Glass Transition and Water Effects on Sucrose Inversion by Invertase in a Lactose−Sucrose System

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Enzymatic changes are often detrimental to quality of low-moisture foods. In the present study, effects of glass transition and water on sucrose inversion in a lactose−sucrose food model were investigated. Amorphous samples were produced by freeze-drying lactose−sucrose (2:1)−invertase (20 mg invertase/49.4 g of carbohydrate) dissolved in distilled water. Sorption isotherms were determined gravimetrically at 24 °C. Sucrose hydrolysis was determined by monitoring glucose content using a test kit and the amounts of fructose, glucose, and sucrose using HPLC. The glass transition temperatures, T_g, at various water contents were measured using differential scanning calorimetry (DSC). The BET and the GAB sorption models were fitted to experimental data up to a_w 0.444 and 0.538, respectively. Water sorption and DSC results suggested time-dependent crystallization of sugars at a_w 0.444 and above. Significant sucrose hydrolysis occurred only above T_g, concomitantly with crystallization. Sucrose hydrolysis and crystallization were not likely in glassy materials.

Keywords: *Crystallization; glass transition; invertase; sucrose hydrolysis; water*

INTRODUCTION

Enzymatic reactions often cause deleterious changes in foods. In some cases, the rate of these reactions may be related to changes in the physical state, e.g., the glass transition. The formation of a glassy state results in a reduction of translational molecular motion and rates of chemical and relaxation rates for various processes, and rates of chemical reactions may become very low, as stated by Cardona et al. (1997).

Transition from the glassy into the rubbery state has the characteristics of a second-order phase transition, but the glass transition occurs over a temperature range. Moisture, the main plasticizer in foods, is an important factor affecting the food stability (Nelson and Labuza, 1994). It is well-known that plasticization decreases the glass transition temperature, T_g, which refers to the temperature range over which the transition occurs (Slade and Levine, 1991; Roos and Karel, 1991; Roos, 1993, 1995). Nelson and Labuza (1994) suggested that reactant mobility and diffusion within a matrix could be related to water activity, a_w, and glass transition. Viscous flow and diffusion above T_g of amorphous sugars, resulting in stickiness and structural collapse, precede crystallization, which occur as a function of temperature, moisture content, and time (Slade and Levine, 1991). However, relationships between glass transition and chemical and enzymatic changes have been difficult to establish. For example, the molecular arrangement of aspartame in solid PVP systems at constant temperature, pH, and buffer concentration were affected more by a_w than T_g (Bell and Hageman, 1994). Bell and Labuza (1994) considered that the T_g was the temperature above which the diffusion of a reactant increases exponentially with moisture, but the glass transition theory did not explain the rate or the rate constant maxima in the 0.6−0.8 water activity range. Buera et al. (1995) found that the rate of acid-catalyzed hydrolysis of sucrose was more dependent on pH of the surrounding medium than the T_g.

A number of studies have been carried out on the hydrolysis of sucrose by invertase. The Michaelis and Menten model is the basis for most of the theories of enzyme kinetics using sucrose/invertase system. Corresponding studies have been conducted with main regard to the enzyme−subtrate−water concentration (Kertesz, 1935; McLaren, 1963; Ruchti and McLaren, 1964; Bowski, 1971). Since then, in some studies, more interest has been paid to the effect of low moisture content on rates of chemical/biochemical reactions (Silver and Karel, 1981; Bell and Labuza, 1994; Buera et al., 1995). As the amorphous state is typical of low-moisture foods, changes from glassy to rubbery states may occur. Therefore, enzyme stability and reactivity in such systems has been studied. Schebor et al. (1995) reported that the extent of sucrose hydrolysis was affected by moisture content but its effect was not attributable to the plasticizing effect of water. Cardona et al. (1997) found that the thermal stability of invertase in reduced-moisture amorphous matrices and invertase inactivation could not be predicted on the basis of T_g. Chen et al. (1999) studied the effects of water activity and T_g on the stability and reactivity of invertase in two PVP systems. However, there are no existing data on a commonly used food system, such as the sucrose/lactose system, in which the effects of T_g and water on enzyme activity have been studied over a large a_w range.

