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Immobilization of *Escherichia coli* novablue γ-glutamyltranspeptidase in Ca-alginate-*k*-carrageenan beads

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Abstract The recombinant *Escherichia coli* γ-glutamyltranspeptidase (EcGGT) was immobilized in Ca-alginate-*k*-carrageenan beads. Effects of alginate concentration, amount of loading enzyme, and bead size on the entrapped activity were investigated. Optimum alginate concentration for EcGGT immobilization was found to be 2% (*w*/*v*). Using a loading enzyme concentration of 1.5 mg/g alginate, maximum enzyme activity was observed. With increase in bead size from 1.9 to 3.1 mm, the immobilization efficiency was decreased significantly because of mass transfer resistance. Thermal stability of the free EcGGT was increased as a result of the immobilization. Ca-alginate-*k*-carrageenan-EcGGT beads were suitable for up to six repeated uses, losing only 45% of their initial activity. Upon 30 days of storage the preserved activity of free and immobilized enzyme were found as 4% and 68%, respectively. The synthesis of L-theanine was performed in 50 mM Tris–HCl buffer (pH 10) containing 25 mM L-glutamine, 40 mM ethylamine, and 1.5 mg EcGGT/g alginate at 40°C for 12 h, and a conversion rate of 27% was achieved.

Keywords *Escherichia coli* · γ-glutamyltranspeptidase · Alginate · Immobilization · Bioconversion · L-theanine

Enzyme immobilization is a technique that confines a catalytically active enzyme within a reactor, preventing its entry into the mobile phase so that it can continuously be reused. It

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also allows the biocatalyst and product/substrate to be segregated, with the possibility of controlling the biocatalyst microenvironment to enhance the stability of biocatalysts [1]. Entrapment is one of the simplest methods for enzyme immobilization under milder conditions, and therefore results in minimum denaturation of the biocatalyst during the process [2]. In search of suitable matrices for enzyme immobilization, alginate has been found to be the top candidate in terms of its biocompatibility and processability [3, 4]. Alginate is a naturally occurring polysaccharide consisting of gluronic acid and mannuronic acid moieties. It is a reversibly soluble-insoluble polymer which changes solubility in the presence of calcium [5]. To date, cross-linked alginate has been successfully used for encapsulation of many biological molecules [6–10], owing to the relatively inert aqueous environment within the matrix, the mild room temperature encapsulation process, and high gel porosity allowing high diffusion rates of macromolecules.

The enzyme γ-glutamyltranspeptidase (GGT; EC 2.3.2.2) catalyzes the cleavage of the γ-glutamyl linkage of glutathione and other related γ-glutamyl compounds, and the transfer of the γ-glutamyl moiety to other amino acids and peptides [11]. GGT is widely distributed in living organisms [11, 12] and plays an important role in the regulation of the intracellular levels of glutathione [13]. The nascent *Escherichia coli* GGT (EcGGT) is synthesized as a 61.8-kDa polypeptide containing sequences coding for a signal peptide of 25 amino acid residues, the large subunit of 365 residues, and the small subunit of 190 residues [14]. The removal of the signal peptide gives rise to a 59.2 kDa precursor polypeptide to be processed by autocatalytic cleavage into large and small subunits in the periplasm [15]. EcGGT utilizes many amino acids and peptides as γ-glutamyl acceptors and inexpensive L-glutamine as a good γ-glutamyl donor in the transpeptidation reaction [16]. For commercial applications, the EcGGT-mediated methods have been developed for the synthesis of γ-glutamyl-L-DOPA, γ-Glu-Phe, γ-Glu-Trp, and L-theanine [17]. L-Theanine is a unique amino acid found in ordinary tea leaves from *Camellia sinensis* [18] and synthesized from glutamate and ethylamine by theanine synthase [19]. The demand for L-theanine has become great not only as a food additive to enhance flavor, but also as a supplement to improve and/or maintain human health because of its favorable physiological functions [20–22]. Many researchers have tried to prepare L-theanine, but their methods are probably impractical because of complexity with low yield and/or the low purity of the product [23–26]. Enzymatic production of L-theanine with glutamine and ethylamine as substrates has been performed successfully to some extent with the γ-glutamyl transfer reaction of glutaminase [27] or GGT [28, 29].

