Electronic Supplementary Information

Osmolyte induced Protein Stabilization: Modulation of Associated Water Dynamics might be a Key Factor

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Section S1: Tagging of fluorescent dye to papain

CPM dye is solvatochromic in nature i.e., it can sense the degree of polarity of its immediate surroundings. CPM dye in buffer has low emissive nature but after the attachment with the protein a huge increment in fluorescent intensity can be seen. It is probably due to the restrictions imposed on the free rotation of the dye by the matrix of the protein and thereby decreasing the possibility of non-radiative decay paths. In addition to this, when excited at 393nm (absorption maxima), the tagged protein emits with emission maxima centered at ~479 nm (shown in fig. 2(i)) whereas emission maxima of free CPM dye in buffer is centered at ~481 nm. The observed blue shift in going from free to tagged state confirms that on tagging it goes to some hydrophobic region. These observations together confirm successful tagging of protein. TMR is not solvatochromic in nature so emission study could not be useful to confirm its tagging to protein. We employed FCS to measure diffusion time of the two. TMR tagged papain diffuses slowly with diffusion time of 130 µs relative to the diffusion time of 39 µs of free TMR dye through the same volume of 0.5 fL. This increase in diffusion time confirms tagging of dye to protein (fig.1(b)). There is no appreciable change in CD signal and normalized emission profile (ex 295nm) of papain on tagging with CPM and TMR as is evident from fig. S1(d) and S1(e) which confirms about no significant alteration of structure of papain on tagging.

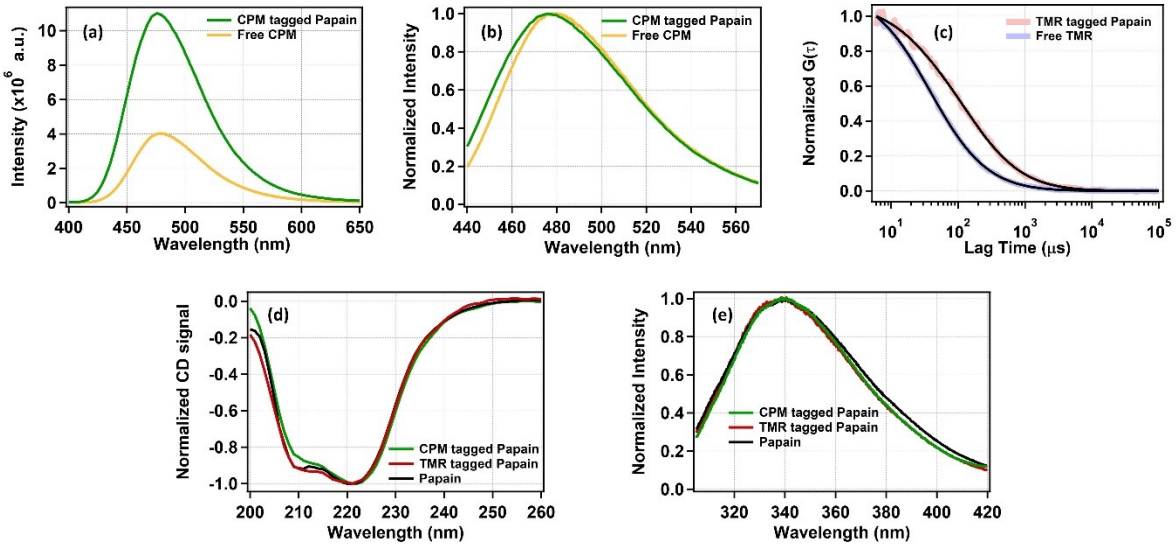


Figure S1. (a) Emission of CPM before and after tagging. (b) Normalized emission spectra of free CPM and CPM-tagged papain on excitation at 390 nm. (c) Normalized autocorrelation traces for free TMR dye and TMR tagged papain. The fitting line for each trace is shown in black. (d) Normalized CD spectra of papain, CPM tagged papain, TMR tagged papain. (e) Normalized emission spectra of papain, CPM tagged papain, TMR tagged papain ($\lambda_{ex} =$ 295nm).

Section S2: Tagging of fluorescent dye to bromelain

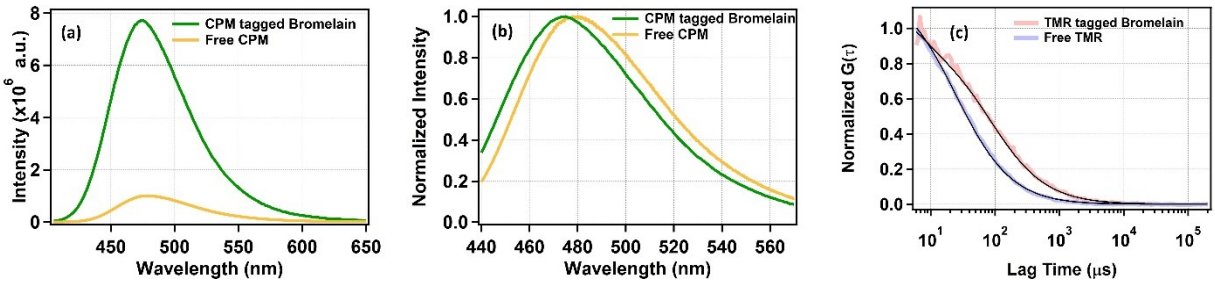


Figure S2. (a) Emission of CPM before and after tagging, (b) Normalized emission spectra of CPM tagged bromelain on excitation at 390 nm, (c) Normalized autocorrelation traces for free TMR dye and TMR tagged bromelain, and here the fitting line for each trace is shown in black.

An increase in intensity and a blue shift of ~6 nm in emission spectra, here also confirms the successful tagging of CPM to bromelain. A slower diffusion of TMR tagged bromelain (114 μs) in fig. 2(c) with respect to the free TMR, confirms the tagging of bromelain with TMR dye. As, we have already shown that no significant perturbation of structure of bromelain occurs upon tagging with CPM and TMR, we restrained ourselves from showing all those here[1].

Section S3: FCS: instrumentation and data fitting procedure

We have performed fluorescence correlation spectroscopic (FCS) measurements on an instrument built in our laboratory. We have used an inverted microscope (IX-71, Olympus, Japan) with a 60X 1.2 NA water-immersion objective (UplanSApo, Olympus, Japan) in this setup. The sample was kept on a cover slip (Blue Star, Polar Industrial Corporation, India) on the microscope sample platform. A 532 nm (MGL-III-532-5mW, DreamLaser, China) laser source was used to create a confocal observation volume 40 μm above the upper surface of the cover slip. The emitted photons, collected using the same objective, travel through a dichroic mirror (ZT532rdc, Chroma Tech. Corp., USA, for 532 nm excitation), emission filter (605/70m, Chroma Tech. Corp., USA), and multimode fiber patch chord (M67L01 25μm 0.10 NA, ThorLabs, USA) before reaching the detector (SPCM-AQRH-13-FC, Excelitas Tech. Inc., Canada). The detected photons were autocorrelated using a correlator card (FLEX990EM-12E, correlator.com, USA) and displayed using the LabVIEW® platform on a computer. Assuming a Gaussian detection volume, for a single component system diffusion without any additional reaction, the fluorescence intensity autocorrelation function can be written as

$$G(\tau) = \frac{1}{N}\left(1 + \frac{\tau}{\tau_D}\right)^{-1}\left(1 + \frac{\tau}{\omega^2\tau_D}\right)^{-1/2} \quad \text{(S1)}$$

In presence of a reaction component that also influence the fluorescence intensity fluctuations in the observation volume in addition to the diffusion, the equation is modified to

$$G(\tau) = \frac{1}{N}\left(1 + \frac{\tau}{\tau_D}\right)^{-1}\left(1 + \frac{\tau}{\omega^2\tau_D}\right)^{-1/2}\left(1 + A\, exp\left(-\frac{\tau}{\tau_R}\right)\right) \quad \text{(S2)}$$

In the above equations, τ_D is the time constant for diffusion, N is the number of particles in the observation volume, $\omega = l/r$ is the ratio of the longitudinal to transverse radius of the 3D Gaussian volume, A is the amplitude of processes other than diffusion that may give rise to fluorescence fluctuations, and τ_R is the timescale of such processes.

