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REPORT

Inelastic neutron scattering study on bioprotectant systems

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We collected inelastic neutron scattering (INS) spectra of homologous disaccharide ($C_{12}H_{22}O_{11}$)/H_2O mixtures at a very low temperature by using indirect geometry time-of-flight spectrometer TOSCA at the ISIS pulse neutron facility (DRAL, UK). The aim of this work is to investigate the vibrational behaviour of trehalose, maltose and sucrose/H_2O mixtures with INS in order to characterize the structural changes induced by these disaccharides on the H_2O hydrogen-bonded network. A higher degree of 'crystallinity' for the trehalose/H_2O system is observed in the vibrational region corresponding to the ice bending modes. This feature could justify the better *crypto*biotic action of trehalose compared with maltose and sucrose. On the other hand, the better bioprotective effectiveness could be explained by the higher destructuring effect of trehalose, emphasized by the analysis of the librational modes region.

Keywords: disaccharides; bioprotectants; water mixtures; vibrational properties; neutron scattering

1. INTRODUCTION

In recent years many efforts have addressed the understanding of the tools used by organisms to survive under environmental stress conditions (Hirsh 1987; Lee *et al.* 1992; Storey & Storey 1992). Examples of these survival mechanisms are found in some species of frogs that are able to survive in relatively low temperatures for weeks by means of natural cryoprotective agents that include low-molecular-weight substances, such as polyhydric alcohols (commonly glycerol) and sugars (e.g. trehalose), and high-molecular-weight proteins that inhibit ice formation (Lee *et al.* 1992; Miller 1978). Tardigrades are another class of organisms able, thanks to the synthesis of trehalose, to live in extreme conditions, such as temperatures near absolute zero and above the boiling point of water, very high pressures, a hard vacuum and high doses of radiation (Miller 1997). For these reasons trehalose has been revealed as being a very effective cryoprotective as well as cryptoprotectant agent. The bioprotective effects of trehalose have suggested manifold promising applications, such as preserving vaccines and biological macromolecules like DNA during radiation exposure (Fahy 1986; Yoshinaga *et al.* 1997).

Although the trehalose cryoprotectant and cryptoprotectant effectiveness is proved, the underlying molecular mechanisms are not yet fully clarified. In this frame Green & Angell (1989) suggest that the higher value of the glass transition temperature of trehalose and its mixtures with water, in comparison with the other disaccharides, is the only reason for its superior bioprotectant effectiveness. In fact, the higher T_g values of the trehalose/H_2O mixtures, in respect to those of the other disaccharides/H_2O mixtures, imply that the glass transition for trehalose mixtures at a given temperature always occurs at a higher water content. As a matter of fact such a hypothesis alone is not entirely satisfactory if one keeps in mind that other similar systems, such as dextran, for example, a linear polysaccharide with α(1-6) glycosidic links, present even higher T_g values but do not show comparable bioprotective action.

Crowe and co-workers (Crowe & Crowem 1984) formulated the hypothesis that a direct interaction between the sugars and the object of protection occurs. More specifically, their 'water replacement hypothesis' justifies the trehalose protective function with the existence of direct hydrogen bonding of trehalose with the polar head groups of the lipids, as water does. This hypothesis is supported by a simulation by Grigera and co-workers, which argued that the structure of trehalose is perfectly adaptable to the tetrahedral coordination of pure water, the structural and dynamical properties of which are not significantly affected by trehalose (Donnamaria *et al.* 1994).

As a matter of fact, experimental findings obtained by several spectroscopic techniques (Magazù *et al.* 1997; Branca *et al.* 1999*a*,*b*, 2001*a*,*b*, 2002; Magazù *et al.* 2001) indicate that the structural and dynamical properties of water are drastically perturbed by disaccharides and, in particular, by trehalose. More specifically, neutron diffraction results (Branca *et al.* 2002) show a strong distortion of the peaks linked to the hydrogen bonded network in the partial radial distribution functions for all disaccharides, and for trehalose to a large extent, which can be attributed to the destruction of pure water's tetrahedral coordination. Analogously, Raman scattering findings (Branca *et al.* 1999*a*,*b*) show that the addition of trehalose, in respect to the other disaccharides, more rapidly destroys the tetrahedral intermolecular network of water, which by lowering the temperature would give rise to ice. These results clearly indicate that disaccharides show a noteable 'kosmotrope' character, namely, the disaccharide–water molecule interaction strength is much higher in respect to that between the water molecules.

