Protein modification by Amadori and Maillard reactions during seed storage: roles of sugar hydrolysis and lipid peroxidation

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Abstract

The non-enzymatic modifications of proteins through Amadori and Maillard reactions play an important role in the loss of seed viability during storage. In the present study, the contribution of sugar hydrolysis and lipid peroxidation to Amadori and Maillard reactions, and to seed deterioration was investigated in mung bean (Vigna radiata Wilczek). The contents of glucose and lipid peroxidation products in seed axes increased significantly during storage. The accumulation of Amadori products in seed axes was correlated to the lipid peroxidation, whereas the accumulation of Maillard products was closely correlated to sugar hydrolysis. The rate of accumulation of Maillard products was not well correlated to the content of Amadori products in both seed axes and protein/glucose model system, reflecting the complex nature of Amadori and Maillard reactions. The content of Amadori products in seed axes increased during the early stages of seed ageing, whereas the content of Maillard products increased steadily during the entire period of storage. The accumulation of Maillard products in seed axes was associated with the decline of seed vigour. These data suggest that, during seed ageing, sugar hydrolysis and lipid peroxidation are coupled with non-enzymatic protein modification through Amadori and Maillard reactions.

Key words: Amadori reactions, lipid peroxidation, Maillard reactions, seed ageing, seed deterioration, seed longevity, sugar hydrolysis, Vigna radiata.

Introduction

Seed deterioration involves many biochemical and biophysical changes, including the loss of enzymatic activities, the loss of membrane integrity and genetic alterations, although the exact cause of seed viability loss is still not well defined (Priestley, 1986; Wilson and McDonald, 1986). Lipid peroxidation and associated free radical oxidative stresses are widely considered to be major contributors to seed deterioration. They affect the structure and function of membranes, including the inactivation of membrane bound proteins and the alteration of membrane permeability (Priestley, 1986; Wilson and McDonald, 1986; Hendry, 1993). However, different mechanisms of seed deterioration may exist under different storage conditions. While at lower temperatures free radical damage may be the primary event of seed deterioration, the loss of seed viability at high temperatures is closely related to thermal inactivation of proteins (Sun et al., 1998). Water content is another important factor affecting the rate of seed deterioration (Justice and Bass, 1978; Priestley, 1986). In dry seeds, enzymatic reactions may play little role in seed ageing, because dry seeds lack active enzymatic metabolism. However, certain non-enzymatic reactions, such as Amadori and Maillard reactions, could occur even at very low moisture content (Priestley, 1986; Eichner et al., 1985; Wettlaufer and Leopold, 1991; Sun and Leopold, 1995).

Amadori and Maillard reactions refer to a series of complex reactions that occur following an initial carbonyl-amine reaction. These reactions generally follow four steps: (1) the non-enzymatic condensation of a reducing sugar, aldehyde or ketone with a free amino group of proteins or nucleic acids to form a glycosylamine (a reversible step), (2) the rearrangement of the glycosylamine to Amadori product, 1-amino-α-deoxyketose, (3) the degradation and dehydration of Amadori products into amino or carbonyl intermediates, and (4) the reaction of carbonyl intermediates with other amino groups as well as the subsequent rearrangement to form advanced...
glycosylation end-products (AGE-products) (Sun and Leopold, 1995). The formation of Amadori and Maillard products and their potential roles in the ageing process of animal and human systems have been reviewed extensively in medical literature (Kristal and Yu, 1992; Monnier, 1990). The non-enzymatic glycation (step 1) reduces the activity of enzymes like Cu-Zn-superoxide dismutase (Tanguchi et al., 1989), ribonuclease (Eble et al., 1983), Na+/K+ ATPase (Garner et al., 1987) and lysozyme (Wettlaufer and Leopold, 1991). It has been reported that non-enzymatic glycosylation of DNA plays an important role in the incidence of DNA strand breaks and intra- or inter-strand cross-linking (Lee and Cerami, 1989). The loss of activity of DNA-repair enzymes such as DNA ligase, is an important factor contributing to the alteration of genetic material and seed mortality during seed ageing (Osborne, 1980; Elder et al., 1987). DNA degradation impairs transcription, causing incomplete protein synthesis that is essential for seed germination (McDonald, 1999).

