A Spin-Label Study on Lipids in Dough Formed at Various Concentrations of Oxygen

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The effect of oxygen on wheat flour lipids during dough mixing was investigated by analysis of the lipid composition and by an ESR technique with a fatty acid spin-label (4,4'-dimethyloxazolidine-*N*-oxyl derivative of 5-ketostearic acid). Dough was prepared in the presence of the spin-label under an atmosphere of air, nitrogen, 95% nitrogen-5% oxygen or oxygen, and the gluten was obtained by washing out the starch. ESR spectra of the spin-label incorporated into the gluten showed decreases in the order parameter, rotational correlation time and activation energy for rotational viscosity with increasing atmospheric oxygen concentration. During dough mixing in oxygen, oxidation of lipids proceeded and bound lipids slightly decreased. These data indicate that modification of lipids by incorporated oxygen leads to an increase in their fluidity and to a decrease in their hydrophobic interaction with protein in dough.

The effects of oxygen on dough properties have received considerable attention. The presence of oxygen gives dough a lower extensibility and greater resistance to extension.^{1,2)} It has been reported that the marked effect of oxygen is caused by its reaction with lipids^{3,4)} and sulfhydryl groups in proteins and peptides.^{5,6)} It is considered that lipid peroxides induce a loss of sulfhydryl groups,^{4,7,8)} and this results in the modification of the dough properties, and also it has been found that lipid binding is influenced by the atmosphere during dough mixing.⁹⁾ Thus, it is conceivable that the lipid-protein interaction contributes to the viscoelastic properties of the gluten matrix in hydrated dough.

In the present study, the effect of oxygen on lipids was investigated by using ESR techniques, in addition to lipid analysis. ESR spectra of the stearic acid spin-label incorporated into the lipid fraction provided useful information about the behavior of oxygen toward dough.

MATERIALS AND METHODS

Materials. A commercially-milled unbleached wheat

flour with 11.9% protein ($N \times 5.7$) and 0.39% ash on a 13.4% moisture basis was used. For doughs mixed under different oxygen concentrations, flour was stored for two days in a desiccator containing nitrogen, oxygen or a gas mixture after evacuation. Nitrogen (99.99%), oxygen (99.5%) and the mixed standard gas (oxygen 4.99% plus nitrogen 95.01%) were of commercial grade. The spin-label, 4,4'-dimethyloxazolidine-*N*-oxyl derivative of 5-ketostearic acid, was the product of Syva Co. (Palo Alto, Calif.). All other chemicals were of reagent grade.

Preparation of dough. Dough was made by mixing 200 g flour, 10 μ mol of the spin-label which had been dispersed in 40 ml of water by sonication, and 135 ml of water in a mixer (Grain Research Laboratory type), with the bowl screened to prevent access of air. The water was deaerated and bubbled with each gas in advance. Mixing was carried out for 6 min at 67 rpm and 30°C in air, or under a nitrogen, oxygen or mixed gas stream at a flow rate of 4 liters per minute after the mixing chamber had been evacuated to 40 mmHg within 20 sec. An aliquot of each dough (5 g) was taken out and the starch was thoroughly washed out with water saturated with nitrogen. The residual wet gluten was put into a 1.2 mm i.d. quartz tube for ESR measurement. The amount of spin-label used was small enough to prevent spin-spin coupling.

Dough was also prepared by the same procedures except for the addition of spin-label. An aliquot of the dough was quickly frozen by immersion in liquid nitrogen followed by lyophilization and pulverization. The powder was used for lipid analysis.

Extraction and separation of lipids. Dough powder or wheat flour (0.1 g) was put into a glass column (200 × 4 mm i.d.). Lipids were eluted with 15 ml of either petroleum ether or a mixture of chloroform and methanol (2:1, v/v). The chloroform-methanol extract was mixed with 0.2 volumes of water and then centrifuged; the lower layer was analyzed for total lipids. Lipids in the petroleum ether extract were regarded as free lipids. The difference between the free and total lipids was taken as the bound lipids.

The petroleum ether or chloroform-methanol-water mixture extract was dried under reduced pressure, and the lipids were developed with a mixture of diethyl ether, benzene, ethanol and acetic acid (40:50:2:0.2, by vol.), or a mixture of chloroform, methanol and water (65:25:4, by vol.) on a silica gel G plate (200 × 200 mm, 0.3 mm thick). Spots were detected with iodine vapor and identified by comparing them with those of standard lipids and ref. 9. Each spot was scraped off and extracted with chloroform and methanol (2:1, v/v).

