phosphate buffer (pH 6.5) containing 1% (w/v) casein and an enzyme level of 20 units per gram of protein substrate. The reaction mixtures were incubated for various times (0, 10, 20, 30, 60 min, respectively), then stopped by directly mixing with the sample buffer (2×) of electrophoresis. The reaction mixtures were analyzed by SDS–PAGE and by measuring the ammonia released (Ngo, Phan, & Yam, 1982).

3. Results and discussion

3.1. Purification of MTG

The culture was harvested when the MTG activity reached its highest value and the specific activity of the enzyme was 0.25 U/mg protein. MTG stability was strongly affected by high concentration of ethanol. In addition, higher temperature is another important factor affecting the enzyme stability in the presence of ethanol (Cui et al., 2006). During the precipitation step, chilled ethanol was used to precipitate the enzyme proteins. The precipitate formed was immediately dissolved in 20 mM phosphate buffer, pH 6.0 and dialyzed against the same buffer overnight at 4 °C. Under these conditions, ethanol concentration in the enzyme solution was very low and a high yield of enzyme activity could be obtained. About 85% of the enzyme activity was precipitated with 70% ethanol with a two fold increase in the specific activity (Table 1). The initial effective and efficient purification step was chromatographed on CM-cellulose. MTG could be eluted between 0.3 and 0.4 M NaCl with a yield of 57.7%, a specific activity of 6.26 U/mg protein and 25-fold purification (Fig. 1a and Table 1). Most impurities were removed in this step according to SDS–PAGE (Fig. 2). Further purification was achieved by subjecting the pooled enzyme fraction from

Table 1
Summary of purification of transglutaminase from *Streptomyces hygroscopicus*

Purification step	MTG (U)	Protein (mg)	Specific activity (U/mg protein)	Purification fold	Yield (%)
Crude enzyme	90	360	0.25	1	100
Ethanol	76.5	132	0.58	2	85
CM-cellulose	52.0	8.3	6.26	25	57.7
Sephadex G-75	19.0	2.5	7.60	30	21.1

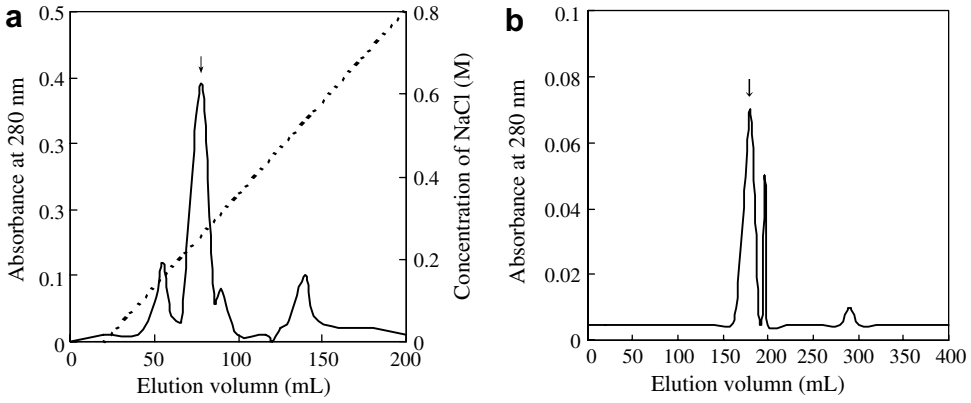

Fig. 1. Chromatography of the crude MTG from *Streptomyces hygroscopicus WSH03-13* on CM-cellulose (a) and Sephadex G-75 (b) column. MTG was purified by CM-cellulose (a) and then Sephadex G-75 column (b). The arrow indicates the active fraction containing MTG activity.

