Reaction Rate Modeling in Cryoconcentrated Solutions: Alkaline Phosphatase Catalyzed DNPP Hydrolysis

Dominique Champion,* Genevieve Blond, Martine Le Meste, and Denise Simatos

Laboratoire de Biochimie, Physico-chimie et Propriétés Sensorielles des Aliments, ENSBANA, Université de Bourgogne, 21000 Dijon, France

The hydrolysis of disodium *p*-nitrophenyl phosphate catalyzed by alkaline phosphatase was chosen as a model to study the kinetics of changes in frozen food products. The initial reaction rate was determined in concentrated sucrose solutions down to −24 °C, and the enzymatic characteristics K_M and V_{max} were calculated. The experimental data were compared to the kinetics predicted by assuming that the reaction was viscosity dependent. Indeed, an analysis of the enzymatic reaction demonstrated that both the diffusion of the substrate and the flexibility of the enzyme segments were controlled by the high viscosity of the media. When the temperature was too low for the viscosity to be measured simply, the Williams−Landel−Ferry equation was used to predict the viscosity, taking, as reference temperature, the glass transition temperature (T_g) corresponding to the concentration of the freeze-concentrated phase at the test temperature. Predicted values of the reaction rate were very close to the experimental ones in the studied temperature range.

Keywords: *Sucrose; enzymatic reaction; viscosity; glass transition; diffusion-controlled reaction; prediction*

INTRODUCTION

One common way to ensure the quality of food products is storage at low temperatures, with chilling or freezing processes. Freezing is generally used for long-term food preservation, and it consists of a reduction in temperature to −18 °C or below. However, although most of deteriorative reactions are slowed with a decrease in temperature, they are not stopped by this storage method. Indeed, irreversible biochemical reactions, for instance, may occur in frozen products and have been widely described in the literature (Maier et al., 1955; Parducci and Fennema, 1978, 1979; Vajda, 1993; Ullmann et al., 1997). Lipid oxidation and enzymatic reactions lead to the development of off-flavor and off-color in the products (Hall and Alcock, 1987). In cellular conditions, components are localized at definite positions. However, on freezing, cellular structure disruption results in the meeting of certain reactive molecules, enzymes with their substrates and/or activators, allowing chemical or enzyme reactions in the frozen state, and they are further enhanced upon thawing of the food. Most often, the rate of these reactions exhibits a very strong temperature effect (Simatos and Blond, 1991).

Under these considerations, frozen foods represent an especially interesting problem in terms of the effects of changes in mobility due to cell damage and the freeze concentration of the liquid phase. During freezing of cellular suspensions, tissues, or aqueous solutions, a part of the water is converted to ice crystals, leaving the constituents of the solution concentrated in a much reduced quantity of unfrozen water. This unfrozen phase may exist as either a very viscous supercooled liquid or a glass. The change from the glassy to the liquid state occurs at a critical temperature known as the glass transition temperature (T_g). The main consequence of the glass−liquid transition is an abrupt decrease in viscosity, which may result in the increase of solute mobility inducing deteriorative changes. Once the system is above its T_g, the viscosity of the unfrozen phase continues to decrease, in a non-Arrhenian way; it has been suggested that the drastic decrease in viscosity as the temperature increases could be responsible for the strong temperature effect mentioned above (Levine and Slade, 1988; Simatos and Blond, 1991). The enzyme reactions in such freeze-concentrated systems are considered to be diffusion controlled (Kerr et al., 1993), the reaction rate being limited by the mobility of either substrate, enzyme, or even the enzyme segments (Karel et al., 1993; Champion et al., 1997a).

The objective of this work was to determine whether the strong temperature effect can be explained (and predicted) on the basis of changes in molecular mobility. The enzymatic reaction catalyzed by the alkaline phosphatase was studied as an example of diffusion-controlled reaction in a frozen food model: sucrose solutions. Simopoulos and Jencks (1994) have demonstrated that the reaction catalyzed by the alkaline phosphatase is diffusion controlled in viscous sucrose solutions at 25 °C; these authors qualified this enzyme as an "almost perfect enzyme", which was not destabilized by the presence of sucrose. Douzou (1977) had reported that the diffusion behavior in frozen systems can be altered because the viscosity is increased by ice formation much more than would be expected from the relationship between viscosity and temperature when no phase change occurs.

Parker and Ring (1995) proposed a model to predict the chemical reaction rate in a frozen system in relation to the effects of the temperature and the concentration of reaction medium, on the basis of the Atkins theory

* Author to whom correspondence should be addressed [e-mail dchamp@u-bourgogne.fr; fax 33(0)380396647].

