Efficient Preservation of Acetylcholinesterase at Room Temperature for Facile Detection of Organophosphorus Pesticide

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A simple and inexpensive strategy is reported to facilitate the detection of an organophosphorus pesticide by acetylcholinesterase (AChE). Pullulan is able to preserve AChE at room temperature, but the activity of conserved AChE varies significantly depending on the time, stir and volume of solution to dissolve it. The reason is that AChE entrapped in pullulan tablet remains in an inactive state to avoid denaturalization and deactivation. There is a reactivation process to gradually recover the enzyme activity during dissolution of the tablet. Stirring would interrupt this procedure and lead to a loss of enzyme activity. Dissolution of the tablet for 5 min with a volume of 15 μ L could facilitate full recovery of AChE activity. The feasibility of activated AChE for organophosphorus pesticide detection was evaluated using malaoxon. These results contribute to the understanding of preservation mechanism by pullulan and the development of easy-to-use enzyme assays.

Keywords Pullulan, preservation, reactivation, enzyme activity, acetylcholinesterase, organophosphorus pesticide

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Introduction

Enzymes are used in many fields including clinical diagnosis, food quality control and environmental monitoring.¹⁻⁶ Acetylcholinesterase (AChE) is an enzyme related to cognitive and neuromuscular processes. AChE is sensitive to organophosphorus pesticides,⁷⁻¹¹ and it shows attractive potentials in the on-site detection of pesticide residuals due to the simple and rapid analysis process. However, the preservation of AChE poses an obstacle for this purpose. Enzymes are instable biocatalysts, and their activity loss in ambient temperature is a severe problem.^{4,12,13} Therefore, it is essential to improve the stability of the enzyme during transportation and storage. Cryopreservation is a conventional, but costly, method. In addition, a switch between the working and the storage temperature leads to repeated thawing and freezing of enzyme, which is harmful to the activity.¹⁴ Thus, preservation of the enzyme at room temperature is attractive from both economic and practical points of view, but it is challenging due to the lack of suitable material for reliable preservation of the labile enzyme.

Pullulan is a biopolymer produced by the *Aureobasidium pullulans*. It is often considered to be an intermediate between amylose and dextran structures. Pullulan is soluble in water, odorless, tasteless, nontoxic, and biodegradable.^{15,16} It shows attractive applications in drug deliveries and preservation of

fruits and bacterias.^{14,17-20} Besides, pullulan offers a facile method for the storage of labile reagents such as biomolecules and oxygen-sensitive species.^{21,22} The process merely involves a mixture of the enzyme with a pullulan solution and a following dry process. The enzyme entrapped in the pullulan tablet can be left at room temperature for several months without loss of activity. Therefore, pullulan greatly facilitates the preparation, preservation and application of bioassays.

Although pullulan exhibits appealing peculiarity, the enzyme behavior in a pullulan tablet is inadequately understood, which hinders the application of pullulan in enzyme assays. We find a unique property of AChE entrapped in pullulan tablets. The activity of preserved AChE varies significantly, and can be very poor occasionally, which is undesired in the usage of a bioassay. Specifically, the enzyme remains in an inactive form in the tablet. There is an important process when the tablet dissolves in water, which exerts a crucial influence on the performance of the enzyme. In this stage, the inactive enzyme restores its activity. Incorrect treatment of the tablet would cause a serious reduction of the activity, resulting in unreliable results. Therefore, a suitable reactivation of the entrapped enzyme is a prerequisite for the application of pullulan.

In this work, we demonstrated the effect of different treatments of the tablet on the enzyme activity and an appropriate way to fully reactivate AChE. The parameters to release AChE from the tablet including the amount of water, time, and stir were explored to disclose the reactivation process. The enzyme showed high and stable activity after reactivation, and we developed a facile method to detect pesticides using the AChE tablet and a home-made screen-printed silver electrode.

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Pullulan meets the demand for the simple, effective and economic preservation of an unstable enzyme. This study reveals the properties of AChE protected by pullulan and addresses the undesired variation of enzyme activity which might occur in analyses, contributing to the development and application of easy-to-use enzyme assays.