the CM-cellulose column to a Sephadex G-75 column (Fig. 1b), which resulted in a 30-fold purification with a yield of 21.1% and a specific activity of 7.6 U/mg protein as compared to the crude extract (Table 1). Though both the purification fold and yield were a little lower after this step, protein purification was successfully achieved to electrophoretical homogeneity on SDS–PAGE. From electrophoretic results the molecular mass of the enzyme was estimated to be about 38,000 Da (Fig. 2). This result shows that the enzyme is much heavier than that from *Bacillus subtilis* (29,000 Da) (Suzuki et al., 2000) and approximately equal to those from *Streptoverticillium mobaraense* (37,000 Da) (Ando et al., 1989), but smaller than other Ca^{2+}-dependent tissue transglutaminases (Icekson & Apelbaum, 1987; Worratao & Yongsawatdigul, 2005).

3.2. Effect of pH on enzyme activity

The effect of pH on MTG activity was determined using the reaction mixtures as described previously at pH 3.7–9.0 and at 37 °C. The enzyme exhibited optimum activity for the catalytic reaction of N-CBZ-Gln-Gly and hydroxylamine in a range of pH 6.0–7.0 and showed some activity at pH 4.5 or 9 (Fig. 3a). The optimum pH of this enzyme was nearly the same as that from *Streptoverticillium mobaraense* (Ando et al., 1989). The activity was found to decrease gradually at alkaline pH, but it decreased rapidly at acidic pH. It was different from *Bacillus subtilis* transglutaminase that has an optimal pH value of 8.2 (Suzuki et al., 2000). The enzyme from mammals and fishes has a pH optimum of 8.0 and soybean TGase has an optimal pH value of 7.6 (Gerber, Jucknischke, Putzien, & Fuchsbauer, 1994; Zhu et al., 1996). The purified MTG was stable within a wide range of pH 5.0–8.0 at 10 °C and there was no activity loss after 30 min (Fig. 3b). At pH 9.0 the relative activity was still over 70%. The enzyme was stable at pH 5.0–7.0 and about 50% activity was retained at pH 8 after 30 min of incubation at 37 °C. The activity decreased greatly outside this pH range.

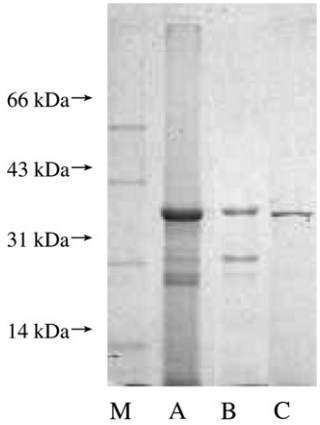

Fig. 2. SDS–PAGE of MTG on acrylamide gel for crude enzyme, after CM-cellulose and after Sephadex G-75. M, standard marker; lane A, crude enzyme; lane B, MTG after CM-cellulose; lane C, MTG after Sephadex G-75.

3.3. Effect of temperature on enzyme activity

The temperature effects on MTG activity were studied by determining the activity between 20 and 70 °C after incubation for 10 min at pH 6.0, with the reaction mixtures as indicated previously. The enzyme exhibited optimum activity at 37–45 °C for the catalytic reaction of N-CBZ-Gln-Gly and hydroxylamine. No enzyme activity was detected at 70 °C (Fig. 4a). Comparing with other TGases from different sources, the optimal temperature of this enzyme almost was the same as that from *Streptoverticillium ladakanum* and completely diverging from *Streptoverticillium mobaraense* and *Bacillus subtilis* transglutaminases, which have optimal temperature of 55 and 60 °C, respectively with the same substrate and incubation period (Ando et al., 1989; Ho, Leu, Hsieh, & Jiang, 2000; Suzuki et al., 2000). The activity increased gradually with temperature up to 40 °C while it declined sharply with temperature over 50 °C. Thermal stability of the purified MTG was investigated between 4 and 60 °C. It almost maintained full activity after incubation for 30 min at 20 °C and retained about 80% of the initial activity after incubation for the same period at 40 °C. When the temperature was above 50 °C, it was inactivated

