JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Antioxidants, Free Radicals, Storage Proteins, Puroindolines, and Proteolytic Activities in Bread Wheat (*Triticum aestivum*) Seeds during Accelerated Aging

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Seeds of bread wheat were incubated at 40 °C and 100% relative humidity for 0, 3, 4, 6, and 10 days. The effects of accelerated aging on seed germinability and some biochemical properties of flour (carotenoid, free radical, and protein contents and proteolytic activity) and gluten (free radical content and flexibility) were investigated. Seed germinability decreased during aging, resulting in seed death after 10 days. A progressive decrease of carotenoid content, in particular, lutein, was observed, prolonging the incubation, whereas the free radical content increased in both flour and gluten. A degradation of soluble and storage proteins was found, associated with a marked increase of proteolytic activity and a loss of viscoelastic properties of gluten. On the contrary, puroindolines were quite resistant to the treatment. The results are discussed in comparison with those previously obtained during accelerated aging of durum wheat seeds.

KEYWORDS: ***Triticum aestivum*; seed aging; free radicals; carotenoids; proteases; gluten; puroindolines**

INTRODUCTION

Seeds deteriorate and, eventually, lose their ability to germinate during periods of prolonged storage following a time-dependent process termed aging. The main factors determining the rate of this process are the temperature and relative humidity at which the seeds are stored and an ill-defined parameter, seed quality. Generally, high moisture levels and temperatures reduce seed longevity and cause profound deteriorative biochemical changes in seed membranes, DNA, and food reserves (*1*−*5*).

Accelerated aging is a method employed to foresee the storage potential of seeds and to obtain seed aging in a short time by incubation at high temperature (∼40 °C) and relative humidity (usually 100%) (*6*). This method has been extensively used by research scientists to derive conclusions about natural aging, even though the debate continues whether accelerated aging produces the same biochemical events as occur during the natural one (*1*−*5*).

In a previous study (*7*), we investigated the effects of accelerated aging on some physiological and biochemical properties of durum wheat (*Triticum durum* Desf. cv. Cappelli) seeds in comparison with those of natural aging (*8*). We found that accelerated aging, performed by incubation of durum wheat seeds at 40 °C and 100% relative humidity, results in a progressive loss of seed viability and in a marked deterioration of the protein components, partially similar to that observed during storage proteins mobilization. On the other hand, biochemical events produced by accelerated aging were quite different from those occurring in durum wheat seeds of the same cultivar during natural aging.

In the present work, we extend the investigation of the effects of accelerated aging to bread wheat seeds. To this purpose, seeds of bread wheat (*Triticum aestivum* L. cv. Centauro) were incubated at 40 °C and 100% relative humidity for periods of up to 10 days. Seed viability was evaluated by germination tests, and the effects of seed deterioration on carotenoids, free radicals, soluble and storage proteins, and proteolytic activities were investigated as a function of aging time by means of chromatographic, electrophoretic, and spectroscopic techniques. The results are discussed in comparison to those previously obtained for durum wheat seeds (*7*), with the aim of pointing out analogies and differences in the storage potential of bread and durum wheat, as well as in the biochemical changes occurring in the two wheat species during accelerated aging. Moreover, we investigated the effects of accelerated aging on puroindolines, proteins belonging to the friabilin family strictly related to kernel softness (*9*−*12*).

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10.1021/jf0353741 CCC: $27.50
Published on Web 05/27/2004

MATERIALS AND METHODS

Materials. Seeds of bread wheat (*Triticum aestivum* L. cv. Centauro) were provided by the Società Produttori Sementi S.p.a. (Bologna, Italy). Seeds were harvested in 2000 and stored in sealed glass containers at 10 °C in the dark.

trans-Epoxysuccinyl-L-leucylamido-4-guanidinobutane (E-64), phenylmethanesulfonyl fluoride (PMSF), pepstatin A, hemoglobin (Hb), azocasein, *N*-carbobenzoxy-L-phenylalanyl-L-alanine (CBZ-Phe-Ala), and 3-maleimidoproxyl (3-MAL) were purchased from Sigma (St. Louis, MO).

Vitavax 200FF was purchased from Uniroyal Chemical Co. Inc. (Middlebury, CT).

All reagents and solvents were of analytical grade and were used without further purification.

Accelerated Aging. Seeds of uniform size were selected by sieving, surface disinfected in 1% NaClO for 5 min, washed 10 times with sterile distilled water, and surface treated with fungicide Vitavax 200FF. Lots of 90 g of seeds (~1400 caryopses) were suspended over 3 L of distilled water on plastic mesh trays within closed plastic boxes (25 × 25 × 14 cm) and maintained at 40 °C in a growth chamber for 3, 4, 6, and 10 days. Then, seeds were rapidly air-dried under a laminar flow hood until they reached their original moisture content (~11% controlled by weighing). Control seeds were subjected to the same procedures except for accelerated aging.

