Biochemical Engineering Journal 121 (2017) 188–195

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Regular article

Synthesis of stimuli-responsive support material for pectinase immobilization and investigation of its controllable tailoring of enzymatic activity

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ARTICLE INFO

Article history:
Received 12 October 2016
Received in revised form 4 January 2017
Accepted 9 February 2017
Available online 13 February 2017

Keywords:
Pectinase
Enzyme immobilization
Stimuli-responsive
Controllable biocatalyst

ABSTRACT

In this study, a novel type of stimuli-responsive support materials was synthesized successfully by atom transfer radical polymerization (ATRP) and ring opening polymerization (ROP) of N-isopropylacrylamide (NIPAAm), *tert*-butyl methacrylate (tBMA) and ε-caprolactone (CL). β-Cyclodextrin (β-CD) and azobenzene (Azo) were employed as the starting groups to form PCL-hv-PNIPAAm by host–guest inclusion complexation, while PCL-PMAA was converted by simple hydrolysis of PCL-PtBMA with trifluoroacetic acid. Both PCL-PMAA and PCL-hv-PNIPAAm formed the microspheres by self-assembly. Their characteristics were confirmed by NMR, TEM, GPC, DLS, etc. Then, the microspheres were used for the immobilization of pectinase by electrostatic interaction. The optimal immobilization reaction parameters were 10 U/mL for pectinase at 25 °C for 4 h at pH = 6. Compared with free pectinase, the immobilized enzymes were found to exhibit better tolerance of variations in pH and temperature, as well as improved storage stability. Furthermore, the relative activity of the immobilized pectinase was controlled at approximately 13% when the temperature was higher than the LCST of PNIPAAm. With different light irradiation, there is approximately a 15% influence on the relative activity of the immobilized pectinase. This new enzyme support material has good environmental adaptability and can make the activity of immobilized enzymes be tailored predictably and enable the enzyme to be used as a biocatalyst for various applications in the food industry.

1. Introduction

During recent decades, biocatalysts such as enzymes have played an important role in different industries [1–3]. In contrast to conventional catalysts, enzymes activity tends to degrade on long-standing storage, and processing conditions also affect the stability of the enzyme [4,5]. Therefore, the techniques used to stabilize enzymes were the keys to the development of a reliable biocatalyst [6,7]. Immobilizing enzymes on a support material is an important strategy of biotechnology that has emerged at the right moment [8]. Various novel immobilization schemes and materials have been developed to improve the storage stability and the catalytic properties of enzymes [9–12].

Due to the excellent bio-compatibility, good stability and multiple – functionalities [13–15], synthetic polymers have been considered an excellent support material for immobilizing enzymes, especially the stimuli-responsive polymers, the famous members of the polymer family that can adjust their morphology with a change of conditions [16,17]. To date, pH, temperature, light, ultrasound, redox agents, and voltage, as well as carbon dioxide (CO_2), have been using as external stimuli for stimuli-responsive systems [18–22]. Because it has good environmental adaptability, a stimuli-responsive polymer has extensive applications in various fields such as affinity separations, immunoassays and drug delivery [23–27].

As an important biocatalyst, enzymes must be in contact with the substrate to exercise its catalytic properties [28]. The quality of the catalytic properties was influenced by the affinity between the enzyme and the substrate [29–31]. If the support material can regulate the affinity between the enzymes and substrates by changing its morphology, the catalytic properties of the immobilized enzymes will be controlled effectively. Based on this conception, we designed a stimuli-responsive support material formed by PCL-PMAA and PCL-hv-PNIPAAm. As shown in Scheme 1, the PCL-PMAA

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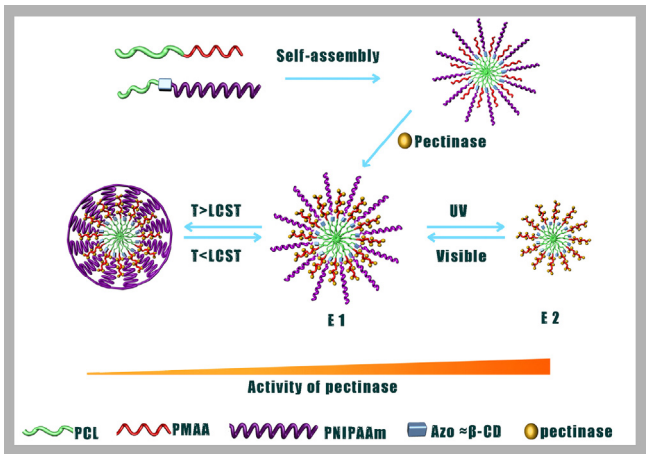
http://dx.doi.org/10.1016/j.bej.2017.02.010
1369-703X/



Scheme 1. Representation of structural changes of the polymer support material under the stimuli of temperature and light and its controlled activity of immobilized pectinases.

chain with the activation of the acid groups provides abundant binding sites for immobilizing pectinase. Furthermore, we took β-CD and azobenzene (Azo) as the starting group to synthesize β-CD-PNIPAAm and azo-PCL by atom transfer radical polymerization (ATRP) and ring opening polymerization (ROP), respectively. Through host–guest inclusion complexation between β-CD and Azo, the stimuli-responsive polymer chain PCL-hv-PNIPAAm was obtained. The support material for enzyme immobilization (E1) was obtained by self-assembly between PCL-PMAA and PCL-hv-PNIPAAm.

In addition, the relative activity, storage stability and recycling rate of immobilized and free pectinase were determined. By regulating the ambient temperature and light, we succeeded in tailoring the activity of the immobilized pectinase.

