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Effects of Cryostabilizers, Low Temperature, and Freezing on the Kinetics of the Pectin Methylesterase-Catalyzed De-esterification of Pectin

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The kinetics of the pectin methylesterase (PME)-catalyzed de-esterification of pectin was studied at 25 °C in the presence of sucrose, fructose, maltodextrin (DE = 16.5−19.5), and carboxymethylcellulose at different concentrations and in the presence of maltodextrin and sucrose at different concentrations in a temperature range between +25 and −4 °C in subcooled and frozen states. The objective was to determine whether the reaction is diffusion-controlled, to gain insight about the factors determining the diffusion of the reactants, and to determine the effect of the carbohydrates, low temperature, and freezing on the structural conformation of the enzyme. The results indicate that the PME-catalyzed de-esterification of pectin is diffusion-controlled. Nevertheless, the diffusion is not controlled by the macroviscosity of the reaction medium, but rather by the microviscosity experienced by the diffusants. Low temperature in the temperature range studied does not affect the structural conformation of the enzyme, while freezing seems to have some effect.

KEYWORDS: Pectin methylesterase; macroviscosity; microviscosity; diffusion-controlled; glass transition

INTRODUCTION

Pectin methylesterase (PME) is a cell-wall-bound enzyme that plays an important role in fruit ripening and food product stability. It catalyzes the de- esterification of pectin to acidic pectin with a lower degree of esterification and methanol. The activity of PME destabilizes pectinaceous materials in fruit juices and concentrates (*1*, *2*) and modifies the texture of fruit and vegetable products (*3*, *4*) as the de-esterified pectin can be easily depolymerized by polygalacturonase (*5*). In addition, the de-esterified pectin precipitates or gels as calcium pectinate or pectate, leading to cloud loss in fruit juices and gelation of juice concentrates (*1*).

Carbohydrate additives such as low-molecular-weight sugars, polyols, and polysaccharide gums are commonly used in the cryopreservation of foods. The physical mechanisms of protection and stabilization by these additives are not well understood (*6*). It has been hypothesized that their cryostabilizing characteristics can be explained in terms of their effect on the glass transition temperature of the maximally freeze concentrated matrix (T_g') of the system and its consequence on molecular mobility and the rates of diffusion-controlled processes (*7*).

Basically, the applicability of the glass transition concept depends on whether the process under consideration is diffusion-controlled or not. For most enzyme-catalyzed reactions, the chemical steps in catalysis are not usually rate-limiting. Rather the formation of the enzyme−substrate complex before the catalytic step or the dissociation of the products after the catalytic step is rate-limiting, and both are diffusion-controlled (*8*). However, the rate-determining step of the enzyme-catalyzed reaction also depends on the properties of the reaction medium such as pH. As a consequence of freeze-concentration in frozen systems and the accompanying high solute concentration, changes in pH, ionic strength, and dielectric constant, and low temperature as such, changes in the conformation of the enzyme may occur (*9*, *10*). Under such conditions, the reaction may not be diffusion-controlled (*10*). The kinetics of a number of enzyme-catalyzed reactions was studied in frozen systems in relation to the glass transition temperature of the freeze-concentrated matrix. Deviations from theory have been reported for the alkaline phosphatase (*11*, *12*, *15*), pectin methylesterase (*14*), and peroxidase and polyphenol oxidase (*13*) catalyzed reactions.

We recently studied the kinetics of the PME-catalyzed de-esterification of pectin in four frozen model systems that consisted of sucrose, fructose, low-viscosity sodium carboxymethylcellulose (CMC), and maltodextrin (DE = 16.5−19.5) (*14*). The kinetics was not consistently correlated with T_g'. The highest reaction rate was observed in the presence of sucrose, followed by fructose, maltodextrin, and CMC in that order. If the reaction rate was correlated with T_g', the fastest reaction rate would have been observed in the presence of fructose ($T_g' = -51.9$ °C),

