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Extraction of microbial transglutaminase from *Amycolatopsis* sp. fermentation broth using aqueous two-phase system

Nan Shi[*,], Hongwei Xu[*], Kaiyuan Guo[*], Chunyu Kang[*], Wei Zhang[*], Yingying Zhang[*], Liping Zhang[**,†], and Jianxin Tan[*,†]**

*College of Food Science and Technology, Engineering Technology Center for Agricultural Products Processing of Hebei Province, Agricultural University of Hebei, Baoding 071001, China
**Key Laboratory of Microbial Diversity Research and Application of Hebei Province, College of Life Sciences, Hebei University, Baoding 071002, China
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Abstract−Partitioning of microbial transglutaminase (MTG) from *Amycolatopsis* sp. in the polyethylene glycol (PEG)/salt-based ATPS was investigated for the first time. The key parameters such as the molecular weight of PEG (PEG 600-6000), the type and concentration of phase-forming salt (ammonium sulfate or phosphates), the pH of system (pH 5.0-8.5), and the concentration of neutral salt (0-6% NaCl, w/w) were determined. The partition coefficient of the enzyme was not linear with PEG molecular weight; PEG1000 gave better yield than others. The concentration of PEG1000, ammonium sulfate and NaCl, and the system pH showed effects with different extents on specific activity (SA) and yield of the enzyme. In the ATPS of 26% w/w PEG 1000 and 19% w/w ammonium sulfate in the presence of 5% w/w NaCl and at pH 6.0, MTG was partitioned into the PEG-rich phase with a maximum yield of 86.51% and SA was increased to 0.83. The results of SDS-PAGE showed the MTG produced by the test strain differed from the enzymes reported before. Thus, this study proves that ATPS can be used as a preliminary step for partial purification of MTG from *Amycolatopsis* sp. fermentation broth.

Keywords: *Amycolatopsis*, Aqueous Two-phase System (ATPS), Extraction, Microbial Transglutaminase (MTG)

INTRODUCTION

Microbial transglutaminase (MTG; protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) is an enzyme originally discovered in *Streptomyces mobaraensis* by Ando et al. [1] and Nonaka et al. [2] that has acquired strong interest because of its application in food industries since 1993 [3,4]. Several food proteins have been reported as substrates for MTG, such as milk and whey proteins, soy globulins, myofibrillar proteins, albumins, and others [5]. Moreover, transglutaminases have been applied in biotechnological areas ranging from material sciences to medicine [6].

MTG has been found in many microorganisms. Besides *Streptomyces mobaraensis* mentioned above, *Streptomyces cinnamoneum* [7], *Bacillus subtilis*, *Physarum polycephalum*, *Streptomyces ladakanum* [8], *Streptomyces platensis* [9], *Streptomyces hygroscopicus* [10], *Bacillus circulans* and other bacteria [11] have been reported as MTG-producing strains. In this study, we extracted MTG in the fermentation broth of *Amycolatopsis* sp. The test strain was isolated from soil samples of Hebei province, China. It is a novel MTG-producing strain having not been reported before (results unpublished).

Over the last decade, the studies of separation and purification of MTG have mainly focused on *Streptomyces* strains, from separation and purification methods variously. Umezawa et al. [12] purified MTG from the culture broth of *Streptomyces libani* by ion-exchange chromatography and size-exclusion chromatography with the final purification of 29.72-fold and a yield of 4.7%. They used ammonium sulfate precipitation as the first step of purification, resulting in a 1.36-fold purification and 96.6% yield of total protein. Similar methods were used by Chen et al. [13] on *Streptomyces griseoverticillatus* and the purification of 5.26-fold and yield of 61.32% were obtained. Cui et al. [14] selected ethanol precipitation as the preliminary step for MTG purification from *Streptomyces hygroscopicus*, followed by successive chromatographies on CM-cellulose and Sephadex G-75 columns with a yield and purification-fold of 21.1% and 30%, respectively. Zhang et al. [15] made MTG in *Streptomyces mobaraensis* purified at 4.22-fold and got the recovery rate of 77.5% just through gel filtration combined with ion exchange chromatography method. Ni-NTA affinity purification was usually used as the final step for analysis and often followed by size exclusion chromatography (SEC) [16]. The latest separation method for MTG, reported by the group of East China University of Science and Technology, was affinity precipitation [17,18]. Crocein orange G and L-thyroxin were used as ligands in the affinity reactions to obtain electrophoretically pure MTG at activity recovery over 95% from crude TGase. Although all these methods mentioned have different disadvantages, respectively, such as being complicated, expensive, and time-consuming, all of them are suitable for the purification of MTG from high-yield strains. In the fermentation broth of *Amycolatopsis* sp. used in this study, which is a wild

