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Research Article

Impact of some additives on the stability of microbial transglutaminase from *Providencia* sp. C1112

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Abstract

Microbial transglutaminase (MTGase) is a transferase that has been applied to many protein containing food products to improve their texture. The stability is an important feasibility of enzyme to achieve the economic trading. *Providencia* sp. C1112 was cultivated to produce MTGase in FPH medium at 37˚C, 150 rpm for 18 h. The purified MTGase of *Providencia* sp. C1112 was formulated with different concentrations of additives including glycerol, maltodextrin, sorbitol and xylitol in liquid form and kept at 37˚C for 12 h. Glycerol and maltodextrin at 20% (w/v) could stabilize the MTGase activity better than sorbitol and xylitol. In the presence of 0.2% glutathione could increase the MTGase stability during storage at 37˚C for 12 h. Glycerol with glutathione showed the most effective on stabilizing of MTGase during storage at 4˚C for 3 months.

Keywords: glutathione, microbial transglutaminase, maltodextrin, *Providencia* sp., stabilizer, food additive, Thailand.

Introduction

Enzymes are biocatalysts that have been importantly used in many fields. Enzymes may be easily denatured by the change of the environmental conditions [1]. Therefore, the resistance of enzyme toward extreme deterioration is one of the most important criteria for commercialization and industrial application [2, 3].

The stabilization of enzyme could be retained by addition of some protective agents such as salts, polyethylene glycol, glycerol, dextran, bovine serum albumin, polyethylenimine, osmolytes (e.g. betaine), organic solvents (e.g. isooctane, cyclohexane, chloroform, benzene) and sugars (e.g. mannitol, sorbitol, xylitol, inositol, erythritol) [4, 5, 6, 7, 8]. The selection of the appropriate

additive depends on the nature of the enzyme [9]. Polyols are often used to increase the thermostability of enzymes, especially during drying [10]. It is known that proteins may alter their three-dimensional structure during drying process. Such changes in structure may or may not be reversible upon rehydration [7]. The polyhydric alcohol protect enzyme activity by altering the hydrate shell of the enzyme, which could be explained by two major mechanisms on stabilizing effect: (1) polyols replacing water interaction with protein to form new hydrogen bonds and (2) polyols providing a glassy matrix to prevent protein from changing in shape in the solid state [11]. The improvement of thermal stability by adding polyols was probably due to the reinforcement of the hydrophobic interactions among nonpolar amino acids inside the enzyme molecules, and thus increased their resistance to inactivation [12]. It had been reported that polyhydroxy compounds modify the structure of water and/or strengthen hydrophobic interactions among nonpolar amino acid inside the protein molecules [13].

Microbial transglutaminase (MTGase, EC 2.3.2.13) is known as the amine transferase or protein glutamine γ-glutamyl transferase [14]. MTGase catalyzes the formation of isopeptide by the exchange of an acyl group from γ-carboxyamide (-C(O)NH_2) group of proteins containing glutamine residue, an acyl donor, to a variety of acyl acceptor which results in cross-linking of protein [14]. MTGase has captured interest due to its attractive potential application in food industry [14], immobilization of enzymes [15] and textile industry [16].

Screening and production of MTGase are important studies for investigation of the novel MTGase. MTGase was reported in cultures of *Streptoverticillium mobaraense* and *Streptomyces* sp. [17]. MTGase could be highly produced by *Sv. ladakanum* [18], *Sv. cinnamoneum* [19], and *S. lividans* [20]. Ajinomoto Co., Ltd., produces the MTGase not only from *Streptoverticillium* species but also from other genus such as *Bacillus* [21, 22]. *Providencia* sp. C1112 was classified almost to *P. stuartii,* a bacteria in the biosafety level 1 [23]. *Providencia* sp. C1112 was isolated from wastewater pond of the seafood factory in Songkhla, Thailand. *Providencia* sp. C1112 possessed as a dominant MTGase producer.

However, preservation of the enzymes during storage is also important. Therefore, this study aimed to investigate the effect of protective agents on preservation of the MTGase activity produced by *Providencia* sp. C1112 during storage.