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followed by sucrose ($T_g' = -43.6$ °C), maltodextrin ($T_g' = -22.0$), and CMC ($T_g' = -16.1$ °C). Note that the T_g' values are measured values in the presence of the substrate buffer. However, the glass transition concept as originally proposed in synthetic polymer science is defined for systems with constant concentration and a fixed T_g, as opposed to frozen systems that undergo continuous evolution of concentration as a function of temperature. Thus, T_g' may not be the appropriate reference glass transition temperature to which the kinetics of diffusion-controlled processes in frozen systems is related. This has led some researchers (*16*) to use concentration-dependent T_g as a reference temperature in the WLF equation to describe kinetics in frozen systems. In this case, the kinetics of the reaction in the four model systems was not described by the WLF equation with the concentration-dependent T_g of the model systems as explained in detail by Terefe et al. (*17*) and Terefe (*18*), which shows that the kinetics is not correlated to the concentration-dependent T_g either.

The objective of this study was to investigate the reasons for the observed deviation from the glass transition concept. Thus, as freezing involves changes in both temperature and concentration (through the removal of water as ice), the kinetics was studied as a function of the concentration of the carbohydrates used in the previous study while keeping the temperature constant and as a function of temperature while keeping the concentration constant. These experiments were designed to deduce whether the reaction is diffusion-controlled or not and to get some insight about the factors that determine the diffusion of the reaction species. The effects of solute additives, low temperature, and ice on the catalytic properties of the enzyme were also investigated.

MATERIALS AND METHODS

I. Effects of Matrix Concentration and Viscosity. The purpose of these experiments was to study the effects of increased matrix concentration and the consequent increase in viscosity, which accompanies freezing at constant temperature and to determine whether the reaction is diffusion-controlled or not.

Reagents. The carbohydrates fructose, sucrose, and low-viscosity CMC were obtained from Sigma (Bornem, Belgium), while maltodextrin with DE = 16.5−19.5 was obtained from Aldrich (Milwaukee, WI). PME was extracted and purified from tomato (*Lycopersicon esculentum* var. Flandria Prince, Belgium, $M = 35$ kDa) as described by Fachin (*19*). PME with an activity of 10.8 U/mL was obtained after purification. Apple pectin with a 70−75% degree of esterification ($M = 30-100$ kDa) was obtained from Fluka (Bornem, Belgium). The substrate solutions were prepared on a weight-by-volume basis by dissolving 3.5 g/L pectin, 0.1 M tris(hydroxymethyl)methane and the carbohydrates at various concentrations in distilled water. Sucrose and fructose (0−70% (m/v)), maltodextrin (0−50% (m/v)), and CMC (0−5% (m/v)) were used in the study. The pH of the substrate solution was adjusted to 7.5 by using 37% HCl solution.

Measurement of Viscosity. The kinematic viscosity of the solutions was measured by capillary viscometers (Ubbelhode capillary viscometers, Schott glass, Mainz, Germany) immersed in a thermostated water bath maintained at 25 °C. The density of the food model solutions was measured with a pycnometer after equilibration in a water bath maintained at 25 °C. The dynamic viscosity was obtained from the product of the kinematic viscosity and density.

Experimental Protocol for the Kinetic Study. A 10 mL sample of the substrate solution was taken and mixed well with 2 units of the PME solution (185 μL). A 1 mL portion of the mixed sample was distributed into 13 mm Pyrex test tubes and placed in a water bath maintained at 25 °C. Samples were removed immediately after temperature equilibration to serve as a zero time reference and at a specified time interval depending on the concentration and type of carbohydrate used. Upon removal from the water bath, the samples were heated for 2 min at 85.5 °C to inactivate the enzyme. Then they were cooled in ice−water. Each sample was diluted 25 times by 0.01 M phosphate buffer (pH 7.5), and the methanol content was analyzed using the method of Klavons and Bennet (*20*). In this method, methanol is oxidized to formaldehyde with alcohol oxidase, followed by the condensation of the formaldehyde with 2,4-pentanedione to the colored product 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine. The absorbance of this colored product is measured at 412 nm and converted to the concentration of methanol using a standard curve. Alcohol oxidase from *Pichia pastoris* with an activity of 25 units/mg (EC 1.1.3.13, Sigma) and 2,4-pentanedione (Aldrich) were used in the analysis. In all cases, the experiments were performed in duplicate.