†To whom correspondence should be addressed.
E-mail: jianxintan@sina.com, zhlping@163.com

type strain, MTG is a kind of ordinary product that has lower production than other *Streptomyces* strains. In this regard, an aqueous two phase system (ATPS) was attempted as preliminary step to enrich, concentrate and extract MTG from the fermentation broth of the test strain.

ATPS can be formulated by mixing appropriate quantity of two hydrophilic polymers or a hydrophilic polymer and a salt. ATPS has been widely used as a rapid and economic method for the separation and partial purification of many intracellular and extracellular enzymes or proteins [19]. Mehrnoush et al. [20] separated pectinase from mango peel using a 14% PEG 4000-14% potassium phosphate system with 3% (w/w) NaCl addition at pH 7.0. Sarangi et al. [21] proved that an ATPS of 15% PEG 6000 and 15% sodium citrate could be used as a preliminary step in purification of alkaline proteases from chicken intestine. Zhao et al. [22] applied multiple ATPS, consisting of PEG 1000 and sodium phosphate, on the extraction of C-phycocyanin from dry *Spirulina platensis*. Nagaraja et al. [23] developed suitable ATPS of 15% (w/w) PEG 2000-22.5% (w/w) sodium sulfate at pH 5 for the crude fish protein purification from the bulk industrial effluent. To the best of our knowledge, there are no available studies about the ATPS for transglutaminase from *Amycolatopsis* actinomycetes. Moreover, parameters of one ATPS are specific for the particular system and not valid for others, and the determination of parameters completely depends on experiment [24]. So, it is necessary to determine and construct a suitable ATPS for the test strain. In this study, the feasibility of using ATPS as a preliminary step for extraction of MTG from the broth of the strain *Amycolatopsis* sp. was investigated for the first time.

MATERIALS AND METHODS

1. Strain and Fermentation Broth Preparation

A wild-type strain *Amycolatopsis* sp. 109.5, isolated from soil samples of Hebei province, China, was used for this study. The strain was cultivated in seed medium (1% glucose, 1% peptone, 1% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.07% K_2HPO_4, and 0.03% KH_2PO_4, pH 7.0) [25] for 30 h at 30 °C, 200 r/min; and then was transferred at 3% (v/v) ratio into the optimized fermentation medium, composed of 2.5% glucose, 3.5% peptone, 0.2% yeast extract, and 0.5% NaCl, at pH 7.0. After cultivation for 70 h at 30 °C, 200 r/min, the fermentation broth was centrifuged at 9,000 r/min for 3 min and clear culture broth was obtained. The cell-free supernatant was referred to as crude enzyme and was used throughout the experiments.

2. Phase Diagrams and Partitioning Studies in ATPS

Phase diagrams of PEG/salt ATPS were determined according to the cloud point method [26]. The PEG solutions of different molecular weight (from PEG 600 to PEG 6000) with mass fraction of 40% were, respectively, placed in a beaker of 50 mL, into which 40% (w/w) salt solutions (ammonium sulfate or phosphates) were added, and stirred till the turbid point appeared. The mass fractions of the PEG solution and salt solution at the turbidity point were calculated by weighing. Then deionized water was added dropwise to the cloudy solution until the solution became clear. After weighing, 40% salt solution was added dropwise to the beaker until the next cloudy point appeared. These procedures were repeated until enough points for the construction of the binodal curves were obtained. Based on the phase diagrams, the appropriate points could be selected for the preparation of PEG/salt aqueous two-phase systems.

According to the points in the phase diagrams, the powder of PEG and salts were mixed with 5 mL crude enzyme in tubes, to achieve the corresponding mass fractions. The phase equilibration was achieved by overnight placement of tubes in 4 °C. The volumes of individual phase were determined and their total protein and MTG activity were analyzed, respectively.

