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Pulsed EPR Spin-Probe Study of Intracellular Glasses in Seed and Pollen

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EPR spectra of 3-carboxy-proxyl (CP) in dry biological tissues exhibited a temperature-dependent change in the principal value A'_{zz} of the hyperfine interaction tensor. The A'_{zz} value changed sharply at a particular temperature that was dependent on water content. At elevated water contents, the break occurred at lower temperatures and appeared to be associated with the melting of the cytoplasmic glassy state. To investigate the reason for the change in A'_{zz}, we employed echo-detected EPR (ED EPR) spectroscopy. The shape of the ED EPR spectrum revealed the presence of librational motion of the spin probe, a motion typically present in glassy materials. The similarities in temperature dependency of A'_{zz} and librational motion of CP in pea seed axes indicated that the change in A'_{zz} arose from librational motion. ED EPR measurements of CP as a function of water content in *Typha latifolia* pollen showed that librational motion decreased with decreasing water contents until a plateau or minimum was reached. ED EPR spectroscopy is a valuable technique for characterizing the relation between molecular motion and storage kinetics of dry seed and pollen. © 2000 Academic Press

Key Words: **seed; pollen; librational motion; spin probe; ED EPR.**

INTRODUCTION

The presence of intracellular glasses in biological systems has been established for over a decade (*1–3*). The formation of glasses has been correlated with the ability to endure the dry quiescent state for a long time, enabling these biological systems to maintain their viability until they resume life when they become hydrated (*3, 4*). The decreased molecular mobility within the glassy cytoplasm has especially been implied to restrict reactions leading to the loss of viability (*4, 5*). To obtain a better understanding of the kinetics of detrimental reactions responsible for the loss of viability, an in-depth study of molecular motions in seeds and pollen is desirable.

A powerful technique for studying molecular motions in seeds and pollens is EPR spectroscopy (*5*). This technique has been applied in the study of rotational and librational motions of spin probes in glass-forming substances, such as supercooled ethanol (*6*), polymers (*7*), sugar–water systems (*8, 9*), and biological materials (*5, 10*). Echo-detected EPR (ED EPR) of nitroxide spin probes in organic glasses revealed that the molecules undergo librational motion (orientational oscillations of a molecule) (*6, 11*). This kind of motion appeared to be a general property of guest molecules in glass-forming liquids (*6, 12*).

Previously, the molecular motion of a polar spin probe, 3-carboxy-proxyl (CP) (Fig. 1), was determined in pea axes using continuous wave EPR (CW EPR) and saturation transfer EPR. This highly polar spin probe was used to exclusively obtain a signal of the spin probe in the cytoplasm. More apolar spin probes have the tendency to partition into the lipid phase with drying (*5*). Saturation transfer EPR revealed that the rotational correlation time of CP was in the order of 10^{-6} to 10^{-3} s (*10*). CW EPR spectroscopy has shown that a change in the principal value A'_{zz} of the hyperfine interaction tensor for CP in pea seed axes and *Typha latifolia* pollen was dependent on water content and temperature (*5*). A relation was found between the A'_{zz} and storage stability of these biological tissues as a function of temperature and water content. This correlation suggested that storage stability might be controlled by molecular mobility. However, the question was raised of what type of molecular motion was responsible for the changes in A'_{zz}, because it is unlikely that rotational motion is involved.

