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Immobilization of aminoacylase on electrospun nanofibrous membrane for the resolution of DL-theanine

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ABSTRACT

The enzyme aminoacylase was used to produce L-theanine from its derivative *N*-acetyl-DL-theanine. For stabilization purposes the aminoacylase was immobilized on polyvinyl alcohol-based nanofibrous membranes generated by electrospinning. The immobilized aminoacylase exhibited better resistance to changes in temperature and pH than the free enzyme with optimal conditions being pH 8 and 52 °C. Under these conditions, K_m values were two to three times higher than those of the free enzyme (3.6 mM). Thermostability was also significantly improved; the activity of the immobilized enzyme was retained at approximately 70% after 6 days at 52 °C. These results indicate potential applications of nanofibrous-immobilized aminoacylase for industrial production of pure L-theanine.

1. Introduction

The secondary metabolite theanine is a free amino acid that is found in tea leaves and has a broad range of biological activities from the reduction of blood pressure [1] to neuroprotection [2]. Like most amino acids, it is chiral with D/L enantiomers. Although natural theanine exists predominantly in the L-form, small amounts of D-theanine are detected in various tea products [3,4]. Surprisingly, five out of six commercial products marketed as pure L-theanine were actually found to be racemic mixtures [5]. Because D-theanine inhibits L-theanine absorption, racemic mixtures drastically decrease theanine's pharmacological benefits [6]. Although there are several methods for producing theanine [7–10], chemical synthesis is the dominant method for industrial-scale production which generates racemic mixtures that require L-enantiomer separation. Thus, there is a need for production of pure L-theanine for nutraceuticals.

Enzymatic resolution of D/L-amino acids has been shown to be an effective approach to produce pure enantiomers [11–13]. For example, the enzyme aminoacylase (*N*-acyl-L-amino acid aminohydrolase, EC3.5.1.14) has been used to resolve some amino acid enantiomers, such as alanine [14], methionine [15], and phenylalanine [16] from their *N*-acyl derivatives. Pure L-theanine was also produced with the lysate of the aminoacylase-producing fungi *Cunninghamella echinulata* 9980, without purifying the enzyme [17]. Due to previous successes of the enzymatic resolution, here, we also use purified aminoacylase for the resolution of D- and L-theanine.

In industrial conditions, free enzymes are frequently not stable or reusable because of high temperatures, extreme pH values, and solvent toxicity. In contrast, enzyme immobilization enhances enzyme activity, specificity, stability, and reusability [18]. Because of their large surface area, high porosity and pore connectivity, nanofibrous membranes have several advantages as support materials for enzyme immobilization, allowing relatively high enzyme loading, low hindrance for mass transfer and high catalytic efficiency [19]. Nanofibrous membranes formed with the synthetic polymer polyvinyl alcohol (PVA) are non-toxic, water soluble, and have been used for cell and enzyme immobilization [9,20–22]. While PVA possesses a large number of hydroxyl groups that could provide a biocompatible microenvironment for the enzyme, PVA in an aqueous environment swells substantially leading to partial or complete loss of the morphological structure of the nanofibrous membranes. To reduce the swelling, PVA membranes were cross-linked with different chemical agents like glutaraldehyde [23]. Cross-linking of glutaraldehyde with gelatin nanofibers could also improve membrane's thermostability and mechanical properties [24]. In addition, genipin and polyethylene glycol diglycidyl ether as crosslinking agents were also used in nanofibrous membrane fabrication to enhance properties of membrane scaffold and immobilized enzymes [25,26].

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Due to the advantages of nanofibrous membranes, here we investigated the properties and activities of aminoacylases on PVA nanofibrous memberanes. After optimizing the immobilization procedure we moved forward to test and optimize aminoacylase separation D- and L-theanine. This procedure will have much utility in the industrial processes of not only theanine enantiomers but other chiral amino acids.

2. Materials and methods

2.1. Materials

PVA (with a degree of polymerization of 1750 ± 50) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Aminoacylase (ACY, EC3.5.1.14) and D/L-theanine were purchased from Shanghai Hanhong Chemical Co., Ltd. Standard L-theanine was provided by Tokyo Kasei Kogyo Co., Ltd. Glutaraldehyde (GA) (50%, AR) was obtained from Shanghai Aibi Chemical Co., Ltd. Genipin (GP) (MW = 226.23, 98% by high-pressure liquid chromatography) was purchased from Linchuan Zhixin Bio-Technology Co., Ltd. Polyethylene glycol diglycidyl ether (PGDE) was supplied by Yifu Chemical Materials Co., Ltd.

2.2. Preparation of PVA/aminoacylase nanofibrous membranes by electrospinning

PVA (0.7 g) was dissolved in 8.33 mL deionized water at 95 °C over a period of 2 h, followed by stirring with 0.3 g aminoacylase at 25 °C to form a homogeneous solution. Then, a crosslinker (GA, GP, or PGDE) was dropped into the solution at a final concentration of 0.5%, 0.5%, or 2% (v/v), respectively. For electrospinning, the mixture was transferred into a 20-mL syringe equipped with a metal needle that was connected to a high-voltage power supply (GDW-A; Beijing Institute of High Voltage Electrical and Mechanical Technology, Inc., China). A syringe pump was used to control the flow rate at 0.8 mL/h, and electrospinning was performed at 15 kV at a distance of 12 cm between the needle tip and the collector. The membranes were collected for 4 h and then dried overnight at 4 °C under vacuum.

