



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.

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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 amino-hydrolase, 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 membranes. 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 $1 \mu\text{mol}$ 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 $29\text{--}72^\circ\text{C}$ 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 $\sim 200 \text{ nm}$ (Fig. 1a). The average diameter of the nanofibers increased to $\sim 300 \text{ nm}$ 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 $29\text{--}72^\circ\text{C}$ 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

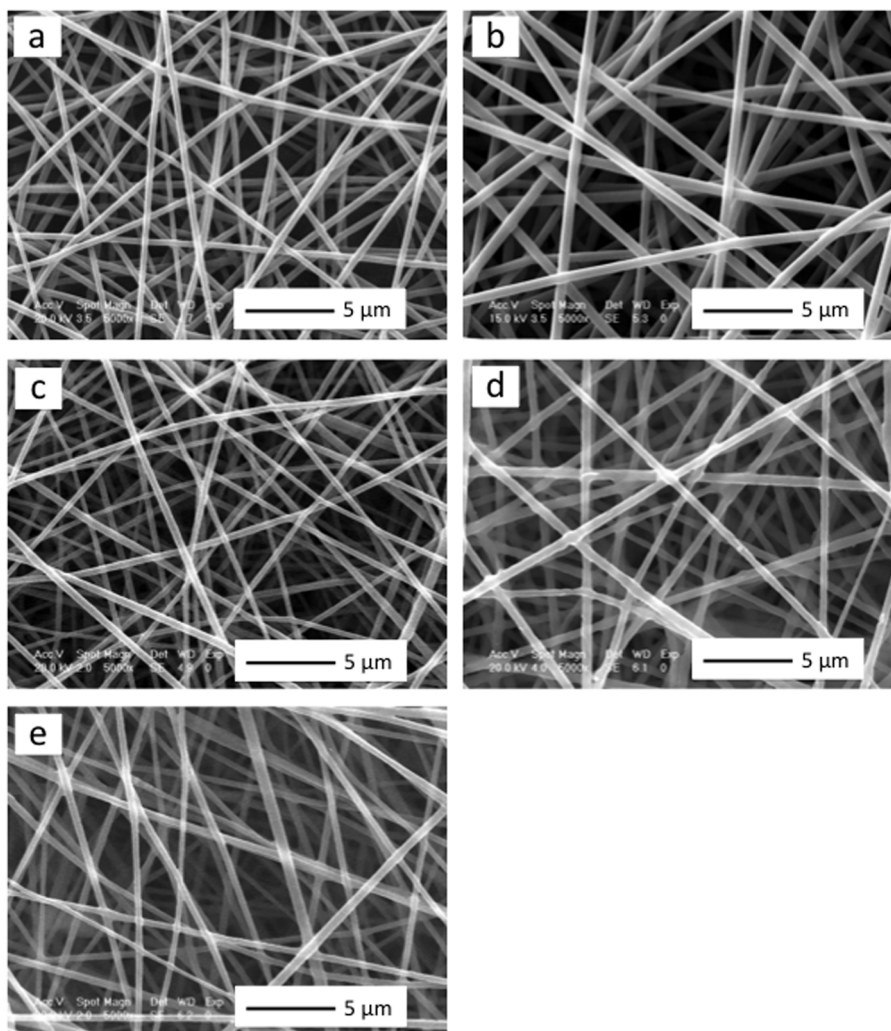


Fig. 1. SEM images of nanofibrous membrane. (a) 7.5% (w/w) PVA, (b) PVA/ACY (70:30) mixture, (c) PVA/ACY (70:30) mixture crosslinked by 0.5% (v/v) GA, (d) PVA/ACY (70:30) mixture with 2% (v/v) PGDE as a crosslinker, (e) PVA/ACY (70:30) mixture crosslinked by 0.5% (v/v) GP.

because of the altered microenvironment between the immobilized enzyme and the solution. In addition, the immobilized enzyme exhibited less sensitivity to changes in pH than did free ACY.

