Free Radicals in Flour, Starch, and Gluten Produced by Ball-Milling, Electric Discharge, and Gamma-Irradiation[1]

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ABSTRACT

Bread flour, dry gluten, and wheat starch, ball-milled under several different conditions, had an electron spin resonance (ESR) spectrum indicating the presence of free radicals. The ESR spectrum of ball-milled gluten was qualitatively similar to the spectrum of ball-milled flour, while that of starch was quite different. The radicals generated by ball-milling and gamma-irradiation were inert to water vapor, but were scavenged quickly by atmospheric oxygen. The ESR spectra of the free radicals generated by electric discharge and gamma-irradiation were quite different from those produced by ball-milling.

Free radicals can be produced in many synthetic (1) and natural (2–5) polymers by mechanical forces. Redman et al. (5) produced free radicals in flour, gliadin, gluten, and wheat starch by ball-milling at -196°C. in a vacuum. They reported that the free radicals were stable at -196°C. but decayed quickly at room temperature, or when oxygen was admitted to the system.

Lee et al. (6–8) generated free radicals in flour, gluten, and starch by gamma-irradiation and studied the effect of moisture on their production and stability. These workers concluded that free radicals could be generated in flour by

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gamma-irradiation if its moisture content was below 8%. In the presence of water vapor these free radicals decayed via a second order reaction.

Our main interest in free radicals in wheat flour and the products obtained from it originates from the hypothesis that they might be involved in the mechanical development of doughs (9). This article reports results obtained with a highly sensitive electron spin resonance (ESR) spectrometer on the production and stability of free radicals in flour under a variety of conditions, and extends the published reports of others (5–9).

MATERIALS AND METHODS

The flour used was milled from Manitou, a variety of Canadian hard red spring wheat. Protein (N × 5.7) and ash contents of the flour were 12.5 and 0.43%, respectively, on a 14% moisture basis. Samples of starch (less than 1% protein on a 14% moisture basis) and gluten were prepared in the laboratory from the flour by the usual hand-washing procedure. A sample of commercial gluten was obtained from Industrial Grain Products, Thunder Bay, Ont.

A high vacuum line was used to degas and introduce desired gases into the sample. The ESR spectrometer was a Varian model E-3 equipped with a multipurpose cavity.

In a typical experiment, flour was placed into a specially fabricated Pyrex bulb containing Pyrex beads and then degassed at room temperature to a vacuum of 10^{-3} mm. of mercury or better. After degassing the sample tube was sealed off. To produce the free radicals, the sample was ground by shaking the bulb with a modified Wig-L-Bug dental amalgamator at approximately 3,000 c.p.m. through an amplitude of 3 cm. The shaking period was usually 1.5 hr. Ball-milling at −196°C. was accomplished by shaking the sample bulb in a large styrofoam tub filled with liquid nitrogen. There was no noticeable temperature change in the samples milled at room temperature.

After grinding, the sample bulb was removed from the shaker and kept immersed in liquid nitrogen (−196°C.) if necessary. A small portion of the ground flour was transferred into the Pyrex finger which was part of the grinding bulb.

ESR spectra were recorded for samples at room temperature (22°C.) or at liquid nitrogen temperature (−196°C.). No special procedure was required to obtain a spectrum at room temperature; however, to obtain spectra at the lower temperature, it was necessary to keep the sample finger in a specially constructed quartz dewar flask which also fitted into the spectrometer cavity.

A cobalt 60 gamma cell was used as a source of gamma-rays. Irradiation at required exposures was made at room and liquid nitrogen temperatures. In the latter case the samples were held in a Pyrex dewar filled with liquid nitrogen, which was irradiated together with the sample. To remove any radiation damage signals that might be present, the quartz sample tubes were constructed to permit heating of the empty end after gamma-irradiation. The irradiated sample was then transferred to the annealed end of the tube prior to recording of the ESR spectra.

A tesla coil was used as a high voltage source in the electric discharge experiments. A copper wire was wrapped around a degassed quartz tube containing the flour sample. This was then placed in a dewar filled with liquid nitrogen. The free end of the wire was then connected to a supported tesla coil in such a manner that the tube was suspended in the dewar by the wire from the tesla coil.

