Research Note

Optimizing the preparation conditions and characterization of cross-linked enzyme aggregates of a monoamine oxidase

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Abstract Monoamine oxidases are useful in determination of biogenic monoamines, particularly histamine and tyramine. In this study, cross-linked enzyme aggregates (CLEAs) technique was applied to improve the stability of a monoamine oxidase from *Arthrobacter aurescens* (AMAO). Under the optimized condition (50% of saturated ammonium sulfate, 5 mM glutaraldehyde, 2.0 mg/mL AMAO, 4 h-cross-linking at 25°C, pH 8.0), CLEAs-AMAO was recovered with a yield of 82% based on the subjected total enzyme activity. Both pH activity and stability at alkaline pHs of CLEAs-AMAO were significantly improved compared to those of the free enzyme, resulting in the shift of optimum pH to pH 8.0 and a broader pH profile. The half-life of the CLEAs at 65°C was elongated by 1.7-fold compared to that of the free enzyme, suggesting the thermal stability of AMAO was also improved by the CLEAs formation.

Keywords: biogenic amine, monoamine oxidase, cross-linked enzyme aggregate, pH stability, thermostability

Introduction

Enzyme immobilization techniques have been considered as useful tools to improve enzyme stability. General methods for enzyme immobilizations are the entrapment of enzymes of interest into polymers or attachment of the enzyme on non-catalytic carrier matrices. In spite of the benefits such as enhanced stability in harsh conditions, reusability of the enzymes, and facile separation of product, such immobilization-on-carrier has drawbacks such as reduced yields of the products because of low volumetric activity due to the presence of the noncatalytic mass of the carrier (1). To address these problems, carrier-free immobilizations have been highlighted. The carrier-free immobilizations are based on direct cross-linking of enzymes using a bifunctionalized cross-linker, such as glutaraldehyde, and dialdehydic pectin (1-3). Of the carrier-free immobilizations, cross-linked enzyme aggregates (CLEAs) technology is the most popular strategy, and so far, various CLEAs biocatalysts have been developed and applied to various industries (3).

Biogenic amines (BAs) in fermented foods and marine products are produced by the action of amino acid-specific decarboxylases from either fermented or contaminated microorganisms using free amino acids as the substrates (4-6). Of BAs, two biogenic monoamines, histamine (HisN) from histidine and tyramine (TyrN) from tyrosine, are recognized as BAs to be controlled because they are responsible of allergy-like symptoms (7-9). For such health problems by BAs, it is

necessary to develop quantitative methods to determine the amounts of BAs in foods to ensure food safety and quality. To date, various instrumental analysis methods have been developed (10-12). In spite of the high sensitivity and individual quantification of BAs, these instrumental methods request pre- or post-column derivatizations (10) and expensive instruments. As a rapid and easy assay for HisN in foods, enzyme-linked immunosorbent assay kits are commercially available (13,14), but they are still semi-quantitative. Enzymatic determination of BAs using amine oxidases conducting an oxidative deamination of biogenic monoamines to the corresponding aldehydes with ammonia and hydrogen peroxide has emerged as a rapid and quantitative assay for BAs (15-17). In addition, the amine oxidases have been successfully applied to enzyme-based amperometric biosensors (18-20). To develop biosensors, the stability of the enzymes is one of the most important factors for extended shelf-life of the sensor chips.

Recently, our group developed an assay method for total biogenic monoamine using a monoamine oxidase from *Arthrobacter aurescens* (AMAO), originally termed AMAO2 (16), and applied the enzymatic assay coupled with a peroxidase to determine the total amount of biogenic monoamines in foods such as commercial cheeses and doenjangs (16,17). Here, we subjected the CLEAs technology to improve the stability of AMAO. The optimization process for the preparation conditions of the CLEAs of AMAO (CLEAs-AMAO) was conducted, followed by the characterization of the enzymatic

properties and comparison with the free enzyme.

Materials and Methods

Preparation of AMAO Recombinant *Escherichia coli* BL21 (DE3) carrying pET28-6xHMAO2 was cultured in Luria-Bertani medium (1% [w/v] tryptone, 0.5% [w/v] yeast extract, and 0.5% [w/v] NaCl) supplemented with kanamycin (20 µg/mL). The culture condition for enzyme production and the purification procedure using Ni-NTA affinity chromatography were followed by the previous work (16). The eluted fractions containing the target protein were combined and dialyzed against 100 mM potassium phosphate buffer (pH 7.0).