We measured several fluorescence intensity autocorrelation curves of rhodamine 6G ($R6G$) at varying concentrations in water and fitted them globally to determine the value of ω. While calibrating the value of ω, the diffusion coefficient of $R6G$ in water is taken to be D_t= 4.14 × 10^{-6} cm^2 s^{-1}.[2] For a particular set of experiments, while fitting the data with equation S1 or equation S2, ω is kept fixed.

The value of transverse radius (r) is calculated using the equation,

$$D_t = \frac{r^2}{4\tau_D} \tag{S3}$$

Where, τ_D is the diffusion time and D_t is the diffusion coefficient of the molecule.

The observation volume of our FCS setup is estimated to be 0.8 fL using equation,

$$V_{eff} = \pi^{\frac{3}{2}} r^2 l \tag{S4}$$

In the presence of additives, the refractive index and the viscosity of the solution may change significantly, which need to be corrected to get the diffusion coefficient. The refractive index change is compensated by changing the objective collar position to achieve the lowest diffusion time value for each of the samples.[3] In this way, we maintain the lowest detection volume attainable for each sample. We have rectified the effects of viscosity changes through performing control experiments at every experimental point taking $R6G$ as the fluorophore. $R6G$ is a rigid molecule and will not undergo any structural changes when exposed to aqueous solution of additives. In this way, any changes in its diffusion time through the detection volume will be solely because of differences in the medium viscosity. Using this information and the reported value of the hydrodynamic radius of $R6G$ (7.7 Å) in pH 7.4 buffer, we have calculated the hydrodynamic radius of papain and bromelain at every experimental point according to the following equation:

$$r_H = r_H^{R6G} \times \frac{\tau_D}{\tau_D^{R6G}} \tag{S5}$$

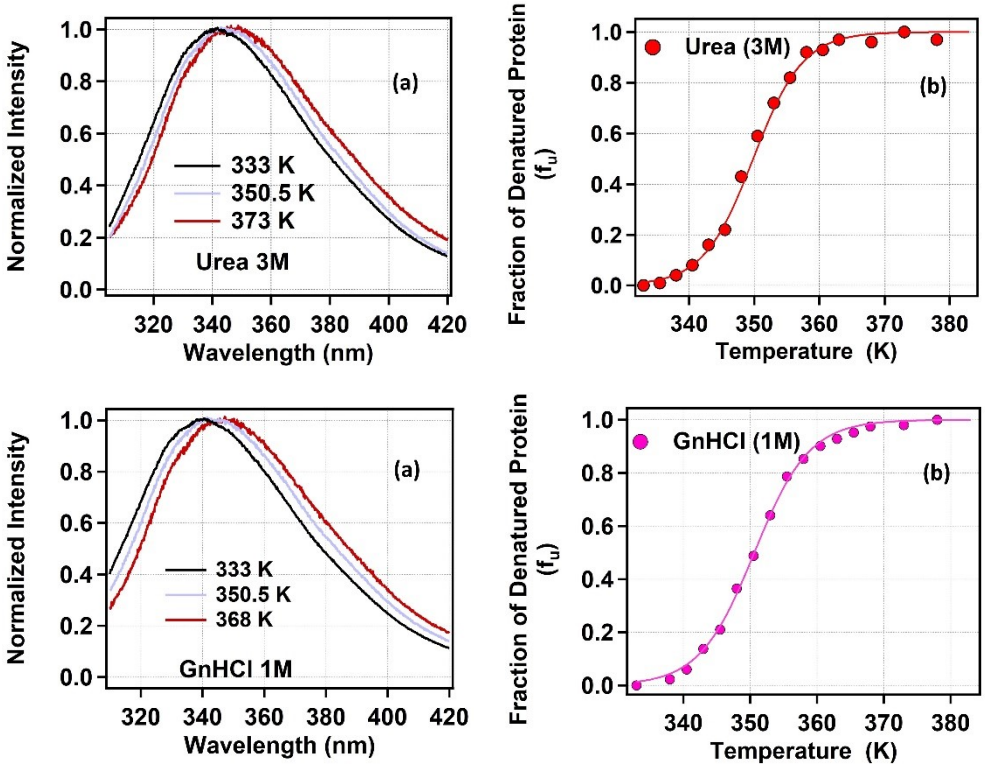
Section S4: Thermal denaturation study of papain in buffer and osmolyte solutions

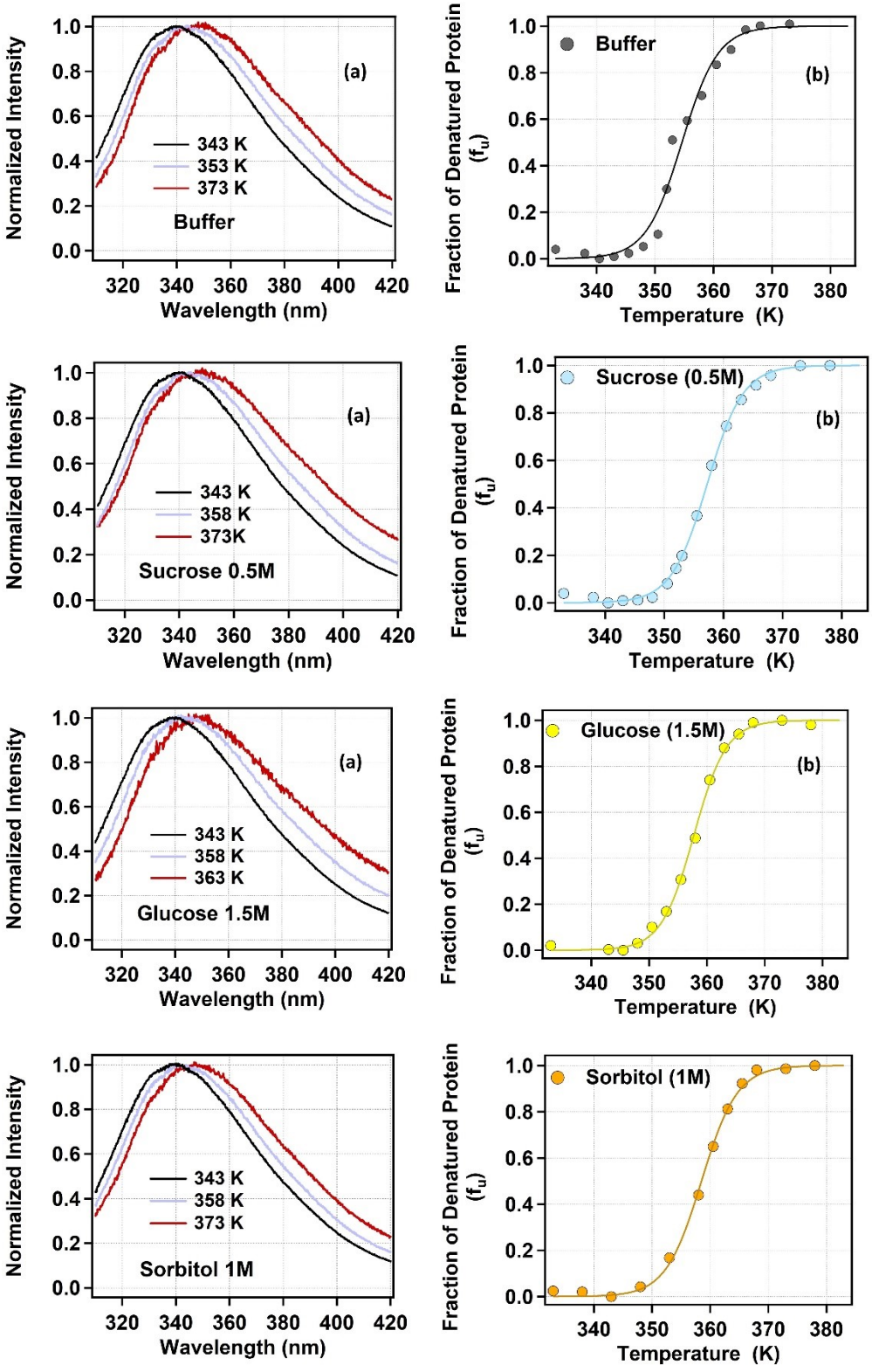
Determination of melting temperature (T_m)

