Biophysical Chemistry, 36 (1990) 71–76
Elsevier

BIOCHE 01434

Modulation of egg-white lysozyme activity by viscosity intensifier additives

Louis Lamy [a], Marie-Odile Portmann [b], Mohamed Mathlouthi [b] and Véronique Larreta-Garde [a]

[a] *Laboratoire de Technologie Enzymatique, Université de Compiègne, BP 649, 60206 Compiègne Cedex France and* [b] *Laboratoire de Chimie Physique Industrielle, Université de Reims, BP 347, 51062 Reims Cedex, France*

Received 13 October 1989
Revised manuscript received 4 December 1989
Accepted 5 December 1989

Enzyme activity; Lysozyme; Viscosity; Water affinity

The activity of egg-white lysozyme was measured in the presence of carbohydrate additives in the reaction medium. These additives show a significant affinity for water. They depress water activity and increase the viscosity of the medium. Solute-solvent interactions in aqueous solutions of the additives are characterized by properties such as the intrinsic viscosity, Huggins constant apparent molar volume and hydration number. It was found that, despite the lowering of enzyme activity when the concentration of additive is increased, the behavior remains Michaelian and neither modification of K_m nor inhibition by excess substrate is observed. On the other hand, the effect of the viscosity of the medium on enzyme activity was determined. This effect is independent of the nature of the additive at high viscosities (> 4 mPa s^{-1}) for which enzyme activity is very low and appears to vary according to the kind of additive in dilute solution at low viscosities (< 2 mPa s^{-1}).

1. Introduction

It was recently [1] pointed out that enzymatic events are highly sensitive to physical and biochemical modifications of their microenvironment. In particular, the nature and state of the solvent greatly influence the reactivity of enzymatic proteins [2]. Small carbohydrates were found [3,4] to induce significant modifications of the water structure on variation of the concentration. When such molecules are used as additives in an enzymatic reaction medium, they are able to modulate enzyme activity [5] and stability [6–8], and also to increase the viscosity of the medium.

The effect of viscosity on enzyme activity has not yet been completely elucidated. Most of the work in this area was performed on invertase dissolved in concentrated aqueous solutions of sucrose [9,10]. Depending on the range of viscosity considered, conflicting effects of the viscosity on enzyme activity were described. For invertase, sucrose can function simultaneously as both substrate and viscosity intensifier, which allows one to approach the problem of whether the inhibition at high concentration is due to viscosity or the result of excess substrate.

We now present results of a systematic study of the effect of viscosity of the reaction medium induced by fructose, glucose, sucrose or sorbitol on the activity of lysozyme. Egg-white lysozyme was chosen as a model enzyme because the carbohydrate additives do not act as substrates for this enzyme. Its usual substrate is a whole cell (*Micrococcus lysodeikticus*) and this should contribute to amplifying the phenomenon of diffusion. In order to interpret the role of each of the

Correspondence address: V. Larreta-Garde, Laboratoire de Technologie Enzymatique, Université de Compiègne, BP 649, 60206 Compiègne Cedex, France.

additives with respect to the enzyme activity, their physico-chemical properties (intrinsic viscosity, apparent molar volume and hydration number) were determined.

2. Experimental

2.1. Catalytic activity

The additives used (fructose, glucose, sucrose and sorbitol) were obtained from Sigma. Solutions of additives were prepared in citrate phosphate buffer (100 mM, pH 6.2) at 25 °C to obtain final concentrations ranging from 0 to 5 M; sugar solubility permitting. 20–80 μl of substrate containing 10 or 30 mg of dried *M. lysodeikticus* cells/ml (Sigma M3770) were added in 3 ml of sugar solution. 50 μl of enzyme solution at 25 mg ml^{-1} was added to the reaction medium. Muramidase activity of lysozyme was assayed spectrophotometrically by determining the decrease in absorbance at 450 nm (A_{450}). The reaction was carried out at 25 °C and enzyme activity expressed in mg dried cells min^{-1} mg^{-1} lysozyme.