Materials and Methods

Production of MTGase

Providencia sp. C1112 (NBRC106720) was isolated and collected in our laboratory. The strain from agar slant culture was activated in nutrient broth at 37˚C, 150 rpm for 12 h before streaking on nutrient agar and incubated at 37˚C for 12 h. A loopful of pure colony was inoculated in 5 ml of FPH medium (3.0% starch, 2.0% fish protein hydrolysate, 0.2% yeast extract, 0.2% Mg_2SO_4, 0.2% KH_2PO_4 and 0.2% K_2HPO_4, pH 6.5) and incubated at 37˚C, 150 rpm for 12 h. Five ml of starter culture was inoculated to 100 ml of FPH medium in 250 ml conical flask and incubated at 37˚C, 150 rpm for 24 h. The cell pellet was removed by centrifugation at 7,500×*g* for 10 min at 4˚C.

Preparation of partially purified MTGase

The supernatant was precipitated using 50-80% $(NH_4)_2SO_4$ by stepwise-precipitation, dialyzed in 200 mM citrate-phosphate buffer (pH 6.5) and eluted through SP-Sepharose using 0.2-0.4 M NaCl in 200

mM citrate-phosphate buffer (pH 6.5). The obtained MTGase was dialyzed, freeze dried and diluted in 200 mM citrate-phosphate buffer (pH 6.5) to 2 U/ml.

Effect of some additives on MTGase stability

MTGase solution was incorporated to glycerol, maltodextrin, sorbitol and xylitol at various concentrations in the presence and absence of 0.2% glutathione in 200 mM citrate-phosphate buffer (pH 6.5) and kept at 37˚C for 12 h. The samples were taken to monitor the residual MTGase activity using hydroxamate assay [24].

Effect of glycerol and maltodextrin on long term stability of MTGase

MTGase stabilized in (i) the liquid form of glycerol (20% w/v) and (ii) the freeze-dried powder form of maltodextrin (20% w/v) with and without glutathione (0.2% w/v) was prepared. The solution of MTGase in maltodextrin solution was freeze-dried under the vacuum using a freeze dryer (Millrock Technology, Germany). The prepared enzyme was kept at 4˚C and the residual MTGase activity was monitored during storage for 90 days. Before use or test, distilled water was added into the freeze-dried sample to the same volume as in the liquid form.

Determination of MTGase activity

The sample (100 µl) was mixed with 200 µl of 200 mM citrate buffer (pH 6.0), 25 µl of 125 mM hydroxylamine, 25 µl of 12.5 mM glutathione and 75 µl of 37.5 mM CBZ-Gln-Gly. The reaction mixture was incubated at 37˚C for 1 h. Thereafter, an equal volume of 5% ferric chloride in 15% TCA solution was added to the reaction mixture and centrifuged at 7,500 ×*g* for 10 min. The absorbance of the supernatant was measured at 525 nm. One unit of MTGase activity was defined as the amount of enzyme needed to produce 1 µmol of hydroxamic acid per min [24].

Statistical analysis

Each experiment was set in duplicate and the sample was collected in triplicate for analysis. The data was analyzed using the analysis of variance (ANOVA) and mean comparison was run by Duncan's multiple range test using the SPSS package (SPSS 10.0 for windows, SPSS Inc, Chicago, IL).

Results and Discussion

Effect of some additives on the MTGase stability

Glycerol, maltodextrin, sorbitol, and xylitol were often used to preserve proteins and enzymes. Table 1 shows the effectiveness of these additives on stabilization of MTGase solution from *Providencia* sp. C1112 during storage at 37˚C for 12 h. Glycerol and maltodextrin at 20% could stabilize MTGase activity higher than sorbitol and xylitol at all concentrations. Glycerol and maltodextrin have long been regarded as proteins stabilizer or additives which have ability to affect the equilibrium between the nature and the unfolded conformational state of the enzymes.