It has been shown that the A'_{zz} temperature dependence may be induced by librational motion (*8*). A'_{zz} is linked to the mean-squared amplitude of motion $<\alpha^2>$ by the relation

$$A'_{zz} = A_{zz} + (A_{zz} - A_{\perp}) < \alpha^2 > , \qquad [1]$$

where A_{zz} and $A_{\perp}$ are the principal values of the hyperfine interaction tensor for immobilized nitroxide (assuming that A_{xx} and A_{yy} are close and therefore may be substituted by their averaged value $A_{\perp}$). It is furthermore assumed in [1] that the motional axis lies in the *xy* plane of the molecular framework (see Fig. 1). Recent studies have shown that the A'_{zz} temperature dependence is a general property of molecular glasses (*11*). In this study, we used ED EPR spectroscopy to confirm this motional model in axes of pea seeds and pollen of *Typha latifolia.*

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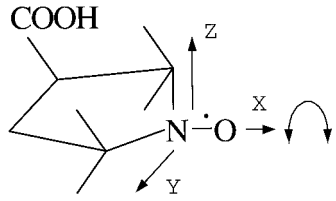

FIG. 1. Structure of the nitroxide spin probe 3-carboxy-proxyl (CP). The principal axes of the nitroxide hyperfine interaction tensor are indicated by *x*, *y*, and *z*. The probable librational motion is indicated.

EXPERIMENTAL

Mature male inflorescences of *Typha latifolia* L. were collected from field populations near Wageningen, The Netherlands, in 1996 and allowed to shed their pollen in the laboratory. Pollen (94% germination) was cleaned by sieving through a fine copper mesh, dried to 0.05–0.08 g water/g dry wt, and stored at 253K until use. Pea seeds (*Pisum sativum* cv Karina) (99% germination) were obtained from Nunhems Zaden BV (Haelen, The Netherlands) and stored at 278K until use.

For spin labeling of these organisms, the polar nitroxide spin probe 3-carboxy-proxyl (Sigma) was used (Fig. 1). Because of its high polarity, CP is present in the cytoplasm of tissues (*5*). *Typha latifolia* pollen and pea axes were labeled according to (*5*). Briefly, the tissues were hydrated in water until their water content reached approximately 1 g water/g dry wt. The tissues were then incubated in a solution of 1 mM CP and 200 mM of the broadening agent potassium ferricyanide for 60 min. After being labeled with CP and dried, samples were subsequently stored over various saturated salt solutions or phosphorus pentoxide (P_2O_5) at 298K for at least 3 days to obtain various water contents. For EPR measurements, samples were hermetically sealed in 2-mm-diameter capillaries to prevent changes in water contents. After the EPR measurements, samples were taken out of the capillary and water contents were analyzed by weighing the samples before and after heating at 369K for 36–48 h and calculating the water loss on a dry weight basis.

Continuous wave EPR spectra were recorded with a Bruker X-band ESP 300E EPR spectrometer. A low microwave power (200 μW) was used to avoid saturation. Temperature was controlled using a temperature controller with liquid nitrogen vapor as the coolant. Samples were rapidly cooled to 123K and allowed to equilibrate for 30 min. Subsequently, spectra were recorded with 10K increments in temperature, equilibrating the sample for 5 min after each increment.

Echo-detected EPR spectra were obtained with a pulsed Bruker X-band ESP-380 FT EPR spectrometer. Electron spin echo was generated by two microwave pulses, with duration of 40 and 80 ns, respectively. The pulse amplitude was adjusted to provide a $\pi/2$–π pulse sequence. ED EPR spectra were taken by scanning magnetic field, while the time delay τ between the two pulses was kept constant. The temperature was maintained with a thermocontrol unit ER4111VT, with an accuracy of $\pm$0.5K. Measurements at 77K were performed in a quartz dewar vessel filled with liquid nitrogen.

RESULTS

Typical CW EPR spectra of CP in dry pea axes are shown in Fig. 2 in their usual form of the first derivative of the resonance absorption with respect to the magnetic field. The A'_{zz} values were determined from the separation between the two outer peaks. This separation decreased when the temperature was increased. The high polarity of the spin probe causes it to be present in the glass-forming aqueous cytoplasm (*5*). However, at high temperatures, a second, small component appeared in the spectrum (Fig. 2, top spectrum) which could be attributed to CP partitioning into the lipid phase present in the pea axes (*5, 13*).