The relevance of Amadori and Maillard reactions to seed deterioration was previously investigated. The accumulation of Maillard products was observed in soybean axes under the accelerated ageing conditions (Wettlaufer and Leopold, 1991). A correlation was established between the accumulation of Maillard products and the loss of seed viability under long-term storage conditions (Sun and Leopold, 1995). Amadori and Maillard reactions may contribute to seed ageing through the chemical alteration of proteins, thus depressing metabolic capability and reducing the ability of the metabolic system to limit the free radical damages and to repair the damages during germination.

In the present study, the roles of sugar hydrolysis and lipid peroxidation in Amadori and Maillard reactions, during seed deterioration, have been investigated. These data indicated that sugar hydrolysis and lipid peroxidation are coupled with Amadori and Maillard reactions, and further confirmed that the non-enzymatic modifications of proteins and enzymes through Amadori and Maillard reactions play an important role in seed deterioration during storage.

Materials and methods

Seed treatment, storage and germination test

Seeds of Vigna radiata (L.) Wilczek (mungbean) were briefly imbibed in water up to 6 h as described previously (Sun et al., 1997). Seeds which had loose and damaged seed coats imbibed water very rapidly and were discarded during the first hour of imbibition. During imbibition, individual seeds were selected for the ageing experiment when the water content of the whole seeds had increased roughly 0.3–0.4 g g⁻¹ dw (g of water g⁻¹ dry weight). The imbibition time for individual seeds to reach this moisture level varies from 2 to 6 h, depending on the water permeability of their seed coats. Seeds which had not imbibed after 6 h accounted for about 10% of the population and were discarded. The preliminary study showed that these hard seeds were still viable, but they took up water very slowly. Selected seeds were immediately placed in a sealed container at 5°C for overnight equilibration to ensure uniform hydration and then dried back at ambient temperature (24±1°C) to water content of 0.149 g g⁻¹ dw (equivalent to 12.9% water content on wet basis). This brief rehydration-dehydration treatment significantly reduces the variation in rates of imbibition and germination due to the difference in seed coat characteristics among individual mungbean seeds and does not affect the desiccation tolerance and seed longevity (Sun et al., 1997). Dried seeds were sealed in laminated aluminium packets and stored at 33°C ±0.4°C. Samples were taken over a period of five months.

To monitor the changes in the seed germination and vigour during the storage experiment, two replicates of seeds (50 seeds each) were imbibed in water for 3 h and then germinated on the top of wet paper in Petri dishes. Seed were allowed to germinate at 24°C (±1°C) for 48 h. The percentage of seed germination and the length of radicles were recorded. The value of percentage germination (%) × average radicle length of germinated seeds were used as a measure of seed vigour, expressed as the percentage of the control seeds.

Measurement of glucose and lipid peroxidation products

The content of glucose in seed axes was determined by using enzymatic assay according to the method described by the manufacturer (Sigma, USA). Embryonic axes (~20 mg) were homogenized with 50% ethanol (0.65 ml) and centrifuged at 15,000 g for 5 min. An aliquot of 0.5 ml supernatant was freeze-dried for 12 h, re-dissolved with 20 μl distilled water, and then 1 ml of glucose assay reagent was added to each sample. The glucose assay reagent without sample was used as a control. The absorbance of the sample solution was measured at 520 nm.