3. Solution properties of additives

3.1. Intrinsic viscosity [η] and Huggins constant k'

The viscometric constants [η] and k' were derived from the time necessary for a given volume to flow through a capillary at a constant temperature of 25 ± 0.02 °C in a semi-automatic Schott-AVS 400 viscosimeter.

The intrinsic viscosity [η] was obtained from triple extrapolation [11] of the reduced specific viscosity $[\eta_{sp/c} = (\eta d - \eta_0 d_0)/\eta_0 d_0 C]$, the inherent viscosity $[\ln(\eta d/\eta_0 d_0)/C]$ and the reduced differential viscosity $[\eta \text{ diff}/c = (\eta d - \eta_0 d_0)/\eta dC]$ towards $C = 0$ where η, η_0 and d, d_0 denote the viscosity and density of solution and solvent, respectively, and C the concentration in g dl^{-1}.

The Huggins constant k' was derived from Huggins' relations [12].

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

Apparent molar volume

The apparent volume of the studied sugars in dilute aqueous solutions is expressed by the relation:

$$V_{app} = \frac{1}{d} - \frac{(d - d_0)}{Cd_0}$$

where d and d_0 are the densities of the solution and solvent, respectively, C being the concentration in g ml^{-1}.

Extrapolation of $V_{app} = f(C)$ towards $C = 0$ permits determination of $\overline{V_0^2}$, the specific partial volume. The apparent molar volume, $\Phi(V_0)^2$, may then be calculated as the product of $\overline{V_0^2}$ multiplied by the molecular weight M:

$$\Phi(V_0)^2 = \overline{V_0^2} M$$

3.3. Viscosity coefficient B

For dilute solutions of small molecules the relation frequently used is that of Jones and Dole [13].

$$\eta_{ref} = \eta/\eta_0 = 1 + BC + DC^2$$

which may be written as:

$$\eta_{sp/c} = B + DC$$

where B is comparable to [η] and D to $k[\eta]^2$. B and D are determined graphically after plotting $\eta_{sp/c}$ (dm^3 mol^{-1}) as a function of C (mol dm^{-3}). The experimental viscosity coefficient B accounts for the overall hydrodynamic volume of the solute. This involves a component B_{size} accounting for the size of the solute [14]:

$$B_{size} = 2.5\Phi(V_0)^2/1000$$

and a component $B_{str} = B - B_{size}$, accounting for the effect of solute on the solvent structure.

3.4. Hydration number

Several methods are used to determine the hydration number (n) of sugars in aqueous solution. We applied procedures based on molar volumes, as given by Herkovits and Kelly [15].

$$n = (1000B/2.5 - M_2\overline{V_2})/M_1\overline{V_1}$$

where $M_1\overline{V_1}$ and $M_2\overline{V_2}$ denote the apparent molar volume of the solvent (water) and solute, respectively, and B the viscosity coefficient.

3.5. Viscosity of concentrated solutions

The dynamic viscosity of concentrated sugar solutions was measured at 25 °C using a Couette-type viscometer supplied by Contraves. This viscometer was a Rheomat 115 equipped with an MS01 cell. Dynamic viscosity of sugar solutions is expressed in mPa s^{-1}.

4. Results and discussion

In order to study the kinetic effect of additive concentration on enzyme activity, sorbitol was chosen as a model for additives. The activity of egg-white lysozyme in the presence of varied concentrations of sorbitol in the reaction medium is shownn in fig. 1. It may be seen from these results that the higher the additive concentration the lower is the enzyme activity. However, at all sorbitol concentrations tested, the enzyme kinetics continued to show Michaelian behavior. Neither change in the K_m value nor inhibition due to excess substrate was observed. Increased additive concentrations do not appear to modify the kinetic behavior of lysozyme, whereas invertase shows

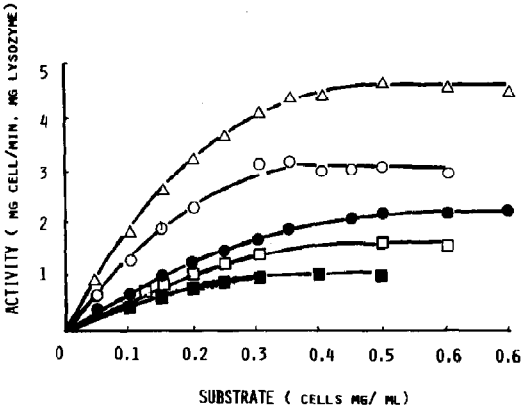

Fig. 1. Effect of sorbitol concentration on the Michaelian kinetics of lysozyme. (△) reference without sorbitol, (○) 0.23 M, (●) 0.45 M, (□) 0.93 M and (■) 1.4 M sorbitol.