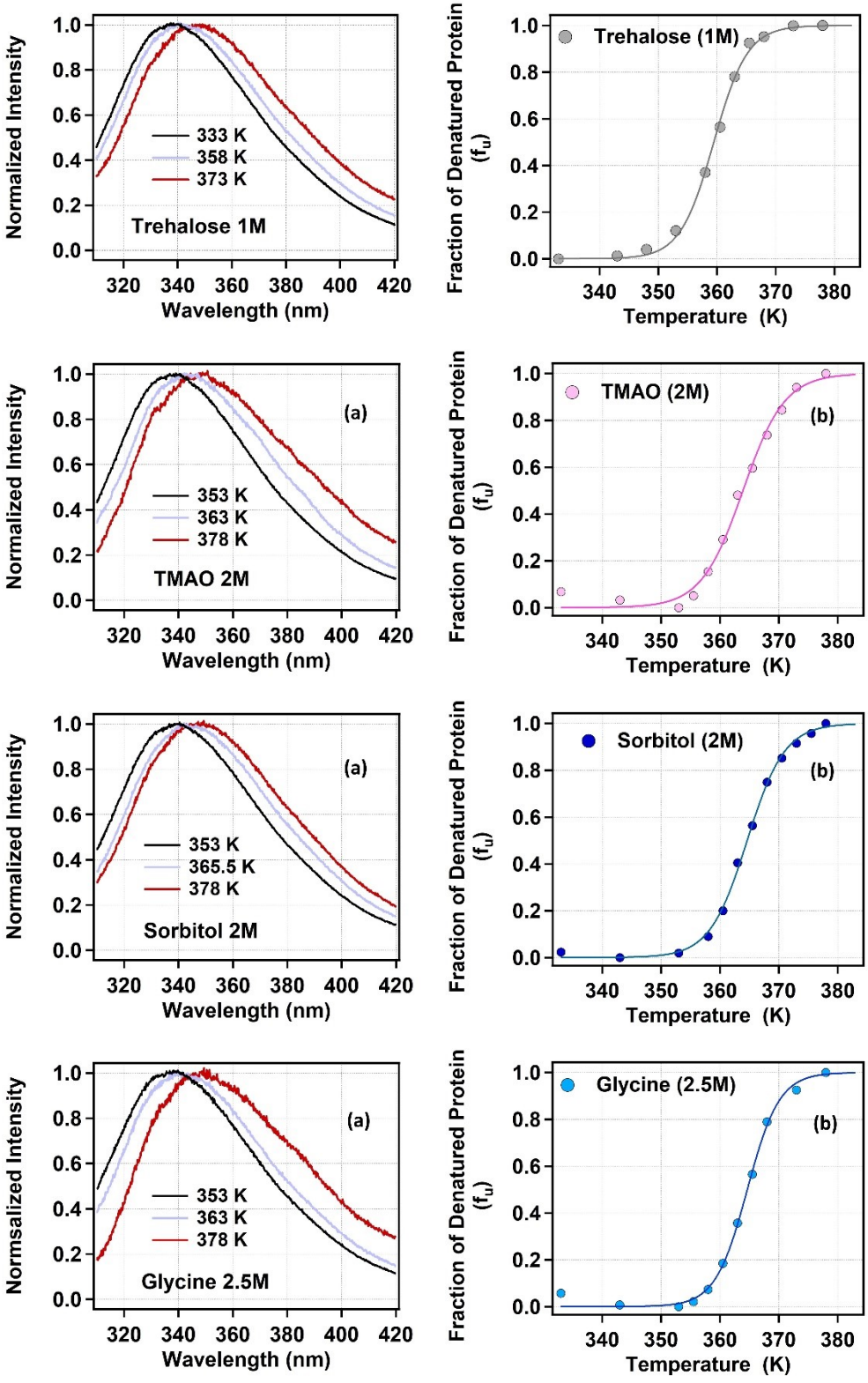
The denaturation of protein is considered as a cooperative phenomenon[4,5] and thus the denaturation pathway follows the sigmoidal nature. Keeping this in mind, here the denaturation plot is fitted with a sigmoidal function[6] given by equation 1,

$$y = a + b/(1 + exp(((x\_half - x))/d)) \quad \text{(S1)}$$

where, a represents the value of lower asymptote which is taken as 0 and signifies the native state (i.e., $f_N = 0$), b represents the distance from a to the upper asymptote which is taken as 1 and signifies the denatured state (i.e., $f_D = 1$), x_half represents the inflection point and signifies the melting temperature (T_m) and d represents the distance from x_half of the zone of high compliance.




Normalized Intensity
Wavelength (nm)
(a)
343 K
353 K
373 K
Buffer
Fraction of Denatured Protein (f_u)
Temperature (K)
Buffer
(b)
343 K
358 K
373K
Sucrose 0.5M
Sucrose (0.5M)
343 K
358 K
363 K
Glucose 1.5M
Glucose (1.5M)
343 K
358 K
373 K
Sorbitol 1M
Sorbitol (1M)


Normalized Intensity
Wavelength (nm)
333 K
358 K
373 K
Trehalose 1M
Fraction of Denatured Protein (f_u)
Temperature (K)
Trehalose (1M)
(a)
353 K
363 K
378 K
TMAO 2M
TMAO (2M)
(b)
365.5 K
Sorbitol 2M
Sorbitol (2M)
Normsalized Intensity
Glycine 2.5M
Glycine (2.5M)

