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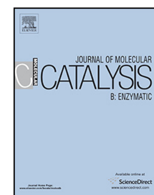

Immobilization of cellulase onto a recyclable thermo-responsive polymer as bioconjugate

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ABSTRACT

Here we report a recyclable thermo-responsive polymer-cellulase bioconjugate, a recoverable, thermo-responsive polymer (P_{NMN}) which was synthesized through the polymerization of N-isopropylmethacrylamide (NIPMA) with methyl acrylate and N-(Hydroxymethyl) acrylamide was used for connection with cellulase to form bioconjugate. The polymer exhibited a thermo-responsive lower critical solution temperature (LCST). The aminooxy group of P_{NMN} provided a handle through which the LCST was adjusted through small-molecule quenching. This allowed the polymer to have a LCST of 51.6 °C and recovery of 98.5%. Cellulase was covalently combined to P_{NMN} by 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide as bioconjugate (P_{NMN}-C). The surface morphologies of P_{NMN} and P_{NMN}-C were obtained by scanning electron microscope (SEM). Under optimized conditions, the highest activity yield of polymer-cellulase bioconjugate construction was 83.2%. Maximum activity of the polymer-cellulase bioconjugate was achieved at 50.0 °C (pH 5.0), while free cellulase exhibited maximum activity at 55.0 °C (pH 5.0). The polymer-cellulase bioconjugate retained 85.2% of its initial activity after repeated five cycles of hydrolysis reaction. P_{NMN}-C could be dissolved and used efficiently during 50.0 °C, and could be collected as precipitate higher than LCST. P_{NMN} is a promising carrier for immobilizing cellulase.

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1. Introduction

In recent years, environmentally friendly polymers have been intensively applied in biocatalysts. Enzymes are highly specific and efficient in most reactions, but their soluble character limits their reusability, which suggests their low stability for long term use [1]. To reduce cost associated with enzymes, various techniques have been developed to enable their repeated use in multiple rounds of processing. To render them reusable, enzymes have been immobilized on solid matrices [2–4] such as sepharose [5], polyester [6], chitosan [7], magnetic affinity sorbent [8] and carbon nanotubes [9], either via covalent attachment or adsorption. However, biocatalysts in solid-liquid interfaces are often accompanied by a reduced activity as the accessibility of the enzymes to the substrate is potentially limited. Furthermore, difficulty in enzyme recovery is not completely eliminated especially if any insoluble substrate remains after the maximal active yield has been reached.

An alternative strategy is the use of stimuli-responsive polymers, which undergo solubility changes in response to external stimuli such as pH or temperature. Some pH-responsive [10–12] and thermo-responsive polymers [13–16] reported have been used to immobilize enzymes. Specifically, polymers with lower critical solution temperature (LCST) behavior are interesting supports for enzymes [11,17,18]. The LCST behavior of certain thermo-responsive polymers can be tuned above the optimum temperature of enzyme activity. Since the reaction temperature for optimum enzyme activity is below the LCST value, the relaxed chains of polymers would provide the immobilized enzymes high accessibility to the substrate. It is well known that cellulase plays a significant role in the bioenergy and biochemicals industry as this enzyme catalyzes the degradation of cellulose to glucose, a well-known precursor for biofuel and platform chemicals production [17]. Herein, a thermo-responsive polymer-cellulase bioconjugate was constructed primarily to render the enzyme reusable. A commercial crude cellulase (Novozymes) from Trichoderma reesei was used as experimental material. The thermo-responsive polymer was designed to have an LCST value above the optimum activity of cellulase which is around 50 °C. However, common thermo-responsive polymers such as poly(N-isopropylacrylamide) and poly(N-vinylcaprolactam) have LCST values between 30.0 °C and 35.0 °C [18–22]. These polymers would be aggregated or precipitated at the desired cellulose degradation temperature hence

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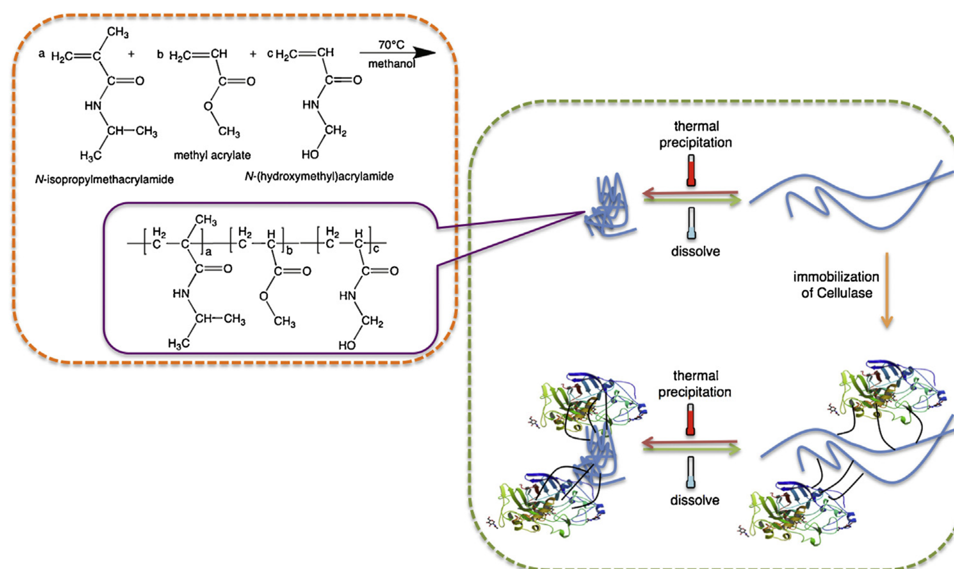

Fig. 1. The process of construction of bioconjugate.

could restrict the substrate-enzyme interaction and reduce cellulase activity due to diffusion limitation.