Germination Tests. Seeds were uniformly spread into Petri dishes with filter paper placed on the top and on the bottom and imbibed with 6 mL of distilled water and incubated at 23 °C in the dark for 24, 48, and 72 h. Four replicates of 25 seeds were considered for each accelerated aging treatment period.

Results of germination tests were expressed through germination capacity, defined as the percentage of completely germinated seeds after 72 h, and germination rate, determined according to the equation

$$\text{germination rate} = \frac{nt_1 \times 100 + nt_2 \times 50 + nt_3 \times 33.3}{N_{total}}$$

where nt_1 is the number of seeds germinated after 24 h, nt_2 and nt_3 are the numbers of seeds germinated between 24 and 48 h and between 48 and 72 h, respectively, and N_{total} is the number of seeds used in the test (*7*, *8*). Seeds were considered to be germinated when the primary root was at least 1 mm long.

Shoot and primary root were measured after 72 h of seed incubation in germination conditions.

Preparation of Flour and Gluten. Seeds were ground in a break roller-mill (Labormill 4 RB), and flour was stored at −20 °C. Gluten was manually extracted from flour according to the procedure reported by Galleschi et al. (*7*), frozen, lyophilized, homogenized in dry conditions with a pestle and a mortar, and stored at −20 °C. Gluten had a moisture content of ~6% and reconstituted readily with water at room temperature. Flour from seeds aged for 10 days was unable to give gluten.

Extraction of Carotenoids and Determination of Lutein. Carotenoids were extracted from flour with CH_3OH following the procedure of Pinzino et al. (*8*). Lutein content in the extracted solutions was determined by isocratic RP-HPLC using a Waters apparatus (model 510) equipped with a spectrophotometric detector (Waters model 441) and a Nucleosil 300-7 C-18 column (4 × 250 mm). Extracts were eluted at 30 °C using 96% CH_3OH as mobile phase at a flow rate of 0.5 mL/min and detected at 445 nm. In these conditions, retention time for lutein was 15 min.

Front-Surface Absorbance Measurements. Front-surface absorbance spectra of flour samples were obtained following the method of Zandomeneghi et al. (*13*, *14*). An ISA Fluoromax II spectrofluorometer was used in the experiments. The samples, 1 mm thick, were enclosed in a Suprasil cell, and the cell windows were tilted 35° with respect to the incident beam to avoid direct reflection of excitation light into the emission monochromator. Powdered $BaSO_4$ was used as the reference light scatterer (*15*).

EPR Measurements. EPR measurements were performed using a Varian (Palo Alto, CA) E112 spectrometer (X-band) equipped with a Varian E257 temperature control unit and interfaced to a 100 MHz personal computer by means of a homemade data acquisition system (*16*, *17*). The content of free radicals was measured at room temperature on dry gluten and flour powders inserted in a quartz tube with an internal diameter of 4 mm. Spectra were recorded using a standard EPR cavity, at a microwave power of 10 mW and a modulation amplitude of 1.25 G. Quantification of organic radicals was performed by comparison of the double integrals of the spectra with that of the standard Varian weak pitch measured under identical instrumental conditions.

3-MAL was used to spin label the sulfhydryl groups of cysteine residues in the gluten samples following the method reported by Capocchi et al. (*18*). For EPR measurements, ~30 mg of the fully hydrated labeled gluten was inserted into a quartz tube closed at both ends to avoid sample loss. EPR spectra were recorded in the range of 0−90 °C on raising the temperature, using a standard EPR cavity, at a microwave power of 2 mW and a modulation amplitude of 1.25 G.

Soluble Protein Extraction and Evaluation. Albumins and globulins, commonly referred to as soluble proteins in flour (*19*), were extracted from flour with 0.1 M Tris-HCl buffer (pH 7.4) (ratio 1:3, w/v), and their concentration was evaluated using the Lowry method as modified by Besandoun and Weinstein (*20*). Bovine serum albumin was used as a standard, and the measurements were performed in a Perkin-Elmer 550S UV−vis spectrophotometer.

Storage Protein Extraction and Analysis. Gliadins and glutenins were extracted as reported by Capocchi et al. (*21*) and freeze-dried.