2. Experimental section

2.1. Materials

β-Cyclodextrin (β-CD) (Shanghai Sinopharm Chemical Reagent Co. Ltd) was dried for 48 h in a vacuum oven before use. Copper(I) bromide (Fluka, 98%) was washed with glacial acetic acid to remove any soluble oxidized species, then filtered, washed with ethanol, and dried. *N*-isopropylacrylamide (NIPAAm, Aldrich, 99%) was recrystallized twice from hexane before use. *tert*-Butyl methacrylate (tBMA, Aladdin, 98%) was dried over calcium hydride and distilled under reduced pressure before use, ε-caprolactone (CL) was purchased from Sigma and purified with CaH_2 by vacuum distillation. Stannous octoate ($Sn(Oct)_2$, Aldrich, 95%), trifluoroacetic acid (TFA, Alfa, 99%), tris[2-(dimethylamino)ethyl]amine (Me_6-TREN) was prepared according to the literature [32,33]. Pectin from citrus peel and the enzyme was polygalacturonase (pectinase), obtained from Fluka Chemical Co. and used without further purification. Dichloromethane (DCM) and triethylamine (TEA) from Sinopharm were distilled over CaH_2 just prior to use. *N*,*N*′,*N*″,*N*‴,*N*⁗-Pentamethyl-diethylenetriamine (PMDETA, Aldrich, 99%), 2-bromoisobutyryl bromide (Aladdin, 98%), tetrahydrofuran (THF, Fisher, GR grade), and other analytical grade reagents from Sinopharm were used as received.

2.2. Synthesis of 2-Hydroxyethyle 2-bromoisobutyrate (HEBiB)

HEBiB was prepared using the synthetic procedure from the literature [34]. Anhydrous ethylene glycol (225 mL, 4.1 mol) was added with magnetic stirring to a 500 mL 2-neck round bottom flask, which had been flame-dried under vacuum and purged 3 times with argon. The flask was then cooled to 0 °C in ice bath. Slowly, 2-bromoisobutyryl bromide (20 mL, 161.8 mmol) was added dropwise to the stirring ethylene glycol. The reaction was kept at 0 °C for 3 h, and then quenched with 100 mL H_2O and extracted with $CHCl_3$ (3 × 100 mL). The combined organic extracts were dried over $MgSO_4$ and filtered, and the $CHCl_3$ was removed by rotary evaporator. The subsequent liquid was purified by distillation to yield a viscous, clear and colorless liquid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.41 (t, 2H, J = 3.5 Hz), 3.87 (t, 2H, J = 3.3 Hz), 3.21 (s, 1H).

2.3. ROP of ε-caprolactone from bifunctional ATRP initiator

The flask was kept under nitrogen. ε-caprolactone (3 mL, 26.1 mmol) was added and dissolved by freshly dried toluene (2.20 mL), then HEBiB (45 mL, 0.285 mmol) and $Sn(Oct)_2$ were added to the flask immediately via syringe. The flask was immersed in an oil bath and maintained at 90 °C with magnetic stirring for 12 h. The reaction was terminated by removing the flask to cold water. The product (PCL–Br) was added dropwise to cold methanol, filtered and dried under vacuum to yield a white solid. The structure was characterized by ^{1}H NMR spectroscopy, and the molecular weight was analyzed via GPC using THF as mobile phase.

2.4. Synthesis of PCL-b-PtBMA

The diblock copolymer of PCL-b-PtBMA was prepared through ATRP of tBMA in toluene by utilizing CuBr as the catalyst. We added 0.8 g (0.2 mmol) PCL-Br and 2 mL (12 mmol) *tert*-butyl methacrylate (tBMA) to 3 mL anhydrous toluene in a Schlenk flask. After adding 43 mg (0.4 mmol) CuBr and 62 mg (0.4 mmol) PMDETA, we sealed the flask. The mixture was degassed three times via a freeze–pump–thaw cycle and then incubated in an oil bath at 80 °C for 12 h. After polymerization, the mixture was cooled to room temperature and dissolved in 50 mL tetrahydrofuran (THF). The resultant solution was passed through a neutral alumina column to remove the Cu^{II} catalyst by absorbing onto the neutral alumina. The eluent was evaporated to remove excess THF and then precipitated in petroleum ether. The resultant solid was dried overnight under vacuum at room temperature.

2.5. Synthesis of PCL-b-PMAA

The typical procedure is as follows: we dissolved PCL-b-PtBMA obtained in the previous step in dichloromethane (C ~100 g L^{-1}) and a fivefold molar excess (tBMA unit) of trifluoroacetic acid (TFA) was added under N_2 atmosphere. After 36 h, TFA and dichloromethane were removed by rotary evaporator. The product was dissolved in a water/THF mixture (v/v, 5/1), transferred into a dialysis tube, and dialyzed against deionized water for 5 days. After lyophilized, a fluffy white solid PCL-b-PMAA was obtained, as shown in Scheme 2.

2.6. Synthesis of 4-hydroxyazobenzene

In a flask, concentrated HCl (8.0 mL) and water (8 mL) were added. This solution was cooled to 0 °C and aniline (2.5 g, 27 mmol) was added. Then, $NaNO_2$ (2.0 g, 29 mmol) in 10 mL water was added to the cooled solution slowly with heat control, keeping the solution temperature below 10 °C. Then, the solution was stirred 20 min in an ice-bath. Then, phenol (2.5 g, 27 mmol) was dissolved in 25 mL of 10% NaOH solution. This prepared solution was added slowly to the diazonium salt –containing solution under stirring, and the temperature was kept below 15 °C. Stirring was continued for 45 min. The yellow-orange solid that was formed was filtered and washed with