10.1021/jf000457s CCC: $19.00 © 2000 American Chemical Society
Published on Web 08/31/2000

(Atkins, 1998) for diffusion-controlled reactions. In a sucrose solution, the cryoconcentration of the reactants induces an increase of the reaction rate, with a maximum between −2 and −3 °C when the initial sucrose concentration is <20%. When the initial sucrose concentration is higher, or at lower temperatures (from −16.5 to −40 °C), a decrease in the reaction rate is induced by a decrease in the temperature and an increase in the cryoconcentrated phase viscosity. Only the influence of the reactant mobility was taken into account in their work for the calculation of the predicted rate constant.

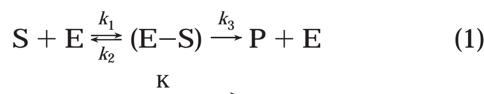
In the case of an enzymatic reaction, however, the freezing process can disturb not only the reactant mobility but also the transformation into product, and the decrease in the reaction rate may reflect the temperature influence on both. The enzyme efficiency can also be changed due to some modifications in the reaction medium induced by low temperatures. Indeed, according to Fennema (Fennema, 1975; Parducci and Fennema, 1979) changes in buffer concentration and composition cause changes in pH. pH as well as ionic strength changes could lead to subunit dissociation and reassociation of oligomeric proteins such as enzymes. Tappel (1966) has also demonstrated that many of the common solutes, such as salt buffers, sugars, and other carbohydrates, are effective inhibitors of enzymatic reactions at high concentrations. Another factor involved in enzyme inactivation at freezing temperatures is the perturbation of sulfhydryl groups essential for the activity of some enzymes (Ashie et al., 1996). For the present study, the assay conditions were chosen so as to limit the perturbation in the enzyme activity caused by changes in pH or ionic strength.

First, the enzymatic characteristics K_M (the Michaelis constant) and k_3 (also called k_{cat}) were explored as a function of the high concentrations of the reaction medium and of the low temperatures. Second, the reaction velocity was studied as a function of temperature. The viscosity effect on the reaction medium was then investigated, and its contribution to both reactant diffusion and enzyme catalytic efficiency (k_{cat}) is discussed.

BACKGROUND

The reaction rate equation for enzymatic reactions is inherently nonlinear, so the relation of kinetic parameters as a function of time cannot be expressed analytically. Some analytical relationships have thus been developed by appropriate approximations of the rate equation and used to qualitatively characterize the kinetic behavior.

A reaction of the simplest Michaelis−Menten (Michaelis and Menten, 1913) type was considered for the approximation of the studied reaction:



$$S + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (E{-}S) \xrightarrow{k_3} P + E \quad (1)$$

E, S, E−S, and P represent the enzyme, substrate, enzyme−substrate complex, and product, respectively. k_1, k_2, and k_3 are the reaction rate constants corresponding to the enzyme−substrate association, the enzyme−substrate dissociation, and the transformation into product (also called k_{cat} for catalytic constant) constants, respectively. The global reaction rate constant K can be calculated from the experimental determination of the initial velocity v_0:

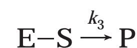
$$v_0 = K[S][E] \quad (2)$$

Indeed, the study of v_0 allows one to neglect the inverse reaction, which can be induced by the presence of a high quantity of the product P. From a theoretical point of view, the initial velocity v_0 can also be expressed in the well-known steady-state expression established by Michaelis and Menten:

$$v_0 = V_{max}[S]/(K_M + [S]) \quad (3)$$

V_{max} denotes the maximum velocity and K_M the Michaelis constant, which represents the inverse of the affinity of the enzyme for the substrate. The values of K_M and V_{max} were experimentally evaluated by determining the initial velocity, v_0, for various substrate concentrations.

In the model established by Michaelis and Menten (eq 1), the reaction can be divided into two steps: the first one corresponds to the diffusion of the molecules toward each other and the formation of the enzyme−substrate complex, which is controlled by the affinity between the two molecules; the second one describes the transformation of substrate into product. The translational mobility of the reactant molecules influences the first step of the reaction and can be a limiting factor of the reaction, especially when the reaction medium is viscous or when the two molecules are separately distributed. However, we have to consider that the viscosity of the medium can also influence the second step of the reaction in decreasing the enzyme flexibility. Taking into consideration the second part of the expression in eq 1 for the enzymatic reaction