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Furthermore, ultrasonic velocity measurements (Magazù *et al.* 1997; Branca *et al.* 1999*a*) highlight that, in respect to the other disaccharides, the trehalose–water system is characterized, in the investigated concentration range, by the highest value of both the solute–solvent interaction strength and the hydration number. As far as dynamics are concerned, quasi-elastic neutron scattering (QENS) results on disaccharides solutions (Magazù *et al.* 2001) indicate that the water dynamics are also strongly affected by the presence of disaccharides and by trehalose in particular, contrary to the predictions of Grigera's simulation (Donnamaria *et al.* 1994).

Furthermore, viscosity measurements on trehalose, maltose and sucrose aqueous solutions point out that trehalose shows, in respect to the other disaccharides, a 'stronger' kinetic character in the Angell's classification scheme (Branca *et al.* 2001*b*). QENS and inelastic neutron scattering (INS) were also employed to investigate the low frequency dynamics across the glass transition of trehalose, maltose and sucrose water mixtures (Branca *et al.* 2001*a*). The obtained experimental findings, through the relaxational to vibrational contribution ratio, confirm that the trehalose/H_2O mixture shows the strongest character as indicated by the highest force pseudo-constant (resilience) value, in comparison with the other disaccharides/H_2O mixtures. Simulation results showed that the 'destructuring' effect on the network of water and the slowing down of the water dynamics are two properties which could play a key role in understanding the microscopic mechanisms of bioprotection (Bordat *et al.* 2004).

On the basis of the performed studies, it is evident that the bioprotective effectiveness of disaccharides, trehalose in particular, reflects a complex array of interactions at the structural, physiological and molecular levels, where many cofactors occur to make trehalose an extraordinary natural bioprotectant (Magazù *et al.* 1997, 2001; Branca *et al.* 1999*a*,*b*, 2001*a*,*b*, 2002; Bordat *et al.* 2004).

The aim of this work is to investigate the vibrational behaviour of trehalose, maltose and sucrose/H_2O mixtures with INS in order to characterize the structural changes induced by these disaccharides on the H_2O hydrogen-bonded network. The obtained neutron scattering findings indicate that disaccharides generally have a destructuring effect on the pure H_2O tetrahedral coordination network. Furthermore, a comparison of the most significant spectral features of trehalose, maltose and sucrose/H_2O mixtures reveals a higher 'crystallinity' degree for the trehalose/H_2O complex.

2. EXPERIMENTAL SECTION

Vibrational spectra of disaccharides/H_2O mixtures have been collected by the indirect geometry time-of-flight spectrometer TOSCA at the ISIS Pulse Neutron Facility (DRAL, UK) (Colognesi & Parker 1999) in the energy range 0–4000 cm^{-1} (0–500 meV) with the best results below 2000 cm^{-1} (250 meV). By this spectrometer both the collective excitations region at the low frequency region and the localized excitations region at the intermediate and higher frequency regions can be covered.

TOSCA's resolution (in the whole energy range of interest, the instrument provides very good energy resolution, about $\Delta E/E \approx 1.5$–2%, which is comparable with optical techniques such as infrared (IR) and Raman spectroscopies) combined with the high intensity of the ISIS source allows us to perform the study of the dynamics with high accuracy, rendering the background noise/signal ratio negligible for strongly scattering samples (Colognesi & Parker 1999).

