doi:10.1246/cl.2010.38

Published on the web December 5, 2009

Effect of Trehalose on Thermal Stability of Bovine Serum Albumin

Roberto Lavecchia* and Antonio Zuorro
Department of Chemical Engineering, University of Rome "La Sapienza," Via Eudossiana 18, 00184 Rome, Italy

(Received October 29, 2009; CL-090960; E-mail: roberto.lavecchia@uniroma1.it)

Trehalose at 10–30% (w/w) greatly stabilized bovine serum albumin (BSA) against thermal denaturation. The highest stabilization was reached in 30% trehalose at 65 °C, when the protein's half life was increased from 72 to 335 min. A kinetic analysis based on the Lumry–Eyring mechanism of inactivation showed that BSA denaturation can be described by a first-order rate expression with an apparent activation energy ranging from 238.1 to 246.4 kJ mol^{-1}.

Trehalose (α-D-glucopyranosyl-[1,1]-α-D-glucopyranoside) is a nonreducing disaccharide widely distributed in organisms facing environmental stress conditions such as osmotic and heat shock, sporulation, and dehydration.[1] Its unique water-structuring properties are believed to be responsible for its protective action on cell membranes and proteins.[2] However, despite the increasing number of reports describing the beneficial effects of trehalose on biomolecules and cellular structures, the underlying molecular mechanisms remain largely elusive.[3] In particular, little is known about how the nature of protein molecules may affect stabilization through the generation of favorable or unfavorable trehalose–protein interactions.[4] Accordingly, the degree of stabilization induced by trehalose is largely unpredictable at present. In order to provide further insight into the ability of trehalose to stabilize proteins, we have investigated the kinetics of thermal denaturation of bovine serum albumin (BSA) in the presence and absence of this compound. Albumin is the most abundant protein in mammalian systems but, despite the similarity among mammalian albumins (the sequence homologies are greater than 70%), remarkable interspecies differences exist in their denaturation behavior.[5] We focused our attention on BSA because of the relatively few studies on this protein as compared with human serum albumin.[6]

Fatty acid-free BSA (>96%) and D-(+)-trehalose (>99%) were purchased from Sigma-Aldrich (Milano, Italy) and used without further purification. Thermal denaturation of BSA was studied by the method of Olsen.[7] Briefly, BSA in aqueous solution (pH 5.5) at 1 g L^{-1}, alone or in the presence of trehalose, was poured into stoppered glass tubes of 10-mL capacity. The tubes were placed in a water bath at the appropriate temperature (±0.1 °C). At selected times, a tube was withdrawn, held in iced water for a few minutes to stop denaturation and centrifuged (30000 × g for 30 min at 15 °C) to remove protein precipitates. Then, an aliquot of the supernatant was passed through a 0.2-μm nylon filter and analyzed spectrophotometrically to determine the residual BSA concentration. Optical measurements were made in the 240–300-nm region with a double beam UV–vis spectrophotometer (Perkin-Elmer Lambda 25).

Trehalose was added at 10 and 30% (w/w), and the temperature was varied between 55 and 75 °C.

Figure 1 shows typical first-derivative spectra for BSA in aqueous solution obtained after subjecting the protein to 2-h heating under given thermal conditions. BSA concentration in the liquid (c) was evaluated from a calibration curve based on the difference of the first-derivative signals ($dA/d\lambda$) at $\lambda_1 = 268.5$ and $\lambda_2 = 288.4$ nm. Since the denatured protein aggregates are removed before analyzing the liquid, the ratio c/c_0 provides a measure of the extent of denaturation. Some plots of c/c_0 versus time are displayed in Figure 2

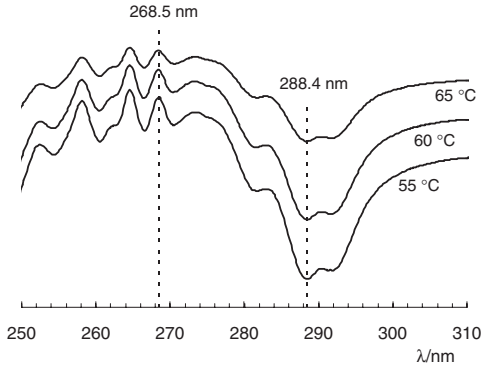

Figure 1. First-derivative spectra of BSA heated for two hours at different temperatures.

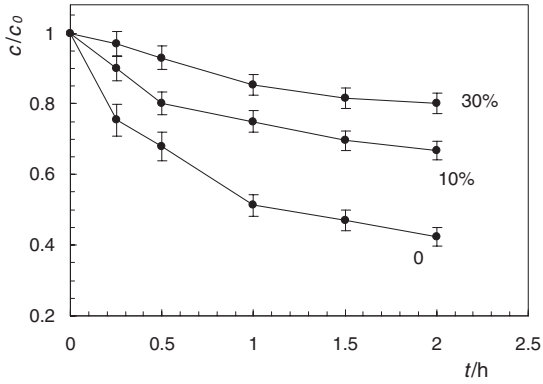

Figure 2. Time course of BSA denaturation at 65 °C in the absence and presence of 10 and 30% w/w trehalose.