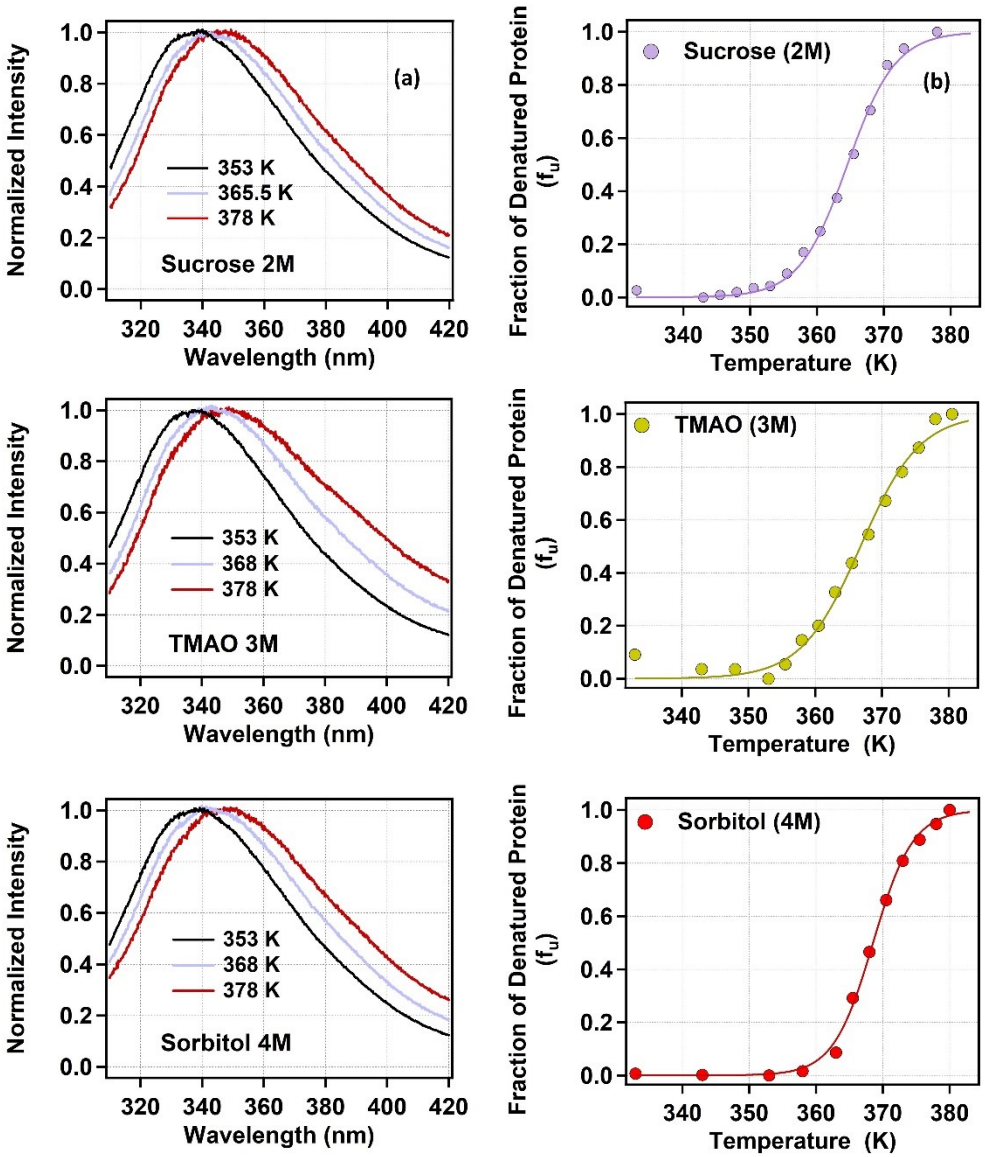


Figure S3. Here the column (a) represents the change in emission spectra of papain (ex. 295 nm) in mentioned osmolyte on raising the temperature. Spectra in black, red and light blue color represent the emission spectra in native state, denatured state and some state in between the two. And (b) represents the variation of emission maxima of papain (ex. 295 nm) with temperature in the same mentioned osmolyte. Line across the points represents the two-state fitting.

Table S1. Melting point of papain in buffer and other osmolyte solution. (Error bar represents the standard deviation of the mean of three independent experiments.)

Osmolyte	T_m**(K)**
Urea (3M)	349.8±0.5
GnHCl (1M)	350.4 ±0.5
Buffer	354.4 ±0.5
Sucrose (0.5M)	357.3 ±0.5
Glucose (1.5M)	358.3 ±1.0
Sorbitol (1M)	358.7 ±1.0
Trehalose (1M)	359.7 ±0.5
TMAO (2M)	363.9 ±0.7
Sorbitol (2M)	364.5 ±0.5
Glycine (2.5M)	364.6 ±0.5
Sucrose (2M)	365.2 ±0.5
TMAO (3M)	366.8 ±0.5
Sorbitol (4M)	368.4 ±0.5

Section S5: Thermal denaturation study of bromelain in buffer and sucrose solution

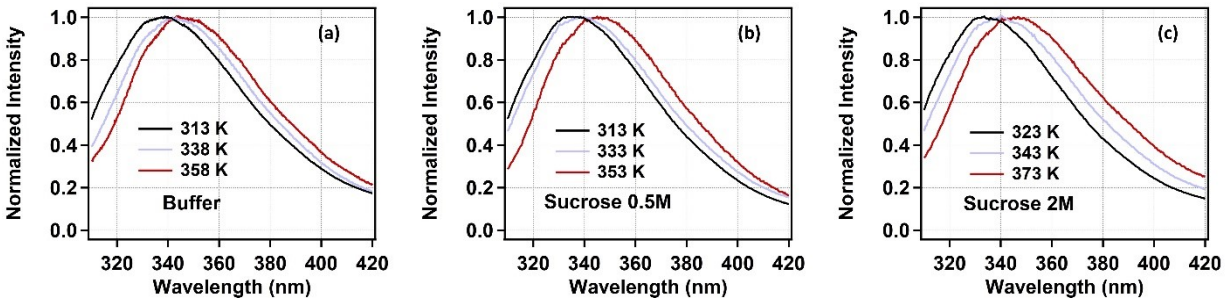


Figure S4. Temperature dependent emission spectra of bromelain (ex. 295 nm) in mentioned osmolyte on raising the temperature. Spectra in black, red and light blue color represent the emission spectra in native state, denatured state and some state in between the two.

Table S2: Melting point of papain in buffer and other osmolyte solution. (Error bar represents the standard deviation of the mean of three independent experiments.)

Osmolyte	T_m **(K)**
Buffer	336.4 ±0.5
Sucrose (0.5M)	334.1 ±0.5
Sucrose (2M)	344.5 ±1.0

Section S6: Solvation study of papain in buffer and other osmolyte solution

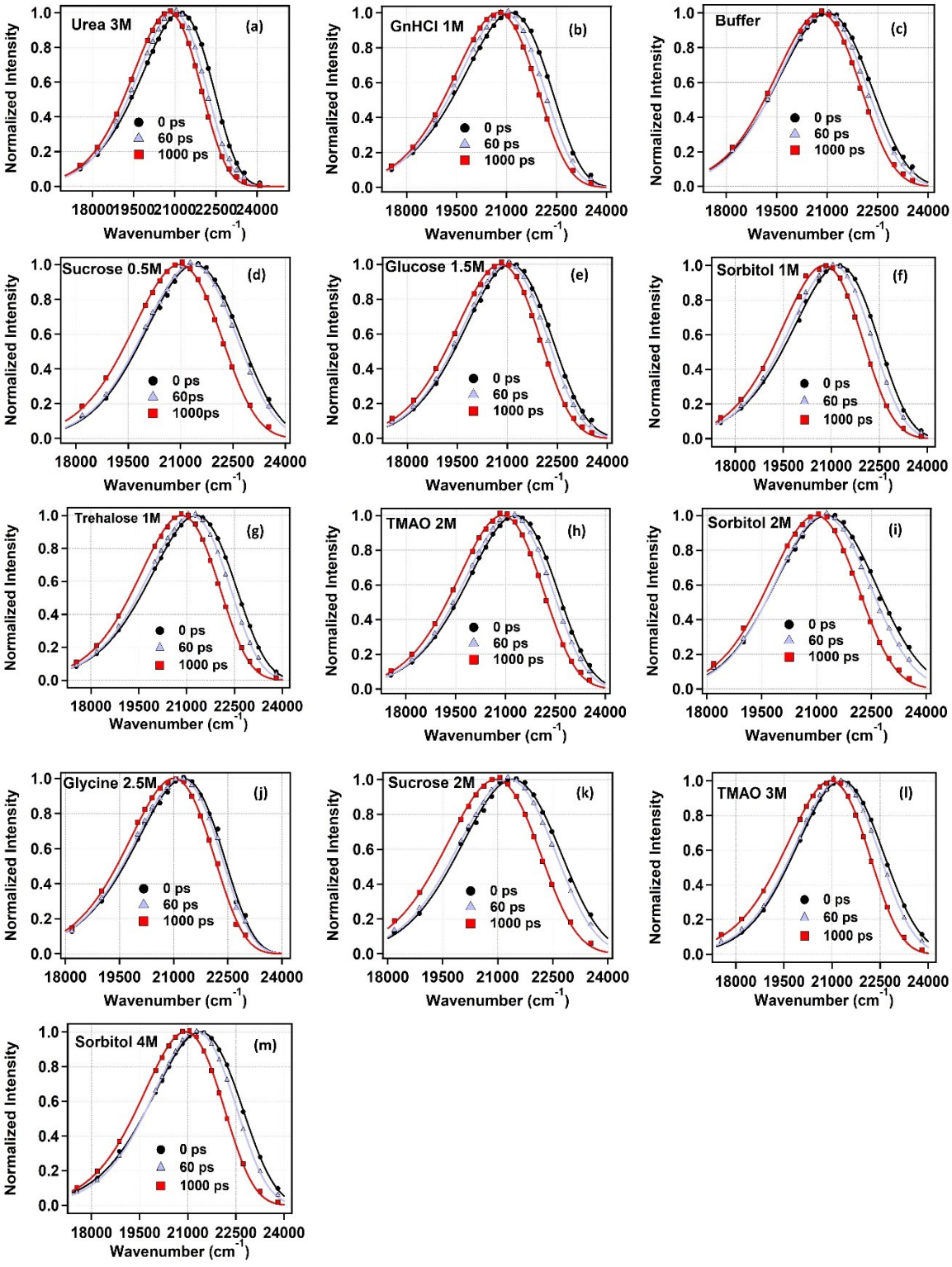


Figure S5. Time resolved emission spectra (TRES) of CPM tagged papain (ex. 375 nm) in mentioned osmolyte.