Analyses were performed by discontinuous SDS-PAGE according to the method of Laemmli (*22*), using a 12% resolving gel and a 4% stacking gel. Freeze-dried proteins were dissolved in 62 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.1% Bromophenol Blue to a final concentration of 1 mg of protein/mL, boiled for 8 min, and loaded (4 μL) on SDS gels. Molecular mass standards used included β-galactosidase (116.3 kDa), phosphorylase *b* (97.4 kDa), ovotransferrin (77.0 kDa), bovine serum albumin (66.25 kDa), glutamate dehydrogenase (55.5 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (30.0 kDa), myoglobin (17.2 kDa), and cytochrome *c* (12.3 kDa). The gels were stained overnight with Coomassie Brilliant Blue R250 (*23*), destained with 7% (v/v) acetic acid, scanned, and processed by a digital image analysis program (SigmaGel, Jandel Corp.).

Enzymatic Assays. Proteases were extracted from flour following the procedure reported by Bottari et al. (*24*) using 2 g of flour and 10 mL of 0.2 M sodium acetate buffer (pH 5) containing 5 mM 2-mercaptoethanol. The proteinase activity was measured as reported in references *7* and *24* using Hb, azocasein, and CBZ-Phe-Ala as substrates and pepstatin A, PMSF, and E-64 as inhibitors. At least three replicates were performed for each analysis.

Puroindoline Extraction and Analysis. Two grams of flour was extracted with 10 mL of extraction buffer following the procedure described by Galleschi et al. (*25*), and the crude extracts were frozen and lyophilized.

For investigation by SDS-PAGE (15% T, 2.7% C), freeze-dried extracts (1 mg) were resuspended in 300 μL of 62 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.1% Bromophenol Blue, boiled for 3 min, and loaded (2 μL) on SDS slab gels. The BDH calibration kit of low molecular mass standards was applied to gels. The gel separation was performed in a mini-gel apparatus (C.B.S. Scientific Co., Solana Beach, CA). Runtime was 30 min at 150 V and, then, 50 min at 200 V. The gels were stained overnight with Coomassie Brilliant Blue R250 and destained with 7.5% acetic acid.

RESULTS AND DISCUSSION

Accelerated aging resulted in a progressive loss of seed viability, associated with both a longer time to radicle emergence and a delay in the time to reach the maximum germination percentage, as previously observed by other authors (*26*−*28*). In particular, the germination capacity decreased by ~30% after 3 days and by >~90% after 4 days of aging. Seeds aged for

Table 1. Germination Parameters of Unaged and Aged Seeds

duration of accelerated aging (days)	germination capacity (%)	germination rate	root length (mm)	shoot length (mm)
0	94 ± 3	86 ± 3	24 ± 2	15 ± 2
3	62 ± 6	47 ± 2	19 ± 2	12 ± 1
4	7 ± 3	7 ± 3	14 ± 2	8 ± 1
6	6 ± 3	3 ± 1	2 ± 1	1.7 ± 0.5
10	0	0	0	0

Table 2. Lutein, Free Radical, and Soluble Protein Content in Flour and Free Radical Content in Gluten

duration of accelerated aging (days)	lutein in flour (ppm)	soluble proteins in flour (mg/g)	free radicals in flour (spins/g)	free radicals in gluten (spins/g)
0	0.98 ± 0.02	4.3 ± 0.1	$(3 \pm 1) \times 10^{14}$	$(5 \pm 1) \times 10^{15}$
3	0.87 ± 0.02	3.1 ± 0.1	$(3 \pm 1) \times 10^{14}$	$(5 \pm 1) \times 10^{15}$
4	0.86 ± 0.01	2.1 ± 0.1	$(7 \pm 1) \times 10^{14}$	$(6 \pm 1) \times 10^{15}$
6	0.79 ± 0.04	1.8 ± 0.1	$(10 \pm 1) \times 10^{14}$	$(9 \pm 1) \times 10^{15}$
10	0.67 ± 0.03	1.5 ± 0.1	$(34 \pm 1) \times 10^{14}$	

10 days did not germinate (**Table 1**). The germination rate followed an analogous trend (**Table 1**). On the other hand, the growth of shoot and primary root (measured on germinated seeds only) was slightly affected by seed aging for short aging times, whereas it rapidly decreased between 4 and 6 days and became null after 10 days (**Table 1**). These data are in agreement with those reported by Krishnan et al. (*29*), who observed loss of germinability of bread wheat seeds (*T. aestivum* cv. HD 2329) stored at 100% relative humidity and 45 °C after 8 days.

When compared with the results reported by us (*7*) during accelerated aging of durum wheat seeds in the same conditions, these data indicate that bread wheat is less resistant to storage at high temperature and humidity, showing loss of germinability at earlier times than durum wheat. In fact, durum wheat seeds died after 14 days of treatment.