The enzymatic method for the production of γ-glutamyl compounds with bacterial GGT is a simple one-step process that does not require any energy source. The most expensive step in this process is the preparation of GGT; therefore, the expression systems for *E. coli ggt* gene have been constructed to obtain a sufficient amount of enzyme for structural studies or industrial utilizations [30, 31]. Recently, we cloned the coding region for the large and small subunits of the *E. coli* novablue GGT lacking the first 16 amino acids of the signal sequence into pQE-30 [29]. On an interesting note, we found that the signal-sequence truncation does not affect the posttranslational modification and active production of EcGGT in the recombinant cells, and the overexpressed enzyme is easily purified from the cell-free extract by nickel-chelate chromatography. In this study, we offer a simple method for the immobilization of EcGGT with Ca-alginate-*k*-carrageenan hydrogel. To the best of our knowledge, the immobilization of microbial GGT by this matrix has not been reported in the literature. This matrix has certain advantages over other materials such as low cost, ease of enzyme accessibility, and hydrophilic character. For this purpose, the Michaelis–Menten kinetic constants, optimum pH and temperature, thermal stability,

storage stability, and reusability for the immobilized enzyme were reported. In addition, the synthesis of L-theanine from glutamine and ethylamine was also employed by the immobilized EcGGT.

Materials and Methods

Materials, Microorganism, and Growth Condition

Luria–Bertani (LB) media for *Escherichia coli* cultivation was acquired from Difco Laboratories (Detroit, MI, USA). Nickel nitrilotriacetate (Ni^{2+}-NTA) resin was obtained from Qiagen, Inc. (Valencia, CA, USA). Sodium alginate, *k*-carrageenan, rhodamine-B-isothiocyanate (RITC), L-glutamine, and ethylamine were purchased from Aldrich-Sigma Fine Chemicals (St. Louis, MO, USA). L-Theanine was acquired from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were commercial products of analytical or biological grade.

Escherichia coli M15 (pQE-EcGGT) [29] was used as the working strain for EcGGT production. The bacterium was grown aerobically at 20°C or 37°C in LB medium supplemented with 100 μg ampicillin per milliliter and 25 μg kanamycin per milliliter.

Enzyme Preparation

To purify EcGGT from the crude extract of *E. coli* M15 (pQE-EcGGT), the recombinant bacterium was grown at 37°C in 100 ml of LB medium containing the above-mentioned antibiotics to an absorbance at 600 nm of 1.0. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM and the cultivation continued at 20 °C for 12 h. *E. coli* cells were harvested by centrifugation at 9,000×*g* for 20 min at 4 °C, resuspended in 30 ml of 50 mM Tris–HCl buffer (pH 9.0) after decanting the supernatant, and disrupted by sonication (Sonicator XL-2000; Misonix, Inc., Farmingdale, NY, USA). The extract was clarified by centrifugation at 12,000×*g* for 30 min at 4 °C, and the resulting supernatant was mixed with Ni^{2+}-NTA resin preequilibrated with binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris–HCl; pH 7.9). The adherent EcGGT was eluted from the column using a buffer containing 0.5 M imidazole, 0.5 M NaCl, and 20 mM Tris–HCl (pH 7.9).

GGT Activity Assay

γ-Glutamyltranspeptidase (GGT) activity was determined at 40 °C according to the method of Orlowski and Meister [32] and the formation of *p*-nitroaniline (*p*-NA) was recorded by monitoring the absorbance changes at 410 nm. Unless otherwise indicated, the reaction mixture contained 1.25 mM L-γ-glutamyl-*p*-nitroanilide (L-γ-Glu-*p*-NA), 30 mM Gly–Gly, 1 mM $MgCl_2$, 50 mM Tris–HCl buffer (pH 9.0), 20 μl of enzyme solution at a suitable dilution, and enough distilled water to bring the final volume of 1 ml. One unit of GGT activity is defined as the amount of enzyme that produces 1 μmol of *p*-NA per minute under the assay conditions.

The kinetic parameters were determined in 50 mM Tris–HCl buffer (pH 9.0) containing 30 mM Gly–Gly, 1 mM $MgCl_2$, and 10 to 1,000 μM L-γ-Glu-*p*-NA. K_m and V_{max} values were calculated from the slope and the *y*-axis intercept, respectively, on the Lineweaver–Burke plot.

Immobilization of EcGGT

The purified EcGGT was immobilized in the Ca-alginate-*k*-carrageenan beads according to the procedures of Şahin et al. [8]. In brief, sodium alginate (0.2 g) and *k*-carrageenan (0.03 g) were dissolved in 20 mM Tris–HCl buffer (pH 9.0), followed by the addition of 1 ml of EcGGT solution (approximately 4.9 U mg^{-1}) to a final volume of 10 ml. Subsequently, the mixture was dropped into 50 mM Tris–HCl buffer (pH 9.0) containing 0.3 M $CaCl_2$ (T/Ca solution) solution using a peristaltic pump to obtain equal size polymeric beads of Ca-alginate-*k*-carrageenan. The entire process was performed aseptically in the laminar airflow chamber. As a result of this protocol, EcGGT was entrapped in the Ca-alginate-*k*-carrageenan spherical beads. After 30 min of hardening, the beads were separated from T/Ca solution by vacuum filtration. They were washed twice with 50 mM Tris–HCl (pH 9.0). The filtered T/Ca solution and the two washings were collected for protein determination. Protein concentration was estimated by the dye-binding method [33] with bovine serum albumin as the standard protein.