$$E{-}S \xrightarrow{k_3} P$$

the catalysis rate constant (k_3) can be calculated using the measured V_{max} and the initial enzyme concentration. Indeed, V_{max} can be measured when all of the enzyme molecules are linked to substrate molecules. Then, the enzyme−substrate complex concentration is considered to be equal to the enzyme concentration as suggested by the Michaelis and Menten model and k_3 is determined with the relation

$$V_{max} = k_3[E] \quad (4)$$

MATERIALS AND METHODS

Sucrose Solutions. Coarse-grained sucrose was dissolved in distilled water. The purity of sucrose solutions was checked by liquid-phase chromatography. As no impurity was detected by this method, the coarse-grained sucrose was used without further purification. The sucrose was just dissolved under agitation at different concentrations from 30 to 65.3% (w/w) in distilled water; the concentrations were checked using a refractometer. The initial concentrations of the sucrose solutions, 43.5, 57.5, and 65.3%, were chosen to be representative of the cryoconcentrated phase of a dilute sucrose solution at the different assay temperatures, −5, −10, and −15 °C, respectively (Champion et al., 1997a).

Enzymatic Reaction. Alkaline phosphatase (EC 3.1.3.1) is a nonspecific phosphomonoesterase. It is a dimeric metalloprotein that has two Zn^{2+} ions and an Mg^{2+} ion in each active site (Simopoulos and Jencks, 1994). Alkaline phosphatase is a hydrolytic enzyme that hydrolyzes disodium *p*-nitrophenyl phosphate (DNPP), the substrate, to form *p*-nitrophenolate ions, the product, and PO_3^{2-} ions. The alkaline phosphatase

Table 1. K_M and V_{max} of the Alkaline Phosphatase in Different Sucrose Solutions as a Function of Temperature

	sucrose concn in the reaction medium (w/w)								
	10%		43.5%			57.5%			
	20 °C	0 °C	20 °C	0 °C	−12.5 °C	20 °C	0 °C	−10 °C	−23 °C
K_M (10^{-4} M)	4.4	1.5	3.2	1.3	5.0	4	1.6	5.4	10.8
V_{max} (10^{-6} M h^{-1})	932	154	297	35	5	99	16.3	6.7	0.5

used was the type 1S obtained from bovine intestinal mucosa with an activity of 50 units mg^{-1} (Sigma). An 0.8 g L^{-1} solution of enzyme was prepared in distilled water with 0.76 mg of $MgCl_2$/mg of enzyme for enzyme activation. A 0.26 g L^{-1} solution of DNPP was prepared in distilled water. Aliquots of 0.1 mL of enzyme solution were added to cryotubes containing DNPP (at different concentrations depending on the studied enzyme-to-substrate ratio) and sucrose preparations. The tubes were immediately capped, mixed for exactly 30 s, and frozen in liquid nitrogen before any substantial DNPP hydrolysis could occur. The samples were stored overnight at −36 °C to ensure that the media were fully frozen. The tubes were put in a water bath for experiments at 20 and 10 °C and in a alcohol bath for ones at temperatures <0 °C. Some samples were removed at once to serve as zero time references. The others were removed at specified times after storage for between 0 and 34 days. Upon removal from the bath, 1 mL of 28 g L^{-1} trichloroacetic acid (TCA) was immediately added to the frozen sample. Therefore, as the sample started to thaw, TCA would denature the alkaline phosphatase and prevent further enzyme-catalyzed hydrolysis of DNPP. After the sample had thawed, the color was developed by increasing the pH with 0.5 mL of 24 g L^{-1} NaOH.

The solution absorbency was measured at 400 nm and converted to *p*-nitrophenol concentration using a standard curve obtained with sucrose solutions. The reaction rate v_0 was taken as the initial slope of the curve on plots of concentration of *p*-nitrophenol versus storage time. This slope was determined from the linear regression of the experimental data between time = 0 and the time when 5% of the total DNPP was hydrolyzed.

The enzyme-to-substrate molar ratio was varied from 250 to 2000 for the determination of the K_M and V_{max} values as a function of temperature and sucrose concentration; this molar ratio was then kept constant at the value of 1045 for the kinetic study of the reaction.

Viscosity Measurements. The viscosity of sucrose solutions was measured with a rheometer (Rheometer 30, Contraves). The viscosity was monitored as a function of temperature in solutions from 30 to 65.3% (w/w) sucrose, with temperature conditions avoiding the formation of ice, that is, from 20 to −15 °C. A temperature-controlled alcohol bath maintained a constant temperature during the experiments.

