# Enzymes That Contribute to the Oxidation of L-Ascorbic Acid in Flour/Water Systems

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Cereal Chem. 63(3):197–200


## ABSTRACT

The enzymes lipoxygenase (LOX), peroxidase (POD), and polyphenol oxidase (PPO) were investigated to determine whether they contribute either directly or indirectly to the oxidation of L-ascorbic acid (AA) in wheat flour. Water extracts of untreated patent Canadian hard red spring wheat flour contained significant amounts of both LOX and POD but no ascorbate oxidase (AOX) activity. However, significant AOX activity was found in flour/water suspensions. When the flour residue from a water extraction was subsequently extracted with 75% saturated $Na_2SO_4$ solution, 60% of the AOX activity of the flour suspension was solubilized, but this extract showed neither LOX nor POD activity. The assay for PPO showed no activity in the patent flour, although activity was detected in a whole wheat flour. It was concluded that patent flour contains an enzyme capable of oxidizing AA that is distinctly different from LOX, POD, or PPO. Water extracts supplemented with both $H_2O_2$ and AA rapidly oxidized AA, indicating a POD species that utilized AA as an H-donor. Because of the negligible $H_2O_2$ content in flour, significant oxidation of AA by this enzyme appeared unlikely. Water extracts supplemented with AA and linoleic acid oxidized AA at one half the rate of a flour/water suspension without any added linoleic acid. The natural levels of free linoleic acid in flour are high enough that indirect oxidation of AA by LOX could account for approximately 30–50% of the total AA oxidation in dough.


The improving effect that results from the addition of small amounts of L-ascorbic acid (AA) to bread flour has been recognized since 1935 (Jorgensen 1935). The data in the literature support the theory that AA is rapidly oxidized to dehydroascorbic acid (DHA) and, in conventional bread dough, the DHA reacts with flour proteins much like bromate or any other oxidative improver. The detailed mechanism of improver action is not clearly understood, but it is generally accepted that these agents oxidize flour sulfhydryl (SH) groups to disulfide cross links and promote sulfhydryl-disulfide interchange reactions (Tsen and Bushuk 1963, Tsen 1965, Meredith 1965, Elkassabany and Hoseney 1980, Elkassabany et al 1980, Nicolas et al 1980).

An expansion on this theory was recently advanced by Ewart (1985), who proposed that low-molecular-weight thiol compounds in flour (e.g., reduced glutathione) interfere with dough development by forming disulfide links with free SH groups in the gluten matrix. This so-called end-blocking effect prevents these SH groups from participating in desirable SH-disulfide interchange reactions that link the gluten subunits together as the dough develops. Oxidative improvers are effective because they react with the low-molecular-weight thiols and prevent their end-blocking effect.

AA also has an effect on short-time doughs that is distinctly different from its effect on conventionally mixed dough. It acts synergistically with cysteine to reduce the amount of mixing that is otherwise required to develop the dough (Chamberlain et al 1966, Coppock 1966, Dahle and Murthy 1970, Johnston and Mauseth 1972). It cannot be replaced by DHA, so this effect apparently does not involve SH group oxidation; and it cannot reduce disulfide bonds (Dahle and Murthy 1970).

There is no generally accepted theory to explain this effect. Two quite different hypotheses have been proposed. Dahle and Murthy (1970) suggested that AA inhibits lipid oxidation and thereby prevents an adverse but unexplained effect of oxidized lipids on dough rheology. In contradiction to this hypothesis, Mann and Morrison (1974) reported that polyunsaturated free fatty acids are the main lipid components susceptible to rapid oxidation and subsequently reported that added AA offers no protective effect (Mann and Morrison 1975). However, it was reported later that for a brief period after mixing, AA does partially inhibit free linoleic acid oxidation, although this inhibition does not persist beyond 30 min (Grant and Sood 1980b).

The second hypothesis attributes the effect of AA on short-time doughs to be a consequence of the way it affects the H-bonding of the water in the dough. This hypothesis is supported by a somewhat similar effect of adding urea and other agents which affect H-bonding (Johnston and Mauseth 1972, Zentner 1968). Obviously, more study is necessary before either hypothesis may be confirmed or rejected.