Immobilization efficiency was defined as follows: Immobilization efficiency (%)$=(a_{\mathrm{imm}}/a_{\mathrm{free}}) \times 100$, where a_{imm} is the specific activity of immobilized enzyme (U mg^{-1} protein) and a_{free} is the specific activity of free enzyme (U mg^{-1} protein).

The mean wet particle diameter was measured, taking random samples of beads and measuring their diameter using a vernier caliper.

Preparation of RITC-EcGGT

The method used for the synthesis of RITC–EcGGT conjugate was essentially that of Fothergill and Nairn [34]. First, RITC was dissolved in 50 mM carbonate–bicarbonate buffer (pH 9.0) and mixed with EcGGT to a final ratio of 0.05 mg dye per milligram of protein. The dye solution was added to the protein over a period of 30 min with gentle stirring, followed by stirring the sample at 4°C for 12 h. After conjugation, the solution was clarified by centrifugation at 10,000×*g* for 20 min. To remove the excess RITC, 10 mg of 20 mM phosphate-buffered charcoal (pH 7.2) was mixed with the conjugate for 60 min and the residual charcoal was removed by centrifugation at 10,000×*g* for 20 min, followed filtering the solution sequentially through 0.8- and 0.2-μm membrane filters. The resulting RITC-EcGGT conjugate was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) analysis and used for enzyme immobilization. Approximately 50 μg of RITC-EcGGT conjugate was used to make spherical beads by the above-mentioned process. The Ca-alginate-*k*-carrageenan-RITC-EcGGT beads were subjected to absorbance measurement with a UV–Vis spectrometer (Hitachi U-1800, Hitachi Ltd., Japan).

Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) was performed with the Laemmli buffer system [35]. Before electrophoresis, the samples were heated at 100°C for 5 min in dissociating buffer containing 2% SDS and 5% 2-mercaptoethanol. The gels were stained with 0.25% Coomassie brilliant blue dissolved in a solution of 30% methanol and 10% acetic acid or visualized with an ultraviolet transilluminator at 280 nm. The protein size markers were phosphorylase *b* (97.4 kDa), bovine serum albumin (66.3 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), and trypsin inhibitor (21.5 kDa).

Characterization of Immobilized EcGGT

To investigate the effect of temperature on the free and immobilized EcGGT, the purified enzyme (0.38 U ml^{-1}) and 10 Ca-alginate-*k*-carrageenan-EcGGT beads were incubated in 50 mM Tris–HCl buffer (pH 9.0) and assayed for GGT activity at temperatures ranging from 20 to 70°C. Thermostability of the free and immobilized enzymes was studied by incubating the samples at 20, 30, 37, 40, 50, 60, and 70°C for 30 min in a water bath shaker (100 rpm). The residual GGT activity was determined under standard assay conditions.

The effect of pH on the free and immobilized EcGGT was also evaluated by incubating the purified enzyme (0.31 U ml^{-1}) and 10 Ca-alginate-*k*-carrageenan-EcGGT beads in 50 mM citrate-phosphate buffer (pH 3–7), 50 mM potassium phosphate buffer (pH 6–8), and 50 mM Tris–HCl buffer (pH 7–10) at 40 °C, and the amount of GGT activity was determined under the standard assay conditions. For the measurement of pH stability, both free and immobilized enzymes were kept at 4 °C for 30 min in different buffers. The residual GGT activity was determined under standard assay conditions.

To evaluate the storage stability, the purified EcGGT (120 U in 200 ml of T/Ca solution) and Ca-alginate-*k*-carrageenan-EcGGT beads (150 beads in 200 ml of T/Ca solution) were stored at 4 °C for 40 days. The aliquots (100 μl for free EcGGT or 10 beads for immobilized enzyme) from both samples were taken in duplicate at the gap of 2 days and were then analyzed for the remaining enzyme activity.

The alginate-entrapped EcGGT was analyzed for its reusability. Repeated batch assay (20 Ca-alginate-*k*-carrageenan-EcGGT beads in 2 ml of reaction mixture) was carried out by decanting the reaction product every 10 min and replacing it by a fresh and prewarmed buffer containing 1.25 mM L-γ-Glu-*p*-NA, 30 mM Gly–Gly and 1 mM $MgCl_2$ after washing the alginate beads with ice-cold Tris–HCl buffer (50 mM, pH 9.0).

