Characterization of Molecular Mobility in Seed Tissues: An Electron Paramagnetic Resonance Spin Probe Study

Julia Buitink,*# Marcus A. Hemminga,# and Folkert A. Hoekstra*
Wageningen Agricultural University, *Laboratory of Plant Physiology, 6703 BD Wageningen, and #Laboratory of Molecular Physics, 6703 HA, Wageningen, the Netherlands

ABSTRACT The relationship between molecular mobility (τ_R) of the polar spin probe 3-carboxy-proxyl and water content and temperature was established in pea axes by electron paramagnetic resonance (EPR) and saturation transfer EPR. At room temperature, τ_R increased during drying from 10^{-11} s at 2.0 g water/g dry weight to 10^{-4} s in the dry state. At water contents below 0.07 g water/g dry weight, τ_R remained constant upon further drying. At the glass transition temperature, τ_R was constant at $\sim 10^{-4}$ s for all water contents studied. Above T_g, isomobility lines were found that were approximately parallel to the T_g curve. The temperature dependence of τ_R at all water contents studied followed Arrhenius behavior, with a break at T_g. Above T_g the activation energy for rotational motion was ~25 kJ/mol compared to 10 kJ/mol below T_g. The temperature dependence of τ_R could also be described by the WLF equation, using constants deviating considerably from the universal constants. The temperature effect on τ_R above T_g was much smaller in pea axes, as found previously for sugar and polymer glasses. Thus, although glasses are present in seeds, the melting of the glass by raising the temperature will cause only a moderate increase in molecular mobility in the cytoplasm as compared to a huge increase in amorphous sugars.

INTRODUCTION

The longevity of seeds is determined by the conditions under which they are stored, major factors being temperature and water content. Predictions of the longevity of seeds or the optimum storage conditions that have to be chosen to obtain maximum longevity are valuable assets in maintaining a seed collection. In desiccation-tolerant organisms, such as seeds and pollen, it has been found that the cytoplasm enters into a glassy state when those organisms are stored at low water contents and/or low temperatures (Williams and Leopold, 1989; Leopold et al., 1994; Sun and Leopold, 1994; Leprince and Walters-Vertucci, 1995; Sun, 1997; Buitink et al., 1998b). A glass is a solid-like liquid with an extremely high viscosity (Franks, 1994a). The formation of glasses in seeds is thought to be responsible for the prolonged survival of these tissues in the dry state. It was found that the life span of biological materials such as seeds and pollen was increased profoundly during storage under conditions in which the cytoplasm was brought into a glassy state (Sun and Leopold, 1994; Sun, 1997; Buitink et al., 1998b).

In many glass-forming substances, melting of the glass results in a dramatic increase in translational and rotational motion (Soesanto and Williams, 1981; Roozen et al., 1991; Steffen et al., 1992; Blackburn et al., 1996; Deppe et al., 1996; Champion et al., 1997; Hemminga and Van den Dries, 1998; Van den Dries et al., 1998). It has been known for a long time that stabilization of many macromolecules is greatly enhanced by the presence of aqueous glasses. The shelf life of food materials has been associated with the presence of a glassy state (see Roos, 1995, for a review). In the pharmaceutical industry, it is currently recognized that the presence of an amorphous phase has very important implications for storage of pharmaceutical dosage forms (Hancock and Zografi, 1997). Recently, Hancock et al. (1995) suggested the use of molecular mobility measurements below T_g in the prediction of shelf lives of amorphous drugs, assuming a direct correlation between the molecular mobility and the degradation of the product.

The physical properties of water have been studied in complex biological systems that are able to survive the removal of their water, such as seeds (Vertucci, 1990; Bruni and Leopold, 1992; Konsta et al., 1996) and *Artemia* cysts (Seitz et al., 1981). The restricted mobility of water at low hydration levels has been attributed to the formation of intracellular glasses (Williams and Leopold, 1989). However, little is known about the viscosity or molecular mobility of molecules other than water in these systems. Detrimental processes associated with aging that take place in the cytoplasm of seeds are likely to be restricted by slow molecular motion of molecules in the cytoplasm. Therefore, characterization of molecular mobility in seeds as a function of temperature and water content might aid in understanding the kinetics of seed aging during storage. Soluble sugars present in the cytoplasm of seeds are thought to be the major component responsible for the formation of intracellular glasses. Comparison of the behavior of intracellular glasses with sugar glasses will shed light on the nature of intracellular glasses.

An elegant technique for the study of rotational motion is electron paramagnetic resonance (EPR) spectroscopy, which measures the rotational correlation time (τ_R) of spin

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Address reprint requests to Dr. Julia Buitink, Laboratory of Plant Physiology, Wageningen Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands. Tel.: +31-317-482452; Fax: +31-317-484740; E-mail: julia.buitink@algem.pf.wau.nl.

