Protective Effect of Biological Osmolytes against Heat- and Chaotropic Agent-Induced Denaturation of *Bacillus licheniformis* γ -Glutamyl Transpeptidase

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In the present study, the stabilizing effect of four different biological osmolytes on *Bacillus licheniformis* γ -glutamyl transpeptidase (BIGGT) was investigated. BIGGT appeared to be stable under temperatures below 40°C, but the enzyme retained less than 10% of its activity at 60°C. The tested osmolytes exhibited different degrees of effectiveness against temperature inactivation of BIGGT, and sucrose was found to be the most effective among these. The use of circular dichroism spectroscopy for studying the secondary structure of BIGGT revealed that the temperature-induced conformational change of the protein molecule could be prevented by the osmolytes. Consistently, the molecular structure of the enzyme was essentially conserved by the osmolytes at elevated temperatures as monitored by fluorescence spectroscopy. Sucrose was further observed to counteract guanidine hydrochloride (GdnHCl)- and urea-induced denaturation of BIGGT. Taken together, we observed evidently that some well-known biological osmolytes, especially sucrose, make a dominant contribution to the structural stabilization of BIGTT.

Keywords: γ -Glutamyl transpeptidase, *Bacillus licheniformis*, osmolyte, sucrose, protein stabilization

Introduction

Most proteins are sensitive to changes in their surrounding environments, and any significant perturbation in thermodynamic conditions can definitely cause alterations in protein structure that consequently lead to a partial or complete loss of biological function [1]. Living organisms are known to adapt to such perturbations through an accumulation of high concentrations of organic molecules (biological osmolytes), which can increase the inherent stability of cellular proteins without compromising their native functionality [2]. A previous report has indicated that biological osmolytes are one of the most potent stabilizers for many disease-associated proteins and are capable of reversing their misfolding and/or aggregation events [3]. Beyond that, biological osmolytes can also

modulate the molecular activity of heat-shock proteins probably due to the promotion of local refolding within the chaperone molecules [4].

Carbohydrates, methylamines, and amino acids are three major classes of biological osmolytes [5]. These biological substances can be further categorized into (i) osmolytes, such as trimethylamine *N*-oxide (TMAO), sarcosine, sorbitol, sucrose and trehalose, that stabilize proteins by raising free energy of both native and denatured states, (ii) osmolytes, such as, glycine, betaine, proline and glycerol, that only moderately enhance the thermodynamic stability of proteins, (iii) denaturing osmolytes, such as urea, and (iv) counteracting osmolytes, such as the mixture of urea and TMAO [5]. Over the last decades, an increasing number of studies have broadened the diverse functions of biological osmolytes pertaining to solubility of proteins [6–10], expression

enhancement of enzymes [11, 12], and protection of living organisms against extreme environmental conditions [13–16].

γ -Glutamyl transpeptidase (GGT, E.C. 2.3.2.2) catalyzes the transfer of a γ -glutamyl group from γ -glutamyl donors to an acceptor that may be water, an amino acid, or a peptide [17]. The initial steps in the reaction catalyzed by GGT enzymes are the cleavage of the γ -carbonyl linkage of γ -glutamyl substrates and the formation of a transient enzyme-substrate (ES) complex [18, 19]. In the ES complex, the γ -carbon of γ -glutamyl substrates forms an acyl bond with the side-chain nucleophile of the catalytic Thr of GGT enzymes [20–22]. Hydrolysis of the ES complex occurs when a water molecule undergoes a nucleophilic attack at the carbonyl carbon of the esterified enzyme [20, 21]. However, in the presence of suitable L-amino acids or peptides, the transpeptidation reaction may actually proceed through intermolecular acylation to resolve the ES complex into new γ -glutamyl compounds [20]. Thus, a straightforward route towards the synthesis of a variety of γ -glutamyl compounds can be established by using GGT and different types of γ -glutamyl acceptors. In fact, GGT enzymes have been applied to the biocatalytic synthesis of a number of γ -glutamyl compounds with great industrial potential [23–26].

Biocatalytic synthesis of diverse types of chemical and biological compounds has become a promising way in chemical and pharmaceutical industries just because the biochemical process usually leads to a profound reduction in process time, number of reaction steps, and amount of waste [27]. Most importantly, biocatalytic processes always offer the most powerful approach to produce enantiomerically pure compounds through high regioselectivity, stereoselectivity, and chemoselectivity of employed enzymes [28]. Although enzyme applications are being developed in full swing [29, 30], breakthroughs are still needed to address their weaknesses in maintaining activities during biocatalytic processes [31]. Osmolyte-mediated stabilization of enzymes may provide a potentially viable solution to overcome the instability problem [32]. Therefore, the objective of the current study is to explore the effect of four different biological osmolytes on the enzymatic activity and structural stability of *Bacillus licheniformis* GGT (*BIGGT*). The stabilizing effect of the tested osmolytes on *BIGGT* may be helpful in industrial applications of this enzyme.