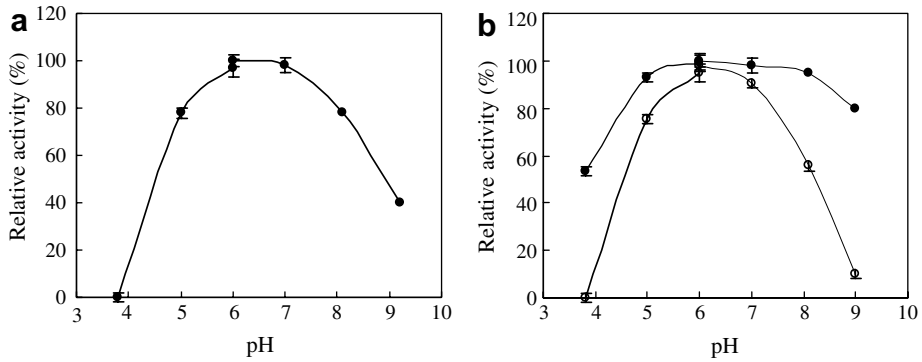

Fig. 3. Effect of pH on the activity (a) and pH stability (b: ● 10 °C; ○ 37 °C) of purified MTG from *Streptomyces hygroscopicus WSH03-13*. The pH of reaction mixture was adjusted with 50 mM acetate buffer (pH 3.7–6.0), 50 mM Tris–HCl buffer (pH 6.0–9.0). The results were the mean values of three experiments.

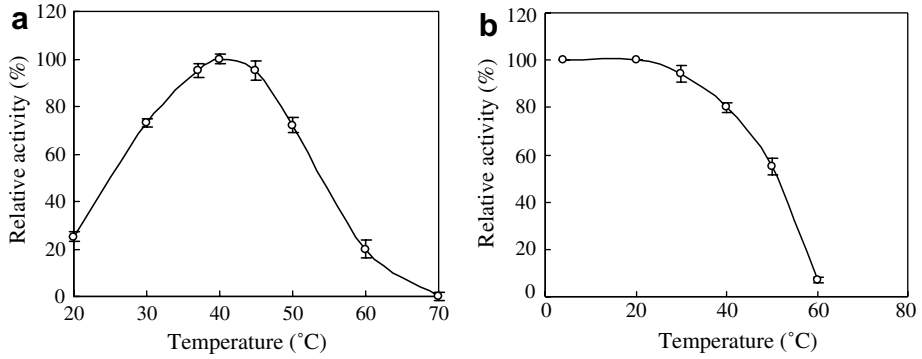

Fig. 4. Effect of temperature on the activity (a) and stability (b) of purified MTG from *Streptomyces hygroscopicus WSH03-13*. The results were the mean values of three experiments.

rapidly and preserved only 7% of the initial activity when it was exposed at 60 °C for 30 min (Fig. 4b).

3.4. Effect of different metal ions and inhibitors

The relative activity of transglutaminase was investigated in the presence of several metal ions and two inhibitors: ethylenediaminetetraacetic acid (EDTA) and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), which were added at different concentrations to enzyme preparations and left for 30 min at 37 °C (Tables 2 and 3). Purified MTG was strongly inhibited by Zn^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+} and Fe^{3+}, not inhibited by Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+} and Co^{2+}. Under the same metal concentration, incubation time period and temperature, the enzyme from *Streptoverticillium mobaraense* was strongly inhibited only by Zn^{2+}, moderately by Pb^{2+} and unaffected by Cu^{2+} (Ando et al., 1989). The purified MTG was not inhibited by $Ca^{2+}$and EDTA, suggesting it was calcium-independent, which was completely different from those of calcium-dependent TGases from animal tissues or organs (Icekson & Apelbaum, 1987; Worratao & Yongsawatdigul, 2005) and was similar to those from microbial sources (Ando et al., 1989; Barros et al., 2003; Ho et al., 2000). This property is very useful in modifying food proteins, as many food proteins—such as milk caseins, soybean globulins and myosins—are sensitive to, and easily precipitated by, Ca^{2+} (Yokoyama, Nio, & Kikuchi, 2004). It was strongly inhibited by DTNB (Table 3) and in this case, the presence of 5 mM N-CBZ-Gln-Gly reduced drastically the rate of MTG inactivation. It is well known that Cu^{2+}, Zn^{2+}, Pb^{2+}, Hg^{2+} and DTNB preferably react with thiol groups. These results in this study indicated that *Streptomyces hygroscopicus* TGase could possess a thiol group at the active site, similar to those of TGases from both tissues and microorganisms (Ando et al., 1989; Barros et al., 2003;