Measurement of Amadori and Maillard products

To extract seed protein, 20 mg embryonic axes were homogenized in 1.2 ml of 10% trichloroacetic acid (in 10% trichloroacetic acid) according to Heath and Packer (Heath and Packer, 1968). Embryonic axes (~20 mg) were homogenized with 1 ml of 50 mM phosphate buffer (pH 7.2) and centrifuged at 10,000 g for 5 min. An aliquot of 0.25 ml supernatant was added to 2.0 ml TBA-TCA reagent and incubated at 95°C for 30 min. The sample was then cooled and centrifuged at 18,000 g for 10 min. The absorbance of the supernatant was measured at 532 nm and corrected by subtracting the absorbance at 600 nm.
The content of Amadori products in seed proteins was measured using the nitroblue tetrazolium (NBT) method, according to Wettlaufer and Leopold (Wettlaufer and Leopold, 1991). One ml of NBT solution (0.5 mM NBT in 100 mM sodium carbonate, pH 10.3) was added to 0.2 mg of extracted proteins and incubated at 40 °C in a water bath. The absorbance was read at 550 nm after 10 and 20 min of incubation. The increase in absorbance (ΔOD) was taken as the measure of Amadori products. The content of Maillard products was measured using the protein fluorescent method. Extracted proteins (0.3 mg ml⁻¹) were scanned with excitation wavelength from 270–400 nm and emission wavelength from 320–500 nm.

In order to gain insight into Amadori and Maillard reactions between seed proteins and reducing sugars, seed proteins were extracted, purified as described above and mixed with glucose (4 mg ml⁻¹ protein + 0.25% glucose). Aliquots of the sample solution were freeze-dried in Eppendorf tubes. Freeze-dried samples were stored at 33 °C, and the progress of Amadori and Maillard reactions was monitored.

Results

The decline of seed germination and vigour during storage

Percentage germination started to decline after 50 d of storage at 33 °C, and this decline became faster as the storage period was extended (Fig. 1). From 50–100 d, seed germinability slowly reduced to 80%, which was then followed by a rapid decline to 20% in the next 40 d (Fig. 1A). However, the change in seed vigour exhibited a different trend (Fig. 1B). Seed vigour, expressed as the percentage of that for control (unaged) seeds, decreased right from the beginning of the storage experiment, and followed more or less a linear manner. Seed vigour was reduced to 50% after 70 d of storage. The leachate conductivity of seeds during the initial 3 h of imbibition was measured (data not shown). The leakage increased linearly during storage and showed an excellent negative correlation with seed vigour.

The changes of glucose content

Figure 2 shows the content of glucose in seed axes after different durations of storage. Before storage (day 0), the content of glucose in seed axes was 11 mg g⁻¹ dw. The content of glucose increased significantly during storage, indicating the occurrence of sugar hydrolysis, presumably from sucrose and oligosaccharide. During the 140 d of the storage experiment, the content of glucose in the axes increased by 75%.

The accumulation of TBA-reactive products

The measurement of TBA-reactive products in the seed axes showed an interesting pattern of change during storage (Fig. 3). The content of TBA-reactive products increased steadily during the first 60 d of storage, and then remained roughly unchanged until 90 d. The TBA-reactive products accumulated again and attained the maximum level at 110 d, which was followed by a rapid decrease. The content of TBA-reactive products increased by 300% within the first 110 d of storage.

The accumulation of Amadori products in seed proteins

The non-enzymatic reaction of reducing compounds with free amino groups of proteins would lead to the formation of glycosylamines, which rearrange to Amadori products. Amadori products in seed proteins accumulated during storage (Fig. 4), with an increase of 400% during the first
The accumulation of TBA-reactive products (lipid peroxidation) in seed axes during storage at 33 °C. The data are means ± SE of three measurements. Bars smaller than symbols are not shown.

Fig. 3.

The change in the content of protein Amadori products (ΔOD) in seed axes during storage at 33 °C. The data are means ± SE of three measurements. Bars smaller than the symbols are not shown.

Fig. 4.

The accumulation of Maillard products in seed axes during storage at 33 °C. The data are means ± SE of three measurements. Bars smaller than the symbols are not shown.

110 d of storage. After this period the content of Amadori products decreased steadily.

The accumulation of Maillard products in seed proteins

Protein fluorescence analysis was used to study protein modification by Maillard reactions during seed storage. Figure 5 shows the fluorescence spectra of proteins extracted from control and stored seed axes; the two fluorescence spectra differed greatly. The fluorescence peak that centred at excitation wave length of 295 nm and emission wave length of 345 nm was identical for proteins extracted both from control and stored seed axes. The nature of this fluorescence peak is well known and is attributed to the presence of certain amino acids in the proteins. Proteins extracted from stored seed axes had an additional fluorescence peak, which was broad and centred at excitation wavelength of 350 nm and emission wave length of 420 nm (Fig. 5B). This additional fluorescence peak is due to the presence of AGE-products in seed proteins (Sun and Leopold, 1995). The fluorescence intensity at excitation wave length of 350 nm and emission wave length of 420 nm was used as a quantitative measure for the content of Maillard products.

