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# Relevance of Amadori and Maillard Products to Seed Deterioration[1]

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## ABSTRACT

**The possible role of Amadori and Maillard reactions in the deterioration of dry seeds was investigated using model systems and whole soybean seeds, *Glycine max* cv Hodgson. In model systems of glucose plus an enzyme (lysozyme), the production of Amadori products was accelerated by higher temperature and relative humidity. The reaction between glucose and lysozyme at 50°C, 75% relative humidity, leads to a progressive decline in enzymatic activity. During accelerated aging of soybean seeds (40°C, 100% relative humidity), a sequence is observed in which the Amadori products increase with time and then decline under conditions in which the Maillard products increase in the axes. Loss of germinability occurs at the time when the Maillard products increase in the soybean axes. These results are suggestive of a role for nonenzymic glycation in soybean seed deterioration during accelerated aging.**

The deterioration of seeds during dry storage is a complex phenomenon involving changes in many seed components (27). Along with a loss of protein integrity, there is a decline in the activity of numerous enzymes (31) and of the respiratory apparatus (2, 26). Several characteristics of the Amadori and the Maillard reactions make these suspect as possible contributors to the deteriorative sequence in seeds (27), particularly with respect to the deterioration of metabolic effectiveness. Both of these reactions are active in dry systems, they have high temperature coefficients, and they are usually not sensitive to oxygen tension (8, 9). These characteristics are shared by deteriorative changes in many seeds (27).

Most of the biochemical understanding of the Amadori and Maillard reactions has arisen from the food technology sector (9, 17) Both play an important part in deterioration of dried food products (11, 21). The Amadori reaction involves a simple, nonenzymic attack on amino groups by reducing sugars to form fructosyl derivatives, or glycated proteins. The Maillard reaction represents subsequent complex interactions between the glycated Amadori products to form polymeric, brown colored products, hence the term "browning reaction" (17).

The deterioration of seeds during storage can be considered to be an aging event (in contrast to a senescence event), in that it is not a programmed developmental process (23, 24). The possible participation of Amadori products in the aging of seeds has an interesting counterpart in the aging of animals. Numerous symptoms of aging in humans have been attributed to the accumulation of glycated proteins due to Amadori products (6). In fact, the acceleration of aging associated with diabetes involves the accumulation of Amadori products as a consequence of the elevation of blood glucose levels (4, 6). One of the notable glycated products is an aberrant hemoglobin that occurs in diabetics (20).

The present report deals with characteristics of the Amadori and Maillard reactions that may be relevant to seed deterioration and provides evidence for the occurrence of these reactions during accelerated aging in soybean seeds.

## MATERIALS AND METHODS

### Model Experiments

Experiments were performed in which lysozyme and glucose solutions (4 mg/ml each) were mixed in a 1:1 ratio by weight and then incubated at temperatures between 20 and 60°C, and/or a full range of RH. The water phase in the samples was lowered to equilibration with the applied RH within 12 to 18 h. Progress of the Amadori reaction was then appraised after incubation by measuring the NBT[2] reaction and/or the loss of enzymatic activity.

#### *Enzymatic Activity*

Lysozyme activity was measured by adding 5 μg of enzyme to 1 mL of a 200 μg/mL solution of *Micrococcus lysodeikticus*. The absorbance was followed at 570 nm (16), and relative activity is expressed as the initial rate of loss of absorbance after the enzyme was added to the bacterial suspension.

#### *NBT Test*

After various times of incubation under controlled RH and temperature, samples of lysozyme plus glucose were taken up in 80 μL of distilled water. The fructosyl product of the Amadori reaction was assayed by the NBT test (13). This involved adding 800 μl of NBT (0.25 mM in 100 mM sodium bicarbonate [pH 10.3]) to the 80 μL of enzyme/glucose solution. Following the method prescribed by the manufacturer (La Roche Co., Nutley NJ), the samples were incubated in a 37°C water bath, and absorbance was read at 550 nm after 10 and after 15 min. The difference between these two absorb-

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[2] Abbreviations: NBT, nitroblue tetrazolium; DMF, dimorpholino fructose; HMF, hydroxymethyl furfural; TBA, 2-thiobarbituric acid.

ances was taken as the measure of the slowly reacting Amadori products and was quantified by comparison to a standard solution of DMF (La Roche) to obtain the amount of glycated product in millimoles DMF/liter.