Materials and Methods

Gene Expression and Enzyme Purification

For the expression of recombinant *BIGGT*, a single colony of

E. coli M15 cells harboring pQE-*BIGGT* [33] was inoculated into 10 ml of Luria-Bertani (LB) broth containing ampicillin (100 μ g/ml) and kanamycin (25 μ g/ml), and the bacterial culture was incubated aerobically at 37°C for overnight. Then, 1 ml of the overnight culture was transferred into 100 ml of the antibiotic-containing LB broth and grown at 37°C. Once an A_{600} of 0.6–0.8 was reached, the bacterial culture was shifted to 28°C and induced for 6 h by the addition of isopropyl thio- β -D-galactoside to a final concentration of 100 μ M. The induced cells were harvested by centrifugation and washed once with 50 mM phosphate buffer (pH 7.0). The harvested cells were subsequently lysed by sonication and the resultant mixture was cleared of precipitates by centrifugation. Thereafter, the recombinant enzyme was purified from the supernatant by affinity chromatography using a nickel-chelating column (Qiagen). The purity of *BIGGT* was monitored by resolution of the eluted sample on 12% SDS-polyacrylamide gel in Tris-glycine buffer followed by staining for 12 h with Coomassie Brilliant Blue G-250. Protein concentration was determined by a quantitative Coomassie dye-binding assay (Bio-Rad) using bovine serum albumin as a concentration standard.

Activity Assay

The transpeptidation activity of *BIGGT* was determined by a colorimeter assay as described previously [33]. Briefly, *BIGGT* and the reaction components (1.25 mM L- γ -glutamyl-*p*-nitroanilide, 30 mM Gly-Gly, 75 mM NaCl, and 20 mM Tris-HCl buffer; pH 9.0) were warmed up separately at 40°C for 10 min. The catalytic action was initiated by adding suitably diluted enzyme solution to the reaction components and enough distilled water to bring the final volume up to 1 ml. The mixture was then incubated at 40°C for 10 min and the enzymatic reaction was stopped chemically by adding 100 μ l of acetic acid (3.5 N). Release of *p*-nitroaniline from L- γ -glutamyl-*p*-nitroanilide was determined by monitoring the absorbance change at 410 nm using a UV-vis spectrophotometer. One unit of *BIGGT* activity is defined as the amount of enzymes that catalyze the conversion of 1 μ mol of substrate per min under the assay conditions.

Effect of Osmolytes on the Thermal Stability of *BIGGT*

To investigate the effect of glycerol, sorbitol, sucrose and TMAO on the thermal stability of *BIGGT*, the deactivation was carried out by incubating the enzyme-osmolyte samples (a final enzyme concentration of 50 μ g/ml and a final osmolyte concentration of 2 M) at various temperatures of 30°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, 80°C, and 90°C for 20 min. The heated samples were taken out after different intervals of time and cooled immediately in ice water. The residual activity was measured using standard assay conditions as described above. An experimental control was simultaneously performed using osmolyte-free enzyme solution.

Circular Dichroism (CD) and Fluorescence Analyses

Protein secondary structure analyses were carried out at two

different temperatures (40°C and 60°C) using a Jasco J-815 spectropolarimeter (Jasco, Japan) equipped with a cell holder thermostatically controlled by circulating water from a bath. The instrument was operated with Jasco's Spectra Manager software and all the measurements were conducted under a nitrogen flow of 10 l/min. The CD spectra were recorded between 190 and 250 nm with a 0.2-nm step resolution and a scanning speed of 20 nm/min. All enzyme samples were diluted with 20 mM Tris-HCl buffer (pH 9.0) to give a protein concentration of approximately 7.6 μ M. After CD measurements, background contribution of 20 mM Tris-HCl buffer (pH 9.0) was subtracted from each spectrum to normalize it. The mean molar residue ellipticity (θ) was calculated according to the following formula: $[\theta] = \theta_{\text{obs}}$ (in mdeg)/(molar concentration of the enzyme $\times$ optical path length $\times$ number of amino acid residues in the enzyme) [34]. The CD intensity is defined in terms of molar residue ellipticity (MRE) with a unit of deg $\cdot$ cm²·dmol⁻¹.

