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BIOCATALYSIS

# Sorbitol counteracts temperature- and chemical-induced denaturation of a recombinant α-amylase from alkaliphilic *Bacillus* sp. TS-23

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**Abstract** Enzymes are highly complex systems with a substantial degree of structural variability in their folded state. In the presence of cosolvents, fluctuations among vast numbers of folded and unfolded conformations occur via many different pathways; alternatively, certain conformations can be stabilized or destabilized. To understand the contribution of osmolytes to the stabilization of structural changes and enzymatic activity of a truncated *Bacillus* sp. TS-23 α-amylase (BACΔNC), we monitored amylolytic activity, circular dichroism, and fluorescence as a function of osmolytes. In the presence of trimethylamine *N*-oxide (TMAO) and sorbitol, BACΔNC activity was retained significantly at elevated temperatures. As compared to the control, the secondary structures of this enzyme were essentially conserved upon the addition of these two kinds of osmolytes. Fluorescence results revealed that the temperature-induced conformational change of BACΔNC was prevented by TMAO and sorbitol. However, glycerol did not provide profound protection against thermal denaturation of the enzyme. Sorbitol was further found to counteract guanidine hydrochloride- and SDS-induced denaturation of BACΔNC. Thus, some well-known naturally occurring osmolytes make a dominant contribution to the stabilization of BACΔNC.

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## Introduction

Folded structures of most proteins are sensitive to changes of environmental conditions, such as temperature, pressure, pH, and salinity [22]. Significant perturbation in thermodynamic and chemical conditions can cause alterations in secondary and tertiary structures of proteins, leading to a partial or complete loss of their activity. Organisms are known to adapt to such perturbations through evolutionary adaptations [19, 49] or accumulation of organic osmolytes [51, 52, 65, 69]. Osmolytes are small molecules that act as chemical chaperones to protect proteins from denaturation by supporting the maintenance of their naturally folded and functional states under adverse environments [4, 10, 60, 66]. Naturally occurring osmolytes belong to diverse chemical families including polyols, amino acids and their derivatives, and methylamines [1, 60, 66]. These molecules have been found to stabilize proteins [24, 32, 54] and are currently used in the field of protein formulation [9, 45]. Although the protective mechanism is not yet completely understood [2, 42, 53], some reports have shown that osmolytes stabilize the native states of proteins as a result of preferential hydration of the denatured states compared with the native states [56, 57]. Other researchers have also proposed that the protein stabilization ability of osmolytes originates from an unfavorable interaction of the chemical chaperone with the peptide backbone, on the basis of transfer Gibbs energy measurements of amino acid side chains and the peptide backbone from water to an osmolyte solution [6, 61]. These two mechanisms support each other

from the point of view that interactions between osmolytes and the denatured states of proteins contribute more to the stabilization.

α-Amylases (1,4-α-D-glucan glucanohydrolases: EC 3.2.1.1) catalyze the hydrolysis of 1,4-α-D-glucosidic linkages in starch and related carbohydrates [41]. Currently, α-amylases are used for the liquefaction of the cereal starch during the production of high fructose corn syrup and fuel ethanol [18, 59]. They are also employed in the sizing/desizing process of the paper and textile industry [17, 25]. In household laundry and automatic dishwashing detergents, α-amylases are usually included to remove starch-containing stains from clothes and dishes [20]. In addition to these traditional applications, the spectrum of amylase usage has widened in many other fields, including clinical, medicinal, and analytical chemistry [41, 50].

Earlier, a raw-starch-degrading α-amylase from thermophilic *Bacillus* sp. TS-23 was cloned and overexpressed in *Escherichia coli* M15 cells [26]. The recombinant enzyme could degrade raw starch to produce maltopentoase as the major end product [29]. Deletion analysis demonstrated that the starch binding domain (SBD) of the cloned enzyme is not essential for the catalytic activity but is involved in the binding of granular starch [30]. A truncated *Bacillus* sp. TS-23 α-amylase (BACΔNC) lacking the N-terminal signal sequence and the SBD was also constructed and functionally expressed in *E. coli* [31]. The primary structure of BACΔNC comprises 489 amino acid residues corresponding to a molecular mass of 55,181 Da, which compares well with the size of α-amylases from *Bacillus stearothermophilus* [38] and *Bacillus licheniformis* [68]. To date, the amylolytic enzymes from *Bacillus* species have been extensively studied [14, 43]; however, the behavior of amylases from moderately thermophilic *Bacillus* spp. in organic osmolytes has not been reported. In this investigation, we performed biophysical analyses of BACΔNC unfolding in the absence and presence of compatible osmolytes.

## Materials and methods

### Materials

Components of Luria–Bertani (LB) medium were obtained from Difco Laboratories (Detroit, MI, USA). Nickel nitrilotriacetate ($Ni^{2+}$-NTA) resin was purchased from Qiagen Inc. (Valencia, CA, USA). The Bradford solution and the reagents necessary for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis were acquired from Bio-Rad Laboratories (Hercules, CA, USA). Soluble starch (98–99.5 % purity), glycerol (99 % purity), trimethylamine *N*-oxide (TMAO; 98 % purity), and sorbitol (98 % purity) were bought from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). All other chemicals were commercial products of analytical grade.

