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Enhancing the thermal stability of inulin fructotransferase with high hydrostatic pressure

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ABSTRACT

The thermal stability of inulin fructotransferase (IFTase) subjected to high hydrostatic pressure (HHP) was studied. The value of inactivation rate of IFTase in the range of 70–80 °C decreased under the pressure of 100 or 200 MPa, indicating that the thermostability of IFTase under high temperature was enhanced by HHP. Far-UV CD and fluorescence spectra showed that HHP impeded the unfolding of the conformation of IFTase under high temperature, reflecting the antagonistic effect between temperature and pressure on IFTase. The new intramolecular disulfide bonds in IFTase were formed under a combination of HHP and high temperature. These bonds might be related to the stabilization of IFTase at high temperature. All the above results suggested that HHP had the protective effect on IFTase against high temperature.

1. Introduction

It is known that most enzymes are easily inactivated at high temperature, therefore, the thermostability of an enzyme is considered to be much more essential for the enzymatic reaction. Thus, it is apparently necessary to take into account for the protection measures against thermal inactivation. The environment factors such as pH and metal ions play important roles in enhancing the thermostability of the enzymes. It has been reported that the stability of β-cyclodextrin glycosyltransferase was much higher at 60 °C in the presence of Ca^{2+} [1]. Some osmolytes such as trehalose and glycerol are also considered to exhibit great protective effects [2]. Much attention has recently been paid to protein engineering. It is acknowledged that disulfide bonds are vital in the conformational stability of proteins, and a large amount of attempts have been taken to introduce new disulfide bonds for the improvement of protein stability over past twenty years [3,4].

Currently, high hydrostatic pressure (HHP) treatment has been attracted considerable research interests. As a novel green technology, it can be applied to modulate the activities and stabilities of the enzymes [5,6]. For example, moderate pressures exerted protective effects against thermal inactivation of β-glucosidase and α-L-rhamnosidase [7,8]. Pressure assisted the stabilization of endo-1,4-β-glucanase at elevated temperature [9]. One of the advantages of pressure application for enzymatic reaction is that the enzyme could resist to unfold under high temperature, and significantly accelerate the reaction [7]. In addition, HHP might be exploited into the reaction at higher temperature for preventing the microbial contamination [10]. However, the conformational stabilization of enzymes induced by HHP has not been fully understood. In this work, inulin fructotransferase (IFTase, EC 4.2.2.18) obtained from *Arthrobacter aurescens* SK 8.001 [11], which was used for producing a novel functional sweetener called difructose anhydride III, was selected to investigate the protective effects of HHP and to elucidate the possible protective mechanism.

2. Materials and methods

2.1. Materials

Purified IFTase solution was prepared with our previous described method [11]. The reagent 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and β-mercaptoethanol (β-ME) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The low molecular weight protein markers were from Takara (Dalian, China). Inulin was from BENEO-Orafti NV (Tienen, Belgium). DFA III standard was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were purchased from Sinopharm (Shanghai, China).

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2.2. IFTase activity assay

The enzyme reaction mixture consisted of 30 μL of the enzyme solution (50 U/mL), 970 μL of acetate buffer (100 mM, pH 5.5) and 1 mL of 20 g/L inulin solution in acetate buffer (100 mM, pH 5.5). The reaction was carried out directly at 60 °C for 15 min. It was followed by heating at 100 °C for 5 min to stop the enzymatic reaction. The enzyme activity was determined by the content of the product DFA III in the solution using HPLC method (column: Waters SugarPakTM1, 6.5 mm id. × 300 mm, Milford, MA, USA; column temperature: 85 °C; mobile phase: deionized water; flow rate: 0.4 mL/min; refractive index detector, Shodex RI101, Shoko Scientific Co., Ltd., Yokohama, Japan). One unit of IFTase was defined as the amount of enzyme that produces 1 μmol DFA III from inulin per min at pH 5.5 and 60 °C [11].

2.3. High pressure combined with high temperature treatments of IFTase

The high pressure machine (MICRO FOODLAB FPG5740; maximum pressure, 900 MPa; Stansted Fluid Power, Ltd., Essex, UK) with an inner cylinder of 20 mL and equipped with temperature regulation (0–90 °C) was used in this study. The enzyme solution in a 2-mL tube was subjected to high pressure and high temperature treatments under a range of isothermal and isobaric conditions. Before the treatments of high pressure, the samples were first preheated to the setting temperature. After the treatment, the sample was immediately cooled down to avoid the reactivation of the enzyme [9]. Then the residual activities of all samples were assayed immediately.