Protein fluorescence intensity increased linearly during storage between 0 and 120 days (Fig. 6), and after 120 d increased even faster. In order to study the relationship between Amadori reactions and Maillard reactions, the rate of protein fluorescence increase in seed axes during storage was calculated and plotted against the content of Amadori products at different times of storage. Surprisingly, the two parameters were not correlated at all (Fig. 6B), even though Amadori reactions were coupled with Maillard reactions. The relationship between Amadori reactions and Maillard reactions were further studied, using the seed proteins/glucose model system. As the ageing of the model system proceeded, the accumulation of Amadori products and Maillard products were observed (Fig. 7A, B). Again, no correlation was observed between the content of Amadori products and the rate of Maillard reactions (Fig. 7B, inset). However, the accumulation of Amadori products showed a different pattern of change (Fig. 7A).
**Protein modification during seed ageing**

Figure 6. (A) The increase in protein fluorescence intensity at excitation wavelength of 350 nm and emission wavelength of 420 nm in seed axes after different duration storage at 33 °C. The fluorescence intensity is a measure of the content of Maillard products in seed proteins. The data are means ± SE of three measurements. Bars smaller than symbols are not shown. (B) The correlation between the content of Amadori products and the rate of increase in protein fluorescence in seed axes.

**Amadori and Maillard reactions in relation to seed deterioration**

Figure 8 shows the correlations between sugar hydrolysis, lipid peroxidation, Amadori and Maillard reactions, and seed deterioration. The increase in the content of glucose, as a result of sugar hydrolysis, and the accumulation of Maillard products in seed axes were correlated excellently with the loss of seed vigour (Fig. 8A, B). The content of TBA-reactive products and Amadori products were also correlated well with the loss of seed vigour during the first 3 months of storage (Fig. 8C, D). A few data points for Amadori products at the end of experiment were not used for the calculation of correlation coefficients, because a reversed trend was observed.

**Sugar hydrolysis and lipid peroxidation in relation to Amadori and Maillard reactions**

To investigate how sugar hydrolysis and lipid peroxidation might contribute to the seed deterioration, a correlation analysis was made between the content of glucose (sugar hydrolysis), TBA-reactive products (lipid peroxidation) and the contents of Amadori and Maillard products in seed axes. (A) The content of glucose. (B) The intensity of protein fluorescence. (C) The contents of TBA-reactive products. (D) The content of Amadori products. For data sets C and D, data points near the dashed lines were not used for the calculation of correlation coefficients.
products (Fig. 9). The content of glucose in seed axes showed a very strong correlation with the accumulation of Maillard products, as measured by protein fluorescence intensity, while the content of TBA-reactive products exhibited a strong correlation with the content of Amadori products. The correlation was much less significant between the content of glucose and the content of Amadori products, and between the content of TBA-reactive products and the accumulation of Maillard products (data not shown).

Discussion

The aim of the present study was to examine the possible coupling of sugar hydrolysis and lipid peroxidation with Amadori and Maillard reactions that were known to alter the structure and function of proteins and DNA. Mature seeds of many species contain only trace amounts of reducing sugars which could initiate Amadori and Maillard reactions. However, sugar composition profile could change during storage (Yaklich, 1985; Petruzelli, 1986; Bernal-Lugo and Leopold, 1992; Sun and Leopold, 1993, 1995; Kalpana and Rao, 1994; Begnami and Cortelazzo, 1996). Mungbean seeds contained considerable amount of glucose. The content of glucose in seed axes increased further during storage (Fig. 2), presumably through the gradual hydrolysis of disaccharide and oligosaccharide. The presence of reducing sugars, such as fructose, galactose and glucose, is a primary driving force of Amadori and Maillard reactions. In soybeans, reducing sugars were formed through the hydrolysis of raffinose and stachyose during storage, and once formed, these reducing sugars were rapidly used up in Maillard reactions (Sun and Leopold, 1995). In the present study, the accumulation of Maillard products in seed axes was found to be highly correlated with the level of glucose (Fig. 9A) and the loss of seed vigour during storage (Fig. 8B). The data suggest that sugar hydrolysis during storage may contribute to seed deterioration through the formation of reducing sugars which in turn initiate Amadori and Maillard reactions.

