Electron Spin Resonance Studies of Isolated Gluten Systems[1]

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Cereal Chem. 65(1):55–58

ABSTRACT

Electron spin resonance (ESR) was used to study paramagnetic species present in commercial gluten powder and the conformational changes of hydrated gluten after heating. Gluten powder had ESR signals attributed to transition metals and to either peroxides or chelated transition metals. The ESR signal was diminished after heating and absent after hydration of the gluten powder. The paramagnetic species found in gluten powder were not affected by room temperature hexane extraction of the gluten. Spin probe studies of gluten-water-TEMPO at room temperature showed TEMPO partitioned into hydrophobic and hydrophilic environments in the protein system. The fraction of TEMPO distributed into hydrophobic regions of the protein system increased after heating to final temperatures ranging from 45 to 95° C. Hexane-extracted gluten-water-TEMPO systems at room temperature and after heating had lower fractions of TEMPO in a hydrophobic environment than the nonextracted systems.

Gluten proteins and the lipids associated with them are constituents of wheat flour important to the final quality of a baked product. Electron spin resonance (ESR) techniques were utilized to identify paramagnetic species present in the components of the wheat kernel (Windle and Evans 1971). With the use of spin probes, ESR was also used to study starch-water and starch-stearic acid interactions (Pearce et al 1985, 1987; Nolan et al 1986; Windle 1985). Spin probes can also be utilized in biological systems to evaluate the hydrophobic and hydrophilic nature of the environment the spin probe is experiencing. TEMPO spin probes were used to study the fluidity of lipid membranes (McConnell 1976) and lipid-protein interactions in lipid bilayers (Grant and McConnell 1974) by estimating the fraction of TEMPO in the hydrophobic fluid lipid environments.

In this study, ESR was used to examine native paramagnetic species present in vital wheat gluten, changes in microenvironments relative to changes in gluten structure upon heating, and the effect of removing some nonpolar lipids by hexane. The relative amounts of TEMPO partitioned between hydrophobic and hydrophilic portions of the gluten system were measured by the free radical activity of the spin probe TEMPO.

MATERIALS AND METHODS

Gluten and Solvents

A commercial vital wheat gluten provided by Continental Baking, Ralston Purina (7.30% moisture, 80.20% protein, 4.00% fat, 0.70% ash) was used. The gluten was also evaluated after a room temperature hexane extraction according to the method of Johnson and Hoseney (1979). The solvents used were glass-distilled water and reagent grade, 99+% hexane (Aldrich Chemical).

[1]Published as paper no. 15337 of the Scientific Journal Series of the Minnesota Agricultural Experiment Station on research conducted under Minnesota Agricultural Experiment Station Projects 18-063 and 18-027.

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Spin Probes

The structure of TEMPO (2,2,6,6-tetramethylpiperidinyloxy) (Aldrich Chemical) is shown in Figure 1. TEMPO in water was prepared by slurrying the spin probe with water (probe/water weight ratio = 1:1,000) at room temperature for 24 hr.

Studies of Native Paramagnetic Species in Gluten

Gluten powder was examined unhydrated, hydrated (gluten/water = 1:1, w/w), unhydrated in corn oil (gluten/oil = 1:0.75, w/w), after hexane extraction, and after metal ion extraction with ethylenediaminetetraacetic acid (EDTA). To extract metal ions, 2 g of gluten powder was stirred for 24 hr in 500 ml of a water and EDTA solution (0.5 g EDTA/1,000 ml water). The suspension was filtered with a Buchner funnel, the gluten washed with 250 ml of glass-distilled water, and the filtering-wash process repeated three additional times. The powder was then dried for 48 hr in a desiccator at room temperature. ESR spectra were recorded for the dry gluten powder and hydrated gluten immediately after preparation at room temperature or after heating for 4 min at the specified temperature, followed by quenching to room temperature. Temperatures from 45 to 95° C at 5° C intervals were utilized. A fresh sample was used for each temperature.

Gluten powder samples were placed in 5-mm nuclear magnetic resonance (NMR) tubes for ESR analysis. Hydrated gluten samples were manually mixed with glass stirring rods until all of the gluten powder was wetted and formed a cohesive mass. Thin strands of gluten sample were wound around the outside of an empty 2-mm glass tube. The 2-mm glass tube was put in a 5-mm NMR tube, and the NMR tube was sealed. Spectra were collected at room temperature using a Varian E-3 spectrometer. The spectra were scanned over a range of 5,000 G with field center in the vicinity of 3.2 kG, modulation amplitude of 10, and attenuation power low enough to avoid saturation.

