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SHORT COMMUNICATION

Immobilization of α-amylase from *Bacillus licheniformis* on developed support using microbial transglutaminase

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Abstract−α-amylase from *Bacillus licheniformis* was successfully immobilized on developed support, which was prepared by coating a chitosan-casein film on silica, at 20 ºC, pH 6.0 for 5 hr with microbial transglutaminase (MTG) as the cross-linking factor. The optimal support was obtained when 1% chitosan and 1% casein were used in the coating mixture. The optimal condition for immobilization catalyzed by MTG was confined to be at MTG concentration of 15 U/mL, pH 6.0, reacting for 6 hr at 20 ºC. The highest specific activity of immobilized α-amylase was achieved as 236 U/g. After immobilization, the obtained enzyme showed broader pH profile and maintained more than 70% of the original activity after 20 reuses.

Key words: α-Amylase, *Bacillus licheniformis*, Microbial Transglutaminase (MTG), Chitosan, Casein

INTRODUCTION

Enzyme immobilization on insoluble supports has been widely studied to achieve various advantages, including enzyme reusability, enhanced stability or rapid separation of the enzyme from the reaction medium. Numerous techniques have been proposed to immobilize enzymes, including adsorption, entrapment and covalent bonding strategies [1]. Preparations obtained by covalent bond formation are generally more robust, exhibiting no enzyme leakage, and have been shown to induce higher resistance to temperature, denaturants, and organic solvents in several cases [2,3]. Nonetheless, some chemicals used as cross-linking agents may inactivate the enzyme, while toxic coupling reagents are generally not recommended for bonding enzymes applied during food processing [4].

Thus, there is growing interest in methods to cross-link enzymes under gentle conditions to preserve the activity of the target enzyme and to avoid the use of toxic coupling reagents to cross-link the enzyme [5,6]. With these considerations, microbial transglutaminase (MTG), which catalyzes acyl transfer reaction between primary amine and γ-carboxyamide group of glutamine (Gln) residues in peptides and proteins may be suitable for the cross-linking process. Compared to chemical methods, the enzymatic strategy with MTG is more appropriate for maintaining the activity of the target enzyme during the immobilization process. Previously, MTG had been broadly studied in fields of biocenology processes, while little was reported on specific enzyme immobilization for practical usage. According to our knowledge, it has not been reported to immobilize α-amylase by MTG [7-9].

In the present study, α-amylase from *Bacillus licheniformis* ATCC 14580 was immobilized on a developed support under mild conditions by microbial transglutaminase-catalyzed cross-linking. To prepare the support, a chitosan-casein mixture was physically coated on non-porous silica gel followed by cross-linking with Epichlorohydrin (ECH). Casein is a compatible substrate for MTG. The polycationic biopolymer of chitosan was used since it could be feasibly layered on the rigid silica support. ECH was selected as the cross-linking agent because it did not eliminate the primary amine groups of casein and chitosan, and thus notably improved the strength of the chitosan films [10,11].

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MATERIALS AND METHODS

α-Amylase was prepared from *Bacillus licheniformis* ATCC14580 by cultivation at 40 ºC for 48 hr as previously described [12,13]. After cultivation, cells were removed from the culture broth by centrifugation at 3,000 g for 20 min, and the supernatant was concentrated at 40 ºC by rotary evaporator (K-1000; Tokyo Rikakikai Co., Ltd., Japan) under vacuum. The obtained preparations were then precipitated with 60% (w/v) ammonium sulfate. The precipitate was re-suspended in 50 mM phosphate buffer (pH 7.0), and this crude enzyme preparation was used for immobilization. The protein concentration of the extract was measured with a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, CA, USA). A calibration curve was prepared with BSA.

To obtain MTG, *Streptomyces mobaraensis* (ATCC 29032) was cultivated at 30 ºC for 4 days (200 rpm) with the culture medium described previously [14]. After cultivation, the culture broth was treated by the same method as for α-amylase preparation. A colorimetric procedure described by Grossowicz [15] was used for determination of MTG activity. One unit of transglutaminase was defined as the formation of 1.0 μmol L-glutamic acid γ-monohydroxamate per minute at 37 ºC.