HPLC Analysis

The analyses were performed using a Hitachi L-7420 series system equipped with a RP18-GP250 column (Kanto Chemical Co., Kanto, Japan) and with a constant flow rate of 1 ml min^{-1}. The procedures for chromatography were essentially as described previously [29]. To optimize the synthesis of theanine, a combination of Gln (5, 10, 15, 20, 25, 30, and 40 mM) and ethylamine (10, 20, 30, 40, 50, and 60 mM) were prepared in 2 ml of 50 mM Tris–HCl buffer (pH 10), and thereafter 20 Ca-alginate-*k*-carrageenan-EcGGT beads were added to the mixture. The samples were incubated with shaking (100 rpm) at 40 °C for 12 h. As controls, the reaction mixtures without EcGGT were incubated similarly. The reaction was terminated by the addition of trichloroacetic acid to 10% and subjected to high-performance liquid chromatography (HPLC). *O*-Phthalaldehyde was used as the detection reagent and the adsorption was detected with a UV detector as the absorbance at 335 nm.

Results and Discussion

Preparation and Immobilization of EcGGT

In an earlier study, we constructed a bacterial expression plasmid, pQE-EcGGT, for the overproduction of recombinant *E. coli* novablue GGT [29]. The mRNA of this construct encodes a His tag at the N-terminus of EcGGT, which facilitates a one-step purification of the recombinant enzyme with Ni^{2+}-NTA resin. As shown in Fig. 1, the molecular masses of

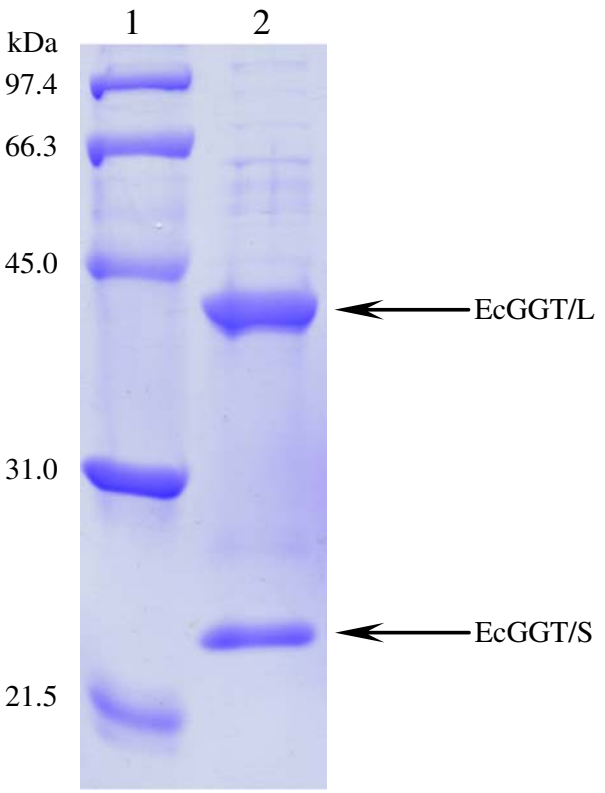

Fig. 1 SDS/PAGE analysis of the purified EcGGT. Lanes: *1*, protein size marker; *2*, purified enzyme. SDS/PAGE was performed in a vertical mini-gel system (Mini-Protean III system; Bio-Rad) with 4% polyacrylamide stacking and 10% polyacrylamide separating gels. Electrophoreses were carried out at ambient temperature and at 100 V constant voltage

the subunits of the purified EcGGT were 41 and 22 kDa, respectively. The specific activity for the purified enzyme was 5.3 U mg^{-1} protein and the purification resulted in a yield of 79%. The purified EcGGT was subsequently used for the preparation of enzyme-RITC conjugate and immobilization.

Sodium alginate in the concentration range of 0.5–4% (*w*/*v*) was used for the preparation of Ca-alginate-*k*-carrageenan beads to immobilize EcGGT. As shown in Table 1, the immobilization efficiency was maximal (~27%) at a sodium alginate concentration of 2%.

Table 1 Immobilization of EcGGT with different concentrations of sodium alginate.

Alginate (%)	*k*-Carrageenan (%)	Immobilization efficiency (%)[a]
0.5	0.3	9.7
1.0	0.3	10.9
1.5	–[b]	9.1
1.5	0.3	18.2
2.0	–	10.1
2.0	0.3	27.1
2.5	0.3	24.3
3.0	0.3	23.7
3.5	0.3	22.9
4.0	0.3	21.2

[a] The relative activity was calculated with respect to the specific activity of free enzyme taking 5.7 U mg^{-1} protein as equal to 100%.