As reported in **Table 2**, during the aging treatment, a gradual reduction of the lutein content in flour was revealed by HPLC measurements, from 0.98 ppm in the unaged sample to 0.67 ppm in the sample treated for 10 days. These results are consistent with the changes observed in the front-surface UV−vis absorbance spectra of flours, where a progressive loss of the typical vibrational structure of the absorption band between 430 and 530 nm, due to lutein and other carotenoids (*13*), was observed as a function of aging time (**Figure 1**). The loss of antioxidants was accompanied by an increase of organic free radicals in flour and gluten, in agreement with the findings of Priestley et al. (*30*) and those of Buchvarov and Gantcheff (*31*) in the axes of soybean seeds subjected to accelerated aging and with the results previously obtained by us in flour and gluten of durum wheat (*7*). In fact, EPR spectra of flours showed a signal with a g value of 2.004−2.005 and a peak to peak line width of 8−9 G, whereas those of glutens showed a main signal with $g \cong 2.006$ and a peak to peak line width of ∼7 G, with a shoulder on the left (data not shown), indicating that nitrogen-centered organic free radicals (*32*) are mainly present in flour and gluten samples, with minor contributions ascribable to peroxyl radicals (*33*). As reported in **Table 2**, levels of free radicals in the control samples of flour and gluten were on the order of 10^{14} and 10^{15} spins/g, respectively. When radical contents in flour obtained from aged and control seeds were compared, no appreciable changes were found for up to 3 days of treatment, whereas the radical content doubled after 4 days

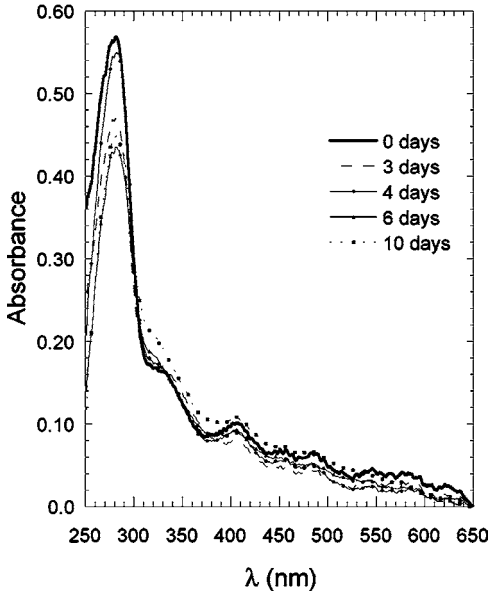

Figure 1. Front-surface absorbance spectra of flour from seeds of bread wheat unaged or aged for 3, 4, 6, and 10 days.

and was 10 times that of the control after 10 days. Radical level in gluten increased with seed aging duration, too, becoming almost 2 times higher than that of the control after 6 days of treatment. g values and relative radical content in flour and gluten samples suggest that free radicals are mainly associated with flour proteins due to bond breakage occurring in endosperm proteins during seed aging because of oxidative processes and enzymatic hydrolysis (*2*, *4*, *5*), whereas starch molecules undergo minor aging-induced scissions or produce radicals that decay or react too rapidly for detection by EPR.

Other findings support the involvement of proteins in seed deterioration. In particular, a decline in the soluble protein content of flour was observed during accelerated aging (**Table 2**), in agreement with the results previously found in seeds of wheat (*7*, *29*, *34*) and other plants (*35*−*42*). In not viable seeds, soluble protein content was reduced by ∼60% with respect to that of unaged seeds. Moreover, an intensity reduction of the lower energy absorption bands of protein aromatic chromophores at ∼280 nm was observed in the front-surface UV−vis absorbance spectra of flours from seeds aged for longer times (**Figure 1**). This behavior can be attributed to protein chromophore losses or to changes of the chromophore environments induced by accelerated aging. In particular, both oxidative radicalic processes and enzymatic hydrolysis may cause protein deterioration (*4*, *5*, *7*, *29*, *35*−*41*) with release of labile amino acids (*36*, *42*) and degradation of amino acids to compounds with different absorption properties (*43*).

