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Cite this: *Phys. Chem. Chem. Phys.*, 2015, **17**, 3599

The effect of sugar stereochemistry on protein self-assembly: the case of β-casein micellization in different aldohexose solutions†

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Received 17th August 2014,
Accepted 8th December 2014

DOI: 10.1039/c4cp03686g

www.rsc.org/pccp

Protein self-assembly applications, such as nanoencapsulation of drugs and nutraceuticals, require deep understanding of the parameters governing the micellization process, including the effects of ionic and non-ionic co-solutes, like salts and sugars respectively, which is often overlooked. Herein, with the aim of shedding light on the effect of nonionic cosolute stereochemistry on protein self-assembly, we studied the ternary system of water–protein–sugar by examining the concentration-dependent effects of three aldohexoses, D-glucose (Glu), D-galactose (Gal) and D-mannose (Man) and that of urea, on the micellization of beta casein (β-Cas), using pyrene as a fluorescent probe for the formation of hydrophobic domains. Pyrene's excitation spectra were recorded for several sets of samples with rising protein concentration (0–5 mg ml^{-1}), each set with a different co-solute type and concentration. Critical micellization concentration (CMC) and cooperativity of micellization were evaluated according to changes in pyrene spectra as it partitioned from the aqueous environment to the hydrophobic cores of β-Cas micelles. All sugars examined lowered the CMC of β-Cas with increasing sugar concentration and with a diminishing degree of effectiveness (Glu > Gal > Man) which correlated well with the sugars' dynamic hydration number, defined by Uedaira, and correlated negatively with their hydrophobic to hydrophilic molecular surface ratio. These results support the hypothesis that sugars affect protein self-assembly through both changes in water structure and by hydrophobic interactions, both of which are evidenced to be highly sensitive to sugar stereochemistry.

Introduction

It is well known that saccharides and polyols act as stabilizers of the native conformation of globular proteins in aqueous solutions,[1,2] and that the presence of sugars has an effect of increasing the denaturation temperature of a protein (T_m).[2–5] In addition, low molecular weight saccharides may cause soluting-in of hydrophilic polymers and gels,[6] but soluting-out of macromolecules having a partial hydrophobic character,[3,7] including most proteins.[2,4,5,8] However, different sugars vary in the intensity of their effect on a given macromolecule,[1,3,7,9,10] and different proteins may have varying responses to the same saccharide.[2,3]

In this study we advanced to explore a yet unstudied phenomenon of the effect of sugar stereochemistry on protein self-assembly, demonstrated through the micellization of β-Cas.[11,12]

Bovine β-Cas has a highly amphiphilic structure, resembling a block copolymer.[11,13–16] The content of hydrophobic amino acids in β-Cas is relatively high, and the great majority of these amino acids are grouped at the C-terminal domain. Conversely, the N-terminal region of the polypeptide chain is rich in polar and negatively charged amino acid residues, including all five phosphate groups attached to seryl residues.[12,13] Just like low-molecular-weight-surfactants, β-Cas tends to self-associate under appropriate conditions to form stable micelle-like structures with a hydrophobic core, a soft exterior and a hydrodynamic diameter of about 12 nm.[17] Both hydrophobic interactions and electrostatic repulsion are suggested to be important for the micellization process, the former being the principal driving force for association, and the latter preventing flocculation of the micelles.[18] At low protein concentrations in aqueous solutions, or at temperatures below 15 °C,[19] β-Cas seems to exist as individual molecules in a rather open, rheomorphic conformation.[11,16] When the protein concentration reaches the critical micellization concentration (CMC), β-Cas micelles start to appear in the system.[17] Dynamic Light Scattering (DLS), Differential Scanning Calorimetry (DSC) and Isothermal Titration Calorimetry (ITC) measurements supported the assumption that up to 30 °C the micellization transition of β-Cas is consistent with the shell

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† Electronic supplementary information (ESI) available: Appendix A – Calculating molar concentrations of the samples. Appendix B – Modeling of I_1/I_3 *vs.* β-Cas concentration sigmoid. See DOI: 10.1039/c4cp03686g

model of Kegeles and can be considered as a cooperative successive association of primary particles.[20–22] The first step (dimerization) of this process is a ''nucleation'' step, and once a dimer forms, growth by association of additional monomers becomes easier. The micellization is a reversible equilibrium process, and it is highly affected by environmental parameters: rising temperature (up to 45 °C)[12] or kosmotropic ions concentration will promote micellization, and so will pH values approaching the pI.[22,23] An aqueous solution of urea, a known protein solubilizing/denaturing agent, is a better solvent for β-Cas than pure water, because urea tends to be preferentially adsorbed to the protein,[24–26] and thus, it reduces the protein's self-assembly propensity.[12,27] Ethanol concentrations up to 2% v/v were found to promote micellization.[22] As the micellization process of β-Cas is highly condition-dependent, it is reasonable to expect that it would be affected by the presence of sugars. We hypothesized that it would even be sensitive to sugar stereochemistry, as in the case of isomeric sugars. Hence we aimed to study whether β-Cas micellization would be differently affected by three highly prevalent stereoisomeric aldohexoses, D-glucose, D-galactose and D-mannose, which differ only[28] by the configuration of their position 2 and 4 OH-groups (Scheme 1).