Ultra-pure powdered trehalose, maltose and sucrose, and H_2O, purchased by Aldrich–Chemie, were used for the experiment. Measurements were performed at a temperature value of 27 K on hydrogenated trehalose, maltose and sucrose ($C_{12}H_{22}O_{11}$) in H_2O at weight fraction values corresponding to 19 water (H_2O and D_2O) molecules for each disaccharide molecule. Trehalose, maltose and sucrose show a different structural arrangement: trehalose and maltose are composed of two glucose rings linked by an oxygen glycosidic bond whose orientation is different for the two sugars, whereas sucrose is formed by a glucose ring and a furanose ring linked by a similar oxygen glycosidic bond (Branca *et al.* 1999*b*). At the temperature value of the measurements, no protons by bulk water are present. More specifically, ultrasonic velocity measurements clearly indicate that the disaccharide–water molecule interaction strength is higher in respect to that between the water molecules and that, in respect to the other disaccharides, the trehalose–water system is characterized, in all the investigated concentration range, by the highest value of both the solute–solvent interaction strength and the hydration number (Magazù *et al.* 1997; Branca *et al.* 1999*b*). For example, at $T = 298$ K, $n_H = 15.2$ for trehalose, $n_H = 14.7$ for maltose and $n_H = 14.1$ for sucrose and such values increase as the temperature decreases (Magazù *et al.* 1997; Branca *et al.* 1999*b*).

For all the investigated hydrogenated samples, the measurement time was 12 h for each run. Thin-walled aluminium cells were used. The samples were cooled to 27 K by a liquid helium cryostat. Care was taken to obtain stable, clear and dust-free samples; ample time was allowed for equilibration.

The raw spectra were manipulated by using the standard GENIE procedures (Colognesi & Parker 1999). The bin size, that is, the number of adjacent points averaged for each data point, has been chosen in order to find the best compromise between accuracy and noise and it was the same for all the investigated samples. A small amount of flat background from the empty cell measured at similar conditions was subtracted from the measured data for the data treatment. The measured INS data were transferred to the dynamical structure factor $S(Q, \omega)$ as a function of energy transfer by using a standard data treatment programme. The multiple scattering contributions have been minimized by using a thin sample in order to obtain a scattering transmission from the sample that is greater than or equal to 90%. The multiphonon neutron scattering (MPNS) contributions has been evaluated by means of an iterative technique based on the assumption that the

