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Stability and conformational change of methoxypolyethylene glycol modification for native and unfolded trypsin

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ABSTRACT

The effect of succinimidyl carbonates activated methoxypolyethylene glycol (mPEG-SC) on the catalytic properties and conformation of native trypsin and dynamic high-pressure microfluidisation (DHPM) induced unfolded trypsin was studied. The thermal stability of unfolded trypsin was enhanced more significantly than that of native trypsin between 45 and 70 °C. The autolysis analysis indicated that modified unfolded trypsin was markedly more resistant to autolysis compared to modified native trypsin between 40 and 180 min. Upon mPEG-SC conjugation, the K_m value of the enzyme decreased by about 2-fold, and the catalytic efficiency (K_{cat}/K_m) increased by about 3–4-fold. Moreover, the increased thermal stability of unfolded trypsin might be due to the lower surface hydrophobicity and the higher hydrogen bond formation after mPEG-SC modification, which was reflected in the decrease of UV absorbance, the quenching and blue shift of fluorescence spectra, as well as the increase of β-sheet content.

1. Introduction

Trypsin, a serine proteinase, which cleaves peptide bonds at the carboxyl-terminal end of arginine, lysine, and ornithine residues, is widely employed in biomedicine, food and biotechnology industry, as well as in protein analysis (Khantaphant & Benjakul, 2010). However, the extensive application of trypsin is limited, due to its autolysis and activity loss at high temperatures. Several methods, such as immobilisation (Rocha, Goncalves, & Teixeira, 2011), chemical modification with PEG (Treetharnmathurot, Ovartlarnporn, Wungsintaweekul, Duncan, & Wiwattanapatapee, 2008) and high hydrostatic pressure (HHP) (Ruan, Lange, Bec, & Balny, 1997), have been reported to investigate the activity and stability of trypsin. In our previous study, we found that the dynamic high-pressure microfluidisation (DHPM) treatment resulted in unfolding of trypsin and could improve the thermal stability of trypsin (Liu et al., 2010). However, according to the thermodynamic hypothesis (Anfinsen, 1973), the unfolded trypsin is in a metastable state and will subsequently aggregate to be in the lowest energy state. Therefore, a chemical modification of unfolded trypsin with polyethylene glycol (PEG) is expected to prevent aggregation and preserve the thermal stability.

Among a variety of polymers for protein conjugation, PEG was the most popular due to its excellent water-solubility, high hydration and flexibility. In addition, PEG has been found to be non-toxic and is approved by the FDA for use in drugs, foods and cosmetics. PEGylation of proteins has been widely studied as a potential approach to add unique properties to proteins, such as shielding the antigenic and immunogenic epitopes, reducing the renal ultrafiltration and the degradation by proteolytic enzymes (Veronese, 2001). It has been reported that native trypsin covalently bound with methoxypolyethylene glycol (mPEG) activated by p-nitrophenyl chloroformate (NPC) (Gaertner & Puigserver, 1992; Zhang, He, & Guan, 1999), succinic anhydride (SA), cyanuric chloride (CC) and tosyl chloride (TC) (Treetharnmathurot et al., 2008) showed a higher thermal stability. Succinylation has been one of the most common chemical modifications for amino acid residues of proteins, particularly lysine (El-Adawy, 2000). In this study, succinimidyl carbonates activated mPEG (mPEG-SC) was employed as a coupling agent to synthesise the PEG-trypsin conjugates. The route included the reaction between the anionic succinate residues of mPEG-SC and the amino groups of lysine residues of trypsin.

To this date, there are few reports that show the effect of PEG on the stability and conformation of unfolded trypsin. In addition, the explanation of enhanced stability, in terms of conformational change, has been rarely provided. Thus, the aim of this study was to investigate the effect of mPEG-SC modification on the stability and conformation of native and unfolded trypsin, and to discuss the stability improvement of modified trypsin in terms of the conformational change.

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2. Materials and methods

2.1. Materials and reagents

Bovine pancreatic trypsin, 8-anilino-1-naphthalene sulfonate (ANS), β-casein and 2,4,6-trinitrobenzene sulfonic acid (TNBS) were provided by Sigma, Germany. Monomethoxy polyethylene glycol-succinimidyl carbonate (mPEG-SC) with a molecular weight of 5000 g/mol was purchased from Kaizheng biotechnology development limited company in Beijing, China. All other reagents and chemicals used were of analytical grade. Buffers were filtered before use.

2.2. DHPM treatment

According to our previous report (Liu et al., 2010), to obtain DHPM-induced unfolded trypsin (DT), native trypsin (NT) solution (1 mg/ml) in 50 mM potassium phosphate buffer (pH 7.4) was treated three passes under the pressure of 80 MPa using a Microfluidizer (M-110EH-30, Microfluidics Co., USA).