Data Analysis. The initial rate, V_o, was calculated as the initial slope of the concentration versus time curve. At low substrate concentration, the initial rate can be expressed as

$$V_o = k[E]_o[S]_o \tag{1}$$

where $[E]_o$ and $[S]_o$ are the initial concentrations of the enzyme and the substrate, respectively, and k is the global reaction rate constant and is given by eq 2 (*21*). k_{cat} is the catalytic rate constant, k_1 is the

$$k = \frac{k_{cat}k_1}{k_{cat} + k_{-1}} \tag{2}$$

association rate constant for the formation of the enzyme substrate encounter complex, and k_{-1} is the dissociation rate constant. The global rate constant k was calculated by substituting the initial rate and the initial substrate and enzyme concentrations in eq 1. If the reaction is diffusion-controlled ($k_{cat} \gg k_{-1}$), the second-order rate constant becomes

$$k = k_1 \tag{3}$$

The simplest way to analyze diffusion-limited reactions is by assuming two uniformly reactive spheres. In this case, the association constant, k_1, for two spherical molecules is given by Smolchowski's equation (eq 4), where D_E and D_S are the diffusion constants and r_E and r_S the

$$k_1 = 4\pi(D_E + D_S)(r_E + r_S)N_A = 4\pi N_A D_r R^* \tag{4}$$

radii of the enzyme and the substrate, respectively. $R^* = r_E + r_S$ is the effective target or interaction diameter, $D_r = D_E + D_S$ is the relative diffusion constant of the two reacting species, and N_A is Avogadro's constant (*22*, *23*).

Many molecules including macromolecules can be considered to have an approximately spherical shape, though there are some extreme deviations (*22*). Assuming that the Stokes−Einstein equation (eq 5) is applicable

$$D_E = \frac{k_B T}{6\pi\eta R_E} \quad \text{and} \quad D_S = \frac{k_B T}{6\pi\eta R_S} \tag{5}$$

where k_B is the Boltzmann constant, T is the absolute temperature, D_E, D_S, R_E, and R_S are the diffusion coefficients and the hydrodynamic radii of the enzyme and the substrate, respectively, and η is the viscosity of the medium.

If the hydrodynamic radii of A and B are assumed to be equal to their interaction radii ($R_E = r_E$, $R_S = r_S$), the association rate constant depends only on the ratio of the radii of the two molecules and viscosity (eq 6). It follows that eq 7 holds, where the superscript "o" refers to

$$k_1 = \frac{2RT}{3\eta}\left(2 + \frac{r_E}{r_S} + \frac{r_S}{r_E}\right) \tag{6}$$

the reaction in the aqueous solution in the absence of viscogenic agent (an additive that changes the viscosity of the reaction medium). Thus,

$$k_1^o \eta^o = k_1 \eta \tag{7}$$

in the absence of other perturbations, the extent to which an enzyme-

catalyzed reaction is diffusion-controlled can be estimated from the dependence of the observed second-order rate constant on viscosity. Accordingly, if the reaction is diffusion-controlled, a plot of k^o/k versus η/η^o will be a straight line with slope equal to 1. To assess if the PME-catalyzed reaction was diffusion-controlled or not, the relative global reaction rate constant was plotted against the relative viscosity.

Such an approach has been used to probe for diffusion control in several enzyme-catalyzed reactions including reactions catalyzed by chymotrypsin (*24*), triosephosphate isomerase (*25*), human C-terminal Src kinase (*26*), alkaline phosphatases from *Echerchia coli* (*27*), human placental alkaline phosphatase (*28*), β-lactamase (*29*), protein kinase (*30*), and gluthione S-transferase A1-1 (*31*).

II. Effects of Low Temperature. The objective of these experiments was to determine the effect of a decrease in temperature and the consequent increase in viscosity that accompanies freezing at a constant concentration condition.

Reagents. Substrate solutions containing 3.5 g/L pectin and 0.1 M Tris buffer were prepared with 30% and 47.6% (m/v) sucrose and 47.6% (m/v) maltodextrin added to them. A substrate solution without additives was also prepared. The pH of the solutions was adjusted to 7.5 using 37% HCl. The same enzyme preparation as in section I was used in the study.

