

INTRAMOLECULAR OH STRETCHING ANALYSIS OF HYDRATED LYSOZYME IN PRESENCE OF TREHALOSE BY IR SPECTROSCOPY

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ABSTRACT. The present work reports the analysis of the intramolecular OH stretching band obtained by InfraRed spectroscopy measurements. In order to characterize the effect of trehalose on the hydration properties of lysozyme the so-called two-state model is adopted for the analysis of the intramolecular OH stretching band. This latter assumes that, provided that the trehalose OH stretching contribution is subtracted, water molecules can be partitioned into two different states of inter-molecular bonding: molecules with two OH groups both hydrogen-bonded within a tetrahedral network, and molecules with one or two dangling OH groups. What emerges from this study is that trehalose significantly influences the hydrogen bond network of water and its temperature behaviour. Such a result confirms that the trehalose induced strengthening of the hydrogen-bond network leads to a stabilization of the lysozyme structure. Moreover, the analysis of the spectra temperature dependence shows a trehalose-induced higher thermal restraint of the lysozyme-trehalose-water system in respect to the lysozyme-water mixture.

1. Introduction

It is well known that, among natural, bioprotectant molecules, an increasing attention has been recently addressed on disaccharides and in particular on trehalose because of its capability to protect biological materials under stress conditions (Crowe *et al.* 1983; Ballone *et al.* 2000; Malins *et al.* 2003; Magazù *et al.* 2008b).

Several hypotheses have been proposed to explain the efficiency of trehalose. In particular some hypotheses involve a direct biomolecule-sugar interaction while other hypotheses are connected with the glass transition properties of the disaccharide-water mixtures together with the influence on the water tetrahedral hydrogen-bond network (Pagnotta *et al.* 2008; Varga *et al.* 2008; Magazù *et al.* 2010). Nowadays the molecular mechanisms by which trehalose stabilizes proteins are still investigated especially concerning the protein thermal behaviour (Magazù *et al.* 2008a; Jain and Roy 2010; Schebor *et al.* 2010). To evaluate the effect of trehalose on a protein on a crowded medium we consider as a model system for protein lysozyme ($M_w=14.3$ kDa) which is a globular protein composed of R-helices (Jollès 1969; Williams and Dunker 1981; Smith and van Gunsteren 1994; Barreca *et al.* 2010; Fenimore *et al.* 2013; Magazù *et al.* 2016). Concerning the investigation technique,

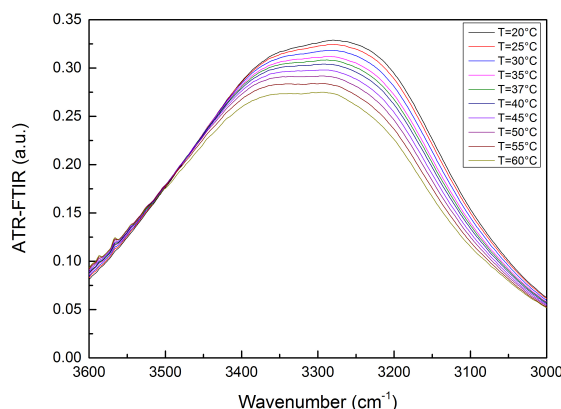


FIGURE 1. OH stretching band intensity as a function of temperature for lysozyme-water mixtures.

InfraRed (IR) spectroscopy represents today an advantageous technique which, directly probing vibrational energy levels and phonons of material systems, provides relevant structural information (Leslie *et al.* 1995; Magazù 1996; Magazù *et al.* 2008a; Hennet *et al.* 2011). On that score, infrared spectra on pure water and on aqueous solutions of lysozyme, also in the presence of trehalose, were collected in the $400 \div 4000 \text{ cm}^{-1}$ wavenumber range in the $20^\circ\text{C} \div 80^\circ\text{C}$ temperature range (Surewicz *et al.* 1993; Aparicio *et al.* 2002; Branca *et al.* 2003; Kong and Yu 2007; Caccamo and Magazù 2017c). As far as the infrared spectra are concerned, Figure 1 reports, as an example, the $3000 < \Delta\omega < 3600 \text{ cm}^{-1}$ spectral range for lysozyme-trehalose-water mixtures at different temperatures, while Figure 2 shows, for a comparison, the OH stretching band at $T=20^\circ\text{C}$ for lysozyme-water and for lysozyme-trehalose-water mixtures (Magazù *et al.* 2011a,b; Peters *et al.* 2016).