The support for immobilization was developed by forming a high density chitosan-casein resin layer on silica particles. The mixed chitosan-casein solution was prepared by dissolving 0-1.0 g of chitosan (Medium molecular weight; Sigma, St. Louis, MO, USA) and 0-1.0 g of sodium casein (Sigma, St. Louis, MO, USA) in 50

mL of 200 mM acetic acid solution. 10 g of silica gel (35-60 mesh; Sigma, St. Louis, MO, USA) was added to the solution and immersed overnight at room temperature before filtration and drying under vacuum. To avoid dispersion of chitosan and sodium casein in aqueous solution, the obtained beads were suspended in 50 mL of dimethylsulfoxide (DMSO) containing 0.1 M NaOH and 0.1 M ECH (Sigma, St. Louis, MO, USA). After incubation at 60 ℃ for 4 hr, the cross-linked matrix was filtered and washed extensively with distilled water three times to remove any residual reagents.

The α-amylase immobilization process was modified for optimal running conditions. To 15 mL of aqueous α-amylase solution (500 U/mL), 5 g of support beads and appropriate amount of microbial transglutaminase from *Streptomyces mobaraensis* were added; the final volume was adjusted to 40 mL with 50 mM phosphate buffer solution (pH 6.0). After incubation at 20 ℃ for 5 hr, the obtained beads were separated by filtration and washed with 1 L of distilled water. For determination of α-amylase activity, the previously described method was modified [16]. Briefly, the soluble starch solution (1%, w/v) was prepared in 50 mM phosphate buffer (pH 7.0) and pretreated for 10 min at 60 ℃ in a water bath before use. 1 g of enzyme-immobilized beads (1 mL free enzyme) were suspended in 4 mL of pretreated starch solution and incubated at 60 ℃ for 30 min (5 min for free enzyme). The reducing sugars were measured by adding 3,5-dinitro salicylic acid reagent, boiling for 5 min, cooling and measuring the absorbance at 540 nm with the spectrophotometer (Mecasys Co., Ltd., Korea) against maltose as a standard. One unit (U) is defined as the amount of enzyme that releases 1 mmol of reducing groups per minute.

RESULTS AND DISCUSSION

The composition of chitosan and casein on the supports greatly affects the immobilization efficiency. Table 1 shows that the most active preparation (236 U/g) was obtained with 1% casein and 1% chitosan in the coating solution when preparing the matrix. No significant α-amylase activity was found when bare silica particles or chitosan-only coated beads were used for enzyme immobilization. This result indicated that a cross-linking network occurred between casein and α-amylase. As a well-known substrate for MTG, casein on the support can provide both the acyl-donor of Gln residues and effective acyl-acceptors such as Gly, Cys, and Lys [8]. Thus, in the case of α-amylase, any of these residues on the α-amylase surface could be bonded to the supports via MTG-catalyzed cross-linking.

Table 1. Effect of chitosan and casein on immobilization efficiency when preparing the support

Casein \ Chitosan	Activity of immobilized α-amylase (U/g)					
	0	0.25%	0.5%	1%	1.5%	2.0%
0	ND	ND	ND	ND	ND	ND
0.25%	ND	35	48	98	75	33
0.5%	ND	31	82	203	96	73
1%	ND	26	51	236	144	112
1.5%	19	23	44	187	149	126
2%	20	21	32	110	102	101

ND, not determined

If the chitosan percent is fixed, the optimal ratio of casein and chitosan in solution is 1 : 1 (Table 1). A larger ratio of casein in the coating mixture may cause poor quality or dispersion of the chitosan-casein resin layer. The chitosan on the silica surface contributes significantly to the stability and strength of the formed film [17]. With a low percent of chitosan, it is difficult for casein to be coated on the silica and no significant immobilized enzyme activity is detected without chitosan in the coating mixture. Furthermore, greater than 2% chitosan caused difficulties in the bead preparation process due to the viscosity of the solution of chitosan in acetic acid.

The concentration of MTG greatly affected the immobilization efficiency during the reaction. Without MTG, no significant α-amylase activity was detected, indicating the immobilization was due to the MTG catalyzed cross-linking. The most active and favorable preparation was obtained at MTG concentration of 14 U/mL in the reaction mixture (Fig. 1). More than this concentration of MTG contributed little to immobilization efficiency.