Experimental

Reagents and chemicals

Pullulan was purchased from Aladdin. AChE (from *electrophorus electricus*, composition: protein $\geq 60\%$), acetylthiocholine chloride (ATCl), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma-Aldrich. Malaoxon was provided and certified by Agro-environmental Protection Institute, the Ministry of Agriculture, China. Silver ink (Electrodag 427SS) and insulating ink (Electrodag 452SS) were obtained from Acheson Co., Ltd. (USA). A silver stain kit was obtained from Biosharp (Hefei, China). All other reagents were of analytical grade and were used as received. MilliQ water ($18.2 \text{ M}\Omega \text{ cm}^{-1}$) was used throughout. All the measurements were performed in 0.01 M PBS unless otherwise stated.

Apparatus

Spectrophotometry was performed on a 96-well microtiter plate by SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA). The instrument for electrophoretic analysis was a DDY-6C (Beijing Liuyi, Beijing, China). Electrochemical measurement was carried out using a CHI440 electrochemical workstation (CH Instruments, USA). The conventional three-electrode system was used. An Ag/AgCl electrode and a platinum wire auxiliary electrode were used as reference and counter electrodes, respectively. The working electrode was a screen-printed silver electrode fabricated using an automatic screen printer 124 (Z-C3050A, Zheng Ting Screen Printing Machine Co., Ltd., Shanghai, China).

Fabrication of AChE tablet

Pullulan (12%, w/v) was mixed with AChE (250 U/mL) at a ratio of 40:1. Aliquots of 15 μL suspension were casted on a plastic sheet, which was then sealed in an air-tight container containing silica desiccant beads and dried at room temperature. The formed AChE tablets were stored at room temperature.

Activation of AChE tablet

AChE entrapped in the tablet remains in an inactive state. Activation is an essential process prior to use. A tablet was added into 15 μL water, and the tablet would gradually dissolve. This process took 5 min and allowed the AChE to rejuvenate. It should be noted that no stirring is allowed during this process.

Activity test

The activity of AChE was examined by Ellman's method.²³ The principle is to detect thiocholine, the catalytic product of AChE. Firstly, the activated AChE was transferred into a glass vial containing 10 mL PBS. DTNB (100 μL , 0.2 M) and ATCl (50 μL , 0.1 M) was added. Then, an aliquot of 200 μL solution was added into the SpectraMax M5 microtiter plate. The absorbance at 412 nm was monitored for 5 min, and the increase reflected the activity of AChE.

Electrophoretic analysis

An electrophoretic analysis of AChE was performed with the non-denaturing polyacrylamide gel electrophoresis (PAGE)

Fig. 1 Initial viability of AChE and residual activities after storage at room temperature for one day under different conditions: in water, dried and in pullulan tablet ($n = 3$).

using a buffer of 37.6 mM Tris and 40 mM glycine, and gels containing 3.125 and 8% acrylamide in stacking and separating gels, respectively.²⁴ AChE on the electrophoresed gel was visualized by staining the gel with a mixture of 5 mg ATCl, 6.5 mL 0.1 M PBS (pH 6.0), 0.5 mL 0.1 M sodium citrate, 1 mL 30 mM CuSO_4 , 1 mL water, and 1 mL 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$, which was based on the hydrolysis of ATCl and the generation of an insoluble copper complex.²⁵ Staining for the total protein was conducted with the silver staining kit.

Detection of malaoxon

Amperometry was used to evaluate the performance of the AChE tablet for the detection of malaoxon, an organophosphorus pesticide. The working electrode was a home-made screen-printed silver electrode. An Ag/AgCl electrode and a platinum wire auxiliary electrode were used as the reference and the counter electrodes, respectively. Fabrication of the screen-printed silver electrode was similar to that of our previous work.²⁶ The substrate was cleaned by ethanol and water. Then silver ink was printed on the substrates and heated at 120°C for 30 min to evaporate the solvent. After that, insulating ink was printed and solidified by ultraviolet light at 254 nm. The working area of the screen-printed electrode was calculated to be 0.025 cm^2 .

