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Short communication

Synthesis and continuous catalytic application of alkaline protease nanoflowers–PVA composite hydrogel

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ABSTRACT

This paper reports a facile approach for the synthesis of alkaline protease nanoflowers–poly(vinyl alcohol) (PVA) composite hydrogel (NPCH) from alkaline protease–$Cu_3(PO_4)_2{\cdot}3H_2O$ nanoflowers and PVA hydrogel through freezing–thawing. During continuous catalytic application, the PVA hydrogel network protected alkaline protease–$Cu_3(PO_4)_2{\cdot}3H_2O$ nanoflowers from damage and helped maintain enzymatic activity at high levels. The enzyme that had been immobilized in nanoflowers and NPCH demonstrated 1027% and 605% higher activity than the free alkaline protease derived from *Bacillus licheniformis*. When used in cyclic catalysis, NPCH exhibited better reusability than nanoflowers and was easily separated from the product.

1. Introduction

A major goal in chemistry is the design of environmentally sustainable processes [1]. Enzymatic biocatalysis is a highly suitable approach toward this goal given its ability to performnumerous chemical biotransformations with astonishing catalytic efficiency and substrate specificity under mild conditions [2–5].

Proteases are notable for their applications in protein hydrolysis in the food industry. The hydrolytic specificity of these enzymes have promoted the continuous development of novel industrial processes. However, protease autolysis encounters the problem of contamination given that one protease molecule becomes the substrate for another protease molecule in this process [6–8]. Enzyme immobilization is an effective method for overcoming the disadvantages posed by free enzymes [9–12]. The main aim of immobilization is to obtain stable and reusable enzymes with resistance to different environmental factors and increased selectiveness and specificity [13–17]. Unfortunately, in most cases, immobilization induces slight distortions in the enzyme structure; these distortions may alter the final properties of the enzyme and ultimately cause activity loss [18, 19]. An encouraging breakthrough in enzyme immobilization is the synthesis of hybrid enzyme–inorganic nanoflowers with enhanced activities and stabilities [20]. In contrast to conventional immobilization methods, the synthesis of hybrid nanoflowers is simple, green, and consumes low amounts of energy. Various enzymes for aqueous-phase catalysis are selected as the organic components of hybrid nanoflowers [21–23]. However, the fragility of hybrid nanoflowers limits their use. The fragile structure of hybrid nanoflowers would be destroyed during catalysis and separation. Therefore, the catalytic activity of hybrid nanoflowers will decrease after several use cycles [24, 25]. Hybrid nanoflowers have to be immobilized into a solid network frame to maintain their structure and catalytic activity. Meanwhile, the network frame can ensure that the hybrid nanoflowers are fully exposed to the substrate and can be removed directly from the reaction fluid without damaging the hybrid nanoflowers.

Hydrogels are three-dimensional polymeric networks and exhibit advantages in diverse biomedical applications given their good biocompatibility, functionality, and mechanical properties [26, 27]. Hydrogel networks can be prepared through physical or chemical cross-linking. These networks allow reactant and product diffusion while preventing biocatalyst migration to the bulk medium [28]. Poly(vinyl alcohol) (PVA) hydrogel is a physically cross-linked hydrogel that can be prepared through freezing–thawing cycles [29, 30]. The preparation of PVA hydrogel does not require the use of factors with adverse effects on enzymes. Thus, it can be used as good carrier for hybrid nanoflowers.

In this study, we prepared alkaline protease–$Cu_3(PO_4)_2{\cdot}3H_2O$ nanoflowers with high catalytic activity. We then immobilized the hybrid

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nanoflowers in PVA hydrogels through freezing–thawing. This method is preferable for immobilizing hybrid nanoflowers into PVA hydrogels because it can prevent damage to nanoflowers and maintain enzymatic activity at high levels. The substrate freely passes through the PVA hydrogel network and can easily come into contact with the nanoflowers. Moreover, the nanoflowers-PVA composite hydrogels can be processed into various physical forms that can facilitate enzyme separation and enable enzyme recycling. The nanoflower-containing composite hydrogels have considerable potential applications in food industry and environmental chemistry.

2. Experimental

2.1. Preparation of alkaline protease -$Cu_3(PO_4)_2{\cdot}3H_2O$ hybrid nanoflowers

Alkaline protease (activity ≥200,000 u/g) derived from fermentation of *Bacillus licheniformis* (material information was provided in supporting information). According to the method in the literature [31]. alkaline protease-$Cu_3(PO_4)_2{\cdot}3H_2O$ hybrid nanoflowers were synthesized as follow: 6 mL of aqueous $CuCl_2$ solution (120 mM) was added to 900 mL of PBS (10 mM, pH 7.4) containing 90 mg of alkaline protease, and the mixture was incubated at 25 °C for 3 days. The obtained blue precipitates were collected by centrifugation (3500 rpm for 5 min) and washed for three times by deionized water. The blue precipitates were dried into the powders by using the technology of the vacuum freeze-drying.

2.2. Preparation of the NPCH

10 wt% PVA aqueous solution (2.5 g) and alkaline protease-$Cu_3(PO_4)_2{\bullet}3H_2O$ nanoflowers (2.5 mg) were mixed under stirring. And then, the mixture was frozen at −20 °C for 4 h and thawed at 4 °C four cycles to form the 1 wt% nanoflowers-PVA composite hydrogels (NPCH1, the mass ratio of nanoflower to PVA is 1 wt%). The NPCH2, NPCH3, NPCH4 and NPCH5 were prepared as the same procedure. The nanoflowers were all immobilized in the hydrogel due to the high crosslinking density. And the mass ratio of nanoflower to PVA of NPCH2, NPCH3, NPCH4 and NPCH5 were 2 wt%, 3 wt%, 4 wt% and 5 wt%, respectively and their feed ratios were provided in supporting information (Table.S1).