Heat-induced unfolding of BIGGT was performed by monitoring the change in ellipticity at a fixed wavelength of 222 nm. Prior to CD measurements, the purified BIGGT was mixed with an appropriate amount of 20 mM Tris-HCl buffer (pH 9.0) and various amounts of each osmolyte stock to generate enzyme solutions with a protein concentration of $\sim$ 12.4 μ M. Enzyme samples were heated at a constant scanning rate of 1°C/min. Ellipticity values at 222 nm wavelength were specifically extracted from the CD spectra to determine the melting temperature (T_m) of enzyme samples. The transition temperatures were calculated by fitting the CD data with Eq. (1) [35].

$$\theta_{222} = \frac{\theta_N + \theta_U \cdot \exp\left[-\frac{\Delta H_U}{RT} \cdot \left(1 - \frac{T}{T_m}\right) + \frac{\Delta C_{PU}}{RT} \cdot \left(T \ln\left(\frac{T}{T_m}\right) + T_m - T\right)\right]}{1 + \exp\left[-\frac{\Delta H_U}{RT} \cdot \left(1 - \frac{T}{T_m}\right) + \frac{\Delta C_{PU}}{RT} \cdot \left(T \ln\left(\frac{T}{T_m}\right) + T_m - T\right)\right]} \quad (1)$$

where θ_{222} is the relative ellipticity at 222 nm wavelength; θ_N and θ_U represent the fraction of enzyme present in the native and unfolded states; ΔH_U is the free enthalpy for BIGGT unfolding; ΔC_{PU} is the heat capacity for BIGGT unfolding; and R represents the universal gas constant.

Counteraction of sucrose against urea-induced denaturation of BIGGT was performed by adding the sugar osmolyte into enzyme samples of 12.4 μ M in 20 mM Tris-HCl buffer (pH 9.0) containing the chaotropic agent to molar ratios of 0.5:1, 1:2, 1.5:3, and 2:4. The denaturation process of BIGGT was determined by recording the CD signal at 222 nm. The change in the T_m value was used to monitor the unfolding of BIGGT.

Steady-state emission spectra were recorded at ambient temperature on a JASCO FP-6500 fluorescence spectrophotometer (Japan) with an excitation wavelength of 295 nm using a 0.1-ml quartz cuvette. The emission spectra of BIGGT and enzyme-osmolyte samples with a concentration of approximately 16.2 μ M were measured from 305 to 500 nm at a scanning speed of 240 nm/min. Thereafter, the emission spectra of BIGGT and

enzyme-osmolyte samples were individually corrected for the contribution of 20 mM Tris-HCl buffer (pH 9.0). GdnHCl-induced denaturation of BIGGT was determined by initially recording fluorescence spectra of protein samples in 20 mM Tris-HCl buffer (pH 9.0) containing 3 M GdnHCl. Counteraction of sucrose against GdnHCl-induced denaturation of BIGGT was performed by individually adding the sugar osmolyte into the aforementioned samples to a final concentration of 2 M. Eventually, the maximal peak of the fluorescence spectrum and the change in the fluorescence intensity were used to monitor the unfolding process of each sample. Both the emission wavelength shift and the change in fluorescence intensity were analyzed together to calculate the average emission wavelength (AEW) (λ) of each sample according to Eq. (2) [36].

$$\langle \lambda \rangle = \frac{\sum_{i=\lambda_1}^{\lambda_N} (F_i \cdot \lambda_i)}{\sum_{i=\lambda_1}^{\lambda_N} F_i} \quad (2)$$

in which F_i represents the fluorescence intensity at the specific emission wavelength (λ_i).

Results

Prevention of BIGGT from Heat-Induced Inactivation by Biological Osmolytes

The transpeptidation activity of BIGGT was examined in response to a number of biological osmolytes and different temperature conditions. As shown in Fig. 1A, the enzyme was inactivated in a temperature-dependent manner regardless of presence or absence of osmolytes. It is noteworthy that all tested osmolytes were able to protect BIGGT against thermal inactivation. Incubation of BIGGT at 60°C lost more than 85% of the enzymatic activity, but this situation was greatly improved by the addition of biological osmolytes. Among the employed osmolytes, the protective effect of sucrose on the thermal inactivation of BIGGT was more obvious than others (Fig. 1A).