In addition, a degradation of seed storage proteins was observed during accelerated aging (**Figure 2**). SDS-PAGE analyses revealed that after 6 days, the aging treatment results in a degradation of gliadins (M_w = 30−75 kDa) and a partial degradation of both high (HMW-GS; M_w = 80−200 kDa) and low molecular mass glutenin subunits (LMW-GS; M_w = 30−70 kDa). After 10 days, the degradation of HMW-GS is more pronounced, whereas LMW-GS do not change anymore. When compared with the electrophoretic analyses reported for durum wheat (*7*), these experiments indicate that degradation of storage proteins begins earlier in bread than in durum wheat. As a result of storage protein deterioration, gluten obtained from the flour of seeds unaged or aged for 3−4 days was yellow and quite abundant (50−70 mg of dry gluten/g of flour), whereas less

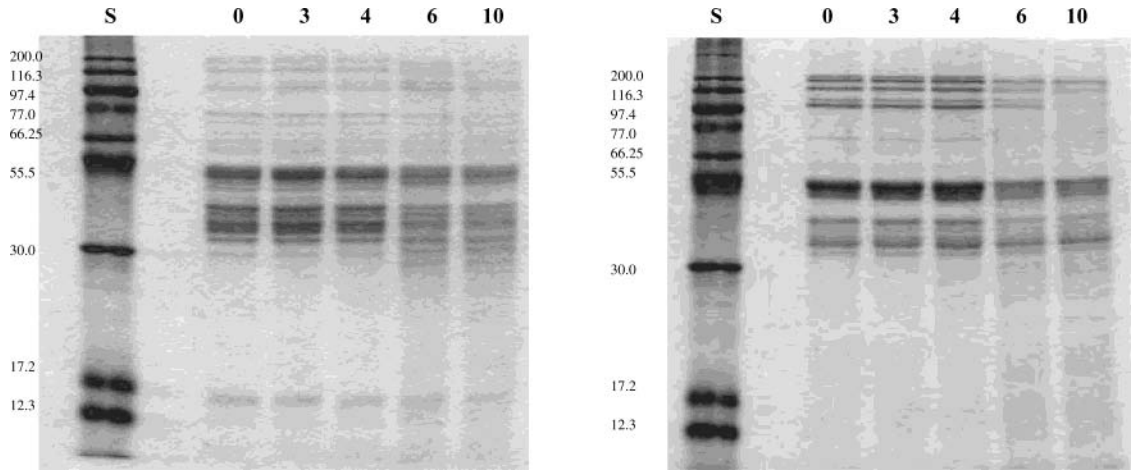

Figure 2. SDS-PAGE of gliadins (left) and glutenins (right) extracted from flour of control seeds (lane 0) and seeds aged for 3, 4, 6, and 10 days (lanes 3, 4, 6, and 10, respectively). Lane S represents molecular mass standards.

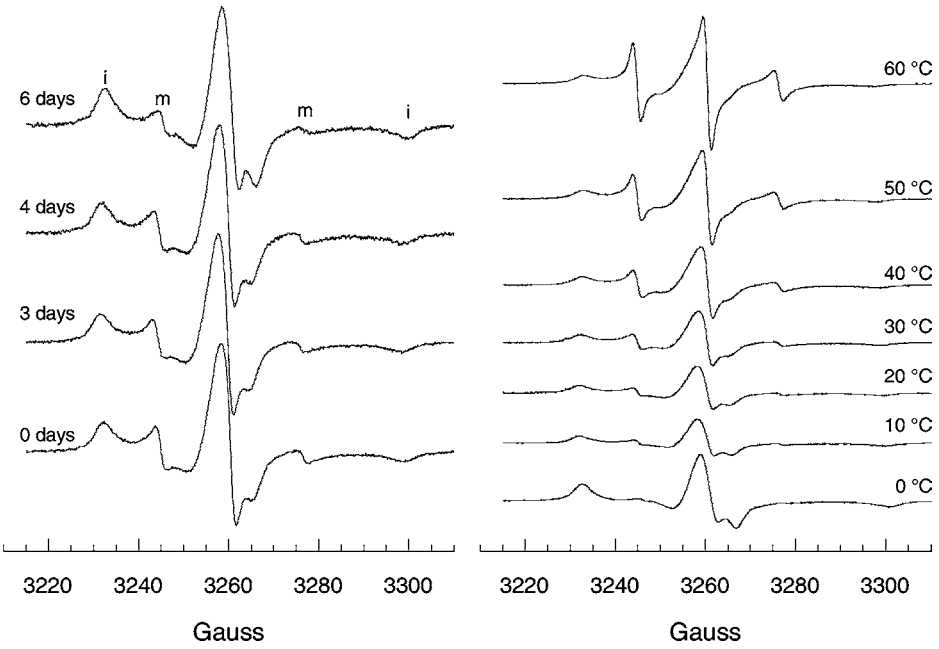

Figure 3. EPR spectra of fully hydrated gluten spin labeled with 3-MAL: (left) spectra recorded at 25 °C on gluten from seeds unaged and aged for 3, 4, and 6 days; (right) spectra recorded on gluten from control seeds at the indicated temperatures. "i" and "m" indicate slow- and fast-moving spin label lines, respectively.

colored gluten was obtained from the flour of seeds aged for 6 days. Flour from seeds aged for 10 days did not give gluten.

