Invertase Storage Stability and Sucrose Hydrolysis in Solids As Affected by Water Activity and Glass Transition

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Research continues to differentiate the impact of water activity (a_W) and the glass transition temperature (T_g) on chemical reactions. Invertase with and without sucrose was incorporated into low and high molecular weight poly(vinylpyrrolidone) model systems (PVP−LMW and PVP−K30, respectively). Invertase activity and sucrose hydrolysis were monitored during storage at a_W = 0.32−0.75 and 30 °C. Pseudo-first-order rate constants for activity loss in PVP−K30 were not different, regardless of the system being glassy or rubbery. In PVP−LMW, invertase stability decreased with increasing a_W. An a_W > 0.62 was required for sucrose hydrolysis to occur in PVP−LMW. PVP molecular weight appeared to affect invertase stability and reactivity. No dramatic change around T_g was found in either invertase stability or sucrose hydrolysis, suggesting that T_g-dictated mobility has a minimal effect on these reactions in amorphous solids.

Keywords: *Glass transition; water activity; invertase; enzyme; stability*

INTRODUCTION

The stability of foods, drugs, and biological materials is of interest to both manufacturers and consumers. Recently, research has increased to better understand the effects of water activity (a_W) and the glass transition temperature (T_g) on the chemical stability of amorphous solids.

A relationship between water activity and chemical/biochemical reactions was recognized more than two decades ago. The effects of a_W on reaction kinetics of food deterioration, food product quality, and enzyme reactivity and stability have been reviewed (Labuza, 1980; Rockland and Nishi, 1980; Schwimmer, 1980). Water activity has proven useful as a parameter for evaluating and predicting the chemical and microbial stability of foods.

Although a_W has traditionally been used to evaluate the effects of water on chemical reactions, it has been suggested that water may influence reactions via a change in the glass transition temperature and thus reactant mobility (Slade and Levine, 1991). The principles of glass transition have been used to explain many physical attributes of food systems; these include cereal product texture (Simatos and Karel, 1988; LeMeste et al., 1992; Nicholls et al., 1995), crystallization (Roos and Karel, 1990; Hagiwara and Hartel, 1996), encapsulated flavor release (Whorton and Reineccius, 1995), and powder stickiness (Chuy and Labuza, 1994). Some of the physical changes were not solely explained by T_g (Hagiwara and Hartel, 1996; Nicholls et al., 1995).

The use of poly(vinylpyrrolidone) (PVP) model systems has allowed the effects of a_W and T_g to be differentiated (Bell and Hageman, 1995). The molecular rearrangement of aspartame in solid PVP systems at constant temperature, pH, and buffer concentration was affected by a_W rather than T_g (Bell and Hageman, 1994). Similarly, acid-catalyzed sucrose hydrolysis was influenced more by the pH of the internal aqueous microenvironment rather than T_g (Buera et al., 1995). However, glycine loss via the Maillard reaction was found to be influenced by T_g, PVP molecular weight, and matrix collapse (Bell et al., 1998). Brown pigment formation appeared to be affected by T_g and PVP type (Bell, 1995; Bell et al., 1998). Other studies have suggested nonenzymatic browning is related to T_g, matrix crystallization, and matrix collapse (Karmas et al., 1992; Karmas and Karel, 1994; Buera and Karel, 1995).

Invertase (EC 3.2.1.26) has been the subject of numerous studies, including those that led to the famous Michaelis−Menten equation. Silver and Karel (1981) evaluated the extent of invertase-mediated sucrose hydrolysis as a function of water activity in microcrystalline cellulose model systems. Hydrolysis occurred at measurable rates at and above a_W = 0.58. Limited data suggested that invertase stability was maintained during the experiments. Monsan and Combes (1984) showed that invertase activity in sucrose solutions at 53 °C decreased with increasing concentrations of sucrose, which was presumably attributable to reduced water activities. Larreta-Garde et al. (1987) demonstrated that invertase thermal stability in solutions at 55−71 °C increased as a_W decreased. In addition, sorbitol (which lowers a_W) had no observed stabilizing effect on invertase below 45 °C but had a stabilizing effect above 45 °C when compared to a solution without sorbitol (Larreta-Garde et al., 1987). These studies occurred prior to the application of glass transition concepts to food chemistry. As such, the effects of glass transition on invertase activity and stability were not evaluated.