Experimental Protocol for the Kinetic Study. The experiments were carried out as described in section I but in this case in a temperature range of +20 to −4 °C depending on the substrate solution. This temperature range was deliberately selected to avoid freezing in the most concentrated solutions. Experiments were carried out in cryostats (Heto CBN18-50, Heto-Holton, Allerød, Denemark) maintained at the experimental temperatures. All experiments were performed in duplicate.

Data Analysis. The initial reaction rate was obtained from the initial slope of the progress curve. The reaction rate constant was calculated using eq 1. The reaction rate versus temperature data were analyzed using the Arrhenius temperature model.

III. Effects of Low Temperature and Freezing on the Catalytic Parameters of PME. The purpose of these experiments was to determine the effects of low temperature and freezing on the catalytic efficiency of PME as both may affect enzymes through reversible protein unfolding and the resulting loss of active sites.

Reagents. A 40% (m/v) sucrose solution was prepared with pectin concentration in a range of 0−8 g/L and 0.1 M Tris buffer. A 20% (m/v) sucrose solution was also prepared with pectin concentration in the same range with 0.05 M Tris to get a 0.1 M concentration at the freezing temperature of the 40% solution (−4.5 °C). The same enzyme preparation as in section I was used in this study.

Experimental Protocol for the Kinetic Study. To investigate the effect of low temperature without freezing, experiments in a range of pectin concentrations were carried out in the presence of 40% sucrose at 25 and −4.5 °C. A 10 mL sample of the solution was taken and mixed with 2 units of enzyme solution. A 1.0 mL portion of the mixed sample was divided into 15 mm plastic tubes and placed in a cryostat at −4.5 °C (the freezing point of 40% (m/v) sucrose solution). Samples were removed immediately after temperature equilibration to serve as a zero time reference and at a specified time interval during storage. Upon removal from the cryostat, the samples were heated for 3 min at 85.5 °C to inactivate the enzyme. Then they were cooled in ice−water. The methanol content was analyzed after dilution 25 times with phosphate buffer using the method of Klavons and Bennet (*24*) as described above. To investigate the effect of freezing, 10 mL of the 20% sucrose solution was taken and mixed with 2 units of the enzyme solution. Eight 1.0 mL samples were taken into 15 mm plastic tubes. These samples were frozen at a rate of 10 °C/min by immersion in a cryostat maintained at −25 °C. Slow freezing was used here to ensure maximal freeze-concentration as the solutions became very viscous at higher pectin concentrations. The samples were then placed in cryostats at −4.5 °C (the unfrozen freeze-concentrated matrix will have 40% (m/v) sucrose). This was followed by the same steps as above.

Data Analysis. The initial rates versus substrate concentrations were fitted to the Michaelis−Menten equation (eq 8). The kinetic parameters

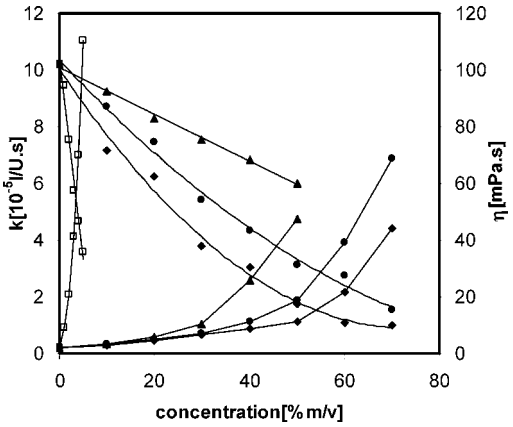

Figure 1. Global reaction rate constant (k) for the PME-catalyzed de-esterification of pectin and the viscosity of the reaction medium (η) at 25 °C versus matrix concentration in the presence of the different carbohydrate additives (□, CMC; ◆, fructose; ▲, maltodextrin; ●, sucrose).