### *Glucose Attachment Experiments*

To determine the extent of protein attack by glucose, lysozyme (20 μL of a 10 mg/mL solution) was combined with 20 μL of a 3:1 mixture of glucose (13.3 mg/mL) and [U-$^{14}$C] glucose (13.5 mCi/mmol, final specific activity = 3.4 mCi/mmol). This mixture was incubated at 50°C, 75% RH. After 5 d, samples were resuspended in 200 μL of 0.1 M Tris (pH 7.6), and centrifuged through a Millipore Ultrafree-MC 10,000 NMWL filter unit at 14,000 rpm in an Eppendorf microfuge for 10 min. The pass through was removed and 200 μL of buffer was once again added to the filter unit. The procedure was repeated four more times to remove the unbound glucose from the filter. The filter was then placed in a scintillation vial with 5 mL of scintillation fluid, and the radioactivity from the glucose now associated with the lysozyme was counted in a Beckman scintillation counter. From the specific activity of the glucose, the number of lysozyme molecules associated with each glucose could be estimated.

## Seed Experiments

Soybean seeds (*Glycine max* cv Hodgson) were given two different accelerating aging regimes, by placing them in sealed jars at 30°C, 75% RH, and 40°C, 100% RH. The 75% RH was obtained using a saturated NaCl solution in the jar. Assays of Amadori and Maillard products were made using excised axes from a portion of the seed population under accelerated aging. Although the NBT assay is effective for model experiments, it was not satisfactory for assays of the seed tissue, presumably because of the various reducing compounds present in the seed. Therefore, the TBA test was used for the seed assays (13).

### *TBA Assay for Glycated Products*

The glycation of proteins increases their hydrophilicity, and for this reason hydrophilic proteins were isolated from the axes of soybeans for analysis. The axes were ground in 50 mM Tris (pH 7.6) and 50 mM $MgCl_2$. The samples were incubated at 80°C for 10 min to coagulate the heat-unstable proteins following the methods of Hincha *et al.* (15), and then were centrifuged in an Eppendorf microfuge to remove them from solution. Aliquots of the heat-stable protein were removed from the supernatant for protein analysis, and the remaining heat stable proteins were precipitated with TCA (final concentration 10%) and pelleted by centrifugation. The precipitated protein pellets were hydrolyzed with 500 μL of 2 N oxalic acid in a sealable vial at 100°C for 5 h. After the hydrolysis, each sample had 600 μL of a 0.025 M solutions of TBA in 20% TCA added to it, followed by incubation for 20 min in a 37°C water bath for color development. The samples were allowed to sit at room temperature for 15 min before taking absorbance readings at 443 and 550 nm. The difference between these two readings can be used to calculate microgram HMF equivalents against a standard dilution curve. Eight replicates were used to calculate the HMF values, each replicate containing the protein from three axes.

### *Maillard Assay*

Axes of soybean were ground in the above extraction buffer and filtered through a Millipore Millex-HA 0.45 filter. The Maillard product was then measured with a modified procedure of Bucala *et al.* (5). Fluorescence was measured at 440 nm using a fluorescence spectrophotometer with excitation at 370 nm. The emission for each time interval was calculated from two replicates of five axes each.

### *Germination*

Percent germination was measured on seed lots containing 30 to 40 seeds that were removed from the seed population undergoing accelerated aging. Seeds were germinated in wet germination paper for 120 h at 24°C. Germination was scored on seeds that had the radicle emerging from the seed coat.

## RESULTS

To characterize the Amadori reaction, a model system was used in which a reducing sugar (glucose) was permitted to react with a protein (lysozyme). A temperature curve was generated for the reaction between them at 75% RH after 5 days. The formation of fructosyl derivatives (DMF) was enhanced with increases in temperature as shown in Figure 1. Similar mixtures were held at various RH, at a uniform temperature of 50°C, for 5 d. The fructosyl derivative was formed best between 45 and 85% RH as shown in Figure 2. A comparable sample in aqueous solution shows almost no Amadori product during the same time period.

The time course for the reaction between glucose and lysozyme can be followed by measurement of the fructosyl product in time. Aliquots of the mixture were held at 50°C and at 75% RH. Using the DMF assay, as illustrated in Figure 3, the fructosyl derivative is seen to accumulate over a period

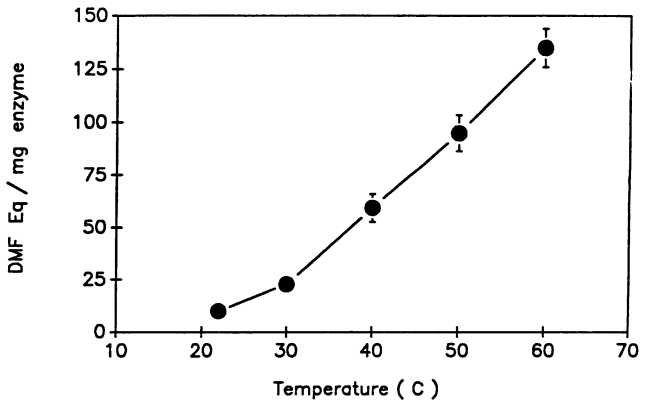


**Figure 1.** Temperature curve for the formation of the Amadori product between lysozyme and glucose. Samples were held for 5 d at 75% RH at the various temperatures. Amadori product was assayed as DMF equivalents. Standard error bars that are not visible fall within the dimensions of the plotted point.