For detection, the activated AChE was added into 10 mL PBS containing known concentrations of malaoxon. Then, incubation was performed for 15 min to ensure the inhibitory effect on AChE. ATCl (50 μL , 0.1 M) was added after incubation. Amperometry was conducted at 0.08 V, and the increase of current in the following 5 min was used to calculate the inhibition of malaoxon on AChE:

$$\text{Inhibition (\%)} = (I_0 - I_t)/I_0 \times 100\%, \quad (1)$$

where I_0 was the increment without malaoxon, and I_t was the increment at the presence of malaoxon.

Results and Discussion

Preservation of AChE

To illustrate the activity loss of AChE at ambient temperature and the ability of pullulan to stabilize AChE, AChE solutions were prepared in water and a pullulan solution (12%, w/v),

Fig. 2 (A) Scheme of two different methods to reactive AChE: (a) pullulan tablet was directly added into 10 mL PBS, and stir was performed to accelerate the dissolution; (b) the tablet was naturally dissolved without stirring (defined as pre-dissolution), and was then made to a volume of 10 mL. (B) Effect of pre-dissolution on the AChE activity ($n = 3$). Pre-dissolutions of 0, 1, 5 and 10 min were performed in 10 mL and 15 μ L PBS, respectively. No stirring was carried out during the pre-dissolution. (C) Non-denaturing PAGE analysis of AChE. Lane 1: AChE in pullulan solution, stained for AChE activity by hydrolysis of ATCl and formation of an insoluble copper complex. Lane 2 and 3: AChE tablet dissolved with and without stirring, stained for total protein by the silver stain kit.

respectively. They were then dried at room temperature. Figure 1 shows their activity after 1 day, and the residual activities varied significantly. The activity of AChE kept in water only remained 57.6%, and its dried form presented an activity as low as 30%. In contrast, the enzyme protected in the pullulan tablet exhibited a high livability of 95.5% and hovered at this level after one month. Clearly, AChE was instable at room temperature and pullulan significantly stabilized AChE.

Behavior of AChE tablet

We observed a strange phenomenon about AChE tablet: sometimes, AChE showed only 40% or even lower activity, and the decrease was not due to the loss of activity during storage. The activity of AChE depended on the process to release the AChE encapsulated in the tablet. Although pullulan is soluble, it still needs a few minutes to fully dissolve the tablet. The appropriate method to release AChE is natural dissolution in a small volume of solution prior to use, which allows a gradual recovery of enzyme activity. As shown in Fig. 2A, the tablet was directly put into the working solution, 10 mL PBS, and was stirred to accelerate the dissolution (method a). This method was found to do harm to the activity of the encapsulated enzyme, because only 39% activity was observed when the solution was stirred upon adding the tablet (Fig. 2B). The activity increased with time and reached 60.4% when the tablet naturally dissolved for 5 min before stirring the solution. The natural dissolution procedure (defined as “pre-dissolution”) is beneficial for the reactivation of AChE. In addition, pre-dissolution in a smaller volume is also preferable for the reactivation of AChE (method b). The tablet naturally dissolved in 15 μ L PBS, and was then made

to a total volume of 10 mL. By doing this, the enzyme presented a higher activity. For the 0-min-pre-dissolution, 15 μ L PBS was added and immediately mixed with the tablet by a micropipettor. Then, the blended solution was transferred into 10 mL PBS, and the activity was 70%. When the pre-dissolution was performed for 5 min, AChE fully rejuvenated with the activity reaching 100%. Non-denaturing PAGE was used to examine whether the loss in activity was due to the aggregation of protein caused by stirring. As shown in Fig. 2C, the same bands for the staining of total protein suggested that stirring did not lead to aggregation of proteins (Lanes 2 and 3). According to activity staining (Lane 1), only one band corresponded to AChE, and other bands could be attributed to the low purity of the enzyme (total protein $\geq 60\%$). These results reveal the existence of a reactivation process for AChE. This procedure is related to dissolution of the pullulan table, *i.e.* pre-dissolution. The appropriate method to use the AChE tablet is dissolution in small volume of water prior to use.