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probes dissolved in samples. Continuous-wave (CW) EPR can detect changes in the τ_R of spin probes ranging from 10^{-12} to 10^{-8} s and has been applied previously to biological systems (Bruni and Leopold, 1990; Buitink et al., 1998a; Leprince and Hoekstra, 1998). Saturation transfer EPR (ST-EPR) can detect τ_R on the order of 10^{-7} to 10^{3} s and has been successfully applied to determine the τ_R of spin probes in sugar glasses (Roozen and Hemminga, 1990; Roozen et al., 1991; Hemminga and Van den Dries, 1998; Van den Dries et al., 1998) and organic liquids at low temperatures (Ito, 1983). It has been shown previously that the τ_R of spin probes provides a unique and simple parameter for the characterization of the state of the cytoplasm of seeds (Buitink et al., 1998a).

For many systems, the temperature dependence of reaction rates or mobility below T_g can be described by the Arrhenius equation (Levine and Slade, 1988; Karmas et al., 1992; Nelson and Labuza, 1994). Above T_g, it has been shown that the temperature dependence of viscosity, translational, or rotational relaxation times of model glasses such as sugar systems and polymers cannot be described by an Arrhenius-like relationship. For such systems it turns out that the effect of increasing temperature on relative relaxation times above T_g can be successfully predicted by the Williams-Landel-Ferry (WLF) equation, an empirical equation, the form of which was originally derived from the free volume interpretation of the glass transition (Williams et al., 1955; Ferry, 1980; Soesanto and Williams, 1981; Chan et al., 1986; Roos and Karel, 1991; Steffen et al., 1992; Champion et al., 1997). Fitting of the rotational mobility in pea axes as a function of temperature according to the Arrhenius and WLF equations will enable us to compare mobility in intracellular glasses with that of model systems. In addition, modeling the temperature effect of mobility could aid predictions of shelf life.

This study was performed to obtain insight into how molecular mobility of molecules in the cytoplasm of seed changes, depending on water content and temperature. Different EPR techniques were used to determine the rotational mobility of spin probes in the fast and very slow motional regions. The temperature dependence of τ_R was assessed in relation to Arrhenius and WLF behavior, and reference was made to other glass-forming substances.

MATERIALS AND METHODS

Plant material and sample preparation

Highly viable pea (*Pisum sativum* L cv karina) seeds were obtained from Nunhems zaden (Haelen, the Netherlands). They were allowed to imbibe for 16 h at 15°C, after which the axes were excised and incubated in 10 ml of a solution of 1 mM 3-carboxy-proxyl (CP) (Sigma). After 45 min, potassium ferricyanide was added to a final concentration of 200 mM, and the axes were incubated for another 15 min. The ferricyanide was added to broaden the signal of CP outside of the cells. Because ferricyanide cannot penetrate intact cells, the signal obtained is derived exclusively from the cytoplasm. Subsequently, the pea axes were dried in dry air (3% RH) for 24 h. After drying, axes were stored over several saturated salt solutions (Winston and Bates, 1960) for 3 days to obtain various water contents.

Two to three axes of the same treatment were sealed in a 2-mm-diameter capillary for EPR measurements. After the measurements, the axes were removed from the capillaries and water contents were determined. For samples that were heated above 50°C during the EPR measurements, similar samples equilibrated to the same water content were taken for water content determination. Water contents were analyzed by weighing the samples before and after heating at 96°C for 36–48 h.

Molecular motion in the fast motional region (CW-EPR)

Spectra were recorded using a Bruker x-band EPR spectrometer (Bruker Analytik, Rheinstetten, Germany, model 300 E). Rotational correlation times in the fast motional region were determined from the lineshapes of CW-EPR spectra according to the method of Freed and Fraenkel (1963) for isotropic tumbling:

$$\tau_R = 6.5 \times 10^{-6} \Delta B_0 \{h_C/h_H)^{1/2} - 1\} \quad (1)$$

where B_0 is the width of the center field component in Teslas, and h_C and h_H are the amplitudes of the central and high field components of the three-line nitroxide radical spectrum, respectively.

Molecular motion in the slow motional region (ST-EPR)

At low water contents and temperatures, τ_R of CP in seed axes becomes slower than 10^{-8} s, resulting in the appearance of a powder spectrum (Buitink et al., 1998a). Under these conditions, τ_R cannot be calculated according to Eq. 1. However, ST-EPR further expands the motional region from 10^{-7} s to 10^{3} s (Hyde and Dalton, 1979; Van den Dries et al., 1998). ST-EPR is based on the diffusion and recovery of saturation between different parts of the powder spectrum in competition with field modulation (Hemminga, 1983). For ST-EPR measurements the second harmonic quadrature absorption signal was detected under the following conditions: field modulation amplitude 0.5 mT, microwave power 100 mW, and field modulation frequency 50 kHz (Hemminga et al., 1984). The phase was set with the self-null method (Thomas et al., 1976).