There are several approaches to explain sugar effects on the behavior of proteins and other polymers in aqueous solution. The preferential interaction theory, by Arakawa and Timasheff, suggests that most sugars are preferentially excluded from the vicinity of a protein, making it preferentially hydrated.[1,29] The consequent decreasing sugar concentration gradient towards the surface of the protein molecule exerts an ''osmotic stress'',[1,30] which may enhance the protein tendency to minimize its contact surface with the solution. Several studies suggested that sugars increase the surface tension of the solution, increasing the energetic penalty for exposing hydrophobic domains, hence enhancing the folding and thermal stability of proteins.[8,31,32]

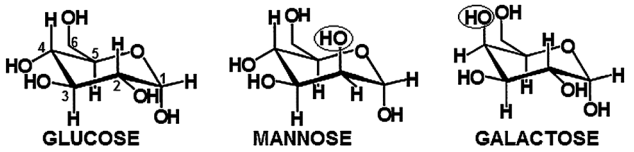
In aqueous environments, sugars act as ''Kosmotropes'' – a term originally coined to describe those ions in the Hofmeister series which interact with water more strongly than bulk water interactions.[33,34] Like ionic kosmotropes, polar nonionic kosmotropes, such as sugars,[2,3] have an exothermic heat of dilution in water.[34,35] This is the basis of another approach for explaining sugar effect on a polymer in a ternary system. If the hydrated sugar is viewed as a ''co-solvent'' for the polymer, its mixing enthalpy with the polymer should be similar to that of water, because the outer layer of the hydrated sugar complex is water. On the other hand, the entropy of mixing of the polymer with the sugar solution should be lower than that with pure water due to the much larger size of the sugar molecule and moreover, the hydrated sugar complex, compared to a water molecule. Therefore, the solvent-quality of a sugar solution for a protein is worse than that of pure water, thereby favoring compacting of the protein into a tighter globular conformation.[3,7,36] Moreover, as kosmotropes, sugars are capable of changing or intensifying hydrophobic interactions,[31,37–39] thus making the solution a more unfavorable solvent than water for the aliphatic and aromatic side chains of a protein. Consequently, more energy would be required for them to be exposed in a sugar solution compared to exposure in pure water.[2,4,30,35] This should similarly lead to promotion of protein self-assembly, in the case of amphiphilic proteins.

Sugars interact with water to an extent which depends upon their molecular structure.[8,31,38,39] Previous studies from our group have proposed that the different set of hydroxyl group orientations, which distinguishes one sugar isomer from another, may form either a better or a worse template for the cooperative arrangement of water molecules around it and consequently have a different extent of effect on vicinal water structure and on polymers in the solution.[3,5,7,36,40]

There is no single unambiguous numerical measure of the kosmotropicity or the chaotropicity of a solute.[2,3] NMR-spin–lattice relaxation times of naturally occurring $H_2{}^{17}O$ in pure water and sugar solutions were used to calculate the Dynamic Hydration Number – n_{DHN}. This hydration number was defined by Uedaira[41] as the number of water molecules around a sugar molecule, whose thermal motion is restricted by the sugar. It is suggested that n_{DHN} could be used as a measure of the sugar's kosmotropicity: the larger the sugar's n_{DHN} value, the more kosmotropic it is. According to this hydration number, the aldohexoses studied herein are scaled: Glu > Gal > Man. We have found a good correlation between size exclusion chromatography (SEC) elusion volume and n_{DHN} for aldohexoses.[10] The number of equatorial OH groups in the molecule, n(e-OH), correlates well with n_{DHN} for many sugars including these three aldohexoses.[41]

Interestingly, saccharides exhibit a combination of kosmotropic and chaotropic characteristics. Certain saccharides exhibit an ability to solubilize lipophilic nonpolar compounds,[41,42] increase the CMC value of surfactants[43] and even destabilize the native conformation of globular proteins.[44] Although these phenomena are much more profound for long chain saccharides, there is some evidence for chaotropic behavior of monosaccharides such as D-mannose, which was shown to promote the solubility of naphthalene and biphenyl to appreciable extents,[45] and glucose, which showed a slight inhibiting effect on the micellization of the surfactant Triton X-100.[43] Some believe that the source for this weak chaotropic character is the hydrophobic regions of the saccharide molecule. Sugars have nearly the same numbers of CH groups as OH groups in a molecule,[44] and thus it was suggested that the modest hydrophobicity of sugars may weaken the hydrophobic association of surfactants.[46]

