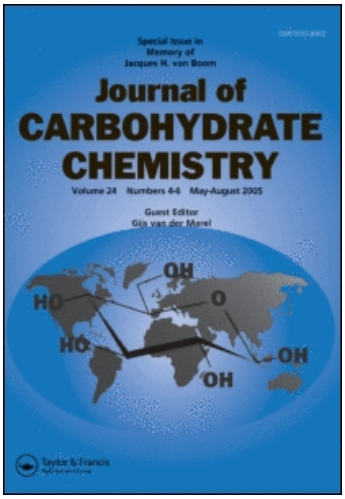

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Solute-Solvent and Water Activity of Small Carbohydrates: Application to the Study of Enzyme Stability in Aqueous Sugar Solutions

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SOLUTE-SOLVENT AND WATER ACTIVITY OF SMALL CARBOHYDRATES : APPLICATION TO THE STUDY OF ENZYME STABILITY IN AQUEOUS SUGAR SOLUTIONS +

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ABSTRACT

Viscometric constants of small carbohydrates are determined and interpreted in terms of specific hydration. Water activity of their saturated solutions is measured. Their effect on water structure is deduced from the deconvolution of the Raman bands in the OH region. The influence of water activity lowering of D-fructose, D-glucose and sucrose on initial lysozyme activity is studied. The effect of these sugars on the preservation of enzyme stability during the time is investigated for yeast alcohol dehydrogenase (YADH). Half-life time of YADH is controlled after the storage of the enzyme in different sugar and polyol solutions. Enzymatic results are in good agreement with the solute-solvent interactions of sugars and polyols in the aqueous medium. The sugar which provokes the most noticeable perturbation of water structure, i.e., D-fructose, causes the denaturation of the enzymes and acts as a destablizer.

INTRODUCTION

Solute-Solvent interactions of small carbohydrates in the aqueous medium together with the knowledge of their structure help in interpreting their properties. We recently[1] based the explanation of differences in sweetness of mono- and disaccharides on their solution properties, especially their effect on water structure. Although the stabilizing effect of concentrated (with low water activities) sugar and polyol solutions against thermal inactivation of enzymes is known,[2] the mechanism by which water activity influences enzyme activity and stability has not yet been elucidated. Because of the preponderance of hydration in aqueous sugar solutions, the activity of water (Aw) deviates from ideality[3] (Raoult's law). The more important the deviation, regardless of whether it is positive or negative, the more it affects enzyme activity.[4] It was noted[5] that for reactions occuring in polyol solutions, the enzymatic reaction proceeds more readily the less the water structure is perturbed by the solute. Having only knowledge of the water activity parameter is not sufficient to predict the behavior of the enzyme.[6] It is also necessary to precisely know the nature of the additive (sugar or polyol) used to lower the value of Aw. The way Aw is controlled is even more important than its value. This means that one should be informed of the spacial arangement of OH groups around the sugar or polyol molecule to interpret its specific hydration. The long-range effect of the additive on water structure is also important to know if it is desired to evaluate the mobility of the solvent. Such properties may be derived from the viscometric parameters for the size of the hydrated carbohydrates and from Raman Spectra for the effect of the solute on water structure.

Different interpretations of enhanced stabilities of enzymes in sugar or polyol solutions were proposed. The protective effect of sugars was correlated with the number of equatorial hydroxyl groups.[7] It was shown[8] that the stability of invertase was increased when the molecular weight of polyethylene glycol (PEG) increased and that dextran showed a maximum stabilizing effect for a molecular weight of 18 000. This work is a part of a systematic study[9] of enzyme stabilization as a function of the structure of the stabilizing sugar or polyol additive and

the modification of the protein microenvironment. Egg-white lysozyme and yeast alcohol dehydrogenase were stored in the presence of different additives and their stability studied with reference to physico-chemical propeties of the storage media.

RESULTS AND DISCUSSION

Solute-Solvent Interactions in Aqueous Sugar Solutions : Intrinsic viscosity [η] and Huggins constant k' are listed in Table 1 for the sugars and polyols investigated. The method of determining [η] involved a test of verification of the results based on the following relation[10] :

$$(\eta_{sp/c} + \eta_{diff/c})/2 = \ln (\eta/\eta_o)/c$$

An example of the triple extrapolation procedure of determination [η] is given in Fig. 1.