ST-EPR spectra can be well characterized by independent lineshape parameters, such as the line-height ratios L''/L and C'/C (see Fig. 1 for details). Using reference material with known viscosity, τ_R values are usually obtained in an empirical way. We used spectra of CP in anhydrous glycerol to construct a calibration curve (Hemminga and Van den Dries, 1998). Because the viscosity for anhydrous glycerol is known over a broad temperature range, τ_R of CP in glycerol can be obtained from the modified Stokes-Einstein equation (Roozen et al., 1991):

$$\tau_R = (\eta V/k_b T)k + \tau_0 \quad (2)$$

where τ_R is the rotational correlation time, η is the solvent viscosity, k_b is Boltzmann's constant, V is the volume of the rotating molecule, T is the absolute temperature, τ_0 is the zero viscosity rotational correlation time, and k is a dimensionless slip parameter. The slip parameter was assumed to be temperature-independent (Hemminga and Van den Dries, 1998). Anhydrous glycerol, containing 1 mM CP, was cooled to −150°C, and after equilibration for 30 min, spectra were recorded at 3°C increments. For each temperature the values of the lineshape parameters L''/L and C'/C were calculated. From the curves representing the lineshape parameters of CP in glycerol against τ_R, the values of τ_R of CP in the seed axes were obtained by interpolation of the corresponding lineshape parameters.

DSC

The glass transition temperature is conventionally the temperature at which a change in the heat capacity can be detected by DSC. Two to three pea axes of different water contents were hermetically sealed into aluminum

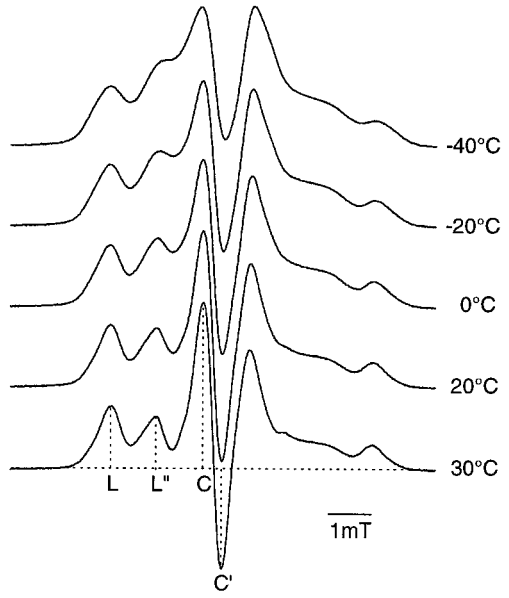

FIGURE 1 ST-EPR spectra of CP in pea axes with a water content of 0.09 g water/g dry weight, recorded at different temperatures.

DSC pans. Second-order transitions of the samples were determined using a Perkin-Elmer (Norwalk, CT) Pyris-1 DSC, calibrated for temperature with indium (156.6°C) and methylene chloride (−95°C) standards and for energy with indium (28.54 J g^{-1}). Baselines were determined using an empty pan, and all thermograms were baseline-corrected. Scans were taken from −100°C to 120°C at a rate of 10°C/min. The T_g values were determined as the onset of the temperature range over which the change in specific heat occurred. All analyses were performed with Perkin-Elmer software.

RESULTS AND DISCUSSION

Rotational motion of CP in pea axes in relation to temperature and water content

ST-EPR spectra of CP in pea axes (0.09 g water/g dry weight) at different temperatures are displayed in Fig. 1. It has been shown previously that to exclusively obtain spectra of the spin probe in the cytoplasm, a nitroxide spin probe of high polarity has to be selected. More apolar spin probes have the tendency to partition into the lipid phase with drying (Buitink et al., 1998a; Golovina et al., 1998). With the use of a broadening agent such as potassium ferricyanide, the CP signal was completely removed from the intercellular spaces and is therefore exclusively of intracellular origin. Although CP is expected to be present in a heterogeneous environment consisting of a mixture of ions, sugars, and proteins, its signal in dry seeds appears to be a single component spectrum (Fig. 1). Only at high temperatures may a small second component appear in the spectrum that can be attributed to CP partitioning into the lipid phase. However, the resulting small distortion in some parts of the spectrum did not influence the calculations of the lineshape parameters from the ST-EPR spectra. Spectra in which a clear distortion of the lineshapes was observed were omitted from the analysis.