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The purpose of this study was to investigate the effects of glass transition and water on the hydrolysis of sucrose by invertase, in an amorphous carbohydrate system.

MATERIALS AND METHODS

Preparation of the Food Model. α-Lactose monohydrate and sucrose (2:1) from Sigma (USA) were successively dissolved in distilled water (200 mL) under mild heating to obtain a clear solution. Invertase V grade from bakers yeast (Sigma, USA) (20 mg of invertase/49.4 g of carbohydrate) was added after cooling at +5 °C and mixed (cooling was essential to minimize sucrose hydrolysis during mixing). Sucrose (2 g) was dissolved in 10 mL of distilled water and 5 mg of invertase was added. After 1 h, the glucose kit was used to test the presence of glucose formed in the solution. This was to check if the invertase used truly possesses its hydrolytic function. The invertase /carbohydrate solution was rapidly prepared in 20 mL vials (5 mL aliquots) and frozen at 20 °C for 2 h. The frozen material was stored at 80 °C about 10 h and freeze-dried at a pressure <0.1 mbar for 3 days using a Lyovac GT 2, Amsco Finn-Aqua GmbH freeze-dryer (Germany). After freeze-drying, the material was stored over P_2O_5 in a vacuum desiccator to keep it anhydrous. The anhydrous samples were used for sorption isotherms, differential scanning calorimetry (DSC), and kinetic studies.

Sorption Isotherms. Sorption isotherms for the lactose/sucrose/invertase food model were determined gravimetrically at 24 °C. Triplicate samples of 1 g of the freeze-dried material in the 20 mL vials were stored over saturated salt solutions until the sample weight leveled off, a sign of the steady-state water content. The salts used were LiCl, CH_3COOH, $MgCl_2$, K_2CO_3, $Mg(NO_3)$, $NaNO_2$, and NaCl (E. Merck, Darmstadt, Germany); the respective relative humidities (RH) were 11.3, 23.9, 33.3, 44.4, 53.8, 66.2, and 76.4% (Labuza et al. 1985), giving water activity values 0.01 × RH % at equilibrium. Sample weights were measured at intervals during storage. The Brunauer−Emmett−Teller (BET) and Guggenheim−Anderson−Deboer (GAB) sorption isotherm models were fitted to the water sorption data, according to Roos (1993).

Differential Scanning Calorimetry (DSC). The glass transition temperatures for lactose/sucrose /invertase model stored at various water activities were determined using DSC (Mettler TA 4000 system with TC 15 TA processor, DSC 30 measuring cell, and STAR Thermal Analysis System version 3.1 software; Mettler-Toledo AG, Switzerland). The instrument was calibrated using *n*-pentane (mp −129.7 °C; $\Delta H = 116.7$ J/g), *n*-hexane (mp (94.0 °C; $\Delta H = 151.8$ J/g), mercury (mp −38.8 °C $\Delta H = 11.4$ J/g), distilled water (mp 0.0 °C; $\Delta H = 334.5$ J/g), gallium (mp 29.8 °C; $\Delta H = 80$ J/g), and indium (mp 156.6 °C; $\Delta H = 28.45$ J/g). Samples were prepared in 40 μL aluminum pans (Mettler ME-2733). Triplicate samples in open pans were stored in vacuum desiccators over saturated salts solutions, as for the sorption isotherm. After 24 or 44 h (depending on time for leveling off) the pans were hermetically sealed and steady-state water contents were determined gravimetrically. The samples (10−20 mg) were scanned at 5 °C/min from at least 50 °C below the glass transition temperature range with an empty pan as the reference. An immediate rescan was run for each sample to verify the endothermic baseline shift associated with the glass transition. The average onset temperature of the change in heat capacity was considered as the glass transition temperature.

Kinetic Studies. The freeze-dried materials in glass vials were ground and the amorphous powder was transferred into Eppendorf polypropylene test tubes (Greiner, Germany). The distribution was performed quickly and vials and test tubes were immediately closed after filling or removal of sample materials, to avoid moisture uptake. Sample weights were between 80 and 100 mg. The tubes were placed on supports made of cardboard and stored in desiccators under vacuum at 24 °C over saturated salt solutions (RH 23.9−76.4%). Two sets of triplicate samples in test tubes equilibrated in closed desiccators were removed at various time intervals for analysis: one set for enzymatic determination of glucose using the glucose Trinder kit, and the other for determination of glucose, fructose, sucrose, and lactose by HPLC.

