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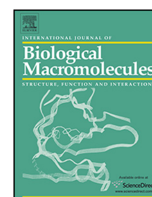

Sorbitol counteracts high hydrostatic pressure-induced denaturation of inulin fructotransferase

Yungao Li, Ming Miao, Miao Liu, Bo Jiang*, Tao Zhang, Xiangyin Chen

State Key Laboratory of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, Jiangsu, PR China

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ABSTRACT

Inulin fructotransferase (IFTase), a novel hydrolase for inulin, was exposed to high hydrostatic pressure (HHP) at 400 and 600 MPa for 15 min in the presence or absence of sorbitol. Sorbitol protected the enzyme against HHP-induced activity loss. The relative residual activity increased nearly 3.1- and 3.8-fold in the presence of 3 mol/L sorbitol under 400 MPa and 600 MPa for 15 min, respectively. Circular dichroism results indicated that the original chaotic unfolding conformation of the enzyme under HHP shifted toward more ordered and impact with 3 mol/L sorbitol. Fluorescence and UV spectra results suggested that sorbitol prevented partially the unfolding of the enzyme and stabilized the conformation under high pressure. These results might be attributed to the binding of sorbitol on the surface of IFTase to rearrange and strengthen intra- and intermolecular hydrogen bonds.

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1. Introduction

There are many interests in the application of high hydrostatic pressure (HHP) for modulating both the stability and activity of the enzymes [1,2]. Similar to the effect of temperature, the destabilization effects on the enzymes induced by HHP are directly related to the loss of the activity [3]. Moreover, elevated hydrostatic pressure has also been used to increase catalytic activity and thermal stability of α-chymotrypsin [1]. In this biological process, HHP as a fascinating and important tool could reveal structural changes of the enzymes. It could provide very useful information such as stabilizing and destabilizing interactions for these molecules [4]. It has been well known that the proteins or the enzymes are unfolded generally by HHP treatments, which is a complex phenomenon depends on the protein structures, solvent compositions and other environmental factors [5,6]. HHP exerts a great influence on the unfolding or aggregation of the proteins or enzymes mainly by rearranging and/or destructing noncovalent bonds such as electrostatic and hydrophobic interactions, hydrogen bonds, van der Waals and disulfide bonds which normally stabilize the secondary and tertiary structure [2,7]. Based on the above mechanism, the enzyme could be stabilized when these noncovalent bonds are strengthened under HHP.

It has been well known that osmolytes could provide abundant noncovalent bonds such as hydrogen bonds. Numerous experiments have established that the osmolytes including glycine betaine, trehalose, sorbitol, sucrose, glycerol and other small organics could stabilize the enzymes or proteins under freezing, pulsed electric field and thermal stresses [3,8–12]. To the knowledge of the authors, the potential mechanisms included: (a) direction interaction between organics and proteins to protect the active sites and to inhibit the aggregates [5,9,10]; (b) the water activity decreased by the osmolytes to prevent water molecules into the interior of the proteins [13]; (c) reduce the ability of the molecular mobility in the rigid matrix [8]; (d) preferential exclusion of solute or preferential hydration of the protein promotes a stabilization of the protein [14–16]. The addition of these low molecular weight osmolytes to an aqueous solution of the protein results in an unfavorable free-energy change, which is used to explain the protein stabilizing action of the osmolytes [15]. This solute exclusion theory is consider to be the primary protecting mechanism accounting for the stabilizing effects of the osmolytes on the protein against denaturing stresses [17].

Increasing the stability of the enzymes against high pressure-denaturation has recently become a great concern for performing the novel enzymatic conversions at high pressure. The sorbitol is generally considered to make the enzymes more stabilized, especially against heat stress [18]. For example, Plaza et al. [11] have reported that the thermostability of pectin methylesterase from *Aspergillus aculeatus* increased with 450 mg/mL of the sorbitol. Other studies with the sorbitol have shown the similar effects

* Corresponding author. Tel.: +86 510 85327859/+86 510 85329055; fax: +86 510 85919161.
E-mail addresses: bjiang@jiangnan.edu.cn, bjiang2001@hotmail.com (B. Jiang).

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on the lysozyme, cytochrome *c*, bovine serum albumin and β-lactoglobulin [18–21]. With regard to other physical stresses such as microsecond pulsed electric field and dehydration, sorbitol could also protect the native conformation of the proteins [12,13,22]. Although the influence of sorbitol on the proteins has been extensively investigated [11,18,23], few studies have plumbed the effects of sorbitol on the stabilization of IFTase against HHP. In this study, the protective effects of the sorbitol on the activity and conformation of IFTase from *Arthrobacter aurescens* SK 8.001, a novel hydrolase for inulin just prepared recently [24], against HHP were focused on. The protective mechanism of sorbitol against the denaturation of IFTase induced by HHP treatments was also explored. In addition, these results might provide a possible method of protecting the enzyme reactions under the high pressure stress condition.