The τ_R of CP in pea axes was derived from spectra as shown in Fig. 1. With increasing temperature, the line height ratios (L''/L and C'/C) decreased, indicating an increase in rotational motion. The saturation transfer in the central part of the spectrum is more extensive than that in the low field region, especially at higher temperatures. If the motion were isotropic, then the two peak height ratios would give the same correlation time, because overall rotation would modulate all of the spectral anisotropies, giving rise to saturation transfer throughout the entire spectrum (Marsh, 1980). However, there appears to be some motional anisotropy around the z axis, which modulates the anisotropy of the g-tensor in the x-y plane, giving rise to saturation transfer in the central part of the spectrum. Because this effect complicates the interpretation of the results from the C'/C measurements, only the τ_R derived from the line-height ratio L''/L will be utilized in the analysis of the ST-EPR spectra.

The temperature dependence of τ_R of CP is shown in Fig. 2 for pea axes. The different curves represent pea axes with decreasing water contents from left to right. The arrows denote the onset of T_g as measured by DSC. Below T_g, τ_R followed a linear behavior with temperature. Around T_g, a change occurred in the relationship between τ_R and temperature. Above T_g, the τ_R increased more sharply with temperature from 10^{-4} s to $\sim 10^{-5}$ s over the next 50°C temperature increase. At temperatures ~50°C above T_g, the increase in τ_R with temperature leveled off.

The increase in mobility of CP in pea axes above T_g is not as dramatic as found for glycerol or other glass-forming sugars (Williams et al., 1955; Soesanto and Williams, 1981; Chan et al., 1986; Roozen and Hemminga, 1990; Roozen et al., 1991). For 20% wt sucrose-water mixtures, τ_R increased by about four orders of magnitude in the first 20°C above T_g (Roozen and Hemminga, 1990). In a maltoheptaose glass, dry or stored at 33% relative humidity, the τ_R increased by

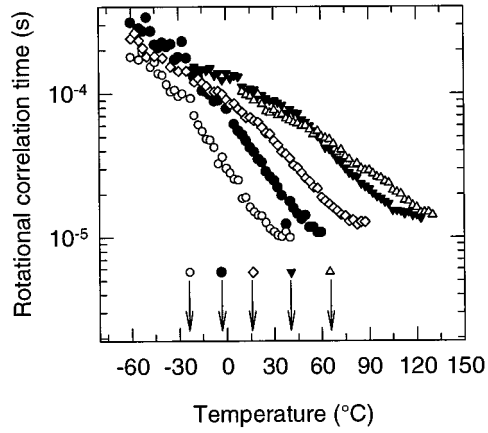

FIGURE 2 τ_R of CP in pea axes as a function of temperature. τ_R was calculated from the line-height ratio L''/L. The different curves represent pea axes with different water contents (g water/g dry weight): 0.048 (△), 0.07 (▼), 0.085 (◇), 0.12 (●), 0.16 (○). Arrows indicate the onset T_g as measured by DSC at a scanning rate of 10°C/min.

three orders of magnitude over the same temperature interval (Roozen et al., 1991). In comparison, the increase in τ_R for CP in pea axes from T_g to 20°C above T_g was only a factor of 6 (Fig. 2). Thus, although glasses are present in seeds, the effect of melting the glass by raising the temperature will not dramatically increase the mobility of molecules in the cytoplasm. Considering the survival of seeds in their natural habitat, the relatively small change in mobility above T_g might render them fairly insensitive to fluctuations in the environmental conditions that drive their cytoplasm out of the glassy state.

During drying of pea axes at 25°C, τ_R of CP in the cytoplasm increased from 7×10^{-11} s at 2.2 g water/g dry weight to 10^{-4} s at 0.07 g water/g dry weight (Fig. 3). Upon further drying τ_R remained constant. The inset shows the decrease in τ_R during drying determined according to Eq. 1, where the water contents were sufficiently high to obtain a sharp three-line spectrum. The rotational mobility decreased when the tissues were dried below 2.2 g water/g dry weight. The dotted line indicates the range of magnitude in which τ_R cannot be measured with either methods. Nonetheless, it can be seen that between 0.3 and 0.2 g water/g dry weight, τ_R strongly increases. This sharp increase in τ_R coincides with the water content range in pea axes at which the water remains unfrozen as measured by DSC (Vertucci, 1990). The change in τ_R upon drying is on the order of more than seven orders of magnitude. The slow molecular mobility at low water contents is thought to have a protective effect on the structural and functional stability of enzymes and other molecules in the cytoplasm (Burke, 1986; Leopold et al., 1994; Leprince and Walters-Vertucci, 1995). Indeed, the considerable decrease in molecular mobility that we found with drying argues in favor of this hypothesis. The preservation of the cellular components in the dry state is likely to prolong the survival of the seeds in the dry state (Leopold et al., 1994; Sun and Leopold, 1994; Sun, 1997; Buitink et al., 1998a,b).