The equilibrium characteristics and partitioning parameters in ATPS were calculated as follows [19,21,27]:

The phase volume ratio (V_R) was defined as the ratio of volume in the enzyme-rich phase ($V_{t\,or\,b}$) to that in the other phase ($V_{b\,or\,t}$) (Eq. (1)).

$$V_R = \frac{V_{t\,or\,b}}{V_{b\,or\,t}} \tag{1}$$

The partition coefficient for MTG (K_{MTG}) was defined as the ratio of MTG activity in the enzyme-rich phase ($A_{t\,or\,b}$) to that in the other phase ($A_{b\,or\,t}$) (Eq. (2)).

$$K_{MTG} = \frac{A_{t\,or\,b}}{A_{b\,or\,t}} \tag{2}$$

The partition coefficient for total protein (K_P) was defined as the ratio of protein concentration in the enzyme-rich phase ($C_{t\,or\,b}$) to that in the other phase ($C_{b\,or\,t}$) (Eq. (3)).

$$K_P = \frac{C_{t\,or\,b}}{C_{b\,or\,t}} \tag{3}$$

The specific activity (SA) was defined as the ratio of MTG activity (A) to protein concentration (C) in the respective phases (Eq. (4)).

$$SA = \frac{A}{C} \tag{4}$$

The yield of MTG is defined as the ratio of MTG activity in the enzyme-rich phase ($A_{t\,or\,b}$) to the total enzyme activity contained in ATPS (A_o) Eq. (5).

$$\text{Yield}\,(\%) = \frac{A_{t\,or\,b}}{A_o} \times 100 \tag{5}$$

3. Parameters Determination

The partitioning of MTG in different ATPS with different molecular weight PEG and different salts was compared. Based on the optimal combination of PEG and salt, the suitable PEG concentration was identified by conducting the experiments at fixed $(NH_4)_2SO_4$ concentration and then varying $(NH_4)_2SO_4$ concentration at fixed PEG concentration. Then the effects of pH and NaCl were studied for the selected system. The system pH of ATPS was adjusted by adding acetic acid or Na_2CO_3 (2.0 mol/L). As a neutral salt, NaCl was added into the system on the concentration varied from 0 to 6% (w/w). All tests were repeated in triplicate.

4. MTG Activity

The enzyme activity was analyzed according to the hydroxam-

ate-based colorimetic method [28,29]. The substrate reagent was composed of 30.0 mmol/L N-CBZ-Gln-Gly, 0.1 mol/L hydroxylamine hydrochloride and 10.0 mmol/L glutathione in 0.2 mol/L Tris-acetic acid buffer, pH 6.0. Substrate reagent was mixed with test samples at equal volume of 0.3 mL, and incubated at 30 °C for 10 min; and then, 0.3 mL of ferric chloride-TCA (5% $FeCl_3$: 12% CCl_3COOH : 3 mol/L HCl=1 : 1 : 1) was added into the mixture immediately to stop the reaction. The mixture was centrifuged at 12,000 r/min for 1 min. The absorbance of the supernatant was measured at 525 nm. One unit of MTG activity was defined as the amount of enzyme producing 1 μmol of hydroxamic acid per minute at 30 °C.

5. Protein Assay

The total protein was determined, as described by Bradford [30], using bovine serum albumin as a standard and Coomassie bright blue G-250 as detection reagent. The absorbance of the reaction mixer was measured at 595 nm.

6. Native-PAGE and SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli buffer system [31] with a 12% separating gel. The gel was stained with 0.1% Coomassie Brilliant Blue R-250. Before being applied on SDS-PAGE, extraction samples obtained from ATPS were precipitated by ammonium sulfate to avoid the influence of PEG. Different amounts (4-24%, w/v) of ammonium sulfate were added to 2 mL top phase obtained after the extraction by optimized ATPS to precipitate MTG. Precipitations were obtained by centrifugation at 9,000 r/min for 3 min, dissolved in 200 μL 0.2 mol/L Tris-acetic acid buffer (pH 6.0), purified by TCA Protein Precipitation Kit (KeyGen Bio TECH, China), and then applied to SDS-PAGE, with commercial enzyme Biobond TG (KINRY, China) (0.05 g/mL) as control. Two hundred microliters crude enzyme was purified by TCA Protein Precipitation Kit and also applied to SDS-PAGE, as another control.