As β-Cas self-associates into micelles *via* hydrophobic interactions,[22] it is reasonable to hypothesize that sugars would enhance its self-assembly propensity. The main question which we focused this study on was: do slight structural differences



Scheme 1 Stereochemical structures of α-D-glucose, α-D-mannose and α-D-galactose. While hydroxyls on carbons 2 and 4 are equatorial in glucose, hydroxyl on C2 in mannose and hydroxyl on C4 in galactose are axial (circled).

between different aldohexoses (D-Glu, D-Gal and D-Man, which are the most prevalent aldohexoses) significantly affect the propensity and cooperativity of protein self-assembly, and if so, how can this be rationalized in terms of sugar stereochemistry and hydration. Therefore, we studied the effects of these three isomeric aldohexoses on the β-Cas micellization process in ternary (water–sugar–protein) systems.

Materials and methods

Materials

β-Casein from bovine milk (Bioultra >98%, PAGE), D(+)-glucose (ACS), and pyrene (98%) were purchased from Sigma-Aldrich, Israel; NaCl (analytical grade) and absolute ethanol were purchased from Frutarom, Israel; $NaH_2PO_4{\cdot}2H_2O$ (Puriss), D(+)-galactose (Puriss), and urea (analytical grade) were purchased from Riedel de Haen, Germany; $Na_2HPO_4{\cdot}H_2O$ (Puriss) was purchased from Merck (Israel), and D(+)-mannose (99%) from Acros Organics, Thermo Fisher Scientific (Holland Moran Ltd, Israel).

Methods

To examine the protein micellization process we used pyrene as a fluorescent probe. The pyrene fluorescent emission spectrum comprises vibronic peaks which show strong solvent dependence, especially to the solvent's polarity.[47,48] The ratio between the emission intensity of the third (~383 nm) and first (~373 nm) peaks in the pyrene spectrum (I_3/I_1) could be used as a quantitative measurement for the polarity of its surroundings.[48] This unique nature of the molecule makes it an excellent probe to accurately determine critical micellization concentrations.[48] Pyrene is a highly hydrophobic probe and its solubility in water is very low (2–3 μM). In the presence of micelles, pyrene is preferentially solubilized in the interior hydrophobic nano-environments of these aggregates. As the number of micelles in the system increases, the I_3/I_1 ratio shifts from the value measured for pyrene in water (~0.63) to a higher value which suits a more hydrophobic environment. It has also been found that the I_3/I_1 ratio in micellar systems is independent of pyrene concentration or excitation wavelength.[49,50]

Solution preparation. β-Casein from bovine milk was dissolved (overnight at 4 °C) in pH 7.0, ionic strength 0.1, phosphate buffer saline (PBS) containing HPLC-grade water, 80 mM NaCl 3.05 mM $NaH_2PO_4{\cdot}2H_2O$ and 5.65 mM $Na_2HPO_4{\cdot}H_2O$. D(+)-Glucose, D(+)-galactose, D(+)-mannose and urea were dissolved in the same buffer and agitated overnight, to obtain sugar stock solutions at various concentrations between 0.15 and 2.2 g ml^{-1} or urea stock solution at a concentration of 0.039 g ml^{-1}. Pyrene was dissolved in cold absolute ethanol to obtain 0.03 M pyrene stock solution and diluted into the co-solute stock solutions to a concentration of 1.5 μM. The final pyrene concentration in all samples was 1.2 μM. Samples were prepared by mixing the two stock solutions (β-Cas and co-solute + pyrene) in addition to pure PBS buffer to obtain a set of final protein concentrations of 0–5 mg ml^{-1} for a constant co-solute concentration. These sets were repeatedly prepared for final sugar concentrations of 0.1–1 M or 0.5 M urea. Molar concentrations were calculated according to the solution densities measured by weighing precise volumes using an analytical balance (Precisa 240A) (see Appendix A, ESI†).

Spectrofluorometry. Pyrene fluorescence spectra were obtained using a spectrofluorometer (Fluorolog 3-22, Jobin Yvon, Horiba Scientific Ltd.). The excitation wave length was 338 nm[51] (slit width 2 nm), and the emission band recorded was 368–387 nm (slit width 2 nm) with an increment of 0.5 nm. All samples measured were kept at 25 °C using a thermal water bath and the spectrofluorometer isothermal water circulation system. All samples were made in duplicates and each duplicate was read twice.