RESULTS

Enzymatic Characteristics. The initial velocity (v_0) was measured for different enzyme-to-substrate molar ratios from 250 to 2000. K_M and V_{max} were graphically determined using linear regression methods for the Lineweaver and Burk ($1/v_0 = f(1/[S])$ or Hanes and Woolf ($[S]/v_0 = f([S])$ representations, using the data of at least three repetitions. Both were used to calculate the enzymatic characteristics (K_M and V_{max}) in sucrose solutions. Table 1 presents the mean values obtained from the different replicates; statistical tests were used to compare these averages.

K_M. The measured Michaelis constant values were not significantly different with changing sucrose concentration or temperature. The mean value was 4.2×10^{-4} M for the alkaline phosphatase using DNPP as the substrate in sucrose solutions. The determined K_M reflects our choice for a nonbuffered reaction medium, to avoid salt concentration or precipitation at sub-ice-melting

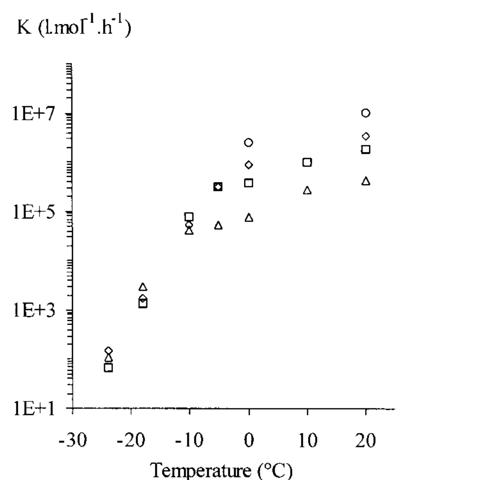


Figure 1. Global reaction rate constant K of alkaline phosphatase as a function of temperature. Experimental values obtained in different sucrose solutions: (○) 10% sucrose (w/w); (◇) 30%; (□) 43.5%; (△) 57.5%.

temperature, which can change the pH and buffer properties (Franks and Hatley, 1991). Indeed, the optimum pH for the alkaline phosphatase is 9.8 (Barman, 1969), but in the sucrose solutions used in this study, the pH of the reaction media was far from the optimal conditions for this enzyme, which explains the low value obtained for K_M. As shown in Table 1, the affinity (the inverse of the Michaelis constant) of the alkaline phosphatase for DNPP was not significantly modified by the presence of sucrose at concentrations up to 57.5%. Any effect of the temperature and ice presence on K_M could also not be observed in the studied media.

V_{max}. The maximum velocity significantly decreased with a decrease in the temperature or an increase of the sucrose concentration (Table 1). It appears that the experimental conditions had a strong effect on the enzymatic catalysis according to the V_{max} evolution.

Global Reaction Rate Constant *K*. The initial velocity (v_0) was determined by keeping the enzyme-to-substrate ratio constant at the value of 1045 in the liquid phase. The concentration in the liquid phase evolves as a function of the temperature due to the cryoconcentration process; however, if the concentrations of the two reactants evolve according to the temperature and the initial sucrose concentration, the ratio of solute concentrations remains constant during the decrease in temperature. To study the influence of the concentration and temperature on the reaction, the global reaction rate constant K (eq 1) was calculated according to equation eq 2, using the measured initial velocity and the calculated concentrations of enzyme and substrate as a function of the temperature (Champion et al., 1997a,b). Each experiment was replicated several times (from three to nine); the average K values are presented in Figure 1.

At room temperature, the reaction rate constant K decreased with an increase in the sucrose concentration.

The viscosity of the sucrose solutions influenced the K values as observed for V_{max} (Table 1).

The reaction rate constant decreased with the decrease in temperature from 3.5×10^6 M^{-1} h^{-1} at 20 °C to 1.5×10^2 M^{-1} h^{-1} at −23 °C for the 30% sucrose solution. This decrease of four decades was due not only to the decrease in temperature but also to the concentration of the reaction media resulting from ice formation. Indeed, the K values obtained at a given subzero temperature, in samples containing various amounts of ice, were not significantly different and were equal to the ones obtained in samples without ice, which had a concentration corresponding to the cryoconcentrated phase of the frozen media. The reaction rate constant seems to be more influenced by the concentration of the liquid phase than by the presence of ice.

DISCUSSION

The reduction of the enzymatic catalysis caused by freezing may be due to several factors: the enzyme structure and mechanism can be modified by the low temperature or the changes in the reaction medium induced by the freezing process can contribute to the decrease in the velocity of catalysis. These two aspects are discussed.