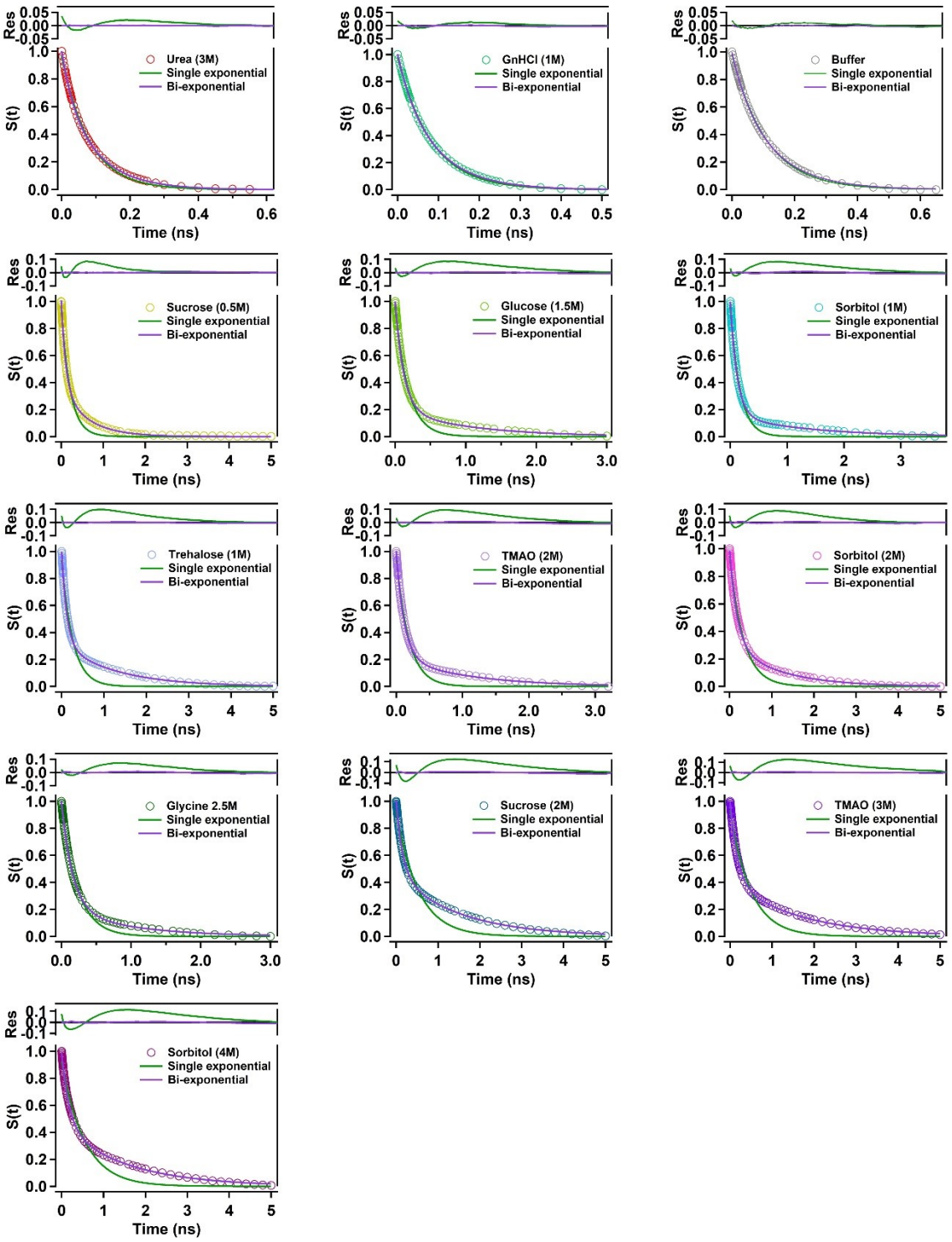


Figure S6. Fitting of solvation data for papain in buffer and other osmolyte solution.

Table S3. Relaxation time constants and their relative weightage in solvation dynamics study of papain in buffer and other aqueous osmolyte solution. (Error bar represents the standard deviation of the mean of three independent experiments.)

Osmolyte	$\mathbf{a_1}$	τ_1 **(ps)**	$\mathbf{a_2}$	τ_2 **(ps)**	τ_{avg} **(ps)**
Urea (3M)	0.30±0.10	30±10	0.70±0.22	100 ±30	79 ±28
GnHCl (1M)	0.17	30	0.83	90	80
Buffer	0.31±0.15	70 ±14	0.69±0.25	130±31	110 ±20
Sucrose (0.5M)	0.64	110	0.36	610	290
Glucose (1.5M)	0.80	120	0.20	1030	300
Sorbitol (1M)	0.84	120	0.16	1380	320
Trehalose (1M)	0.68±0.02	120±7	0.32±0.01	1290±58	490 ±31
TMAO (2M)	0.82	130	0.18	1260	330
Sorbitol (2M)	0.63±0.01	160±6	0.37±0.01	980±43	460±20
Glycine (2.5M)	0.74	150	0.26	770	310
Sucrose (2M)	0.56±0.01	170±6	0.44±0.01	1560±53	780±27
TMAO (3M)	0.59	170	0.41	1650	830
Sorbitol (4M)	0.54	180	0.46	1540	800

Section S7: Solvation study of CPM-tag bromelain in buffer and sucrose solution

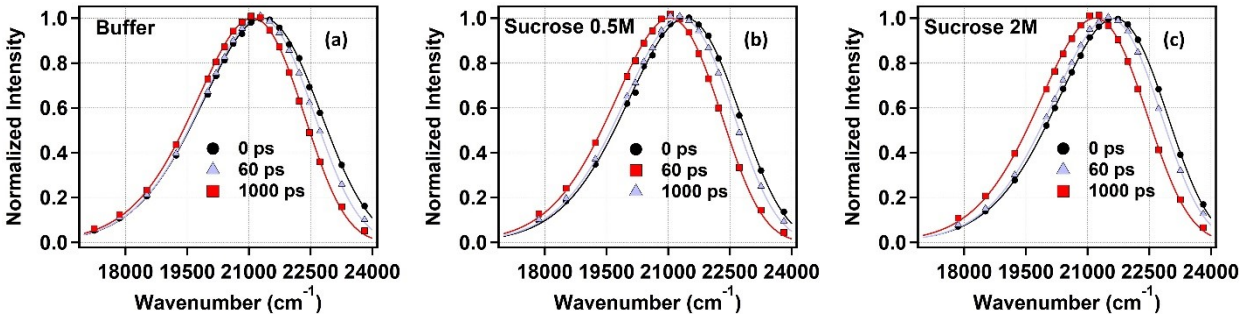


Figure S7. Time resolved emission spectra (TRES) of CPM-tag bromelain (ex. 375 nm) in mentioned osmolyte.

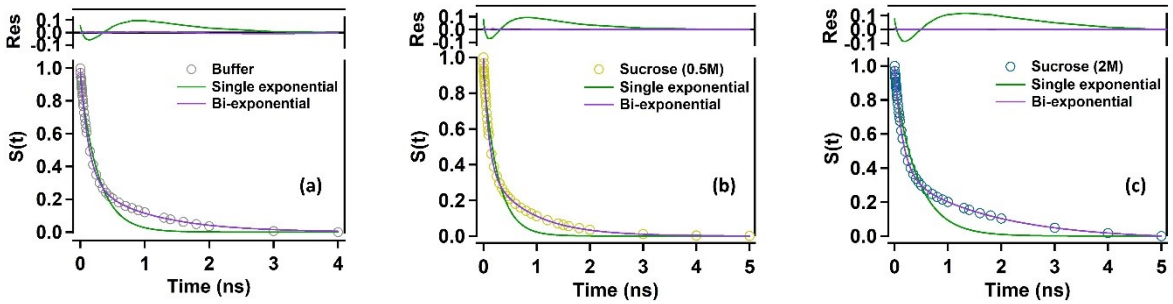


Figure S8. Fitting of solvation data for bromelain in buffer and other osmolyte solution.