[b] –Without the addition of *k*-carrageenan.

Although approximately the same efficiency was obtained with 2.5–4.0% sodium alginate, but for further experimental processes these concentrations were not used as the high viscosity of Ca-alginate-*k*-carrageenan-EcGGT mixture led to nonuniformity in the size of beads. Consistently, sodium alginate in the concentration of 2–3% has been conducted in the immobilization of *Aspergillus oryzae* keratinase [36], *Candida rugosa* lipase [37], and goat brain dipeptidylpeptidase IV [6].

Interpenetrating polymer networks (IPNs) are mixtures of two cross-linked polymers. Generally, IPNs are formed either by simultaneous parallel reactions according to various mechanisms or by swelling one network in monomers from which the second network can be produced [38]. IPNs are preferred in a number of biotechnological and biomedical applications because of their certain biophysical properties such as ease of fabrication to various geometrical forms, soft and rubbery texture, and unusual stability to biofluids [39]. A research group has demonstrated the potential of *k*-carrageenan-related IPNs as supports for the immobilization of biocatalysts [8, 40, 41]. In this study, Ca-alginate-*k*-carrageenan polymer blend has also shown to entrap more GGT activity than that of Ca-alginate polymer (Table 1). Accordingly, 2% alginate–0.3% *k*-carrageenan polymer blend was selected for the further experiments.

Another factor affecting the immobilization is initial enzyme loading of beads. To determine the effect of EcGGT loading in Ca-alginate-*k*-carrageenan beads on the activity, various concentrations of immobilized enzyme (0.2, 0.5, 1, 1.5, 2, 3, and 4 mg/g alginate) were investigated. As expected, the increases in enzyme concentration raised the activity of immobilized enzyme. A saturation of the immobilization support around enzyme concentration of 1.5 mg/g alginate was observed (data not shown).

As with most heterogeneous catalysts, immobilized enzymes generally experience mass transfer limitations. The size of beads in which EcGGT is entrapped may be one of the most important parameters for enzyme immobilization. It has been reported that enzymes entrapped in the smaller beads will show higher catalytic activity because of the reduced substrate transfer resistance [42–44]. To make smaller alginate beads, an electrostatic droplet generation technique has been used [44]. In this study, Ca-alginate-*k*-carrageenan-EcGGT beads of three different sizes were generated reproducibly by changing the size of a needle through which a mixture of enzyme and matrix was dripped into the Ca/T solution. By this approach, the average bead sizes were determined to be 1.2±0.2, 1.9±0.3, and 3.1± 0.2 mm, respectively. Maximum enzyme activity was observed with a bead size of 1.9 mm (data not shown). The enzyme activity of *Aspergillus sclerotiorum* α-amylase also show a significant decrease when the size of Ca-alginate beads over 3 mm [42].

To examine covalently bound RITC-EcGGT, the conjugate was subjected to SDS/PAGE analysis. As shown in Fig. 2A, precharcoal conjugate exhibited both free dye and dye–protein conjugate, and the subunits of EcGGT were still conjugated with RITC after the charcoal treatment step. To confirm the presence of EcGGT in the immobilization matrix, both Ca-alginate-*k*-carrageenan-EcGGT and Ca-alginate-*k*-carrageenan-RITC-EcGGT beads were analyzed by a UV–Vis spectrometer. As shown in Fig. 2B, the unmodified beads did not display any characteristic absorbance between 300 and 700 nm. However, Ca-alginate-*k*-carrageenan-RITC-EcGGT beads possessed strong absorbance at 560 nm, which corresponds to the absorbance of RITC and verifies the successful immobilization of EcGGT in the beads.

Kinetic Parameters, Optimum Temperature, and Optimum pH of Immobilized EcGGT

The kinetic parameters, K_m (Michaelis constant) and V_{max} (maximum reaction rate) for free and immobilized EcGGT were determined by varying the concentration of L-γ-Glu-*p*-NA