Table 2
Effect of metal ions on MTG from *Streptomyces hygroscopicus WSH03-13*

Metal ion (5 mM)	Relative activity (%)
None	100 ± 3.00
NaCl	105.2 ± 5.78
KCl	100.5 ± 3.98
$CaCl_2$	107.9 ± 3.12
$MgSO_4$	109.1 ± 4.20
$ZnSO_4$	4.5 ± 1.05
$BaSO_4$	110.6 ± 5.12
$MnSO_4$	111.0 ± 5.10
$CuSO_4$	9.5 ± 2.00
FeCl3	10.3 ± 2.05
$HgCl_2$	6.5 ± 1.78
$CoSO_4$	90.8 ± 3.65
$Pb(CH_3COO)_2$	19.4 ± 5.90

The results were the mean values of three experiments.

Table 3
Effect of two chemicals on MTG from *Streptomyces hygroscopicus WSH03-13*

Relative activity (%) Concentration (mM)	EDTA	DTNB	
		Substrate (5 mM)	No substrate
0	100 ± 3.00	100 ± 3.00	100 ± 3.00
0.1	114 ± 5.30	–	13.4 ± 3.50
0.5	112 ± 4.35	–	–
1.0	105 ± 4.00	40.7 ± 2.78	8.5 ± 2.90
5.0	103 ± 6.00	–	4.9 ± 2.00
10.0	–	38.5 ± 4.05	4.9 ± 3.00

The results were the mean values of three experiments.

Ho et al., 2000; Icekson & Apelbaum, 1987; Worratao & Yongsawatdigul, 2005).

3.5. Determination of kinetic parameters

The effect of substrate concentration on the velocity of the enzymatic reaction was determined at pH 6.0 and 37 °C. The purified enzyme presented a K_m of 54.69 mM and a V_{max} of 1.28 U/ml for N-CBZ-Gln-Gly in the hydroxamate procedure derived from a Lineweaver–Burk plot (results not shown here).

3.6. Effect of ethanol on MTG activity

Ethanol is often used in food industry (Donnell, 1987). Ethanol can modify the water holding capacity, compete with water during the formation of chemical bonds and alter the water activity (a_w) of the media (Carlo & Lara, 2000). Accordingly the conformational change of the enzyme protein or substrate protein can be caused in aqueous–ethanol solvent mixtures. So the effect of ethanol on MTG activity is one of the important characterizations and is useful for its application in the food industry.

MTG stability was strongly affected by ethanol concentration (Fig. 5). MTG activity was slightly elevated with a lower concentration of ethanol at 25 °C. With increasing ethanol concentration, the enzyme activity decreased, and in the presence of 50% ethanol the enzyme activity was almost lost after incubation for 30 min at 25 °C. To determine the effect of ethanol on the transglutaminase cross-linking reaction, a casein-based system was used and the SDS–gel electrophoretic patterns obtained after cross-linking and the net amount of ammonia produced were shown in Figs. 6 and 7, respectively. The presence of 10% ethanol in the mixture did not influence the cross-linking of casein by TGase from *Streptomyces hygroscopicus* evidently (Fig. 6). Under the two conditions, a majority of protein constituents of casein declined continuously with increasing the incubation time from 0 to 60 min, and correspondingly, new high MW biopolymers increased on the top of separating and stacking gel. The net amount of ammonia produced by cross-linking casein with transglutaminase in the presence of 10% ethanol was more than the control during the initial 60 min. The data indicated that a little of ethanol could improve the susceptibility of casein to cross-linking by MTG and accelerate the catalytic reaction. This may result from the structural changes of the enzyme and the substrate protein induced by ethanol.