Results

Pyrene fluorescence

The third peak of the pyrene excitation spectrum showed high sensitivity to β-Cas concentration in a non-linear manner, as can be seen in Fig. 1, in which the fluorescence intensity values were normalized by the intensity of the first peak. A slight redshift in the spectra was also observed with rising β-Cas concentration. A similar redshift can be observed upon rising casein concentrations in a study by Liu *et al.*[23]

When plotted against β-Cas concentration, the I_3/I_1 ratio was found to increase sigmoidally. A mathematical model was developed to describe the sigmoid and to find its parameters: CMC, K = cooperativity parameter (when higher than 1, the process is considered cooperative[52]), $\left(\frac{I_3}{I_1}\right)_{C=0}$ = minimum I_3/I_1 ratio value and $\left(\frac{I_3}{I_1}\right)_{C=\infty}$ = maximum I_3/I_1 ratio value (see Appendix B, ESI†). Fitting the model to experimental data was carried out using OriginPro 9.1 software, and the adjusted R^2 value was 0.99 and above for all sigmoidal fits (see example for D-Gal in Fig. 2).

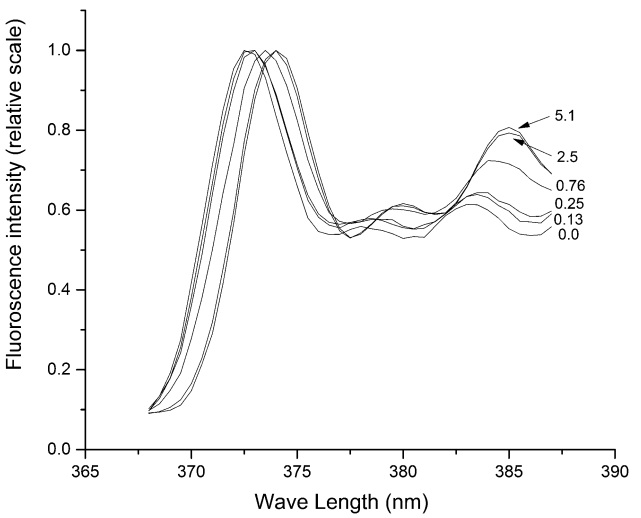


Fig. 1 Pyrene excitation spectrum (λ_{Ex} = 338 nm) in the presence of rising β-Cas concentration (indicated in mg ml^{-1} to the right of each spectrum), [PBS pH = 7.0, ionic strength 0.1 M, 25 °C, pyrene concentration 1.2 μM].

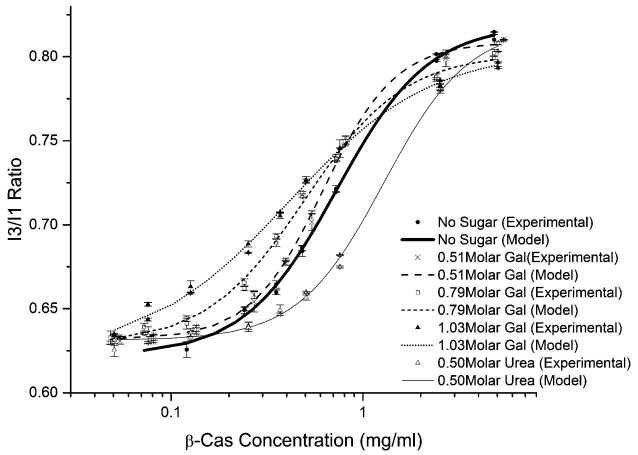


Fig. 2 Calculated sigmoids of I_3/I_1 *vs.* β-Cas concentration in the presence of various concentrations of D-galactose or urea. [PBS pH = 7.0, ionic strength 0.1 M, 25 °C, pyrene concentration 1.2 μM].

As can be seen in Fig. 2, an increasing concentration of sugar stretched the sigmoidal curve to the left and changed the slope non-monotonously. For comparison, the addition of urea, a nonionic chaotropic solute, stretched the curve to the right and decreased the slope. The changes induced by the sugar could indicate an increase in the protein propensity to self-associate, while the urea affected the system in an opposite way. Differences in the sigmoidal slope indicate a change in the cooperativity of the micellization process: an increased slope stands for higher cooperativity.

Comparison between the effects of different sugar isomers on CMC of β-Cas

To investigate the effect of different aldohexoses, and that of urea, on the propensity of β-Cas to self-associate, calculated CMC values were plotted against co-solute concentration (Fig. 3).