Lipid peroxidation is frequently cited as the cause of seed deterioration (McDonald, 1999). It was suspected that the secondary products of lipid peroxidation might participate in non-enzymatic protein and DNA degradation through Amadori and Maillard reactions. Lipid oxidation produces a variety of lipid hydroperoxides, which can be further degraded into reactive ketones, aldehydes and alcohols. The possible mechanism as to how lipid peroxides and their secondary products may reduce seed storage life remains unclear. Lipid peroxides and their secondary products can react with terminal groups of amino acids in proteins (Feeney and Whitaker 1982; Ory and St Angelo, 1982). The formation of Schiff bases was observed between peroxidized phospholipids and membrane proteins (Castilho et al., 1994). This reaction was similar to the first step of Amadori reactions. Therefore, the possible coupling between lipid peroxidation, Amadori and Maillard reactions was investigated. The determination of TBA-reactive products was used as a convenient way to quantify the extent of lipid peroxidation in seed axes (Fig. 3). The content of Amadori products in seed proteins increased by 400% during storage (Fig. 4), and showed a strong correlation with the content of TBA-reactive products (Fig. 9B), supporting the hypothesis that a possible coupling existed between lipid peroxidation and the initiation of Amadori reactions. It is noted that the accumulation of Amadori products showed different patterns of change between the seed protein/glucose model (Fig. 7A) and proteins in the seed axes (Fig. 4). This difference is probably due to the involvement of lipid peroxidation products in Amadori reactions in the seed axes.

A few studies, however, reported that there was no consistent association between lipid peroxidation and seed ageing (Priestley and Leopold; 1983; Powell and Harman, 1985; Kalpana and Rao, 1994). The mechanism of lipid peroxidation in seeds depends on moisture content,
storage temperature, and oxygen concentrations. Since the products from both sugar hydrolysis and lipid peroxidation may initiate non-enzymatic protein and DNA degradation through Amadori and Maillard reactions, it is possible that under certain conditions seed ageing might not be associated with both lipid peroxidation and sugar hydrolysis. For example, at intermediate moisture content between 8% and 12%, both lipid auto-oxidation and enzyme-mediated lipid peroxidation are significantly retarded (McDonald, 1999). In such cases, reducing sugars could probably play a prominent role in the initiation of Amadori and Maillard reactions. At moisture contents below 6%, lipid auto-oxidation becomes increasingly common (McDonald, 1999), while sugar hydrolysis is practically prevented because the seed exists in a glassy state (Sun and Leopold, 1993, 1995, 1997). Therefore, at such a low moisture content, lipid peroxidation products might become the primary cause for the initiation of Amadori and Maillard reactions when reducing sugars are absent in seeds.

Since Maillard reactions represent complex interactions between Amadori products following the non-enzymatic attack on proteins by reducing sugars or aldehydes (Amadori reactions), an attempt was made to examine the relationship between the initial Amadori reactions and subsequent Maillard reactions during seed ageing. Surprisingly, the rate of Maillard reactions was not correlated with the content of Amadori products (Fig. 6B). A similar result was obtained with proteins/glucose model system (Fig. 7B). This result could be a reflection of the complex nature of Amadori and Maillard reactions.

In conclusion, sugar hydrolysis and lipid peroxidation may be coupled with Amadori and Maillard reactions during seed ageing. The accumulation of Amadori products in seed axes was strongly correlated to lipid peroxidation, whereas the accumulation of Maillard products was closely correlated to sugar hydrolysis. Amadori and Maillard reactions were associated to the loss of seed viability during storage.

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