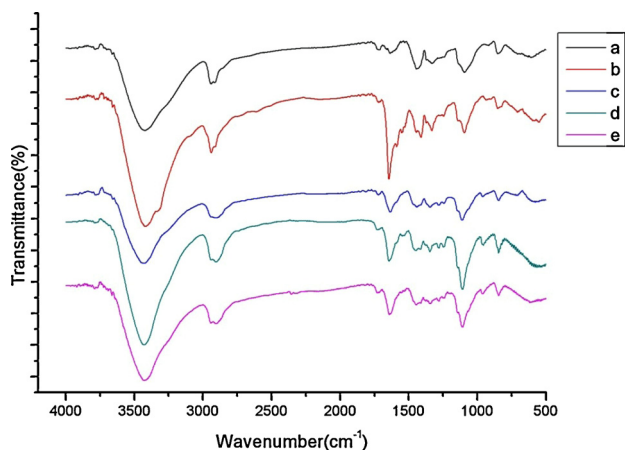


Fig. 2. FT-IR spectra of (a) pure PVA nanofibrous membrane, (b) PVA/ACY nanofibrous membrane, (c) PVA/ACY nanofibrous membrane with GA as a crosslinker, (d) PVA/ACY nanofibrous membrane with PGDE as a crosslinker, (e) PVA/ACY nanofibrous membrane with GP as a crosslinker.

3.3. Activity of immobilized aminoacylase

Enzymatic activities of free and immobilized aminoacylases for the resolution of D- and L-theanine under optimal conditions are shown in Table 1. To improve the retention of enzyme activity during electrospinning, three crosslinkers GA, GP, and PGDE were added separately to form the PVA/ACY nanofibrous membranes. The activity retention varied, with residual values over the range of 52.9–77.8%. Overall, the K_m values of the immobilized aminoacylases were two to three times higher than that of the free enzyme (3.6 mM). With immobilization, the K_m values of enzymes usually increase, as reported previously [29,30], indicating a decrease of affinity between the immobilized enzyme and the substrate after immobilization. This may be caused by structural changes of immobilized aminoacylase that provide less accessibility of the substrate to the active catalytic site.

3.4. Stability of immobilized aminoacylase

Thermostability of immobilized ACY at the optimal reaction temperature of 52 °C was investigated. As shown in Fig. 4, the activity of free ACY decreased sharply compared with that of the immobilized ACY. The free ACY lost ~70% of its activity over 2 days and only 10% of the activity was left after 6 days in storage. However, the immobilized enzyme retained a relatively high activity,

Table 1
Activity of free and immobilized ACY under optimal reaction conditions.

Sample	Temperature (°C)	pH	Specific activity (U g ⁻¹ protein)	Activity retention (%)	K _m (mM)
Free ACY	42	7.0	127.7	100	3.6
PVA + ACY + GA	52	8.0	67.6	52.9	10.7
PVA + ACY + GP	52	8.0	99.4	77.8	6.4
PVA + ACY + PGDE	52	8.0	91.8	71.9	7.2

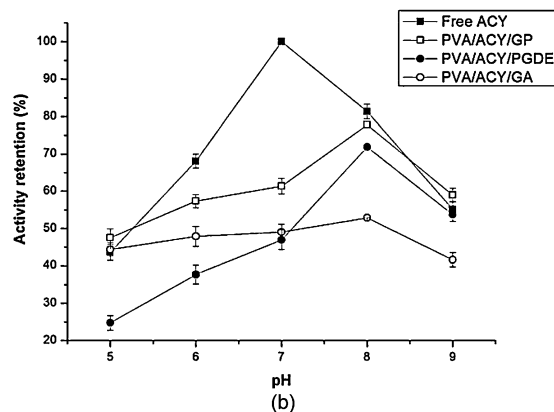
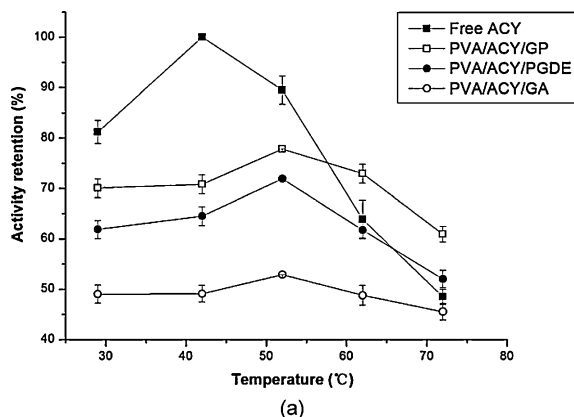


Fig. 3. Effect of temperature (a) and pH (b) on free and immobilized aminoacylase activity.

with a final value of ~70%, over 6 days at 52 °C. The enhanced thermostability of immobilized ACY probably occurs because of covalent bonding between the enzyme and the supports that prevent conformational alteration and denaturation of the enzyme at high temperatures. This effect has also been shown for other immobilized enzymes [31,32].