Determination of dry weight of lipids. An aliquot of a lipid extract (~150 µg of lipids) was put into a test tube and completely dried by flushing with nitrogen, followed by colorimetric determination using potassium dichromate.¹⁰ Linoleic acid was used for the standard curve.

Determination of the peroxide value (PV). Dough powder (20 g) was mixed with 170 ml of deaerated water-saturated butanol for 1 hr in a nitrogen atmosphere. After centrifugation at 2,000 rpm for 5 min, the precipitate was washed with 50 ml of the same solvent and the supernatants were combined. After this lipid extract had been dried, the PV test was performed by the standard titrimetric method.¹¹

ESR analysis. ESR measurements were performed with a Hitachi 771 ESR spectrometer in the X-band mode at various temperatures (15~50°C). This study was concerned with the relative changes in motion of the spin-label bound to lipid in wet gluten. The order parameter (*s*),

rotational correlation time (τ_c) and activation energy (*E*) of rotational viscosity were calculated from the ESR spectra as described previously.¹²

RESULTS

Effect of oxygen on lipid binding and oxidation

Table I shows the amounts of total, free and bound lipids in dough mixed under different atmospheres. Compared with the lipids in flour, the process of mixing in nitrogen increased the proportion of bound lipids from 25 to 71%. In the oxygen atmosphere bound lipids were 3% less than in the nitrogen atmosphere.

Table II shows the compositions of the total lipids fractionated by TLC. The spots from 1 to 9 were of non-polar lipids and spot 10 was of polar lipids. In the non-polar lipid fraction of the dough mixed under an oxygen atmosphere, the amount of spot 4, corresponding to free fatty acid, decreased; and concomitantly spot 6 became larger. Spot 6 reacted positively with KI-starch reagent, so it was identified as a peroxidation product. The compositions of polar lipids for doughs mixed under oxygen and nitrogen atmospheres were almost the same.

The PV of the total lipids from dough mixed under oxygen, air and nitrogen atmospheres were 14.7, 7.6 and 0.4, respectively. Peroxidized lipids were not detected in flour lipids (PV=0). Mixing in a nitrogen atmosphere depressed lipid oxidation; oxygen accelerated lipid oxidation.

TABLE I. FREE AND BOUND LIPIDS IN DOUGH MIXED UNDER VARIOUS ATMOSPHERES

Atmosphere		Lipid (mg/0.1 g dough powder)		
		Total lipid	Free lipid	Bound lipid
Dough	Nitrogen	1.45	0.42 (29.0)	1.03 (71.0)
	95% Nitrogen + 5% oxygen	1.42	0.42 (29.6)	1.00 (70.4)
	Air	1.47	0.45 (30.6)	1.02 (69.4)
	Oxygen	1.38	0.44 (31.9)	0.94 (68.1)
Flour		1.58	1.18 (74.7)	0.40 (25.3)

In parentheses are given the percentages of free or bound lipids of the total lipids.

TABLE II. COMPOSITIONS OF DOUGH LIPIDS FRACTIONATED BY TLC

Spot No. on TLC	Atmosphere		<i>R_f</i> on TLC	Remarks
	Oxygen	Nitrogen		
Spots 1 to 9	0.96 mg	1.06 mg		Non-polar lipids
1	(10.2)	(10.4)	1.00	SE
2	(51.9)	(46.0)	0.90	TG
3	(11.4)	(11.2)	0.77, 0.73	DG
4	(4.0)	(11.0)	0.56	FA
5	(1.8)	(2.0)	0.46	FS
6	(1.6)	(0.3)	0.42	Peroxidized product
7	(5.7)	(6.9)	0.25	MG
8	(4.5)	(3.4)	0.15	MG
9	(8.6)	(8.8)	0.06	Unknown
Spot 10	0.39 mg	0.36 mg	Origin	Polar lipids

Lipid weight is shown per 0.1 g of dough powder, and the percentages of each spot of the total non-polar lipids are given in parentheses. Abbreviations: SE, sterol ester; TG, triglyceride; DG, diglyceride; FA, fatty acid; FS, free sterol; MG, monoglyceride.

Effect of oxygen on ESR parameters

Figure 1 shows the ESR spectra of gluten obtained from dough mixed under an oxygen or nitrogen atmosphere. The spin-probe, which was selectively incorporated into the lipid position of the gluten, reflects the environment at a depth of 5 carbon atoms of stearic acid. The ESR spectra of the gluten had separated parallel and perpendicular hyperfine splittings, and alternations with temperature. Gluten prepared in a nitrogen atmosphere showed a broader spectrum than that of gluten in oxygen.