Modeling of molecular mobility in pea axes

If the rate of reactions were controlled by the mobility of molecules in the cells, characterizing the temperature dependence of molecular mobility would aid in predicting shelf life. In addition, it allows us to compare the behavior of rotational motion in intracellular glasses with that of sugar and polymer glasses. The temperature dependence of reaction rates is often described by the Arrhenius equation:

$$k = k_0 \exp(-E_a/RT) \quad (3)$$

where k is the rate constant at temperature T, k_0 is a preexponential factor, R is the ideal gas constant, and E_a is the activation energy. Fig. 4 shows Arrhenius plots of τ_R of CP in pea axes at two water contents. The arrows indicate the onset of T_g as measured by DSC. The temperature dependence of τ_R of CP in pea axes followed Arrhenius behavior below T_g, with a break in the plot at T_g. Above T_g, Arrhenius behavior with a higher activation energy was found compared to below T_g. At temperatures ~40–60°C above T_g, the data deviated from Arrhenius behavior, as became apparent from the deviation of the data points from a straight line. Activation energies from the slopes of the Arrhenius plots below T_g and for the first 40–60°C above T_g for pea axes containing different water contents are summarized in Fig. 5. For τ_R of CP in pea axes above 0.07 g water/g dry weight, activation energies below T_g were lower (7–11 kJ/mol) than above T_g (25 kJ/mol), as generally expected (Levine and Slade, 1988; Karmas et al., 1992; Nelson and Labuza, 1994). This indicates that the rotational motion below T_g changes at a slower rate than above T_g. Below 0.07 g water/g dry weight, activation energies above T_g decreased substantially (Fig. 5). The activation energy values of rotational motion of CP in pea axes below T_g are in the same range as those found for glassy sugar systems (Roozen et al., 1991).

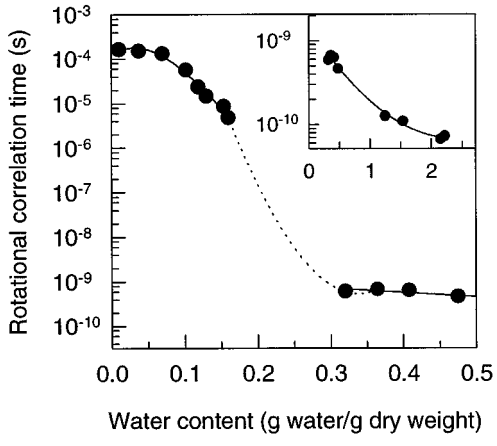

FIGURE 3 τ_R of CP in pea axes as a function of water content at 25°C. Values of $\tau_R > 10^{-6}$ s were calculated from the L''/L ratio of ST-EPR spectra. For $\tau_R < 10^{-9}$ s, Eq. 1 was used with the EPR spectra. The inset shows the decrease in τ_R during drying from high water contents.

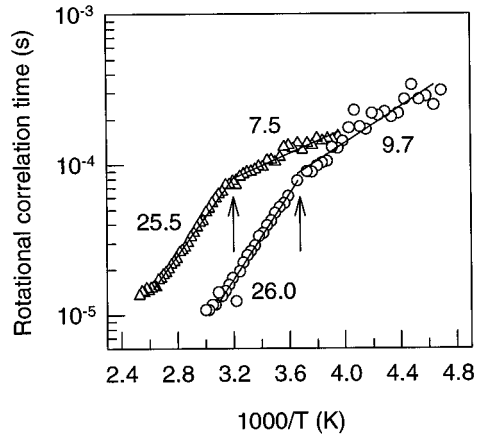

FIGURE 4 Arrhenius plots of the temperature dependence of τ_R of CP in pea axes. The τ_R was calculated from the line-height ratio L''/L. The different curves represent pea axes with different water contents (g water/g dry weight): 0.07 (△), 0.12 (○). Arrows indicate the onset T_g as measured by DSC at a scanning rate of 10°C/min. Lines represent linear regressions. Values represent the activation energy expressed as kJ/mol.

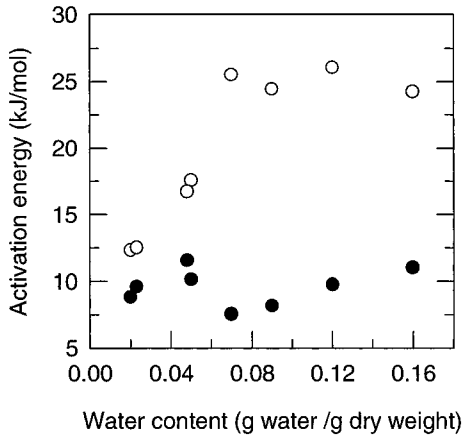

FIGURE 5 Activation energies (kJ/mol) for the temperature dependence of τ_R below (●) and above (○) T_g for pea axes of different water contents. The data points were derived from the Arrhenius plots as shown in Fig. 4.