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The thermal inactivation of invertase in dry amorphous systems at elevated temperatures was not sufficiently explained using glass transition theory (Schebor et al., 1996, 1997). Invertase thermal stability in reduced-moisture trehalose, maltodextrin, and PVP model systems was reported recently (Cardona et al., 1997). Again, invertase inactivation at high temperatures could not be predicted from T_g data. However, the moisture content was not held constant in their systems and the water activity at 90 °C was not specified, making the effects of a_W and T_g difficult to distinguish.

The primary invertase study utilizing ambient storage conditions evaluated only the effect of a_W on sucrose hydrolysis (Silver and Karel, 1981). The other studies, which provided limited data on the effect of a_W on invertase stability in solutions (Monsan and Combes, 1984; Larreta-Garde et al., 1987), were carried out at elevated temperatures. The effects of T_g on invertase stability were also evaluated at high temperatures (Schebor et al., 1996, 1997; Cardona et al., 1997). Invertase may not respond similarly to an acute thermal stress as compared to the stresses associated with longer term storage at lower temperatures. As shown by Larreta-Garde et al. (1987), invertase reacts differently to the presence and absence of sorbitol depending on whether the temperature is 25 or 65 °C. Conformational entropy and hydrophobic interactions change as temperature increases. At 70−80 °C, significant structural changes occur in most proteins, and reactions can occur that do not normally occur at lower temperatures (Stanley and Yada, 1992). Thus, the effects of a_W and T_g on invertase stability may differ between ambient and high-temperature conditions.

The stability and activity of enzymes in reduced-moisture solids under storage conditions as impacted by a_W and T_g need a more comprehensive investigation. The objective of our project was to systematically evaluate both the storage stability and the reactivity of invertase at ambient temperature in reduced-moisture solid PVP model systems, in which the effects of a_W and T_g can be determined independently.

MATERIALS AND METHODS

Model System Description. PVP, a polar, water-soluble polymer, was used as the inert matrix for evaluating the impact of a_W and T_g on the stability and activity of invertase. Bell and Hageman (1995) showed that the moisture sorption isotherms for different molecular weight PVPs were virtually identical. At a given a_W, different PVP types have similar moisture contents but different T_g values, depending upon molecular weight. Thus, the impact of a_W and T_g on enzyme stability and activity can be examined by using PVP model systems in which a_W and moisture contents can be maintained while the T_g is altered by using PVPs of different molecular weights.

Enzyme and Chemicals. Invertase (EC 3.2.1.26, grade VII, from baker's yeast, 500 units/mg), PVP−K30 (average molecular weight ≈40000), PVP−K15 (average molecular weight ≈10000), 1-methyl-2-pyrrolidone, glucose Trinder kit, KOH pellets, and sucrose were purchased from Sigma (St. Louis, MO). $NaH_2PO_4 \cdot H_2O$ was obtained from Fisher (Fair Lawn, NJ).

Preparation of Model System. The model system was prepared according to the method of Bell and Hageman (1994). PVP−K30 and PVP−K15 were dissolved separately in deionized water. The solutions were dialyzed overnight in deionized water through a 3500 MWCO dialysis bag to separate the low molecular weight components. The dialyzed PVP−K30 and dialysate (i.e., PVP−LMW; molecular weight <3500) were lyophilized for 4 days at room temperature under a vacuum of <13 Pa. The dialyzed PVP−K15 was not used in this study. The lyophilized material was ground into powder and stored in a desiccator containing anhydrous calcium sulfate.

To prepare PVP/invertase systems, 25 g of lyophilized PVP−LMW or PVP−K30 powder was dissolved in 50 mL of deionized water. To this solution was added 1 mL of invertase solution (4 mg/mL). The resultant solution was flash frozen by dripping into liquid nitrogen using a 22 gauge needle. The frozen pellets were lyophilized for 3 days at room temperature under a vacuum of <13 Pa. The lyophilized material was ground into a powder and stored in a vacuum desiccator containing anhydrous calcium sulfate.

To prepare PVP/invertase/sucrose systems, 23 g of lyophilized PVP−LMW or PVP−K30 powder was dissolved in 50 mL of deionized water. To this solution was added 1 mL of invertase solution (4 mg/mL). A sucrose solution (0.2 g/mL) was prepared separately. Both of the above solutions were kept on ice to reduce sucrose hydrolysis during mixing. The PVP/invertase solution was quickly mixed with 10 mL of the sucrose solution. The resultant solution was flash frozen and freeze-dried as discussed above.