### Enzyme expression and purification

BACΔNC was produced using an expression plasmid pQE-AMYΔNC [31] transformed into *Escherichia coli* M15. The recombinant bacterium was grown in 100 ml LB medium supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin, and induced with 0.1 mM isopropyl-*β*-D-thiogalactopyranoside at 28 °C for 12 h. Cells were harvested by centrifugation at 6,000×*g* and 4 °C for 10 min and the pellet was resuspended in a buffer (pH 7.9) containing 20 mM Tris-HCl, 300 mM NaCl, and 10 mM imidazole. Subsequently, cells were lysed by sonication (30-s bursts and pulses for 5 min) on ice and the debris was removed by centrifugation. The $His_6$-tagged protein was then purified on a $Ni^{2+}$-NTA agarose column (Qiagen) under native conditions according to the manufacturer's protocol. Protein purity was analyzed by 12 % SDS-PAGE stained with Coomassie blue R-250 and was estimated to be greater than 96 %.

### Determination of amylase activity

Amylase activity was routinely determined by measuring the release of reducing sugars using the dinitrosalicylic acid (DNS) method [34]. A 100-μl appropriately diluted enzyme sample was added to 900 μl of starch solution (1 %, w/v), previously gelatinized in 20 mM Tris-HCl buffer (pH 7.9). The reaction mixture was incubated at 60 °C for 10 min and stopped by the addition of 500 μl of DNS reagent. Absorbance at 540 nm was then measured. One unit of amylase activity is defined as the amount of enzyme that produces reducing sugar equivalent to 1 μmol of glucose per minute.

The effect of osmolytes on BACΔNC activity was studied by incubating 12 mg/ml enzyme solution (20 mM Tris-HCl, pH 7.9) in the absence and presence of 3 M glycerol, 3 M TMAO, or 3 M sorbitol, at 4 °C for 2 h to reach complete equilibrium. The samples were then filtered with a 0.45-μm disposal filter (Millipore) through a syringe before the activity assays. After incubation of the prepared samples at the indicated temperatures for 10 min, their residual activities were determined under the standard assay conditions.

### Circular dichroism (CD) and spectrofluorimetric analyses

Far-UV CD spectra of BACΔNC with or without osmolytes were acquired on a JASCO model J-815 spectropolarimeter

(JASCO Inc., Japan) from 210 to 240 nm in cuvettes at 30 °C using a 1.0-nm bandwidth, 0.1-nm resolution, 0.1-cm path length, 1.0-s response time, and a 100-nm/min scanning speed. The photomultiplier absorbance was always below 600 V in the analyzed region. Each scanning was repeated ten times and an average was reported. All spectra were corrected by subtraction from buffer and each respective osmolyte, and the results were expressed as molar ellipticity $[\theta]$ in the units of degrees $cm^2$ $decimol^{-1}$ according to Eq. 1.

$$[\theta] = \frac{\theta}{10 \cdot C \cdot l} \tag{1}$$

where $l$ represents the light path length (cm), $C$ is the molar concentration of protein (mol/l), and $\theta$ represents the observed ellipticity (mdeg).

Thermal denaturation experiments were performed by monitoring the ellipticity at 222 nm. The temperature was increased from 20 to 100 °C at a heating rate of 0.5 °C/min. The transition midpoint temperature, $T_m$, is defined as the temperature at which half of the protein molecules in the system are unfolded. To calculate $T_m$ values, the data were fitted to a simple two-state thermodynamic model of unfolding with Eq. 2.

$$\theta_{222} = \frac{\theta_N + \theta_U \cdot \exp\left[-\frac{\Delta H_U}{RT} \cdot \left(1 - \frac{T}{T_m}\right) + \frac{\Delta C_{PU}}{RT} \cdot \left(T \ln\left(\frac{T}{T_m}\right) + T_m - T\right)\right]}{1 + \exp\left[-\frac{\Delta H_U}{RT} \cdot \left(1 - \frac{T}{T_m}\right) + \frac{\Delta C_{PU}}{RT} \cdot \left(T \ln\left(\frac{T}{T_m}\right) + T_m - T\right)\right]} \tag{2}$$

where $\theta_{222}$ is the relative ellipticity at 222 nm, $\theta_N$ and $\theta_U$ are the calculated ellipticities of the native and unfolded states, respectively, $\Delta H_U$ is the free enthalpy of unfolding, $\Delta C_{PU}$ is the heat capacity of unfolding, $T_m$ is the transition midpoint temperature, $T$ is temperature, and $R$ represents the universal gas constant.