Testing of Invertase Inhibition by Acetonitrile. In order to monitor the activity of invertase, it was essential to inhibit its action at any desired time, so that the sugar concentration did not change further. According to Folkes and Jordan (1996), acetonitrile deactivates any enzyme. This has been proved in this study for invertase. Thus, 20 mg/mL of sucrose in distilled water was prepared. One milliliter of freshly prepared invertase (0.5 mg/mL) solution was added to 2 mL of the sucrose solution, and 2 mL of acetonitrile (99.7%, HPLC grade, Merck, Germany) was immediately added, so that the solution concentration was acetonitrile:water (40:60). Two other mixtures were made with the same amount of the invertase solution and acetonitrile:water (50:50) and (60:40), respectively. The three preparations were kept for 30 min and filtered through a 0.45 μm Sparttan 30/B filter (Dassel, Germany). Triplicate aliquots of each filtrate were used for detection of glucose using the Trinder kit, and for detection of fructose and glucose, products of sucrose hydrolysis using HPLC. No hydrolysis product was detected by the Trinder kit analysis as well as from the HPLC. This showed that a concentration of acetonitrile higher than 40% stopped the activity of invertase.

Determination of Sucrose Hydrolysis by the Trinder Kit. Six solutions of glucose of known concentrations over the concentration range of sucrose in samples were used. A 10 μL aliquot of the solution was mixed with 3 mL of Trinder kit. The mixture was kept at room temperature for 18 min; thereafter the absorbance was measured using a Perkin-Elmer Lambda 2 UV−vis spectrometer at 505 nm. Linear regression analysis of the absorbance vs glucose concentration was made. The R^2 values were between 0.96 and 0.99. Triplicate samples of lactose/sucrose/invertase stored over saturated salt solutions were removed at intervals and dissolved each in 2 mL of acetonitrile:H_2O (60:40) (as proved to stop totally invertase action). 3 × 10 μL aliquots were taken for determination of the glucose content using the Trinder kit solution as described above. The amount of glucose in the sample was determined according to the equation

$$\frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{standard}} - A_{\text{blank}}} \times \text{concentration of standard}$$

where A_{sample}, A_{blank}, and A_{standard} are the absorbance of the sample, the absorbance of a standard solution of known concentration, and the absorbance of a blank, respectively. The coefficient of variation between samples was less than 8%.

Determination of Sucrose Hydrolysis by HPLC. High-performance liquid chromatography (HPLC) 1090 (Hewlett-Packard) with a RI detector (Hewlett-Packard) was used for the determination of fructose, glucose, sucrose, and lactose. The column used was 25 × 4.6 mm $S5NH_2$ (Spherisorb, U.K) thermostated at 40 °C. Folkes and Jordan (1996) suggested acetonitrile−water (75:25)−(85:15) as an appropriate mobile phase. We observed that at this concentration range, although the sugars eluted rapidly, peaks tailing was very significant. Therefore, acetonitrile:water (60:40) was used as mobile phase, as it provided a better peak symmetry. The flow rate was 1.8 mL/min. The external standard method was used to determine the content of fructose, glucose, sucrose, and lactose in the samples. Six solutions of known concentration of these sugars were used and linear regression analysis of each sugar was done. R^2 values of glucose, fructose, sucrose, and lactose were 0.98, 0.99, 0.97, and 0.99, respectively. Triplicate samples of the lactose/sucrose/invertase in Eppendorf tubes were taken from desiccators over saturated salt solutions, at various RH, and dissolved into 2 mL of acetonitrile:water (40:60). This solvent system was chosen for two reasons. First, lactose did not dissolved easily when the amount of acetonitrile in the solvent was higher than 40%. Second, we tested that this solution stopped the action of invertase. The injection volume