The molecular properties and structure-function relationships of BIGGT have been extensively studied in our laboratory [37–40]. The results of these investigations clearly demonstrate that the recombinant enzyme has an optimal activity at 40°C. It is noteworthy that BIGGT was quite stable at 40°C even in the absence of 2 M sucrose (Fig. 1B). However, a dramatic loss in GGT activity was observed after incubating the enzyme at either 60°C or 70°C for 10 min. This phenomenon was greatly improved by 2 M sucrose, with more than 80% retention of the enzymatic activity over an incubation time of 6 h (Fig. 1B). The stabilizing effect will be very valuable for the BIGGT-

Fig. 1. Stabilization of *BIGGT* by biological osmolytes.

(A) Effect of four different osmolytes on the thermal stability of the enzyme. (B) Thermal inactivation curves of *BIGGT* in the absence and presence of 2 M sucrose. Aliquots of each sample were immediately cooled and assayed for GGT activity under the standard assay conditions.

mediated synthesis of γ -glutamyl compounds at elevated temperatures. Advantages for a biocatalytic process operated at high temperatures have been documented [31, 41]. Besides, the stabilizing effect definitely renders the *BIGGT*-catalyzed process more economical due to the high cost of enzyme production and purification [31].

Our previous studies have shown that the CD spectrum of *BIGGT* exhibits two strong peaks of negative ellipticity at 208 and 222 nm [37, 42], an indication of substantial α -helical content. These spectrometric characteristics were essentially conserved in enzyme-osmolyte samples when the denaturation temperature was set at 40°C (Fig. 2A). However, upon heat treatment of *BIGGT* at 75°C for 20 min,

Fig. 2. Far-UV CD spectra of *BIGGT* in the absence and presence of four different biological osmolytes.

The spectra were recorded over a wavelength range of 190–250 nm at temperatures of 40°C (A) and 75°C (B).

the CD signal at 222 nm was profoundly diminished in osmolyte-free samples (Fig. 2B). It is interesting to note that the representative peaks for the α -helix of *BIGGT* were mostly reserved in the presence of biological osmolytes. Thus, the tested osmolytes, especially sucrose, can apparently protect *BIGGT* against heat-induced conformational change.

The conformational stability of *BIGGT* was also investigated by recording the molar ellipticity at 222 nm. Visual inspection of the transition curves indicated that the thermal unfolding of all tested samples followed a two-state process (Fig. 3). *BIGGT* exhibited one well-defined unfolding transition of 61.1°C. Notably, in the presence of 2 M osmolytes, the T_m value was individually increased to 64.9°C (sorbitol), 64.8°C (glycerol), 79.7°C (sucrose), and

Fig. 3. Effect of biological osmolytes on the thermal stability of *BIGGT*.

The protein samples in 20 mM Tris-HCl buffer (pH 9.0) were monitored with the CD signal at 222 nm.

73.3°C (TMAO). This observation allows us to confirm that the employed osmolytes are able to protect *BIGGT* against temperature-induced denaturation.

Counteraction of GdnHCl-Induced Denaturation of *BIGGT* by Sucrose

The chaotropic agent GdnHCl is commonly used as a protein denaturant to generally bring about unfolding of proteins by disrupting their secondary and tertiary structures [43]. In this respect, unfolding of *BIGGT* at different concentrations of GdnHCl has already been performed [44]. The enzyme starts to unfold at a denaturant concentration of 1.92 M and reaches an unfolded intermediate, $[\text{GdnHCl}]_{0.5, \text{N-U}}$, at 3.07 M. Based on this information, the transpeptidation activity of *BIGGT* was determined in the presence of 3 M GdnHCl to examine the correlation between conformational dynamics and enzyme catalysis. As shown in Fig. 4, GdnHCl displayed a time-dependent inhibitory effect on the catalytic activity of *BIGGT*. Upon the incubation of *BIGGT* in aqueous solutions of 3 M GdnHCl for 25 min, the enzyme retained $15.2\% \pm 1.5$ of its initial activity. The transpeptidation activity was almost abolished when the incubation time was extended to more than 6 h. It is quite interesting to note that the functional inactivation of the enzyme by 3 M GdnHCl was tremendously inhibited in the presence of 1.5 and 2.0 M sucrose (Fig. 4). This observation clearly indicates the potent ability of this common sugar to counteract the GdnHCl-induced denaturation.

Fig. 4. Protective effect of sucrose against GdnHCl-induced inactivation of *BIGGT*.