2.3. Weight percentage of alkaline protease in hybrid nanoflowers

For determining the enzyme content (Table S2), the dried hybrid nanoflowers were calcined at 700 °C for 2 h by muffle furnace. After removing the organic enzyme in hybrid nanoflowers and water of crystallization, the remaining part was inorganic metal salt $Cu_3(PO_4)_2$ (green powders). The calculation of weight percentage of enzyme in nanoflowers was given as the following equation:

$$W = \frac{G_N - G_0}{G_N} \times 100\% \tag{1}$$

where W is the weight percentage of alkaline protease (%), G_N is the weight of hybrid nanoflowers, and G_0 is the weight of $Cu_3(PO_4)_2{\cdot}3H_2O$.

2.4. Determination of enzymatic activity and reusability

The enzymatic activity of alkaline protease, alkaline protease-$Cu_3(PO_4)_2{\cdot}3H_2O$ nanoflowers and NPCH were measured by using N-benzoyl-L-arginine ethylester (BAEE) as substrate under the same conditions (25 °C, pH 7.4). Alkaline protease could catalyze the hydrolytic cleavage of the easter linkage in BAEE and produce N-α-benzoyl-L-arginine (BA) which can be detected at 253 nm by UV spectrometer (Detailed process was in supporting information). The calculation of enzymatic activity was shown in the following equation:

$$U = \frac{\Delta A_{253}}{0.001{\bullet}G_E{\bullet}T} \tag{2}$$

where U is the enzymatic activity of alkaline protease (U/mg), $\triangle A_{253}$ is the absorbance changed at 253 nm, G_E is the amount of alkaline protease (free alkaline protease, alkaline protease in nanoflowers or NPCH, mg), T is the reaction time (min).

In addition, the reusability of nanoflowers and NPCH1 was also evaluated. 1 mL of 30 mM DTT was added to 2 mL of 10 mM PBS (pH 7.4) containing nanoflowers (2.5 mg) or NPCH1 (2.5 g). And after 10 min, 3 mL of BAEE (2 mM) was added to the above mixture. This reaction mixture was incubated at 25 °C for 5 min to hydrolyze BAEE. Upon completion of one cycle, the nanoflowers were then separated by centrifugation and NPCH1 was taken out directly. The recovered nanoflowers and NPCH1 were rinsed three times with deionized water and then suspended again in a fresh reaction mixture. The residual enzyme activity of each cycle was calculated by taking the enzyme activity of free alkaline protease as 100%.

3. Results and discussion

3.1. Structure of alkaline protease–$Cu_3(PO_4)_2{\cdot}3H_2O$ nanoflowers and NPCH

We synthesized NPCH, the multiscale immobilized alkaline protease used in this study. Its formation mechanism is illustrated Fig.1. The whole preparation process is environmentally friendly given that it does not require the use of organic solvents and toxic chemicals. The nanoflowers were almost 100% immobilized into PVA hydrogels because of the high viscosity of the aqueous PVA solution. We subjected the NPCH5 sample with the highest doping amount to structural and morphological characterization. The structures of alkaline protease-–$Cu_3(PO_4)_2{\cdot}3H_2O$ nanoflowers and NPCH5 were confirmed through FT-IR and XRD. IR spectra (Fig. S2) showed NPCH5 had the characteristic absorptions of both the PVA hydrogel and alkaline protease-

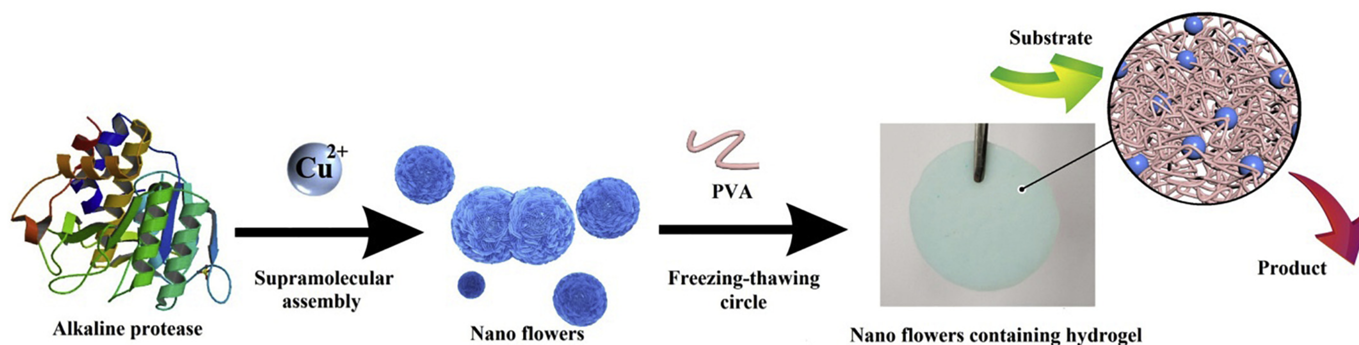

Fig. 1. Proposed growth mechanism of schematic representation of the synthesis of NPCH.

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