From a general viewpoint, the IR intra-molecular OH stretching band contribution in aqueous solutions shows two major broad bands where by lowering temperature the low-frequency sub-band centered at about 3220 cm^{-1} becomes more prominent than the high frequency sub-band centered around 3390 cm^{-1} ; in the meantime, the total OH stretching spectrum shifts to lower frequencies (Lazarev *et al.* 1985; Oberg *et al.* 2003; Caccamo and Magazù 2016, 2017a,b). In this framework collected data on lysozyme/water and lysozyme/water/trehalose mixtures were analyzed in terms of a two state model; what emerges from the present investigation is that trehalose significantly influences the hydrogen bond network of water as well as its temperature behaviour (Lokotosh *et al.* 2000; Manning 2005; Zhang and Aksan 2010).

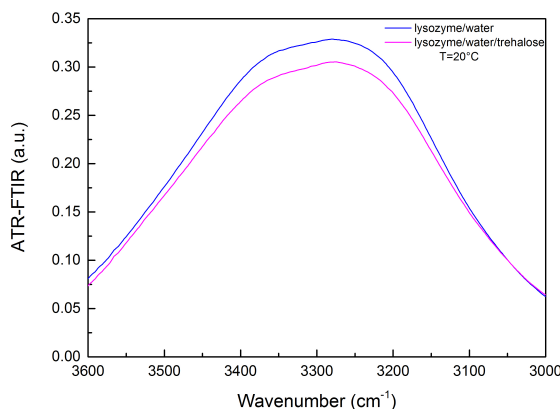


FIGURE 2. OH stretching band intensity at $T=20^{\circ}\text{C}$ for lysozyme-water and for lysozyme-trehalose-water mixtures.

2. Experimental section

Lysozyme (14.3 kDa) was purchased from Sigma as lyophilized powder (purity minimum 98 %). High-purity trehalose dihydrate was supplied from Fluka and Sigma. Double-distilled pure water was used to prepare aqueous mixtures. Lysozyme/water mixtures and lysozyme/water/trehalose mixtures were prepared. Measurements were carried out in the $20\div 80^{\circ}\text{C}$ temperature range. To avoid evaporation the sample holder was equipped by a proper polymeric cap. ATR-FTIR measurements as a function of concentration and temperature were performed just after preparation. In order to detect small changes in the sample spectral features for the present analysis we have chosen an energy resolution of 0.4 cm^{-1} while 48 scans have been collected for each temperature.

3. Results and discussion

It is well known that the analysis of the OH stretching contribution in water and in aqueous solutions is usually performed following different interpretative models that make reference to different arrangements of water molecules. In particular, two main classes of models, usually termed continuum and mixture, can be mentioned. Continuum models assume a continuous distribution of interactions whereas mixture models refer to water molecule arrangements differing in the extent of the hydrogen bonding. Some of the earlier spectroscopic investigations appeared to support continuum models, but recent IR, Raman scattering, stimulated Raman scattering, hyper-Raman, inelastic harmonic light scattering, and x-ray spectroscopy are in favor of mixture models where a consecutive hydrogen-bond disruption is assumed. In the following in order to characterize the hydration properties of lysozyme/water/trehalose, we adopt the so-called two-state model; this assumes that, provided that the trehalose OH stretching contribution has been subtracted, water is a mixture of two different states of inter-molecular bonding (Magazù *et al.* 2012; Migliardo *et al.* 2013,