Mineral Analysis of Gluten

Inductively coupled plasma analysis was done on the gluten powder. Gluten powder was ashed, dissolved in 2*N* HCl, and analyzed using an Applied Research Laboratories spectrometer model QA 137.

Gluten-TEMPO-Water Studies

Gluten powder or hexane-extracted gluten powder was prepared for studies using TEMPO by mixing gluten and the slurried TEMPO-water mixture (gluten/water/spin probe = 1:1:0.001). Samples were prepared and heated by the same method used for the hydrated gluten samples. Gluten-water-TEMPO samples were scanned in the ESR over a 100-G range at a modulation amplitude of 1. When three-line spectra with a high-field doublet as shown in Figure 1B were recorded, the fraction (f-value) of spin probe representative of TEMPO in a more hydrophobic environment was estimated by the procedure used by McConnell (1976) to examine the fluidity of lipid membranes where $f = H/(H + P)$; H is the amplitude of the line representing the more hydrophobic environment and P the amplitude of the line representing the more hydrophilic environment of the system.

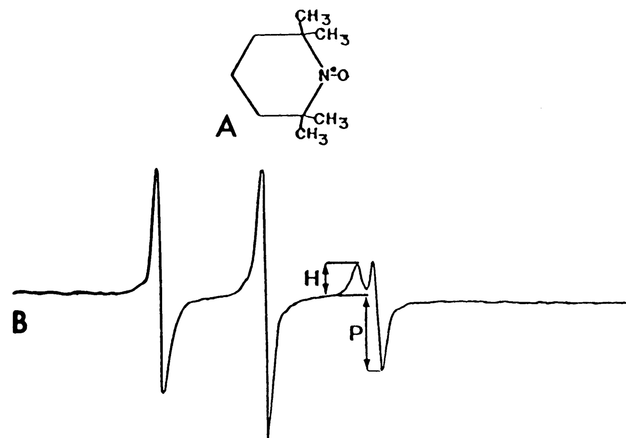

Fig. 1. A, The structure of TEMPO. **B,** An electron spin resonance spectrum with a high-field doublet where line H is due to the spin probe in a more hydrophobic environment and line P is due to the spin probe in a more hydrophilic environment. The fraction of spin probe in a more hydrophobic environment (f-value) can be estimated by measuring the amplitudes of lines H and P and calculating $f = H/H + P$.

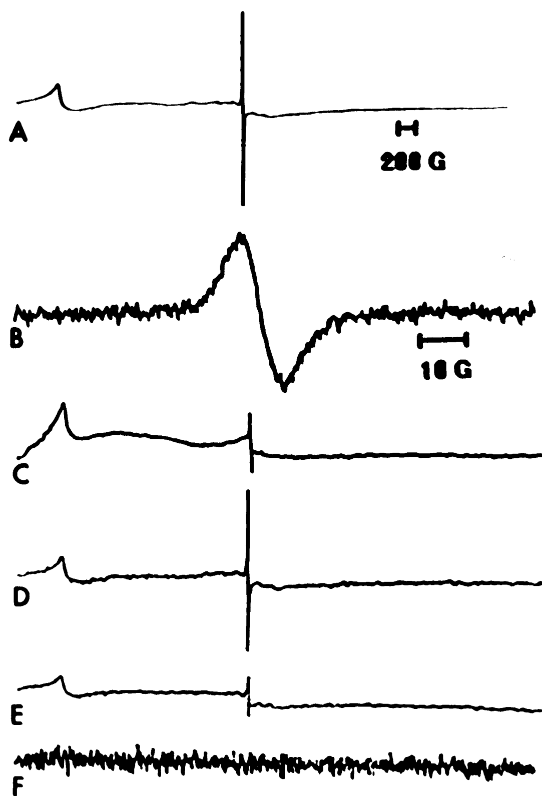

Fig. 2. Electron spin resonance spectra recorded at room temperature for gluten powder **A,** unhydrated; **B,** unhydrated, over a 100-G scan range; **C,** slurried in ethylenediaminetetraacetic acid, washed, and dried; **D,** after heating at 50° C; **E,** after heating at 95° C; **F,** hydrated. Spectra A, C, D, E, and F were recorded over a 5,000-G scan range.