Moreover, glycerol and maltodextrin can evidently increase the viscosity of the enzyme solution, which may cause reduction of chemical or biological reaction rate which could result in inactivation of the enzymes [25, 26]. Therefore, the resulting activity was preserved by the addition of glycerol and maltodextrin, which is essential to maintain the natural structure of the protein molecule. When 0.2% (w/v) glutathione was added with other additives at 2.5-20% to stabilize the liquid form of MTGase, the results showed that 0.2% (w/v) glutathione could improve the effectiveness of sorbitol, xylitol and control as shown in Table 2. However, addition of glutathione to maltodextrin and glycerol could not help to improve the stability of MTGase.

Table 1. Effect of some additives on the residual relative MTGase activity after incubating at 37˚C, 12 h.

Additive	Relative activity (%)			
	2.5% (w/v)	5% (w/v)	10% (w/v)	20% (w/v)
Glycerol	96.26 ± 1.67^{abB}	92.83 ± 2.97^{aB}	99.66 ± 1.74^{bB}	103.64 ± 3.83^{cC}
Maltodextrin	104.67 ± 2.21^{aC}	103.08 ± 3.01^{aC}	104.31 ± 5.69^{abB}	109.30 ± 1.23^{bD}
Sorbitol	72.90 ± 1.45^{aA}	77.26 ± 5.31^{abA}	90.03 ± 2.69^{cA}	77.26 ± 2.31^{bA}
Xylitol	75.70 ± 2.21^{aA}	77.76 ± 2.58^{aA}	90.34 ± 3.38^{bA}	95.64 ± 2.02^{bB}
Control*	40.02 ± 1.20			

The different superscripts in the same row (a-c) and column (A-D) denoted the significant different ($P<0.05$)
* Citrate-phosphate buffer

Table 2. Effect of glutathione on the residual relative MTGase activity after incubating at 37˚C, 12 h.

Additive	Relative activity (%)			
	2.5% (w/v)	5% (w/v)	10% (w/v)	20% (w/v)
Glycerol + GSH	94.66 ± 2.53^{aB}	97.26 ± 2.86^{aC}	98.84 ± 1.28^{bB}	109.62 ± 1.31^{bD}
Maltodextrin + GSH	101.38 ± 2.54^{aC}	99.48 ± 1.91^{aC}	105.81 ± 1.24^{bC}	102.01 ± 1.25^{aC}
Sorbitol + GSH	83.00 ± 2.53^{aA}	86.17 ± 0.65^{aB}	93.95 ± 3.48^{bA}	93.77 ± 0.65^{bA}
Xylitol + GSH	80.22 ± 0.40^{aA}	80.47 ± 1.27^{aA}	93.32 ± 1.57^{bA}	97.26 ± 1.59^{cB}
GSH*	54.81 ± 0.81			
Control**	40.87 ± 0.95			

The different superscripts in the same row (a-c) and column (A-D) denoted the significant different ($P<0.05$)
* GSH is 0.2% glutathione
** Citrate-phosphate buffer

GSH alone could not play a great role in protecting MTGase, the combination of maltodextrin or glycerol with GSH resulted in significantly higher activity preservation [27]. The anti-oxidation of GSH played the role of protecting MTGase, indicating that besides conformational changes of proteins, probably there were some chemical groups such as cysteine residue located at the active center of MTGase [28], which tend to oxidize during thermo-denaturation. Cui *et al.* [27] reported that the combined addition of maltodextrin and GSH is an effective way to protect MTGase from denaturating.

Stability of MTGase during long term storage at 4˚C

Based on the residual MTGase activity, glycerol and maltodextrin were used for further long term preservation of MTGase (in the powder form) at 4˚C. During the storage of MTGase at 4˚C, the addition of glutathione could synergist to 20% glycerol and maltodextrin for retaining of MTGase activity. The addition of 0.2% glutathione with glycerol increased the MTGase stability during storage (Figure 1). However, preservation of MTGase in glycerol although without glutathione was better than maltodextrin with glutathione. Glycerol has been used as a cryoprotectant for preservation of many biological substances. Due to glycerol had small structure therefore it easier to access the protein molecule than other polyhydric alcohols [29]. Moreover, the addition of 10-100% glycerol led to increase in thermal stability by strengthening the hydrophobic interactions among non-polar amino acid residues leading to protein rigidification and resistance to thermo-deactivation [30].