Figure 3 shows the temperature dependence of the A'_{zz} values of CP in pea axes with water contents of 0.07 or 0.12 g water/g dry wt. At high temperatures a remarkable departure from a linear dependence was observed. When the water content of the pea axes was lower, the break at which the deviation occurred commenced at higher temperatures. Arrows indicate the glass transition temperature (T_g) as measured by differential scanning calorimetry (DSC), obtained from (*10*). The sharp decrease in A'_{zz} coincided with the T_g (Fig. 3), as was found previously (*5*). At the temperatures investigated, the possible influence of rotational molecular motion on the CW EPR spectra can be ruled out because the rotational motion it is too slow in this region (*5, 14*). Therefore, the abrupt change in A'_{zz} around T_g is likely to be associated with librational motion. To

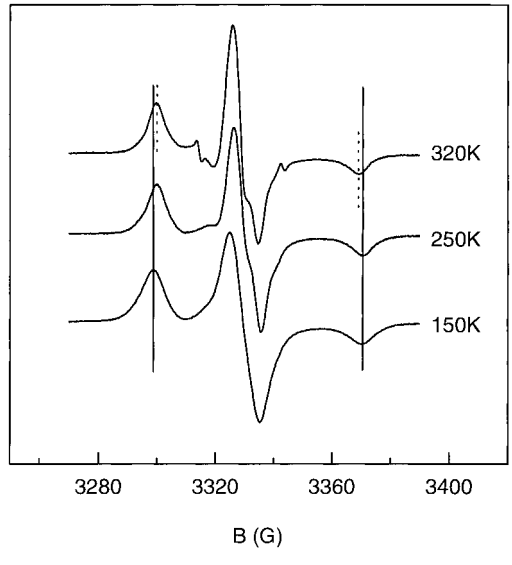

FIG. 2. CW EPR spectra of CP in pea seed axes containing 0.07 g water/g dry wt at different temperatures. Two vertical lines are given to illustrate the decrease of the principal value $2A'_{zz}$ of the hyperfine interaction tensor with increasing temperature.

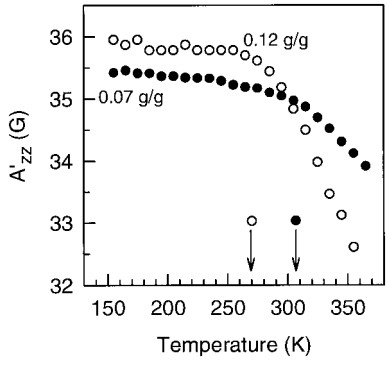

FIG. 3. Temperature dependence of the A'_{zz} values of CP in pea seed axes having water contents (g/g) of 0.07 g water/g dry wt (closed symbols) or 0.12 g water/g dry wt (open symbols). Arrows indicate the onset T_g measured by DSC, obtained from (*10*).

further investigate this phenomenon, we performed ED EPR spectroscopy on similar biological samples.

ED EPR spectra are obtained by detecting the electron spin echo signal as a function of the external magnetic field. The time separation τ between two echo-forming pulses was kept constant during the measurement but was varied from one measurement to another. In this way, the relaxation rate for different field positions was studied. Figure 4 depicts the echo-detected spectra of CP in dry *Typha latifolia* pollen at different times τ (136 and 272 ns) between the echo-forming pulses. The spectra were normalized to their maximum amplitude in order to exclude all field-independent relaxation mechanisms. Notable changes were observed in the shapes of the low-field and high-field components. These changes are determined by the different rates of magnetic phase relaxation of the nitroxide for the different orientations with respect to the external magnetic field (*6*). The reason for such a behavior has been suggested to be the result of molecular librations (*6, 8*).