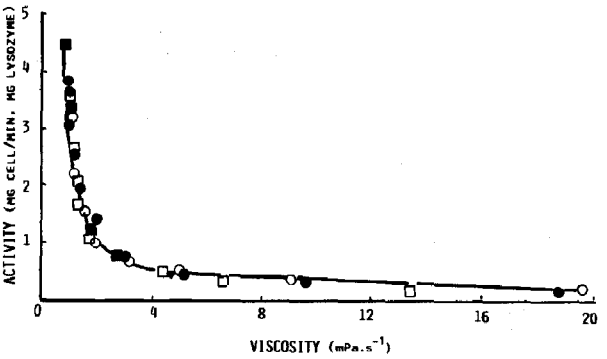

Fig. 2. Evolution of enzyme activity as a function of viscosity of the medium obtained with (■) glucose, (□) fructose, (●) sucrose or (○) sorbitol.

significant inhibition due to excess substrate in the presence of concentrated aqueous sucrose solutions [16]. The invariance of K_m shows that the enzymatic reaction in the presence of additives even at high concentration is not diffusion-controlled. It is then possible to compare its activity in the presence of different additives contributing to the increase in viscosity of the reaction medium.

We recently [17,18] discussed the relation between lysozyme activity and its degree of hydration. It was shown that the organization of water around the enzyme and the sugar molecules in the reaction medium play major roles in the enzymatic activity. Lysozyme catalysis was found [17] to increase exponentially with the water activity of the reaction medium. A thermodynamic parameter may thus control enzyme activity.

In order to determine whether a physicochemical parameter may also be of importance, enzyme activity was measured as a function of the viscosity of the reaction medium which was modified by addition of small carbohydrates. This correlation is shown in fig. 2. Enzyme activity appears to be very sensitive to the viscosity of the medium, especially on attaining a threshold value of 2–4 mPa s^{-1}. Indeed, fig. 2 shows a curve with two asymptotic parts to the activity and viscosity axis, respectively.

For viscosities above 4 mPa s^{-1}, very low enzyme activity was observed irrespective of the additive present in the medium and its concentration. It should be noted that for such viscosities,

the concentration of sugars ranged from 2.5 to 4 M and water activity from 0.8 to 0.9, a range over which lysozyme activity was found to increase rapidly [17]. In the range of high viscosity, the chemical structures and conformations of the saccharides do not seem to affect the initial lysozyme activity. Under these conditions, a macromolecular effect governs the reaction, the influence of solvent appearing to be rate determining whatever the medium composition. Water molecules in the medium are involved firstly in the hydration of additive, substrate and enzyme and are no longer available for the enzymatic reaction path.

For viscosities below the threshold of about 2 mPa s^{-1} a slight modification of the composition of the medium induces a marked change in catalytic activity of the enzyme (see fig. 2). The enzymatic response appears to depend not only on additive concentration, but also on the nature of the saccharide. For example, for the same concentration 0.5 M, lysozyme activity has a value of 2.3 or 3.4 units when the additive is sorbitol or glucose. The effect of viscosity on invertase activity depends on the nature of the viscosigen additive even at high concentration [9]. An increase in the concentration of carboxymethylcellulose or sucrose evokes a decrease in enzyme activity, the effect of sucrose being more significant probably because it is the result of the combination of a viscosity effect and inhibition by excess substrate as described recently [10].