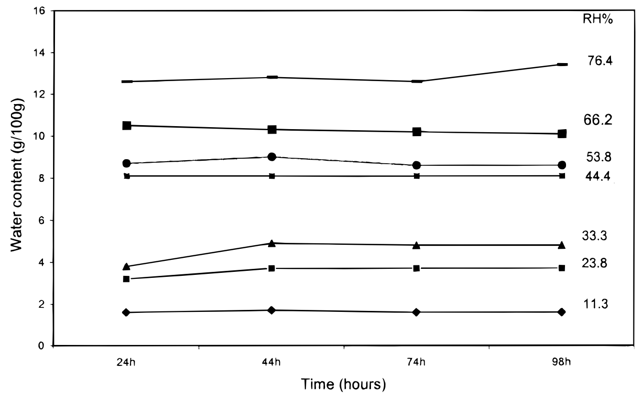

Figure 1. Water content of samples at various RH as a function of storage time.

was 10 μL. The amounts of glucose and fructose released by hydrolysis of sucrose, the remaining sucrose, and lactose per 100 g of solids were calculated according to the equation $X = (Y - b)/a$ obtained from the linear regression analysis, where X represents the unknown amount of sugar, Y is its peak area, and a and b are the slope and the intercept of the linear regression curve, respectively. The coefficient of variation was less than 5.5%. The analytical lactose content was compared to the initial concentration in samples. This was to evaluate the accuracy of the analytical procedure. Rate constants were calculated from first-order plots (Villota and Hawkes, 1989) using single regression analysis with 95% confidence limit. Points below 0.75 were not considered for the determination of these rate constants.

RESULTS AND DISCUSSION

Sorption Isotherms. Figure 1 shows the water sorption at 24 °C at various RH. The water sorption leveled off within a day at 11.3, 44.4, 53.9, and 66.2% RH. At 33.3% RH the system leveled off after 2 days. Water sorption at RH ≥ 44.4% showed a reduction in sorbed water after 24−44 h, as an indication of crystallization (Linko et al., 1982; Roos and Karel, 1992). Below this RH, water sorption indicated steady-state water contents as also observed by Jouppila and Roos (1994). This agreed with Vuataz (1988) who reported that α-lactose monohydrate above a_w 0.57 crystallized at room temperature. As the system contained 2/3 of lactose, this sugar might be responsible for the crystallization suggested by the sorption curves at RH > 44.4%. This observation is in agreement with Jouppila and Roos (1994) who reported crystallization of lactose in milk powders within 24 h at RH > 50%.

Average steady-state water contents of all samples were used in fitting the BET and GAB sorption models to the data over a_w 0.113−0.444 and 0.113−0.538, respectively. The BET constant, C, was 15.16 and the monolayer value, m_m, 3.58 g of H_2O/100 g of solids. The GAB constants, C' and K were 15.16 and 0.53, respectively, and the GAB monolayer value, m_m, was 2.85 g of H_2O /100 g of solids. The GAB model has been shown to fit experimental data over almost the whole a_w range (Van den Berg et al., 1975), and to be applicable to predict water sorption of most foods (Roos, 1995; Jouppila and Roos, 1994; Maskan et al., 1997). However, Bizot (1993) pointed out that the GAB model does not apply to all shapes of isotherms, and indicated the example of starch for which this model did not fit the data at a_w 0.6−0.7 due to crystallization. Our results seem to obey the same restriction. Indeed, crystallization seemed to occur at room temperature at a_w 0.4−0.7, causing a discontinuity during the sorption process as observed in the shape of the sorption isotherm (Figure 2).

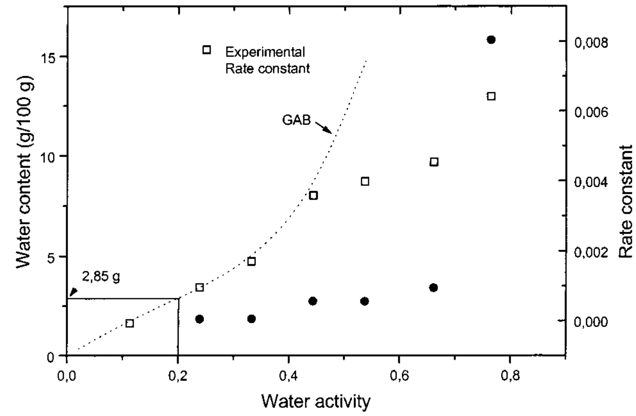

Figure 2. Relationships between water activity, rate constant, and water content.