Enzyme Structure. From the results of K_M (Table 1), no modification of the affinity of this enzyme for DNPP was observed whatever the temperature or sucrose concentration. It could be assumed that there was no significant change in conformation of the active site of the alkaline phosphatase due to the presence of ice or high concentrations of sucrose (up to 73% at −24 °C). The same result was observed in buffered highly concentrated sucrose solutions at 25 °C (Simopoulos and Jencks, 1994). The decrease of the water content of the liquid phase did not disturb the specificity of alkaline phosphatase during the cryoconcentration and the specificity of invertase during the desiccation of the reaction media (Silver and Karel, 1981). The change of slope of K versus temperature (observed around −5 °C for the 43.5% sucrose solution) cannot be attributed to a cold denaturation of the enzymatic protein due its aggregation or dissociation as can be observed for several proteins (Hänsler and Jakubke, 1996) but reflected the concentration change in the reaction media. Indeed, for the 57.5% sucrose solution down to −10 °C, in which there was no cryoconcentration, the reaction kinetics followed a continuous evolution.

Molecular Mobility. A frozen product is constituted of ice crystals surrounded by a concentrated amorphous matrix (in the liquid or glassy state depending on the temperature). Every reaction in this type of system occurs in this amorphous phase, and the presence of ice can be considered inert on the reaction process, as confirmed by our results. For a theoretical analysis of the kinetics, one of the main difficulties is the determination of the viscosity of the liquid phase. The rheological measurements of the viscosity in frozen products are hindered by the presence of ice. When possible, in solutions without ice, the viscosity was measured using a classical apparatus of rheology, but the modeling was required to evaluate the viscosity of the unfrozen phase in frozen products. To do that, the solute concentration as a function of the storage temperature has to be known. The concentration of the liquid phase of a frozen system below its ice-melting temperature T_m can be obtained from the corresponding state diagram. For the

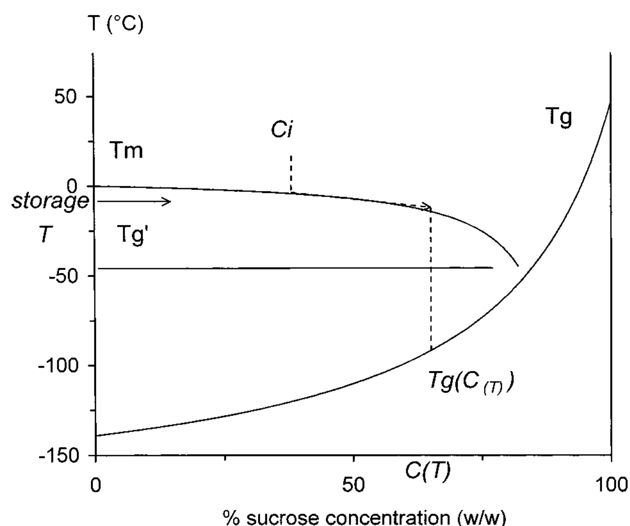


Figure 2. State diagram of the sucrose−water system. T_m = equilibrium ice formation-and-melting curve calculated from UNIFAC−UNIQUAC models; T_g = onset glass transition temperature curve calculated with the Gordon−Taylor equation (Blond et al., 1997).

sucrose−water system, the state diagram was widely studied and described in the literature (Luyet and Rasmussen, 1968; Roos and Karel, 1991; Le Meste and Huang, 1992). We used the state diagram given by Blond et al. (1997) (Figure 2), improved with the latest computer developments. For instance, a 30% sucrose solution at room temperature, if stored at −10 °C (T) in thermodynamic equilibrium, is constituted of a liquid phase at the concentration of 57.5% sucrose [$C(T)$].

Once the system is above its T_g, the viscosity of the liquid solution can be calculated using the Williams−Landel−Ferry relation (WLF) (Williams et al., 1955):

$$\log \eta_T/\eta_{T_g} = C_{g1}(T - T_g)/[C_{g2} + (T - T_g)] \quad (5)$$

where η_T and η_{T_g} are viscosities at the storage temperature T and at T_g, respectively; Cg_1 and Cg_2 are phenomenological coefficients.

The well-adapted WLF coefficients for our sucrose solution systems were first determined from the fitting of the experimental viscosity data measured in the absence of ice to the WLF equation. This determination allowed us then to calculate the viscosity of a solution as a function of its T_g (Champion et al., 1997a,b). Indeed, the viscosity of the cryoconcentrated phase was predicted as a function of temperature T, using eq 5, taking for $T_g(c_T)$ the glass transition temperature corresponding to the concentration at the storage temperature T. Our calculated values were in agreement with the viscosity data given by Kerr and Reid (1994) obtained for partially frozen sucrose solutions (Champion et al., 1997a).