The tesla coil was then discharged for 45 min. After the discharging time had elapsed, the upper (empty) end of the tube was annealed as described above.

All grindings and irradiations were made under specific atmospheres depending on the type of experiment. Actual atmospheres used will be indicated later.

RESULTS AND DISCUSSION

Free Radicals Produced in Flour by Fine Grinding

Experiments were carried out under a variety of conditions to gain information in addition to that already published (5) about the stability and reactivity of free radicals produced by ball-milling flour.

Flour ground in a vacuum for 1.5 hr. at −196°C. gave a strong ESR signal at a field strength of 3,280 gauss indicating the presence of free radicals (Fig. 1A). A control (not ground) gave no signal at the highest gain that could be obtained on the instrument used. Experiments in which the flour was replaced with powdered quartz showed that fragmented Pyrex beads were not responsible for the signal.

The spectrum of flour ball-milled in a vacuum at −196°C. strongly resembled a gaussian spectrum for a 1:4:6:4:1 distribution (10). This spectrum is similar to that reported by Redman et al. (5), except that the peaks are considerably better resolved.

When the ground sample was allowed to warm up to room temperature there

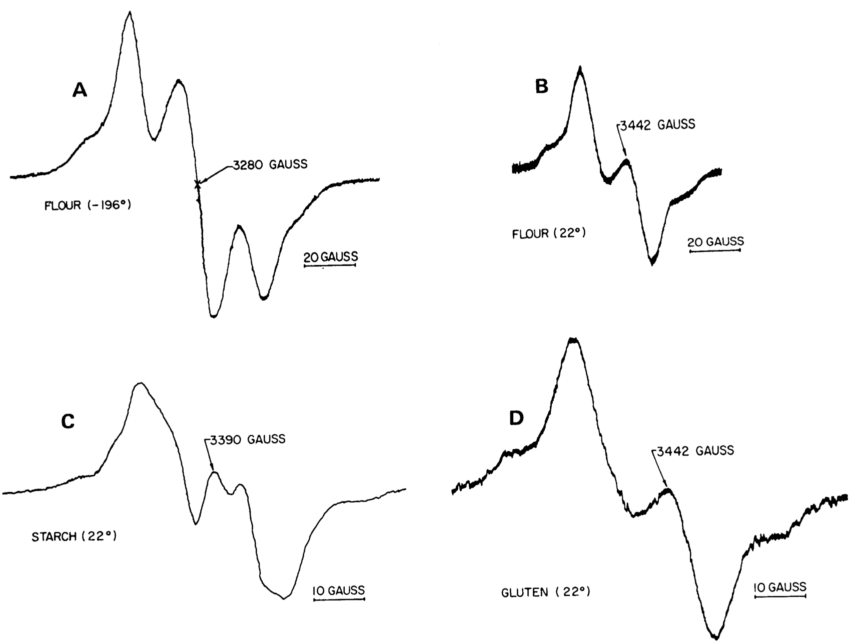

Fig. 1. ESR spectra: A, flour ball-milled at −196°C. for 1.5 hr., recorded at −196°C. at a relative gain of 1; B, flour ball-milled at −196°C. for 1.5 hr. and then kept at room temperature for 1 day, recorded at 22°C. at a relative gain of 4; C, starch ball-milled at −196°C. for 1.5 hr. and recorded at 22°C. at a relative gain of 1; and D, gluten ball-milled at −196°C. for 1.5 hr., recorded at 22°C. at a relative gain of 3.2.

was a decrease of the original signal intensity, but the remaining free radicals were quite stable. This decrease can be accounted for by the Boltzmann effect. Figure 1B illustrates the stabilized spectrum at room temperature at a gain setting four times the gain used for Fig. 1A (recorded at −196°C.). On warming to room temperature, the spectrum was shifted to 3,442 gauss and transformed into a doublet with some hyperfine splitting at both ends of the spectrum. The ratio of the peak intensities was 1:1. The observed doublet was transformed to a singlet (spectrum not shown) when the sample was cooled to −196°C.

The transformation of free radical species present at −196°C. to those stable at 20°C. may be explained by any of the following changes:

1. The site at which the unpaired electron is located at −196°C. becomes altered at higher temperatures.
2. The unpaired electron moves to a site of lower potential energy.
3. The highly reactive free radicals produced at −196°C. abstract hydrogen atoms from adjacent molecules to produce the less reactive free radicals observed at room temperature.