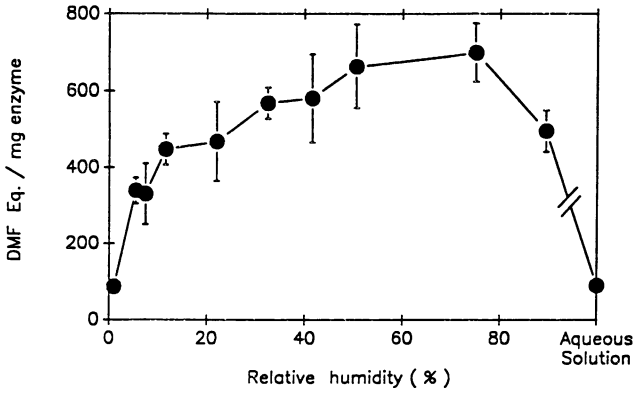


**Figure 2.** RH curve for the formation of the Amadori product. Samples of glucose plus lysozyme were held for 5 d at 50°C at the various RH. The Amadori product obtained in a liquid sample is indicated at far right. Standard error bars that are not visible fall within the bounds of the plotted point.

of a week or more. As the fructosyl derivative accumulates, the enzymic activity of the lysozyme declines.

The fructosyl derivative is itself reactive toward other proteins, and consequently its formation often leads to a polymerization of sugar molecules with several protein molecules (25). An estimate of the number of protein molecules attacked by each glucose can be made as the ratio of glucose to glycated protein, using radiolabeled glucose. In the same model system of glucose plus lysozyme, incubated at 50°C and 75% RH, when lysozyme lost 78% of its enzymic activity, the radioactivity associated with the protein indicated a ratio of about 0.37 mol of glucose per mole of lysozyme. Thus, approximately 3 molecules of enzyme were sufficiently glycated by 1 molecule of glucose to reduce enzymic activity by 78%.

Having established that the reaction of glucose with a representative enzyme is a function of the degree of hydration, and that the consequent product shows a depression of enzymic activity, we sought to establish the possible relevance of such reactions to the deterioration of seeds during accelerated aging. Seeds of soybean were given conditions which accelerated aging. Axes were excised at various time intervals, and evidences of Amadori products and Maillard products were assayed using the TBA test and the fluorescence assay, respectively. Seed samples were also tested for germinability. When the seeds were subjected to an accelerated aging regime of 30°C and 75% RH, a gradual rise in Amadori products was seen, but no rise in the Maillard products as shown in Figure 4, A and B. With this regime there was no decrease in seed germinability (Fig. 4C). When the seeds were subjected to a harsher regime of 40°C, 100% RH, there was a substantial increase in apparent HMF products at 5 d, followed by a decline after 10 d. The fluorescence assay for Maillard products over the same time period showed a gradual increase for 15 d of aging. Germination tests during this period showed that germinability was falling at 10 d, and dropped to zero at 14 d of accelerated aging (40°C, 100% RH). It is apparent that the progress of the Amadori and the Maillard reactions proceed more rapidly under conditions of higher temperatures and relative humidity, and that the rise in Maillard products is associated with the loss of seed germinability.

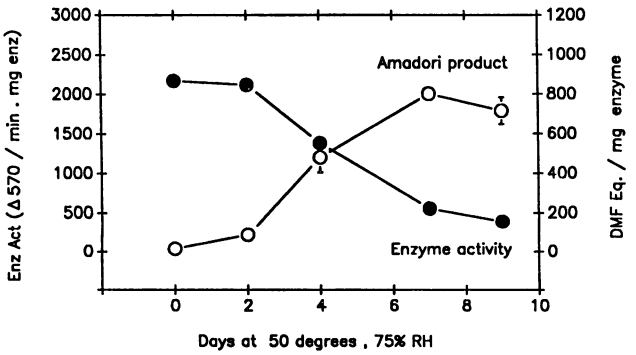


**Figure 3.** Comparison of the progress of accumulation of Amadori product and of the change in lysozyme enzymatic activity. Samples were held at 50°C, 75% RH. Closed circles (●): enzymatic activity. Open circles (○): Amadori product. Standard error bars that are not visible fall within the bounds of the plotted point.