Gluten flexibility was investigated using spin-labeling EPR spectroscopy (*44*, 45). Spectra of fully hydrated gluten (**Figure 3**) were of a composite type, with line shapes resulting from the superposition of at least two subspectra each belonging to spin labels differing in their mobility (*7*, *8*, *18*, *46*, *47*), the mobility of spin labels being determined by the flexibility of the protein segments to which they are bound and the steric hindrance of their environments. Slow-moving spin labels, that is, labels bound to sulfhydryl groups localized in somewhat restrictive, crevice-like regions of the proteins, gave broad lines in the spectra, whereas sharp lines were attributed to fast-moving spin labels, that is, spin labels bound to shallow, solvent-exposed protein sulfhydryl groups. Small but significant differences were observed between the EPR spectra recorded at the same temperature on gluten obtained from control and aged seeds (**Figure 3**). In particular, the proportion of fast-moving spin labels decreased with the aging time, especially between 4 and 6 days. Spectra with line shape evolutions indicative of a general increase of mobility with increasing the temperature were observed (**Figure 3**). Two parameters were calculated from the spectra to obtain quantitative information on spin label dynamics and, in turn, on gluten flexibility. The first one is the rotational correlation time (τ) of the mobile spin labels, determined according to the Freed and Fraenkel equation (*48*)

$$\tau = (6.65 \times 10^{-10})\Delta h_{+1}[(I_{+1}/I_{-1})^{1/2} - 1] \qquad (1)$$

where Δh_{+1} is the peak-to-peak width of the low-field line, in gauss, and I_{+1} and I_{-1} are the amplitudes of the low- and high-field lines, respectively. The second parameter that can be calculated from the EPR spectra is R (*46*, *47*), defined as the ratio

$$R = i/m \qquad (2)$$

where i and m are the amplitudes of the low-field peaks of the slow-moving and fast-moving spin labels, respectively. R reflects the ratio between slow- and fast-moving spin label populations.

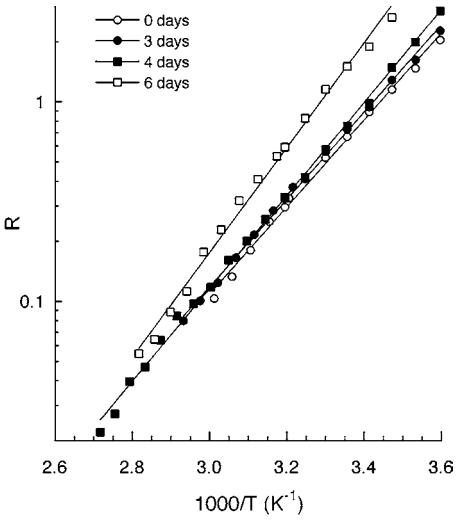

Figure 4. *R* ratio versus 1000/*T*. Lines represent fits of experimental values in terms of the Arrhenius equation.

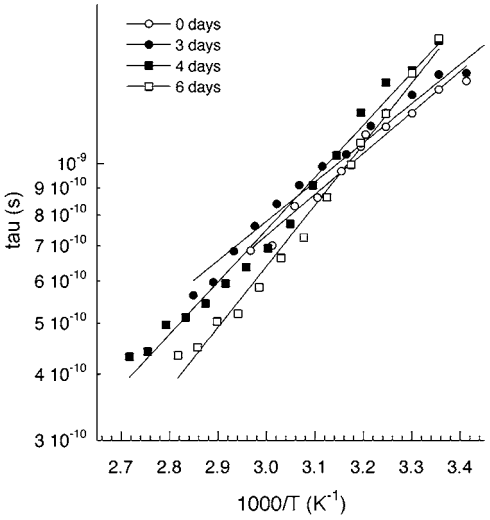

Figure 5. Rotational correlation time τ versus 1000/*T*. Lines represent fits of experimental values in terms of the Arrhenius equation.