Equilibration of PVP/Invertase and PVP/Invertase/Sucrose Systems. Triplicate samples of 100 mg of PVP/invertase or 200 mg of PVP/invertase/sucrose were equilibrated in evacuated desiccators at 30 °C for 1 week over saturated salt solutions at the following water activities: 0.32 [$MgCl_2$], 0.44 [K_2CO_3], 0.53 [$Mg(NO_3)_2$], 0.62 [$CoCl_2$], 0.70 [$SrCl_2$], and 0.75 [NaCl]. At various time intervals after equilibration, samples were removed from the desiccators for analysis, with the first one designated time 0. Desiccators were re-evacuated immediately after sample removal. The moisture contents were determined gravimetrically as moisture adsorbed into the dry system.

Invertase Assay. PVP/invertase samples were dissolved in 1 mL of 0.36 M phosphate buffer at pH 4.2. One milliliter of sucrose solution (40 mg/mL, freshly prepared) was added to start the reaction. After 6 min, 3 mL of 0.1 N ice-cold KOH was added to inactivate the enzyme, and the solution was kept on ice. A 10 μL aliquot of the above solution was mixed with 1 mL of Trinder solution. The mixture sat at room temperature for 18 min, after which time absorbance was read at 505 nm using a DU 640 spectrophotometer (Beckman, Fullerton, CA). A standard glucose curve (A_{505nm} versus [glucose]) was also determined for each day's analysis. One unit is designated as 1 μmol of glucose formed/min at 25 °C. The coefficient of variation was <6% for this analysis.

Invertase Stability in Solutions. To further study the effects of PVP-type on invertase stability, invertase solutions were prepared in 1-methyl-2-pyrrolidone (similar to the PVP monomer), PVP−LMW, PVP−K30, and buffer. Two grams of 1-methyl-2-pyrrolidone, PVP−LMW, PVP−K30, or water (control) was added to 20 mL of 0.36 M phosphate buffer at pH 4.2. One milliliter of invertase solution (1 mg/mL) was then added to each solution. One milliliter of the resultant solution was assayed immediately for invertase activity and recorded as day 0. The flasks containing the solutions were sealed with Parafilm and placed at 30 °C. Invertase activity was assayed daily for 6 days. Water activities of these solutions were determined using an AquaLab CX-2 (Decagon, Pullman, WA).

Determination of Sucrose Hydrolysis Rate. PVP/invertase/sucrose samples were dissolved in 2 mL of 0.1 N ice-cold KOH. Samples were kept in an ice−water bath during dissolving to minimize any additional hydrolysis. Glucose concentration was determined the same way as in the invertase assay above. The amount of glucose formed per gram of solid was calculated. The coefficient of variation was <6% for this analysis.

Data Analysis. The loss of invertase activity and the production of glucose were modeled using pseudo-first-order kinetics. Rate constants with 95% confidence limits (CL) were calculated using computerized least-squares analysis (Labuza and Kamman, 1983).

Differential Scanning Calorimetry (DSC). T_g values for the PVP systems were determined using a 2910 differential

Table 1. Rate Constants for Invertase Loss in PVP Systems at 30 °C As Influenced by Water Activity (a_W), Moisture, and Onset Glass Transition Temperature (T_g)

system	a_W	moisture (% db)	T_g (°C)	state of the system at 30 °C	rate constant with 95% CL (d^{-1})
PVP−LMW	0.32	9.1	41	glassy	0.018 ± 0.004
PVP−K30	0.32	11.3	68	glassy	0.005 ± 0.002
PVP−LMW	0.44	13.4	31	near T_g	0.019 ± 0.004
PVP−K30	0.44	16.0	62	glassy	0.005 ± 0.002
PVP−LMW	0.53	17.0	12	rubbery	0.029 ± 0.003
PVP−K30	0.53	18.8	54	glassy	0.005 ± 0.002
PVP−LMW	0.62	23.1	5	rubbery	0.040 ± 0.005
PVP−K30	0.62	23.0	33	near T_g	0.006 ± 0.002
PVP−LMW	0.70	29.7	2	rubbery	0.051 ± 0.021
PVP−LMW	0.75	34.9	−19	rubbery	0.049 ± 0.007
PVP−K30	0.75	34.9	7	rubbery	0.007 ± 0.002

scanning calorimeter (TA Instruments, New Castle, DE) calibrated with indium. Samples (10-20 mg) were hermetically sealed into aluminum DSC pans. The scan was run at 5 °C/min over an appropriate temperature range. Duplicates and rescans verified the endothermic baseline shift associated with T_g. Although the glass transition occurs over a temperature range, the results were reported as the onset temperature and were similar to those obtained previously (Karmas et al., 1992; Buera et al., 1992; Bell and Hageman, 1995).