One well-established fact is that AA is rapidly oxidized to DHA in doughs and flour/water suspensions, and most of this oxidation appears to be enzymatically catalyzed (Kuninori and Matsumoto 1963, Grant 1974, Elkassabany et al 1980, Nicolas et al 1980). Nonenzymatic oxidation accounts for a small fraction of the total reaction, as there are components in flour that inhibit nonenzymatic oxidation (Sandstedt and Hites 1945, Grant 1974, Nowicka and Wojciak 1984). Reports of slow oxidation rates in aqueous extracts of flour are probably explained by the difficulty in solubilizing the responsible enzyme (Grant and Sood 1980a).

Progress toward isolating this enzyme was reported by Grant and Sood (1980a) and by Pfeilsticker and Roeing (1980, 1982), who used the technique of isoelectric focusing and reported that the main ascorbic acid oxidase (AOX) isozyme had a high specificity for AA.

The extent of enzymatic oxidation implies an important role for this enzyme insofar as the rheological effects of AA on dough are concerned. However, there is no evidence to support the suggestion that a specific AOX is required in dough or that the rate of AA oxidation in any way limits the effectiveness of the additive. In fact, several reports suggest that nonenzymatic oxidation, or indirect oxidation as a side reaction to the activity of other oxidative enzymes, can produce all the DHA that is needed for the improving effect of AA to be observed (Carter and Pace 1965, Meredith 1965, Morimitsu 1975, Elkassabany et al 1980, Lillard 1980). That substances can be indirectly oxidized by an enzyme acting upon another substrate is well known, for example, the loss of the yellow pigment in durum semolina as a result of the action of lipoxygenase (Matsuo et al 1970).

We investigated the possibility of indirect oxidation of AA by lipoxygenase (LOX), peroxidase (POD), and polyphenol oxidase (PPO), three well-known oxidative enzymes that are found in wheat (Fox and Mulvihill 1982).

## MATERIALS AND METHODS

Samples of untreated patent flour milled from Canadian hard red spring wheat were obtained from a local commercial mill.

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Sample A contained 9.6% moisture, 0.35% ash, and 12.1% protein (N × 5.7). Sample B contained 9.2% moisture, 0.36% ash, and 14.7% protein (as is basis).

AA was purchased from Aldrich Chemical Co. Linoleic acid, guaiacol, 4-methylcatechol, $H_2O_2$, and Tween-20 were purchased from Sigma Chemical Co. All other chemicals were reagent grade or better. Reagent solutions and flour extractants were prepared with glass-distilled water.

Flour extracts were prepared by stirring flour suspensions with water or other appropriate extractant for 30 min at room temperature (22–25° C) followed by centrifugation at 15,000 × *g*. LOX was extracted with water at pH 6.9 (Al-Obaidy and Siddiqi 1981). POD was extracted with 0.1 *M* phosphate buffer, pH 7.8 (Gaspar et al 1977), or with water at pH 5.5. PPO was extracted with 0.1 *M* phosphate buffer, pH 6.9 (Interesse et al 1980). AOX was extracted with 75% saturated $Na_2SO_4$ solution at pH 8.3 or measured directly in a flour/water suspension (Grant 1974, Grant and Sood 1980a). In all cases, the flour suspensions were adjusted to the specified pH at the beginning of the extraction period using either dilute NaOH or dilute $H_2SO_4$ solutions.

LOX activity was measured with a Pye Unicam spectrophotometer (model 1705) from the increase in absorbance at 234 nm resulting from the oxidation of linoleate to conjugated diene hydroperoxide (Vliegenthart and Veldink 1982). The substrate was prepared according to Yoon and Klein (1979), except that the pH was adjusted to 10.0 before final adjustment to 6.5, which aided in achieving optically clear solutions.

POD activity was also measured spectrophotometrically at 470 nm using guaiacol as the secondary substrate (Honold and Stahmann 1968).

PPO was assayed spectrophotometrically using 4-methylcatechol as substrate and measuring the appearance of the *o*-quinone according to Interesse et al (1980). The procedure was modified in that the spectrophotometer readings were at 400 rather than 395 nm.

AOX was measured in both extracts and suspensions by following the disappearance of AA by the indophenol-xylene extraction method described previously (Grant and Sood 1980a).