K_m and V_{max} were estimated from the nonlinear regression of the data using SAS statistical software.

$$V = \frac{V_{max}[S]}{K_m + [S]} \tag{8}$$

RESULTS AND DISCUSSION

I. Effects of Matrix Concentration and Viscosity. The global reaction rate constant of the PME-catalyzed de-esterification of pectin versus the concentration of the different carbohydrates is presented in **Figure 1**. In all cases, k decreased with increasing solute concentration. CMC caused the largest decrease per unit mass of added solute followed by fructose, sucrose, and maltodextrin in that order. The viscosities of the solutions as a function of concentration are also shown in the same figure. The effect of the different matrix solutes on the kinetics did not correlate with their molecular weight and viscosity. Though CMC caused the greatest decrease in the kinetics, this was not followed by maltodextrin, sucrose, and fructose as can be expected on the basis of the viscosity and the molecular weight of the respective matrixes.

Solute additives can affect the structural conformation of macromolecules such as enzymes through direct interaction, by indirect action through their effects on the structure and properties of the solvent, or by a combination of both mechanisms (*32*). Simpoulos and Jencks (*27*) observed that the alkaline phosphatase-catalyzed hydrolysis of disodium *p*-nitrophenyl phosphate in the presence of glycerol decreased by a degree much larger than expected for a diffusion-controlled reaction and attributed this to the decrease in the catalytic activity of the enzyme due to the binding of the glycerol molecules to the active site or the perturbation of its structure by a solvent effect. In our case, the logarithm of the rate constant versus concentration is reasonably linear in the presence of all the solutes (see **Figure 2**), which indicates that the solutes do not affect the structural conformation of the enzyme (*33*).

The monotonic decrease in the rate constant with the concentration of the carbohydrates gives an indication that the reaction is diffusion-controlled and that the solutes affect the diffusion of the reactants rather than the catalytic efficiency of the enzyme (*27*, *28*). As the average molecular size of the substrate pectin (M = 30−100 kDa) is much larger than that of the enzyme (M = 35 kDa), it is likely that $D_E \gg D_S$ and the diffusion rate of the enzyme determines the frequency of enzyme−substrate encounters and the reaction rate. However,

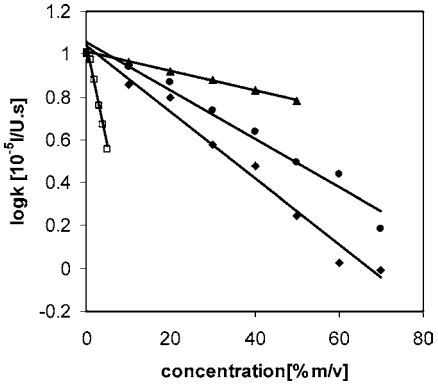

Figure 2. Logarithm of the global reaction rate constant (k) for the PME-catalyzed de-esterification of pectin versus the concentration of the solutes (□, CMC; ◆, fructose; ▲, maltodextrin; ●, sucrose).

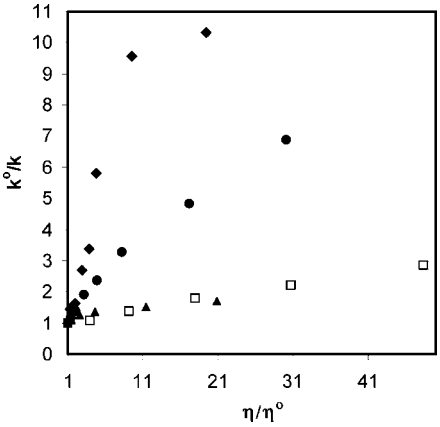

Figure 3. Relative reaction rate constant for the PME-catalayzed de-esterification of pectin at 25 °C versus relative viscosity in the presence of the different carbohydrate additives (□, CMC; ◆, fructose; ▲, maltodextrin; ●, sucrose).

