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The microbial transglutaminase immobilization on carboxylated poly(*N*-isopropylacrylamide) for thermo-responsivity

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ABSTRACT

Microbial transglutaminase (mTG) is widely utilized in the PEGylation of pharmaceutical proteins. mTG immobilization can be achieved via covalent bonding on solid supports. However, the catalytic efficiency of mTG immobilized on solid supports was significantly reduced by mass transfer limitation. To overcome this limitation, mTG was covalently immobilized on the thermo-responsive carboxylated poly(*N*-isopropylacrylamide) (pNIPAM). The pNIPAM-mTG conjugate exhibited reversibly solubility in aqueous solution with a low critical solution temperature (LCST) at 39 °C, i.e., it was insoluble above 39 °C and soluble below 39 °C. The pH dependence of the pNIPAM-mTG conjugate was similar with that of the native mTG. Upon conjugation to pNIPAM, the optimal temperature of mTG shifted down from 50–55 °C to 40–45 °C, and the thermal stability of the conjugate was elevated. The easy separation of the pNIPAM-mTG conjugate with its substrate and the catalytic efficiency of the pNIPAM-mTG conjugate were demonstrated by employing the pNIPAM-mTG conjugate to cross-link bovine serum albumin (BSA) and catalyze PEGylation of therapeutic protein, cytochrome c (Cyt C), respectively. The thermo-responsive mTG is suitable to modify proteins in food processing and biomedical engineering.

1. Introduction

Transglutaminase (EC 2.3.2.13, TG) catalyzes an acyl transfer reaction between the γ-carboxyamide group of glutamine residues (acyl donor) and a variety of primary amines (acyl acceptor), thus promoting the formation of isopeptide bonds [1]. TG is a large family of enzymes widely distributed in mammals, plants and microorganisms. Nowadays, microbial TG (mTG) is one of the most useful enzymes because of its excellent properties, including calcium-independence, lower molecular weight (about 38 kDa), lower substrate specificity requirements, higher reaction rate and easy obtainment [2,3]. mTG has been extensively utilized for food treatment and textile processing to improve the functionality and texture of products via protein crosslinking [4–6]. Another important application of mTG has been intensively focused on protein modification, such as the PEGylation of pharmaceutical protein [7–12], the formation of the oligonucleotides-protein conjugation [13,14], the introduction of fluorescent probes into the target protein [15], the glycosidation of protein with aminated polysaccharides [16] and the crosslinking of enzyme protein[17]. Such enzymatic approach to the preparation of protein derivatives can be mild, highly selective and retain the protein's bioactivity compared with the chemical methods [18]. Recently, mTG has been used to make site-specific antibody drug conjugates (ADCs) and radioimmunoconjugates [19,20], which offers promise for cancer diagnosis and therapy.

However, at the end of these enzymatic modification processes, mTG should be denatured by heating or adjusting pH value through adding acid or base to terminate the reaction; at the same time, the denaturation step unavoidably leads to the bioactivity compromise of the substrate protein [11,16,21], which limits the application of mTG on protein modification. Such limitation can be overcome by using the immobilized mTG. The immobilized enzyme can be easily separated from solution, which eliminates the requirement for an inactivation step and allows the reuse of enzyme. Nonetheless, with macromolecular (protein) as substrates, the catalytic efficiencies of the TG immobilized on insoluble supports were limited by mass transfer compared with that of the native enzyme. For example, Oh et al., reported that the catalytic efficiency, k_{cat}/k_m, is reduced roughly 8-fold by immobilization TG on the porous glass with poly(lysy1)-α_S-casein as spacer [22], and an 84-fold reduction was observed for the immobilized TG on the biotinylated aminopropyl glass [23].

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To overcome the above problem, mTG was covalently immobilized on the thermo-responsive carboxylated poly(*N*-isopropylacrylamide) (pNIPAM) in this work. The low critical solution temperature (LCST) of pNIPAM is known to be at about 32 °C, namely soluble in water at temperature lower than 32 °C and insoluble when temperature higher than 32 °C. The mTG conjugated with pNIPAM will also become sensitive to the change of temperature. Thus, it can be applied to catalyze protein modification or cross-linking in soluble form at low temperature, and separated by increasing temperature and centrifugation for recycling. In this study, the pNIPAM-mTG conjugate was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), ultraviolet scanning spectroscopy and fluorescence spectroscopy. Then the LCST and the properties of the pNIPAM-mTG conjugate, such as optimum pH and temperature, were investigated. In addition, to demonstrate the catalytic efficiency of the pNIPAM-mTG conjugate and the practicability of the complete separation of the pNIPAM-mTG conjugate with its substrate, the pNIPAM-mTG conjugate was used to cross-link bovine serum albumin (BSA) and catalyze PEGylation of therapeutic protein, cytochrome c (Cyt C), respectively.

2. Materials and methods

2.1. Materials

The mTG product (10% enzyme in maltodextrin, from *Streptoverticillium mobaraense*) was kindly provided by Yiming Biological Products Co., Ltd. (Taizhou, China). The carboxylated pNIPAM (Mw = 5100 Da, polydispersity 1.366, gel permeation chromatographer (GPC), Fig. S2, Supporting Information. Fourier transform infrared (FTIR) spectrum, Fig. S1, Supporting Information.) was synthesized according to the method described by Li et al. [24]. Carboxybenzyl-glutaminyl-glycinyl-methoxypolyethylene glycol (CBZ-QG-mPEG) (Mw ~5300) was prepared according to the method of Tominaga et al. [13]. Carboxybenzyl-glutaminyl-glycine (CBZ-Gln-Gly), glutathione (reduced), L-glutamic acid γ-monohydroxamate, and bovine serum album (BSA) were purchased from Sigma-Aldrich (Shanghai, China). Hydroxylamine was from Alfa Aesar (Tianjin, China). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was from Yacoo Chemical Reagent Co., Ltd. (Suzhou, China), *N*-hydroxysuccinimide (NHS) was from Aladdin (Shanghai, China). Other chemicals were purchased from Sangon Biotech Co., Ltd. (Shanghai, China).

