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Determination of the Phase Change in the ^{1}H–NMR Relaxation Behavior of Dehydrating Soybean Seed Using the AIC Method

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The physical state of water in dehydrating soybean seeds determined by the NMR relaxation times (T_1) was analyzed using AIC and a three phase regression model was the most appropriate. The first transition occurred at a 44% water content and the second at 18%. In the first phase, the water content 60% at the "physiological maturity" stage, at which time developmental processes in the majority of seeds either cease or slow down. In the second phase, the seeds quickly lost loosely bound water from the cytoplasm during a very short period. In the third phase, a glassy state could represent a useful mechanism to trap residual water molecules and to prevent damaging interactions between cell components and thus T_1 might increase at the glass transition point. The three phases demonstrating cytoplasmic water might thus correspond to desiccation tolerance with a different dehydration process.

INTRODUCTION

Water plays an important role not only as a solvent for biochemical reactions, but also as a stabilizer of macromolecular structure. Seeds provide a useful model for studies on water–assisted mechanisms in cellular function, due to their ability to tolerate desiccation for long periods of time (Clegg, 1986). The late maturation of seeds is marked by a dynamic change in the synthesis of proteins, which are correlated with desiccation tolerance and have been called either "maturation" proteins (Rosenberg and Rinne, 1986, 1988; Blackman *et al.*, 1991), α–TIP (Johnson *et al.*, 1989, 1990) or late embyogenesis abundant (LEA) proteins (Hsing *et al.*, 1995). On the other hand, previous studies (Vertucci and Leopold, 1984, 1987) have reported the importance of "bound" water in the tolerance of organisms to dehydration stresses. The degree of physiological activity in the tissue reflects the level of water binding. Bruni and Leopold (1991) have shown that the cytoplasm of anhydrobiotic organisms exists in a glassy state using the ESR technique, thus suggesting that the ability to withstand dehydration is associated with glass formation. Glass is a liquid of high viscosity, such that it stops or slows down all chemical

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** Present address: Kagoshima Prefectural Flower Center, Kagoshima 891–0513, Japan reactions requiring molecular diffusion (Franks, 1982). Although the presence of intracellular aqueous glasses has been established in seeds by the group of Leopold (Williams and Leopold, 1989; Bruni and Leopold, 1991; Leprince and Vertucci, 1995), the changes of water status determined by NMR longitudinal relaxation times (T_1) in relation to water loss have not yet been studied during seed maturation. The purpose of this study is thus to interpret the dehydration properties of seeds, in terms of the phase changes of bound water estimated T_1 using the AIC method, and to discuss such causative factors as protein synthesis and a glassy state during seed development and dehydration.

MATERIALS AND METHODS

Plant materials and seed development

Summer soybean (*Glycine max* (L.) Merril cv. Koganedaizu) was planted in April 1994. Two plants were grown in a plastic pot (diameter: 25 cm, height: 30 cm) filled with paddy soil containing 8 g of compound fertilizer (N–P_2O_5–K_2O: 3%–10%–10%) and 5 g of slaked lime. Each flower was tagged at flowering. Ten pods were harvested at 5–day intervals from 5 days after flowering (DAF) to harvest maturity (HM), and in each harvest time both fresh seeds and empty pods were weighed. The seeds were dried for 2 h at 100 °C followed by 48 h at 80 °C and weighed to determine seed moisture content (fresh weight bases). From about a week before physiological maturity (PM), five pods were collected daily. Seed and pod color, fresh and dry weight, and the seed moisture content were determined. The time of PM was determined following a procedure similar to that used by Crookston and Hill (1978). For the measurements of NMR relaxation time (T_1), the seed samples were collected from 30 DAF to HM.

Measurements of proton T_1 relaxation times

The T_1 values of summer soybean seeds were measured using a $180^\circ - \tau - 90^\circ$ pulse sequence (Farrar and Becker, 1971) at 20 MHz with a Bruker Minispec PC 20 pulsed NMR spectrometer. Short T_1, which was assumed to be cytoplasmic water (Ishida *et al.*, 1987), was determined in this experiment. The probe temperature (20 °C) was controlled by a thermostat (Lauda Kryo–SK65) connected to the sample chamber of the spectrometer. The T_1 relaxation behavior in relation to the water content of the seed was thus statistically investigated using Akaike's Information Criterion (AIC) (Akaike, 1974).

Statistical models

In the present work, we investigated the most adequate model among one–phase, two phase, three–phase regression models and a quadratic curvilinear model. The basic model for the one–phase regression model tries to fit the observed of the theoretical line $Y=a+bx$.

The two–phase regression model tries to fit the observed values to two half–lines: one before and one after the break point.