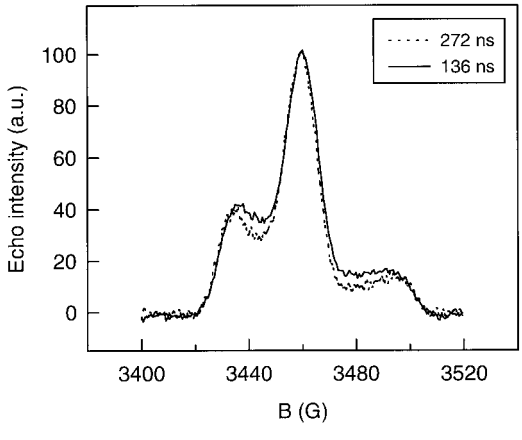

FIG. 4. ED EPR spectra of CP in *Typha latifolia* pollen at different times τ between the echo-forming pulses, recorded at room temperature. Water content of the pollen was 0.04 g water/g dry wt. τ = 136 ns, solid line; τ = 272 ns, dashed line. Spectra were normalized to the same maximum amplitude.

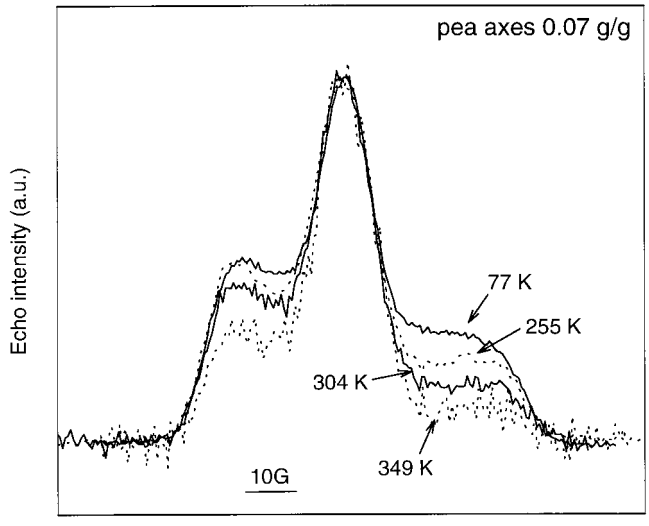

FIG. 5. ED EPR spectra of CP in pea seed axes with 0.07 g water/g dry wt (g/g), recorded at different temperatures. All spectra were obtained for the same time separation between microwave pulses (τ = 136 ns) and were normalized to the same maximum amplitude.

Qualitatively this may be understood as follows. The orientational dependence of phase relaxation arises as a result of different variations of the resonant magnetic field, B_{res}, when the nitroxide librates. Molecular motion moves a spin off resonance providing a phase relaxation pathway. The larger the effect of motion on B_{res}, the more effective the relaxation pathway. This variation, $dB_{res}/d\theta$, where θ is the angle between the molecular z axis and the external magnetic field, is larger for the low-field and high-field components. Also, it tends to zero for canonical orientations of the nitroxide (*6, 8*). So for these orientations the relaxation must be slower than for others. This is indeed observed for the outer edges which correspond to the parallel orientation (Fig. 4).

Changing time τ may result in an additional field-dependent relaxation mechanism, so-called "instantaneous spectral diffusion," which also influences the ED EPR lineshape (*15*). Another way of performing ED EPR experiments, which is free of this influence, is keeping the time τ constant for all measurements but varying the temperature, as shown in Fig. 5. The ED EPR spectra were strongly influenced by temperature. All spectra were obtained for the same time separation between microwave pulses (τ = 136 ns) and were normalized to their maximum amplitude. Analogous data sets were also obtained for CP in pea axes containing 0.12 g water/g dry wt and for CP in *Typha latifolia* pollen (data not shown).

