Analysis of Factors Influencing Lipid Oxidation of Almond Seeds during Accelerated Ageing

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Almond seeds age rapidly during storage at high relative humidity (80%) and moderate temperature (20°C). The content of lipid decreased during accelerated ageing up to 40 d. The levels of linoleic (C18:2) and linolenic (C18:3) acids in almond lipid fraction also decreased during ageing up to 20 d. The aged seeds contained high levels of malondialdehyde, a product of the peroxidation of unsaturated fatty acids. Lipooxygenase activity, which oxidizes unsaturated fatty acids, was detected in almond seeds. Increased activity of this enzyme was observed during accelerated ageing. It is suggested that accelerated ageing leads to peroxidative changes to lipids. No changes in peroxide-scavenging enzymes such as superoxide dismutase and peroxidase were detected in accelerated-aged seeds. The accelerated ageing treatment could be a useful tool to investigate the mechanism of natural seed ageing.

Introduction

Almond seeds age rapidly during storage at high relative humidity (80%) and moderate temperature (20°C). The content of lipid decreased during accelerated ageing up to 40 d. The levels of linoleic (C18:2) and linolenic (C18:3) acids in almond lipid fraction also decreased during ageing up to 20 d. The aged seeds contain high levels of malondialdehyde, a product of the peroxidation of unsaturated fatty acids. Lipooxygenase activity, which oxidizes unsaturated fatty acids, was detected in almond seeds. Increased activity of this enzyme was observed during accelerated ageing. It is suggested that accelerated ageing leads to peroxidative changes to lipids. No changes in peroxide-scavenging enzymes such as superoxide dismutase and peroxidase were detected in accelerated-aged seeds. The accelerated ageing treatment could be a useful tool to investigate the mechanism of natural seed ageing.

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Materials and Methods

Seed ageing

Dry seeds of almond (Prunus dulcis c.v. Fra Giulio), harvested in October, 1994, were used for all experiments. After preliminary investigations at different temperatures (10, 20, 30, 45°C) and 80% relative humidity (RH) the experiments on accelerated ageing were performed by incubating the seed in a closed plastic box at 20°C and 80% relative humidity for up to 40 d. Rapidly-aged seeds were sampled at 10-d intervals, then air-dried for 2 d at 25°C in order to restore the initial weight. Seeds incubated at 20°C, 40% RH were used as control (non aged seed).
Lipid extraction

Lipids were extracted by comminuting 10 seeds (about 10 g) for 30 s in a Waring Blender in the presence of chloroform–methanol, and were quantified according to the method of Bligh and Dyer (13). To minimize oxidation of solvent extracts during extraction, 0.05 g/L butylated hydroxytoluene was added (4, 14). For the extraction of fatty acids, samples were saponified for 1 h at 90 °C with ethanol, acidified and fatty acids extracted (15). Fatty acid methyl esters (FAME) were prepared as described previously (16). Gas chromatography analysis was performed using a 10% SP2330 on Chromosorb WAW 100/120 column (2 m × 2 mm i.d.); the carrier gas was nitrogen at a flow rate of 20 mL/min. The column was run isothermally at 130 °C for 2 min, with the temperature then programmed to increase to 200 °C in 3 °C steps; injector and detector were programmed at 220 °C and 200 °C, respectively. Fatty acids were identified by retention time relative to standard FAMEs (Sigma Chemicals). Three esterifications and nine GC analyses were performed for each almond lipid sample (17). Values were expressed as area percentages.

Malondialdehyde (MDA) determination

Seeds (2 g) were homogenized at 4 °C in a Waring Blender with 20 mL of 50 g/L trichloracetic acid to precipitate proteins. The precipitate was pelleted by centrifugation and an aliquot of supernatant was reacted with an equal volume of 5 g/L TBA in a boiling water-bath for 30 min. To prevent additional lipid peroxidation and subsequent aldehyde formation, air present in the reaction tubes was removed by a stream of nitrogen gas and the tubes were capped (18). After cooling the absorbance was read at 532 nm. The concentration of MDA was calculated by using an extinction coefficient 153 mmol (L·cm⁻¹), corrected for nonspecific turbidity by subtracting the absorbance at 600 nm (19).

Enzyme extraction and assays

Lipoxygenase. Lipoxygenase was prepared from six seeds which were finely ground in a blender for 30 s; 0.5 g of the powder was extracted with 5 mL distilled water and incubated at 25 °C for 1 h. The extract was then filtered through four layers of cheesecloth and washed with 5 mL of distilled water. Extracts were immediately assayed and the reaction followed by observing the increase in absorbance at 234 nm. The buffer systems employed to develop the pH curve were all 50 mmol and consisted of potassium acetate pH 4–5.5, potassium phosphate pH 6–7 and sodium borate pH 8–9. One unit was defined as the quantity of enzymes generating 1 μmol of conjugated diene per min under standard assay conditions. The extinction coefficient for the diene was taken to be 2.5 × 10⁴ mol/cm for the linoleic acid product at 234 nm (20, 21).