2.4. Thermostability determination and thermal inactivation kinetics

To determine the stability of IFTase, the purified enzyme in 100 mM acetate buffer (pH 5.5) was incubated at temperatures ranging from 70 to 80 °C and at pressures ranging from 0.1 MPa to 200 MPa for several time intervals in a thermostatic water bath.

The inactivation of IFTase, which is expressed as A/A_0, is described adequately by a nth-order reaction model (Eq. (1)):

$$\frac{dA}{dt} = -k * A^n \quad (1)$$

where A is the residual IFTase activity, A_0 is the initial activity, n is the reaction order and k is the inactivation rate constant. Then the values of k were calculated from non-linear regression of (A/A_0) vs. t.

2.5. Molecular modeling

The crystal structure of IFTase from *Bacillus* sp. snu-7 (PDB: 2INV), which was used as the template, was obtained from the Protein Data Bank (http://www.rcsb.org). The molecular model of IFTase from *Arthrobacter aurescens* SK 8.001 was generated using SWISS-MODEL workstation (http://www.expasy.ch/swissmod/SWISS-MODEL.html) [12]. The model was further visualized using the Maestro 9.8 software (Schrödinger, NY, USA).

2.6. Circular dichroism (CD)

The samples were scanned at the far-UV range (200–250 nm) using a model Mos-450 CD spectropolarimeter (Biologic, Claix, France) with cells of 10 mm path lengths at 25 °C. The scan rate was 5 nm/s. Three scans were averaged to obtain one spectrum. The acetate buffer solution (100 mM, pH 5.5) was used as the blank for all samples. The CD data were expressed in terms of molar ellipticity, $[\theta]$, in deg cm^2/dmol, which was calculated as described by Chen et al. [13].

2.7. Intrinsic fluorescence

Fluorescence spectra of the samples were measured at 25 °C with a Hitachi F-7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The excitation wavelength was set at 295 nm (slit width, 5 nm) and the emission wavelength range was from 300 to 450 nm, with 12,000 nm/min of scanning speed. The acetate buffer solution (100 mM, pH 5.5) was used as the blank for all samples. Three scans were averaged to obtain one spectrum. All samples after HHP treatments were measured immediately.

2.8. The thermostability of IFTase affected by the content of disulfide bonds

For the determination of the disulfide bonds content, the method of Petruccelli and Añón was used [14]. The absorbance of the solution was measured at 412 nm by a UV-2102PC spectrophotometer (Unico, Shanghai, China).

Reducing and non-reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed to explore the formation of the intermolecular disulfide bonds in IFTase after the treatment of HHP. According to the method of Li et al. [15], the separating gel was 12%, and the stacking gel used was 4%. 10 μL of the sample was loaded, and the gel was stained with Coomassie Brilliant Blue after the electrophoresis. For non-reducing SDS-PAGE, the samples without β-ME were treated with the above method.

In order to inhibit the formation of disulfide bonds under HHP, β-ME was added to the enzyme solution. The enzyme solution with β-ME (10 mM) was incubated at 80 °C for 15 min, and then cooled down immediately. This process was also performed at 200 MPa. The both residual activity was assayed by the above method to investigate the effect of the disulfide bonds generated under HHP on the thermostability of IFTase.

2.9. Data analysis

The nonlinear regression analysis was carried out using software Origin 8.5 (OriginLab Corporation, MA, USA). All the experiments were performed under the same conditions in triplicates and the data were represented as mean values ± standard errors in the tables or in the figures.

3. Results

3.1. Thermal inactivation

The effects of temperature on the activity of IFTase are shown in Fig. 1(A). It was observed that the activity of IFTase increased with the increase in the temperature in the range of 40–60 °C. The activity was enhanced by 27.4% at 60 °C compared with that at 40 °C. The enzyme activity decreased significantly above 60 °C, where was the optimum temperature. At 80 °C, about 60% of the activity was lost. Detailed thermal inactivation of IFTase was investigated at temperatures ranging from 70 to 80 °C. According to the Eq. (1), the thermal inactivation of IFTase was described by a typical first-order model, and the fitting curves ($R^2 > 0.95$, for all thermal experiments) are illustrated in Fig. 2. The inactivation rate constants (k) were estimated as 0.011, 0.013 and 0.030 min^{-1} at 70, 75 and 80 °C, respectively. The inactivation rate increased with the increasing the temperature at the studied conditions.