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Improvingthecatalytic properties and stability of immobilized γ-glutamyltranspeptidase by post-immobilization with Pharmalyte^{MT} 8–10.5

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ABSTRACT

γ-Glutamyltranspeptidase (GGT) is a dimeric protein that specifically catalyzes the transfer of γ-glutamyl in the optimum pH range of 8.5–9.0, but has poor *in vitro* stability under the alkaline conditions. In the present work, GGT was immobilized on a mesoporoustitania oxide whisker (MTWs) carrier to afford MTWs-GGT that was further modified with Pharmalyte^{MT} (Phar) 8.0–10.5 to yield MTWs-GGT-Phar. Phar absorbed on MTWs-GGT to form a buffering layer with an isoelectric point of ~9.2 that isolated the immobilized enzyme from the liquid bulk and significantly in proved the pH tolerance and stability of the immobilized GGT. The MTWs-GGT-Phar exhibited a stable enzyme activity in the pH range of 6.0–11.0 and an optimum temperature 10 °C higher than GGT. Its pH stability at pH 11.0 and thermal stability at 50 °C were respectively 23.7 times and 19.4 times higher than those of GGT. In addition, the affinity constant of MTWs-GGT-Phar towards G*p*NA (K_m) was 0.597 mM, slightly lower than that of free GGT, indicating that Phar had a protective effect on the structure of GGT.

1. Introduction

Biocatalysis has been widely used in modern chemical, pharmaceutical [1–5], biofuel [6–9] and food [10–12] industries due to its high specificity and selectivity, as well as mild reaction conditions. However, the industrial catalytic conditions, such as solvents, pH, temperature, ionic strength, and so on, are significantly different from those of the physiological environment, which may deactivate enzymes and thus limitstheir industrial application [13,14]. Therefore, efforts have been made to improve the stability of industrial enzymes.

The most widely used method is to screen high tolerance extremophiles to obtain industrial enzymes or modify the natural enzymes by protein engineering technologies, such as site-directed mutagenesis [15–17] and random mutations [18–20], to improve the stability of the enzymes. In addition, the conventional enzyme immobilization has also been proved an effective method to improve the stability of natural enzymes. The most commonly used enzyme immobilization technique is adsorption, by which the enzyme molecules physically adsorb on a solid support via weak forces, such as hydrogen bond, ionic bond or Van der Waals force. Although this method might alter the properties of natural enzyme, such as conformations and activities [21,22], it is simple and can be realized under very mild conditions. Thus, enzyme adsorption has been widely applied in industrial biocatalysis [23–25]. However, the stabilities of the physically immobilized enzymes, especially those of the multimeric enzymes, such asdehydrogenases, aldolases, oxidases, catalases and galactosidases, and so on, are poor. The subunits of multimeric enzyme are usually on different planes, and thus are difficult to bind to the carrier surface simultaneously. The unbound subunits of the immobilized enzyme may dissociate and fall off from the carrier as they are exposed to *in vitro* catalytic conditions, leading to irreversible deactivation. Therefore, post-immobilization was proposed to stabilize the binding between the multimeric enzyme and carrier. The unbound subunits are cross linked via organic polymers, such as aldehyde-dextran and polyethyleneimine (PEI), to prevent theirdissociation and falling-off [26–29]. However, the cross linking can affect the conformation of multimeric enzymes and increase the mass transfer resistance, resulting in decreasedcatalytic activities of the immobilized enzymes [30].

γ-glutamyltranspeptidase (GGT) is a dimeric protein of a large and a small subunit that can specifically catalyze the transfer of γ-glutamyl group to an acceptors, namely GGT reaction. GGT reaction has been widely used in the synthesis of several valuable γ-glutamylcompounds [31–38] due to its facile reaction condi-

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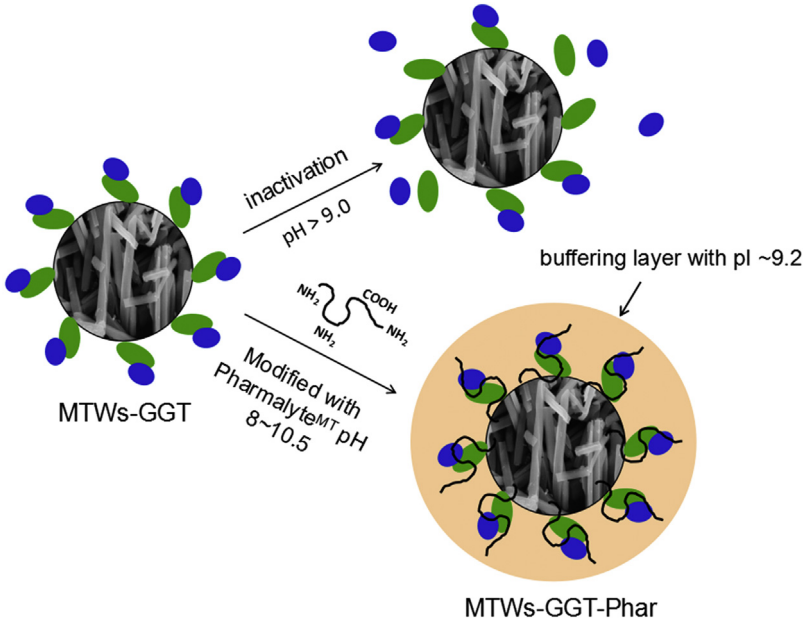