RESULTS AND DISCUSSION

Invertase Storage Stability. Table 1 shows the water activity, moisture content, and T_g of the experimental systems, which were similar to those reported previously (Bell and Hageman, 1994). At constant water activity, both PVP−LMW and PVP−K30 had similar moisture contents but different T_g values. Thus, the individual effects of water activity and glass transition temperature on invertase storage stability can be studied.

Figure 1 shows the pseudo-first-order kinetic plots for invertase stability at a_w = 0.53 and 30 °C as affected by PVP type. Pseudo-first-order rate constants with the 95% CL as determined from the kinetic plots are listed in Table 1. Lower rate constants correlate to greater enzyme stability. Thus, invertase was more stable in PVP−K30 than in PVP−LMW (Figure 1 and Table 1), suggesting mobility may be affecting enzyme stability.

Figure 2 shows the pseudo-first-order rate constants for invertase activity loss as affected by the distance from the glass transition temperature. Mobility-dependent reactions are expected to have a sharp increase in rate as the immobile glassy system converts into a more rubbery system, based on ESR mobility data (Roozen et al., 1991). However, neither PVP system showed a dramatic change in the rate constant around the glass transition ($T - T_g = 0$). Rate constants were not different in PVP−K30, regardless of whether the system was glassy or rubbery. In PVP−LMW, rate constants increased smoothly in the rubbery state, with the overall change being ∼3-fold. Although there was a 100-fold increase of the rotational mobility of a small ESR probe (tempol) at T_g (Roozen et al., 1991), our results are more similar to the 4-fold increase of the aspartame degradation rate constant around T_g (Bell and Hageman, 1994). The rate constant appeared to plateau around $T - T_g$ = 25 °C or a_W = 0.62. The small change in the rate constant near the glass transition suggests that T_g may not directly explain invertase storage stability, which is similar to that found for invertase thermal stability in solids at 90 °C (Schebor et al., 1996, 1997; Cardona et al., 1997). The mobility of small molecules, such as glucose and glycine, increases as glassy systems become rubbery, as indicated by the increase in reaction rates for glycine loss and Maillard browning (Bell et al., 1998). For larger molecules, such as invertase, one may be required to move further into the rubbery state (e.g., $T - T_g$ = 20 °C) before molecular mobility is sufficient to promote enhanced enzyme inactivation. Thus, although T_g may not be an absolute indicator of invertase activity loss, molecular mobility may still be influencing the enzyme's stability.

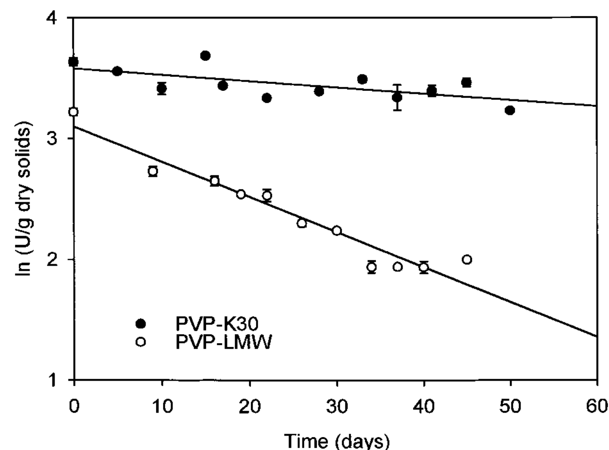

Figure 1. Pseudo-first-order plots for invertase activity loss at a_W = 0.53 and 30 °C.