The morphologies of the PVA/aminoacylase nanofibrous membranes were characterized with scanning electron microscopy (SEM, XL-30; Philips, Eindhoven, Netherlands) and Fourier transform infrared spectroscopy (Nicolet 5700, Thermo Fisher Scientific, USA).

2.3. Activity assay of free and immobilized aminoacylase

Aminoacylase activity was measured by determining the production of L-theanine from *N*-acetyl-D/L-theanine *via* the ninhydrin colorimetric method described by Rosen [27], with slight modifications. To resolve theanine enantiomers, a reaction mixture of *N*-acetyl-D/L-theanine (0.02 M, pH 7) and aminoacylase solution (5 mg/mL) was incubated at 52 °C for 30 min. The chromogenic reaction was initiated by addition of 0.2 mL acetate buffer (2 M, pH 5.4) and 0.2 mL ninhydrin solution (1%, w/v) to a 0.2 mL resolution mixture. This mixture was heated in boiling water for 15 min, followed by cooling to room temperature. Afterwards, 0.2 mL was mixed with 3 mL 60% ethanol for the colorimetric measurement at 570 nm. A standard calibration curve was used to determine the aminoacylase activity. One activity unit (U) of aminoacylase was defined as the amount of enzyme required to generate 1 μmol of L-theanine per minute. The retention of aminoacylase activity was defined as the ratio of the activity of the immobilized enzyme to that of the same amount of free enzyme. All activity assays were performed in triplicate.

2.4. Effect of temperature and pH on aminoacylase activity

The effect of temperature on the activities of free and immobilized aminoacylase was examined for the range of 29–72 °C at pH 7. The effect of pH was tested over the range pH 5–9 at 52 °C.

2.5. Stability of immobilized aminoacylase

The thermostability of free and immobilized aminoacylase was determined by measuring the residual enzyme activity, as described above, every other day during storage in phosphate buffer (0.1 M, pH 7) at 52 °C.

The free and immobilized aminoacylase were stored in phosphate buffer (0.1 M, pH 7) at 25 °C and 4 °C for 6 d and 30 d, respectively. The residual enzyme activities were measured at different time intervals.

The reusability of immobilized aminoacylase was studied by measuring the residual enzyme activities repeatedly for 5 times. After each reaction, the enzyme was washed with fresh phosphate buffer (0.1 M, pH 7) and the retention activity was performed under optimal conditions.

3. Results and discussion

3.1. Characterization of nanofibrous membranes

In order to characterize the PVA electrospun nanofibrous membranes with and without aminoacylase (ACY), SEM images were taken (Fig. 1). The nanofibrous membranes have a uniform morphology with an average diameter of ~200 nm (Fig. 1a). The average diameter of the nanofibers increased to ~300 nm when aminoacylase (ACY) was added (Fig. 1b). This is probably because the PVA/ACY mixture becomes viscous after the formation of intermolecular hydrogen bonds between PVA hydroxyl groups and ACY amino groups. From Fig. 1c–e, it is clear that crosslinkers caused the formation of nonuniform nanofibers with different diameters.

FTIR spectra for nanofibrous membranes of pure PVA, PVA/ACY, and PVA/ACY/crosslinkers were acquired to characterize the immobilization of aminoacylase on the membranes (Fig. 2). Pure PVA exhibited a wide band from 3100 to 3600 cm^{-1} that was assigned to O–H stretching and intermolecular hydrogen bonds [28]. All immobilized ACY membranes exhibited a new peak at 1652 cm^{-1}, indicating O=C–NH bonds formed between the enzyme and PVA [20]. Thus, ACY was immobilized on the PVA membranes *via* chemical bonding.

3.2. Effect of temperature and pH on aminoacylase activity

Fig. 3 shows the aminoacylase activity over the temperature range of 29–72 °C and pH 5–9. Under optimal conditions (42 °C and pH 7) enzyme activity retention was maintained at 100%. With immobilization, the ACY activity retention decreased to 49.2–70.5% relative to that of free ACY at 42 °C, most likely due to activity loss during the immobilization process. When the temperature was increased to 52 °C, the free ACY activity decreased to 90% of that at 42 °C. In contrast, all immobilized ACYs had their highest activities at 52 °C. Thus, the optimal temperature shifted from 42 °C for free ACY to 52 °C for immobilized ACY, suggesting that immobilized enzymes could remain in a stable conformation and remain robust at high temperatures.

For free ACY, pH 7 was optimal, whereas pH 8 was optimal for the immobilized ACYs, with 52.9–77.8% activity retention. Thus, the immobilized enzyme required a higher pH for optimal activity than that of the free enzyme. This is most likely