Thermal denaturation of BSA was assumed to proceed according to the Lumry–Eyring model: F $\rightleftarrows$ U $\rightarrow$ I, where the reversible partial unfolding is followed by an irreversible denaturation step. In the above scheme, F and U represent the folded and unfolded protein forms, respectively, while I denotes the irreversibly denatured protein.

Assuming the following rate law for the irreversible step:

$$r = kc_U{}^m \quad (1)$$

and performing a mass balance for the irreversibly denatured BSA over the solution volume yield:[8]

Table 1. First-order rate constants for BSA denaturation in the absence (k^*_0) and presence (k^*_{10}, k^*_{30}) of 10 and 30% w/w trehalose

T/°C	k^*_0/h^{-1}	k^*_{10}/h^{-1}	k^*_{30}/h^{-1}
55	0.039 ± 0.004	—	—
60	0.123 ± 0.015	0.102 ± 0.012	0.050 ± 0.007
65	0.579 ± 0.062	0.235 ± 0.032	0.124 ± 0.014
70	1.957 ± 0.236	0.929 ± 0.108	0.504 ± 0.068
75	6.432 ± 0.855	3.987 ± 0.521	2.194 ± 0.220

Table 2. Estimated parameters from the Arrhenius equation (E, T^*) and standard error for the dependent variable (σ_Y)

Trehalose	E/kJ mol^{-1}	T^*/K	σ_Y
0	246.4 ± 6.4	340.6 ± 8.9	0.106
10% w/w	238.1 ± 22.1	343.0 ± 32.0	0.256
30% w/w	245.4 ± 20.3	345.5 ± 28.9	0.235

$$\frac{dc_I}{dt} = k\left(\frac{K_U}{K_U + 1}\right)^m (c_0 - c_I)^m \quad (2)$$

where k is the specific denaturation rate constant, m is the apparent reaction order, and K_U is the equilibrium constant for partial unfolding. Integration of eq 2 with the initial condition: $c_I(0) = 0$ allows evaluation of the time course of BSA denaturation. The model contains two parameters: m and $k^* = k[K_U/(K_U + 1)]^m$, which were estimated by minimizing the sum of square errors between experimental and calculated BSA concentrations in the liquid.

At all temperatures and in all media the best results were obtained for the first-order assumption ($m = 1$), which provided the apparent rate constants listed in Table 1.

The activation energy on denaturation (E) was then evaluated by the following Arrhenius-type equation:

$$k^*(T) = \exp\left[-\frac{E}{R}\left(\frac{1}{T} - \frac{1}{T^*}\right)\right] \quad (3)$$

where T^* is the temperature at which k^* assumes a unitary value. The results are presented in Table 2 and Figure 3.

Examination of k^* values indicates that trehalose stabilizes BSA under all the temperature and concentration conditions. In the presence of 30% trehalose, the protein's half life (calculated as $\tau = \ln(2/k^*)$) exhibited an average 3.5-fold increase. The highest stabilization was achieved at 65 °C, when τ increased from 72 to 335 min.

The fact that the apparent reaction order and the activation energy (243.3 ± 4.5 kJ mol^{-1}) were independent of the presence and concentration of trehalose suggests that the basic phenomena involved in BSA denaturation are essentially independent of the nature and composition of the medium. A physical explanation for the protective action of trehalose could be found in its ability to enhance solvent ordering through the formation of networks of structured water[2] and, to a lesser extent, in the induced increase in the surface tension of the medium.[9] If the protein behaves as an inert component, that is, does not interact specifically with trehalose, the above mechanisms cause the additive molecules to be excluded from the domain of the protein, which becomes preferentially hydrated.[10]

The uneven distribution of an additive between the protein domain and the bulk solvent is a reflection of the mutual

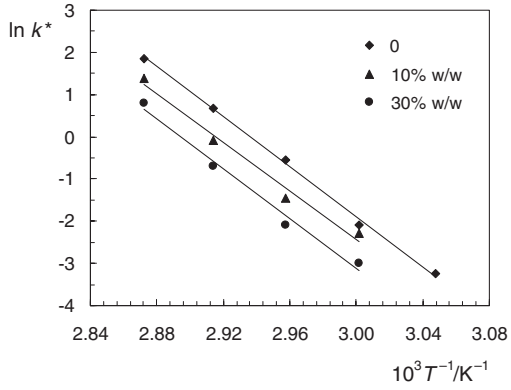

Figure 3. Arrhenius plot for BSA denaturation in the absence and presence of 10 and 30% w/w trehalose.

perturbation of their chemical potentials. According to the Wyman theory, a preferentially excluded additive can displace the unfolding equilibrium (F ⇌ U) toward the native form, stabilizing the protein.[11] Turning to the trehalose–BSA system, such a displacement would cause the equilibrium constant K_U and, consequently, k^* to decrease. Of course, trehalose could also affect the irreversible denaturation step (U → I). Although these effects are much more difficult to assess,[10] the trehalose-induced reduction of k^* suggests that they proceed in the same direction as those on K_U or, alternatively, that their influence is not too strong.

Overall, these results add further support to the view that the stabilizing properties of trehalose do not arise from a direct interaction of this compound with the protein but, rather, from the entropically driven mechanism of solvent ordering, making it a fairly universal protein stabilizer.

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