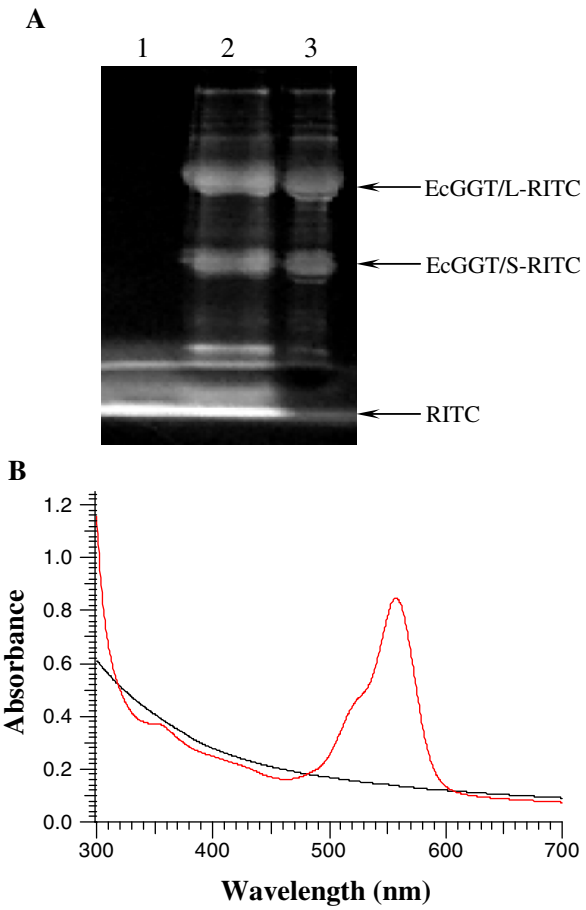

Fig. 2 SDS/PAGE analysis of RITC conjugated EcGGT (A), and UV–vis absorbance spectrum of Ca-alginate-*k*-carrageenan-EcGGT (black line) and Ca-alginate-*k*-carrageenan-RITC-EcGGT (red line) (B). Lanes: *1,* RITC; *2*, precharcoal conjugate of RITC and EcGGT; *3*, after-charcoal conjugate RITC and EcGGT

in the reaction mixture. The K_m values for free and immobilized enzymes were 40.3±3.4 and 52.2±2.7 μM, respectively. The change in the enzyme affinity to its substrate is probably caused by structural changes in the enzyme introduced by the immobilization procedure or by lower accessibility of the substrate to the active site of the immobilized enzyme [45]. As expected, V_{max} value was also significantly affected after entrapment of EcGGT in Ca-alginate-*k*-carrageenan beads, changing from $52.9 \pm 6.3 \times 10^{-3}$ to $37.2 \pm 4.1 \times 10^{-3}$ mM min^{-1}. The decrease in reaction rate might be attributed to the interaction of enzyme with the functional groups on the surface of beads or large areas of contact between enzyme and support causing large deformation of enzyme conformation. Similar results involving change in K_m and V_{max} values of enzyme after immobilization have also reported in the literature [8, 46–48].

As shown in Fig. 3A, optimum temperature was found at around 40 °C for both free and immobilized enzymes, but immobilization resulted in slower decline of the activity as the assay temperature was increased.

The effect of operational pH on the activity of free and immobilized enzyme was performed as described in "Materials and Methods". The immobilized enzyme had a similar pH range to that of free enzyme (data not shown).

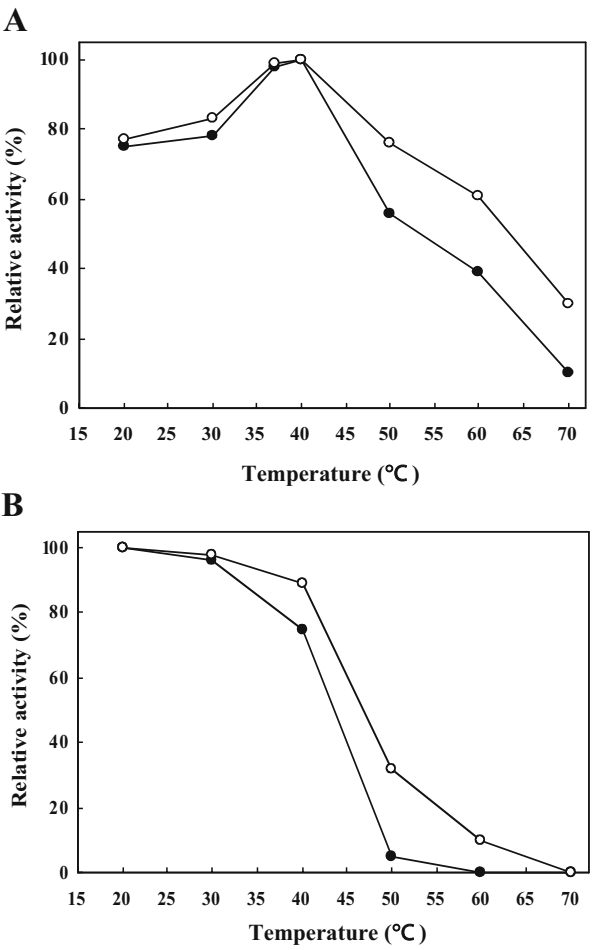

Fig. 3 Effect of temperature on activity (A) and stability (B) of free and immobilized EcGGT. The experiments were performed in triplicate and the standard derivations were lower than ±4.5%. The 100% relative activity corresponds to 6.8 U ml^{-1} for Ca-alginate-EcGGT and Ca-alginate-*k*-carrageenan-EcGGT, respectively. (*Filled circle*) free EcGGT; (hollow circle) immobilized EcGGT