RESULTS AND DISCUSSION

Studies of Native Paramagnetic Species

The ESR scan for unhydrated gluten is shown in Figure 2A and consists of three lines: a broad low-field line, a larger and narrower line centered at approximately 3.3 kG, and a smaller line having a sextet hyperfine pattern superimposed on the larger central line.

The large narrower ESR line centered at approximately 3.3 kG is attributed to the presence of transition metals as discussed below. Inductively coupled plasma analysis done on the vital wheat gluten used in this study showed that the gluten contained the transition metals Mg, Fe, Mn, Zn, and Cu. The individual paramagnetic metals contributing to the center field ESR signal could not be identified due to lack of hyperfine line structure and spin broadening. Although the central transition metal line appeared very narrow in the 5,000-G scan (Fig. 2A), a 100-G scan (Fig. 2B) shows the line to be spin broadened because of the different paramagnetic species present. Metal ion extraction of the gluten powder with EDTA resulted in reduction of the 3.3-kG line height (Fig. 2C), indicating that the center line was due to paramagnetic transition metals.

The low-field line and center-field sextet of the ESR scan were like those found in ESR studies of wheat and bread. These lines were attributed to the Fe^{3+} ion and Mn^{2+} ion, respectively, because of their line position and characteristic line shape (Windle and Evans 1971, Windle et al 1976).

The low-field line, however, did not diminish in intensity after slurrying with EDTA (Fig. 2C). This indicated that if the low-field line is due to Fe, it is either strongly chelated with the gluten system, or else it is due to some other free radical species such as peroxides.

The intensity of the transition-metal line of dry gluten powder was diminished after heating. ESR spectra of gluten powder heated at 50° C and 95° C are shown in Figure 2D and E. In other biological studies the disappearance of ESR spectra of paramagnetic metals was attributed to loss of an unpaired electron in the d-orbitals, changes in protein-metal interactions, or altered interaction between transition metals (Hyde et al 1979).

Gluten powder hydrated at a ratio of gluten to water equal to 1:1 had no detectable ESR activity (Fig. 2F). Loss of ESR activity could result from one or more factors. In hydrated samples, smaller sample sizes were required because of the high dielectric absorption of water. Consequently, in these smaller samples the concentration of paramagnetic species was also reduced. ESR activity was not lost, however, in samples of gluten and corn oil. Thus the loss of ESR activity in hydrated samples was not because of the smaller sample sizes utilized. Hydration of the gluten-forming proteins also could result in hydration of transition metals and consequently alter protein conformation and protein-metal interactions. This can result in loss of ESR activity. Loss of ESR activity with gluten hydration was reversible. When hydrated gluten was desiccated, ground, and rescanned in the ESR, the typical spectrum seen earlier for dry gluten powder (Fig. 2A) was restored. Hexane extraction of gluten powder gave ESR spectra similar to unextracted gluten (Fig. 2A).

Gluten-Water-TEMPO Studies

Loss of detectable ESR activity in hydrated gluten samples eliminated the problem of spectral overlap with nitroxides in spin probe studies of hydrated gluten. A typical ESR spectrum for TEMPO-water systems is shown in Figure 3. ESR absorption lines for nitroxides are centered at approximately 3.2 kG; therefore, scans for TEMPO systems ranged over 100 G.

The sharp three-line spectrum of equal line height for TEMPO-water (Fig. 3) is typical of TEMPO in a low-viscosity aqueous environment. Gluten-water-TEMPO (Fig. 4A) has a three-line spectrum with reduced high-field line height typical of a spin probe having slowed motion because of different microenvironments within the gluten proteins. In addition to reduced high-field line height, the high-field line of gluten-water-TEMPO systems was a doublet composed of two components, *H* and *P*, as shown in Figure 4. The sharp, well-defined nature of the doublet indicates that there is not a wide distribution of differences in aqueous microenvironments. If such a distribution were present, then the peak would merely be broadened. Each of the three lines in gluten-water-TEMPO systems was a doublet; however, for a spectrometer operating at 9.33 GHz only the high-field doublet is resolved because of the effects of solvent polarity on line splitting. Gluten-water-TEMPO spectra recorded at room temperature before heating and after undergoing heating at 50° C and 95° C are shown in Figure 4A, B, and C. These spectra show that the amount of TEMPO in a more hydrophobic environment increased as the gluten-water-TEMPO was heated at higher temperatures, as indicated by the increased amplitude of the high-field *H* component.