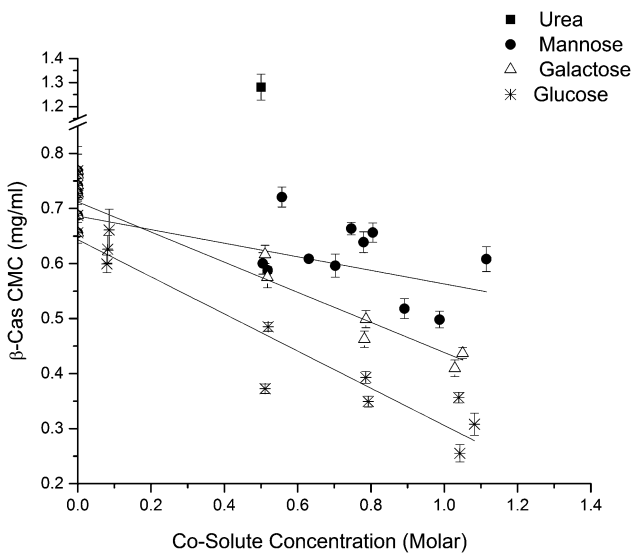


Fig. 3 CMC of β-Cas *vs.* co-solute concentration for the different aldohexoses and for urea, [PBS pH = 7.0, ionic strength 0.1 M, 25 °C].

As can be seen in Fig. 3, while urea increased the CMC, all of the three aldohexoses tended to lower the CMC of β-Cas with their rising concentrations (for all sugars, the slope was found to be significantly different from zero at the 0.05 level), however, to different extents. The most potent CMC reducer was found to be glucose, followed by galactose, and mannose was found to be the least potent. The results for each pair of sugars were found to be significantly different ($p < 0.05$), according to an *F*-test performed using the Origin 9.1 software.

The main aim of plotting the linear trendlines was to evaluate the differences between the effects of these three aldohexoses. The adjusted R^2 values for the linear trend lines were 0.80, 0.93 and 0.26 for glucose, galactose and mannose, respectively, (one should bear in mind that R^2 values are sensitive not only to the scatter, but also to the slope, hence the low value for mannose is not only due to the somewhat larger scatter, but also due to the fact that the slope is the lowest). Results for low sugar concentrations (approx. 0.08 M) were obtained only for glucose to demonstrate the consistency of the linear trend. All three *y*-intercept values were not statistically different from the average CMC value calculated for the no-sugar–PBS system (0.72 ± 0.04 mg ml^{-1}).

When we used a molal sugar concentration scale or a percentage on a weight basis instead of the presented molar values, the results obtained were numerically different, but the trends and the order of sugars were the same.

Micellization cooperativity parameter

Fig. 4 presents the effects of the three sugars on the β-Cas micellization cooperativity parameter (K).

All of the calculated K values in the concentration range studied were higher than 1, indicating a cooperative micellization process. Interestingly, the three sugars examined seemed to have a non-monotonous effect on the cooperativity of β-Cas micellization. Up to a sugar concentration of about 0.5 M, cooperativity rose, and above these concentrations, the cooperativity decreased with rising concentration. Therefore, it can be deduced that at

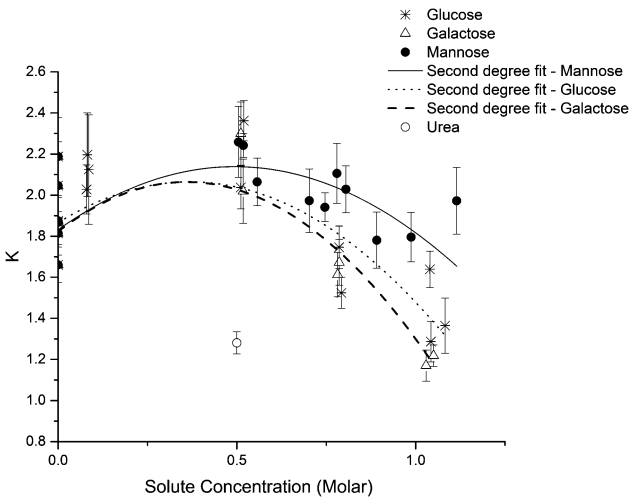


Fig. 4 The cooperativity parameter (K) *vs.* solute concentration [PBS pH = 7.0, ionic strength 0.1 M, 25 °C, pyrene concentration 1.2 μM].

low concentrations, sugars promote cooperativity, while at higher concentrations (above 0.5 M) they diminish it. The second degree polynomial fit matched the experimental data to various extents (adjusted R^2 = 0.52, 0.87 and 0.47 for glucose, galactose and mannose respectively), but the three data sets were not significantly different from one another. Urea at 0.5 M had a strong suppressive effect on the cooperativity of the micellization process, compared to its value in the (''pure'') buffer, and compared to the sugars.