Table 3. Activation Energy of *R* (E_a^R) and τ (E_a^τ) in Gluten from Unaged and Aged Wheat Seeds

duration of accelerated aging (days)	E_a^R (kJ/mol)	E_a^τ (kJ/mol)
0	41.8 ± 0.8	14.8 ± 0.9
3	41.9 ± 0.7	14.2 ± 0.8
4	44.6 ± 0.7	18.9 ± 1.4
6	50.3 ± 0.9	22.1 ± 0.8

Both *R* and τ decreased with increasing the temperature (**Figures 4** and **5**), indicating that a progressive transfer of less mobile radicals to a more mobile population occurs and that the rotational diffusion of mobile spin labels becomes faster. The trends of *R* and τ as a function of temperature were described in terms of the Arrhenius equation, and activation energies were determined. As shown in **Table 3**, values determined in samples from seeds aged for 4 and 6 days were higher. Moreover, slightly higher values of *R* were found at each temperature for gluten from seeds aged for up to 4 days with respect to gluten from unaged ones (**Figure 4**), whereas significantly higher values were determined for the sample from seeds aged for 6 days. As far as τ values are concerned, small differences were found between untreated and treated samples.

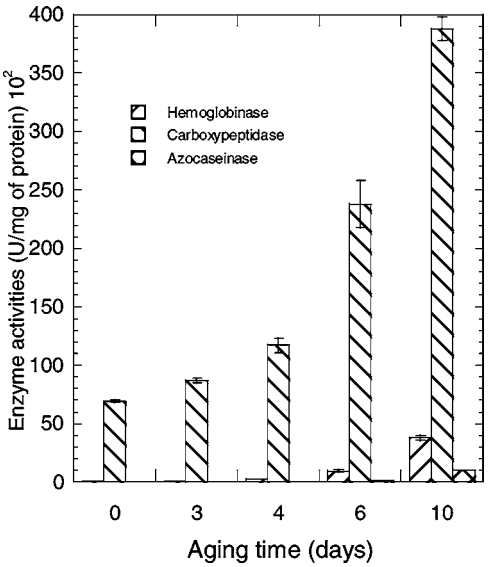

Figure 6. Proteolytic activities in flour from unaged and aged wheat seeds. Each value is an average of three replicates of at least two extractions.

Table 4. Inhibition Percentages of Proteolytic Activities

duration of accelerated aging (days)	CBZ-Phe-Ala	hemoglobin	azocasein		
	PMSF	pepstatin A	E-64	pepstatin A	PMSF
0	92	83		24	18
3	93	82		43	20
4	95	74		51	22
6	95	81		38	42
10	89	75		16	76

In particular, the unaged sample and that aged for 3 days presented very similar values of τ at all temperatures, whereas samples aged for 4 and 6 days had values of τ slightly lower at temperatures $\geq$50 °C and slightly higher at temperatures $\leq$35 °C (**Figure 5**).

As already observed for durum wheat (*7*), the findings on storage protein degradation and gluten flexibility are similar to those obtained during in vitro gluten degradation by proteases from dry and germinating wheat seeds (*18*). This prompted us to investigate the effects of accelerated aging on seed proteolytic activities, with the aim of understanding whether the changes in protein and enzymes occurring during seed aging and germination are similar, as stated by Chauan et al. (*49*).

Among proteins extracted from flour, three proteolytic activities were detected by utilizing exogenous substrates, that is, Hb, CBZ-Phe-Ala, and azocasein (**Figure 6**). The activity degrading CBZ-Phe-Ala was strongly inhibited by PMSF, whereas that hydrolyzing hemoglobin was inhibited by pepstatin A. The azocaseinase activity was inhibited by both pepstatin A and PMSF. In particular, for samples aged for up to 4 days, azocaseinase activity was mainly inhibited by pepstatin A, whereas PMSF was the main inhibitor in samples aged for 6 and 10 days (**Table 4**). E-64 had no effect on azocaseinase activity, indicating that no proteinase activity of cysteine type was present in seeds. These properties allowed us to assign the proteolytic enzymes degrading Hb and CBZ-Phe-Ala as the aspartic proteinase and carboxypeptidase previously isolated from wheat seeds (*50*, *51*). As far as enzymes degrading azocasein are concerned, they could be identified as aspartic and serine proteinases. To the best of our knowledge, the serine protease activity was observed for the first time in wheat seeds in our previous study on accelerated aging of durum wheat (*7*).

On the other hand, serine activity was found to be involved in the first step of storage protein mobilization in germinating *Vigna mungo* seeds (*52*). In our opinion this protease, together with carboxypeptidase and hemoglobinase, could be involved in storage protein degradation during accelerated aging.