as can be seen in **Figure 3**, k^o/k versus η/η^o curves are nonlinear in all cases, contrary to what is expected for diffusion-limited reactions. Nevertheless, it has to be noted that the inverse relation between viscosity and the rate constant depends on the applicability of the Stokes−Einstein equation, which is limited. The Stokes−Einstein relationship holds when the diffusing molecules are much larger than the molecules that constitute the medium and when the viscosity is low (η < 10 mPa·s) (*34*, *35*). As can be seen in **Figure 3**, in the cases where the polymers CMC and maltodextrin were used as additives, the relative change in the reaction rate constant as a function of the change in the relative viscosity is very low (the slope is almost zero), which is most probably due to the relatively small difference between the molecular sizes of the enzyme and these additives. The initial parts of the curves in the presence of sucrose and fructose are linear. The concentration regime where linearity is observed corresponds to the relative viscosity and concentration range where many investigations that reported diffusion control in the biochemistry literature were carried out (*25*, *27*, *28*, *31*). In fact, in the case of fructose, the curve is linear up to a relative viscosity of 9.5 with a slope of 1.03. The deviation from linearity at higher viscosity values and in the presence of the polymeric additives is clearly due to the inapplicability of the Stokes−Einstein relation under these conditions.

Similarly, the relative reaction rate constant for the triose-phosphate-catalyzed reaction was linearly correlated with the relative viscosity in the presence of sucrose and glycerol, while no effect was observed when the polymeric additives poly(ethylene glycol) (PEG), polyacrylamide, and ficoll (a copolymer of sucrose and epichlorohydrin) were used in the same relative viscosity range (*25*). PEG at a concentration up to 6.3% w/w (η/η^o = 3) did not affect the reaction catalyzed by human C-terminal Src kinase, while sucrose caused a significant decrease in the kinetics at the same η/η^o value (*30*). Such deviations from the Stoke−Einstein relationship are common in the literature (*34*−*38*). These observations indicate that it is the microviscosity (the resistance to motion experienced by the diffusing molecules) rather than the bulk viscosity which determines the rate of diffusion (*25*, *34*, *39*). The difference between the two variables increases with increasing concentration and molecular weight (*38*), and that is what we observed in this case.

The question is exactly what constitutes the microviscosity experienced by diffusing molecules. There are several models that describe the diffusion of molecules in polymer melts and solutions based on the theories of inert obstruction, hydrodynamic interactions, and free volume (*40*, *41*). However, there is no single theory which consistently explains all these effects (*39*). Clearly, molecular diffusion depends on frictional effects that may arise from obstruction, hydrodynamic drag, etc. These in turn depend on the size, shape, and flexibility of the diffusing solute and the molecules that constitute the background matrix, their possible interaction, physical or chemical (e.g., electrostatic), and the concentration of the matrix among other things (*35*, *36*, *39*−*41*). Gao and Fagerness (*42*) observed that the obstruction to the diffusion of the drug adinozolam mesylate in aqueous solutions of glucose, lactose, maltoheptaose, and hydroxypropyl methylcellulose (HPMC) of different viscosity grades increased with the molecular weight of the background molecules from glucose to HPMC, which they attributed to the increase in the degree of polymerization. This was not consistently observed in our case, which points to the involvement of other factors such as electrostatic and hydrodynamic effects in addition to obstruction.

Several studies indicate that PME from higher plants such as tomato catalyze the de-esterification of pectin through multiple attack mechanisms; i.e., the enzyme catalyzes a limited average number of residues for every active enzyme−substrate complex formed, giving rise to negatively charged blocks on the pectin molecule (*43*−*45*). This has an impact on the diffusion of the enzyme toward the substrate in the presence of charged matrix molecules such as the polyanion CMC. The electrostatic repulsion between the negatively charged pectin blocks and CMC can reduce the relative diffusion of PME toward its substrate and may explain the highest reduction in the kinetics per unit mass of added solutes in the presence of CMC while the other macromolecular additive maltodextrin had the least effect.

The magnitude of the hydrodynamic drag experienced by diffusing molecules depends on the dynamics of the solvent water (*40*), which in turn depends on the type of the carbohydrate additives, as the effects of different carbohydrates on water dynamics differ on the basis of their structure (*46*, *47*). This may partly explain the higher effect of fructose as compared to sucrose and maltodextrin. Wang and Haymet (*46*) on the basis of their thermal study of aqueous solutions of different sugars concluded that the interaction between water and sugar molecules depends on the structural features of the sugars and explains the superior quality of trehalose as a cryopreservative as compared to other sugars. The higher effects of the low-molecular-weight additives as compared to maltodextrin on the reaction kinetics could also be due to the difference in the degree