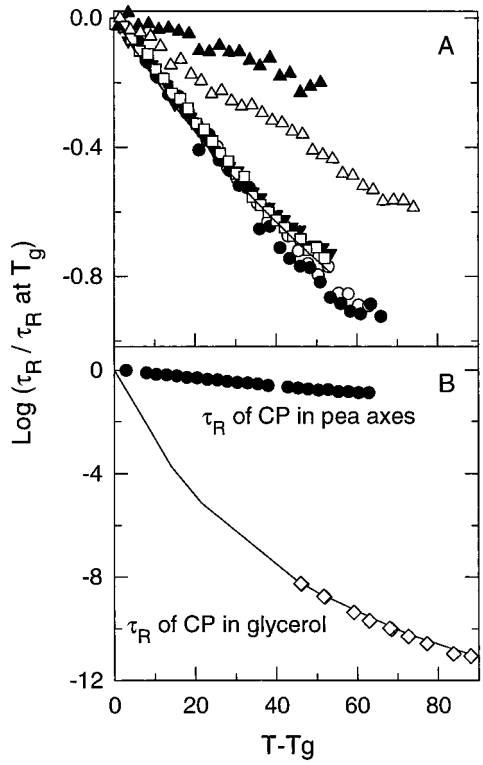

FIGURE 6 (*A*) WLF plot of the temperature dependence of τ_R of CP in pea axes. τ_R was calculated from the line-height ratio L''/L. The different curves represent pea axes with different water contents (g water/g dry weight): 0.01 (▲), 0.048 (△), 0.07 (▼), 0.085 (◇), 0.12 (●), 0.16 (○). The solid line is a WLF fit with $C_1 = 3.4$ and $C_2 = 150$. (*B*) WLF plot of the temperature dependence of τ_R of CP in anhydrous glycerol and in pea axes of 0.12 g water/g dry weight. The solid line is a WLF fit with the universal constants ($C_1 = 17.44$ and $C_2 = 51.6$).

For model glasses, such as sugar-water systems and polymers, it has been shown that the temperature dependence of viscosity or mobility above T_g cannot be described by an Arrhenius-like relationship (Williams et al., 1955; Ferry, 1980; Soesanto and Williams, 1981; Chan et al., 1986; Roos and Karel, 1991; Steffen et al., 1992; Champion et al., 1997). Instead, it can be described by the WLF equation (Williams et al., 1955; Ferry, 1980):

$$\log a_r = -C_1(T - T_{ref})/\{C_2 + (T - T_{ref})\} \qquad (4)$$

where C_1 and C_2 are system-dependent coefficients (Ferry, 1980) and a_T is defined as the ratio of the relaxation phenomenon at T to the relaxation at the reference temperature T_{ref}. Average values for the WLF coefficients ($C_1 = 17.44$ and $C_2 = 51.6$) were calculated by Williams et al. (1955), using the available values for many synthetic polymers. The universal constants have been shown to also apply to molten glucose (Williams et al., 1955), amorphous glucose-water systems (Chan et al., 1986), amorphous sucrose and lactose powders at low moisture (Roos and Karel, 1991), and concentrated solutions of mixed sugars (Soesanto and Williams, 1981). However, several problems are associated with the use of the average coefficients in the WLF equation (Peleg, 1992), and other values have been used to obtain a better fit (Ferry, 1980; Slade and Levine, 1991; Champion et al., 1997).

The τ_R values for CP in pea at different water contents were fitted to the WLF equation (Fig. 6 *A*). The curves of τ_R from CP in pea axes with water contents higher than 0.07 g water/g dry weight followed approximately the same relationship (Fig. 6 *A*). To obtain a reasonable fit, the WLF constants had to be changed considerably by decreasing C_1 and increasing C_2 compared to the universal constants. This becomes apparent from Fig. 6 *B*, in which the τ_R of CP in pea axes is compared to the τ_R of CP in glycerol, fitted with the universal constants. These data were also obtained using ST-EPR spectroscopy (Van den Dries et al., 1998; Buitink et al., 1998a). The curve fitted to the data in Fig. 6 *A* was calculated using $C_1 = 3.4$ and $C_2 = 150$. In samples with water contents below 0.07 g water/g dry weight, there was a further decrease in C_1 or increase in C_2 needed to obtain a good fit.