BACΔNC in 20 mM Tris-HCl buffer (pH 7.9) or in this buffer plus sorbitol was unfolded with 1 M guanidine hydrochloride (GdnHCl) at room temperature. After 30 min incubation, the degree of BACΔNC unfolding was monitored by fluorescence. Fluorescence spectra of this enzyme were monitored at 30 °C in a JASCO FP-6500 spectrophotometer at an excitation wavelength of 280 nm. All spectra were corrected for the contributions of buffer and each osmolyte. The fluorescence emission spectra of protein samples with a concentration of 12 μM were recorded from 305 to 400 nm at a scanning speed of 240 nm/min. The maximal peak of the fluorescence spectrum and the change in fluorescence intensity were used in monitoring the unfolding processes of the enzyme. Both the red shift and the change in fluorescence intensity were analyzed together using the average emission wavelength (AEW) ($\lambda$) according to Eq. 3 [44].

$$\langle \lambda \rangle = \frac{\sum_{i=\lambda_1}^{\lambda_N} (F_i \cdot \lambda_i)}{\sum_{i=\lambda_1}^{\lambda_N} F_i} \tag{3}$$

in which $F_i$ is the fluorescence intensity at the specific emission wavelength ($\lambda_i$).

### Preparation of SDS-modified BACΔNC

To study the effects of SDS on BACΔNC activity and structure, the enzyme (0.328 mg/ml) was incubated in 20 mM Tris-HCl buffer (pH 7.9) without and with 0–200 mM SDS for 20 h, and then the samples were subjected to activity assays and spectroscopic measurements. To investigate the counteracting effect of osmolyte on the SDS-denatured BACΔNC, the enzyme in 20 mM Tris-HCl buffer (pH 7.9) containing 3 M sorbitol and different concentrations of SDS was incubated at 25 °C for 20 h before amylase assays and spectroscopic analyses.

## Results

### Effect of osmolytes on BACΔNC activity at elevated temperatures

We compared the amylolytic activity of BACΔNC in the absence and presence of three different osmolytes, glycerol, TMAO, and sorbitol, using soluble starch as the substrate. BACΔNC was incubated at the indicated temperatures for 10 min before performing the amylase assay. It was evident that the hydrolytic capability of the enzyme was significantly retained at elevated temperatures in the presence of osmolytes, especially the addition of sorbitol (Fig. 1). At 70 °C, the counteraction against thermal inactivation by 3 M sorbitol was still profound with greater than 80 % retention of the initial activity even extending the incubation beyond more than a week (data not shown). However, glycerol had only little protective effect on the heated enzyme (Fig. 1). Taken together, these data indicate that TMAO and sorbitol facilitate the catalytic activity of

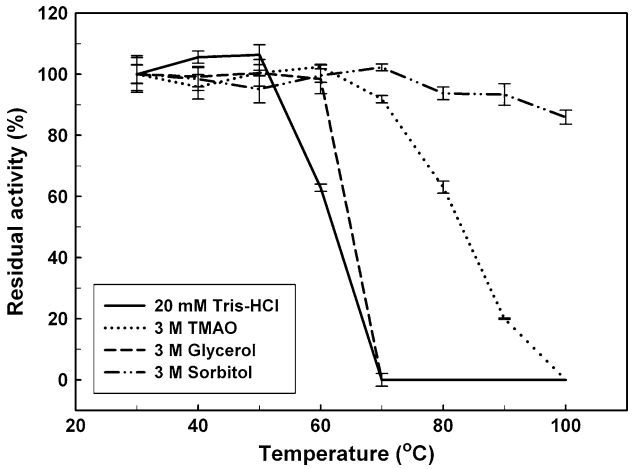


**Fig. 1** Effects of naturally occurring osmolytes on the thermostability of BACΔNC

BACΔNC and glycerol seems to have a limited protection on the enzyme.

## Thermal stabilization of the BACΔNC structure by TMAO and sorbitol

Chemical chaperones have been shown to stabilize proteins in their native states and to protect them against thermal denaturation and aggregation in vitro [3, 15]. There are several reports dealing with the function of osmolytes in the unfolding process of some proteins [11, 16, 27, 28, 55, 67]. Many potential osmolytes have also been investigated and they mainly affect the solvent properties of water as related to the structural stability of proteins [47]. To evaluate the effect of osmolytes on the secondary structures of BACΔNC, far-UV CD measurements were performed under various conditions. As shown in Fig. 2a, the CD spectra of BACΔNC in the absence and presence of osmolytes all displayed the strong peaks of negative ellipticity when the monitored temperature was set at 30 °C. The negative ellipticity of BACΔNC was retained in the presence of either TMAO or sorbitol at 70 °C, whereas no obvious ellipticity was observed in osmolyte-free and glycerol-containing samples (Fig. 2b). The content of secondary structural elements was further estimated by using the online DICHROWEB server (http://public-1.cryst.bbk.ac.uk/cdweb/htm1/) using the implemented CDSSTR program. In the osmolyte-free condition, BACΔNC contained 52 % α-helix, 22 % β-strand, 9 % β-turn, and 17 % random structure at 30 °C (Table 1). Together with a profound increase in the amount of β-turn and random structure, the helical content decreased to approximately 1 % when the enzyme sample was incubated at 70 °C. At the same temperature, TMAO and sorbitol had the ability to prevent the change of secondary structural elements of the enzyme (Fig. 2b and Table 1). These results