Activity assay of free AMAO and CLEAs-AMAO The enzymatic activity of either free AMAO or CLEAs-AMAO was determined using the method by Lee and Kim (16). The assay was conducted using 0.1 mM TyrN as the substrate in 100 mM potassium phosphate buffer (pH 6.5). After the initiation of the reaction by the addition of free or CLEAs enzyme, the reaction mixtures were incubated at 55°C for 10 min, followed by boiling for 5 min to terminate the reaction. The amount of H₂O₂ in the reaction mixture was measured using peroxidase assay method. The measurement of absorbance was conducted using a plate reader (VERSAmax™, Molecular devices, Sunnyvale, CA, USA) at a wavelength of 540 nm.

Optimization of condition for CLEAs-AMAO formation To determine the best precipitating agent, 10 mL of different protein precipitating agents, including saturated ammonium sulfate (AS), acetone, acetonitrile, tert-butyl alcohol (t-BtOH), dimethoxyethane (DME), dimethylformamide (DMF), dimethyl sulfoxide (DMSO), ethanol, isopropanol, and methanol, was added into 10 mL of AMAO solution (25 mg/mL). After aggregation with shaking for 1 h at 25°C, add 900 mL of 100 mM sodium phosphate buffer (pH 7.0) to resolubilize AMAO, followed by enzyme assay to select proper precipitating agents. Upon varying the concentration of the chosen agent, the supernatants and the pellets were collected after the aggregation, followed by enzyme assay to determine the minimum concentration of the precipitant. At a fixed concentration of ammonium sulfate for maximum precipitation, glutaraldehyde (GA) concentration was varied in a range from 2–8 mM and incubated at 25°C for 4 h. After the collection of the pellets by centrifugation at 12,000×g for 10 min, the pellet was washed twice and resuspended in 1 mL of phosphate buffer (100 mM, pH 7.0) for the activity assay of CLEAs-MAO using TyrN as the substrate. After determining the optimum GA concentration, the reaction mixtures were incubated at different reaction times of 1–24 h and a fixed GA concentration of 50 mM. Then, the reactions were carried out at various enzyme concentrations ranging from final 0.25 to 2.5 mg/mL at 25°C to identify the optimum enzyme concentration. The pH values of the reactions were varied from pH 3.5 to pH 10 using 100 mM sodium citrate buffer (pH 3.0–6.0), 100 mM potassium

phosphate buffer (pH 6.0–8.0), and 100 mM Tris-HCl buffer (pH 8.0–9.0) for 17 h at 50 mM GA and 2.0 mg/mL AMAO to determine the optimum pH.

Preparative scale production of CLEAs-AMAO At a large scale, CLEAs-AMAO was prepared in a 25-mL flask with shaking at 100 rpm on a large scale. Initially, 20 mg of AMAO was incubated in 10 mL of the reaction buffer (50% of sat. AS, 50 mM GA in 100 mM Tris-HCl buffer/pH 8.0) for 17 h at 25°C for cross linking. After centrifugation, the pellet was washed three times with 3 mL of 100 mM potassium phosphate buffer/pH 8.0 and stored at 4°C for further experiments.

Effects of pH and temperature on the activity of free AMAO and CLEAs-AMAO The optimal temperatures of the free AMAO and the CLEAs-AMAO were determined by measuring the initial reaction rate in the temperature range of 35–65°C in phosphate buffer (50 mM, pH 7.0). The thermostabilities of free AMAO and the CLEAs-AMAO were evaluated as follows: the properly diluted enzymes in 50 mM phosphate buffer (pH 7.0) were incubated at specific temperatures (55, 60, and 65°C) at different time intervals for heat-inactivation. After heat-inactivation, the residual activities were measured in 50 mM phosphate buffer (pH 7.0) at 55, 60, and 65°C. The first-order rate constant of irreversible thermal denaturation (k_d) was obtained from the slope of the plots of $\ln$ (residual activity/initial activity) versus time, and the half-lives were calculated as $\ln 2/k_d$. The optimal pH of the free AMAO and the CLEAs-AMAO was determined using TryN as a substrate in the pH range of 4.5–9.0 at 55°C. The pH buffers listed above were used for pH profiling. Moreover, the pH stabilities of the free AMAO and the CLEAs-AMAO were investigated by measuring the residual activity after incubating at 4°C for 24 h in one of the above buffer systems.

Results and Discussion

Optimization of preparing condition for CLEAs-AMAO First, ten kinds of protein precipitating reagents were tested for the precipitation and activity recovery of AMAO. After precipitating at 50% of each precipitating reagent, followed by recovery by dilution of the buffer, the activity recovery yields of the precipitated AMAO by saturated AS and t-BtOH were almost 100%, whereas those by isopropanol, acetone, and DME were lower than 70% (Fig. 1A). Of two precipitating reagents, the sat. AS solution was chosen. The minimum concentration of the sat. AS solution was determined as 50% at which all used enzyme were precipitated and the precipitated enzyme was fully refolded after removing the ammonium sulfate (Fig. 1B). Based on these results, the basic condition for the CLEAs formation was determined as the system consisting of 50% of sat. AS, 5 mM GA, and 2.0 mg/mL AMAO for 1 h-aggregation and 4 h-cross-linking at 25°C. Upon adding GA with different final concentrations (2–8 mM), the maximum activity of the CLEAs was 58% of the subjected enzyme