Table 1. Glass Transition Temperature (T_g) and Crystallization Temperature at Various a_w

a_w	water content (g/100 g)	T_g (°C) onset	T_g (°C) midpoint	crystallization onset (°C)
0	0	72.60 ± 0.44	81.6 ± 0.38	145.30 ± 0.47
0.239	1.63 ± 0.23	35.38 ± 2.57	40.07 ± 2.57	102.01 ± 2.30
0.333	3.14 ± 0.05	21.32 ± 2.67	29.46 ± 3.06	87.63 ± 2.02
0.444	4.85 ± 0.05	4.82 ± 0.50	12.70 ± 0.23	63.81 ± 0.23
0.538	8.17 ± 0.19	0	8.18 ± 0.16	
0.662	8.86 ± 0.12			
0.764	12.99 ± 0.14			

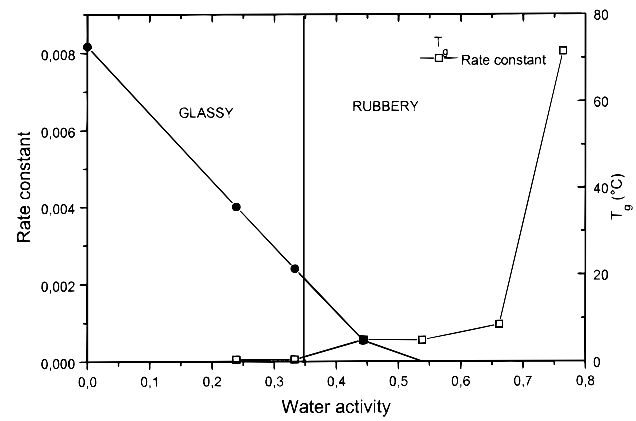

Figure 3. Relationships between water activity, glass transiton temperature T_g, and reaction rates.

DSC Analysis. The onset temperature of the glass transition (T_g) of the lactose/sucrose/invertase system, and the onset of the crystallization temperature decreased with increasing water activity and water content (Table 1). Above a_w 0.538 it was very difficult to determinine the T_g. This was probably due to interference from crystallization that probably had taken place during sample storage under saturated solutions as suggested by the water sorption isotherm. This is in agreement with Hagiwara and Hartel (1996) who reported that crystallization took place during sample storage. Thus, at this a_w range, no exact values of T_g and crystallization could be found. Values of T_g and crystallization temperatures of single sugars, such as lactose, sucrose, and glucose, have been reported (Roos, 1991; Roos, 1993). The system studied here contained lactose and sucrose and eventually fructose and glucose formed by sucrose inversion. Figure 3 shows the rela-

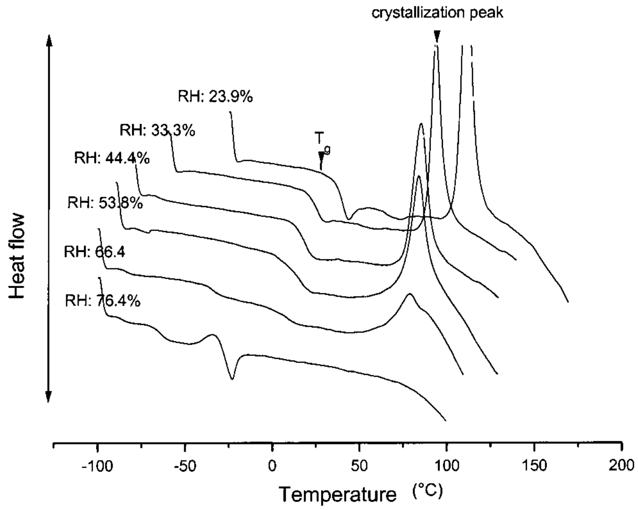

Figure 4. DSC thermal curves for amorphous lactose/sucrose/invertase samples, after equilibration at different water activities (24 °C).

tionships between water activity, T_g, and reaction rates, and Figure 4 shows DSC thermal curves of samples at various a_w.