Removal of nonpolar lipids from gluten by hexane extraction gave ESR spectra for hexane-extracted gluten-water-TEMPO systems that displayed high-field doublets (Fig. 5A, B, and C) similar to those of the unextracted gluten in Figure 4. The three-line spectrum resulting from TEMPO did not show any binding or interaction between TEMPO and gluten except the interaction of the spin probe with more hydrophobic molecules as the sample underwent heating at progressively higher temperatures. However, the actual amplitude of the portion of the doublet attributed to the hydrophobic component was lower in the hexane-extracted gluten than in the unextracted samples.

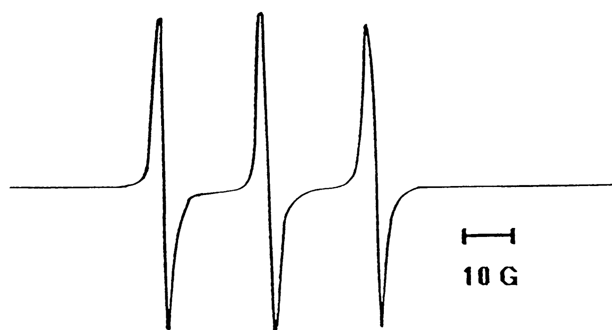

Fig. 3. Typical electron spin resonance spectra for water-TEMPO.

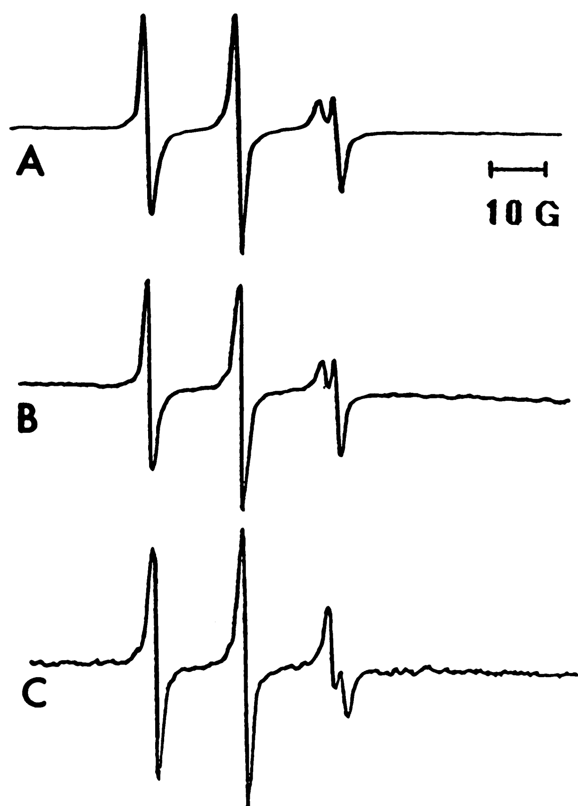

Fig. 4. Electron spin resonance spectra recorded at room temperature for gluten-water-TEMPO: **A,** unheated; **B,** after heating at 50° C; **C,** after heating at 95° C.

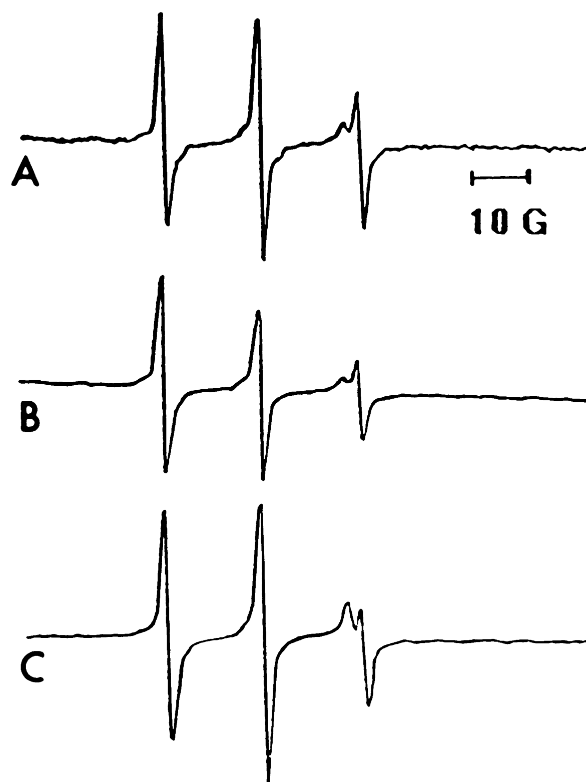

Fig. 5. Electron spin resonance spectra recorded at room temperature for hexane-extracted gluten-water-TEMPO: **A,** unheated; **B,** after heating at 50° C; **C,** after heating at 95° C.