Fig. 1. (A) Selection of protein precipitating reagent. The activity of AMAO was measured after aggregation by adding a protein precipitating reagent, followed by renaturation by adding 100 mM Tris-HCl (pH 7.0) buffer for the dilution. The relative activity was based on the total activity of the enzyme used initially. (B) Optimum concentration of ammonium sulfate. The enzyme activities in the supernatant and the pellet after aggregation of AMAO using ammonium sulfate. The supernatant and the pellet were diluted by 10-fold using 100 mM Tris-HCl (pH 7.0) buffer to remove ammonium sulfate.

Fig. 2. Effects of reaction conditions on the yield of CLEAs-AMAO. (A) Glutaraldehyde concentration, (B) cross-linking time, (C) AMAO concentration, (D) pH.

activity at 5 mM of GA (Fig. 2A). Elongation of the cross-linking time yielded a saturation curve with 80% activity of the CLEAs at 17 h (Fig. 2B). The time interval between addition of AS and GA for the aggregation before cross-linking was not as significant as to improve the CLEAs yield (Data not shown). In the case of enzyme concentration, 2.0 mg/mL of AMAO was better than 2.5 mg/mL (Fig. 2C). The pH of

cross-linking yielded a bell-shaped curve with a maximum yield of 87% at pH 8.0 (100 mM Tris-HCl) (Fig. 2D). There was no detection of the enzyme activity in the supernatants after the cross-linking and the washing steps, suggesting that all proteins used formed the CLEAs, but a part of the enzyme was inactivated during the cross-linking. At a preparative scale preparation (1 mL scale), CLEAs-AMAO

Fig. 3. Effect of pH and temperature on activity and stability of free AMAO and CLEAs-AMAO. (A) Activity profile over pH, (B) stability profile over pH, (C) activity profile over temperature, (D) inactivation over time at 55°C (circles), 60°C (triangles), and 65°C (squares). Closed and open symbols represent free AMAO and CLEAs-AMAO, respectively.

was recovered with a yield of 82% and was used for the characterization.

Effect of pH on activity and stability of free AMAO and CLEAs-AMAO

AMAO showed an optimum pH of 6.5 and a rapid decline in the activity in alkaline pHs (16). By contrast, CLEAs-AMAO exhibited a broad pH profile of the activity over pH values (Fig. 3A). The optimum pH was shifted to pH 8.0, and the activities at an alkaline pH (pH 8-10) were much higher than those of the free enzyme. Such high activity of the CLEAs at alkaline pHs would be caused from the stabilization at the pH region. The CLEAs showed higher pH stability than the free enzyme (Fig. 3B). Interestingly, in spite of the shift of the pH profile to the alkaline pH, the activity of the CLEAs at pH of 6.0-8.0 was almost maintained (Fig. 3A). The shift of the optimal pHs and stabilization of the CLEAs at alkaline pHs are often reported in literatures (21-23).

Effect of temperature on activity and stability of free AMAO and CLEAs-AMAO

Although both free AMAO and CLEAs-AMAO had the same optimal temperature (55°C), the activity of the CLEAs at 70°C was significantly higher than that of the free enzyme (Fig. 3C). Such higher activity at high temperatures might be caused by the improvement of the thermostability of the CLEAs than that of the

free enzyme (Fig. 3D). The half-life of the CLEAs at 65°C was 40 min, whereas that of the free enzyme was 23 min, which means that the thermostability of the CLEAs at 65°C was improved by 1.7-fold compared to that of the free enzyme. Such thermostabilization of CLEAs-forms of other enzymes has been observed in many previous studies (22,23), and the stabilization of the enzyme conformation by rigidification of the enzyme structure through multipoint covalent attachment has been expected as the main cause for the stabilization of CLEAs (24,25).

In conclusion, the condition for the formation of CLEAs-AMAO was optimized with a yield of 83%. The CLEAs exhibited higher activity and stability at the alkaline pH region than the free enzyme. The optimal temperatures of the free AMAO and CLEAs-AMAO did not differ, whereas the improvement in the thermal resistance of CLEAs-AMAO was recorded. Currently, the application of AMAO to a biosensor system is progressing. To achieve successful performance of the biosensors equipped with AMAO, the stability of the enzyme is very important for resistance against the enzyme denaturation during chemical immobilization on the biosensor chip as well as the shelf-life of the chip during circulation. The findings of this study may provide the stability-improved amine oxidase as a biological element suitable for biosensors to determine BAs.

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