2. Materials and methods

2.1. Materials

IFTase was purified with the previously described method [24], which was demonstrated to be a single band on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme powder, obtained by frozen-drying, was dissolved in the acetate buffer (100 mM, pH 5.6), and the enzyme activity was about 50 U/mL.

Sorbitol was purchased from Sinopharm (Shanghai, China). Inulin was from BENEO-Orafti NV (Tienen, Belgium). DFA III standard was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals used were of analytical grade from Sinopharm (Shanghai, China).

2.2. HHP treatments

Samples of the enzyme solution or mixtures of the enzyme and sorbitol were sealed in a 2 mL capacity of Eppendorf tube. Then these samples were treated at different pressures (0.1–600 MPa) for 5–20 min using a high pressure device (MICRO FOODLAB FPG5740, respectively; Stansted Fluid Power Ltd., Essex, UK) equipped with temperature regulation, respectively. The temperature was kept at 60 °C in the pressure processing. After the treatments, all samples were kept in a 0 °C an ice–water bath before analysis.

2.3. The preparation of the enzyme–sorbitol solution

The enzyme powder was first dissolved in the acetic acid–sodium acetate buffer (HAc-NaAc, 100 mM, pH 5.6) to make the enzyme activity about 50 U/mL. Different amounts of the sorbitol were added to prepare enzyme–sorbitol solution. The concentration of the sorbitol in the mixed solution was 1, 3 and 5 mol/L, respectively.

2.4. IFTase activity assay

The enzyme reaction was carried out for 15 min. The reaction mixture contained 0.9 mL of sodium acetate buffer (100 mM, pH 5.5), 2% (w/v) inulin solution (1 mL) and 0.1 mL of the enzyme solution or the enzyme–sorbitol mixture. The reaction was quenched immediately by heating at 100 °C for 5 min once the pressure decompressed. The activity in the presence or absence of the sorbitol was both determined by measuring the content of α-D-fructofuranose-2′,1:2,3′-β-D-fructofuranose dianhydride (DFAIII) with HPLC method (column: Waters SugarPak^{TM} 1, 6.5 mm id × 300 mm, Milford, MA, USA; column temperature: 85 °C; mobile phase: water; flow rate: 0.4 mL/min; a 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 [24]. The relative residual activity was defined as a percentage of activity of the HHP-treated IFTase solution relative to that of the native enzyme.

2.5. Viscosity analysis

The viscosities of different concentrations of the sorbitol were measured by Ostwald viscometer (Longtuo, Shanghai, China) at 60 °C.

2.6. Water activity analysis

The water activities of the enzyme–sorbitol solution were directly performed on the 4TEV dew point water activity meter (Natoli, MO, USA) at 25 °C. All the measurements were performed at ambient pressure.

2.7. Circular dichroism (CD) spectrum

The samples treated or untreated were scanned at the far-UV range (200–250 nm) using a model Mos-450 CD spectropolarimeter (Biologic, Claix, France) with the rate of 5 nm/s at 25 °C. The path length of a quartz cuvette used for far-UV CD was 1.0 mm. The CD data, average of three scans, were expressed in terms of molar ellipticity, $[\theta]$, in deg cm^2/dmol, which was calculated as described by Chen et al. [25].

2.8. Fluorescence measurements

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, at which only Trp residues are excited [26], as well as the intensity was measured over the range 300–450 nm, with the speed of 12,000 nm/min. In this case, center of spectral mass $[\nu]$ could be used appropriately for the analysis of the changes of tertiary structure of the enzyme on the different condition [4]. The calculation formula was as follows:

$$[\nu] = \frac{\sum \left(\lambda_j \times I_j\right)}{\sum I_j} \quad (1)$$

where λ_j was the wavelength from 300 to 400 nm and I_j was the fluorescence intensity at λ_j [3].

2.9. UV absorption spectrum

The UV absorption spectra were scanned from 220 to 320 nm using a UV-2102PC spectrophotometer (Unico, Shanghai, China). Three scans were averaged to obtain one spectrum. The phosphate buffer solution (10 mM, pH 7.0) was used as the blank.

2.10. Statistical analysis

Measurements were performed in triplicates and the data were presented as mean values ± standard errors. The results were assessed by a one-way analysis of variance with a significance level of 95% ($p = 0.05$) using the Student–Newman–Keuls test.

3. Results

3.1. The effects of sorbitol on the IFTase activity under HHP

IFTase was subjected to the pressure in the range of 0.1–600 MPa for 15 min. As shown in Fig. 1, IFTase activity increased nearly by

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