Thermal Stability of Immobilized EcGGT

The effect of temperature on stability of immobilized EcGGT is illustrated in Fig. 3B. Free enzyme was almost inactive after a 30-min incubation at 50 °C. Immobilized EcGGT has shown high thermal stability at 20–40 °C, whereas free enzyme only kept 73% of the activity at 40 °C. Based on these results, we can say that immobilization of EcGGT in Ca-alginate-*k*-carrageenan beads preserve tertiary structure of the enzyme and it protects the enzyme from conformational changes caused from environmental effect. It is often observed that immobilized enzyme has a higher thermal stability than the corresponding free enzyme because of the reduction of conformational flexibility in the immobilized enzyme [49].

Storage and Operational Stability of Immobilized EcGGT

Free EcGGT retained about 50% of its activity within 15 days and lost its remaining activity within 40 days (data not shown). The half-life of immobilized enzyme was found to

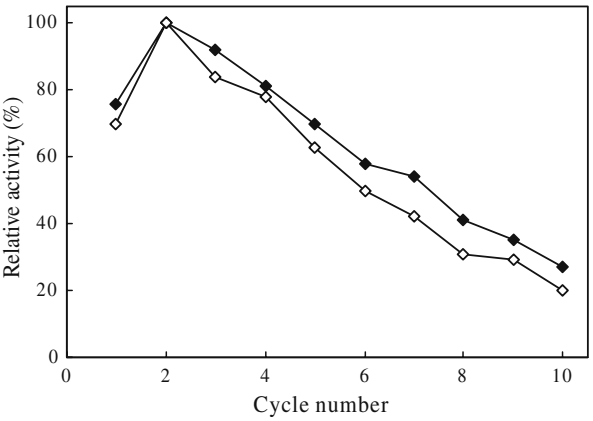

Fig. 4 Effect of reused cycle on immobilized GGT activity. The experiments were performed in triplicate and the standard derivations were lower than ±3.1%. The 100% relative activity corresponds to 7.3 U ml^{-1} for Ca-alginate-EcGGT and Ca-alginate-*k*-carrageenan-EcGGT, respectively. (*Filled diamond*) immobilization of EcGGT by Ca–2% alginate–0.3% *k*-carrageenan; (*hollow diamond*) immobilization of EcGGT by Ca–2% alginate

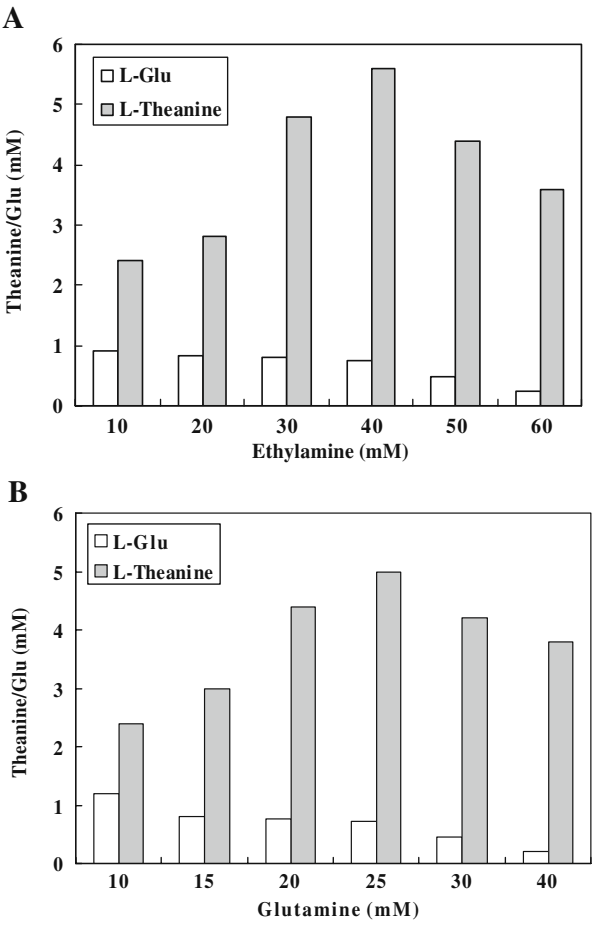

Fig. 5 Effects of ethylamine (A) and glutamine (B) concentrations on the synthesis of theanine. The experiments were performed in triplicate and the standard derivations were lower than ±2.5%