To understand the influence of the medium on the reaction rate, the variations of K and viscosity were compared. In Figure 3, we observe a good correlation between the viscosity and the reaction rate constant (r^2 = 0.999).

The effect of temperature has to be emphasized. On the one hand, the concentration of the liquid phase in frozen systems is a function of the storage temperature according to the ice formation (cryoconcentration); on the other hand, the viscosity is dependent on the "WLF effect" [the difference between the storage temperature

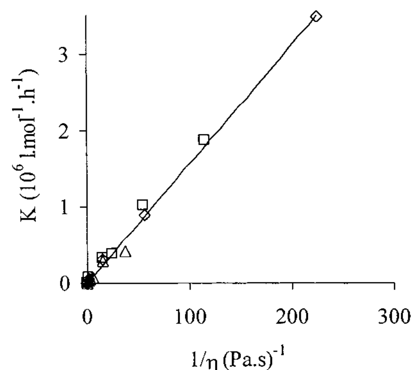


Figure 3. Comparison of the reaction rate constant K of the hydrolysis of DNPP by alkaline phosphatase with the inverse of the viscosity (measured or calculated with WLF).

and the T_g of the corresponding solution [$C(T)$]]. The storage temperature effect contributes to both the concentration and the viscosity. These two parameters are inseparable and result in a drastic temperature effect on reaction kinetics.

The two steps of the reaction can be analyzed separately to describe the viscosity action on the studied reaction. On the one hand, k_1 can be dependent on the viscosity, which contributes to the reactant mobility and association of molecules; on the other hand, the viscosity can also disturb the enzymatic catalysis efficiency causing a change in k_3.

Despite the fact that substrate and enzyme are mixed together before freezing, the mobility of the substrate can be a limiting factor due to its molecular environment. Indeed, the molar ratio of substrate to sucrose molecules is of $1/10^4$ and may explain the possible contribution of the reactant mobility on the global velocity.

The viscosity influences the diffusion of the molecules, and the mobility of the substrate may control the global reaction rate, limiting the first step of the reaction (k_1). The higher the viscosity is, the lower the translational diffusion coefficient is expected to be, as described by the Stokes–Einstein relation:

$$D = RT/6\pi r\eta \quad (6)$$

It was already shown that viscosity and diffusion of a small molecule are linked with this relation in sucrose solutions if the temperature is not too close to T_g (Champion et al., 1997b).

Atkins (1998) has proposed a theory for bimolecular diffusion-controlled reactions, which is based on the Stokes–Einstein relation and allows the determination of the reaction constant using the viscosity of the medium as a variable. His reaction model

$$K = k_1 = 8RT/3\eta \quad (7)$$

allows the prediction of the global reaction rate constant when the latter is equal, or approximately equal, to k_1, the association constant. The Atkins model was developed for a bimolecular reaction in which the reactant molecules have a similar size, and this characteristic is not generally observed for an enzymatic reaction. Moreover, it is difficult to take into account the size of an enzyme molecule because just a small part of it is active for the reaction. However, the expression established by Atkins can be simplified when it is considered that the diffusion of the enzyme can be neglected relative to the substrate diffusion. The Atkins prediction can be used for the approach of the first step of the reaction, taking into account the relative size and diffusivity of the reactant molecules.

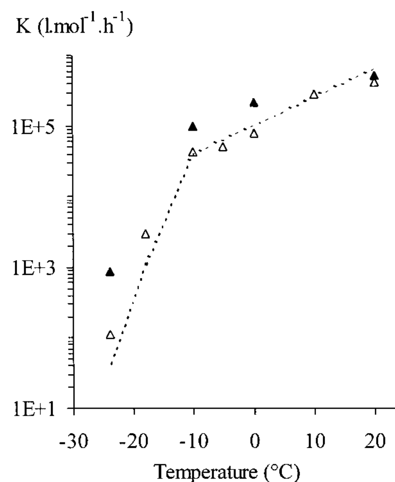


Figure 4. Comparison of the global reaction rate constant K (△) and the catalysis efficiency k_3/K_M (▲) in an initial 57.5% sucrose solution as a function of temperature. The dotted line represents the prediction of K according to eq **8**.

Considering that only the substrate was able to diffuse and that the catalytic site of the enzyme was similar in size to the substrate molecule, eq 7 becomes

$$K = RT/\eta \quad (8)$$

The K values thus predicted are in good agreement with our experimental data (Figure 4).