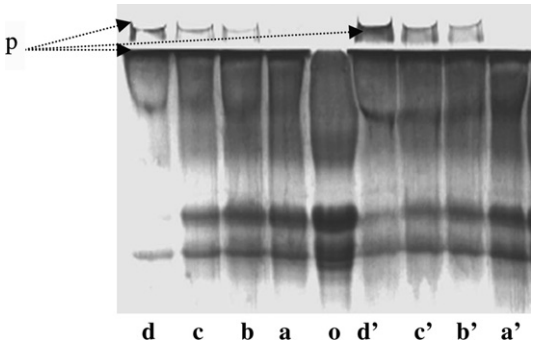

Fig. 6. SDS–PAGE analysis of casein incubated with MTG at 25 °C for various times: (o) casein solution at 0 min, (a, b, c, d) casein solution without ethanol at 25 °C for 10, 20, 30, 60 min, (a′, b′, c′, d′) casein solution with 10% ethanol at 25 °C for 10, 20, 30, 60 min, p indicates the cross-linked biopolymers.

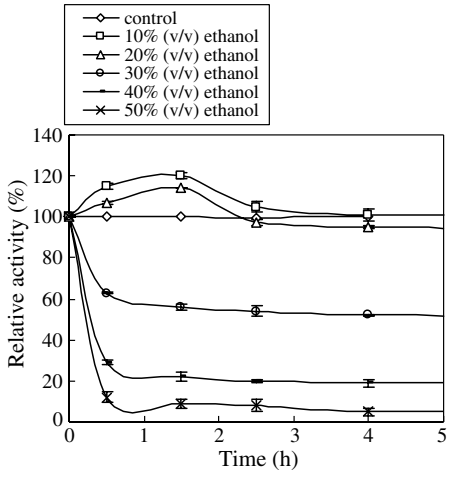

Fig. 5. Effect of different ethanol concentration on stability of MTG at 25 °C. The results were the mean values of three experiments.

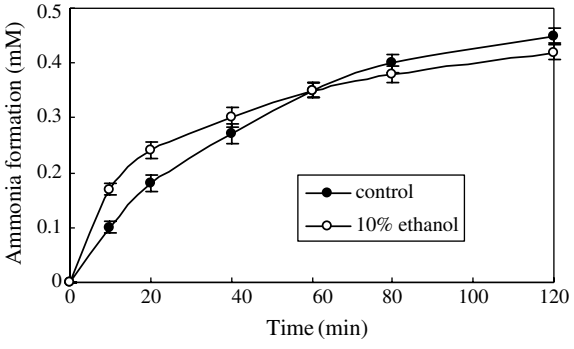

Fig. 7. Effect of ethanol on the reaction rate of MTG by determining the amount of ammonia produced. The results were the mean values of three experiments.

4. Conclusions

This paper reports on the purification and characterization of transglutaminase from a newly isolated *Streptomyces hygroscopicus*. The purified MTG was gained after two purification steps and the molecular mass was about 38,000 Da. The enzyme exhibited optimum activity in a range of pH 6.0–7.0 and at 37–45 °C for the catalytic reaction of hydroxylamine and N-CBZ-Gln-Gly. It was stable over a broad pH (5–8) and up to 50 °C. The purified MTG was independent of Ca^{2+}, and in this respect is quite different from tissue enzymes. The enzyme activity was slightly elevated in the presence of a little ethanol (at 25 °C). These properties make this enzyme a good candidate for application in the food industry. However, additional work is required to increase activity yield during extraction and purification for commercialization of MTG from this strain.

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