The purified enzyme was mixed with 3.0 M GdnHCl and different concentrations of sucrose. Aliquots of each sample were withdrawn at desired time intervals to test the residual activity.

The protective effect of sucrose against GdnHCl-induced denaturation of *BIGGT* was further verified by CD and fluorescence spectroscopy. As shown in Fig. 5A, the negative ellipticity of GdnHCl-treated *BIGGT* was profoundly decreased within the first 30 min in the absence of sucrose, reflecting a greatly diminished native secondary structure of the enzyme. A similar phenomenon was observed with 0.5 M sucrose/ 3 M GdnHCl-treated *BIGGT*. However, the decrease in the negative ellipticity of GdnHCl-treated *BIGGT* was slowed down in the presence of 1.0 M sucrose. GdnHCl-induced secondary structural changes of *BIGGT* were almost inhibited by 1.5 and 2.0 M sucrose even after prolonged incubation (Fig. 5A). Therefore, the protective effect of sucrose on the secondary structure of *BIGGT* is very obvious at sugar concentrations of above 1.5 M.

A valuable feature of intrinsic protein fluorescence is the high sensitivity of tryptophan to its local environment. Changes in the fluorescence spectra of proteins often occur in response to substrate binding, subunit association, conformational transitions, or denaturation [45]. Therefore, AEW that reports on the changes in both emission wavelength and fluorescence intensity can be suitably used to monitor the conformational change of *BIGGT*. As shown in Fig. 5B, upon the treatment of *BIGGT* with 3 M GdnHCl, the AEW value seemed to level off over a relatively short period of time. In the presence of 0.5 M sucrose, the rate of conformational change for GdnHCl-treated *BIGGT* increased linearly for the first 20 min and then began to level off after

Fig. 5. Counteraction of GdnHCl-induced denaturation of BIGGT by sucrose as monitored changes in the negative ellipticity at 222 nm (A) and in the AEW value (B).

50 min. Consistent with the above-mentioned CD data, there was no significant change in the AEW value of GdnHCl-treated BIGGT in the presence of 1.5 and 2 M sucrose (Fig. 5B). Apparently, the results of CD and fluorescence measurements confirm the counteracting ability of sucrose against the GdnHCl-induced denaturation of BIGGT.

Sucrose Offsets Urea's Effect on BIGGT Structure

Fig. 6 shows the change in T_m values, which correspond to the transition of BIGGT to the unfolded state, as a function of sucrose or urea. It can be seen that sucrose increased the T_m value of BIGGT with different increments of osmolyte concentrations (changing from 61.1°C in the absence of osmolyte to 79.7°C in the presence of 2.0 M sucrose). However, urea lowered the T_m value of BIGGT by

Fig. 6. Counteraction of urea-induced denaturation of BIGGT by sucrose.

The point 61.1°C is the control, which represents the BIGGT transition temperature in buffer solution (the solid line). The dashed lines show the region of sucrose + urea.

a concentration-dependent manner (changing from 61.1°C in the absence of urea to 41.8°C in the presence of urea). The experimental results explicitly indicate that the sugar osmolyte increases the transition temperatures and keeps the folding form beyond 79°C, which is obviously higher than the control. Fig. 6 also depicts that the T_m values of BIGGT in sucrose-plus-urea mixtures (sucrose and urea at molar ratios of 0.5:1, 1:2, 1.5:3, and 2:4) stably increased and these mixtures had almost the same trend as sucrose-alone action on BIGGT. Based on the fact that sucrose-plus-urea mixtures substantially increase the T_m value of BIGGT, sucrose can indeed offset the urea-induced denaturation of the enzyme.

Discussion

Protein structure is stabilized mainly by a combination of hydrophobic interactions, hydrogen bonding and electrostatic interactions with additional contributions in particular proteins arising from metal complexing, cross-linking, and specific bindings of ions and cofactors. Some previous studies have shown that protein stability is essentially established through a balance between intramolecular

interactions of protein functional groups and their interaction with solvent environment [46–48]. Accordingly, the effects of sugars and polyols on the thermal stability of proteins depend upon how they influence the overall balance of interactions. Our results clearly indicate that sucrose is the most effective osmolyte for preventing BIGGT from thermally induced functional inactivation (Fig. 1). As a naturally occurring sugar, sucrose has also been shown to increase the conformational stability of the native state of several globular proteins against temperature-induced denaturation [49–51]. Although the molecular-level mechanism of sucrose-mediated stabilization is not yet fully understood, the solvent-excluded volume effect may play a fundamental role to stabilize the N-state of globular proteins [52].