Table S4. Relaxation time constants and their relative weightage in solvation dynamics study of bromelain in buffer and other aqueous osmolyte solution. (Error bar represents the standard deviation of the mean of three independent experiments.)

Osmolyte	$\mathbf{a_1}$	τ_1 **(ps)**	$\mathbf{a_2}$	τ_2 **(ps)**	τ_{avg} **(ps)**
Buffer	0.68±0.13	130±10	0.32±0.20	980±35	400±22
Sucrose (0.5M)	0.61	100	0.39	0.81	0.38
Sucrose (2M)	0.59±0.02	150±8	0.41±0.01	1440±50	680±31

Section S8: Emission spectra of CPM-tag papain and bromelain in buffer and osmolyte solution.

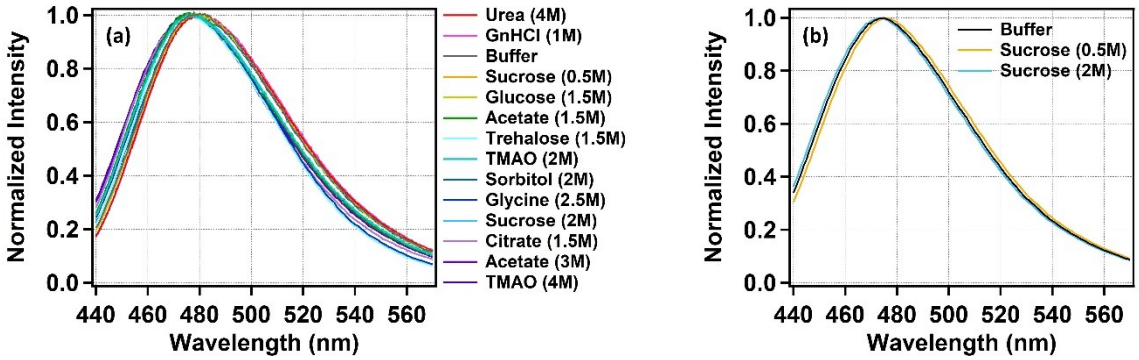


Figure S9. Normalized emission spectra (λ_{ex}=375 nm) of (a) CPM-tag papain and (b) CPM-tag bromelain in buffer and other osmolyte solution.

Section S9: CD spectra of papain and bromelain in buffer and other osmolyte solution.

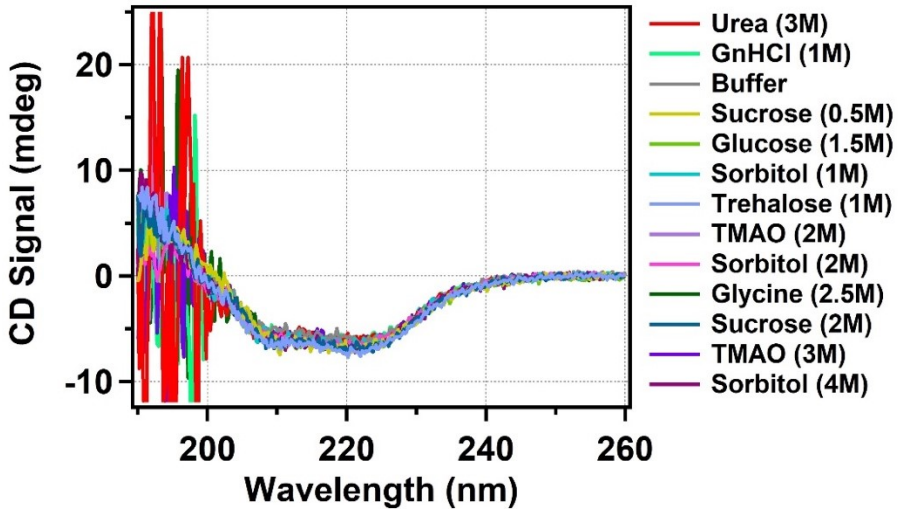


Figure S10. CD spectra of papain in presence and absence of osmolytes.

Table S5. Percentage secondary structural content of papain in buffer and other osmolyte solutions[#]

Osmolyte	α-helix (%)	β-sheet (%)	β-turn (%)	Random coil (%)
Buffer*	25.8±1.3	22.0±1.1	13.6±0.7	38.6±1.9
Sucrose (0.5M)	25.7±1.2	20.7±1.0	12.8±0.6	40.9±2.0
Glucose (1.5M)	26.9±1.4	20.4±1.0	11.7±0.6	41.0±2.1
Sorbitol (1M)	25.9±1.3	19.5±0.9	13.7±0.7	40.7±2.0
Trehalose (1M)	25.3±1.2	16±0.8	13.5±0.7	45.3±2.3
Sorbitol (2M)	26.4±1.3	20.3±1.0	14.5±0.7	38.8±1.9
Sucrose (2M)	27.8±1.4	17.4±0.9	14.9±0.7	40.0±2.0
Sorbitol (4M)	25.4±1.3	17.2±0.8	13.1±0.6	44.3±2.2

*The secondary structural content of papain in buffer matches well with that reported for the solved papain structure.[7]

[#]CD analysis were not carried out in the case of 5 osmolyte solutions i.e., urea 3M, GnHCl 1M, glycine 2.5M, TMAO 2M, TMAO 3M as in these cases the CD spectra suffered from low S/N ratio in the wavelengths shorter than 210 nm, as the PMT voltage goes beyond the threshold level.

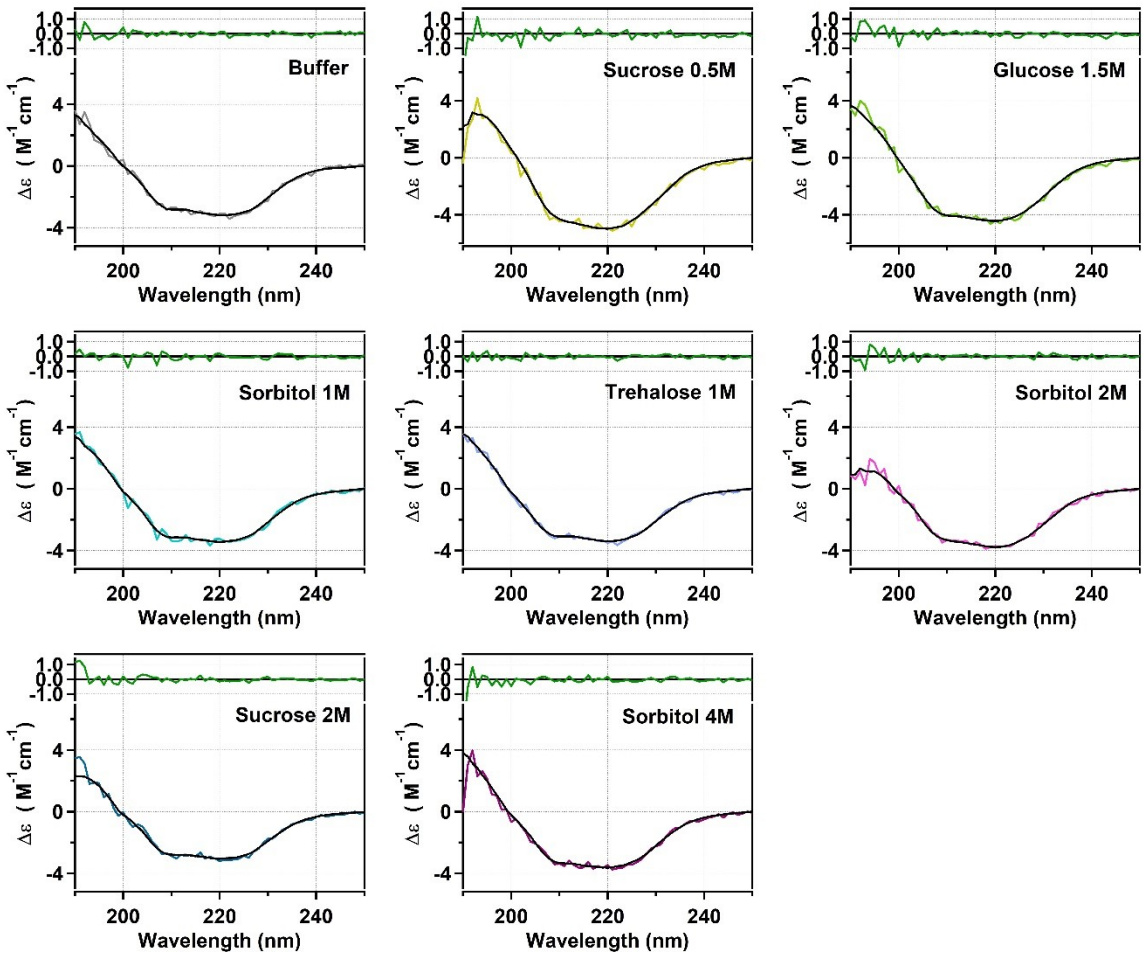


Figure S11. Processed CD spectra of papain in presence and absence of osmolyte along with the fitting line (black) and residuals (light green).