However, the significant decrease of the maximal velocity (V_{max}) with an increase in sucrose concentration or a decrease in temperature demonstrates the action of the viscosity on the enzymatic efficiency. The k_3/K_M parameter represents the catalysis efficiency of an enzyme (Lambert et al., 1997). This value was calculated under our drastic concentration and temperature conditions. The evolution of the catalysis rate constant k_3 (or k_3/K_M) as a function of the temperature can be assumed to follow Arrhenius law when only the temperature is the varying factor, but under our experimental conditions, this parameter evolved according to the viscosity, as checked for the global reaction rate constant K. This observation emphasized the viscosity influence on the enzymatic catalysis, which is probably due to the decrease in the enzyme segmental flexibility.

To analyze the relative importance of the two steps of the reaction, the global rate constant K was compared to k_3/K_M (Figure 4). At room temperature, K was nearly the same as the k_3/K_M value, meaning that the reaction-controlling step was the catalysis of the reaction. On the contrary, at −23 °C, K was significantly lower than k_3/K_M, possibly through the large importance of the reactant mobility. Between these extreme temperatures, the two steps of the reaction were in balance with an increase of the diffusion influence as the temperature was decreased.

Conclusions. Experimental data about an enzymatic reaction studied in frozen sucrose solutions down to −23 °C are reported in this paper. The results are discussed in relation to the temperature effect. The viscosity seems to be the main parameter, which controls the studied reaction. In fact, the viscosity acts on the two steps of the reaction: on the mobility of the reactants that

control the enzyme substrate complex formation and on the product formation, probably through an action on the enzyme segmental flexibility. However, although the viscosity allows a satisfactory prediction of the reaction rate, the complexity of the phenomena taking place in frozen food products cannot be overemphasized, for example, changes in the order of reaction and the complexity of the concentration effect such as the pH change during freezing processes (Simatos and Blond, 1993).

Further investigations are being carried out in model systems in which enzyme and substrate are initially separated. The measured initial velocity will be analyzed as a function of both reactant diffusion and enzymatic catalysis. The two steps of the reaction will be inserted in the prediction of the reaction rate constant in terms of the translational diffusion coefficient for the former and in terms of viscosity and the catalysis constant for the latter.

ACKNOWLEDGMENT

We thank Prof. V. Larreta Garde for her help and stimulating discussion on the subject.

LITERATURE CITED

Ashie, I. N. A.; Simpson, B. K.; Smith, J. P. Mechanisms for controlling enzymatic reactions in foods. *Crit. Rev. Food Sci. Nutr.* **1996**, *36*, 1–30.

Atkins, P. W. Molecular reaction dynamics. In *Physical Chemistry*, 6th ed.; Oxford University Press: Oxford, U.K., 1998; pp 819–848.

Barman, T. E. In *Enzyme Handbook II*; Springer-Verlag: New York, 1969; pp 83–84.

Blond, G.; Simatos, D.; Catte, M.; Dussap, C. G.; Gros, J. B. Modeling of water-sucrose state diagram below 0 °C. *Carbohydr. Res.* **1997**, *298*, 139–145.

Champion, D.; Blond, G.; Simatos, D. Reaction rates at subzero-temperatures in frozen sucrose solutions: a diffusion-controlled reactions. *Cryo-Lett.* **1997a**, *18*, 251–260.

Champion, D.; Hervet, H.; Blond, G.; Le Meste, M.; Simatos, D. Translational diffusion in sucrose solutions in the vicinity of their glass transition temperature. *J. Phys. Chem. B* **1997b**, *101*, 10674–10679.

Douzou, P. In *Cryobiochemistry: An Introduction*; Academic Press: London, U.K., 1977; p 286.

Fennema, O. Activity of enzymes in partially frozen aqueous systems. In *Food Science and Technology. A Series of Monographs*; Academic Press: London, U.K., 1975; pp 397–412.

Franks, F.; Hatley, R. H. M. Stability of proteins at subzero temperatures: thermodynamics and some ecological consequences. *Pure Appl. Chem.* **1991**, *63*, 1367–1380.

Hall, L. P.; Alcock, J. The effect of microbial enzymes on the quality of frozen foods. *Food Microbiol.* **1987**, *4*, 209–219.

Hänsler, M.; Jakubke, H. D. Reverse action of hydrolases in frozen aqueous solutions. *Amino Acids* **1996**, *11*, 379–395.

Karel, M.; Buera, M. P.; Roos, Y. Effects of the glass transitions on processing and storage. In *The Glassy State in Foods*; Blanshard, J. M. V., Lillford, P. J., Eds.; Nottingham University Press: Nottingham, U.K., 1993; pp 3–34.