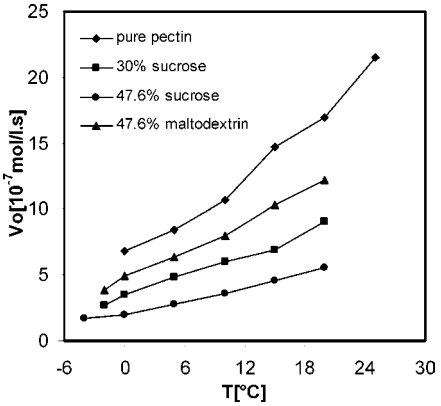

Figure 4. Initial rate of the PME-catalyzed de-esterification of pectin versus temperature in the presence of carbohydrates at different concentrations.

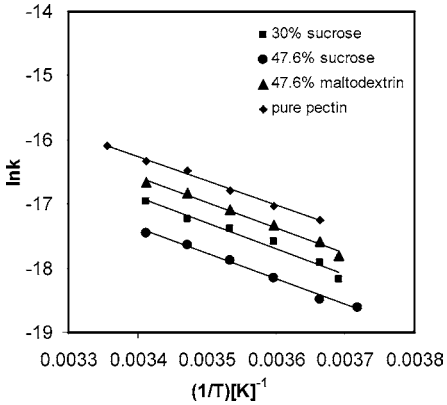

Figure 5. Arrhenius curves for the PME-catalyzed de-esterification of pectin in the presence of carbohydrates at different concentrations.

of compactness of the random packing of the molecules, which may affect the resistance to the diffusion of the reactants. Tromp and others (*48*) studied the diffusion of water in highly concentrated matrixes of maltodextrins and maltose. A higher diffusion rate was observed in the maltodextrins at the same water content as compared to that in maltose. They explained this to be due to the less dense random packing of the molecules of the maltodextrins as compared to maltose, leaving more pathways for water diffusion.

The observations at ambient conditions were also reflected in the study of the kinetics in frozen model systems mentioned earlier (*14*). In that study, the lowest reaction rate in the frozen systems was observed in the CMC model system followed by maltodextrin, fructose, and sucrose. The same trend was observed in this study with the exception of maltodextrin. However, on the basis of its phase diagram, the maltodextrin model system has the highest solute concentration at any given subfreezing temperature (*17*). Thus, the observed low reaction rate in the frozen maltodextrin system does not contradict the result at ambient conditions. This observation shows that the characteristics of the reacting species and the matrix solutes such as molecular size, electrostatic charge, their effect on water dynamics, and the equilibrium freezing curve determine the diffusivity of reacting species and hence kinetics in frozen systems rather than the macroviscosity of the reaction medium and the related concept of glass transition. These and the additional complexity due to the heterogeneity of the freeze-concentrated unfrozen matrix, with the ice crystals presenting an additional obstacle to molecular diffusion, may explain the reported deviations of kinetics in frozen systems from the glass transition concept.

II. Effects of Low Temperature. The initial rate versus temperature in the presence and absence of the food model solutes is presented in **Figure 4**. In all cases, the same trend as in the concentration study was observed with the fastest reaction in the absence of added solutes followed by 47.6% maltodextrin, 30% sucrose, and 47.6% sucrose solutions. The Arrhenius plots for the reaction in the different conditions are shown in **Figure 5**. As can be seen, linear Arrhenius plots were observed in the whole temperature range studied. No break was observed as the temperature decreased to the subzero temperature range, indicating that low temperature does not affect the specific activity of the enzyme through conformational change, at least in the temperature range studied. A break in the Arrhenius plots is considered as one indication of a change in the specific catalytic activity of an enzyme due to shifts in the distribution of conformational isomers (*49*). The Arrhenius plots are nearly parallel. The activation energies obtained under the different conditions are presented in **Table 1**.

Table 1. Activation Energies Calculated for the PME-Catalyzed De-esterification of Pectin in the Presence of Different Solutes

solute	E_A (kJ/mol)	r^2
pure pectin	31.5 ± 1.2[a]	0.9945
30% sucrose	33.5 ± 2.9	0.971
47.6% sucrose	33.1 ± 3.6	0.968
47.6% maltodextrin	33.1 ± 1.9	0.982

[a] Standard error of regression.