Section S10: Autocorrelation curves of TMR tagged papain in buffer and osmolyte solution.

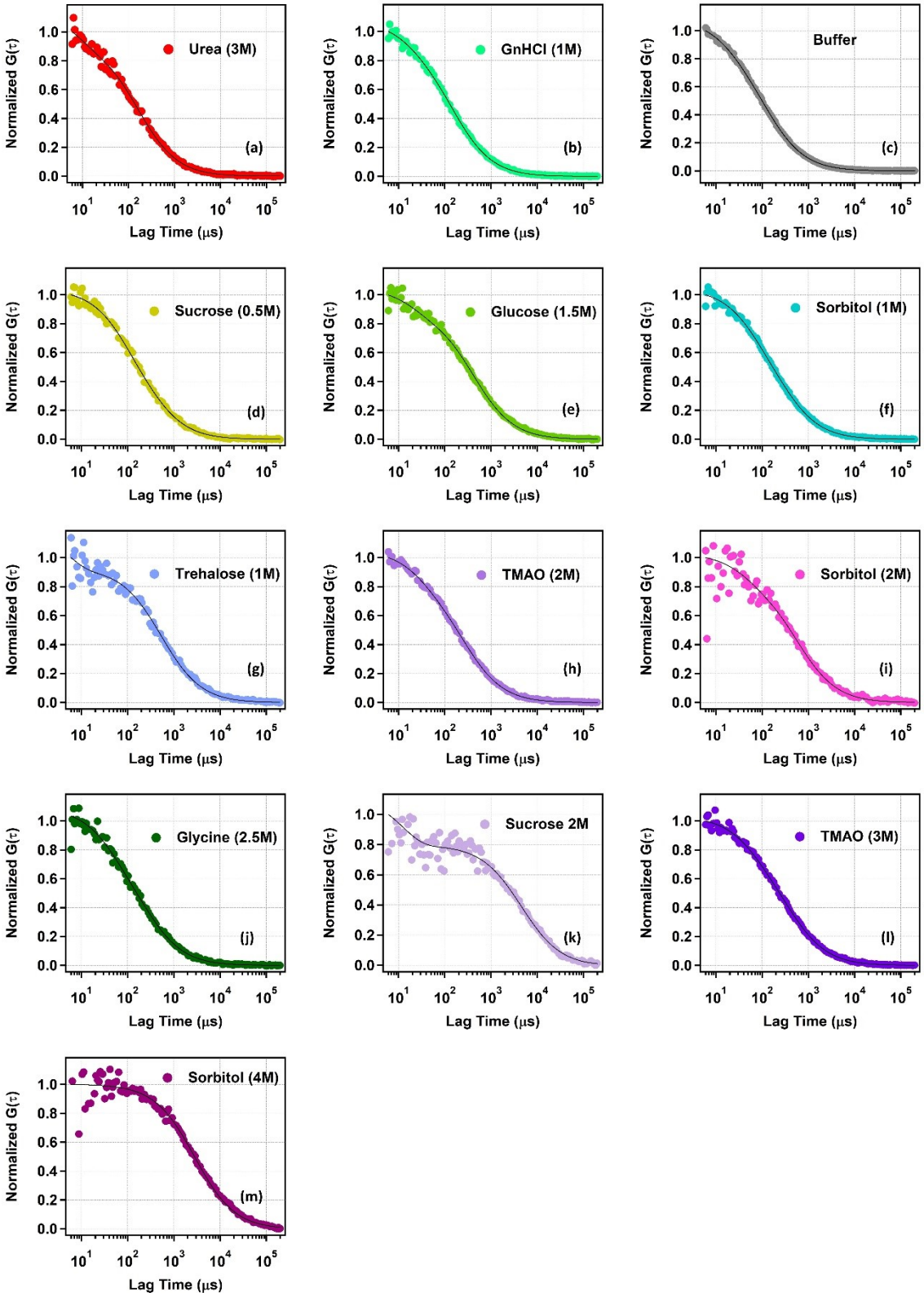


Figure S12. Normalized traces of FCS autocorrelation curves of TMR-tag papain in mentioned osmolyte. Black colored line represents the fitting line.

Table S6. Variation of hydrodynamic radii of papain in buffer and other osmolyte solution (Error bar represents the standard deviation of the mean of three independent experiments.)

Osmolyte	Hydrodynamic radii (Å)
Urea (3M)	25.1±2.3
GnHCl (1M)	22.9±1.5
Buffer	22.8±2.3
Sucrose (0.5M)	23.0±1.8
Glucose (1.5M)	22.6±1.2
Sorbitol (1M)	23.0±1.8
Trehalose (1M)	22.8±1.5
TMAO (2M)	23.0±1.5
Sorbitol (2M)	23.4±1.4
Glycine (2.5M)	24.6±3.1
Sucrose (2M)	23.2±3.0
TMAO (3M)	21.8±2.0
Sorbitol (4M)	22.5±3.0

Section S11: Autocorrelation curves of TMR tagged bromelain in buffer and sucrose

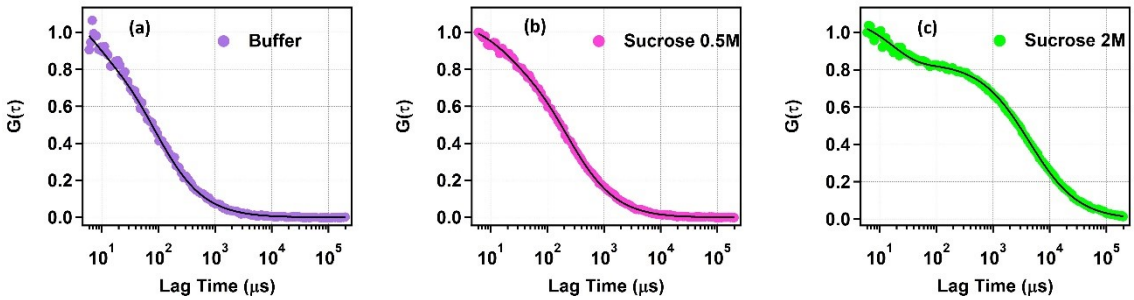


Figure S13. Normalized traces of FCS autocorrelation curves of TMR tagged bromelain in mentioned osmolyte. Black colored line represents the fitting line.

Table S7. Variation of hydrodynamic radii of bromelain in buffer and other osmolyte solution (Error bar represents the standard deviation of the mean of three independent experiments.)

Osmolyte	Hydrodynamic radii (Å)
Buffer	22.5±3.0
Sucrose (0.5M)	24.5±2.0
Sucrose (2M)	16.9±4.0

Section S12: Fluorescence Up-Conversion study of CPM-tag papain

We have used femtosecond resolved up-conversion spectroscopy for examining the time scale of different events of solvation for CPM tagged papain. We merged the time dependent wavenumber maxima shift obtained from fs-fluorescence up conversion and TCSPC to get the information on the whole range from fs to ns relaxation processes involved in the protein solvation. On fitting S(t) obtained from time dependent wavenumber maxima shift we got 4-time constants as 905 fs (47 %), 7.8 ps (27 %) ps, 77.6 ps (13 %), 135 ps (13 %).