A sample of degassed flour ball-milled at room temperature gave an ESR spectrum qualitatively similar to that produced by grinding at −196°C. and studied at room temperature. However, the concentration of free radicals was slightly higher in this case than in the sample that was ball-milled at −196°C. and studied at room temperature.

Free radicals were also detected in a flour sample ball-milled at −196°C. in the presence of air (spectrum not shown). This spectrum was quite different from that obtained for the flour ground in a vacuum at −196°C. Also, the concentration of free radicals was considerably lower than in the samples ground in a vacuum. This signal faded on warming, and no free radicals could be detected after a few days' storage at room temperature.

Free radicals could not be produced in flour by grinding in air at room temperature. Presumably, the free radicals formed under these conditions are quickly scavenged by atmospheric oxygen.

Free Radicals in Wheat Starch and Gluten Produced by Fine Grinding

Wheat starch ground at −196°C. in a vacuum and subsequently warmed to room temperature showed a somewhat different ESR spectrum at 3,442 gauss than that obtained for flour subjected to identical treatment (Fig. 1C). The main difference is qualitative.

The sample of commercial gluten used in this study showed a weak ESR signal before grinding (spectrum not shown). This spectrum was a singlet with no hyperfine splitting. The origin and nature of these free radicals were not investigated. On grinding at −196°C. and after warming to room temperature, this signal disappeared and a new signal at 3,442 gauss appeared (Fig. 1D). The spectrum is a broad doublet with a peak intensity ratio of 1:1. The ESR spectra of gluten, ball-milled at −196°C. and 20°C. and recorded at −196°C. and 20°C., were similar to the spectra of flour which had been ball-milled and studied at these temperatures.

Gluten prepared in the laboratory had no detectable free radicals before grinding. The ESR spectrum produced by grinding at −196°C. or 20°C. was qualitatively similar to that produced in flour and commercial gluten. However, the intensity of the signal for the laboratory gluten was about twenty times higher than the signal for the flour or the commercial gluten. These results suggest that it is the protein rather than the starch component of the flour that is the major source of free radicals when flour is ground by ball-milling.

These results pose an interesting question. Why should proteins, which form only about 12% of the flour, be the major source of free radicals while starch forms 70 to 80% of the flour? The answer could be in the physical structure of the flour particles. It is known that in flour from hard wheats the starch granules are covered with layers of proteins (11). Accordingly, the protein would be more subject to abrasive action during ball-milling than the starch. This speculation remains to be verified.

Effect of Water Vapor

Two experiments were carried out to determine the effect of water vapor on the free radicals produced in flour by ball-milling. In the first, oxygen-free water was added to a degassed flour sample before ball-milling at $-196°$C. In the second, oxygen-free water was added to the flour which had been ball-milled at $-196°$C. in a vacuum. Results of both experiments showed that the free radicals produced by ball-milling in vacuum were not affected by the presence of water.

Free Radicals Produced by Electric Discharge

Figure 2A shows the ESR spectrum of flour exposed to an electric discharge of 75,000 v. for 45 min. in a vacuum at $-196°$C. Upon warming to 20°C. the peak intensities decreased by 90% (Fig. 2B), but the field strength (3,280) remained constant for both samples. The spectra of samples subjected to electric discharge were qualitatively different from the spectrum of ground floor (compare Figs. 1B and 2).

Free Radicals Produced in Flour and Gluten by Gamma-Irradiation

Experiments similar to those of Lee et al. (6–8) were carried out to compare the free radicals produced in flour and gluten by gamma-irradiation with those produced by ball-milling or by electric discharge.

The spectra for flour and gluten recorded at $-196°$C. were almost identical and appeared as broad singlets at the field strength of 3,280 gauss (spectra not shown). Upon warming to room temperature the signals were shifted to 3,442 gauss, became narrower, and the intensities in both cases decreased by approximately 30%. The flour signal remained slightly weaker than the gluten signal.

The free radicals produced by gamma-irradiation of flour and gluten were quite stable. Free radical concentrations remained constant for several months under vacuum at room temperature. In air there was a slow but continuous decay with time. In one week the intensities decayed by 80%.