All assays were performed at room temperature. Enzyme activities are expressed as micromoles per minute per gram of flour. In calculating LOX activities, the value of 26.6 $cm^{-1} \cdot mM^{-1}$ was used for the extinction coefficient of the oxidation product as recommended by Chan and Maehly (1964). In calculating POD activities, the value of 28.0 $cm^{-1} \cdot mM^{-1}$ was used for the extinction coefficient for tetraguaiacol (Privett et al 1955), and the calculation took into account that 4 mol of guaiacol are oxidized for each mole of tetraguaiacol produced.

In control experiments performed regularly, the enzyme extracts were boiled before adding them to the assay medium to correct the data for any nonenzymatic oxidation. In other experiments, flour samples were extracted sequentially, twice with water (pH 5.5) or phosphate buffer (0.1 *M*, pH 7.8), followed by a final extraction with 75% saturated $Na_2SO_4$ solution (pH 8.3). Each extract was analyzed for AOX and either LOX or POD.

**TABLE I**
**Peroxidase (POD), Lipoxygenase (LOX), Ascorbic Acid Oxidase (AOX), and Polyphenol Oxidase (PPO) Activities of Patent CRS Wheat Flour**

| Enzyme | Enzyme Source | Activity ($\mu mol \cdot min^{-1} \cdot g^{-1}$ of flour) Flour A | Flour B |
|---|---|---|---|
| POD | 0.1 *M* Phosphate buffer extract, pH 7.8 | 21.5 ± 1.0[a] | 19.0 ± 1.0 |
| LOX | Water extract, pH 6.9 | 2.25 ± 0.23 | 1.94 ± 0.19 |
| AOX | Flour/water suspension,[b] pH 6.3 | 0.063 ± 0.008 | 0.060 ± 0.005 |
| AOX | Flour/water suspension,[c] pH 6.3 | 0.055 ± 0.002 | 0.047 ± 0.001 |
| PPO | 0.1 *M* Phosphate buffer extract, pH 6.9 | 0 | ... |

[a] Average ± standard deviation of two or more replicates.
[b] Assayed within six months of milling.
[c] Assayed more than nine months after milling.

Indirect oxidation of AA by LOX was measured by adding LOX extracts to the LOX assay medium, which was modified to include AA in addition to the normal linoleic acid substrate. Oxidation of AA by POD was measured similarly using a water extract as the POD source, and the POD assay medium was modified by substituting AA for guaiacol. In both cases, the disappearance of AA was followed by the indophenol-xylene extraction method referred to above.

Procedures for assaying LOX, POD, and PPO were tested by performing a series of experiments in which the substrate concentrations and the pH were varied systematically.

Salt fractionation with $Na_2SO_4$ was performed as described previously (Grant and Sood 1980a).

## RESULTS AND DISCUSSION

When enzyme activities were measured over a range of pH and substrate concentrations, the results confirmed that 6.5 was the optimum pH for both POD and LOX. Plots of activity versus substrate concentrations (not shown) confirmed that 0.48 m*M* linoleate in the LOX assay medium and 5.9 m*M* $H_2O_2$ plus 26 m*M* guaiacol in the POD assay medium were all within the range of concentrations where the substrate was neither rate limiting nor inhibiting. Similar testing, using whole wheat flour as the enzyme source, confirmed that the conditions recommended for the PPO assay were also appropriate.

The levels of activity of the four enzymes being investigated in the test flours are shown in Table I. The POD activity in a phosphate buffer extract was approximately 10 times greater than the LOX activity, which in turn was approximately 30 times greater than the AOX activity in 1:3 flour/water suspensions. No PPO activity could be detected. Although AOX was low by comparison to POD and LOX activities, it was high enough that normal levels of added AA in dough would be almost completely oxidized within 10 min.

The data in Table I were compared to data reported in the literature. The AOX activities were similar to the results obtained previously with similar flour (Grant and Sood 1980a). They were 10 times higher than the level reported by Pfeilsticker and Roeung (1980), but the method of extraction they used probably did not solubilize all of the enzyme. Kruger and LaBerge (1974) reported POD levels in Manitou flour that were very similar to the values we observed, although Honold and Stahmann (1968) reported POD levels in hard red spring wheat flour that appear to be about one-half that amount. The LOX activities agree well with values reported by Irvine and Anderson (1953). They are about four times higher than those determined by Graveland (1970); he used a much different procedure, namely quantitative thin layer chromatography of the individual oxidation products formed from linoleate as recovered by chloroform-methanol extraction from dough.