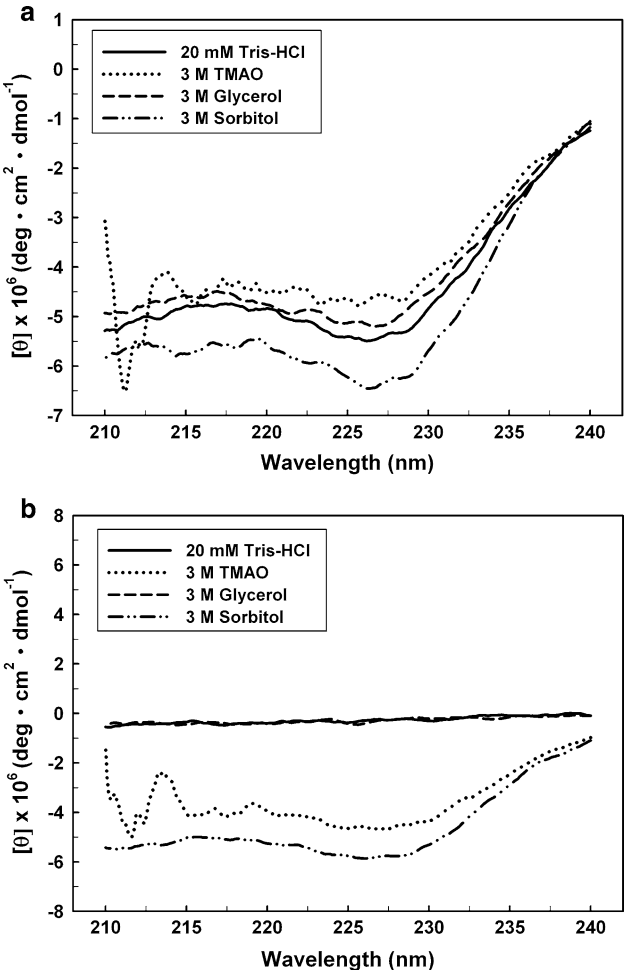


**Fig. 2** Effects of naturally occurring osmolytes on the secondary structures of BACΔNC. The far-UV CD spectra were recorded at 25 °C (**a**) or 70 °C (**b**)

explicitly indicate that TMAO and sorbitol have beneficial effects that counteract the temperature-perturbing actions on the secondary structures of BACΔNC.

Thermal denaturation of BACΔNC in the absence and presence of osmolytes was further evaluated by monitoring the ellipticity at 222 nm. Figure 3 shows the transition curves obtained with BACΔNC solutions at a heating rate of 0.5 °C/min. Osmolyte-free BACΔNC had an ellipticity of $-8.5 \times 10^6$ deg cm$^2$ dmol$^{-1}$ at 20 °C. The ellipticity was not appreciably changed up to 60 °C, whereas a significant decrease in the negative ellipticity was observed beyond this temperature. It should be noted that glycerol exhibited little counteraction against thermal denaturation. However, in the presence of either TMAO or sorbitol, the change of BACΔNC structure was retarded beyond 60 °C (Fig. 3). The $T_m$ value for glycerol-containing BACΔNC was calculated to be 75.2 °C, which is similar to that of the osmolyte-free sample. As expected, the transition midpoint temperature increased to 79.3 and 86.3 °C, respectively, in

**Table 1** Effects of three different osmolytes on the content of secondary structural elements of BACΔNC

| Conditions | α-Helix (%) | | β-Strand (%) | | β-Turn (%) | | Random (%) | |
|---|---|---|---|---|---|---|---|---|
| | 30 °C | 70 °C | 30 °C | 70 °C | 30 °C | 70 °C | 30 °C | 70 °C |
| 20 mM Tris-HCl | 52 | 1 | 22 | 31 | 9 | 23 | 17 | 45 |
| 3 M TMAO | 51 | 45 | 24 | 29 | 7 | 6 | 18 | 20 |
| 3 M Glycerol | 53 | 2 | 18 | 33 | 10 | 17 | 19 | 48 |
| 3 M Sorbitol | 52 | 51 | 20 | 21 | 8 | 8 | 20 | 20 |