It should be borne in mind that for lysozyme, a common relation was determined for three different sugars and a polyol with different chemical structure, none of which was a substrate for the enzyme. In order to interpret the subtle influence

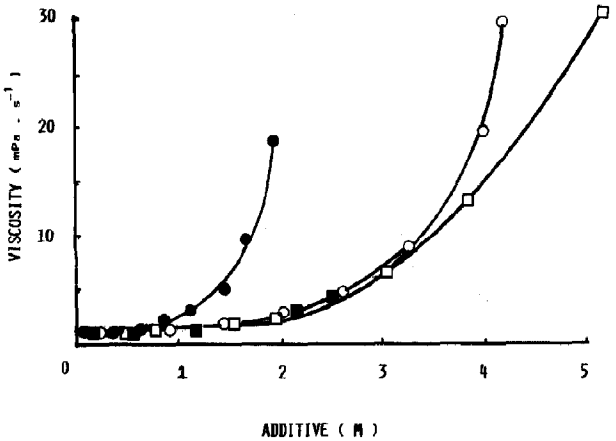

Fig. 3. Dynamic viscosity of aqueous solutions of (■) glucose, (□) fructose, (●) sucrose or (○) sorbitol.

on enzyme activity of additive at low concentration, knowledge of its solution properties is needed. The first of these factors is the viscosity of aqueous solutions. Fig. 3 shows variations in viscosity plotted vs concentration for fructose, glucose, sucrose and sorbitol. It may be observed that fructose is the lowest viscosigen, sucrose the highest, and glucose and sorbitol show approximately equal values when comparison is made between identical concentrations.

For dilute solutions at concentrations lower than 1 M, no difference was observed in the viscosity of aqueous solutions of the four additives (fig. 3). In this concentration range, the specificity of the saccharides influences enzyme activity. Interpretation of the data on the activity requires the determination of specific physico-chemical properties of the additives in dilute solutions. The

Table 1

Enzymatic activity in 0.5 M sugar, viscometric constants, molar volume and hydration number of fructose, glucose, sucrose and sorbitol

Sugar	Activity in 0.5 M sugar	$[\eta]$ (dl g^{-1}) ($\times 10^3$)	k'	B	B_{size}	B_{str}	$\Phi(V_0)^2$ (ml mol^{-1})	n
Fructose	3.6	23.40	0.75	0.41	0.27	0.14	108.8	3.24
Glucose	3.4	24.30	0.64	0.43	0.28	0.15	112.8	3.45
Sucrose	2.5	24.65	0.95	0.84	0.52	0.31	210.2	7.03
Sorbitol	2.3	23.85	0.94	0.43	0.30	0.13	119.2	2.98

parameters determined mainly concern the degree of hydration of the molecules. These are summarized in table 1. Intrinsic viscosity $[\eta]$ is generally considered as a shape factor accounting for the hydrodynamic diameter of the solvated molecule. Due to their hydrophilic hydration and similar shape, the value of $[\eta]$ is comparable for the studied saccharides.

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 solute with water structure, the higher is the value of k'. The viscosity coefficient B accounts for the overall hydrodynamic volume of the solute, its components B_{size} and B_{str} being related to the size of the solute and its effect on water structure. While the $[\eta]$ values, expressed in volume per g are comparable, values of B expressed in volume per mol show a difference between monosaccharides and the disaccharide sucrose. The apparent molar volume $\Phi(V_0^2)$, apart from differentiating between monosaccharides and the studied disaccharide, is a good indicator of the difference between linear polyol, sorbitol and ring-shaped molecules, glucose and fructose. Values of the apparent molar volumes and hydration numbers are in good agreement with those reported by Miyajima et al. [14].

Comparison of enzyme activity in the case of low sugar concentration (0.5 M) in the medium is also reported in table 1. The lowest activity is observed with sorbitol in the reaction medium. This is probably due to the conformation of sorbitol which is incompatible with the tetrahedral organization of water molecules [19]. The linear form of the sorbitol molecule is capable of folding in dilute solution, which forms the basis for the increase in size of the hydrated solute as may be deduced from the values of B_{size} and $\Phi(V_0^2)$. The hydration of the other carbohydrates investigated is of the same nature. As mentioned previously [18], fructose increases water mobility and this 'structure breaking' effect may reduce the stability of the enzyme in the medium used for preservation. In dilute solutions, without storage before reaction, fructose and glucose seem to have the same effect on the initial enzyme activity. The difference with sucrose probably arises from a difference in the water activity of the medium. Indeed, 0.5 M glucose or fructose corresponds to half the mass concentration of 0.5 M sucrose, which signifies that the water activity of the sucrose solution is lower than that of glucose or fructose. The effect of water activity on enzyme activity was studied [18] and a rapid decrease in enzyme activity was found to occur on decrease in the water activity.