In addition, glycerol could increase the protease tolerance of protein by lowering the free water for catalysis, retarding of biological reaction [31]. Whereas, some particular efficiency of maltodextrin

was lower than glycerol. Moreover, the MTGase in 20% (w/v) maltodextrin was able to lose in the activity during freeze-drying process [32]. Therefore, glycerol possessed better efficiency than maltodextrin on preservation of MTGase during storage at 4˚C.

However, the role of glutathione on stabilizing of MTGase activity was loosening at 3 months of storage. The result suggested that glutathione could effectively reserve the relative MTGase activity in a short period and in the solution or aqueous status. The addition of 0.2% of glutathione did not significantly develop the ability of maltodextrin to retain the MTGase activity throughout 3 months of storage. The drying of maltodextrin could remove water to form the solid powder. Glutathione needed water for dissociate the molecule into the active form [33, 34]. Therefore, the addition of glutathione into the maltodextrin and then freeze-drying could not effectively stabilize MTGase activity.

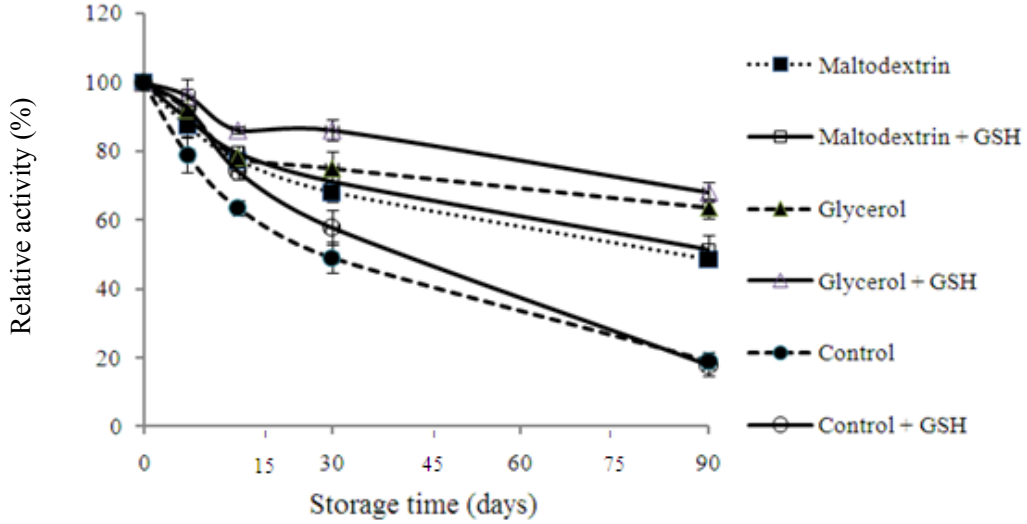

Figure 1. The protective effect of maltodextrin and glycerol with glutathione on retained MTGase activity during storage at 4˚C.

However, the storage of MTGase in the powder form could prevent the action of MTGase auto-crosslinking themselves during storage. Moreover, maltodextrin could entrap MTGase inside the particles which could resist to the temperature deterioration [27]. Commercially, MTGase was stabilized in the powder of maltodextrin and kept at 4˚C.

Conclusion

The result suggested that MTGase could be preserved in 20% (w/v) glycerol with 0.2% (w/v) glutathione which could retain MTGase nearly 80% of the initial activity at 4˚C for 3 months. However, the use of glutathione would no longer effective to stabilize MTGase after 3 months. Although maltodextrin could retain MTGase activity lower than the ability of glycerol, however, maltodextrin in the powder form might retard the auto-crosslinking of MTGase during storage which could affect to the lowering of MTGase activity. This point would have to more study in the more details.

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