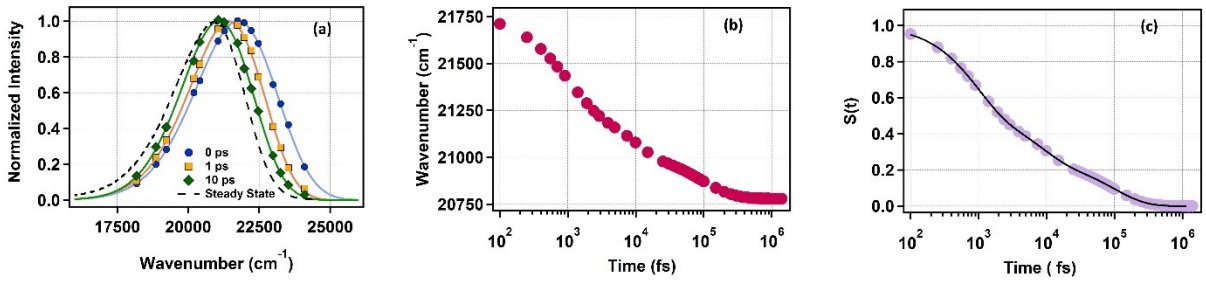


Figure S14. (a) Normalized time resolved emission spectra of papain in buffer (TRES) (b) variation of emission maxima (wavenumber) of papain in buffer with time. Emission maxima are obtained by employing TRES. (c) solvent response function (S(t)) for papain in buffer.

From literature we know that fs component is coming from bulk water as the probe is at the surface of protein thus can sense bulk water, fast ps component is coming from water reorientation in PHL, moderately slow ps component is coming from restructured water in the vicinity of protein with coupled motions of side chain, and slowest ps component is coming from internal motions of protein itself. We are successfully getting a moderately slow ps component from TCSPC as 70±14 ps and we are interested in the event associated with this time scale.

Section S13: Variation of size and structure of bromelain in buffer and osmolyte solution

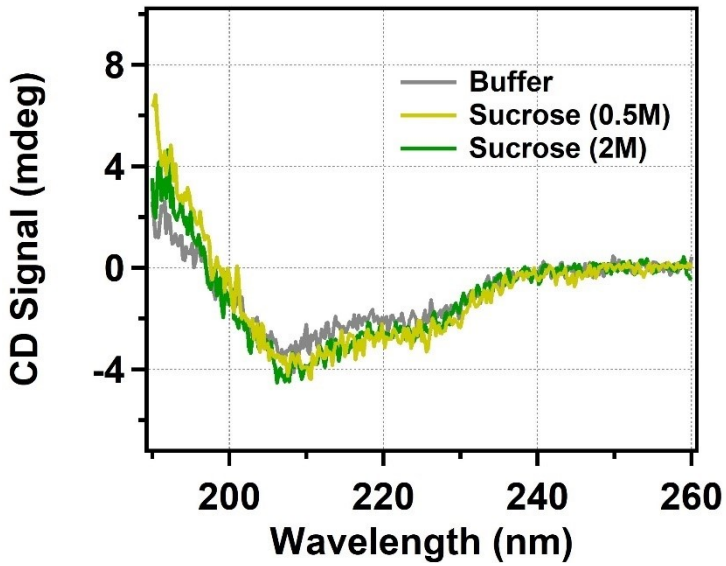


Figure S15. CD spectra of bromelain in presence and absence of osmolytes.

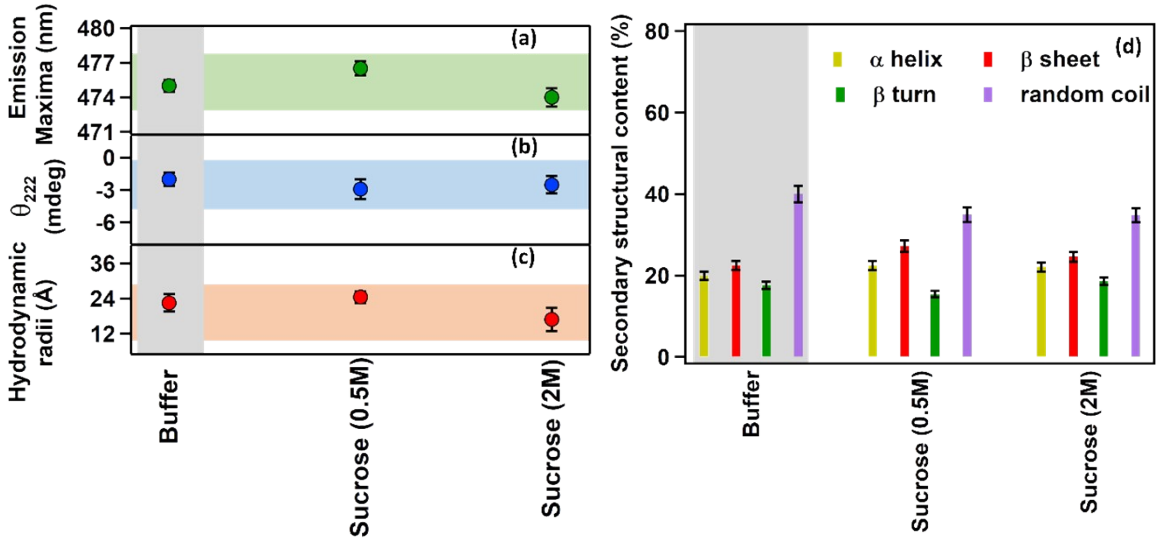


Figure S16. Variation of (a) emission maxima of CPM-tag bromelain ($^{\lambda_{ex}}$ = 375 nm) (for raw data see section S8 of the ESI), (b) CD signal at 222 nm ($^{\theta_{222}}$) of bromelain (see figure S15 of the ESI), (c) hydrodynamic radii of bromelain (see section S11 of the ESI for details). (d) Secondary structural content of bromelain in the presence of various osmolytes and buffer. Error bar represents the standard deviation of the mean of three independent experiments.

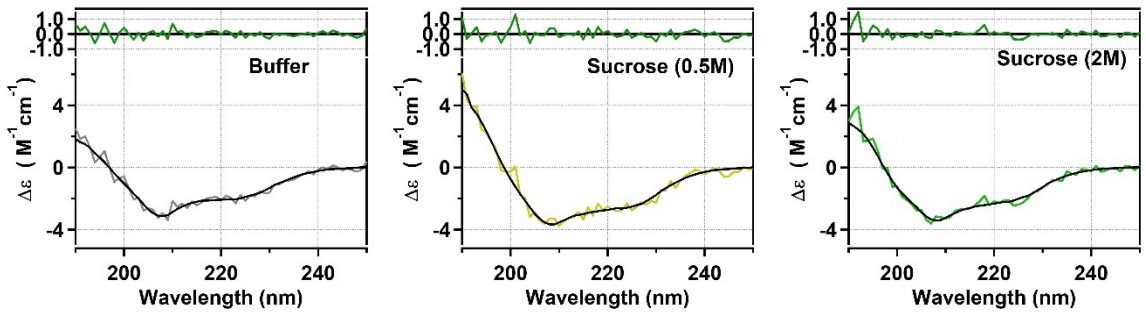


Figure S17. Processed CD spectra of bromelain in presence and absence of osmolyte along with the fitting line (black) and residuals (green).

Table S8. Percentage secondary structural content of bromelain in buffer and other osmolyte solutions

Osmolyte	α-helix (%)	β-sheet (%)	β-turn (%)	Random coil (%)
Buffer*	19.9±1.0	22.5±1.2	17.6±0.9	40±2.0
Sucrose (0.5M)	22.4±1.1	27.2±1.4	15.4±0.8	34.9±1.7
Sucrose (2M)	22.1±1.1	24.6±1.2	18.6±0.9	34.8±1.7

*The secondary structural content of bromelain in buffer matches well with that reported in the literature.[1]

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