After immobilization, the activities of free and immobilized α-amylase for starch hydrolysis were determined from pH 3 to 8 at 60 ℃. The result showed that the immobilization did not change

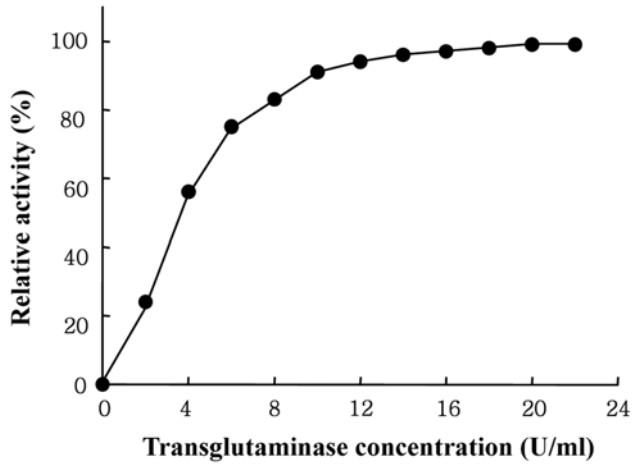

Fig. 1. Effect of transglutaminase concentration on α-amylase immobilization efficiency.

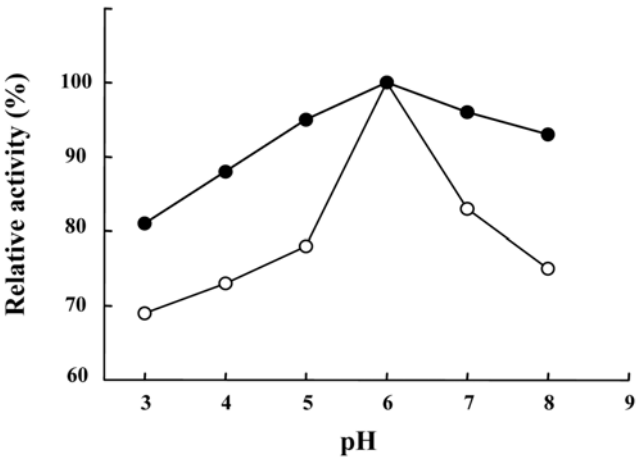

Fig. 2. Effect of pH on free α-amylase (○) and immobilized α-amylase (●) activity during soluble starch hydrolysis.

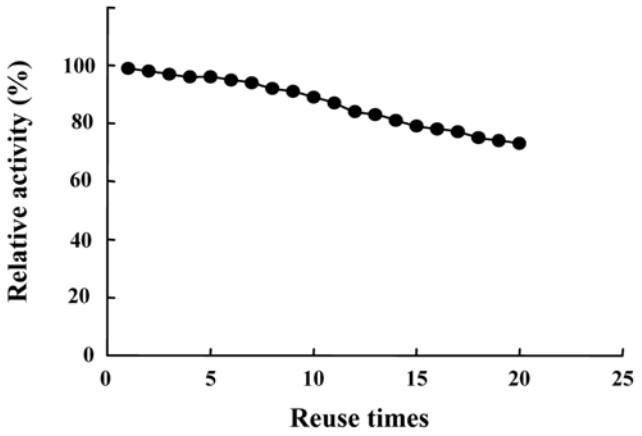

Fig. 3. Reuse of immobilized α-amylase cross-linked by microbial transglutaminase.

the optimum pH (pH 6.5) for starch hydrolysis. However, the immobilized enzymes displayed a broader pH profile than the free enzyme (Fig. 2). Similar observations, for immobilization of α-amylase and other enzymes, have been reported by other researchers [18-20]. Samples of immobilized and free enzyme were also incubated at various temperatures (30-100 ºC) for 1 hr, and the residual activity was identified to evaluate the thermostability. The immobilized enzyme exhibited almost the same thermostability with that of free enzyme (data not shown).

In general, enzymes in solution are not stable during storage and the activities will gradually decrease over time. The immobilized α-amylase was stored in 50 mM phosphate buffer (pH 7.0) at 4 ºC, and its activity was measured for a period of 30 days and compared to that of stored free enzyme (data not shown). The free enzyme lost almost all its activity within 15 days. On the other hand, 71.0% residual activity was preserved in the immobilized beads even after 30 days.

One of the most important reasons for enzyme immobilization is to allow repeated use of the target enzyme. In this study, 1 g of immobilized sample was used for soluble starch hydrolysis as described in the enzyme assay. After 20 reuses with an operation time of 30 min, the beads maintained about 70% of their original activity (Fig. 3).

In summary, α-amylase was successfully immobilized on solid support under mild conditions. This report marks the first time that α-amylase was immobilized by an enzymatic method with MTG. A support was developed for enzyme immobilization via MTG-catalyzed cross-linking. This model can be used to covalently immobilize proteins that are substrates for MTG. After immobilization, the α-amylase showed a better pH resistance profile. The immobilized enzyme also had favorable storage stability and reusability. These results suggest that the α-amylase immobilized on this developed support is very robust and suitable for continuous use in industrial processes.

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