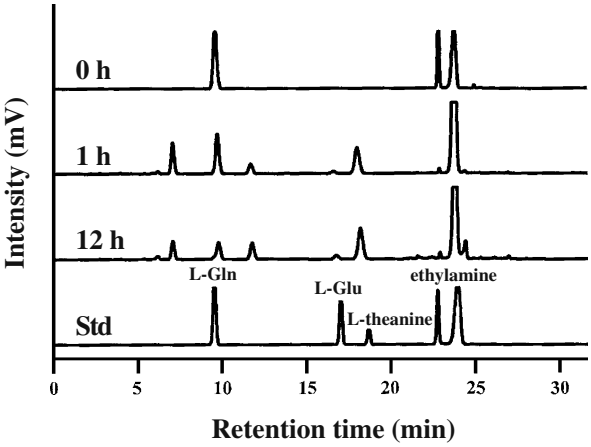

Fig. 6 Chromatograms of the reaction products analyzed by HPLC with UV detector. The retention times for L-Gln, L-Glu, L-theanine, and ethylamine were 9.9, 16.4, 18.3, and 22.8 min, respectively

be 35 days. The decrease in activity can be explained as a time-dependent natural loss and this is prevented to a significant degree by immobilization. Consistently, it has been demonstrated that hydrogel carriers, such as alginate, gelatin and polyacrylamide, provide a protective microenvironment for the immobilized enzymes and therefore yield a higher stability during storage [8, 48, 50].

One of the advantages for enzyme immobilization is its reusability during the operation [49]. Operational stability of immobilized EcGGT was determined for 10 successive batch reactions at 40 °C for 10 min each time. As shown in Fig. 4, the immobilized enzyme retained about 50% of initial activity after five to six cycles. The initial diameter of Ca–2% alginate–0.3% *k*-carrageenan-EcGGT beads was 2.1±0.2 mm. A significant increase in the mean diameter of beads was observed for the first run of recycle. The bead diameter continued to swell and reached 3.6±0.8 mm at the end of operation. The bead swelling might contribute to the leakage of immobilized enzyme, leading to a dramatically increase of the entrapped activity in the first few runs and thereafter the sequential decrease in retention activity. Stability of the alginate beads and prevention of their rupture are important determinants of long-term proper function of entrapped enzymes. A significant barrier to successful use of alginate in cell/tissue encapsulation is the phenomenon of capsule swelling, which may lead to disintegration of beads and rapid destruction of entrapped islet cells [51, 52]. The swelling phenomenon is thought to be caused by increased hygroscopy of the microcapsule caused in part by the divalent cations used for gelling the microbeads [52–54].

Table 2 The reusability of immobilized EcGGT for L-theanine synthesis.

Cycle number	Conversion rate (%)[a]
1	23
2	20
3	18
4	15
5	14
6	10

[a] The experiments were performed in triplicate and the data were expressed as mean values.

Synthesis of L-theanine by Immobilized EcGGT

Because of the commercial importance of L-theanine, the reaction conditions for the synthesis of this compound by the immobilized EcGGT were investigated. As shown in Fig. 5, the optimum concentrations for L-glutamine and ethylamine were 25 and 40 mM, respectively. When 25 mM L-Gln was used, 40 mM ethylamine, 1.5 mg EcGGT/g alginate, pH 10, and incubation at 40 °C for 12 h were the optimum conditions. Under the optimum conditions, the conversion rate of L-Gln as to L-theanine was determined to be approximately 25% (Fig. 6).

The immobilized enzyme was repeatedly used to synthesize L-theanine in batch reactions. Twenty Ca-alginate-*k*-carrageenan-EcGGT beads in 2 ml of 50 mM Tris–HCl buffer (pH 10) containing 25 mM L-Gln and 40 mM ethylamine were shaken (100 rpm) at 40 °C for 30 min each time, and the synthesized L-theanine was determined by HPLC. After each cycle of reaction, the beads were washed once with 2 ml of 50 mM Tris–HCl buffer (pH 9.0) and reused for another run. As shown in Table 2, the conversion rate for the first run reached to 23% and the rate was still kept at 14% after five cycles of reuse. These observations make the immobilized EcGGT more valuable for industrial applications.

Conclusion

The present study provides that a simple process to immobilize EcGGT on Ca-alginate-*k*-carrageenan beads can be of potential interest for the biosynthesis of L-theanine. Alginate is a support of low cost, not toxic, and easily available, and the immobilization provides optimum conditions for enzymatic reactions similar to those of free enzyme. Furthermore, the entrapped EcGGT has shown excellent stability on repeated reuse for five cycles without any significant loss in the enzyme activity. Finally, further research is still needed to improve the conversion rate of the system for practical applications.

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