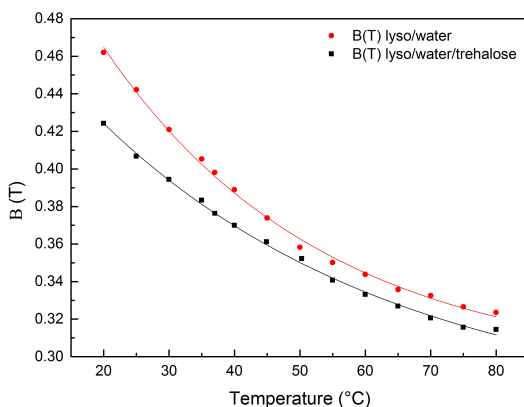


FIGURE 3. Behaviour of fractional open band intensity, $B(T)$, as a function of temperature for lysozyme-water and lysozyme-trehalose-water mixtures in the 20÷80°C temperature range. The integrated area of the “open” band is always smaller in lysozyme-trehalose-water mixtures, revealing a higher destructuring effect induced by trehalose on the tetrahedral hydrogen bonded network.

2014). In particular we partition the water molecules into two distinct classes: molecules with two OH groups both hydrogen-bonded within a tetrahedral network (fully hydrogen-bonded water molecules), and molecules with one or two dangling OH groups (non-fully hydrogen-bonded water molecules). For lysozyme in water mixtures and lysozyme in trehalose water mixtures temperature scans were performed at $T=20^{\circ}\text{C}$, 25°C , 30°C , 35°C , 40°C , 45°C , 50°C , 55°C , 60°C , 65°C , 70°C , 75°C and 80°C . Then the isotropic spectrum can be decomposed into an “open” and a “closed” Gaussian contribution:

$$I_{iso}(\omega, T) = \alpha(T)I_{open}(\omega) + [1 - \alpha(T)]I_{closed}(\omega). \quad (1)$$

The open Gaussian component is placed at 3200 cm^{-1} and the closed contribution is positioned at 3400 cm^{-1} . In order to extract numerical curve-fit coefficients for the lysozyme in water mixtures and for the lysozyme in trehalose water mixtures we calculated the fractional open band intensity:

$$B(T) = \frac{\int I_{iso}^{open}(\omega, T)d\omega}{\int I_{iso}^{ot}(\omega, T)d\omega} \quad (2)$$

where I_{iso}^{ot} is the sum of the open and closed contributions. Figure 3 shows the behaviour of the fractional open band intensity as a function of temperature for lysozyme-water and lysozyme-trehalose-water mixtures in the 20÷80°C temperature range.

As it can be seen, the integrated area of the “open” band is always smaller in lysozyme-trehalose-water mixtures, revealing a destructuring effect induced by trehalose on the tetrahedral hydrogen bonded network of water; furthermore the temperature dependence of fractional open band intensity $B(T)$ is less strong for the ternary system so revealing a higher

thermal restraint of the lysozyme-trehalose-water system in respect to the lysozyme-water mixture.

4. Conclusions

The present study has been addressed to the study of the thermal stability of hydrated lysozyme as induced by trehalose by employing IR spectroscopy measurements. The study has been mainly focused to the intramolecular OH stretching region. The IR findings show that trehalose affects the hydrogen bond network of water. This result confirms that the disaccharide reduces the protein dynamic fluctuations thanks to a strengthening of the intermolecular O-H interactions which in turn leads to a stabilization of the lysozyme structure. In addition the analysis of the band temperature dependence shows a higher thermal restraint of the lysozyme-trehalose-water system in respect to the lysozyme-water mixture.