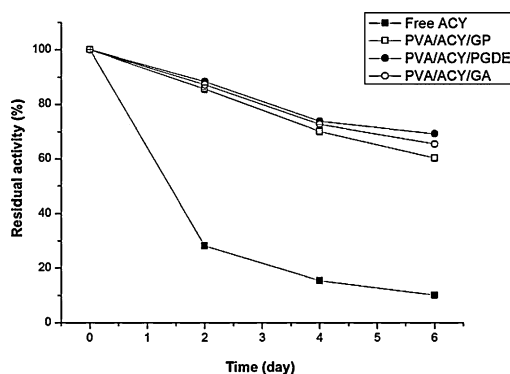


Fig. 4. Thermostability of free and immobilized aminoacylase.

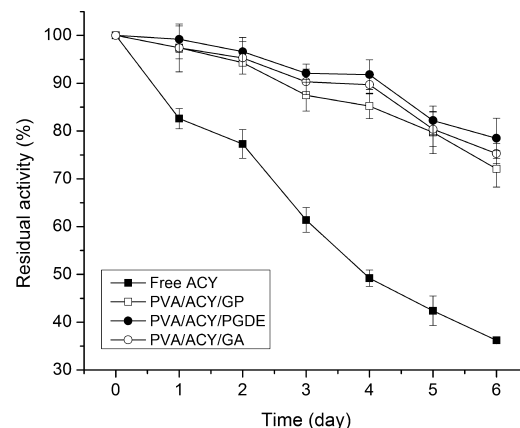


Fig. 5. Storage stability of free and immobilized aminoacylase at 25 °C.

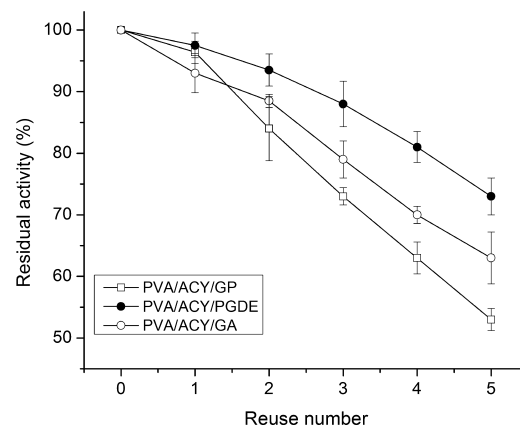


Fig. 6. Reusability of immobilized aminoacylase.

Fig. 5 shows the storage stability of immobilized ACY under 25 °C for 6 days. Clearly, immobilized ACYs remained much higher residual activities of ~70% after 6 days than the free ACY activity dropped significantly to ~30%. This result indicated that the storage stability of immobilized ACY was better than that of free enzyme, which might be attributed to the immobilization of enzyme to a matrix. While after 30 days of storage at 4 °C, immobilized and free ACY could keep about 90% and 82% of their original activities, respectively.

The reusability of immobilized ACY was examined and the result indicated that all immobilized ACYs retained over 50% of their initial activities after five reuses (Fig. 6). This reusability is an advantage for the continuous use of the immobilized enzyme in industry applications.

4. Conclusions

In this study, the enzyme aminoacylase was immobilized onto PVA nanofibrous membranes that were produced via electrospinning. For the immobilized enzyme, the optimal reaction temperature was 52 °C and the optimal pH was 8. Additionally, the

immobilized aminoacylase was relatively less sensitive to changes in temperature and pH than the free enzyme. Under these optimal conditions, the K_m values of the immobilized aminoacylases increased by a factor of two to three times that for the free enzyme (3.6 mM), while a high residual activity could be maintained longer (up to 6 days) than that of the free aminoacylase. In conclusion, robust aminoacylase immobilized on nanofibrous membranes has potential applications in the production of pure L-theanine.

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