Although WLF behavior is expected for glass-forming substances above T_g, we found that the Arrhenius equation can well describe the temperature dependence above T_g for the first 50°C (Fig. 4). Deterioration kinetics in complex food systems (Karmas et al., 1992; Nelson and Labuza, 1994) and aging kinetics in pollen (Buitink et al., 1998b) were also found to follow Arrhenius behavior both above and below T_g. Apparently, kinetics in complex systems such as food materials and seeds can be described by the Arrhenius equation for the first 50°C above T_g. The activation energy of relaxation kinetics increases by a factor of ~3 when the pea axes are brought to conditions in which the intracellular glass melts. Interestingly, a comparable change in activation energy for aging kinetics around T_g has been shown for cattail pollen (Buitink et al., 1998b).

Mobility at any temperature depends primarily on the free volume present (Ferry, 1980). Some information regarding the free volume of the system can be derived from the

constants of the WLF equation. C_1 is proportional to the inverse of the free volume of the system at T_g, and C_2 is proportional to the ratio of free volume at T_g over the increase in free volume due to thermal expansion above T_g (i.e., the ratio of free volume at T_g to the difference between the volumes of the rubbery liquid and glassy solid states, as a function of temperature above T_g) (Williams et al., 1955). A lower C_1 would indicate that the free volume associated with the intracellular glass is larger than that found for glycerol. Free volume is related to the packing irregularities caused by the side chains of the glass-forming molecules. This finding reinforces the notion that intracellular glasses are composed of many different molecules, such as ions, amino acids, sugars, and proteins, that are responsible for the increase in free volume because of imperfect packing. The implication of a higher C_2 constant is that the difference in thermal expansion coefficient between the glassy and liquid states would be smaller than that for other glass-forming substances.

When spin probe techniques are used, the rotational mobility of probes in glassy systems is not only determined by the free volume of the host system, but also by the specific interaction of the spin probe with the chain molecules. Hydrogen bonds are often the most important interaction (Roozen and Hemminga, 1990). The deviating behavior of rotational mobility of the spin probe in intracellular glasses compared to sugar glasses can therefore also be mediated by the spin probe's interaction with the surroundings. To learn which of the two factors is determining the rotational mobility, the τ_R values at the T_g (measured by DSC) of CP in pea axes with different water contents were plotted (Fig. 7). With decreasing water content, the onset T_g from pea axes increases from -50°C at 0.26 g water/g dry weight to 90°C when they are completely dry, as measured by DSC. The τ_R is constant at T_g, on the order of 10^{-4} s (Fig. 7). These results would imply that it is indeed the free volume that is the major factor in influencing the rotational mobility. If hydrogen bonding would affect the rotational mobility, it would be unlikely that τ_R at T_g would remain constant, considering the wide range of water contents (0.01–0.20 g water/g dry weight) and temperatures (-50°C to 90°C) studied (Fig. 7).

The use of the WLF and other models in predicting temperature dependence of viscosity allows the establishment of state diagrams that show isoviscosity states above T_g as a function of water content. Such diagrams may be used in the evaluation of changes in water content on relaxation times at constant temperature in establishing critical temperatures for the stability of amorphous materials (Roos, 1995) or may possibly aid in predictions of seed longevity. Slade and Levine (1991) demonstrated that parallel to the T_g curve of sucrose, isoviscosity lines can be found. Fig. 8 shows a state diagram in which isomobility lines are drawn above the T_g curve. Isomobility lines were found to be parallel to the T_g curve.

Implications for seed storage

The presence of intracellular glasses in seeds is thought to be of considerable significance for the storage longevity of the seeds (Leopold et al., 1994; Sun, 1997; Buitink et al., 1998b). The role of intracellular glasses in longevity is derived from the dramatic increase in viscosity or decrease in the molecular mobility of molecules in other glass-forming substances, thus decreasing the rate of detrimental reactions (Soesanto and Williams, 1981; Roozen et al., 1991; Steffen et al., 1992; Blackburn et al., 1996; Deppe et al., 1996; Champion et al., 1997; Hemminga and Van den Dries, 1998; Van den Dries et al., 1998). Detrimental processes associated with aging that take place in the cytoplasm of seeds are likely to be restricted by slow molecular motion of molecules in the cytoplasm. The low rotational mobility

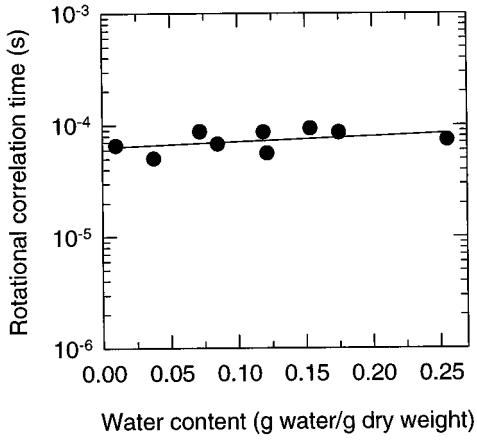

FIGURE 7 τ_R of CP in pea axes of different water contents at T_g. τ_R was calculated from the line-height ratio L''/L. T_g was determined as the onset of the second-order transition measured by DSC at a scanning rate of 10°C/min. T_g for the different water contents ranged from -50°C at 0.26 g water/g dry weight to 90°C at 0.01 g water/g dry weight.