The procedure of native-PAGE is familiar to SDS-PAGE, without SDS in the preparation of gel and electrophoresis buffer, and accomplished in pH 8.8 buffer (0.025 mol/L Tris, 0.2 mol/L glycine). Extraction samples were ultrafiltrated (VIVAFLOW 200, 10000 mwco HY) to remove PEG before applied on native-PAGE. Fifty milliliter of top phase was concentrated to 10 mL by ultrafiltration and 20 μL of which was loaded on native-PAGE, with Biobond TG as control. After electrophoresis, the gel of native-PAGE was divided into two parts and stained with 0.1% Coomassie brilliant blue R-250 and the substrate reagent mentioned above [28], respectively, to confirm the band of MTG in samples. The specific bands of MTG were recovered by gel cutting, purified by TCA Protein Precipitation Kit and then applied to SDS-PAGE to estimate the molecular weight.

RESULTS AND DISCUSSION

1. Phase Diagrams for PEG/Salt Systems

The isolation mechanism of ATPS is based on the differential partitioning of the target biomolecule and other components in different phases. Single phase and two-phase systems were separated by the binodal curves in phase diagram. Two phases were formed only above the binodal curve. A chosen point too far or too near the binodal curve might result in a failed operation of ATPS [32]. To determine the appropriate ATPS for the extraction of MTG from fermentation broth, different combines of PEG and salts were experimented. Binodal curves were obtained for the PEG/$(NH_4)_2SO_4$ ATPS formed by PEG600-6000 and $(NH_4)_2SO_4$ (Fig. 1); and for the PEG/phosphate ATPS formed by PEG2000, PEG6000 and $NaH_2PO_4{\cdot}2H_2O$-$K_2HPO_4{\cdot}3H_2O$ (Fig. 2). In all these systems, PEG was rich in the top phase while the salts in the bottom. With the increase of PEG molecular weight, the binodal curves were closer to the Y-axis. Table 1 shows the compositions of PEG/salts ATPS used in this study.

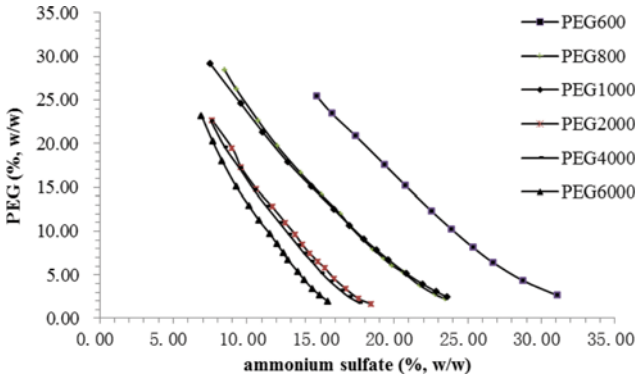

Fig. 1. Phase diagrams for PEG/ammonium sulfate ATPS at room temperature.

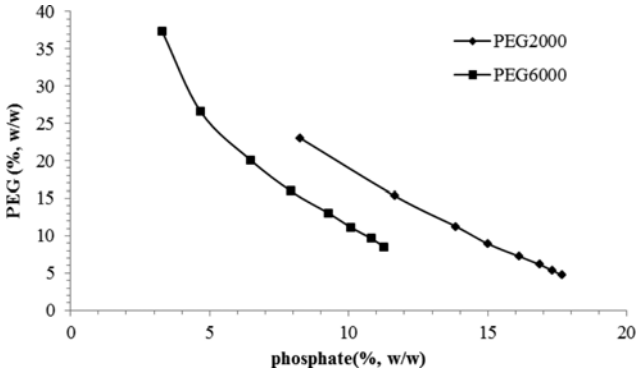

Fig. 2. Phase diagrams for PEG/phosphates ATPS at room temperature. The phosphates solution consists of 1.1 mol/L $NaH_2PO_4{\cdot}2H_2O$ and 2.3 mol/L $K_2HPO_4{\cdot}3H_2O$.

2. Partition of MTG in PEG/Salt ATPS

In Table 1, eight PEG/salt ATPS, thirty-five proportions were compared and the partitioning parameters of MTG in these systems are shown. In the systems of PEG/phosphate, the MTG were mostly partitioned into the salt phase; however, the yields of MTG were extremely low or even none, except for the last system in Table 1, in which the MTG went into the PEG phase. Compared with ammonium sulfate, phosphates showed the least activity in both top and bottom phase, which indicated that the enzyme activity was lost largely in this system. Although phosphates were used as widely as ammonium sulfate in ATPS for the extraction of many enzymes, such as β-galactosidase [33], elastase [34], alkaline protease [21,35], glucoamylase [19], and alkaline xylanase [36], the results