Two theoretical lines, $Y=a_1+b_1x$ and $Y=a_2+b_2x$, should meet at a break point x_0 and the following relation must be satisfied:

$$a_1+b_1x_0 = a_2+b_2x_0 \qquad (1)$$

The three–phase regression model tries to fit the observed values by three trisected-lines:

the first before one break point, the second between one break point and the first, and the third after the first. Three theoretical lines, $Y=a_1+b_1x$, $Y=a_2+b_2x$ and $Y=a_3+b_3x$ should meet at the break point x_0 and x_1 ($x_0>x_1$), the following relation must be satisfied:

$$a_1+b_1x_0 = a_2+b_2x_0 \text{ and } a_2+b_2x_1 = a_3+b_3x_1 \qquad (2)$$

The quadratic curvilinear regression model tries to fit the observed values to the theoretical curve $Y=a+bx+cx^2$.

Estimation procedure

We assumed that the errors are independently, normally distributed with mean of 0 and variance σ^2, with the least square estimates equal to the maximum likelihood estimates. The parameter for the one-phase and quadratic curvilinear regression models (Models 1 and 4) can be estimated by a least square estimate.

In Model 2, let $\hat{x}_0$ assuming to be an estimate of the break point, namely the intersection of the two regression lines, the following equation must be satisfied:

$$\hat{a}_1+\hat{b}_1\hat{x}_0 = \hat{a}_2+\hat{b}_2\hat{x}_0 \qquad (3)$$

where x_0 is given by x_m, 1/2 (x_m+x_{m+1}) or x_{m+1}, and the difference of their value was negligible in this study.

For all l and m, calculate the residual sum of squares,

$$S(m) = \sum_{i=1}^{m} (y_i-a_1-b_1x_i)^2 + \sum_{i=m+1}^{n} (y_i-a_2-b_2x_i)^2 \qquad (4)$$

Let m_0 be the value of m which attains the minimum of S (m). Namely,

$$S(m_0) = \text{Min } S(m) \qquad (5)$$

where the minimum is for $2 \leqq m \leqq n\text{-}2$, and let $\hat{x}_0 = \hat{x}_0\ (m_0)$.

In Model 3, for the intersection of the two regression lines, then the following equation must be satisfied:

$$\hat{a}_1+\hat{b}_1\hat{x}_0 = \hat{a}_2+\hat{b}_2\hat{x}_0 \text{ and } \hat{a}_2+\hat{b}_2\hat{x}_1 = \hat{a}_3+\hat{b}_3\hat{x}_1 \qquad (6)$$

where x_0 were given by x (l), 1/2 (x_l+x_{l+1}), and x_1 were given by x_m, 1/2 (x_m+x_{m+1}) or x_{m+1}.

For all i, l and m, ($2 \leqq l < m \leqq n$–2), calculate the residual sum of squares,

$$S(m) = \sum_{i=1}^{l} (y_i-a_1-b_1x_i)^2 + \sum_{i=l+1}^{m} (y_i-a_2-b_2x_i)^2 + \sum_{i=m+1}^{n} (y_i-a_3-b_3x_i)^2 \qquad (7)$$

Let l_0 and m_0 be the value of l and m which attains the minimum of S (l, m). That is,

$$\text{S } (l_0, m_0) = \text{Min S } (l, m) \qquad (8)$$

where the minimum is for $2 \leqq l < m \leqq n$–2, and let $\hat{x}_0 = \hat{x}_0\ (l_0)$, $\hat{x}_1 = \hat{x}_1\ (m_0)$.

AIC method

The fitness of the models was evaluated by the AIC method, and each set of data was subjected to four models. For d=1, 2, 3 and 4, let L $(\hat{\theta}_d \mid d)$ be the maximum likelihood for Model d, AIC value, AIC (d) for the Model d is given by,

$$\mathrm{AIC}\ (d) = (-2)\log \mathrm{L}\ (\hat{\theta}_d \mid d) + 2r \qquad (9)$$

where $\hat{\theta}_d$ is the maximum likelihood estimate of the unknown parameter θ_d contained in the Model d and r is the number of independent parameter in θ_d. If any errors occurred $\{e_i,\ i=1,\ldots,n\}$ in the model, they were normally, and independently distributed with mean 0 and an unknown variance of σ^2, then

$$\mathrm{AIC}\ (d) = n\log\{Se^{(d)}/n\} + 2r + n\ (\log 2\pi + 1) \qquad (10)$$

where n, the number of data, $Se^{(d)}$ is the sum of the squares of bias between the observed values Y_i and the expected values $\hat{f}_i^{(d)}$ in Model d. That is,

$$Se^{(d)} = \sum_{i=1}^{n} \left\{ y_i - \hat{f}_i^{(d)} \right\}^2 \qquad (11)$$

Therefore, if $Se^{(d)} < Se^{(d')}$, then Model d fits better than Model d'.