Assumption and verification

Generally, the activity of an enzyme is related to its conformation. Increased flexibility of the enzyme molecule leads to higher activity.²⁷ We thus assume that AChE in the tablet is in an inactive form. Pullulan is capable to ensure the reversible conversion of enzyme configuration, and the reactivation procedure depends on the dissolution of pullulan. Specifically, pullulan becomes solidified when water gradually evaporates in drying process, during which the AChE is entrapped into the pullulan and becomes inactive. When the tablet redissolves, the trapped AChE is gradually released,

Fig. 3 Activities of AChE tablets with 1-min-pre-dissolution in 15, 200 µL, 2.5 and 10 mL PBS ($n = 3$).

Fig. 4 0-min-pre-dissolution in 15 µL and 10 mL solution. Activities of AChE tablets were monitored at 0, 10, 25 and 50 min ($n = 3$).

experiencing a conformational transition and reviving its activity. If this process is disturbed, the AChE would not fully acquire its activity.

A series of experiments were conducted to validate this assumption. Firstly, pre-dissolution was performed in different amount of PBS for 1 min. Then, the solutions were stirred and the volumes were brought to 10 mL; the activities are shown in (Fig. 3). The AChE tablet pre-dissolved in 15 µL PBS exhibited 96% activity. The activity of enzyme decreased with the increase of solution volume for the pre-dissolution, and the AChE tablet pre-dissolved using 10 mL PBS only presented 54% activity. Therefore, pre-dissolution in too much solution is harmful to the activity of AChE, and the optimal volume is 15 µL.

In another experiment, 15 µL and 10 mL PBS were used for the 0-min-pre-dissolution of AChE tablets, respectively. Solutions were stirred upon adding the tablets to dissolve the tablets, and then the activities were measured at 0, 10, 25 and 50 min (Fig. 4). The AChE activated by 10 mL PBS showed 39% activity at the beginning, and the activity decreased with time in the following 50 min. As for the AChE activated by 15 µL PBS, the activity was 70% at first, and was 66% in 50 min later. Obviously, the activity of AChE showed no improvements with time. This phenomenon was attributed to

Fig. 5 Residual activities of AChE tablets after incubation at 25, 50, 80 and 100°C for 1 h ($n = 3$). Diamond (◆), square (■), triangle (▲) and dot (●) were the AChE in pullulan tablet, in pullulan solution, naturally dried and in water, respectively.

the interruption of pre-dissolution by stirring. This implied the existence of the reactivation procedure, and the activity of AChE stopped growing when the reactivation process was disturbed.

Studies concerning in organic solvent show a similar phenomenon,^{28,29} which is attributed to water molecules. There is a gradual hydration process of enzyme that involves three stages. Firstly, the ionizable groups are hydrated. Then, these polar patches are surrounded by water clusters. After that, water covers the less interacting surface elements. The enzyme exhibits activity when it reaches total mobility. A change in protein mobility occurs in the second step, and the activity can keep rising even when the enzyme is fully hydrated. This theory may be suitable for the present study as well. Pre-dissolution of AChE table can be considered as the gradual hydration process of the enzyme, involving the interaction between the AChE and water molecules. Appropriate hydration process is natural dissolution of the AChE tablet in 15 µL PBS to rejuvenate the enzyme step by step. Both the larger volume of the solution and stirring would accelerate the dissolution of pullulan and the contact between AChE and water molecules to disturb the natural renaturation process. As a result, the AChE could not revive to the appropriate conformation, and showed limited biocatalysis ability.