Table 1. Partitions of MTG in eight PEG/salt ATPS

Systems	PEG (%, w/w)	Salt (%, w/w)	V_R	K_{MTG}	K_P	SA	Yield (%)
PEG600/$(NH_4)_2SO_4$ ATPS	29	20	1.68*	N/A*	N/A*	0.38*	75.42*
	22	22	0.88*	N/A*	3.57*	0.46*	60.08*
	17	25	0.47*	N/A*	3.08*	0.36*	51.61*
	11	29	0.18*	N/A*	1.23*	0.14*	26.44*
PEG800/$(NH_4)_2SO_4$ ATPS	30	12	0.34	17.37	N/A	0.24	27.96
	25	14	0.50	20.24	N/A	0.18	29.15
	18	18	0.97	1.43	1.23	0.28	34.83
	13	21	2.00	3.40	0.56	0.38	44.91
	9	23	3.29	3.52	0.96	0.44	44.15
PEG1000/$(NH_4)_2SO_4$ ATPS	22	17	1.52*	N/A*	16.28*	0.31	89.81*
	21	18	1.20*	78.50*	8.40*	0.41	76.77*
	17	21	0.78*	N/A*	8.26*	0.39	71.23*
	14	22	0.53*	16.22*	7.10*	0.43	60.80*
	8	22	0.15*	2.97*	1.33*	0.46	44.82*
PEG2000/$(NH_4)_2SO_4$ ATPS	23	11	2.00	19.26	5.04	0.32	42.38
	17	13	1.30	46.91	5.18	0.36	42.05
	15	13	0.90	N/A	6.40	0.38	52.00
	10	15	0.44	N/A	6.79	0.35	53.22
	5	18	0.20	22.75	5.15	0.41	59.33
PEG4000/$(NH_4)_2SO_4$ ATPS	23	9	5.00	6.16	1.63	0.75	37.65
	17	11	1.75	24.81	3.85	0.42	52.57
	13	13	0.97	N/A	8.41	0.40	57.29
	10	15	0.47	N/A	6.35	0.39	64.06
	5	18	0.23	10.68	4.92	0.41	62.67
PEG6000/$(NH_4)_2SO_4$ ATPS	24	8	5.00	39.45	3.04	0.43	35.37
	19	10	1.75	N/A	26.77	0.35	49.71
	13	12	0.81	N/A	52.24	0.30	53.95
	8	14	0.33	N/A	16.23	0.30	58.52
	5	16	0.24	N/A	17.08	0.31	56.32
PEG2000/phosphate ATPS	20	11	0.11	N/A	0.63	0.05	2.18
	16	13	0.54	0	4.84	0	0
	9	17	2.41	0	5.11	0	0
PEG6000/phosphate ATPS	35	7	0.14	N/A	N/A	0.04	3.45
	25	8	0.17	N/A	N/A	0.07	5.64
	15	11	1.86*	N/A*	0.44*	0.52*	13.27*

N/A, the parameters of the enzyme-poor phase could not be detected
*The enzyme was rich in the top phase; while the others in the bottom

of this study revealed that the phosphates were not suitable for extracting MTG from fermentation broth of the test strain.

As another widely used salt in ATPS, ammonium sulfate showed appropriate phase-forming capabilities with PEG and variety of partition capabilities on MTG (Table 1). Among the systems of PEG/$(NH_4)_2SO_4$ ATPS, the enzyme enriched into the top phase (PEG phase) in the PEG 1000 and PEG 600 ATPS, while went into the bottom phase (salt phase) in the ATPS of PEG800, PEG2000, PEG4000 and PEG6000. The partition of total protein was familiar to the enzyme. Molecular weight and concentration of PEG influences the partition behavior of MTG, since the hydrophobic interaction and effective excluded volume of the ATPS greatly depends on it.

In these systems, the yields of MTG varied from 26.44% to 89.81%, the SA ranged from 0.14 to 0.75. The highest yield (89.81%) was obtained in the ATPS of 22% (w/w) PEG 1000-17% (w/w) ammonium sulfate, at the SA 0.31; while the highest SA was 0.75, with the yield of 37.65% obtained in the 23% (w/w) PEG 4000-9% (w/w) ammonium sulfate. Although the latter system could give better purity, the loss of enzyme is unacceptable. Thus, the following procedures were carried out on the basis of PEG1000/$(NH_4)_2SO_4$ ATPS to obtain a more optimized system.