**Table 2** Counteraction of SDS-induced denaturation of BACΔNC by 3 M sorbitol

| SDS (mM) | Relative activity (%) | Secondary structural elements | | | | Relative fluorescence intensity (%) |
|---|---|---|---|---|---|---|
| | | α-Helix (%) | β-Strand (%) | β-Turn (%) | Random (%) | |
| Without sorbitol | | | | | | |
| 0 | 100.0 ± 4.8 | 51 | 22 | 10 | 17 | 98.0 |
| 1 | 85.1 ± 6.2 | 53 | 23 | 8 | 16 | 92.7 |
| 2 | 62.8 ± 5.8 | 54 | 23 | 9 | 14 | 68.3 |
| 10 | 21.4 ± 5.1 | 57 | 20 | 13 | 21 | 33.9 |
| 20 | 19.5 ± 4.6 | 56 | 20 | 11 | 13 | 29.4 |
| 50 | 5.4 ± 0.6 | 65 | 19 | 9 | 7 | 26.1 |
| 100 | –[a] | 69 | 14 | 8 | 9 | 23.9 |
| 200 | – | 67 | 14 | 10 | 9 | 22.5 |
| With sorbitol | | | | | | |
| 0 | 100.0 ± 7.2 | 53 | 22 | 7 | 18 | 100.0 |
| 1 | 97.7 ± 6.2 | 51 | 23 | 10 | 16 | 92.9 |
| 2 | 90.1 ± 5.3 | 52 | 20 | 11 | 17 | 83.1 |
| 10 | 52.3 ± 4.2 | 57 | 16 | 9 | 18 | 50.3 |
| 20 | 45.2 ± 4.5 | 56 | 18 | 10 | 16 | 35.4 |
| 50 | 10.3 ± 0.4 | 59 | 15 | 9 | 17 | 30.6 |
| 100 | – | 64 | 14 | 7 | 15 | 27.4 |
| 200 | – | 66 | 17 | 9 | 8 | 23.9 |

[a] –, BACΔNC activity was not detected

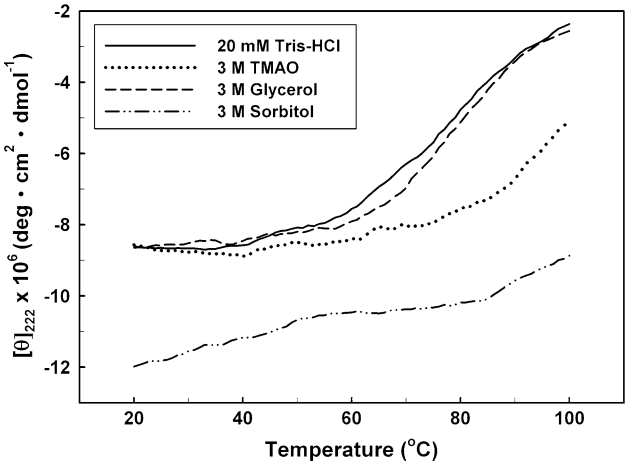


**Fig. 3** Unfolding of BACΔNC in 20 mM Tris-HCl buffer (pH 7.9) containing different osmolytes. Transition curves were obtained by recording the negative ellipticity of the enzyme sample at 222 nm

the presence of TMAO and sorbitol. These observations evidently reveal that the thermal stability of BACΔNC is significantly enhanced upon the addition of TMAO or sorbitol.

Additionally, we were interested in testing whether the osmolyte-modulated amylolytic activity is positively correlated with the conformational change of BACΔNC. Figure 4 shows the fluorescence emission spectra of the enzyme measured upon excitation at 295 nm. At elevated temperatures, the quantum yield and a blue shift in wavelength maxima of the emission spectra were obtained in aqueous solution or in the presence of 3 M glycerol (Fig. 4a, c). These fluorescence changes are typical of those accompanying the removal of aromatic residues from polar, aqueous solution into a more hydrophobic environment within the protein. However, at 70 °C, the temperature-induced conformational change was almost prevented

