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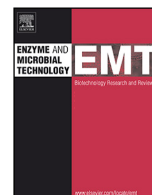

Synthesis and characterization of thermo-responsive poly-*N*-isopropylacrylamide bioconjugates for application in the formation of galacto-oligosaccharides

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ABSTRACT

The study demonstrates the properties of conjugation of β-galactosidase with a thermo-responsive polymer, poly-*N*-isopropylacrylamide (PNIPAAm) in comparison to a non-responsive polymer, poly-acrylamide (PAAm). The maximum formation of bioconjugate (PNIPAAm-β-galactosidase) was 75% (yield) with 50% chemically modified enzyme (using itaconic anhydride). The process of bioconjugation (bioconjugate concentration: 7.4%) decreases lower critical solution temperature from 32.5 °C (with pure PNIPAAm) to 26.5 °C. The effect of temperature on the activities of PNIPAAm-β-galactosidase, PAAm-β-galactosidase and native enzyme was also compared. At 70 °C, the maximum activity was observed for PNIPAAm-β-galactosidase while for others it was at 60 °C. However, the effect of pH was insignificant on activities of both the bioconjugates than the native enzyme. The addition of ethylene glycol (20%, v/v) enhances the activity (by 45%) of PNIPAAm-β-galactosidase with no loss in stability; however; the trend is reversed with the addition of ethanol. Further, employing bioconjugates even up to 24 cycles of precipitation (at 40 °C) followed by re-dissolution (4 °C) around 90% of activity could be retained by PNIPAAm-β-galactosidase. The PNIPAAm-β-galactosidase also showed much-improved thermal and storage stabilities. A lower Michaelis–Menten constant (K_m) was estimated with the PNIPAAm-β-galactosidase than the native enzyme as well as PAAm-β-galactosidase. Finally, PNIPAAm-β-galactosidase was tested to synthesize galacto-oligosaccharides from lactose solution.

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1. Introduction

Enzyme immobilization has always received a great deal of attention in research for its use in industrial-scale enzymatic processes. Enzyme immobilization may overcome the drawbacks associated with enzymatic processes under free conditions [1]. The advantages of immobilization include easy separation of the enzyme from the reaction mixture [2], reuse of the enzyme, continuous product formation, better product quality, and improved stability [3]. Extensive studies have been reported in relation to enzyme immobilization on various solid supports [2–5], however, with certain drawbacks [6]. Incorporating the concept of soluble–insoluble polymer bioconjugates may eliminate the problem associated with immobilization solid supports [7,8]. Responsive polymers, also known as smart/intelligent/environmentally sensitive/reversible polymers, have recently received a great deal of attention from researchers regarding their applications to the fields of biotechnology, medicine, and engineering [9–12]. Responsive polymers respond to slight changes in environmental stimuli such as pH, temperature, and ionic strength. The polymers then regain their previous form when the stimulus is removed [13,14].

Thermo-responsive polymers are the most widely studied class of responsive polymers [15,16]. A slight change in temperature around the lower critical solution temperature (LCST) causes the polymer to undergo a reversible phase change from soluble to insoluble or vice versa [13]. Several polymers with different LCSTs show thermo-responsive properties, such as poly-*N*-isopropyl-acrylamide (PNIPAAm; LCST: ∼32 °C) [8], poly-*N*-vinyl-caprolactam (LCST: 31–37 °C) [17], poly-*N*,*N*-diethyl-acrylamide (LCST: 32–34 °C) [18], poly-acrylicacid-*co*-acrylamide (LCST: 25 °C) [19], and poly-*N*-ethyl-oxazoline (LCST: 62 °C) [16]. PNIPAAm is the most preferred thermo-responsive polymer [20–24] because of its solubility in water, sharpness of phase transition (coil-globule conformation) at its LCST (32 °C), and easy separation from the solution phase by the addition of salt or surfactants above the LCST [25]. Owing to its soluble–insoluble property,

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Notations and abbreviations

K_m	Michaelis–Menten constant
M_w	weight-average molecular weight
M_n	number-average molecular weight
M_z	*Z*-average molecular weight
U	unit of enzyme
V_m	maximum reaction rate
AAm	acrylamide
APS	ammonium persulphate
BCA	bicinchoninic acid
DC	denaturation capacity
EG	ethylene glycol
GOS	galacto-oligosaccharides
GPC	gel permeation chromatography
HPLC	high performance liquid chromatography
LCST	lower critical solution temperature
NIPAAm	*N*-isopropylacrylamide
ONP	*o*-nitro phenol
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
PAAm	poly-acrylamide
PDI	polydispersity index
PNIPAAm	poly-*N*-isopropylacrylamide
TEMED	*N*,*N*,*N*′,*N*′-tetra-methylethylenediamine
THF	tetrahyrofuran

using these polymers as supports for enzyme immobilization does not impose any mass transfer limitations on the substrate and products [8]. Furthermore, the LCST of PNIPAAm is almost independent of the concentration or molecular weight of the polymer [16]. PNIPAAm-based bioconjugates are most suitable for biomedical applications because their LCST is close to normal human body temperature [14], which is the optimum temperature for most enzymes.