3. Effect of PEG Concentration

The $(NH_4)_2SO_4$ concentration was fixed as 17% (w/w), the PEG1000 concentrations ranged from 18% (w/w) to 30% (w/w). The effects of different PEG1000 concentrations on the MTG par-

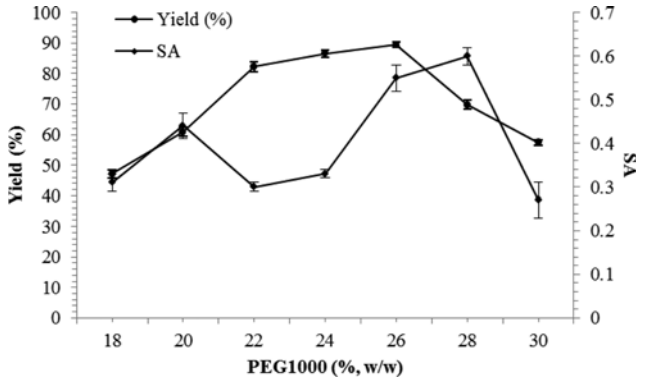

Fig. 3. Effect of PEG concentration on both MTG specific activity and yield (%) in presence of 17% (w/w) ammonium sulfate.

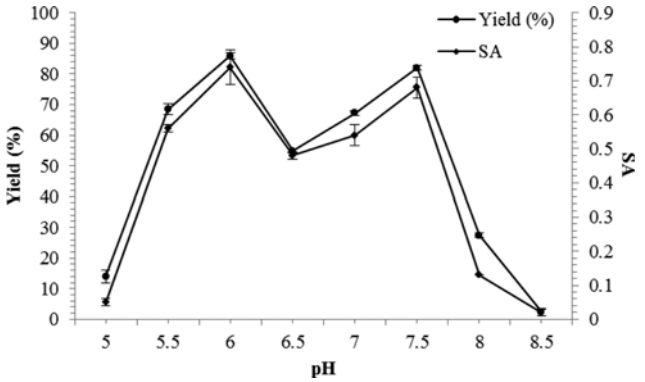

Fig. 5. Effect of pH on MTG specific activity and yield (%) in presence of 26% (w/w) PEG and 19% (w/w) ammonium sulfate.

tition are shown in Fig. 3. With the increase of PEG1000 concentration, the yields of MTG first increased and then decreased, the SA fluctuated up and down. Among these results, 26% PEG gave the highest enzyme yield value (89.50%) with the SA was 0.55; 28% PEG gave the highest SA of 0.60 at the yield of 69.82%. The results indicated more loss of enzyme in 28% PEG system. Considering the two parameters together, the highest yield was considered preferentially, and 26% (w/w) PEG 1000-17% (w/w) ammonium sulfate was identified as the system to be further studied.

4. Effect of Salt Concentration

The PEG1000 concentration was fixed as 26% (w/w), the $(NH_4)_2SO_4$ concentrations ranged from 15% (w/w) to 29% (w/w) (Fig. 4). The enzyme activity and total protein content were found to be very little in the salt phase, even not to be detected at all. The enzyme yield in the top phase was highest (88.16%) for 23% ammonium sulfate system, while the highest SA was obtained in 19% ammonium sulfate system (0.69), which gave the enzyme yield of 84.11%. It revealed that fewer non-target proteins entered into the top phase in the system of 19% ammonium sulfate. Higher purity is the goal of this study and the loss of a small amount of enzyme is unavoidable and acceptable. At this stage, the SA is more important than the yield. Therefore, the system of 26% (w/w) PEG 1000-19% (w/w) ammonium sulfate was selected to be further optimized.

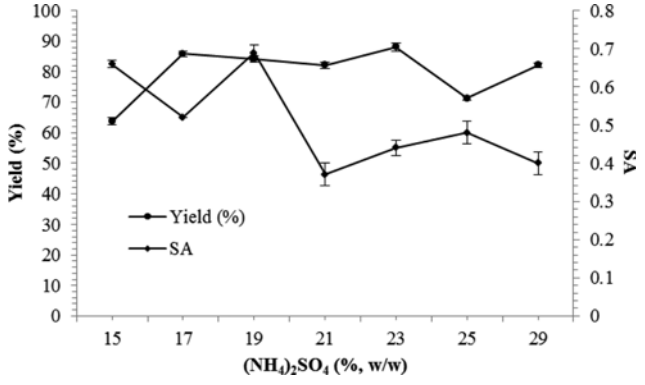

Fig. 4. Effect of ammonium sulfate concentration on MTG specific activity and yield (%) in presence of 26% (w/w) PEG.