A number of mechanisms were proposed to explain the relationships between the solution properties of the reaction medium and enzymatic behavior [17,20]. Among these, we may also include additive enzyme interactions, electrostatic interactions, diffusion control, exclusion effects, collisions and water availability. Bowski et al. [16] demonstrated the effects of substrate diffusion, solution viscosity, water concentration and substrate inhibition on invertase activity. Hinsch and Kula [20] described relationships between the collisional activation mechanism and exclusion effects on the one hand, and Michaelis constant on the other. The influence of viscosity intensifier additives cannot be generalized for application to all enzymes as some of the viscosigens employed can also function as substrates for certain types of enzymes.

Our results show that differentiation should be made between low and high viscosities of the medium. The threshold value lies at about 2–4 mPa s^{-1}. Above this value, solvent molecules are used for hydration and are thus no longer available for enzymatic reaction. Enzyme activity remains very low irrespective of the nature and concentration of the additives. Furthermore, this decrease in enzyme activity cannot be attributed to increased diffusion, even for a high molecular weight substrate such as a whole cell. At low concentrations, for viscosity below 2 mPa s^{-1}, the specific effect of each additive and its influence on enzyme activity are evident; it decreases as the viscosity increases. It may be interpreted by reference to its solution properties, particularly intrinsic viscosity, apparent molar volume and hydration number.

References

1 J. Watterson, Biochem. J. 248 (1987) 615.
2 A. Klibanov, Trends Biochem. Sci. 14 (1989) 141.
3 M. Mathlouthi, C. Luu, A.M. Meffroy-Biget and D.V. Luu, Carbohydr. Res. 81 (1980) 213.
4 M. Mathlouthi, Carbohydr. Res. 91 (1981) 113.
5 R. Drapron, in: Properties of water in foods, eds. D. Simatos and J.L. Multon, (Martinus Nijhoff, Dordrecht, 1985) p. 171.
6 K. Gekko and S. Timasheff, Biochemistry 20 (1981) 4667.
7 P. Monsan and D. Combes, Ann. N.Y. Acad. Sci. 434 (1984) 48.
8 E. Anderson and B. Hahn-Hagerdal, Biochim. Biophys. Acta 912 (1987) 317.
9 B.C. de Almeida Cunha and M. Vitolo, Biotechnol. Bioeng. 26 (1984) 811.
10 D. Combes and P. Monsan, Carbohydr. Res. 117 (1983) 215.
11 M. Mathlouthi and A.M. Seuvre, J. Chem. Soc. Faraday Trans. 1, 84 (1988) 2641.
12 M. Huggins, J. Am. Chem. Soc. 64 (1942) 2716.
13 G. Jones and M. Dole, J. Am. Chem. Soc. 51 (1929) 2950.
14 K. Miyajima, M. Sawada and N. Nakagaki, Bull. Chem. Soc. Jap. 56 (1983) 1954.
15 T. Herkovits and M. Kelly, J. Phys. Chem. 77 (1973) 381.
16 L. Bowski, R. Saini, D.Y. Ryu and W. Vieth, Biotechnol. Bioeng. 23 (1971) 641.
17 V. Larreta-Garde, Z.F. Xu, L. Lamy, M. Mathlouthi and D. Thomas, Biochem. Biophys. Res. Commun. 155 (1988) 816.
18 M. Mathlouthi, V. Larreta-Garde, Z.F. Xu and D. Thomas, J. Carbohydr. Chem. 8 (1989) 233.
19 R. Jasra and J. Ahluwalia, J. Solution Chem. 11 (1982) 325.
20 B. Hinsch and M.R. Kula, Biochim. Biophys. Acta 525 (1978) 392.