As can be seen, the activation energies obtained in all cases are not significantly different. The observed activation energy values are much higher than the activation energy for the viscosity of water (17.47 kJ/mol), which gives another indication that inert obstruction is not the only factor governing the diffusion of the reactants. If that were the case, the same activation energy as that for diffusion in free water would have been observed (*42*, *50*, *51*). Brown and Johnsen (*50*) observed similar activation energies for the diffusion of glycerol and PEG in polyacrylamide gels, and the inverse viscosity of water from which they concluded that the frictional resistance experienced by the diffusing molecules solely depends on the solute−solvent interaction and the polymer segments simply impose a more tortuous path in diffusion. Similar observations for the diffusion of the drug adinozalm mesylate in glucose, lactose, HPMC of different viscosity grades, and water (*42*) and the diffusion of glucose in pure water and carrageenan gels of different concentrations (*51*) led to the same conclusion.

As the reaction was found to be diffusion-controlled, the observed activation energy reflects the activation energy for diffusion. As the temperature varies, only the fluid phase viscosity, i.e., the microviscosity, sensed by the diffusing molecules in the absence of interaction with the molecules in the background matrix (*52*) varies, as the matrix concentration (and the obstruction effect by the matrix molecules) remains constant. Thus, the observed activation energy refers to this component of the microviscosity. The fact that the same activation energy was observed in all cases implies that the fluid phase viscosity is the same in all cases at the studied concentrations. The most probable cause for the difference in the kinetics in the different solutions is the difference in the obstruction effects, which can be related to the difference in the compactness of the packing of maltodextrin and sucrose molecules as well as the difference in concentration in the case of the sucrose solutions.

Table 2. Comparison of the Catalytic Parameters of PME Obtained in the Presence of 40% Sucrose at 25 and –4.5 °C in the Liquid State and at –4.5 °C in the Frozen State

conditions	K_m (g/L)	V_{max} (10^{-6} mol/(L·s))
40% sucrose, 25 °C	4.63 ± 0.82[a]	3.17 ± 0.25
40% sucrose, –4.5 °C, unfrozen	4.81 ± 1.88	0.408 ± 0.008
40% sucrose, –4.5 °C, frozen	3.53 ± 0.74	0.22 ± 0.020

[a] Standard error of regression.

III. Effects of Low Temperature and Freezing on the Catalytic Parameters of PME. The kinetic data in all cases could be described well using the Michaelis−Menten equation. The Michaelis−Menten parameters obtained in the presence and absence of ice are presented in **Table 2**. As can be observed in **Table 2**, V_{max} decreased with temperature as normally expected. In addition a decrease in V_{max} was observed in the presence of ice, indicating that freezing may affect the structural conformation of the enzyme and its catalytic efficiency. The presence of ice may also cause additional obstruction to the diffusion of the reacting molecules and may contribute to the decrease in V_{max} with freezing. Champion and others (*53*) observed the same trend in their study of the alkaline phosphatase-catalyzed hydrolysis of disodium *p*-nitrophenyl phosphate. This may contribute to the observed deviation of the kinetics of the reaction in the frozen systems (*14*) from the glass transition concept. No significant change was observed in K_m with temperature or in the presence of ice.

ABBREVIATIONS USED

η, η^o, Viscosity in the presence or absence of viscogenic additives, respectively (mPa·s); CMC, carboxymethylcellulose; D_E, D_S, diffusion coefficients of the enzyme and the substrate, respectively; D_r, sum of the diffusion coefficients of the enzyme and the substrate; D_E, dextrose equivalent; DSC, differential scanning calorimetry; k_1, k_2, k_{cat}, reaction rate constants corresponding to the enzyme−substrate association, the enzyme−substrate dissociation, and the transformation of the enzyme−substrate complex into product; k, overall reaction rate constant; PEG, poly(ethylene glycol); PME, pectin methylesterase; T, temperature (°C or K); T_g, glass transition temperature; T_g', glass transition temperature of a maximally freeze concentrated system; V_o, initial rate of de-esterification (mol/(L·s)); WLF, William, Landel, and Ferry.

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