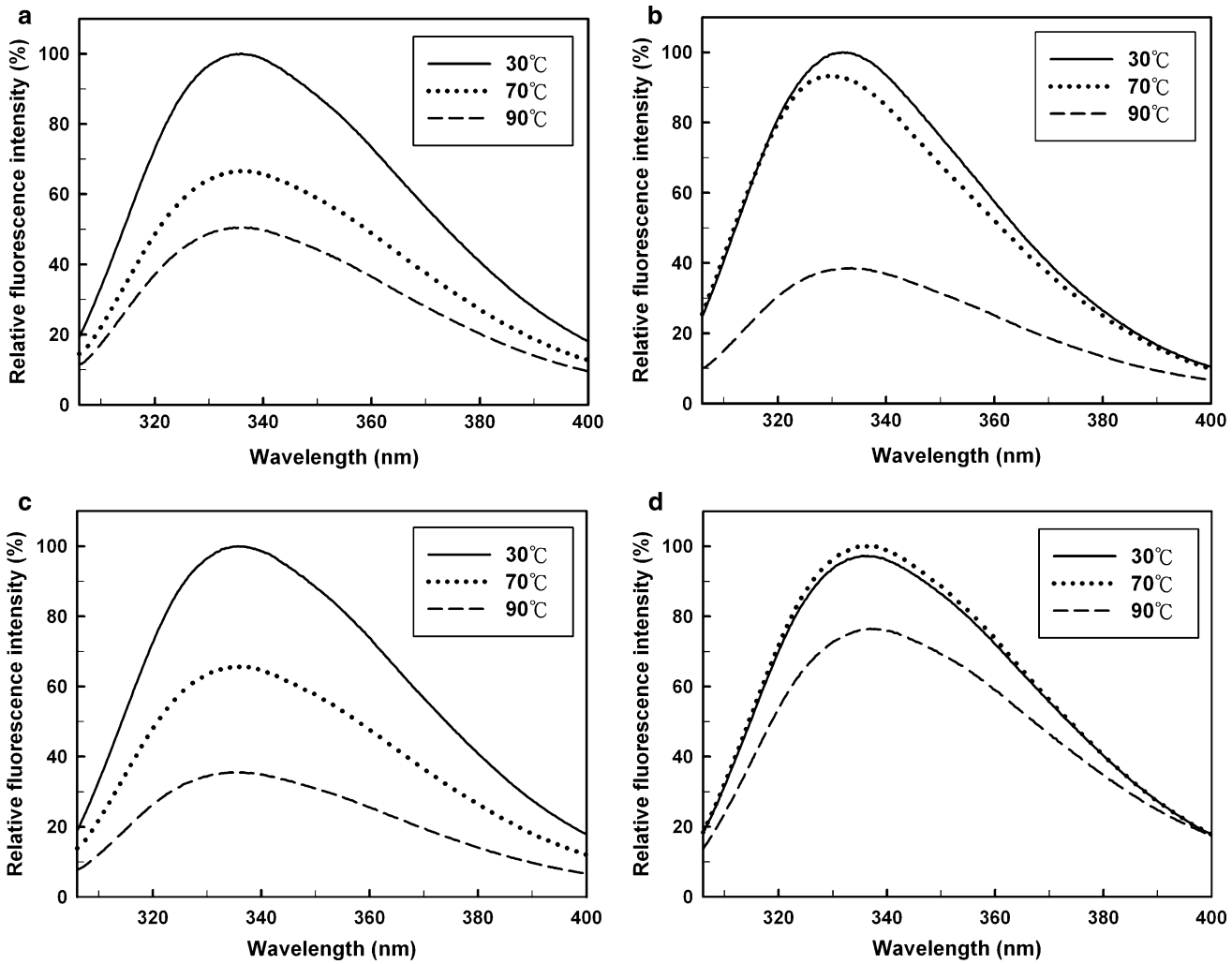


**Fig. 4** Relative fluorescence intensity of BACΔNC in the absence and presence of osmolytes. Each spectrum was corrected for the contribution of respective osmolytes. **a** BACΔNC in 20 mM Tris-HCl buffer (pH 7.9); **b** BACΔNC in 20 mM Tris-HCl buffer (pH 7.9) containing 3 M TMAO; **c** BACΔNC in 20 mM Tris-HCl buffer (pH 7.9) containing 3 M glycerol; **d** BACΔNC in 20 mM Tris-HCl buffer (pH 7.9) containing 3 M sorbitol

by 3 M TMAO. It is worth noting that there was little conformational change (less than 25 % changes in the relative fluorescence intensity) for BACΔNC supplemented with 3 M sorbitol, even when the temperature increased up to 90 °C (Fig. 4d). These observations strongly suggest that BACΔNC folds into a more compact structure in the presence of TMAO and sorbitol.

## Counteraction of GdnHCl-induced denaturation by sorbitol

The function of a protein depends on its ability to acquire a unique three-dimensional structure. GdnHCl is commonly used as a protein denaturant, which generally brings about unfolding of proteins by disrupting their secondary and tertiary structures. Earlier, the biophysical characteristics of BACΔNC were studied [13]. The enzyme started to unfold beyond approximately 0.2 M GdnHCl and reached an unfolded intermediate at 1.14 M. To investigate whether sorbitol would counteract GdnHCl-induced denaturation of BACΔNC, the structural properties of the denatured enzyme were analyzed in the absence and presence of this osmolyte. As shown in Fig. 5a, the negative ellipticity of the GdnHCl-treated BACΔNC decreased in a time-dependent manner, which indicates a significant disruption in the secondary structures upon denaturant treatment. Eventually, the change in the negative ellipticity of GdnHCl-treated enzyme was slowed down by the addition of sorbitol. These results clearly indicate that sorbitol has a counteracting effect on the GdnHCl-induced denaturation of BACΔNC.

Moreover, changes in both fluorescence wavelength and intensity were recorded to calculate the counteracting effect of the GdnHCl-induced unfolding. In the absence of