The order parameters and correlation times were calculated from the spectra obtained at different temperatures (Fig. 2). The order parameter changed from 0.7 to 0.5 with increasing temperature and the presence of oxygen made the order parameter lower (Fig. 2a). The rotational correlation time increased rapidly as the temperature decreased (Fig. 2b), and it decreased with an increase in oxygen concentration.

Andrade's plots¹³⁾ of rotational viscosity of the spin-labeled glutes are shown in Fig. 3. The plots were all composed of two straight lines with a break point. The breaks in the plots appeared at 28, 30, 32 or 36°C for the gluten obtained from dough mixed under a ni-

FIG. 1. ESR Spectra of Spin-labeled Gluten Prepared in Nitrogen or Oxygen.

trogen, 95% nitrogen–5% oxygen, air or oxygen atmosphere, respectively. The break point indicates the temperature at which the lipid phase at the depth of 5 carbon atoms from the polar head group changes from solid to liquid. The activation energies of rotational viscosity calculated from the tangent of the plots are shown in Table III. The activation energies decreased with increasing oxygen concentration; they were from 9 to 4 kcal/mol in the liquid phase (LP) and from 23 to 13 kcal/mol in the solid phase (SP).

FIG. 2. Changes in the Order Parameter (A) and Rotational Correlation Time (B) of Spin-labeled Gluten at Various Temperatures.

FIG. 3. Andrade's Plots for Activation Energies of Rotational Viscosity of Gluten.

TABLE III. EFFECT OF THE ATMOSPHERE ON ROTATIONAL ACTIVATION ENERGIES OF SPIN-LABELED GLUTEN

Atmosphere	E (kcal/mol)	
	SP	LP
Nitrogen	22~25	8~10
95% Nitrogen + 5% oxygen	16~18	9
Air	14~16	5~6
Oxygen	13~14	3~5

DISCUSSION

Air gives dough desirable rheological properties. Several workers have reported the

effect of oxygen on the lipids found in dough.³⁾ Smith and Andrews¹⁾ reported that the actual oxygen uptake in a patent flour dough was 26 $\mu\text{eq/g}$; uptake was less in defatted flour dough. Graveland⁹⁾ found that linoleic acid, which accounted for 60% of the free fatty acids, decreased rapidly when dough was mixed under an oxygen atmosphere instead of nitrogen. We found here that the oxidation of lipids, especially of free fatty acids, was accelerated by mixing in oxygen, which is compatible with the findings of Graveland.⁹⁾ Daniels *et al.*^{14,15)} who used a rapid mixer and worked with a differently composed dough containing lipoxigenase and fats, showed that bound lipids were released in the dough and suggested that this might result from the weakening of the hydrophobic interaction between bound lipids and proteins owing to coupled oxidation at the hydrophobic area of the proteins caused by oxidized lipids. Though the decrease in bound lipids was less than that Graveland⁹⁾ and Daniels *et al.*¹⁴⁾ observed, we think that lipid oxidation leads to a decrease in the hydrophobic interaction of lipids with protein, as predicted by Daniels *et al.*¹⁴⁾

The lipid-protein interaction in the gluten matrix has been studied by many techniques,^{16~18)} and we have previously observed by ESR measurement that a strong interaction of protein with lipid occurs in the gluten.¹²⁾ In the present study the spin-label technique was

first employed for the investigation of the effect of oxygen on lipids in the gluten matrix. The decreases in the order parameter and rotational correlation time with the introduction of oxygen reflect the increase in molecular motion of the spin-probe, that is, the increase in the fluidity of lipids. Oxygen, even at a concentration of 5%, produced clear changes in the activation energy and the break point of the Andrade's plot. The break point indicated the transition temperature. The oxidized products of lipids such as *trans* and/or hydroxy fatty acids, which were produced in dough mixed in oxygen,⁹⁾ show higher melting points and higher polarities than free fatty acids. These products will have a tendency to increase the activation energy and the transition temperature. However, the activation energy seems to be influenced by the magnitude of the interaction of lipid with protein more than the production of oxidized lipids. The decrease in the activation energy in gluten formed under oxygen reflects the decrease in the hydrophobic interaction of lipid with protein related to the release of bound lipids. Though such a simple explanation should be considered with care, the above results indicate that oxygen made lipids in the gluten matrix more fluid and interact less hydrophobically with protein.

The values of parameters for dough mixed under air were intermediate between those for doughs under nitrogen and oxygen atmospheres. The results obtained at a high concentration of oxygen indicate the dough-improving action of lipoxygenase in air.¹⁹⁾

We investigated qualitatively the change of lipids as a target of oxygen by ESR measurement. It is noteworthy that the ESR results can be employed in investigation of the physical

properties of lipids and the mode of their interaction with protein in hydrated dough prepared under different atmospheric conditions.

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