Fig. 1. Schematic diagram of the modification of the immobilized GGT with Phar.

tions, no ATP consumption, and easy operation. To reduce the side reaction of auto-transpeptidation, GGT reaction is usually performed at pH 8.5–9.0. However, the microbial GGTs, such as *Escherichia coli* GGT and *Bacillus subtilis* GGT are unstable and subjects to subunit dissociation under alkaline conditions, leading to irreversible declines in enzyme activity [39]. In order toovercome this drawback, the enzyme GGT was reported covalentimmobilization onto chitosan microspheres [40] and the functionalized magnetic nanoparticles [41,42]. Although these methodswere proved effective for improving the stability ofGGT, the recovery of enzymeactivity was poor. In our previous work, the GGT from *Bacillus subtilis* was physically immobilized on the silylated mesoporous TiO_2 whiskers [39]. The recovery of GGT activity was above 97% and the stability of immobilized GGT was also satisfactory, but a decrease in the enzyme activitywas observed.

Pharmalyte^{MT} (Phar) is a carrier ampholyte composed of amino acid polymers of different isoelectric points (pI). Its buffer range is 8.0–10.5 with pI ~9.2 that is close to the optimum pH of GGT. In the present work, Phar was used to modify the immobilized GGT on mesoporousTiO_2whiskers(MTWs-GGT). Pharwith high hydration ability and buffering capacity formed a buffering layer on MTWs-GGT, which not only stabilized the quaternary structure of GGT, but also provided a favorable micro-environment for GGT (Fig. 1). The stability and catalytic properties of the immobilized GGT were significantly improved.

2. Materials and methods

2.1. Materials

Recombinant strain BL21(DE3)-pET22b-*Bggt* for *Bacillus subtilis* GGT was constructed in-house. The Mesoporous TiO_2 whisker (MTWs) with a width of 100–300 nm, a length 1–10 μm and a pore diameter of 7–30 nm was provided by Prof. Xiaohua Lu of Nanjing Technology University, China. Pharmalyte^{MT} 8–10.5 (Phar) and Ni Sepharose 6 Fast Flow (GE Healthcare, USA) were purchased. BCA protein quantification kit was purchased from Shanghai Jierui Biological Engineering Co. (Shanghai, China). γ-glutamyl-*p*-nitroanilide (G*p*NA) and *N*-glycylglycine were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Other reagents were of analytical grade.

2.2. Instruments

UV spectra were recorded on an Ultrospec 7000 UV spectrophotometer (GE Healthcare, USA). pH was measured with a Mettler-Toledo pH meter (Switzerland). GGT was purified by ÄKTA FPLC (GE Healthcare, USA).

2.3. Methods

2.3.1. Fermentation and purification of GGT

The recombinant strain *E.coli* BL21 (DE3)-pET22b-Bggt was inoculated in a fermentation medium composed of 10.0 g/L sucrose, 20.0 g/L yeast extract, 9.0 g/L NaCl, 3.0 g/L K_2HPO_4 and 1.0 g/L $MgSO_4$ (pH 7.0), and cultured at 37 °C for 4 h. Lactose was then added to obtain a final concentration of 2.5 g/L to induce the fermentation at 24 °C for 12 h. The activity of GGT was measured to be 3.34 U/mL. GGT was purified by an affinity chromatography on anÄKTA FPLC using 500 mM imidazole in Tris-HCl buffer (50 mM, pH 8.0)as the eluent. The elute was further dialyzed against Tris-HCl buffer (pH 8.0) to yield electrophoresis pure GGT with a specific activity of 43.1 U/mg.

2.3.2. Enzyme immobilization

GGT was immobilized on MTWs by Wang's method [39]. MTWs was soaked and washed thoroughly with deionized water, mixed with GGT solution at a concentration of 224 U/g carriers in a sand-core tube and oscillated at 4 °C for 1.5 h. The produced particles were washed for three times with 10 volumes of Tris-HCl buffer (50 mM, pH 8.0) to afford MTWs-GGT with a specific activity of 184 U/g.

2.3.3. Modification ofMTWs-GGT

MTWs-GGT and Phar were mixed at a solid-to-liquid ratio of 1:5 (w/v) in a sand-core tube and oscillated at 4 °C for 20 h. The produced particles were washed with 50 mMTris-HCl (pH 8.0)for three times and the washing buffer wasthen extruded out to afford MTWs-GGT-Phar.

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