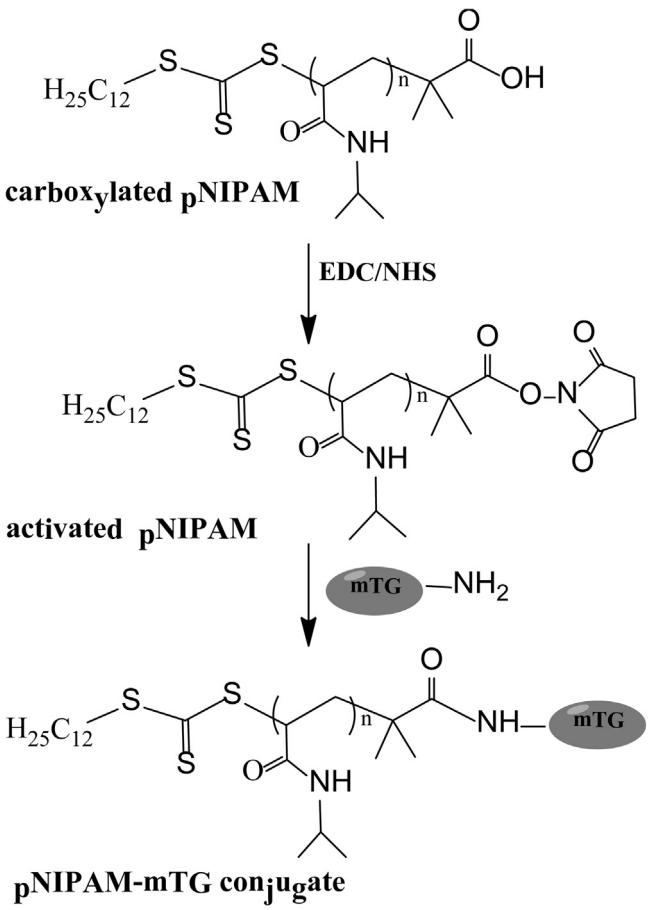
2.2. Methods

2.2.1. Determination of mTG activity

mTG was purified using a Sephadex G-75 column (1.2 × 40 cm). The eluent buffer was 100 mM Tris-HCl buffer (pH 6.0) at a rate of 0.5 mL/min. The eluate was fractionated into 1.5 mL aliquots. The fractions containing mTG were pooled and stored at 4 °C. The activity of mTG and the pNIPAM-mTG conjugate was assayed using 30 mM CBZ-Gln-Gly and 100 mM hydroxylamine in 100 mM Tris-HCl buffer, pH 6.0 at 37 °C [25]. A unit (U) of activity was defined as the formation of 1 μmol of L-glutamic acid γ-monohydroxamate per min.

2.2.2. Conjugation of pNIPAM with mTG

The carboxylated pNIPAM was activated by EDC and NHS according to the method described by Liang et al. [26] with some modifications. pNIPAM (13 mg), EDC (250 mg) and NHS (145 mg) were dissolved in 20 mM phosphate buffer (3 mL, pH 8.0). The solution was stirred for 24 h at 4 °C to completely activate the carboxylated pNIPAM. Then mTG (120 mg) was added to the solution, and then the mixture was kept stirring at 4 °C for 24 h. The resulting pNIPAM-mTG conjugate was stored at 4 °C for further use. The general scheme for conjugation is illustrated in Scheme 1.



Scheme 1. Activation of the carboxylated pNIPAM and conjugation of the activated pNIPAM to mTG.

2.2.3. Analysis of pNIPAM and the pNIPAM-mTG conjugate

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a mini-protein tetra system electrophoresis apparatus (PowerPac Basic, Bio-Rad, USA) according to the method of Laemmli [27]. The gels consisted of a resolving gel (12% acrylamide) covered by a stacking gel (4% acrylamide) and were stained with Coomassie Brilliant Blue. Generally, 10 μL of the respective samples and molecular weight marker were added to the SDS-PAGE gel. After destaining, the gels were taken photos by gel imager (Gene genius, Syngene, USA).

Ultraviolet (UV) scanning spectra of the solution of mTG (0.22 mg protein/mL) and the pNIPAM-mTG conjugate (0.22 mg protein/mL) were scanned at the range of 200–400 nm using a UV–vis spectrophotometer (UV-2600, Shimadzu, Japan).

Fluorescence scanning spectra of the solution of mTG (0.22 mg protein/mL) and the pNIPAM-mTG conjugate (0.22 mg protein/mL) were measured using a luminescence spectrometer (LS55, PerkinElmer, USA) at 300–400 nm with the excitation wavelength at 280 nm (2.5–5.0 nm slits).

2.2.4. Determination of LCST

The LCST was determined by transmittance measurements of the pNIPAM solution or the pNIPAM-mTG conjugate solution as a function of temperature at 500 nm on a spectrophotometer (Lambder 35, PerkinElmer, USA) equipped with a temperature controlled cell holder.