Maximal and minimal I_3/I_1 ratio values

Lastly, the calculated values of $\left(\frac{I_3}{I_1}\right)_{C=0}$ and $\left(\frac{I_3}{I_1}\right)_{C=\infty}$ were found to be completely independent of the solute identity or concentration, with average values of 0.625 ± 0.005 and 0.81 ± 0.01 respectively for all the sigmoidal fits (n = 37) obtained in this study (each corresponds to a data point in Fig. 3) taken together.

Discussion

Pyrene fluorescence

The pyrene spectrum and its response to increasing β-Cas concentration (Fig. 1 and 2) concurred with earlier studies.[23,48,50] The gradual increase of I_3/I_1 ratio values implies a successive association process which is compatible with the shell model of Kegeles.[12,22,53] CMC values determined for the no-sugar–PBS system at 25 °C and ionic strength of 0.1 M (0.72 ± 0.04 mg ml^{-1}) were a bit smaller than the CMC value reported for β-Cas under identical conditions using ITC ($\sim$0.8 mg ml^{-1})[12] and slightly higher than the CMC value determined using the sedimentation equilibrium technique ($\sim$0.7 mg ml^{-1}) at 20 °C in a 0.2 M sodium phosphate buffer of pH = 6.7.[54] Overall our results are thus in good agreement with the literature, and small differences may be due to different raw materials used, to differences in the methods for CMC determination and in the mathematical determination of CMC.[50]

The effect of decreasing the CMC of β-Cas by the three aldohexoses, shown in Fig. 3, suits our earlier hypothesis that sugars, acting as non-ionic kosmotropes, would enhance the propensity of the amphiphilic β-Cas to self-associate into micelles. Moreover, the trend of these results is in good agreement with some earlier dynamic light scattering (DLS) results of β-Cas CMC obtained by our group (Alina Shapira, unpublished): The bimodal distribution (monomeric protein and micelles) was followed at increasing protein concentrations, and CMC was estimated as the protein concentration at which the micelles fraction starts increasing at the expense of the monomer fraction, as we have previously reported in PBS only;[55] however, it was repeated with rising glucose or urea concentrations. In PBS the CMC of β-Cas was 0.5 mg ml^{-1}, and it decreased to about 0.4 and 0.2 in the presence of 0.5 and 1 molar glucose respectively. In contrast, the CMC increased to about 0.7 and 0.85 mg ml^{-1} in the presence of 1 and 2 molar urea respectively. The results reported herein are in quite a good agreement with these DLS results, considering that different methods were used. The strengthening of a protein propensity to self-assemble into micelles in the presence of sugars is an indication of the kosmotropic effect of sugars, which serve as worse co-solvents for the protein by imbibing and structuring the water around the sugar molecule.[3,5,7,27,36] The observation of an opposite effect induced by the presence of urea (Fig. 2 and 3) may support this explanation, as urea is a known chaotrope with strong soluting-in capabilities for β-Cas and other proteins.[2,27]

Peculiarly, in a study by Belyakova *et al.* (2003) using static and dynamic light scattering, the presence of sucrose seemed to exert a dissociative effect on sodium caseinate aggregates at pH > pI of caseinate.[56] We suppose that these different results originated from methodological differences or from differences between pure β-Cas and sodium caseinate. Furthermore, that study focused on sucrose, a disaccharide, and did not compare it to urea, nor to different sugar isomers.

The effect of sugar isomers on the CMC of β-Cas

According to Fig. 3, it can be observed that, albeit their identical molecular weight and similar structure, these aldohexoses have significantly different propensity to promote β-Cas micellization. These differences imply a more essential difference in the extent to which each of the three aldohexoses affects water structure. In an attempt to correlate the observed phenomenon with the dynamic hydration number (n_{DHN}) scale of Uedaira,[41] the slope of each of the trend lines in Fig. 3 (designated ''CMC increment'') was plotted against the n_{DHN} value of the corresponding sugar (Fig. 5).

Fig. 5 demonstrates a correlation between the potency of the three sugars to promote the micellization of β-Cas and their n_{DHN} values. Although a description of the exact mechanism through which the examined sugars affect the micellization of β-Cas is beyond the scope of this work, it could be concluded that for the examined aldohexoses, the more water molecules the sugar restricts, the stronger is its propensity to promote the micellization of β-Cas.