RESULTS AND DISCUSSION

The Minimum AIC Estimate (MAICE) is a versatile method for model building (Iwaya-Inoue *et al.*, 1989). The most adequate model among the one-phase (Model 1), two-phase (Model 2), three-phase regression models (Model 3) and a quadratic curvilinear model (Model 4) is shown in Table 1. The order of the AIC values in the four models was as follows: Model 3<4<2<1 (Table 1). Accordingly, Model 3 (AIC value: -520.9) was judged to be the most appropriate model and was thus assumed to be a three phase regression model. Three types of relaxation behaviors associated with dehydration were observed. A close relationship was observed between the T_1 and water content in three individual phases (Fig. 1).

Table 1. The AIC values and the occurrence of a break point in the water content for seeds.

Models	AIC values	Water contents of break point (%)	Water contents range of break point (%)*
Model 1	-417.4	—	
Model 2	-508.7	32.5	28.4～35.1
Model 3	-520.9	44.2 17.6	42.3～49.2 14.8～23.3
Model 4	-514.5	—	—

* The break points were determined based on the difference in the minimum AIC values within 2.

Based on these findings, two break points for the minimum AIC value were observed at about a 44% water content and 0.11s of T_1, and at 18% and 0.06 s in Model 3 (shown as broken lines). When the difference in the AIC value exceeds 2, the model showing a smaller AIC value is considered to be significant and can be more highly evaluated than the model having a larger one (Sakamoto *et al.*, 1986). This indicates that breaks occurred at a water content range of from 42 to 49% in the former and at a range of from 15 to 23% in the latter (Table 1).

Phase 1 – There was no break point in the stage at a water content range of 75 and 44% (Phase 1). Ishida *et al.* (1987) showed a closed relationship between two components of water states and physiological changes in maturing soybean seeds using ^{1}H–, ^{13}C– and ^{31}P–NMR: longer T_1 was assumed to indicate vacuolar free water with an obvious changing point at a water content of around 60%, it is thus reasonable that the shorter T_1, which is assumed to be the cytoplasmic water component vs the water content plots in this experiment, thus showed no phase change.

The maximum seed fresh weight occurred about 40 DAF (days after flowering; data not shown), and the water content then declined to about 60% water content at the

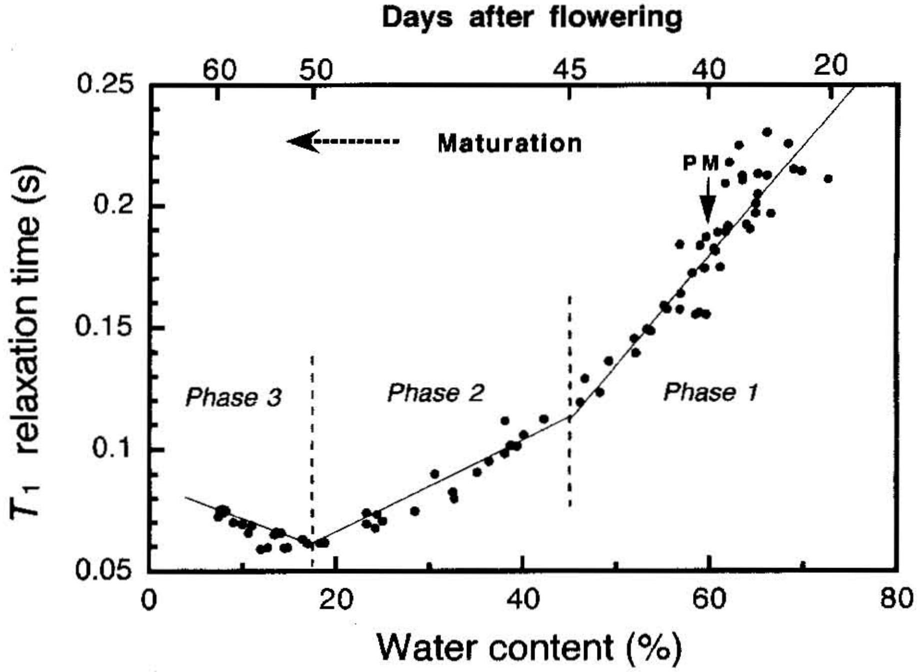

Fig. 1. The relationship between the water content and the T_1 values for soybean seed and the occurrence of the break points during the maturation period. PM; physiological maturity. $Y=-0.081+0.0041x$, r=0.909 (Phase 1), $Y=0.022+0.0020x$, r=0.961 (Phase 2), $Y=0.087-0.0016x$, r=0.0856 (Phase 3). Arrow shows PM. Broken lines show phase transition points.