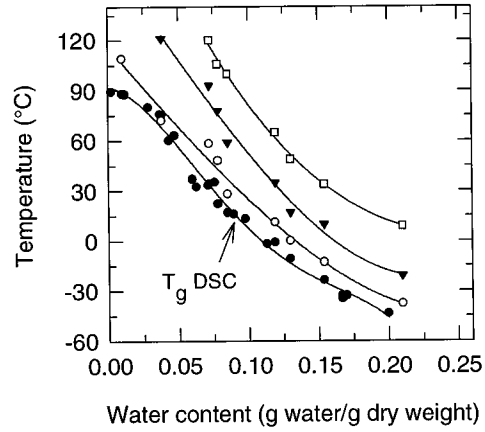

FIGURE 8 State diagram of pea axes. The solid circles represent the onset T_g measured by DSC at a scanning rate of 10°C/min. Lines represent the temperature/water content combinations at which τ_R is equal. $\tau_R = 5 \times 10^{-5}$ s (○), $\tau_R = 2 \times 10^{-5}$ (▲), $\tau_R = 1 \times 10^{-5}$ (□). Lines are fourth-order polynomial regressions.

of CP in dry pea axes, when the cytoplasm is in a glassy state, indicates that formation of a glassy matrix is indeed benefical to the preservation of molecules during storage in the dry state.

The significance of glasses in seeds can be assessed by comparing the changes in mobility of molecules as a function of temperature and relating them to those found previously for other glass-forming substances. In particular, comparison with sugar glasses will ascertain whether the formation of intracellular glasses can be attributed to the soluble sugar present in the cytoplasm, as previously suggested (Williams and Leopold, 1989; Leopold et al., 1994). Intracellular glasses behave in a manner somewhat similar to that of other glass-forming substances in that the temperature dependence of the rotational motion follows WLF behavior above 0.07 g water/g dry weight, which gives rise to isomobility lines parallel to T_g. However, the rate of change of the rotational motion with temperature is not as large as seen in sugar glasses. Thus, although glasses are present in seeds, the effect of melting the glass by raising the temperature will not have a tremendous effect on changes in mobility of molecules in the cytoplasm. Nonetheless, although the difference in activation energy is relatively small below and above T_g, the change in activation energy warns us not to simply extrapolate kinetic data obtained by aging seeds or pollen above T_g (brought about by high humidity and temperature) to aging conditions below T_g (Franks, 1994b; Buitink et al., 1998b; Duddu and Dal Monte, 1997). Considering the different kinetics between sugar glasses and intracellular glasses, sugars may aid in the formation of the intracellular glass in seeds, but other molecules will also participate in the formation of intracellular glasses (Leopold et al., 1994; Leprince and Walters-Vertucci, 1995).

Below 0.07 g water/g dry weight, mobility starts to deviate from the general behavior. The activation energy above T_g decreases with decreasing water content, and the temperature dependence of τ_R of CP in pea axes changes for each water content. It is interesting to note that below this water content, the storage behavior of pea seeds also changes (Vertucci et al., 1994). Instead of an increase in shelf life with decreasing water contents, the shelf life decreases. The factors that cause the change in kinetics of mobility and aging rates are unknown. It has been suggested that the shelf life of foods and seeds or pollen is associated with the Brunauer–Emmett–Teller monolayer, derived from isotherms (Labuza et al., 1970; Labuza, 1980; Buitink et al., 1998b). Removal of this structural water may create holes in the cytoplasm, resulting in increased mobility (Buitink et al., 1998a) and increased aging kinetics. Alternatively, removal of the last water changes the hydrogen-bonding properties of molecules, thereby changing the behavior of the spin probe, which could account for the increase in activation energy at these low water contents (Roozen et al., 1991).

CONCLUDING REMARKS

A spectroscopic method was successfully employed to characterize the rotational motion of small spin probes incorporated into cells of biological materials. The results indicate that intracellular glasses can be formed in dry seeds, but that soluble sugars are not the only determining factor in the glass formation. The complex composition of the intracellular glass is suggested to be responsible for the moderate increase in mobility when the glass is melted, compared to other glass-forming substances. Our work shows that the use of a physical approach in obtaining detailed molecular information will be crucial to predicting the stability of food and biological materials.

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