Because PPO could not be detected in patent flour, the assay method was tested on a sample of whole wheat flour. Significant activity was detected, proving the method to be reasonably sensitive. Honold and Stahmann (1968) were not able to detect PPO in flour samples either and noted that most of this activity is localized in the bran fraction.

Results in Table I are consistent with our earlier observation that AOX activity declines as flour ages (Grant and Sood 1980a).

The pH 7.8 phosphate buffer extract which was used for POD analysis was also tested for AOX activity. It was found to contain 30% of the AOX measurable in a flour-water suspension. This result agreed with the previous observation in our laboratory, that extracting flour at a pH near 8 could solubilize a part of this activity (Grant and Sood 1980a). The previous study also indicated that flour/water extracts below pH 7 contained negligible AOX activity.

In an attempt to obtain an extract that contained POD but no AOX, a water extract was prepared at pH 5.5, and after adjustment to the optimum pH, aliquots were tested for both activities. Our earlier work was confirmed in that no AOX was detected. To our surprise, the POD activity was more than twice that found in the pH 7.8 phosphate buffer extracts (Table II). When the water extracts were diluted 1:1 with 0.2 *M* phosphate buffer at pH 7.8, a

TABLE II
Comparison of Peroxidase (POD) Activity in Water and Phosphate Buffer Extracts of Flour[a]

| Extract | POD Activity ($\mu$mol·min$^{-1}$·g$^{-1}$ of flour) |
|---|---|
| 0.1 *M* Phosphate buffer, pH 7.8 | 19.0 ± 1.0[b] |
| Water, pH 5.5 | 56.1 ± 8.1 |
| Water, pH 5.5, diluted 1:1 with phosphate buffer[c] | 6.9 ± 0.4 |
| Water, pH 5.5, diluted 1:1 with water | 43.2 ± 5.5 |

[a]Flour B: 9.2% moisture, 0.36% ash, and 14.7% protein (as is basis).
[b]Average ± SD of three or more replicates.
[c]Diluted to a final concentration of 0.1 *M* phosphate, pH 7.8, and centrifuged to remove resulting precipitate prior to assay.

TABLE III
Peroxidase (POD), Lipoxygenase (LOX), and Ascorbic Acid Oxidase (AOX) Activities of Sequential Extracts of Flour[a]

| Sequence | Extract | Activity ($\mu$mol·min$^{-1}$·g$^{-1}$ of flour) POD | LOX | AOX |
|---|---|---|---|---|
| 1 | Water, pH 6.9 | ··· | 1.94 ± 0.19[b] | 0 |
| 2 | Water, pH 6.9 | ··· | 0.13 ± 0.13 | 0.002 ± 0.001 |
| 3 | $Na_2SO_4$ solution[c] | ··· | 0 | 0.032 ± 0.002 |
| 1 | Water pH 5.5 | 56.1 ± 8.1 | ··· | 0 |
| 2 | Water pH 5.5 | 0 | ··· | 0 |
| 3 | $Na_2SO_4$ solution[c] | 0 | ··· | 0.029 ± 0.003 |

[a]Flour B: 9.2% moisture, 0.36% ash, and 14.7% protein (as is basis).
[b]Average ± SD, two or more replicates.
[c]Solution of 75% saturated $Na_2SO_4$.

considerable amount of precipitation was observed with much loss of POD activity. There was a much smaller but significant loss of specific activity on dilution with water. In general, the POD activity levels of water extracts were consistently higher, but also more variable, than those of phosphate buffer extracts. These results clearly show that water is superior to the pH 7.8 phosphate buffer recommended by Gaspar et al (1977) as an extractant for POD in wheat flour.