The thermo-responsive polymer was synthesized through co-polymerization of Methyl acrylate (MA), N-Isopropylmethacrylamide (NIPMA), and N-methylolacrylamide (N-MAM) while the bioconjugation of cellulase was performed post-polymerization via covalent attachment on the LCST-type polymer. The thermo-responsive polymer and bioconjugated polymer-cellulase were thoroughly characterized to confirm the immobilization of cellulase on the polymer. Reactions were performed at various conditions to observe the influence of the thermo-polymer on the performance of cellulase, and finally a recycling test was conducted to demonstrate the reusability of the thermo-responsive polymer-cellulase bioconjugate.

2. Materials and methods

2.1. Materials

Azobisisobutyronitrile (AIBN), methyl acrylate (MA), N-Isopropylmethacrylamide (NIPMA), and N-methylolacrylamide (N-MAM) were purchased from Sinopharm Chemical Co., Ltd (Shanghai, China). 1-Ethyl-3-(3-dimethyllaminopropyl) carbodiimide (EDC) was purchased from Sigma (St. Louis, USA). Whatman No.1 filter paper was obtained from GE Ltd. (Shanghai, China). Cellulase with 75.00 FPU/mL was provided by Novozymes (Denmark). All other chemicals used were reagent grade.

2.2. Preparation of polymer P_{NMN}

In a 150 mL conical flask containing 40 mL MeOH, the three monomeric components NIPMA (3.81 g), MA (0.19 mL), and N-MAM (0.3 g) were slowly added under constant stirring. Shortly after, 0.025 g AIBN initiator was added; copolymerization (Fig. 1) was carried out for 8 h under N_2 blanket at 70 °C in a water bath with stirring. After the reaction, MeOH was removed via vacuum distillation. The crude residue was dissolved in acetone and the polymer product was extracted by adding excess hexane. The extract was dried to collect the final thermo-responsive polymer denoted as P_{NMN}.

2.3. Construction of P_{NMN}-C bioconjugate

A P_{NMN} solution was dissolved in deionized water at a final concentration of 0.5–3.0% w/v. About 0.1–0.5 g EDC was then added hereafter to activate the hydroxyl groups of P_{NMN} [23]. After gentle agitation for 10–15 min at 25 °C, cellulase was added to the P_{NMN}-EDC solution at varied concentrations (50–300 mg protein); the solution was continuously stirred for 1–6 h at 25 °C. When immobilization was completed, the polymer was precipitated by heating the solution to a temperature above the LCST point of the P_{NMN} and then was washed thoroughly three times with 0.02 mol/L acetic acid (containing 1 mol/L NaCl and $CaCl_2$) to remove the unbound enzyme and residual EDC [10]. The P_{NMN}-cellulase bioconjugate was dissolved in 0.1 mol/L acetate buffer (pH 4.8) and stored at 4 °C, denoted as P_{NMN}-C.

2.4. Determination of the properties of P_{NMN}-C

The activities of free cellulase and P_{NMN}-C bioconjugate were determined according to the method from the International Union of Pure and Applied Chemistry (IUPAC) [24]. One unit of filter paper cellulase (FPU) is defined as the amount of enzyme, which produced 2.0 mg reducing sugar from a 50 mg filter paper strip within 60 min. The experiment was carried out by using the mixture systems containing 0.5 mL diluted free cellulase solution, and 50.0 mg Whatman No.1 filter paper (1 × 6 cm). The mixture was incubated at 50 °C for 60 min. The amount of released reducing sugar was determined by the 3, 5-dinitrosalicylic acid (DNS) method [24]. Polymer-cellulase bioconjugate solution was added to the same medium and incubated at 50 °C for 60 min. The resulting mixture was separated through centrifugation at 7104g for 10 min, and the supernatant was collected and determined. The activity yield of polymer-cellulase bioconjugate was determined by the percentage of activity of P_{NMN}-C bioconjugate relative to the activity of free cellulase.

In order to observe the pH dependency of the free cellulase and P_{NMN}-C bioconjugate, the activities were tested at different pH from 3.0 to 7.0 at 50 °C; filter paper was used as the substrate. Separate buffers were employed depending on the pH (pH 3–5: 50 mM acetate buffer (acetic acid/sodium acetate), pH 6–7: 50 mM phosphate buffer (Na_2HPO_4/NaH_2PO_4).

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