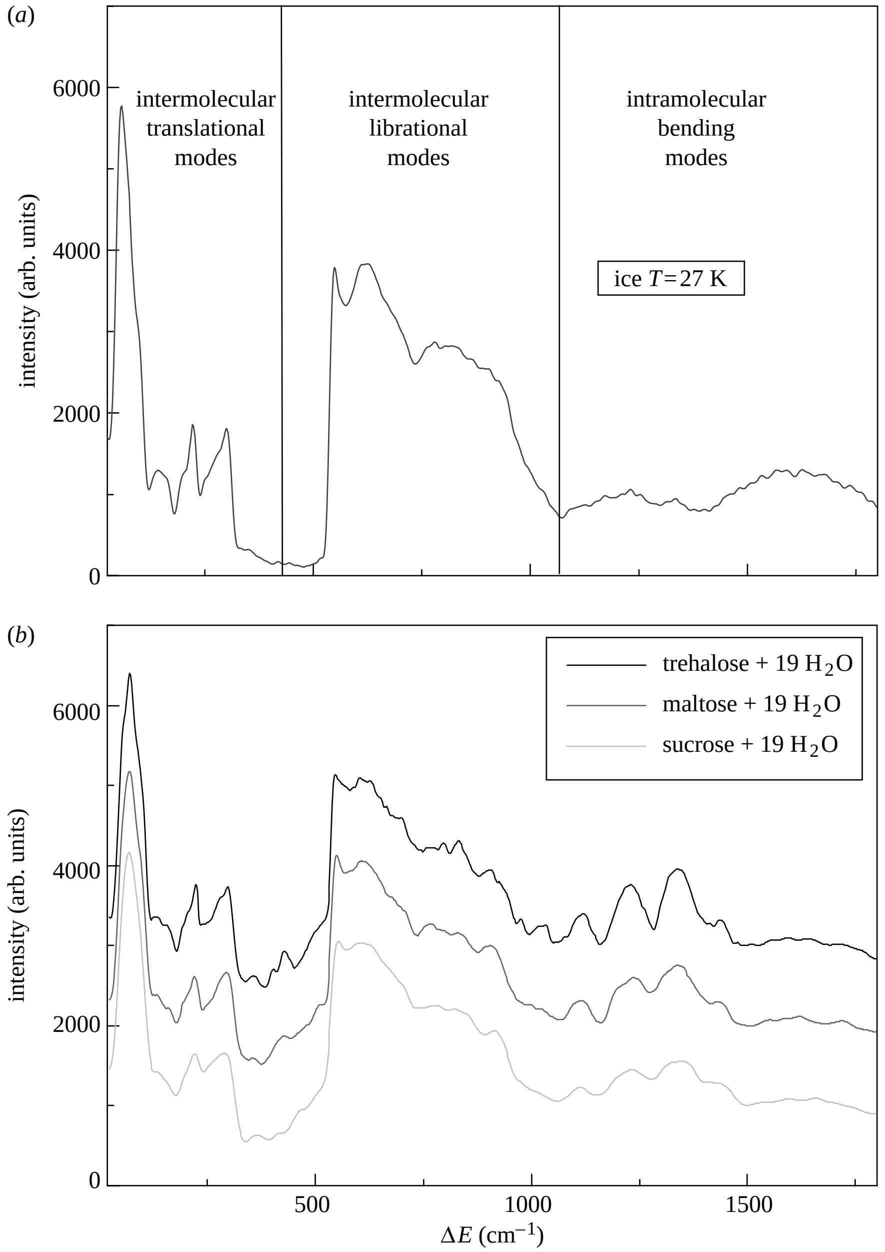

Figure 1. INS spectra of (*a*) ice and (*b*) disaccharide/H_2O mixtures at $T=27$ K in the range 0–1800 cm^{-1}.

one-phonon term describes the INS spectrum at the lower energy transfers and the subsequent calculation of the first MPNS term for the whole energy region from this first step. The result is a new one-phonon spectrum that allows one to obtain MPNS terms with successive iterative calculations until good convergence is obtained. The multiphononic correction has been performed for all the spectra. The density of states has been calculated by extrapolating the data to a low Q, after performing the MPNS and Debye–Waller factor corrections and assuming a squared-Q regime for the intensity.

3. RESULTS AND DISCUSSION

It is well known that the INS spectra of ice can be subdivided into two different regions: (i) the region of the H_2O intermolecular vibrations up to approximately 1060 cm^{-1}, in which one distinguishes a ‘translational’ part up to approximately 400 cm^{-1} and a ‘librational’ part and (ii) the region of the H_2O intramolecular vibrations up to approximately 3600 cm^{-1}, which presents the bending modes at approximately 1600 cm^{-1} and the stretching modes at approximately 3360 cm^{-1} (Li 1996; Kolesnikov *et al.* 1999*a*,*b*). In figure 1*a*,*b* the INS spectra of ice and disaccharide/H_2O mixtures at $T=27$ K in the range 0–1800 cm^{-1} are shown, respectively. The error bars are approximately 2% in all of the figures.

As far as the INS spectra of trehalose/H_2O mixtures are concerned, figure 2 shows, within the spectral frame of ice, the spectrum of trehalose/H_2O mixture at a concentration value corresponding to two H_2O molecules for each disaccharide molecule together with the multiphononic correction. As can be seen, this correction does not significantly affect the translational modes region at low frequency but increases at larger energy transfers.