The free radicals produced by ball-milling, electric discharge, and gamma-irradiation are quite different. Further work is required to identify the nature of the free radicals produced under these conditions.

In another experiment, a flour sample at 14% moisture was irradiated for 1 hr. in an open vessel. Although the concentration of free radicals produced under these conditions was low, their existence was readily detectable. This treated sample was subsequently covered with distilled water and the rate of decay of the free radicals was monitored. The free radicals were observed to remain quite stable

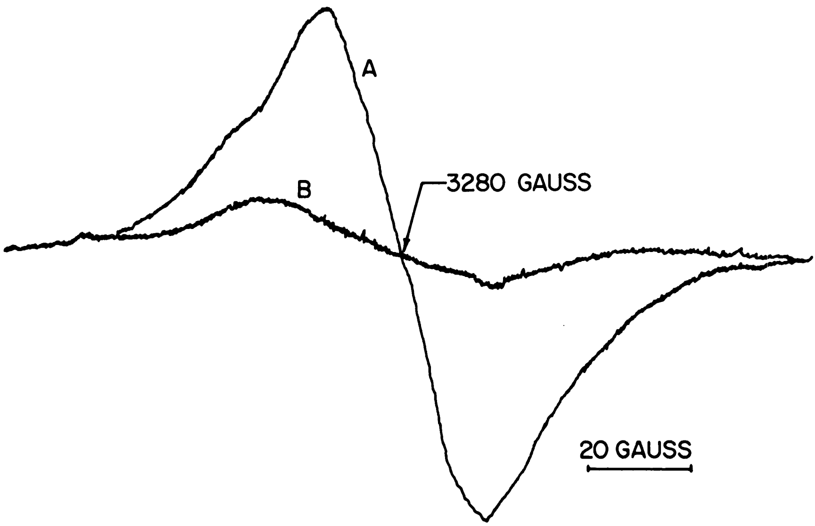

Fig. 2. ESR spectra: A, flour subjected to high voltage discharge at −196°C. for 45 min., recorded at −196°C. at a relative gain of 1; and B, flour subjected to electric discharge as in A and subsequently warmed to 22°C., recorded at 22°C. at a relative gain of 6.

under these high moisture conditions. These observations appear to be contrary to the findings of Lee et al. (6–8), who reported that the presence of water inhibited the production of free radicals by gamma-irradiation. It is possible that the results obtained by Lee et al. could arise from incomplete removal of oxygen.

GENERAL DISCUSSION

Free radicals can be produced in flour by fine grinding at −196°C. in the absence of oxygen. A large portion of the free radicals produced by this action disappeared quite rapidly during storage at room temperature after grinding, but a relatively strong ESR signal persisted after long period of storage. It is speculated that the radicals produced by grinding at −196°C. react to form a more stable free radical with a lower energy state. Both species of free radical are readily scavenged by oxygen but not by water.

The free radicals produced by gamma-irradiation of flour and gluten appear to be relatively inert to atmospheric oxygen compared to the free radicals produced by ball-milling. The free radicals produced by gamma-irradiation are also quite inert to water vapor.

Results of the present study showed that the free radicals produced in flour by ball-milling are different from those produced by gamma-irradiation. This observation is contrary to that of Redman et al. (5) who reported that the free radicals produced under similar conditions were identical.

The gamma-irradiation results of the present study differ from those of Lee et al. (6–8) in one important aspect. The previous workers reported that the free radicals they detected were readily scavenged by water vapor. In contrast, the present study showed that the free radicals produced in flour and dry gluten by gamma-irradiation were quite stable in the presence of water at normal moisture levels. The results of Lee et al. (6–8) can be explained on the assumption that the flour samples used were not completely degassed and contained small amounts of oxygen which quickly scavenged the free radicals produced.

The finding that free radicals produced by electric discharge were different from the free radicals produced by ball-milling is interesting. According to published evidence (12, 13) it would seem that the free radicals in flour produced by electric discharge result from ionization of hydrogen atoms from substrate molecules. Since the spectrum produced by electric discharge is different from the spectrum produced by ball-milling, it is probable that free radicals produced by ball-milling flour do not result from abstraction of hydrogen atoms.

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