Sucrose Hydrolysis. The extents of sucrose hydrolysis (g/100 g solids/h) were determined at various a_w. Figure 5 shows the curves of the extent of sucrose hydrolysis A, B, C, and D corresponding to glucose content by enzymatic assay (test kit), glucose, fructose, and sucrose remaining, by HPLC, respectively. Figure 6 shows an example of the first-order plot of glucose content vs time. The curves for sucrose hydrolysis as determined by glucose kits and HPLC followed the same trend. No hydrolysis was observed at a_w 0.239 and 0.333. Above a_w 0.333 sucrose hydrolysis was moderate with a corresponding rate at a_w 0.444 and 0.538. Above a_w 0.538 the rate of sucrose hydrolysis increased with a_w. At a_w 0.764, the rate was highly significant, and the reaction completed after 25 days approximately. The variation between the initial amount of lactose in sample and the calculated values from the HPLC was between 0.36 and 4.9%. This low variation indicated that the analytical procedure was relevant. The first-order reaction rate and rate constants determined from the kinetics plots with 95% confidence limits are displayed in Tables 2 and 3, respectively. The rate constants were very low in the a_w 0.239−0.538 range. Above a_w 0.538 the rate constant increased markedly with increasing water activity. Silver and Karel (1981) found that not sucrose hydrolysis was measurable in Avicel/sucrose/invertase, agar/sucrose/invertase, and starch/sucrose/invertase model systems at a_w lower than 0.58. That is the impression that emerges at first sight from the curve of kinetics of hydrolysis products vs time. However, the values of the reaction rates and the rate constants, showed that a slight hydrolysis occurred at a_w 0.444 and 0.538 concomitantly with sugars crystallization. This result did not totally meet those reported by Chen et al. (1999), who indicated that no sucrose hydrolysis was found at a_w < 0.62. Above a_w 0.662, our results agreed with those of Silver and Karel (1981) who