β-Galactosidase (EC 3.2.1.23) is an important and well-established enzyme for the production of galacto-oligosacchatides (GOS) [3,26–28]. The enzyme β-galactosidase converts lactose via two simultaneous reactions: the trans-galactosylation reaction, which produces GOS; and the hydrolysis reaction, which forms glucose and galactose [2,29,30]. GOS is an important nutraceutical with prebiotic properties. It has useful applications in the dairy, food, pharmaceutical, and beverage industries. Synthesis of GOS with enzymes under free condition [29] provides maximum conversion (GOS yield: 39.0%, lactose conversion: 60.0% with lactose concentration of 525 g/L, enzyme concentration 2.27 kU/L at 6-pH and 40 °C), however, with this approach one additional step is required for separating enzymes from reaction mixture. In this regard, immobilization of enzymes saves such an additional step, though, the conversion may get affected [2]; still, this approach may be a preferred mode. Most studies utilize solid supports which have mass transfer limitations [6] under immobilization. It is in this regard, the present work was conceived with a belief that bioconjugates may prove useful because of its special properties. Relating to our earlier work [2,29], it is felt that bioconjugates in its soluble state may be considered equivalent to freeing enzyme [29] while in its insoluble state it may be considered equivalent to enzymes under immobilized state [2]. Though, similar to any kind of immobilization approach, the conjugation of enzymes may also reduce the activity of enzymes. Still, there may be distinct possibility of retaining enzyme activity for repeated use as compared to immobilization on membrane as support [2] where a maximum of 3-times the membrane could be utilized. Further, the bioconjugates can be separated easily from the reaction mixture by heating above its LCST.

Bioconjugates are generally synthesized through covalent or non-covalent approaches by using different physical forms such as soluble cross-linked gels, and nanoparticles. To achieve better conjugation, the polymer, enzyme, or both need to be modified. Random conjugation is the most frequently used technique in covalent approaches, where enzymes are randomly attached to the polymer backbone via covalent bonding [31]. Modifying the enzyme with itaconic anhydride introduces an acryl amide group, which is stable over a wide pH (1–12) range [32]. Enzymes generally possess lysine residues, which are the easiest and most preferred site for conjugation with polymers through the amine terminal [33]. The choice of the conjugation site of the enzyme depends on several factors such as the pH, temperature, and ionic strength of the medium [31]. Random conjugation provides structural rigidity to the enzyme by multipoint attachment. Structural conformations of enzymes are flexible in an aqueous solution and rigid in an organic medium [34]. Studying the change in properties of PNIPAAm-β-galactosidase compared to the native enzyme and the enzyme conjugated with non-responsive poly-acrylamide (PAAm) would be of interest.

The present study focused on the synthesis and characterization of thermo-responsive PNIPAAm-β-galactosidase to produce GOS from lactose. The properties of PNIPAAm-β-galactosidase were compared with those of the native enzyme as well as those of PAAm-bioconjugates. The bioconjugates were synthesized from their respective monomers using the copolymerization approach. The effects of operating parameters such as the pH, temperature, and organic solvent on the catalytic activity of enzyme were studied. The PNIPAAm-β-galactosidase was utilized to form GOS from lactose under soluble conditions.

2. Experimental

2.1. Materials and chemicals

N-Isopropylacrylamide (NIPAAm; assay 97%), 2,4,6-trinitrobenzenesulfonic acid (TNBS; 5% aqueous solution), and *o*-nitrophenyl-β-D-galactopyranoside (ONPG; assay 98%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Itaconic anhydride (assay 97%) was obtained from Alfa Aesar (Heysham, Lancashire, UK). Acrylamide (AAm) was supplied by Sisco Research Laboratories (Mumbai, India). Lactose (monohydrate), D-galactose (assay 99%), and *N*,*N*,*N*′,*N*′-tetra-methylethylenediamine (TEMED) were procured from Loba Chemie Pvt. Ltd. (Mumbai, India). Ammonium persulphate (APS) and D-glucose (anhydrous) was purchased from Qualigens Fine Chemicals (Mumbai, India). Commercial grade β-galactosidase (EC 3.2.1.23; commercial name: Biolacta FN5) from *Bacillus circulans* was provided by Daiwa Kasei K.K. (Osaka, Japan). Bicinchoninic acid (BCA) protein reagent kit was purchased from Novagen (WI, USA). Ethanol and ethylene glycol (EG) were procured from Marck KGaA (Darmstadt, Germany). Milli-Q (Millipore Corporation, India) water was utilized for the preparation of all solutions. All of the chemicals were used without any further purification.

2.2. Enzyme assay and protein estimation

The activities of both the bioconjugates and native enzyme were measured by using ONPG as the substrate [29]. The reaction was carried out by mixing 0.5 mL of 10 mM ONPG with 400 mM citrate buffer (pH 6.0) and 0.1 mL enzyme solution. The mixture was incubated at 30 °C for exactly 10 min and to stop the reaction 3.0 mL of 200 mM borate buffer (pH 9.8) was added. The amount of *o*-nitrophenol (ONP) released by the reaction between the enzyme and ONPG was estimated by measuring the absorbance at 410 nm in an UV–vis spectrophotometer (Parkin Elmer, Lambda 35). The

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