References

- Aparicio, S., Doty, S. B., Camacho, N. P., Paschalis, E. P., Spevak, L., Mendelsohn, R., and Boskey, A. L. (2002). "Optimal methods for processing mineralized tissues for Fourier transform infrared microspectroscopy". *Calcified Tissue International* **70**(5), 422–429. DOI: [10.1007/s00223-001-1016-z](https://doi.org/10.1007/s00223-001-1016-z).
- Ballone, P., Marchi, M., Branca, C., and Magazù, S. (2000). "Structural and vibrational properties of trehalose: A density functional study". *The Journal of Physical Chemistry B* **104**(26), 6313–6317. DOI: [10.1021/jp994346b](https://doi.org/10.1021/jp994346b).
- Barreca, D., Laganà, G., Ficarra, S., Tellone, E., Leuzzi, U., Magazù, S., Galtieri, A., and Bellocchio, E. (2010). "Anti-aggregation properties of trehalose on heat-induced secondary structure and conformation changes of bovine serum albumin". *Biophysical Chemistry* **147**(3), 146–152. DOI: [10.1016/j.bpc.2010.01.010](https://doi.org/10.1016/j.bpc.2010.01.010).
- Branca, C., Magazù, S., Maisano, G., Bennington, S., and Fåk, B. (2003). "Vibrational studies on disaccharide/H₂O systems by inelastic neutron scattering, Raman, and IR spectroscopy". *Journal of Physical Chemistry B* **107**(6), 1444–1451. DOI: [10.1021/jp026255b](https://doi.org/10.1021/jp026255b).
- Caccamo, M. T. and Magazù, S. (2016). "Tagging the oligomer-to-polymer crossover on EG and PEGs by infrared and Raman spectroscopies and by wavelet cross-correlation spectral analysis". *Vibrational Spectroscopy* **85**, 222–227. DOI: [10.1016/j.vibspec.2016.04.017](https://doi.org/10.1016/j.vibspec.2016.04.017).
- Caccamo, M. T. and Magazù, S. (2017a). "Ethylene Glycol - Polyethylene Glycol (EG-PEG) mixtures: Infrared spectra wavelet cross-correlation analysis". *Applied Spectroscopy* **71**(3), 401–409. DOI: [10.1177/0003702816662882](https://doi.org/10.1177/0003702816662882).
- Caccamo, M. T. and Magazù, S. (2017b). "Multiscaling wavelet analysis of infrared and Raman data on polyethylene glycol 1000 aqueous solutions". *Spectroscopy Letters* **50**(3), 130–136. DOI: [10.1080/00387010.2017.1291524](https://doi.org/10.1080/00387010.2017.1291524).
- Caccamo, M. and Magazù, S. (2017c). "Thermal restraint on PEG-EG mixtures by FTIR investigations and wavelet cross-correlation analysis". *Polymer Testing* **62**, 311–318. DOI: [10.1016/j.polymertesting.2017.07.008](https://doi.org/10.1016/j.polymertesting.2017.07.008).
- Crowe, J. H., Crowe, L. M., and Jackson, S. A. (1983). "Preservation of structural and functional activity in lyophilized sarcoplasmic reticulum". *Archives of Biochemistry and Biophysics* **220**(2), 477–484. DOI: [10.1016/0003-9861\(83\)90438-1](https://doi.org/10.1016/0003-9861(83)90438-1).