Another possible source for differences between the sugars may be their different hydrophobicities. Some sugars with the same molecular formula have shown different hydrophobic character, and consequently, a measure for the hydrophobicity

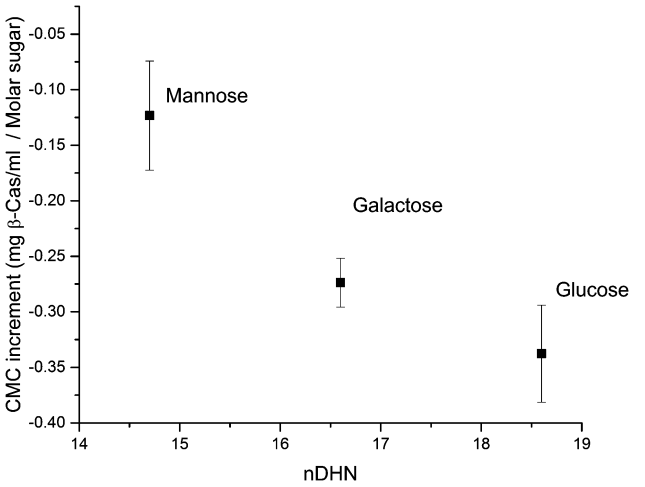


Fig. 5 β-Cas CMC increment *vs.* n_{DHN} for glucose, galactose and mannose.

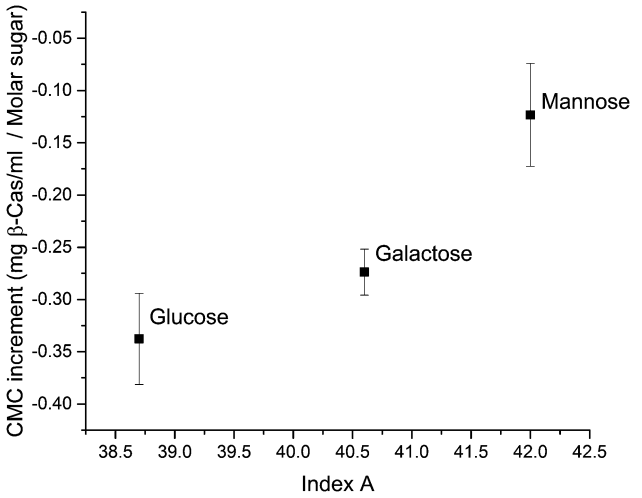


Fig. 6 β-Cas CMC increment *vs.* Index A[57] for glucose, galactose and mannose.

level of a sugar molecule was proposed by Miyajima *et al.*[57] in the form of index A (the ratio of the hydrophobic to hydrophilic surface areas of the sugar molecule, weighted by the equilibrium anomer proportions in solution). Index A has been shown to be in good correlation with the sugars' partition coefficients between polystyrene gel and water, which means that the larger the index, the stronger the hydrophobicity of the molecule, and presumably it will be less kosmotropic. According to index A, our sugars of interest were scaled: Glu = 38.7 < Gal = 40.6 < Man = 42.0.[57]

As shown in Fig. 6, the higher the sugar's hydrophobic to hydrophilic molecular surface ratio (hydrophobicity index A[57]) the weaker is its effect of decreasing the CMC of β-Cas.

Higher levels of hydrophobicity of a sugar molecule[57] could result in slightly more favorable hydrophobic interactions with hydrophobic groups of the protein. Consequently, the hydrophobic interactions of a sugar molecule with hydrophobic domains of β-Cas may physically interfere with-, and thus have a weakening effect on-the hydrophobic association leading to protein self-assembly,[57] and so, a sugar with a higher level of hydrophobicity would have a weaker promoting effect on β-Cas micellization.

Cooperativity parameter

As seen in Fig. 4, the effect of sugars on the cooperativity of micellization seems to be concentration dependent. Sugars, like other kosmotropes, are expected to lower the cooperativity of self-assembly processes due to their interference with the cooperativity of water escape during the assembly.[36] This expected decrease in the cooperativity was only observed here for sugar concentrations higher than approximately 0.5 M, unlike the observed effect of a monotonous decrease of the cooperativity of PNIPA phase-transition described by Shpigelman *et al.*[36] An effect of increasing the cooperativity, however, is rather surprising and may indicate the existence of at least two mechanisms with opposite effects whose relative dominance is concentration dependent. It is likely that at low sugar concentrations, the main effect of sugar is of decreasing solvent quality for the protein, which enhances protein association (and its cooperativity), while at higher sugar concentrations, sugar–protein interactions become more frequent, and diminish protein micellization cooperativity. At higher sugar concentrations also sugar–water interactions may hinder cooperative water escape upon protein association. Notably, unlike the CMC increment induced by the three aldohexoses, the cooperativity parameter was not as sensitive to sugar stereochemistry, and we observed no significant differences in the effect of the different sugars on the micellization cooperativity.