5. Effect of pH

In this study, the ATPS was formed by the direct addition of PEG and salt to the crude enzyme solution. However, the addition of the phase-forming compounds had influence on the pH of the system, i.e., the pH of ATPS was not equal to that of crude enzyme solution. In general, the pH value directly affects the ionization and charge properties of biomolecules in the fermentation broth. Especially for proteins, if the pH of the fermentation broth is equal to the isoelectric point of the protein, the protein will precipitate; and if the pH is altered greatly, the protein may denature and solidify. So, pH adjusting is one of the common methods in pretreatment of fermentation broth. Thus, the ATPS was subjected to pH changing from 5.0 to 8.5, to determine how this factor governs the partition of proteins in the system. The variation of partition coefficients with respect to the pH of the system is shown in Fig. 5. The change in pH significantly affected the partition coefficient of MTG in ATPS. Very low enzyme activity was detected in systems at pH 5.0 or pH 8.5, while protein existed in the top and bottom phase. Varying degrees of loss of enzyme activity were observed in other systems except for system of pH 6.0 and pH 7.5. The low stability of MTG at different pH could be one of possible reasons for this reduction. Another possible reason for the reduction could be that one of these pH values was adjacent to the isoelectric point of MTG, which should be determined by further experiments.

The system of pH 7.5 was formed by the addition of PEG and salt to the crude enzyme solution without pH adjusting. While, the system of pH 6.0 had the better partition coefficient and yield, which reached 0.74 and 85.95%, respectively. The decrease in pH made the enzyme more positively charged and led to stronger interaction between it and polymer, which caused more migration of enzymes to the PEG-rich phase. Similar results were observed on glucoamylase [19].

6. Effect of NaCl

Neutral salt is one of important factors acting on the partitioning behavior of protein by changing the electrostatic potential difference between the phases or by increasing the hydrophobic interactions [37]. To examine the effect of neutral salt on the partition of MTG, NaCl was added into the selected system (26% w/w PEG1000-19% w/w ammonium sulfate system, pH 6.0) on the

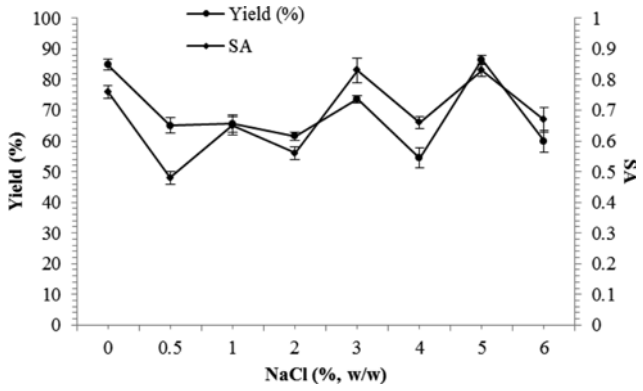

Fig. 6. Effect of NaCl concentration on MTG specific activity and yield (%) in presence of 26% (w/w) PEG and 19% (w/w) ammonium sulfate at pH 6.0.

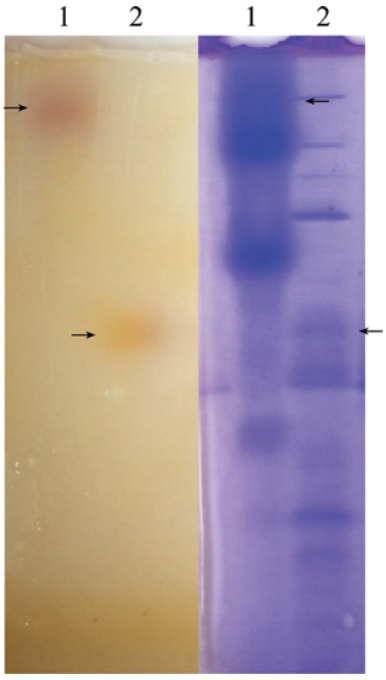

Fig. 7. Native-PAGE of ATPS extraction sample (Lane 2) and Biobond TG (0.01 g/mL) (Lane 1). Electrophoresis was carried on one 10% gel and samples were loaded twice in the same order. Then the gel was cut into two parts from the middle and stained separately. The left part was stained with the substrate reagent and the right one was stained by 0.1% coomassie brilliant blue R-250. The two arrows at the top of figure are pointing to band of transglutaminase in Biobond TG; two arrows at the middle of figure are pointing to band of MTG in fermentation broth.