- Fenimore, P. W., Frauenfelder, H., Magazù, S., McMahon, B. H., Mezei, F., Migliardo, F., Young, R. D., and Stroe, I. (2013). “Concepts and problems in protein dynamics”. *Chemical Physics* **424**, 2–6. DOI: [10.1016/j.chemphys.2013.06.023](https://doi.org/10.1016/j.chemphys.2013.06.023).
- Hennet, L., Cristiglio, V., Kozaily, J., Pozdnyakova, I., Fischer, H. E., Bytchkov, A., Drewitt, J. W. E., Leydier, M., Thiaudière, D., Gruner, S., Brassamin, S., Zanghi, D., Cuello, G. J., Koza, M., Magazù, S., Greaves, G. N., and Price, D. L. (2011). “Aerodynamic levitation and laser heating: Applications at synchrotron and neutron sources”. *The European Physical Journal: Special Topics* **196**(1), 151–165. DOI: [10.1140/epjst/e2011-01425-0](https://doi.org/10.1140/epjst/e2011-01425-0).
- Jain, N. K. and Roy, I. (2010). “Trehalose and protein stability”. *Current Protocols in Protein Science* (SUPPL. 59), 4.9.1–4.9.12. DOI: [10.1002/0471140864.ps0409s59](https://doi.org/10.1002/0471140864.ps0409s59).
- Jollès, P. (1969). “Lysozymes: A chapter of molecular biology”. *Angewandte Chemie International Edition* **8**(4), 227–239. DOI: [10.1002/anie.196902271](https://doi.org/10.1002/anie.196902271).
- Kong, J. and Yu, S. (2007). “Fourier transform infrared spectroscopic analysis of protein secondary structures”. *Acta Biochimica et Biophysica Sinica* **39**(8), 549–559. DOI: [10.1111/j.1745-7270.2007.00320.x](https://doi.org/10.1111/j.1745-7270.2007.00320.x).
- Lazarev, Y. A., Grishkovsky, B. A., and Khromova, T. B. (1985). “Amide I band of IR spectrum and structure of collagen and related polypeptides”. *Biopolymers* **24**(8), 1449–1478. DOI: [10.1002/bip.360240804](https://doi.org/10.1002/bip.360240804).
- Leslie, S. B., Israeli, E., Lighthart, B., Crowe, J. H., and Crowe, L. M. (1995). “Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying”. *Applied and Environmental Microbiology* **61**(10), 3592–3597. URL: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC167656/>.
- Lokotosh, T., Magazù, S., Maisano, G., and Malomuzh, N. (2000). “Nature of self-diffusion and viscosity in supercooled liquid water”. *Physical Review E* **62**(3), 3572–3580. DOI: [10.1103/PhysRevE.62.3572](https://doi.org/10.1103/PhysRevE.62.3572).
- Magazù, S. (1996). “IQENS - Dynamic light scattering complementarity on hydrogenous systems”. *Physica B: Condensed Matter* **226**(1-3), 92–106. DOI: [10.1016/0921-4526\(96\)00255-4](https://doi.org/10.1016/0921-4526(96)00255-4).
- Magazù, S., Calabrò, E., Caccamo, M. T., and Cannuli, A. (2016). “The shielding action of disaccharides for typical proteins in aqueous solution against static, 50 Hz and 1800 MHz frequencies electromagnetic fields”. *Current Chemical Biology* **10**(1), 57–64. DOI: [10.2174/2212796810666160419153722](https://doi.org/10.2174/2212796810666160419153722).
- Magazù, S., Maisano, G., Migliardo, F., and Benedetto, A. (2008a). “Mean square displacement evaluation by elastic neutron scattering self-distribution function”. *Physical Review E* **77**(6). DOI: [10.1103/PhysRevE.77.061802](https://doi.org/10.1103/PhysRevE.77.061802).
- Magazù, S., Migliardo, F., Affouard, F., Descamps, M., and Telling, M. T. F. (2010). “Study of the relaxational and vibrational dynamics of bioprotectant glass-forming mixtures by neutron scattering and molecular dynamics simulation”. *The Journal of Chemical Physics* **132**(18). DOI: [10.1063/1.3407428](https://doi.org/10.1063/1.3407428).
- Magazù, S., Migliardo, F., and Benedetto, A. (2011a). “Elastic incoherent neutron scattering operating by varying instrumental energy resolution: Principle, simulations, and experiments of the resolution elastic neutron scattering (RENS)”. *Review of Scientific Instruments* **82**(10). DOI: [10.1063/1.3641870](https://doi.org/10.1063/1.3641870).
- Magazù, S., Migliardo, F., Benedetto, A., Mondelli, C., and Gonzalez, M. A. (2011b). “Thermal behaviour of hydrated lysozyme in the presence of sucrose and trehalose by EINS”. *Journal of Non-Crystalline Solids* **357**(2), 664–670. DOI: [10.1016/j.jnoncrysol.2010.06.075](https://doi.org/10.1016/j.jnoncrysol.2010.06.075).
- Magazù, S., Migliardo, F., and Caccamo, M. T. (2012). “Innovative wavelet protocols in analyzing elastic incoherent neutron scattering”. *Journal of Physical Chemistry B* **116**(31), 9417–9423. DOI: [10.1021/jp3060087](https://doi.org/10.1021/jp3060087).
- Magazù, S., Migliardo, F., and Telling, M. T. F. (2008b). “Structural and dynamical properties of water in sugar mixtures”. *Food Chemistry* **106**(4), 1460–1466. DOI: [10.1016/j.foodchem.2007.05.097](https://doi.org/10.1016/j.foodchem.2007.05.097).