Urea is known to hinder self-association of amphiphilic molecules by preferentially binding to them,[22,58] and indeed we found it to diminish the cooperativity of the assembly process. The presence of partly adsorbed urea may disturb dimer formation and the joining of additional β-Cas molecules to the dimer, and so, micellization would only commence at higher concentrations and the process would be less cooperative. This mechanism is nicely demonstrated in the stretching of the urea sigmoid to the right in Fig. 2, and in the much lower cooperativity parameter, K, obtained for 0.5 M urea: $K = 1.3 \pm 0.05$ compared with $K = 1.9 \pm 0.2$ for pure-PBS system.

Maximal and minimal I_3/I_1 ratio values

Because pyrene is sensitive to water in its nanoenvironment,[47] the calculated $\left(\frac{I_3}{I_1}\right)_{C=\infty}$ values may indicate the water content in the micelle and thus indicate its compactness. In this experiment we observed a rather constant $\left(\frac{I_3}{I_1}\right)_{C=\infty}$ value with an average of 0.81 ± 0.01. This value indicates a certain level of water penetration and matches the ''fluffy particle'' description of β-Cas micelles.[27]

$\left(\frac{I_3}{I_1}\right)_{C=0}$ value is, in fact, a parameter of the binary water–sugar system which can be used as a measure of the polarity of the solvent (aqueous sugar solution). All of the solutions in this experiment had a very similar $\left(\frac{I_3}{I_1}\right)_{C=0}$ value, at an average of 0.625 ± 0.005, and thus seem to have similar polarity levels. The observation of $\left(\frac{I_3}{I_1}\right)_{C=0}$ value, which is very similar to the I_3/I_1 of pyrene in water (approx. 0.63), and is completely independent of the presence of co-solutes, supports the assumption by which all of the sugar molecules are completely hydrated and there are no significant pyrene–sugar interactions.

Conclusions

The presence of D-glucose, D-galactose or D-mannose in β-Cas solution decreased the CMC of the protein at pH = 7, 25 °C and ionic strength of 0.1 M. This effect was in a roughly linear correlation with sugar concentration for all the three sugars studied. Urea at 0.5 M expectedly had an opposite effect. Remarkably, the slight stereochemical structural differences between the three examined aldohexoses, resulting in significant differences between each pair of sugars, in their propensity to

lower the CMC of β-Cas. The most potent CMC reducer was found to be D-glucose, followed by D-galactose, and D-mannose was found to be the least potent. The potency of an aldohexose to lower the CMC of β-Cas correlated with the number of water molecules around a sugar molecule, whose thermal motion is restricted by it (n_{DHN}). This indicates the susceptibility of the micellization process to water structure changes induced by a sugar molecule. The effect of the sugars also negatively correlated with their hydrophobic-to-hydrophilic molecular surface ratio (index A). This modest but important hydrophobicity of sugars may either be partly responsible for their different dynamic hydration numbers (the higher the hydrophobicity ratio index A, the lower the n_{DHN}), but may also have an impact on their interaction with hydrophobic domains of open-structured proteins, such that the more hydrophobic the sugar, the more it may have interacted with hydrophobic domains of the protein, hence interfering with its association and self-assembly. The examined sugars had a non-monotonous concentration dependent effect on the cooperativity of the protein micellization process (they enhanced cooperativity below ~0.5 M and diminished it above ~0.5 M). This mixed effect may suggest the existence of at least two opposing concentration dependent mechanisms, by which sugars affect β-Cas micellization. These exact mechanisms remain to be identified, although it may be that at low sugar concentrations the effect is predominantly kosmotropic, through sugar hydration which enhances the cooperativity of protein association, while at higher sugar concentrations, direct sugar–protein interactions, partly hydrophobic, may interfere with, and hence diminish association cooperativity. Even at high protein concentrations β-Cas micelles contained a notable concentration of water in their cores, but their compactness was independent of the presence of sugars or urea under the condition ranges examined. The presence of the examined sugars had no effect on the polarity of their aqueous solution, as assessed by pyrene $\left(\frac{I_3}{I_1}\right)_{C=0}$ values. This may support the assumed absence of significant sugar–pyrene interactions.

In light of the presented results, we suggest that sugars could be useful in controlling the behavior, and particularly the self-assembly, of proteins in aqueous environments, analogously to the well-known Hofmeister series, for a "finer tuning" or as an alternative, where salts should be avoided.

Acknowledgements

The authors thank Dr Alina Shapira for the DLS results quoted for comparison, and Prof. Raoul Zana, whose studies and discussions inspired us to use pyrene for this study.

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