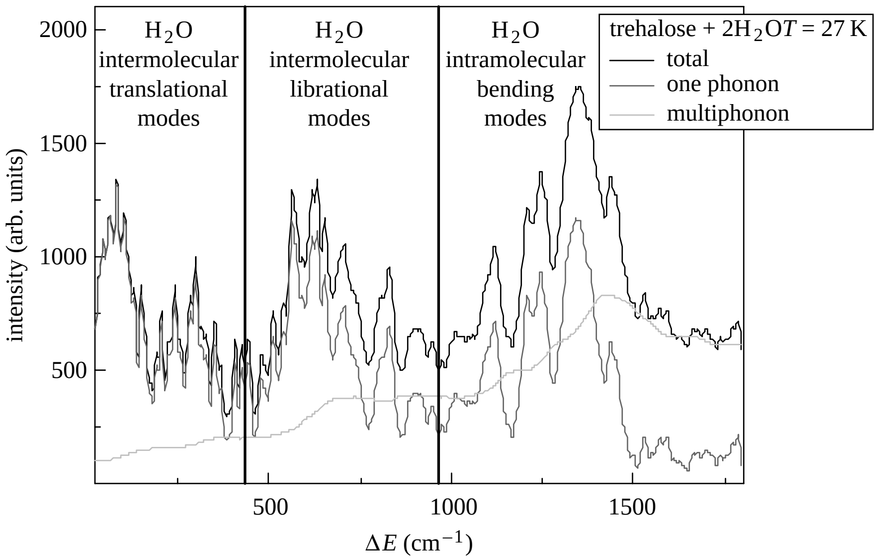

Figure 2. INS spectrum of trehalose/H_2O mixture at a concentration value corresponding to two H_2O molecules for each disaccharide molecule with the multiphononic correction.

Furthermore, it appears that the single phonon contribution reproduces each experimental peak and, in particular, indicates that the three peaks substituting the bending region of water are to be attributed to characteristic motions of the trehalose molecule.

Taking into account the different spectral regions, the following features can be observed.

3.1. 0–200 cm^{-1} region

This spectral region is concerned with low energy collective modes. No significant difference is observed for the trehalose/H_2O mixture in comparison with the other disaccharides mixtures: as an example, the peak at approximately 54 cm^{-1} in the ice spectrum, that is, the first Van Hove singularity in the dynamics of acoustic phonons in crystalline ice, is equivalently depressed by the three disaccharides.

3.2. 200–1060 cm^{-1} region

As already mentioned, two different contributions to the intermolecular modes can be distinguished in this region for ice: translational and librational modes, respectively. The spectra of the disaccharides/H_2O mixtures in the region 200–400 cm^{-1} are all equivalently dominated by ice.

It was found that the characteristic value for the librational band in the INS spectrum for different ice forms is the position of its low-energy cut-off (Li 1996; Kolesnikov *et al.* 1999*a*,*b*). Assuming the moment of inertia for water molecules in the different ice forms is about the same, the observed shifts of the cut-off position indicate that the transverse forces between the water molecules are of different intensity for the different ice forms. Furthermore, these shifts have also been observed in IR measurements on the same ice forms (Givan *et al.* 1997).

In order to point out the differences among the disaccharides/H_2O mixtures in the librational spectral region, the density of states for the investigated mixtures are shown in figure 3. Comparing the trehalose, maltose and sucrose/19 H_2O mixture spectra in this spectral region, we note a shift and depression of the librational cut-off intensity for ice in the presence of disaccharides. These findings suggest that disaccharides modify the spectral features of ice in this spectral region, affecting the intermolecular interaction forces and the arrangements of the H_2O molecules, as already highlighted by Raman and IR spectroscopy (Branca *et al.* 1999*a*,*b*).

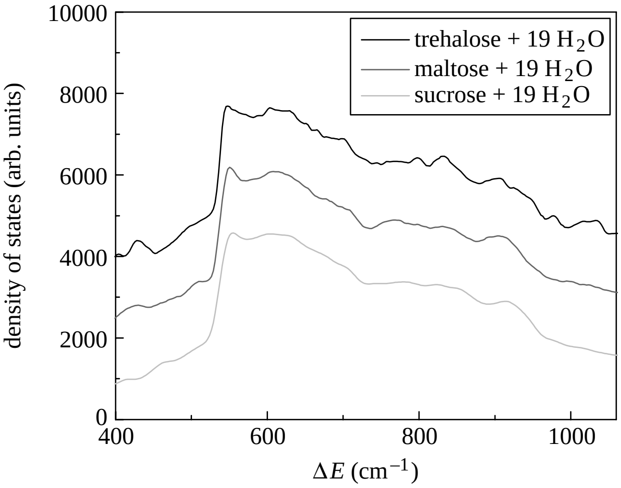

Figure 3. Densities of different states of trehalose, maltose and sucrose/H_2O mixtures in the librational spectral region. The disaccharides mixtures' profiles are shifted for clarity.