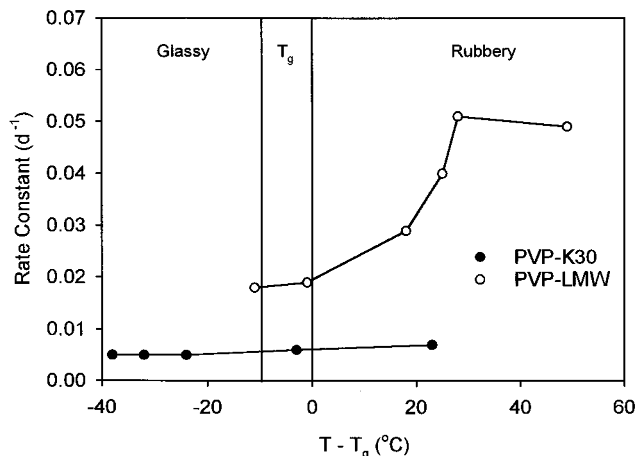

Figure 2. Rate constants for invertase activity loss at 30 °C as a function of the distance from the onset glass transition temperature.

Figure 3 shows invertase stability as affected by water activity in the form of a Q_A plot, where the natural log of half-life in days is plotted as a function of a_W. From the slope of the plot, the Q_A value can be calculated, which gives an indication of the effect of water activity on invertase stability. The reaction in PVP−LMW has a Q_A of 1.3, which means for each 0.1 a_w increase, the stability decreases by 30%. The Q_A value in PVP−K30 was only 1.07 (7% decrease in stability per 0.1 a_W increase). Different invertase stabilities at constant a_W and Q_A values in different PVP types indicate a_W, like T_g, is not sufficient to explain invertase storage stability. These results suggest that the type of PVP may be influencing enzyme stability.

To further study the effect of PVP type on invertase stability, invertase activity was monitored in solutions

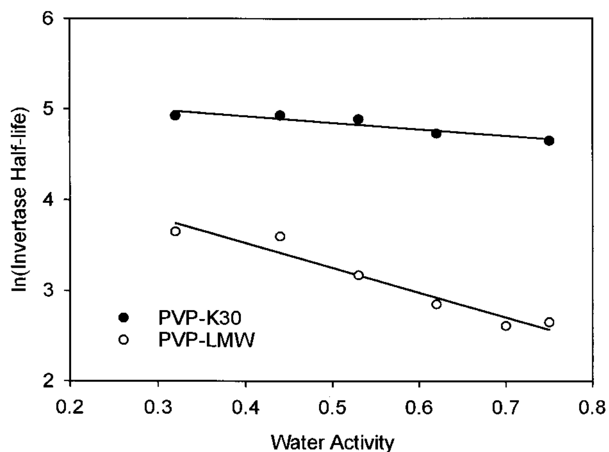

Figure 3. Q_A plot for invertase activity loss in PVP at 30 °C.

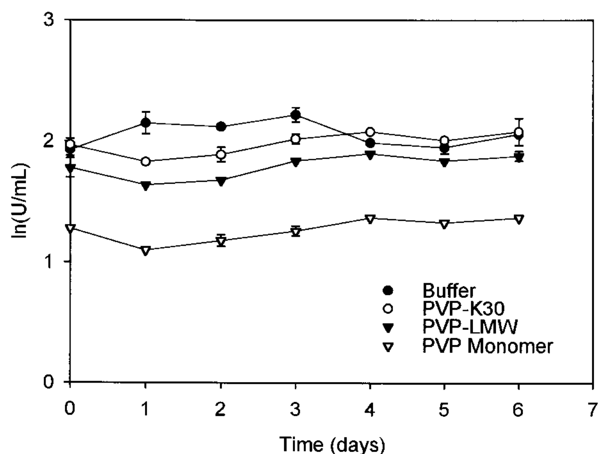

Figure 4. Invertase activity in solution at 30 °C.

of 1-methyl-2-pyrrolidone (similar to the PVP monomer and will subsequently be called PVP monomer), PVP−LMW, PVP−K30, and buffer (Figure 4). The water activities of the buffer, PVP−LMW, and PVP−K30 solutions were all between 0.982 and 0.986; the water activity of the PVP monomer solution was lower, being 0.971. At day 0, invertase activity was significantly lower in the monomer (MW = 99) than in the other solutions. Although the lower enzymatic activity in the PVP monomer solution could be attributed to its lower water activity, as shown by Monsan and Combes (1984) for invertase in sucrose solutions, water activity does not explain the difference between invertase activities in PVP−LMW and PVP−K30 solutions. The enzyme activities in PVP−K30 and control were similar but decreased with decreasing PVP molecular weight. It appears that PVP molecules may be interacting with invertase to partially deactivate the enzyme. However, in all four solutions, similar invertase activity profiles were observed, with no significant change over time.