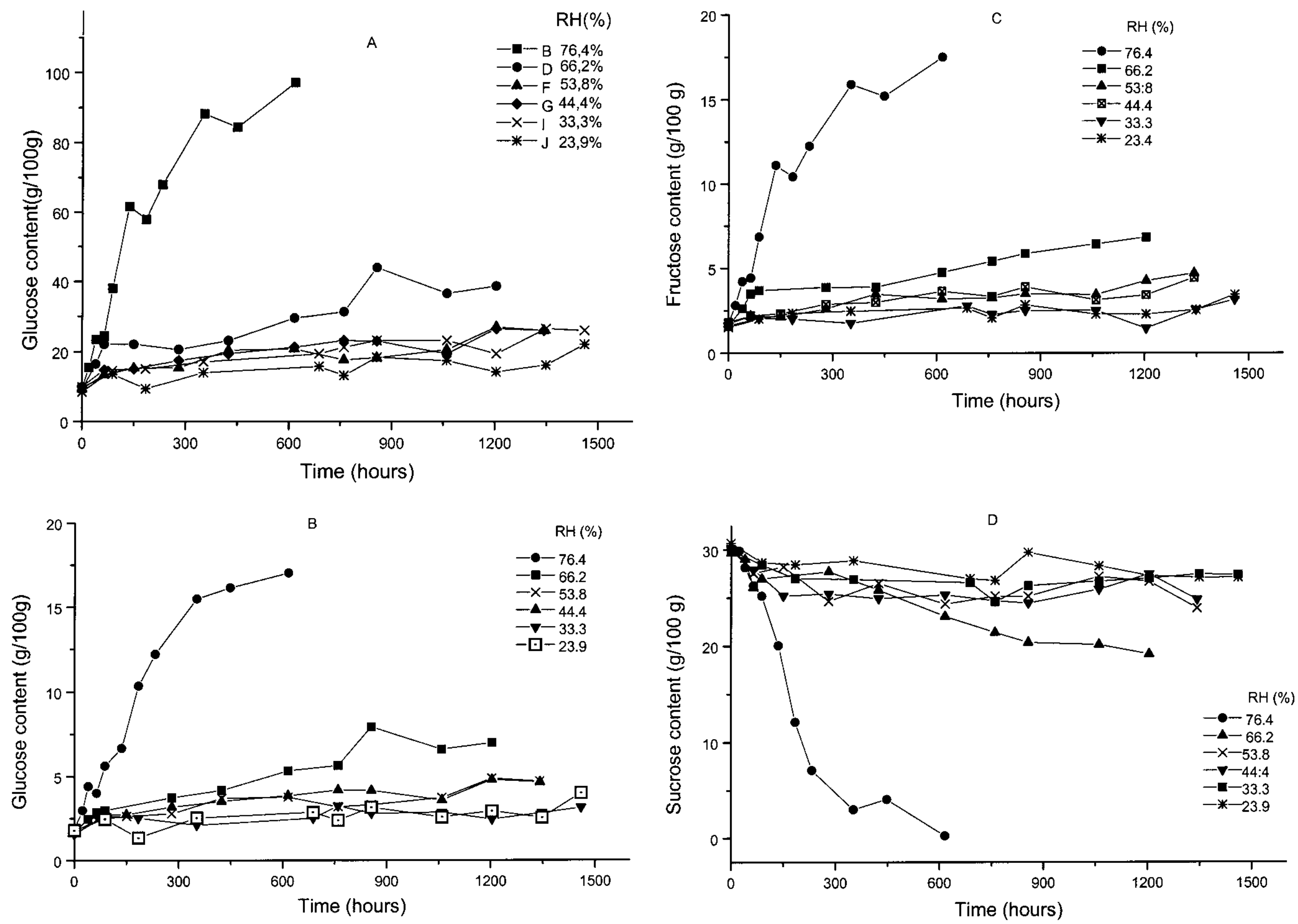

Figure 5. Extent of sucrose hydrolysis in g/100 g (dry weight) as a function of time for glucose by test kit (A), and by HPLC for glucose (B), fructose (C), and sucrose (D).

Table 2. Rate Constants with 95% CL for Sucrose Hydrolysis at Various a_w

	rate constant (h^{-1})			
a_w	glucose kit	glucose HPLC	fructose HPLC	sucrose HPLC
0.239	0.00005 ± 0.00001	0.00005 ± 0.00001	0.00005 ± 0.00002	0.00006 ± 0.00001
0.333	0.00005 ± 0.00001	0.00005 ± 0.00002	0.00005 ± 0.00001	0.000060 ± 0.00001
0.444	0.0005 ± 0.00002	0.00050 ± 0.0001	0.0006 ± 0.0001	0.00060 ± 0.0001
0.538	0.0005 ± 0.0001	0.0006 ± 0.0001	0.0006 ± 0.0001	0.0005 ± 0.0002
0.662	0.0009 ± 0.0002	0.0009 ± 0.0002	0.0009 ± 0.0001	0.0009 ± 0.0001
0.764	0.0079 ± 0.0003	0.0089 ± 0.0003	0.0081 ± 0.0003	0.0070 ± 0.0004

Table 3. Rate of Sucrose Hydrolysis Calculated from Glucose, Fructose, and Sucrose Concentrations at Various a_w with 95% CL

			HPLC					
	glucose kit		glucose		fructose		sucrose	
a_w	g/100 g h^{-1}	mmol/100 g h^{-1}	g/100 g h^{-1}	mmol/100 g h^{-1}	g/100 g h^{-1}	mmol/100 g h^{-1}	g/100 g h^{-1}	mmol/100 g h^{-1}
0.239	1.59×10^{-3}	8.87×10^{-3}	1.16×10^{-3}	6.45×10^{-3}	1.017×10^{-3}	5.65×10^{-3}	3.026×10^{-3}	8.884×10^{-3}
0.333	1.31×10^{-3}	7.30×10^{-3}	1.35×10^{-3}	7.50×10^{-3}	1.051×10^{-3}	5.83×10^{-3}	3.053×10^{-3}	8.917×10^{-3}
0.444	3.75×10^{-2}	2.08×10^{-2}	3.65×10^{-2}	2.03×10^{-2}	3.750×10^{-2}	2.08×10^{-2}	8.116×10^{-2}	2.608×10^{-2}
0.538	4.64×10^{-2}	2.57×10^{-2}	3.75×10^{-2}	2.08×10^{-2}	3.314×10^{-2}	1.73×10^{-2}	8.928×10^{-2}	2.608×10^{-2}
0.662	22.98×10^{-2}	7.28×10^{-2}	34.375×10^{-2}	8.41×10^{-2}	31.250×10^{-2}	6.87×10^{-2}	54.00×10^{-2}	6.886×10^{-2}
0.764	58.98×10^{-1}	3.22×10^{-1}	53.544×10^{-1}	2.97×10^{-1}	42.697×10^{-1}	2.37×10^{-1}	79.546×10^{-1}	2.32×10^{-1}