Kerr, W. L.; Reid, D. S. Temperature dependence of the viscosity of sugar and maltodextrin solutions in coexistence with ice. *Lebensm.-Wiss. Technol.* **1994**, *27*, 225–231.

Kerr, W. L.; Lim, M. H.; Reid, D. S.; Chen, H. Chemical reaction kinetics in relation to glass transition temperatures in frozen food polymers solutions. *J. Sci. Food Agric.* **1993**, *61*, 51–56.

Lambert, L.; Pourplanche, C.; Larreta-Garde, V. Comparison of macromolecular and molecular effects of cosolvents on the catalytic behavior of soybean lipoxygenase-1. *Biocatal. Biotransform.* **1997**, *15*, 39–55.

Le Meste, M.; Huang, H. Thermomechanical properties of frozen sucrose solutions. *J. Food Sci.* **1992**, *57*, 1230–1233.

Levine, H.; Slade, L. Principle of "cryostabilization" technology from structure/property relationships of carbohydrate/water systems. A review. *Cryo-Lett.* **1988**, *9*, 21–63.

Luyet, B.; Rasmussen, D. Study by differential thermal analysis of the temperatures of instability of rapidly cooled solutions of glycerol, ethylene glycol, sucrose and glucose. *Biodynamica* **1968**, *10*, 167–191.

Maier, V. P.; Tappel, A. L.; Volman, D. H. Reversible inactivtion of enzymes at low temperatures. Studies of temperature dependence of phosphatase- and peroxidase-catalyzed reactions. *J. Am. Chem. Soc.* **1955**, *77*, 1278–1280.

Michaelis, L.; Menten, M. L. The kinetics of invertase. *Biochem. Z.* **1913**, *49*, 333–341.

Parducci, L. G.; Fennema, O. Rate and extent of enzymatic lipolysis at subfreezing temperatures. *Cryobiology* **1978**, *15*, 199–204.

Parducci, L. G.; Fennema, O. Extent of invertase-catalyzed sucrose hydrolysis at subfreezing temperatures. *Cryobiology* **1979**, *16*, 578–581.

Parker, R.; Ring, S. G. A theoretical analysis of diffusion-controlled reactions in frozen solutions. *Cryo-Lett.* **1995**, *16*, 197–208.

Roos, Y.; Karel, M. Water and molecular weight effects on glass transitions in amorphous carbohydrates and carbohydrate solutions. *J. Food Sci.* **1991**, *55*, 1676–1681.

Silver, M.; Karel, M. The behavior of invertase in model systems at low moisture content. *Food Chem.* **1981**, *5*, 283–311.

Simatos, D.; Blond, G. DSC Studies and stability of frozen foods. In *Water Relationships in Foods*; Levine, H., Slade, L., Eds.; Plenum Press: New York, 1991; pp 139–156.

Simatos, D.; Blond, G. Some aspects of the glass transition in frozen foods systems. In *The Glassy State in Food*; Blanshard, J. M. V., Lillford, P. J., Eds.; Nottingham University Press: Nottingham, U.K., 1993; pp 395–415.

Simopoulos, T.; Jencks, W. Alkaline phosphatase is an almost perfect enzyme. *Biochemistry* **1994**, *33*, 10375–10380.

Tappel, A. L. Effects of low temperatures and freezing on enzymes and enzyme systems. In *Cryobiology*; Merymann, H. T., Ed.; Academic Press: New York, 1966; pp 163–177.

Ullmann, G.; Haensler, M.; Gruender, W.; Wagner, M.; Hofmann, H. J.; Jakubke, H. D. Influence of freeze concentration effect on proteinase-catalysed peptide synthesis in frozen aqueous systems. *Biochim. Biophys. Acta* **1997**, *1338*, 253–258.

Vajda, T. Cryochemistry. Large acceleration of the oxidation of hydroxylamine by iodate in frozen solutions. *Int. J. Chem. Kinet.* **1993**, *25*, 1015–1018.

Williams, M. L.; Landel, R. F.; Ferry, J. D. The temperature dependence of relaxation mechanisms in amorphous polymers and other glass forming liquids. *J. Am. Chem. Soc.* **1955**, *77*, 3700–3706.

Received for review April 11, 2000. Revised manuscript received July 10, 2000. Accepted July 10, 2000. This work was supported by the Burgundy Region (99-5112-10-13011) and the European Communities, Agriculture and Fisheries (FAIR) specific RTD program CT96.1085 (Enhancement of Quality of Food and Related Systems by Control of Molecular Mobility).

JF000457S