Invertase is a glycoprotein with 50% (w/w) carbohydrate. The protein moiety of the enzyme has two identical subunits of ∼60 kDa (Taussig and Carlson, 1983). The smaller PVP molecules may interact with the surface and/or active site of the enzyme through hydrogen bonding. These interactions may disturb the enzyme such that its activity is reduced. More interactions with the enzyme surface and/or active site could be established with smaller molecules. This would explain the lower initial invertase activity in monomer solution than in PVP−LMW solution. Being a bulky molecule, steric hindrance may prevent PVP−K30 from interacting with the enzyme active site; thus, the invertase activity was maintained at the same level as in the buffer solution. Once the quick interactions between PVP and enzyme molecules were established, the enzyme maintained its activity at the new level, and no further activity loss was observed. More research, however, is necessary to prove this hypothesis.

Table 2. Rate Constants for Sucrose Hydrolysis in PVP Systems at 30 °C As Influenced by Water Activity (a_W), Moisture, and Onset Glass Transition Temperature (T_g)

system	a_W	moisture (% db)	T_g (°C)	state of the system at 30 °C	rate constant with 95% CL (d^{-1})
PVP−LMW	0.32	7.7	32	near T_g	−0.003 ± 0.001
PVP−K30	0.32	10.2	65	glassy	0.002 ± 0.001
PVP−LMW	0.44	13.2	23	rubbery	−0.003 ± 0.001
PVP−K30	0.44	13.3	50	glassy	0.001 ± 0.001
PVP−LMW	0.53	16.0	8	rubbery	−0.003 ± 0.001
PVP−K30	0.53	16.2	35	near T_g	0.002 ± 0.001
PVP−LMW	0.62	21.0	2	rubbery	0.000 ± 0.001
PVP−K30	0.62	21.4	26	rubbery	0.001 ± 0.001
PVP−LMW	0.70	28.2	−3	rubbery	0.023 ± 0.004
PVP−K30	0.70	28.3	20	rubbery	0.002 ± 0.003
PVP−LMW	0.75	33.5	−24	rubbery	0.048 ± 0.006
PVP−K30	0.75	32.9	−2	rubbery	0.012 ± 0.003

In reduced-moisture systems, the hypothesized initial effect of PVP molecular size was again observed (Figure 1). During the preparation of the experimental systems, PVP and invertase solutions were mixed before freeze-drying, which allows for the initial interactions between PVP and invertase as discussed above. Thus, higher invertase activity was found in PVP−K30 than in PVP-LMW after freeze-drying. However, on the basis of solution data, it appears that after the initial interaction, this PVP−invertase interaction does not impact residual stability. Rate constants in PVP−K30 were always lower than those in PVP−LMW at constant a_W, which implies that destabilizing invertase in solids during storage may require some movement (conformational change). Although T_g may not be directly impacting inactivation, the bulky PVP−K30 matrix may be physically blocking enzyme movement that promotes inactivation. This hypothesis is consistent with that made by Bell et al. (1998), which stated that PVP−K30 appeared to block glucose and glycine movement and subsequent reactivity and the observed reduction of recrystallization rates in ice cream by high molecular weight gums (Hagiwara and Hartel, 1996).

Sucrose Hydrolysis. Table 2 shows the water activity, moisture content, and T_g of the experimental systems containing sucrose, which are similar to their counterparts in invertase stability studies. The slightly lower T_g can be attributed to sucrose acting as a plasticizer. It is noted that water is in excess even at a_W = 0.32 for sucrose hydrolysis.

Figure 5 shows the pseudo-first-order kinetic plots for sucrose hydrolysis at a_w = 0.75 as affected by PVP type. Pseudo-first-order rate constants with the 95% CL were determined from the kinetic plots and are listed in Table 2. It should be noted that rate constants in some systems were small negative numbers due to the negligible reaction rates and experimental variability; these do not suggest that glucose was decomposing or recombining to form disaccharides.