In order to quantify the temperature dependence of the shape of the spectra, we determined the ratio of the line height in the low-field region at 77K (h_{77K}) and at higher temperatures (h) (see inset, Fig. 6). Note that ED EPR lineshape depends on the parameter $<\alpha^2>\tau_c$ where τ_c is the correlation time of motion (*6, 8*). This parameter increases when the low-field component and high-field component decrease. The line height of the low

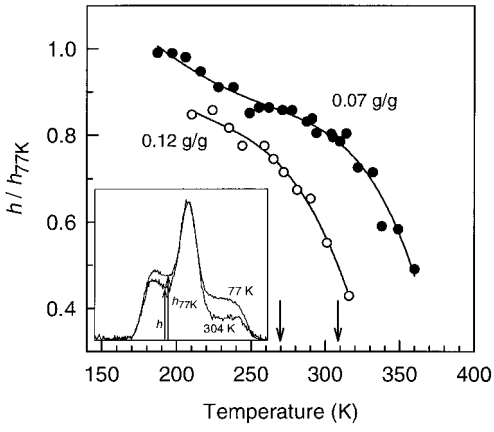

FIG. 6. Changes in the line height ratio (h/h_{77K}) in dependence of temperature for CP in pea seed axes at two water contents. Water contents (g/g) were 0.07 g water/g dry wt (closed symbols) or 0.12 g water/g dry wt (open symbols). Inset shows the calculation of changes in librational motion as a function of temperature, using the difference between the height of the low-field region at 77K (h_{77K}) and the height of the low-field region of spectra recorded at temperatures above 77K (h).

field of ED EPR spectra at 77K was taken as 1, assuming that at this temperature motion was frozen out. This was confirmed by recording a CW EPR spectrum at the same temperature and digitally integrating the spectrum. Comparison of the CW EPR spectrum with the ED EPR spectrum showed that they coincided, implying that motion was frozen out at 77K (data not shown). The ratio of h/h_{77K} of CP in pea axes decreased with increasing temperature, indicating an increase in librational motion during warming (Fig. 6). Arrows indicate the T_g as measured by DSC. The sharp decrease in the ratio occurred at a similar temperature as that found for the A'_{zz} (Fig. 3), implying that both parameters were related to the same type of molecular motion. Note that the effect of motion in ED EPR is much more pronounced compared with CW EPR (compare Figs. 3 and 6).

The A'_{zz} is also known to change in dependence of the water content of the sample, when measured at a constant temperature (*5*). To ascertain whether these changes in A'_{zz} were also due to changes in libration motion, ED EPR spectra were recorded for CP in pollen at different water contents and three constant temperatures (298, 233, and 77K) (Fig. 7). At 298K, the ratio h/h_{77K}, calculated as shown in the inset in Fig. 6, increased with decreasing water contents from 0.53 at 0.22 g water/g dry wt to 0.83 at 0.05 g water/g dry wt. At water contents lower that 0.05 g water/g dry wt, the ratio remained constant. At 233K, the data were more scattered (Fig. 7). From 0.22 to approximately 0.15 g water/g dry wt, the ratio h/h_{77} appeared to increase slightly. Below approximately 0.15 g/g, the ratio decreased or remained constant. In general, the water content at which a minimum in librational motion (max. h/h_{77}) was found, shifted to higher values with decreasing temperature.

DISCUSSION

In this study, we report a change in the principal values of the hyperfine interaction of CP in the axes of pea seeds in dependence of temperature as was found previously for *Typha latifolia* pollen (*5*) and *Impatiens* seeds (unpublished data). The characteristic time of rotational molecular motion for spin probes near or above T_g is in the order of 10^{-6} to 10^{-4} s (*10*). These motions are too slow to account for changes observed in the principal values of the hyperfine interaction that are determined from CW EPR spectra. Modeling of ED EPR spectra of TEMPONE in glucose and trehalose glasses has shown that the nitroxide molecules undergo orientational oscillations about some axis fixed in the molecular frame (librational motion) (*8*). The analogous shape of the echo-detected EPR spectra obtained in this study revealed that it was indeed librational motion that was detected in biological tissues. This type of spectral transformation was found to be common for disordered systems and supports the concept that the local microscopic structure in glassy biological cells is comparable to other glass forming substances (*3–5, 15*).