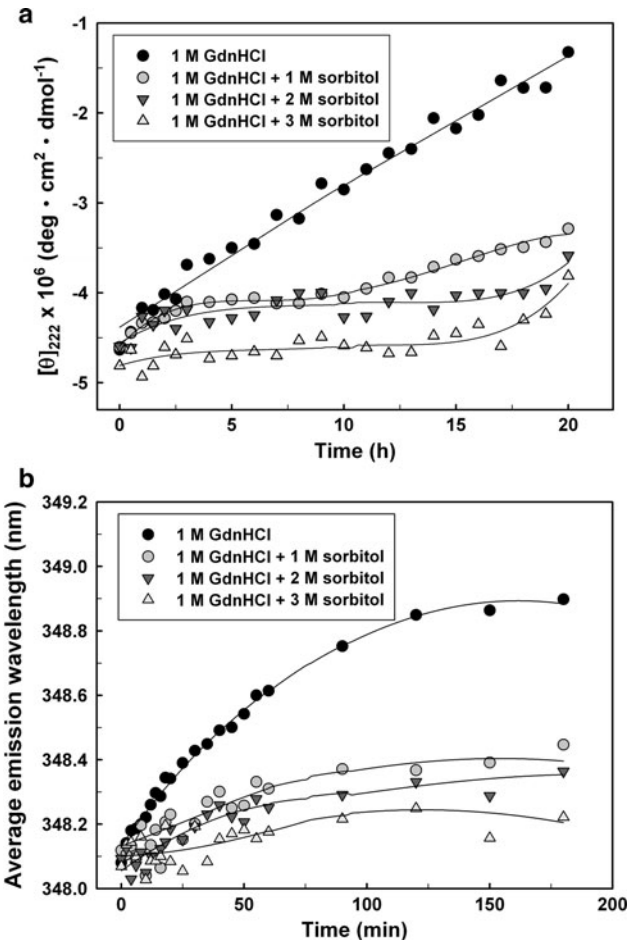


**Fig. 5** Counteraction of GdnHCl-induced denaturation of BACΔNC by sorbitol. **a** GdnHCl-induced changes in the secondary structures of BACΔNC as monitored by the negative ellipticity at 222 nm; **b** GdnHCl-induced changes in the AEW value of BACΔNC

sorbitol, the AEW value of GdnHCl-treated BACΔNC was 348.1 nm (Fig. 5b). The transition occurred at 60 min and a plateau region existed beyond 100 min. It is quite clear that the AEW value of GdnHCl-treated enzyme was kept below 348.4 nm in the presence of sorbitol. This finding further indicates that sorbitol strongly counteracts the GdnHCl action on the denaturation of BACΔNC.

Suppression of SDS-induced denaturation by sorbitol

SDS is an anionic surfactant that is frequently used in denaturing proteins, such as in the preparation of samples in SDS-PAGE. Time course experiments indicated that BACΔNC inactivation by 1–200 mM SDS reached equilibrium in less than 20 h (data not shown). Thus, the extent of BACΔNC inactivation was measured by incubating the enzyme solutions with various concentrations of SDS for 20 h at 25 °C, and then the residual activity was measured. The concentration-dependent inactivation of BACΔNC by SDS exhibited a typical two-state process (figure not shown). The purified BACΔNC had a specific activity of 157.3 ± 7.8 U/mg protein, whereas approximately 78.6 % of the activity was abolished in the presence of 10 mM SDS (Table 2). The enzyme was fully inactivated in the presence of greater than 50 mM SDS and the midpoint of BACΔNC inactivation was at 4.0 ± 0.2 mM denaturant. It is worth noting that the SDS-mediated inactivation of BACΔNC was slowed down upon the addition of 3 M sorbitol and the protective action of this osmolyte resulted in a 1.5-fold increase in the midpoint.

Far-UV CD and intrinsic tryptophan fluorescence experiments were also performed to evaluate the secondary and tertiary structural changes in the SDS-mediated denaturation of BACΔNC. Table 2 shows the percentages of secondary structural elements of the enzyme in different concentrations of SDS. As compared with the control, a nearly identical proportion of the $\alpha$-helix and $\beta$-strand elements indicated that the presence of up to 2 mM SDS did not appreciably affect the secondary structures of the enzyme. Beyond 10 mM of SDS, the helical contents of BACΔNC were significantly increased with the increasing concentrations of this detergent. It should be noted that 3 M sorbitol could prevent the changes in the secondary structures of BACΔNC in the presence of SDS, especially when the concentration of this detergent was below 10 mM. This might be the reason for the activity retention upon the addition of low concentrations of SDS.

The primary sequence of BACΔNC contains 19 tryptophan residues [12], which provide a useful probe to detect the microenvironments around these aromatic residues by intrinsic fluorescence. The fluorescence spectrum of the native enzyme reached its emission maximum at a wavelength of 336.5 nm (Fig. 4a). With the increase of SDS concentrations, a clear decrease of fluorescence intensity was observed (Table 2). This observation implies that the Trp residues are more exposed to the solvent upon the addition of SDS. Interestingly, the fluorescence quenching was prevented significantly in the presence of 3 M sorbitol (Table 2). These results lead to the conclusion that the osmolyte could act as folding assistants during the detergent-induced unfolding of BACΔNC.