In order to separate AOX activity from that of the other enzymes, flour was extracted sequentially, twice with water and finally with 75% saturated $Na_2SO_4$ solution. The extracts were analyzed for POD, LOX, and AOX. Data are in Table III. The first water extract contained LOX and POD but no detectable AOX. The second water extracts contained very low or undetectable amounts of any activity, thus indicating that a single water extract was quite efficient in solubilizing both LOX and POD. The $Na_2SO_4$ solution extract contained no LOX or POD, but it contained an average of 60% of the AOX activity of a flour/water suspension, a percentage similar to that observed previously (Grant and Sood 1980a). Further experiments in which water extracts of both LOX and POD were salt fractionated with $Na_2SO_4$ showed that at 75% saturation the supernatants had lost 90% or more of both these activities. Therefore, on the basis of the differences in solubility and in response to $Na_2SO_4$ concentration, we concluded that AOX exists in flour as an enzyme species that is distinctly different from either POD or LOX. Furthermore, because no PPO was detected in these flour samples, it appears that AOX is also distinct from PPO.

The results in Table III do not preclude that some of the oxidation of AA in dough may occur as a consequence of the activity of oxidative enzymes other than AOX. AA may be oxidized indirectly by either the products or the intermediates generated by LOX, acting on endogenous polyunsaturated fatty acids. The observation by Faubion and Hoseney (1981), that AA inhibits carotene bleaching by LOX, is in accord with this suggestion. AA may also serve as an H-donor for POD activity.

When a water extract that contained LOX but no AOX activity was incubated with both linoleate and AA, the AA was oxidized at an optimum initial rate of 0.029 ± 0.001 mol·min$^{-1}$·g$^{-1}$ (average of five replicates ± SD). This rate was approximately 60% of the rate of oxidation of AA in a flour/water suspension without any added linoleate. The optimum pH for the indirect oxidation of AA by LOX was 6.1, not greatly different from pH 6.5, the optimum for linoleate oxidation. The optimum linoleate concentration was 0.57 m*M*, with evidence of substrate inhibition at higher concentrations. The rate was independent of AA concentration down to 0.06 m*M*, the lowest concentration at which it was possible to measure AA oxidation with reasonable precision. Above 0.30 m*M*, AA had an inhibiting effect on this reaction.

When a water extract that contained POD but no AOX activity was incubated with both $H_2O_2$ and AA, the AA was oxidized at an optimum initial rate of 0.74 ± 0.08 mol·min$^{-1}$·g$^{-1}$ of flour (average of seven replicates ± SD). This rate is more than 10 times faster than the rate of AA oxidation in a flour/water suspension without added $H_2O_2$. The pH optimum for this POD activity was 3.5, although the pH activity profile was broad, and substantial activity persisted as high as pH 7.0. This optimum pH was markedly different from the value of 6.5, which is optimum when guaiacol is used as the H-donor, and suggests that different POD isozymes are present. The rate of AA oxidation by POD reached a plateau at 1.2 m*M* $H_2O_2$ concentration and remained constant up to 1.8 m*M*. The optimum AA concentration was 0.23 m*M*, and there was evidence of substrate inhibition at higher concentrations.

AA-dependent peroxidases have been reported in other species (e.g., Kelly and Latzko 1979, Shigeoka et al 1980), but this is the first report of such an enzyme in wheat flour. Although this enzyme appears to have the potential to oxidize AA in doughs, it is questionable whether such oxidation takes place to any significant extent, because the endogenous level of $H_2O_2$ in flour is very low (Lillard 1980).

A level of free linoleate of 73.9 mg per 100 g of flour was reported by Mann and Morrison (1974). This level would provide a substrate concentration in dough that is the same order of magnitude as that found to be optimum for the indirect oxidation of AA by LOX. Thus, it is estimated that 30–50% of the AA in dough may be oxidized by this mechanism. Indirect oxidation of AA by LOX would also explain the earlier observation that supplementing flour with a lipid fraction isolated from another portion of the same flour results in a small increase in the rate of AA oxidation in a flour/water suspension (Grant 1974).

## ACKNOWLEDGMENTS

Our thanks to Z. Labach of CSP Foods for generously providing samples of untreated patent flour. We gratefully acknowledge the Natural Sciences and Engineering Research Council of Canada for financial support and the Government of Saskatchewan for a graduate scholarship to the first author.

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[Received August 7, 1985. Revision received November 6, 1985. Accepted November 8, 1985.]