Superoxide dismutase (SOD). Seeds (20 g) were homogenized with 100 mmol/L potassium phosphate, pH 7.8, containing 1 mmol/L EDTA in a Potter homogenizer cooled in ice. The homogenate was squeezed through four layers of cheesecloth (gauze) and centrifuged at 12,000 × g for 30 min. The supernatant was then precipitated in 80% saturated ammonium sulphate and the resulting precipitate resuspended in 20 mmol/L phosphate buffer, pH 7.8, and dialysed against the same buffer. The dialysed material was centrifuged at 10,000 × g for 10 min and the supernatant was used for determination of SOD and proteins. SOD activity was measured spectrophotometrically according to the procedure modified by Furusawa et al (22). One unit of SOD is defined as the amount of enzyme which inhibits the reduction rate of cytochrome C by 50%. Unit of enzyme activity was proportional to V/v – 1 where V and v are the reduction rates of cytochrome C in the absence and in the presence of SOD, respectively (22).

Peroxidase (POD). Freshly dissected seed coats were frozen in liquid nitrogen, ground with a mortar and pestle, and extracted in buffer containing 10 mmol/L NaHPO₄, pH 6.0, 5 mmol/L dithiothreitol, and 10 g/L polyvinylpyrrolidone 40. After centrifugation at 15,000 × g for 5 min, an aliquot of the supernatant fraction was used to estimate peroxidase activity. Peroxidase activity was assayed spectrophotometrically using a final concentration of 0.05% H₂O₂ and 12 mmol/L guaiacol in 10 mmol/L NaHPO₄, pH 6.0. The absorbance was recorded at 490 nM and expressed as a unit. One unit is equivalent to the Δ490 min⁻¹ × mg protein⁻¹ (23).

Protein

Protein concentration was estimated by the Bradford method (24) using the Bio-Rad reagent with BSA as a standard.

Results

Table 1 shows that the content of soluble proteins and lipids in the nonaged seeds of almond cv. Fra Giulio was 151 and 41 mg/g dry weight (DW), respectively. This lipid content is lower (20–30%) than that obtained in different varieties by Romojaro et al (25). Lipid content decreased with time in aged seeds; the decrease was about 14% in seeds aged for 10 d and 31% at the end of the experiments (40 d). Palmitic methyl esters (C16:0 and 16:1) did not differ significantly during ageing. The level of stearic acid (18:0) was found to be significantly lower (20% and 29%, respectively) in seeds exposed for 20 and 40 d at 20 °C and 80% RH, than in seeds held at 20 °C and 40% RH. The concentration of oleic acid (C18:1) was higher in aged than in nonaged seeds. The concentration of linoleic (C18:2) and of linolenic acid (C18:3) was 39% and 41% lower, respectively, in accelerated aged...
Table 1 Effect of accelerated ageing on lipid and protein contents of almond seeds

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<th>Non aged</th>
<th>Aged (d)</th>
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<tr>
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<td></td>
<td>10</td>
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<tr>
<td>Protein content (mg/g dry weight)</td>
<td>151±2.0</td>
<td>148±2.0</td>
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<tr>
<td>Lipid content  (mg/g dry weight)</td>
<td>41.1±1.1</td>
<td>35.3±0.7</td>
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*Results are the mean ± S.D. of five experiments.

The activities of radical- and peroxide-scavenging enzymes such as superoxide dismutase and peroxidase remained unchanged throughout the duration of accelerated ageing.

Discussion

Our observations indicate high RH (80%) and moderate temperature (20 °C) results in accelerated ageing in almond seeds. After 20 d of ageing there was a significant decrease of polyunsaturated fatty acids and an increase of lipoxygenase activity and MDA content, suggesting that lipid peroxidation had increased.

Most frequently acceleration of ageing is induced by storing seeds at high temperatures and humidity (typically 40–45 °C and 100% RH) (3). Almond seeds age rapidly during storage at high humidity, but are unaffected by high temperatures (26). Experiments conducted on almond seeds stored at room temperature and in dry conditions (data not reported here) demonstrated that no chemical differences were detectable before 12 month ageing, but accelerated ageing in our experiments induced the development of lipid peroxidation and thus it is possible that it occurs in partially hydrated (i.e., high RH) seeds. Our study agrees with other reports (3, 10, 27) which indicate that the major peroxidation damage occurs during storage, but that this is expressed only upon imbibition. It appears likely that peroxidation of unsaturated fatty acids occurs during accelerated ageing at high RH after 20 d, linolenic and linoleic declining appreciably and the product of peroxidation (malonaldehyde) increasing. This result is not surprising as we found a decrease in total lipid content (Table 1) during the ageing of almond seeds and similar results were also reviewed by Priestly (28) in aged seeds. The data also agree with previous reports that lipoxygenase is capable of catalysing lipid peroxidation which could promote the formation of activated oxygen species (4).

As reported by Priestly and Leopold (9) in partially hydrated seeds undergoing accelerated ageing, the status of lipid is evidently determined by a complex balance between degradative and protective mechanisms that could scavenge the peroxidatively produced free radicals and peroxide. Since the aged almond seeds are incapable of activating the scavenging enzymes, it seems that POD and SOD do not have a role in restricting peroxidation damage after seed imbibition (at high degree of humidity). The biochemical consequence is that accelerated ageing not only stimulated lipid peroxidation, but also inhibits the free radical and peroxide scavenging enzymes. Our results indicate that...
changes in the degree of fatty acid saturation and in lipoxygenase occur in artificially aged almond seeds. Although there are several short-comings in our study the accelerated ageing procedure is a simulation of natural ageing and a means of understanding the mechanism that underlies seed deterioration.

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