As shown in **Figure 6**, all of the proteolytic activities increased with prolonged duration of seed aging. A rise in proteinase activity during accelerated aging of seeds was also reported by other authors in sorghum (*53*), maize (*40*), pigeonpea (*35*), and barley and wheat (*54*) and by us in durum wheat (*7*). These data support our opinion that during storage at high temperature and humidity wheat seeds undergo some, but not all, biochemical changes needed for germination; in fact, synthesis of cysteine proteinase, a fundamental process for seed germination (*55*, *56*), was not observed during accelerated aging of either durum (*7*) or bread wheat.

When the proteolytic activities of bread wheat are compared with those of durum wheat (*7*), similar hemoglobinase and azocaseinase specific activities are observed after 10 days of accelerated aging, whereas carboxypeptidase activity is 2.6 times higher in bread than in durum wheat. This difference in proteolytic activity could be responsible for the earlier loss of viability of bread wheat seeds during accelerated aging. However, the influence of oxidative processes involving free radicals on seed deterioration cannot be disregarded. Lipid peroxidation is indeed the most cited cause of degradative reactions that occur during incubation of seeds at high temperature and moisture levels and contribute to loss of viability in seeds (*1*−*5*). Antioxidants, such as carotenoids, exert a protective effect against free radical attacks. In this context, the lower content of carotenoids in bread wheat seeds [as an example, 0.98 and 2.6 ppm of lutein have been determined by us in flour of bread (**Table 2**) and durum (*7*) wheat, respectively] could render these seeds prone to more rapid deterioration of organs and depletion of stored reserves. This hypothesis is supported by the much stronger increase of free radicals observed as a function of accelerated aging duration for bread wheat flour (**Table 2**) with respect to the durum wheat one (*7*). Nevertheless, further investigations are required to draw general conclusions on the different resistances of bread and durum wheat seeds to accelerated aging, because genetic factors, conditions during seed maturation and harvest, state at which seeds are harvested, and postharvest treatments may affect seed longevity (*5*).

The contribution of microorganisms to the deterioration of seeds artificially aged for 10 days cannot be ruled out (fungus growth was indeed observed on the surface of seeds treated from 10 days, although a fungicide was used). Different classes of proteases (i.e., aspartic proteinases, aminopeptidases, carboxypeptidases, and prolyl dipeptidyl peptidases) produced by pathogens (*57*) could have increased the rate of seed deterioration. Unfortunately, the fact that high moisture content and temperature favor both seed deterioration and microbial growth makes it difficult to ascertain which aspects of deterioration are microbially induced and which are self-inflicted by seeds (*1*).

Finally, accelerated aging did not deeply affect puroindolines for up to 6 days of duration, whereas a sudden disappearance of these proteins was observed after 10 days (**Figure 7**). To explain these results, it is worth noting that puroindolines belong to the family of pathogenesis-related proteins, which also includes lipid transfer proteins (LTPs) (*58*) and thionins (*59*). These proteins share some relevant characteristics such as stability at low pH values and resistance to proteolysis (*60*), which can be related to their folding properties. Puroindolines and LTP1 are structurally related and, most probably, display similar folds (*61*). This could explain the high stability of puroindolines during accelerated aging.

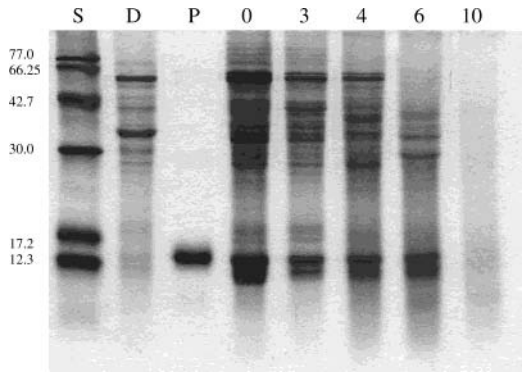

Figure 7. SDS-PAGE of puroindolines: (lane S) molecular mass standards; (lane D) crude extracts from durum wheat flour; (lane P) purified Pin-b obtained by preparative A-PAGE (*25*); (lanes 0, 3, 4, 6, and 10) crude extracts of flour from unaged and aged bread wheat seeds.

ABBREVIATIONS USED

EPR, electron paramagnetic spectroscopy; 3-MAL, 3-maleimidoproxyl; CBZ-Phe-Ala, *N*-carbobenzoxy-L-phenylalanyl-L-alanine; E-64, *trans*-epoxysuccinyl-L-leucylamido-4-guanidinobutane; Hb, hemoglobin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride.

SAFETY

Precautions were taken to prevent any contact of 3-MAL and labeled gluten with skin and eyes.

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Received for review November 21, 2003. Revised manuscript received April 21, 2004. Accepted April 25, 2004. This work has been partially financed by CNR (Progetto Giovani Agenzia 2000).

JF0353741