- Malins, D. C., Johnson, P. M., Barker, E. A., Polissar, N. L., Wheeler, T. M., and Anderson, K. M. (2003). "Cancer-related changes in prostate DNA as men age and early identification of metastasis in primary prostate tumors". *Proceedings of the National Academy of Sciences of the United States of America* **100**, 5401–5406. DOI: [10.1073/pnas.0931396100](https://doi.org/10.1073/pnas.0931396100).
- Manning, M. C. (2005). "Use of infrared spectroscopy to monitor protein structure and stability". *Expert Review of Proteomics* **2**(5), 731–743. DOI: [10.1586/14789450.2.5.731](https://doi.org/10.1586/14789450.2.5.731).
- Migliardo, F., Caccamo, M. T., and Magazù, S. (2013). "Elastic incoherent neutron scatterings wavevector and thermal analysis on glass-forming homologous disaccharides". *Journal of Non-Crystalline Solids* **378**, 144–151. DOI: [10.1016/j.jnoncrysol.2013.06.030](https://doi.org/10.1016/j.jnoncrysol.2013.06.030).
- Migliardo, F., Caccamo, M. T., and Magazù, S. (2014). "Thermal analysis on bioprotectant disaccharides by elastic incoherent neutron scattering". *Food Biophysics* **9**(2), 99–104. DOI: [10.1007/s11483-013-9322-3](https://doi.org/10.1007/s11483-013-9322-3).
- Oberg, K. A., Ruysschaert, J. M., and Goormaghtigh, E. (2003). "Rationally selected basis proteins: A new approach to selecting proteins for spectroscopic secondary structure analysis". *Protein Science* **12**(9), 2015–2031. DOI: [10.1110/ps.0354703](https://doi.org/10.1110/ps.0354703).
- Pagnotta, S. E., Ricci, M. A., Bruni, F., McLain, S., and Magazù, S. (2008). "Water structure around trehalose". *Chemical Physics* **345**(2-3), 159–163. DOI: [10.1016/j.chemphys.2007.07.052](https://doi.org/10.1016/j.chemphys.2007.07.052).
- Peters, J., Luczak, A., Ganesh, V., Park, E., and Kalyanaraman, R. (2016). "Protein secondary structure determination using drop coat deposition confocal raman spectroscopy". *Spectroscopy (Santa Monica)* **31**(10), 31–39.
- Schebor, C., Mazzobre, M., and Buera, M. d. P. (2010). "Glass transition and time-dependent crystallization behavior of dehydration bioprotectant sugars". *Carbohydrate Research* **345**(2), 303–308. DOI: [10.1016/j.carres.2009.10.014](https://doi.org/10.1016/j.carres.2009.10.014).
- Smith, P. E. and van Gunsteren, W. F. (1994). "Translational and rotational diffusion of proteins". *Journal of Molecular Biology* **236**(2), 629–636. DOI: [10.1006/jmbi.1994.1172](https://doi.org/10.1006/jmbi.1994.1172).
- Surewicz, W. K., Mantsch, H. H., and Chapman, D. (1993). "Determination of protein secondary structure by Fourier transform infrared spectroscopy: A critical assessment". *Biochemistry* **32**(2), 389–394. DOI: [10.1021/bi00053a001](https://doi.org/10.1021/bi00053a001).
- Varga, B., Migliardo, F., Takacs, E., Vertessy, B., Magazù, S., and Mondelli, C. (2008). "Neutron scattering studies on dUTPase complex in the presence of bioprotectant systems". *Chemical Physics* **345**(2-3), 250–258. DOI: [10.1016/j.chemphys.2007.07.004](https://doi.org/10.1016/j.chemphys.2007.07.004).
- Williams, R. W. and Dunker, A. K. (1981). "Determination of the secondary structure of proteins from the amide I band of the laser Raman spectrum". *Journal of Molecular Biology* **152**(4), 783–813. DOI: [10.1016/0022-2836\(81\)90127-3](https://doi.org/10.1016/0022-2836(81)90127-3).
- Zhang, L. and Aksan, A. (2010). "Fourier transform infrared analysis of the thermal modification of human cornea tissue during conductive keratoplasty". *Applied Spectroscopy* **64**(1), 23–29. DOI: [10.1366/000370210790571918](https://doi.org/10.1366/000370210790571918).

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