Given the above results, it is certain that AChE remained in an inactive form in the tablet, and there was a reactivation process during the dissolution of pullulant tablet. This may also help to reveal the mechanism for the preservation of AChE at room temperature. Water is essential for the activity of enzyme.²⁸ It ensures the flexibility of enzyme by forming bonds with functional groups present on the protein. In the absence of water, these groups interact with each other and turn into an inactivated conformation. The protein structure becomes rigid and results in significantly enhanced thermostabilization. In a dry pullulan tablet, AChE may experience a similar process, loses water and become rigid. Improved rigidity of AChE offers outstanding protection against thermal denaturation which results in loss of activity. Therefore, the thermal stability of AChE was analyzed to validate this assumption. The dry AChE, AChE dissolved in water, AChE preserved in pullulan tablets and AChE dissolved in the pullulan solution were placed in an oven at different temperatures for 1 h and their residual activities were presented in Fig. 5. AChE in water and in pullulan solution

Fig. 6 Detection of malaoxon by amperometry ($n = 3$). Insert shows the amperometric curves in (a) 0, (b) 10, (c) 20, (d) 40 and (e) 80 ppb malaoxon. Potential: 0.08 V vs. Ag/AgCl. ATCl: 50 μ L, 0.1M.

lost activity totally after heating at 50°C. The naturally dried AChE was still active with residual activities of 81.3% at 50°C and 49.4% at 100°C. As for the AChE preserved in pullulan tablet, there was only a slight fluctuation when the temperature increased. Its activity maintained at 98.6% even when the temperature reached 100°C. Thus, the ability of pullulan to preserve AChE was related to water, and only dry pullulan could stabilize AChE. The mechanism for the preservation is that the rigid structure of dry enzyme improves its thermostabilization, and dry pullulan provides outstanding protection of dry AChE against thermal denaturation. In addition, irreversible inactivation may arise from a number of covalent processes such as deamidation, peptide hydrolysis and cystine decomposition. But these processes are extremely slow in the water-deficit pullulan tablet. Besides, pullulan is an excellent oxygen barrier.²⁰ Therefore, AChE in the pullulan tablet is in an inactive state with excellent resistibility to harsh surroundings. The inactive AChE becomes active when the pullulan tablet dissolves again, and pre-dissolution is essential for the gradual recovery process.

Application in pesticide detection

The performance of AChE preserved at room temperature in pullulan tablet was evaluated by detection of malaoxon, a commonly used organophosphorus pesticide.³⁰ The principle is based on the inhibition on enzyme and electrochemical oxidation of thiocholine, the catalytic product of AChE. A screen-printed silver electrode was used as the working electrode for the measurement, for the silver electrode showed high sensitivity and selectivity to thiocholine, and could detect thiocholine at a low potential to avoid the effect of interferences.^{7,23} For detection, the tablet was naturally dissolved for 5 min with the volume of 15 μ L to recover the activity of AChE. Then, it was added into 10 mL PBS containing different concentrations of malaoxon, and incubated for 15 min. The substrate, ATCl, was introduced to generate thiocholine after the incubation. Figure 6 presents the results obtained in 0 to 80 ppb malaoxon. As shown in the inset, the amperometric curves increased upon the addition of the substrate, but the increments decreased with the concentration of malaoxon due to the inhibition on AChE. According to Fig. 6, 10 ppb malaoxon

showed an inhibition of 21%. The limit of detection is usually defined as the concentration with an inhibition efficiency higher than 5%.^{23,31} The maximum residue limits (MRL) of organophosphorus pesticide in the European Union pesticides database was 10 μ g L⁻¹.^{32,33} Therefore, the AChE tablet was capable to meet the requirement for pesticide analyses. Commonly, AChE has to be preserved in a refrigerator to extend the shelf life. In contrast, the pullulan achieve preservation of AChE at room temperature, presenting promising potential for the development and application of AChE-based pesticide analyses.

Conclusions

The activity of AChE entrapped in the pullulan tablet was analyzed. An inappropriate method to dissolve an AChE pullulan tablet would lead to a severe loss of enzyme activity. There is a reactivation process when the AChE tablet redissolves. It involves the release of AChE from the pullulan tablet and gradual hydration of AChE, thereby increasing the activity of an enzyme. The activity cannot fully recover if this process is disturbed. Pullulan offers a facile, effective and economic strategy to preserve enzyme. This research offers a more detailed understanding on the theory of enzyme preservation by pullulan, which is essential for the application of pullulan and the development of easy-to-use enzyme assays.

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