Intrinsic viscosity [η] is generally considered as a shape factor accounting for the hydrodynamic diameter of the solvated molecule. Because of the quasi-spherical shape of sugar molecules and the hydrophilic nature of their hydration, the values of [η] are comparable (see Table 1). The lowest value of [η] is observed for D-fructose, probably because of a marked difference between its hydrophobic and hydrophilic sides. The Huggins constant k' is taken as an interaction factor accounting for the mobility of water around the solute. The higher the compatibility of the hydration of the solute with water structure, the higher is the value of k' as observed for D-glucose. Viscometric constants [η] and k' may be used as an indicator of the extent and stability of hydration of the investigated carbohydrates.

However, in the case of enzyme stabilization, water activity (Aw) seems to be the preponderant parameter. To compare Aw of the different sugars, it was most convenient to compare saturated sugar solutions. Aw and saturation solubilities at 25°C are given in Table 2. It may be observed that the higher the sugar solubility the lower the water activity. D-fructose could than be taken as the best Aw depressor. However, the saturation concentration is the standard state to take into account for comparing the behavior of enzymes in concentrated aqueous solutions.

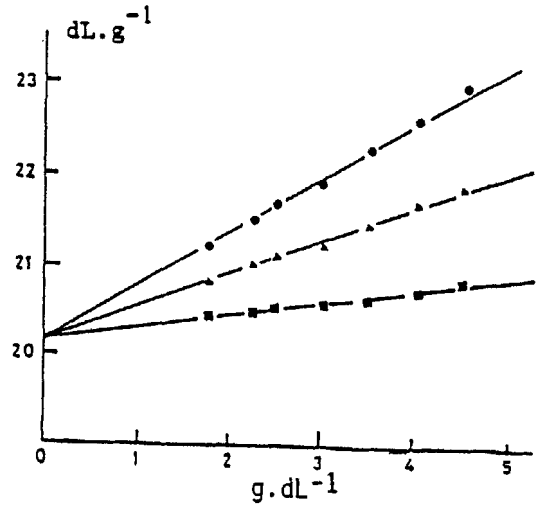

Fig.1. Determination of intrinsic viscosity [η] of D-glucose by extrapolation of η_{sp}/c (●) : $(\ln\eta/\eta_o)/c$ (▲) and η_{diff}/c (■).

Table 1 : Intrinsic viscosity [η] and Huggins constant k' of the sugars and polyols used in enzyme stability experiments.

sugar	$[\eta]x10^3 dL.g^{-1}$	k'
D-Glucose	20.15	1.50
D-Galactose	19.80	1.04
D-Fructose	19.00	1.14
Sorbitol	20.70	1.04
Mannitol	21.35	1.51
Sucrose	20.35	1.19
Maltose	22.50	1.01

Table 2 : Water Activity (Aw) and concentration C(g°/ₒg) of Saturated Aqueous Solutions at 25°C.

Sugar	Aw	C(g°/ₒg)
D-Glucose	0.89	50.6
D-Galactose	0.93	40.0
D-Fructose	0.63	79.8
Sorbitol	0.77	70.1
Mannitol	0.98	18.0
Sucrose	0.86	67.5
maltose	0.95	45.7

It was demonstrated from laser-Raman[11] and X-ray[12] results that water-sugar interactions in aqueous solutions of D-fructose, D-glucose and sucrose show discontinuities at about 1/3 and 2/3 of saturation concentrations. These discontinuities were assigned to the preponderance of water-water, water-sugar and sugar-sugar interactions respectively. In dilute solutions the intrinsic effect of the solute on the solvent may be derived from the Raman Spectra. In order to minimize the effect of the OH stretching from the sugars on the Raman Spectrum of water, only the laser-Raman Spectra of the solutions containing less than 1 % of D-fructose, D-glucose or sucrose were analysed. It was shown[1] that low concentations of D-glucose and sucrose do not provoke a noticeable change in the Raman spectrum of water, whereas an important modification of the Raman intensity is observed for the spectra of D-fructose solutions. This is interpreted as a "structure breaking" effect of traces of D-fructose on water. The chaotropic effect of D-fructose on the structure of water may be derived from the deconvolution of the experimental Raman band of water on the one hand and that of D-fructose, D-glucose and sucrose solutions on the other. The results are shown in fig. 2. Application of a semi empirical[13] method of deconvolution of the Raman bands leads to find four Gaussian components noted a, b, c and d on the spectra.