concentration varied from 0 to 6% w/w. As shown in Fig. 6, the increase in NaCl concentration initially decreased partitioning of enzyme in top phase and then increased at 3% and 5% NaCl concentration, at which highest SA (0.83) was obtained, while the highest yield (86.51%) appeared at 5% NaCl concentration. So, 5% NaCl concentration was determined as the suitable neutral salt concentration.

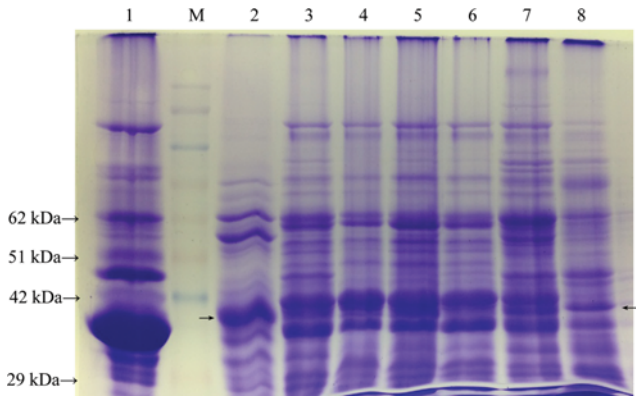

Fig. 8. 12% SDS-PAGE of Biobond TG, gel cutting sample and precipitation samples from top phase. Lane 1, Biobond TG; Lane M, Marker (Multicolor Protein Marker, CWBIO, China); Lane 2, gel cutting sample (arrow pointing to is the band of MTG); Lane 3, precipitation sample obtained by 19% $(NH_4)_2SO_4$ addition; Lane 4, precipitation sample obtained by 4% $(NH_4)_2SO_4$ addition; Lane 5, precipitation sample obtained by 9% $(NH_4)_2SO_4$ addition; Lane 6, precipitation sample obtained by 14% $(NH_4)_2SO_4$ addition; Lane 7, precipitation sample obtained by 24% $(NH_4)_2SO_4$ addition; Lane 8, crude enzyme (arrow pointing to is the band of MTG).

7. PAGE Analysis

As shown in Fig. 7, reactive dyeing band of MTG differed from Biobond TG. Proteins separated in native-PAGE are based on their different electrophoretic mobility and the molecular sieve effect in gel. This result illustrates that MTG was exactly extracted through ATPS extraction, and the transglutaminase produced by *Amycolatopsis* sp. differed from Biobond TG, which comes from *Streptomyces mobaraensis*.

The molecular weight of Biobond TG is about 38 kDa (Lane 1 in Fig. 8), so it could be inferred that the main band in Lane 2, whose molecular weight is about 40 kDa, should be considered as MTG, i.e., the MW of MTG is about 40 kDa. Compared the bands in Lane 3 to 7, different $(NH_4)_2SO_4$ concentration gave similar results, but not the same. In Lane 5, MTG band was the most significant. It indicates that 9% (w/v) $(NH_4)_2SO_4$ addition could precipitate more enzyme from the top phase after ATPS extraction than other $(NH_4)_2SO_4$ concentrations. These results would be helpful for the further purification of MTG.

CONCLUSION

The extraction of MTG from *Amycolatopsis* sp. using ATPS is reported for the first time. The influence of different parameters has been revealed. The PEG 1000/ammonium sulfate system was found to be efficient for the extraction of MTG, which partitioned to the PEG-rich top phase. The optimized system was determined as 26% w/w PEG 1000 and 19% w/w ammonium sulfate in the presence of 5% w/w NaCl and at pH 6.0. The said conditions provided a maximum yield of 86.51% and SA of 0.83. Compared with other MTG producing strains, *Amycolatopsis* sp. is wild type and has relatively lower production. For this kind of weak solution of enzyme, ATPS extraction is a relatively mild method, with less loss

of enzyme activity and requires fewer reagents and instruments. Overall, the results demonstrated the feasibility of using ATPS as a preliminary step for the partial purification of MTG. There is a need for further research to elevate the ratio of MTG in the fermentation broth by strain breeding.

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