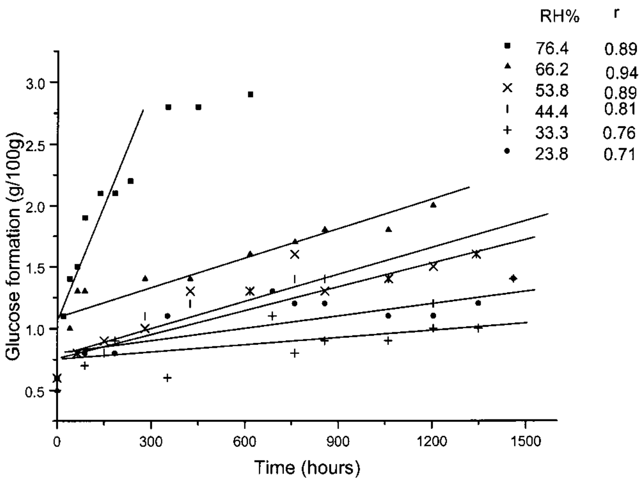

Figure 6. Example of first-order plots of sucrose hydrolysis.

reported a maximum reaction rate or rate constant at 0.6−0.7 water activity range.

The BET m_m we found corresponded to a_w between 0.25 and 0.33. As Roos (1993) emphasized, the critical water content is often slightly higher than the BET m_m, the critical water content corresponds to a_w close to 0.33, value above which hydrolysis becomes noticeable. Whitaker (1995) considered the same water activity when he described the effect of water on enzyme activity. Similarly, Acker (1969) reported that phospholipase activity on lecithin was not noticeable after almost 2 months until a_w reached 0.35. Our results suggest that a correlation between water activity and invertase activity exists, and sucrose hydrolysis and crystallization take place mainly in the rubbery state.

Whitaker (1995) stated that water plays at least four important functions in all enzyme-catalyzed reactions: folding of the protein, acting as a transport medium for the substrate and enzyme, hydration of the protein, and ionization of prototropic groups in the active sites of the enzyme. It is also known from the polymer science approach (Slade and Levine, 1994) that the glassy state of the amorphous matrix in which the reactants are embedded could be responsible for the diffusion-limited rates of the chemical reactions that take place at low moisture content. This diffusional limitation disappears and a drastic increase in the reaction rate may be noticeable as the glass−rubber transition occurs due to increased moisture content (Buera et al., 1995). Water, therefore, may facilitate the structural organization and provide a better diffusibility to the enzyme and the substrate through the matrix. Figure 3 shows the reaction rates as related to the T_g and a_w. Our results show that there is no sudden change in reaction rate at $T - T_g = 0$, corresponding approximately to a_w 0.333, but the effective change appears above 0.538 a_w, concomitant with crystallization. Our T_g determined below a_w 0.538 confirmed this tendency. Crystallization may favor the diffusibility and mobility of the enzyme or the substrate, increasing, therefore, the rate of sucrose hydrolysis in the water solute phase.

CONCLUSIONS

Sucrose hydrolysis was perceptible at a_w 0.444. Above this value sucrose inversion increased with water activity and reached a maximum at a_w 0.764. It was also observed that sucrose inversion did not occur in the glassy state but rather in the rubbery state where crystallization also occurred. The increased sucrose hydrolysis may be presumably due to increase molecular mobility of the enzyme or substrate molecules in the phase-separated, partially crystalline system. Further studies should be addressed to mobility of the enzyme and substrate through the glassy−rubbery transition.

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