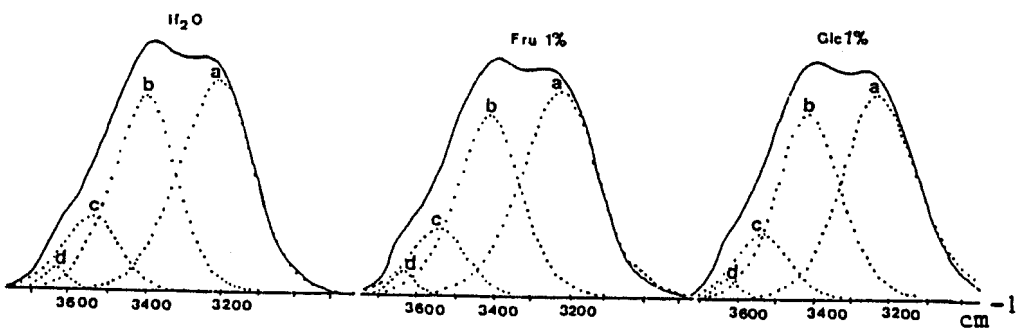

Fig. 2 : Experimental plot (-) and calculated Gaussian components (....) of the Raman spectrum of water and 1 % aqueous solution of D-fructose and D-glucose.

An increase of (d) component, assigned to unassociated H_2O molecules together with a decrease in (c) component, the liquid-like amorphous fraction of water (see Table 3 and 4), is interpreted as evidence of the chaotropic effect of D-fructose. D-glucose and sucrose do not contribute to increase the mobility of water as no modification is observed in the vibrational energy of unassociated molecules [component(d)] for both sugars (see Table 5 for D-glucose). The most sensitive component to the structure breaker effect is the one which reveals the existence of free H_2O molecules localized at 3638 cm^{-1}. The difference in chaotropic action between D-fructose on the one hand and sucrose and D-glucose on the other is more important when the concentration is lowered to 10^{-6} (w/w) (1).

These results agree with previous work[14] on the depolarisation ratios of Raman bands of H_2O and dilute solutions of D-fructose, D-glucose and sucrose. They lead to the conclusion that D-glucose and sucrose have a structure making effect on water, whereas D-fructose acts as a structure breaker.

The effect of sugars on water structure should be considered when other properties like water activity (Aw) depression are interpreted. It is expected from the Aw values, that D-fructose which contributes to the largest Aw depression should be a good enzyme protector during the storage. However, the effect of this sugar on water structure explains why Aw depression cannot be taken as the sole parameter for enzyme preservation.

Enzyme Stability in Sugar Solutions.- The muramidase activity of egg-white lysozyme was measured in aqueous solutions of carbohydrates with different Aw values. The results are represented in Fig. 3. It may be observed that the initial enzyme activity increases with Aw. A hyperbolic relation between enzyme activity and water activity is obtained when the reaction medium is composed of buffer, or aqueous solutions of D-glucose, sucrose or sorbitol. However, when Aw depressing results from the addition of D-fructose in the medium, initial enzyme activity is lowered (see Fig. 3). This special behavior in

Table 3 : Position of maximum (ν), intensity (I), width, area and assignments of the Gaussian components of the Raman band of water.

Component	ν/cm^{-1}	I	width	area	total area(%)	assignments
(a)	3223	268	188	50384	48.18	quasi-cristalline
(b)	3413	248	159	39432	37.70	solid-like amorphous
(c)	3547	94	140	13160	12.58	liquid-like amorphous
(d)	3638	27	59	1593	1.59	unassociated H_2O, free OH
total area				104569	100	

Table 4 : Position of maximum (ν), intensity (I), width, area, shifts in frequencies ($\Delta\nu$) and variation of area ($\Delta a\%$) for the Gaussian components of water with 1 % D-fructose.

Component	ν/cm^{-1}	I	width	area	total area (%)	$\Delta\nu/cm^{-1}$	$\Delta a(\%)$
(a)	3228.3	259	193	49968	49.90	+5.3	-0.78
(b)	3417.4	228	162	36936	36.87	+4.4	-6.30
(c)	3547	84	138	11592	11.57	0	-11.90
(d)	3638	28	59	1652	1.64	0	+ 3.70
	Total area			100148			- 4.20

Table 5 : Position of maximum (ν), intensity (I), width, area, shifts in frequencies ($\Delta\nu$) and variation of area ($\Delta a\%$) for the Gaussian components of water with 1 % D-Glucose.