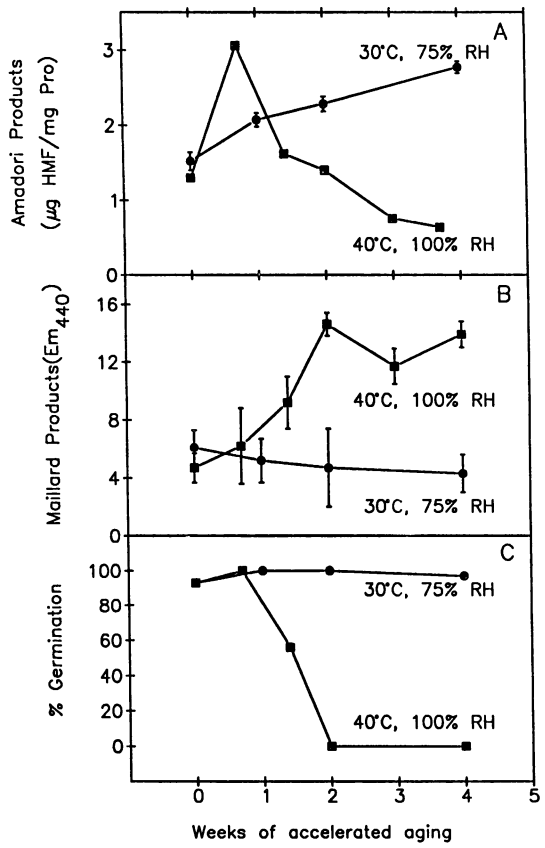


**Figure 4.** Changes in Amadori and Maillard product and percent germination during accelerated aging of whole soybean seeds. A, Amadori product, as HMF equivalents, from isolated axes of seeds held at 30°C, 75% RH and 40°C, 100% RH; B, Maillard product, as fluorescence at 440 nm, from isolated axes of seeds held at the same two conditions; C, percent germination of soybean seeds held at the same two conditions. Standard error bars in A and B that are not visible fall within the bounds of the plotted point.

These results suggest that the Amadori reactions occur in soybean seed during accelerated aging and that subsequent accumulation of products of the Maillard reaction may be associated with the loss of seed germinability.

## DISCUSSION

Through the use of a model system of a reducing sugar plus a protein, our experiments have illustrated the ability of Amadori products to accumulate in a relatively dry medium. The attack by the reducing sugar is shown to be associated with a depression of enzymic activity. These experiments are intended to illustrate the potential for nonenzymic reactions contributing to the deterioration of dry seeds in storage. Attempts to measure the products of such reactions in soybean seeds have yielded evidence that such products do in fact occur during accelerated aging. The accumulation of Maillard products shows correlation with the loss of germinability.

The occurrence of Amadori and Maillard products in dry foods at low moisture activities has been clearly established (8–10). The actual moisture levels at which they occur vary among different foodstuffs, but they range from 6% to 15% $H_2O$ on a dry weight basis (8, 29). Soybean axes achieve these moisture levels when equilibrated at 20 to 70% RH (30). One may well deduce that the presence of reducing sugars in dry seeds could threaten the viability of the seeds through the Amadori and Maillard reactions. A striking feature of orthodox seeds (*i.e.* seeds that can survive desiccation) is the near absence of reducing sugars. Amuti and Pollard (3) reported on the sugar components of seeds of 31 species, representing 10 different families; sucrose was uniformly the most widespread and abundant sugar in seeds and was usually associated with lesser amounts of oligosaccharides. Reducing sugars such as glucose, fructose, or galactose were notably absent or present only in trace amounts. The absence of reducing sugars is characteristic of several types of orthodox pollen (18, 19), nut crops (12), desiccation-tolerant yeast (AC Leopold, R Glenister, unpublished results) and fungal spores (14).

There are situations in which reducing sugars may increase with seed age. For example, aged seeds contain substantial amounts of glucose (1). In fact Takayanagi (28) has suggested that the amount of glucose and fructose leakage from imbibing seeds was proportional to loss of vigor and could even be used to estimate the extent of lowered vigor. A gradual hydrolytic release of reducing sugars during seed aging may well serve to threaten enzymic functions in the imbibing seed.

In the experiments reported here, there is a notable difference between the reactions in the model system and the intact soybean. In the former the optimal RH for Amadori formation was between 50 and 75% RH, whereas the intact seeds produced the most Amadori and Maillard products at 100% RH. Because the mature soybean seed has very small amounts of reducing sugars (22), we might expect that the rate of formation of reducing sugars in the seed experiments would strongly influence the rate of these nonenzymic reactions. The enzymes which would produce reducing sugars, *i.e.* invertase and $\alpha$-galactosidase, require relatively high hydration levels for their enzymic activities (7). Therefore it is reasonable that a higher hydration level would enhance the Amadori and Maillard reactions in the seeds.

The evidence reported here establishes that under conditions of moderate to high relative humidity, the Amadori reaction can occur and bring about a lowering of enzymic activity in a model system such as lysozyme plus glucose. Accelerated aging of seeds has provided evidence that the Amadori and Maillard reactions can occur in the seed, and the accumulation of Maillard products shows correlation with the loss of germinability.

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