The temperature dependence of ED EPR spectra of CP in pea axes revealed that librational motion increased with increasing temperature. An abrupt change in A'_{zz} was observed above a temperature corresponding to the melting of the glassy matrix (Fig. 3). The association with the glassy state was further corroborated by the increase in temperature at which the break was observed when the water content of the pea axes was decreased from 0.12 to 0.07 g water/g dry wt. The abrupt change in the h/h_{77K} ratio was comparable to that observed for the change in A'_{zz} (compare Figs. 6 and 3). Because the parameter $<\alpha^2>\tau_c$ increases when the ratio of the low-field component decreases, melting of the glass results in a sharp increase in $<\alpha^2>\tau_c$. Similar observations were made on spectra of nitroxide spin probes present in trehalose and glucose glasses (*8*).

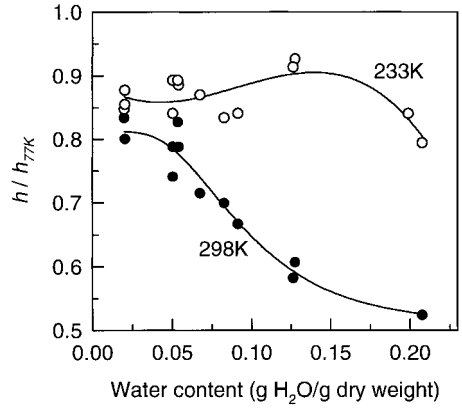

FIG. 7. Changes in the line height ratio (h/h_{77K}) in dependence of water content for CP in *Typha latifolia* pollen. Line height ratios were determined at 233K (open symbols) and 298K (closed symbols).

The A'_{zz} is also known to change in dependence of the water content of the sample when measured at a constant temperature (*5*). In order to determine the change in A'_{zz} as a function of water content instead of temperature, a correction was made for the polarity change of the environment in which CP is present in *Typha latifolia* pollen for each water content (*5*). The resulting mobility parameter, expressed as the difference between the A_{zz} (at 123K, where the spin probe is assumed to be immobilized) and the A'_{zz} measured at the desired temperature (ΔA_{zz}), was shown to exhibit a minimum when plotted as a function of water content. The water content corresponding to this minimum mobility shifted to higher values with decreasing temperatures. In this study, similar results were found by ED EPR measurements (Fig. 7). Although it is not clear whether there is a plateau in librational motion at low water contents rather than a minimum, both ED EPR and CW EPR measurements confirm that below a certain water content, librational motion does not decrease anymore.

The implications of these findings are important for seed storage preservation. Aging rates are thought to be influenced by the molecular motion in the cytoplasm (*3–5, 10*). A decrease in librational or rotational motion in the cytoplasm is likely to result in decreased aging rates that take place in this cytoplasm. Determination of molecular motions under various conditions of temperature and water content might reveal the storage conditions under which molecular mobility is minimized. ED EPR measurements show that there is a sharp increase in librational motion when the cytoplasm is heated above its T_g. Therefore, storage of seeds is recommended below T_g. In addition, we found that at a low temperature (233K), the water content at which the librational motion is minimized is as high as 0.1–0.15 g water/g dry wt. A further reduction in the water content did not decrease the librational motion any further. This finding implies that when tissues are stored at these ultralow temperatures, they do not have to be dried to very low water contents in order to maintain their viability (*16, 17*).

In this study, ED EPR measurements revealed the occurrence of librational motions of CP in biological tissues. As this kind of motion is thought to be a general property of molecular glasses (*8, 11, 15*) the data obtained provide additional evidence for the existence of intracellular glasses in seed and pollen. The librational motion changed along with changes in A'_{zz}, indicating that A'_{zz} measurements reflect librational motion. The change in librational motion in dependence of temperature and water content of the biological samples can be used to obtain information on the rate of detrimental aging reactions that take place in these organisms during storage.

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