Component	ν/cm^{-1}	I	width	area	total area (%)	$\Delta\nu/cm^{-1}$	$\Delta a(\%)$
(a)	3229	256	188	48128	48.72	+6	-4.48
(b)	3418	233	162	37746	38.21	+5	-4.27
(c)	3547	82	138	11316	11.45	0	-14.0
(d)	3638	27	59	1593	1.61	0	0
				98783			-5.53

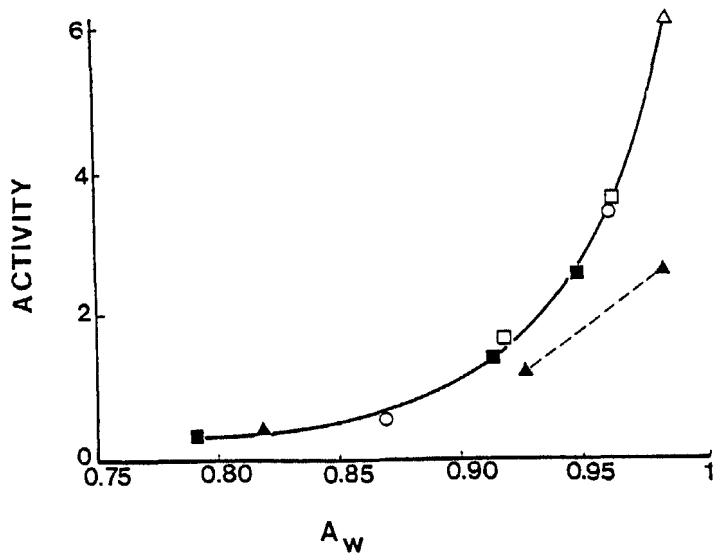

Fig. 3 : Initial lysosyme activity as a function of water activity (Aw) of the reaction medium adjusted with sucrose (o), sorbitol (■), D-glucose (□) or D-fructose (▲), and in the buffer (△).

D-fructose solutions seems to be in relation with the effect of the solute on water structure. Moreover, the role of water mobility induced by the sugar additive on enzyme activity is not only observed when this activity is measured in the reaction medium containing the additive, but also after a storage of the enzyme in a carbohydrate solution.[15]

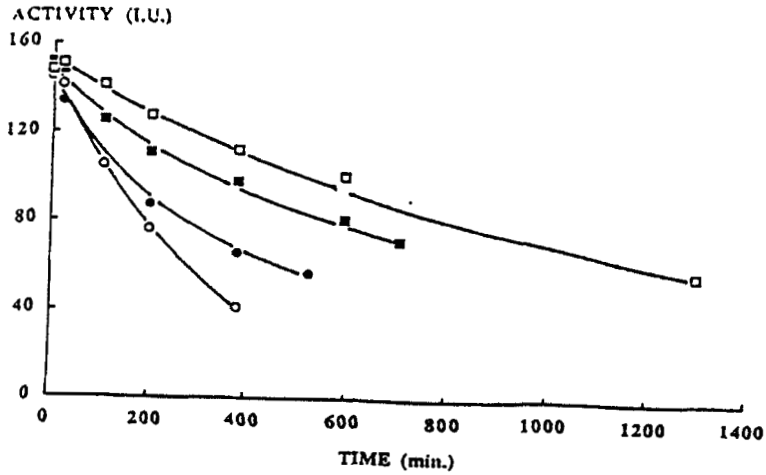

Fig. 4 : Residual activity of YADH as a function of incubation time in buffer (●), D-glucose (■), D-fructose (○) and sucrose (□) solutions with 0.9 M concentration.

Carbohydrates were also used to preserve YADH activity against time denaturation. D-fructose, D-glucose and sucrose were added to the enzyme storage medium and enzyme stability measured. Evolution of enzyme activity, after a storage at 25°C in the buffer or in presence of each of the three sugars, is represented in Fig. 4.

It may be observed that while D-glucose and sucrose exhibit a protective effect, D-fructose shows a destabilizing action on the enzyme. For sugars acting as enzyme protectors, initial activity of YADH is increased[9] by 10 %.

Comparison of the action of D-glucose and sucrose on the one hand, and D-fructose on the other, on enzyme stability may be correlated with their solute-solvent interactions in the aqueous medium. Indeed, the low intrinsic viscosity [η] of D-fructose, its high solubility and its chaotropic effect on water structure derived from Raman spectra deconvolution, show that although water activity depression is achieved with this sugar, the modification of hydration and conformation of the enzyme in the presence of this additive do not fit with an improved enzyme activity. The structure making effect of the other

Table 6 : Half-life time ($t_{1/2}$) of YADH in presence of different carbohydrate additives with 0.9 M concentration in the storage medium

Storage Medium	$t_{1/2}$ (min.)
buffer	320
D-Glucose	660
D-Galactose	612
D-Fructose	273
Sorbitol	1040
Mannitol	1874
Sucrose	1010
Maltose	683

sugars on water structure, seems to fit with the more active conformation and with the stabilizing type of hydration of the enzyme.