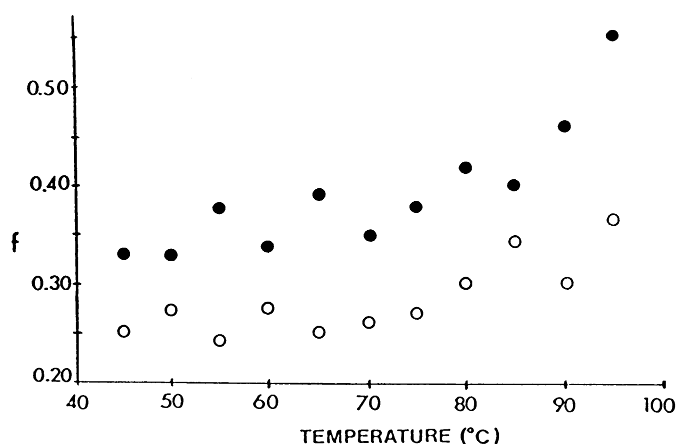

Fig. 6. The fraction of TEMPO in a hydrophobic environment (f) versus temperature (° C) to which the sample was heated for gluten-water-TEMPO (●) and hexane extracted gluten-water-TEMPO (o).

To illustrate further the effects of heating and hexane extraction, the f-values for a series of samples heated to temperatures from 45 to 95°C are summarized in Figure 6 for both unextracted and hexane-extracted samples. For unheated samples, f-values for unextracted and hexane-extracted samples were 0.32 and 0.23, respectively. The standard deviation for eight replicate determinations of hexane-extracted samples at room temperature was 0.039.

The f-value for unextracted gluten-water-TEMPO was 0.33 compared with 0.25 for hexane-extracted gluten-water-TEMPO after both systems were heated at 45°C. After heating at 95°C, f-values were 0.55 for unextracted and 0.36 for hexane-extracted gluten-water-TEMPO.

These data show that the spin probe TEMPO can be used to differentiate between microenvironments in hydrated gluten proteins. The hydrophilic portion of the spectra, *P*, resulted from TEMPO being closer to more hydrophilic groups in the system, whereas the hydrophobic portion of the spectra, *H*, resulted from TEMPO being closer to more hydrophobic regions of the protein or lipids in the system. McConnell (1976) observed increased partitioning of TEMPO into hydrophobic environments in aqueous lipid systems resulting from increased lipid fluidity and TEMPO solubility as the ESR spectra were recorded at elevated temperatures. ESR spectra were recorded at room temperature in this study for gluten-water-TEMPO. Therefore, the increased partitioning of TEMPO into hydrophobic environments reflected by increased f-values after the gluten samples were heated and cooled could not be attributed to increased TEMPO solubility or lipid fluidity at those elevated temperatures. Conformational changes that occur upon heating in protein systems can expose more of the hydrophobic protein groups to the aqueous system (Tanford 1980). Therefore, there is the possiblity that the changes in f-values were a result of conformational changes. The hydrophobic TEMPO interaction could even be a result of lipid associated to protein.

TEMPO partitioned to a proportionally greater hydrophobic environment versus a hydrophilic environment for either hexane-extracted or unextracted systems after undergoing heating at progressively higher temperatures. In addition, the removal of some nonpolar lipids by hexane extraction resulted in lower f-values that persisted throughout heating relative to f-values before lipid removal.

The behavior of the gluten protein complex contrasts with that of wheat starch systems. No partitioning of TEMPO into hydrophobic and hydrophilic environments or differences between hexane-extracted and nonextracted wheat starch were observed in starch-water-TEMPO systems at room temperature or after heating (Pearce et al 1985). The differences between the starch and gluten systems suggest that the response of starch to hydration and heating as evaluated by ESR is quite different from that of proteins as they undergo conformational changes with heat.

In summary, this study demonstrated that ESR is a sensitive method for the characterization of native paramagnetic species found in gluten powders. This study also demonstrated that TEMPO spin probes can be used to differentiate between microenvironments that are hydrophilic or hydrophobic in nature. Changes in microenvironments as the protein is heated can also be measured.

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[Received March 31, 1987. Revision received September 18, 1987. Accepted September 21, 1987.]