2.3. mPEG-SC modification

The native and unfolded trypsin solution (1 mg/ml) was prepared by dissolving 50 mg of sample in 50 mM potassium phosphate buffer (pH 7.4). mPEG-SC dissolved in 10 ml of 50 mM potassium phosphate buffer (pH 7.4) was added to a trypsin solution with a molar ratio of 20:1. The mixed solution was stirred with a magnetic stirrer, at room temperature, for 1 h and at 4 °C for 16 h. The solution was then dialysed at 4 °C, for 48 h, against a potassium phosphate buffer using a dialysis membrane of molecular weight cut-off 12,400 (Treetharnmathurot et al., 2008). Finally, the aqueous solutions of mPEG-modified native trypsin (NT_P) and mPEG-modified unfolded trypsin (DT_P) were obtained and lyophilised. In the following process, the modified enzymes solutions were prepared by dissolving the lyophilised sample, in 50 mM potassium phosphate buffer (pH 7.4) and diluted to a final volume of 50 ml. The concentration of the NT_P and DT_P solution can be expressed as 1 mg enzyme equiv./ml.

The degree of modification was estimated by determining the number of free amino groups using the TNBS assay, as described by Snyder and Sobocinski (1975). Briefly, the TNBS (25 μl, 0.03 M) was added to 1 ml of sample dissolved in 0.1 M sodium tetraborate, pH 9.3. The solution was then rapidly mixed and incubated at room temperature, for 30 min. The absorbance was determined at 420 nm. The blank consisted of 25 μl of 0.03 M TNBS in 1 ml of 0.1 M buffer solution.

2.4. Determination of trypsin activity

The activity of trypsin was measured using β-casein as a substrate, as described by Purcena, Caramori, Mitidieri, and Fernandes (2009). The enzyme solution (1 ml) (NT, DT, NT_P and DT_P) was added to the reaction mixture containing 2.0 ml of 50 mM phosphate buffer saline (pH 7.6) and 5.0 ml of 7.5 mg/ml β-casein solution. The final mixture was incubated at 37 °C, for 30 min and the reaction was subsequently stopped by addition of 5.0 ml of 5% (w/v) trichloroacetic acid. After centrifugation at 10,000 rpm for 10 min, the UV absorbance of the supernatant was measured at 275 nm using a UV–Vis spectrophotometer (2510PC, Shimadzu Corporation, Japan). One enzyme unit (U) is defined as the amount of the enzyme that catalyses the conversion of 1 μmol of β-casein per minute, at 37 °C and pH 7.6. In this study, the relative activities of the samples were estimated, by considering the activity of native trypsin determined at 37 °C and pH 7.6, as 100%.

2.5. Evaluation of the thermal stability

According to the method of Zhang et al. (1999) and Treetharnmathurot et al. (2008), the thermal stability of enzymes (NT, DT, NT_P and DT_P) was estimated by incubating trypsin solutions in aqueous buffer, for 10 min, at different temperatures between 30 and 80 °C. The relative activity of trypsin was then assayed using β-casein as substrate, as described above and compared to the activity of native trypsin.

2.6. Evaluation of autolysis

The autolysis of enzymes was monitored by incubating the samples (NT, DT, NT_P and DT_P), at 40 °C, for 180 min in aqueous buffer (50 mM phosphate buffer saline, pH 7.6). Aliquots of native, unfolded trypsin and modified enzyme (0.5 ml, 0.5 mg enzyme equiv.) were taken out every twenty minutes and the activity assayed using β-casein as described above.

2.7. Kinetics analysis

The kinetic parameters (K_m, V_{max}, K_{cat} and K_{cat}/K_m) were determined from the raw data of trypsin activity against a range of substrate concentrations (0.01–0.30 mM) based on the equation of Lineweaver–Burk.

2.8. Fluorescence spectra analysis

The fluorescence spectra of ANS binding to NT, DT, NT_P and DT_P were measured by exciting at 380 nm, with scanning between 400 and 600 nm (Naseem & Khan, 2003). The concentration of ANS and enzyme was 0.018 and 0.2 mg/ml, respectively.

The intrinsic fluorescence emission spectra of enzymes (NT, DT, NT_P and DT_P) were recorded at room temperature, in a quartz cuvette of 1 cm of optical pathway using a spectrofluorometer (F-4500, Hitachi Ltd., Japan). Enzyme solutions (0.5 mg/ml) were excited at 280 nm and the emission spectra recorded from 300 to 400 nm. The excitation and emission slit were both 2 nm by width.

2.9. UV absorption spectra analysis

The absorption spectra of enzymes (NT, DT, NT_P and DT_P) were recorded with a UV–Vis spectrophotometer (2510PC, Shimadzu Corporation, Japan). The quartz cuvettes used for the sample were 1 cm in length. UV absorption spectra were scanned from 200 to 400 nm.

2.10. Circular dichroism spectra analysis

All circular dichroism (CD) measurements were obtained with a MOS-450/AF-CD spectrometer (BioLogic, Claix, French), using quartz cells of a 1 mm path length with a band width of 1 nm, a response time of 1s and a scanning speed of 50 nm/min at ambient temperature. Aliquots from all lyophilised samples were dissolved in PBS (20 mM, pH 7.4) which was used as a blank to a final enzyme concentration of 0.16 mg/ml. CD spectra were scanned in the far-ultraviolet range from 190 to 250 nm to investigate the conformational changes in the secondary structure of trypsin. All the measurements mentioned above were performed in a controlled sample chamber under a constant nitrogen flow. The CD data was expressed as a mean residual ellipticity (θ_{MR}), in deg cm^2/dmol or mdeg. The predictions of different secondary structures were performed using the SELCON3 algorithm, one of five popular and freely available analysis algorithms supported by DICHROWEB, which is a software package freely available online (http://