Specific effect of small carbohydrates on enzyme stability may also be derived from half-life time ($t_{1/2}$). Results are given in Table 6. Comparison of the effect of monosaccharides on half-life time of YADH show a destabilizing effect of D-fructose, while D-glucose and D-galactose contribute to double $t_{1/2}$ as compared to a storage in the buffer medium. Although disaccharides maltose and sucrose may be considered as stabilizers, their effects on $t_{1/2}$ are different probably because of their different interactions with water. The more striking result is that of the large difference in $t_{1/2}$ values when hexitols are added with the same concentration to the storage medium of YADH. The hexitol which depresses Aw to lower values, i.e., sorbitol, does not show the better enhancement of half-life time for YADH. Retention of water around the sugar or polyol molecules, which is at the origin of water activity lowering, does not occur with the same procedure.

Depending on whether, the solute presents to the solvent its hydrophobic or its hydrophilic side, retention of water occurs differently. This is more critical for alditols as they may have different conformations[16] depending on the axes and centers of symmetry of the molecule.

CONCLUSION

Dilute solution conditions are not needed for lysozyme activity to occur. It is only necessary to assure a hydration level of about 0.2 water/g protein. This hydration level allows flexibility in the helical region, which contributes to restoring the active conformation.[17] When Aw is obtained with a sugar, like D-fructose, which increases water mobility , the nature of the hydration of the enzyme is perturbed, hence its conformation and this may contribute to a decrease of initial activity.

Storage stability of YADH is also influenced by the mobility of water. When this enzyme is stored in different carbohydrates and polyols, the effect of D-fructose on half-life time is negative, probably because of the perturbation of water structure. Moreover, for polyols, one should also consider the conformation of the additive itself if it is desired to explain the effect of the polyol on storage stability of the enzyme.

EXPERIMENTAL

The sugars and polyols studied are Sigma products. Distilled water was used for the preparation of the solutions used in viscosity measurements. Viscosity results were derived from the time necessary for a given volume to flow through a capillary at constant temperature of 25 ± 0.02°C in a semi-automatic Schott AVS 400 viscometer. The intrinsic viscosity $[\eta]$ is obtained from the triple extrapolation of the reduced specific viscosity ($\eta_{sp}/c = (\eta-\eta_o/\eta_o c)$, the inherent viscosity $[\ln (\eta/\eta_o)/c]$ and the reduced differential viscosity $[\eta_{diff} = (\eta-\eta_o)/\eta c]$ towards c=o, where η and η_o are, respectively the viscosities of the solution and the solvent and c the concentration in $g.cm^{-3}$. The Huggins constant k' was derived from Huggins' relation :

$$\eta_{sp}/c = [\eta] + k' [\eta]^2 c + \ldots$$

Raman spectra were recorded according to a method previously described,[11] and the procedure of deconvolution of Raman bands of water was published[13] elsewhere.

Egg-white lysozyme (Sigma, L6876) was used as a model hydrolase. A concentrated solution (25 mg. mL^{-1}) was prepared in citrate-phosphate buffer pH = 6.2. Sugars and polyols were solubilized in the buffer. Water activity was measured with a Novasina unit equipped with an En BS4 Sensor. Microcus lysodeikticus suspensions containing 0.5 g dried cells per mL. were prepared in the buffer or the carbohydrate solution. 20 µL of the enzyme solution was added to 2.5 mL of the bacterial suspension and the muramidase activity of lysosyme measured by following the O.D. decrease at 450 nm. The reaction was carried out at 20°C and enzyme activity expressed in mg. min^{-1}. of hydrolyzed cells per mg of lysozyme.

Yeast alcohol dehydrogenase (E.C.1.1.1.1., Sigma) 0.1 $mg.ml^{-1}$ was stored at 25 or 40°C in veronal buffer (0.04 M sodium phenolbarbital, pH 8.5) or in the buffer with 0.9 M concentration of sugar or polyol. The residual activity in an aliquote (25 µL) of enzyme solution was measured in 3 mL of reaction medium as a function of incubation time. The medium contained ethanol (0.1 M) and NAD (3.6 mM) in pyrophosphate buffer (0.06 M, pH = 8.5). Semicarbazide hydrochloride (0.06 M) and glycine (0.02 M) were used to draw the reaction to the direction of ethanol oxidation and NAD reduction. The kinetics of YADH was measured through the spectrophotometric absorbance of the produced NADH at 340 nm using ε_{340} = 5.8 $\mu mole^{-1}.cm^{2}$. Stability was expressed from half-life time ($t_{1/2}$) obtained when the enzyme exhibits the half of its initial activity.

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