## Discussion

A major limitation to the industrial use of enzymes is their relative instability under operational conditions, which involve exposure to extremes of temperature, pressure, pH, denaturants, and organic solvents. Given that enzyme stability is a key issue in most industrial and pharmaceutical applications, many efforts have been made in the development of highly stable enzymes that can perform efficiently under severe working conditions of industrial

processes [8, 21, 35]. Among the enzymes used for biocatalytic processes, thermostable ones are highly attractive and have received increasing attention because of their higher reactivity, higher process yield, lower viscosity, and fewer contamination problems [36]. In general, there are two options to thermally stabilize a protein: the first is to modify the solution conditions and the second is to engineer the enzyme itself. Because of the cost factor associated with protein engineering, the second option is not basically recommended. Thus, understanding of the protein stabilization exerted by different solution conditions is critical for the development of ideal formulations to increase enzyme stability. In this investigation, the sorbitol-containing BACΔNC was active toward 1 % soluble starch after incubating the enzyme sample at up to 100 °C for 10 min (Fig. 1). Far-UV CD and intrinsic fluorescence data confirmed that sorbitol or even TMAO could stabilize the secondary and tertiary structures of BACΔNC at elevated temperatures. In this regard, the enzyme seems to keep its flexibility under the tested conditions, which might provide the structural basis for a higher catalytic activity of this enzyme at temperature near 100 °C. Consistently, thermal stabilization by sorbitol has been observed in ribonuclease [63] and papain [46].

Osmolytes can stabilize proteins against unfolding under environmental stresses such as high temperatures, desiccation, or chemical denaturants [66]. The stabilizing property of osmolytes has been shown to correlate with the preferential exclusion of osmolytes from unfolded protein domains, leading to the preferential hydration near an unfolded protein [63]. This implies a net repulsive interaction between the stabilizing osmolytes and proteins, and indeed preferential exclusion has been shown to arise from repulsive interactions between osmolytes and the backbone of proteins [7]. Eventually, repulsive osmolyte–backbone interactions would raise the enthalpy of a protein, and the increase in enthalpy would be larger for the unfolded state owing to its larger solvent-exposed backbone area. In contrast to protecting osmolytes, protein unfolding by the classical chemical denaturants, such as urea and GdnHCl, has long been considered to arise because of the favorable interactions of the chemical agents with the normally buried segments of a protein [58]. The basis of biomolecular interactions for destabilization by these denaturants has been generally attributed to direct ligand binding with the protein surface or the influence of the denaturants on the structure and dynamic of water molecules [39, 48]. In our case, sorbitol was found to offset the denaturing effect of GdnHCl on BACΔNC (Fig. 5). It is widely argued that the counteracting ability of organic osmolytes does not arise from the stabilization of the native state but arises primarily from the destabilization of the unfolded state of proteins upon osmolyte addition [5, 6, 61]. Thus, the counteractive effect of sorbitol on the GdnHCl-induced unfolding of BACΔNC is likely due to destabilization of the unfolded state of the enzyme by this osmolyte. Timasheff also demonstrated a similar mechanism to explain the counteractive abilities of osmolytes [57]. In fact, the mechanism proposed for sugar osmolytes such as sorbitol [7] and sucrose [28] shows a predominant effect of the osmolyte on the unfolded state. In molecular terms, the number of non-thermodynamically neutral contacts with loci on the protein surface decreases relative to those with water when the protein unfolds. The idea that species with smaller surface areas are favored over species with larger surface areas in the presence of sugars was proposed previously. Liu and Bolen [28] proposed that the decreased exposure of the backbone on folding is the major driving force for sucrose-induced stabilization. The increase of hydrophobic interactions induced by water-structuring sugars may favor more compact states.

In this study, at SDS concentrations above 10 mM, a profound increase in the helical content together with a significant decrease in the $\beta$-strand and random coil values was observed for osmolyte-free BACΔNC sample (Table 2). Consistently, SDS has been shown to increase the helical content of a variety of enzymes, including aminoacylase [23], inorganic pyrophosphatase [37], and cellulase [62]. Despite years of study, the mechanism of surfactant–protein interactions is not completely understood [33, 40, 64]. However, our results definitely indicate that 3 M sorbitol can modulate the inactivation of BACΔNC by SDS. Stabilization of the secondary and tertiary structures of the enzyme by this osmolyte might be responsible for the modulation, especially at lower concentrations of SDS (Table 2). Furthermore, sorbitol has been previously shown to protect proteins from chemical denaturation because it is less effective at solvating the protein backbone relative to water [5]. This stabilizing effect is described as preferential hydration, in which the osmolyte destabilizes the unfolded state relative to the native state because the unfolded state has a higher degree of backbone exposure to the solvent [2]. In this regard, sorbitol might affect the structure of BACΔNC, which leads to the preferential hydration of the peptide backbones and would thus be expected to rescue the action of SDS on the enzyme.

The present study allows us to conclude that TMAO and sorbitol are able to strongly counteract the effects of temperature-induced destabilization on BACΔNC. Spectroscopic studies indicated that the secondary and tertiary structures of the enzyme could be accomplished with the help of these two osmolytes. Moreover, sorbitol exerts a powerful counteraction against the effects of the chemical perturbants GdnHCl and SDS on the enzyme. In this regard, this osmolyte might be useful for the design of new

stabilization and counteraction studies on the amylolytic enzymes from *Bacillus* species.

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