In trehalose vibrational spectrum obtained by simulation (Ballone *et al.* 2000) we note that the peak attributable to the C–O stretching mode at approximately 1000 cm^{-1} does not appear or, at least, is not detectable, indicating that these kind of modes in the rings are constrained because of the presence of hydrogen bonds with water. In general terms, however, below 960 cm^{-1} it is not possible to assign a well defined character to all the bands (Ballone *et al.* 2000). While the stretching of the C–O bonds occurs in a range between 970 and 1100 cm^{-1}, it is difficult to assign a well-defined character to most of the modes below 950 cm^{-1}; only the oscillations of the OH groups around the minimum of the C–C–O–H torsional potential are well localized and fairly easy to identify. These modes have energy between 420 and 570 cm^{-1}, with the exception of the OH group giving rise to the intramolecular hydrogen bond, whose torsional mode has an energy of 700 cm^{-1} (Ballone *et al.* 2000).

Neutron diffraction results confirm the changes induced by disaccharides on water tetrahedral structure (Branca *et al.* 2002). It is well known that the peak at 4.5 Å in $g_{OO}(r)$ of pure water is associated with the 'degree of tetrahedrality' (Branca *et al.* 2002). In $g_{XX}(r)$, (where X are atoms distinct from hydrogen) for trehalose +40 H_2O at $T=300$ K this peak is absent and the general trend is significantly distorted (shown in figure 4).

3.3. 1060–2000 cm^{-1} region

The intensity profile of trehalose/H_2O mixture in this spectral region, which corresponds to the range of the bending vibrational modes of ice, appears more 'structured' in comparison with the other disaccharides and with sucrose in particular, the latter showing a smoother trend.

In figure 5 the densities of different states of trehalose, maltose and sucrose/H_2O mixtures in the spectral bending region are reported. Since it is not possible to extract quantitative information about the changes induced by trehalose on the H_2O structural arrangement from these results, we take the DFT simulation results into account in order to assign the observed peaks to significant modes of the investigated system. Following the simulation results (Ballone *et al.* 2000), the stretching of the C–O bonds occurs in the range between 960 and 1104 cm^{-1}, whereas the band of the hybridized H–C–H, C–C–H and C–O–H bending modes, which in the trehalose vibrational spectrum occurs in the range between 1175–1433 cm^{-1}, is present between 1160 and 1440 cm^{-1}. A striking feature characterizing the simulation results is found in the evaluation of the conformational freedom in the trehalose molecule: the results show that a relatively low energy is required for the torsions around the oxygen linking the two rings to occur, whereas the bending of the same angle is much less likely, being energetically unfavourable. When the interaction with water is introduced through the simulation of the trehalose crystal with two trehalose monohydrate molecules in the unit cell, apart from some expected distortions in the O–H bonds of water molecules, the most apparent difference between the vibrational spectra of the crystal and the molecules concerns the modes localized on the OH groups. The C–H stretching band is relatively unchanged in frequency but the results are broader. At 1630 cm^{-1} the isolated peak of the H–O–H bending of water appears. Below this frequency, the differences between the vibrational frequencies of the crystal and the molecule are quantitatively small and difficult to identify. The most apparent effect is an increase in the energy of the OH rotations around the minimum of the torsional potential, due to the formation of the intermolecular hydrogen bonds (Ballone *et al.* 2000).