Biological osmolytes have been reported to push the reversible folding equilibrium of a protein toward natively folded conformation by raising the free energy of unfolded state [53]. As they predominantly affect the protein backbone, the balance between osmolyte-backbone interactions and amino acid side chain-solvent interactions is surely an important *criterion* of protein folding [53]. It is well known that the stabilizing osmolytes are preferentially excluded from the immediate vicinity of the protein surface, leading to the formation of a solvophobic interaction between peptide backbone and side chain on the protein surface and the protecting osmolytes [54]. The tendency to exclude osmolytes from the protein surface forces polypeptides to adopt a folded conformation with a minimum possible exposed surface area [55]. Thus, protein-osmolyte compatibility depends on the degree of freedom a protein backbone has to bury itself during the folding of polypeptides into a native-like conformation [56]. Because of their preferential hydration, biological osmolytes have been shown to increase the respective T_m values of many proteins [57–59]. Apparently, the tendency of increased T_m value of BIGGT by biological osmolytes is consistent with previous studies. This allows us to conclude that the tested biological osmolytes, especially sucrose and TMAO, can efficiently protect BIGGT against temperature-induced denaturation.

Protein unfolding by the classical denaturants, such as GdnHCl and urea, has long been considered to arise from the favorable interactions of the chaotropic agents with the normally buried segments of proteins [60]. The basis of bimolecular interactions for the destabilization of chemical denaturants are generally attributed to either a direct ligand binding with the protein surface or the effect of denaturants on the structure and dynamic of water

molecules [61, 62]. In our case, sucrose was found to counteract the GdnHCl-induced unfolding of BIGGT (Fig. 5). Some previous studies have already provided possible interpretations for the protective effect of sucrose on GdnHCl-induced denaturation of proteins [63–66]. It has been shown that GdnHCl disrupts the structure of ordered water of a phosphoglycerate kinase and this conformational change can be counteracted by sucrose [63]. A comprehensive investigation of the sucrose-mediated effect on the GdnHCl-induced denaturation of proteins further reveals that this sugar is able to inhibit conformation fluctuations within the native state of proteins as well as increase the free energy of unfolding [66]. Thus, the aforementioned elucidations might be appropriate to interpret the protective effect of sucrose on the GdnHCl-induced denaturation of BIGGT.

The evolutionary advantage of biological osmolyte systems is compatible with macromolecular structure at high and variable osmolyte concentrations without modifying the functionality of cellular proteins [67, 68]. Although compatible osmolytes are largely accumulated to stabilize protein and enzyme systems, nature has not ignored the use of protein-destabilizing osmolytes to act as efficient osmoprotectants. Urea, a metabolic waste of living organisms, and amino acid osmolytes (for example, arginine, histidine, and lysine) are familiar compounds for use as an osmoprotectant [67, 68]. It has been reported that urea can disrupt the noncovalent interactions responsible for the molecular structure of proteins [69]. The denatured protein with exposed hydrophobic residues is probably stabilized by enclosure of its hydrophobic sectors in a cage-like structure, which minimizes the contact with the aqueous phase and the entropy loss of the system. Hua and her co-workers has proposed a direct interaction mechanism in which urea has a stronger dispersion interaction with proteins than water [70]. In order to counteract the denaturing effect of urea on proteins *in vivo*, living organisms or cells produce several types of biological osmolytes, such as TMAO [71], betaine [72], and polyols [67] to stabilize protein structure and to maintain its functionality under high levels of urea. Furthermore, biological osmolytes TMAO, betaine, and sarcosine have also been shown to counteract urea-induced denaturation of protein [73]. Giving the fact that urea-induced denaturation of BIGGT can be counteracted by sucrose, the experimental data thus provide more insights into osmolyte-mediated protein stabilization, and the findings are expected to further facilitate the practical use of this biological osmolyte on the recovery of recombinant

proteins from inclusion bodies.

In conclusion, the potential of protective biological osmolytes as protein stabilizers has been further extended by this study. Our experimental results have shed some light on the structural alterations and loss of functionality of BIGGT upon exposure of this enzyme to heat and denaturants. Furthermore, sucrose-mediated stabilization of the BIGGT structure is surely helpful in the retention of enzymatic activity at elevated temperatures and in the presence of denaturants. From an economic viewpoint, the stabilization strategy is simpler and less expensive than other methods like protein engineering, chemical modification, and immobilization. A further advantage is that the sugar stabilizer could be added to the enzyme solution just when it is desired.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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