"physiological maturity" stage (PM: shown by an arrow), at which time developmental processes in the majority of seeds either cease or slow down. The dehydration was thus markedly enhanced from 0.8%/day to 3.2%/day after the PM. The PM thus indicated the maximum sensitivity of the seed to be damaged by the absorption of rain (Tekrony *et al.*, 1980; Noda *et al.*, 1997). Johnson *et al.* (1989, 1990) found that tonoplast intrinsic protein with molecular mass 25–27 kDa called TP25 or α–TIP which accumulated around that stage might thus play a role in maintaining the integrity of the tonoplast in midmatured soybean cotyledons during the dehydration sequence of the seeds. Therefore, the dehydration process in the vacuoles around the PM in seeds is disturbed by water absorption, and thus results in a breakdown of the dry and inactive membrane organizations and also a decline in the seed vigor (Vertucci and Leopold, 1984). At this stage, soybean seeds synthesize abundant proteins (LEA proteins) that are hydrophilic (Hsing *et al.*, 1995), and dehydrins (LEA D–11 protein) were the most conspicuous of the soluble proteins induced by the dehydration process in pea seeds (Robertson and Chandler, 1994). These proteins have been postulated to play a role in protecting the cytoplasm from desiccation damage. Such water-protein interaction may thus reflect the phase change in the water status of the soybean seeds.

Phase 2 – The signals of inorganic phosphate in the cytoplasm only remained while those of sugar phosphate in the vacuole disappeared when the water content decreased from 55 to 28% (Ishida *et al.*, 1987). These results suggest that a phase change in the water status seems to occur in the water content range. The AIC method showed the first transition point was at about 44% water content.

In the second water phase between 44 and 18% (Phase 2), the T_1 in response to the water gradually rose compared to that of phase 1. From the results of the highest desiccation rate (5.2%/day), the seeds quickly lost loosely bound water from the cytoplasm during a very short period. Furthermore, at this stage, a marked accumulation in neutral lipid and changes in lipid class composition occurred during soybean seed dehydration (Privett *et al.*, 1973). Therefore, a metabolic pathway to synthesize the oil body which contains a hydrophobic triacylglycerol matrix surrounded by a monolayer of phospholipids with embedded proteins (Huang, 1992 references therein) might be essential to induce the T_1 change in the cytoplasmic water component.

Phase 3 – The second break point occurred at a water content of 18%, while the T_1 contrary increased as the water content decreased (Phase 3). In the region of water affinity below 24% in soybean seed, there appears to be very low rates in O_2 consumption (Vertucci and Leopold, 1984). Furthermore three regions of water binding of the seed axes were also observed between 0.09–0.25 g H_2O/g dry wt. using differential enthalpy (Vertucci and Leopold, 1987), and the water content per fresh wt ranged from 8 to 20%. Although the water content of the various plant tissues is a main factor reflecting the signal intensity of water proton (Iwaya–Inoue *et al.*, 1993 references therein), the present results showed that the changes in Phase 3 are not simply attributable to the change in water content and factors described above. Glass transitions were also observed at this stage at a water content between 3 and 31% (Leprince and Vertucci, 1995). Such a "glass transition" state thus seems to be directly related to the frost resistance of the seeds and their ability to survive desiccation (Franks, 1982; Bruni and Leopold, 1991). Glass is protected by an amorphous metastable state that resembles a solid, brittle material but retains the disorder and physical properties of the liquid state (Leprince and Vertucci, 1995). Therefore, glass formation, or vitrification, is the creation of a liquid solution with the viscosity of a solid (Williams and Leopold, 1989). Viscosity of the cellular protoplasm was previously demonstrated by Keith and Snipes (1974); a log-log plot of rotational correlation time of water molecules (τ_c) vs viscosity linearly correlated. T_1 decreased to 18% of the water content and thereafter again increased, and this result was due to the characteristics of tightly bound water (Farrar and Becker, 1971); the relationship between τ_c and T_1 abruptly changes at a long τ_c, and thus T_1 might increase at the glass transition point.

Based on the above findings, the transition of the water status can be predicted from the NMR relaxation times at a water content of 18 and 44% using by AIC method. We therefore propose that the three phases of the T_1 assumed as cytoplasmic water in a drying soybean seed might thus correspond to the desiccation tolerance of different types of dehydration behavior.

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