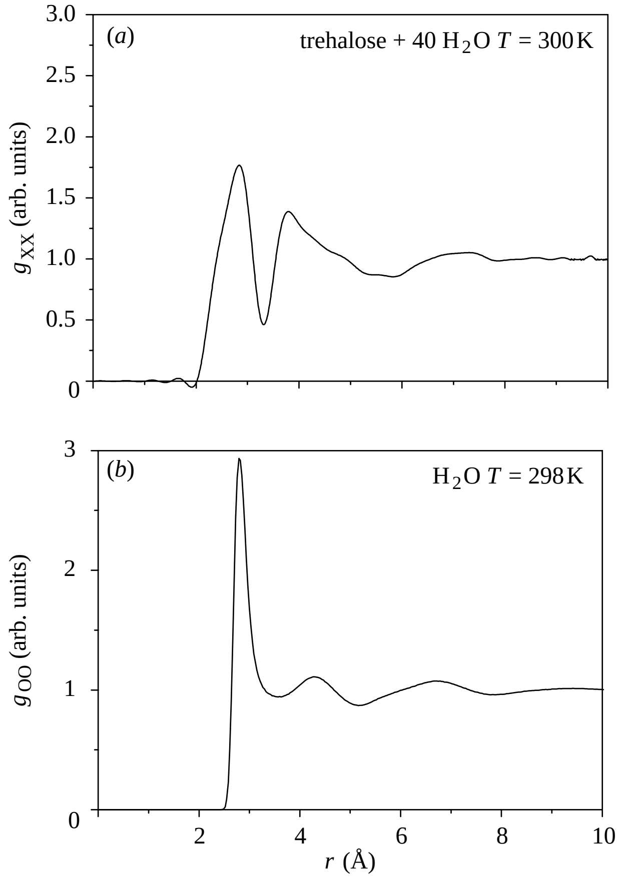

Figure 4. $g_{XX}(r)$ for trehalose aqueous solution for a concentration value corresponding to 40 H_2O molecules for each of trehalose at $T=300$ K. For comparison, the $g_{OO}(r)$ of pure water is reported.

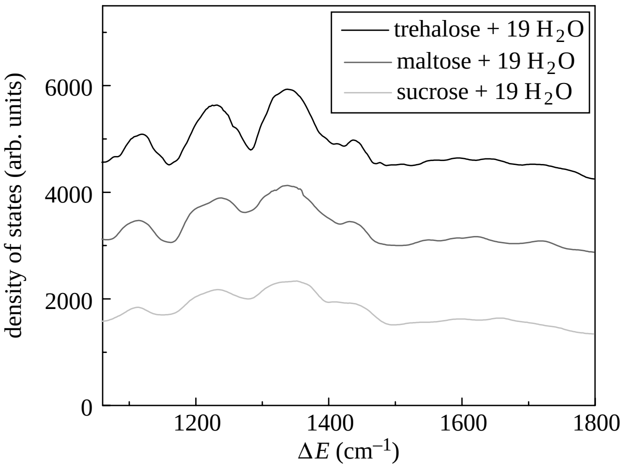

Figure 5. Densities of different states of trehalose, maltose and sucrose/H_2O mixtures in the bending spectral region. The disaccharides mixtures' profiles are shifted for clarity.

It is evident, with an inspection of figure 5, that trehalose shows more distinctly, in respect to the other disaccharides, the characteristic peaks typical of the crystalline state described above. In other terms the trehalose/H_2O complex shows a more 'crystalline' behaviour, namely, a locally more ordered structure, which can justify the higher rigidity of this system (Branca *et al.* 2001*a*). Trehalose, besides significantly modifying the structural and dynamical properties of water, forms with H_2O a more rigid unique entity able to encapsulate biological structures and protect them in a more rigid environment. In our opinion this circumstance is relevant because it implies a better *crypto*-biotic effect and, hence, a higher bioprotection capability at high disaccharide concentrations.

4. CONCLUSIONS

In this paper INS results on disaccharides/H_2O mixtures are shown. The experimental data are evidence of the effects of disaccharides on the ice vibrational properties. The most relevant result is that trehalose more significantly affects the structural arrangements of the neighbouring H_2O molecules by means of a more 'crystalline' conformation. This circumstance could explain the trehalose *crypto*protectant effectiveness: by interacting with water, trehalose is able to create more rigid structures that are able to adapt the trehalose/H_2O complex to the irregular surface of the biostructures.

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