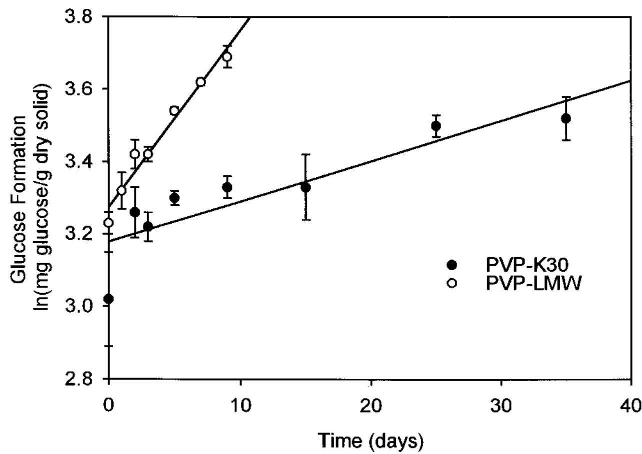

Figure 5. Pseudo-first-order plots for sucrose hydrolysis at $a_W = 0.75$ and 30 °C.

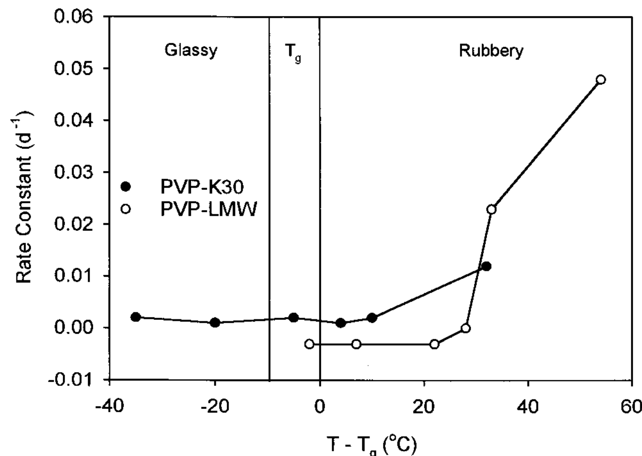

Figure 6. Sucrose hydrolysis rate constant at 30 °C as a function of the distance from the onset glass transition temperature.

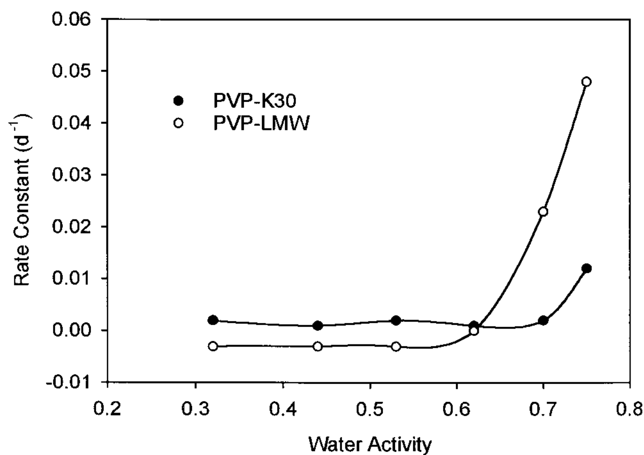

Figure 7. Rate constants for sucrose hydrolysis at 30 °C as affected by water activity.

Figure 6 shows the pseudo-first-order rate constants for sucrose hydrolysis as affected by the distance from the glass transition temperature. Again, no dramatic change of rate constant around the glass transition ($T - T_g = 0$) was observed. An increase in rate constant occurred well into the rubbery state ($T - T_g = 30$ °C). Figure 7 shows the pseudo-first-order rate constants of sucrose hydrolysis as affected by water activity. There was no reaction up to $a_W = 0.62$. Once a_W was >0.62, rate constants increased greatly with increasing water activity. Whereas Silver and Karel (1981) showed that measurable sucrose hydrolysis occurred at $a_W = 0.58$, our results suggest a higher water activity is required. Differences in sucrose concentration, moisture content, or model system morphology (i.e., crystalline versus amorphous) may account for the differences between the two studies.

Figures 6 and 7 suggest that sucrose hydrolysis is impacted more by water activity than T_g. As a_W increases, the amount of sorbed water also increases, resulting in more sucrose dissolution. Dissolved sucrose can move to the invertase active site more easily and reaction rates increase. However, it appears that PVP type also affects sucrose hydrolysis rate, because rate constants at $a_W = 0.70$ and 0.75 in PVP−LMW were higher than in PVP−K30. Again, large molecular weight PVP may be blocking the diffusion of sucrose to the invertase active site. Substrate dissolution and polymer physical size appear to be more significant factors than T_g.

CONCLUSION

Invertase storage stability and sucrose hydrolysis appeared to be affected minimally by T_g, some by a_W, and most by polymer size. More research is needed to further evaluate the roles of molecular mobility and polymer size on enzyme stability and reactivity.

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