

# Effect of Mixing Conditions on the Behavior of Lipoxygenase, Peroxidase, and Catalase in Wheat Flour Doughs

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## ABSTRACT

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The effect of mixing has been tested on the extractable activities of lipoxygenase, peroxidase, and catalase from dough after 2, 5, and 20 min of mixing, and 30 min of rest period after 20 min of mixing. Different mixing conditions have been studied including temperature, atmosphere, speed, amount of water added to the dough, buffer solutions between pH 3.6 and 7.5 added to the dough, and different additives (linoleic acid, guaiacol, hydrogen peroxide, ascorbic acid, cysteine, yeast, and sodium chloride). In all the mixing conditions tested, the dough peroxidase

activity remains equivalent to the initial flour activity, whereas losses in lipoxygenase and catalase activities largely varied according to mixing conditions. The results show that a self-destruction mechanism as well as physicochemical denaturation are responsible for these losses. Lipoxygenase losses seem mainly associated with the former mechanism, whereas catalase losses are highly increased in acidic conditions (physicochemical denaturation). Therefore, the relative impact of the three oxidoreducing enzymes may be largely modulated by mixing conditions.

Oxidoreducing enzymes in wheat flour, as well as in baking additives, have always been of interest to cereal chemists as illustrated by numerous reviews (Nicolas and Drapron 1983, Grosch 1986, Drapron and Godon 1987, Stauffer 1987, Van Dam and Hille 1992). The effect of lipoxygenase during flour maturation and dough mixing is well known (Nicolas 1979, Faubion and Hosney 1981, Nicolas and Potus 1994). This enzyme catalyzes the oxidation of polyunsaturated fatty acids in the free and mono-glyceride forms by molecular oxygen (Drapron et al 1969, Mann and Morrison 1974, Galliard 1986, Tait and Galliard 1988). By coupled oxidation mechanisms, the lipoxygenase is also able to degrade carotenoid pigments (Nicolas 1978) and to promote a loss of flour sulfhydryl groups (Tsen and Hlynka 1963, Shiiba et al 1991). The former action results in dough bleaching (Kruger and Reed 1988), whereas the latter action is probably the causal agent of the increase of the amount of free lipids in dough (Daniels et al 1970) and of the increase of mixing tolerance and relaxation times of dough that results in enhanced loaf volume (Frazier et al 1973, 1977; Hosney et al 1980; Kieffer and Grosch 1980; Shiiba et al 1991; Cumbee et al 1997). Finally, the hydroperoxides, the primary products formed by lipoxygenase during dough mixing, are partly destroyed during dough baking with formation of volatile compounds such as *n*-hexanal, inducing a modification of the bread aroma (Drapron et al 1974).

The effects of peroxidase, and catalase are much less documented, although they have a high level of activity in wheat flour (Kruger and LaBerge 1974a,b; Kruger 1977; Kieffer et al 1982). Thus, both enzymes increased the rate of carotenoid bleaching during dough mixing (Hawthorn and Todd 1955, Nicolas 1978), whereas Kieffer et al (1981) claimed that the baking properties of wheat flour can be improved by addition of horseradish peroxidase. Moreover, among numerous oxidizing agents, peroxidase in the presence of hydrogen peroxide appears as the most efficient catalyst for the oxidative gelation of wheat flour water-soluble pentosans (Neukom and Markwalder 1978; Izydorczyk et al 1990, 1991). Two mechanisms have been proposed for the oxidative crosslinking of pentosans. The first is based on the formation of a diferulate bridge between two adjacent ferulic acid residues caus-

ing crosslinking of two arabinoxylan chains (Geissmann and Neukom 1973). The second involves the addition of a protein radical to the activated double bond of ferulic acid esterified to the arabinoxylan fraction, resulting in a crosslinking between a protein and a pentosan chain (Hosney and Faubion 1981). However, the effective action of peroxidase and catalase in breadmaking is uncertain because the formation of hydrogen peroxide, their primary substrate, during dough mixing remains questionable. Nevertheless, addition of glucose oxidase to wheat flour results in an improvement of the dough properties (Van Dam and Hille 1992). The latter effect could be due to the formation of hydrogen peroxide through the oxidation of glucose by this enzyme leading to the activation of peroxidase (and catalase).

Most of the experiments concerning the enzyme activities are obtained from enzyme reactions in model systems and special care must be taken when these *in vitro* results are applied to the *in vivo* phenomena observed during dough mixing. The dough conditions are entirely different because the water, and sometimes the substrates, are present in limited amounts. The particular hydrophobic environment, due to the presence of gluten, might also modify the reaction mechanism. Such a phenomenon was observed for lipoxygenase by Graveland (1970). Also concerning lipoxygenase, Pourplanche et al (1992, 1994) have shown that addition of polyols and sugars to aqueous media resulted in a modification of both the enzyme activity and stereospecificity. Similarly for other enzymes, Rothfus and Kennel (1970) noticed that wheat  $\beta$ -amylase gave an insoluble complex with glutenin and this nonreversible adsorption due to hydrophobic bonding did not alter the kinetic properties of the enzyme, but the optimum of activity was shifted from pH 5.5 for the free enzyme to pH 4.5 for the bound one. Wang and Grant (1969) found also that the proteolytic activity of an aqueous wheat flour suspension was higher than that of an aqueous flour extract with a pH optimum shift from 4.4 to 3.8.

The purpose of the present work is to study the effect of different mixing conditions on the extractable activities of wheat lipoxygenase, peroxidase, and catalase from dough. Such a study could give valuable information on the functionality of these enzymes during mixing.

## MATERIALS AND METHODS

The wheat flour used in this work was an untreated, improver-free straight-grade flour, commercially milled by les Moulins Soufflet (Nogent-sur-Seine, France). It contained 9.7 % protein ( $N \times 5.7$ ) and 0.61% ash, on 15.3% moisture basis. Linoleic acid (>99%), Tween 20, guaiacol, L-ascorbic acid and L-cysteine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO).

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All other chemicals were of reagent grade from Prolabo (Paris). Dry yeast was obtained from Lesaffre (Marcq-en-Bareuil, France).

### Dough Preparation

In routine experiments, doughs were mixed with a thermostated alveograph mixer from 150 g of flour and 90 mL of deionized water for 20 min at 75 rpm and 25°C. At the end of the mixing period, the doughs were left to rest for 30 min in the mixing bowl which was covered to prevent surface drying. Aliquots of doughs were taken at 2, 5, and 20 min of mixing (15–20 g). These aliquots and the dough after the rest period were quickly frozen in liquid nitrogen and stored at –20°C until analysis. In some experiments, deionized water was replaced by buffer solutions. At pH 3.6 and 4.2, an acetate buffer (0.1M) was used and at pH 5.6 and 7.5, a phosphate buffer (0.1 M) was used. For the doughs mixed in nitrogen atmosphere, the flour and the deionized water were separately deprived of oxygen before mixing under a stream of nitrogen. Thus, the flour was flushed for 10 min by nitrogen gas, whereas the deionized water was deaerated by bubbling nitrogen gas for 15 min.

Linoleic acid (1.8 mmol) was slowly dispersed in the 150 g of flour gently agitated in the mixer before adding water. Hydrogen peroxide (0.85 mmol), guaiacol (3.6 mmol), ascorbic acid (0.15 mmol), cysteine (0.15 mmol), and sodium chloride (51.3 mmol) were dissolved in the 90 mL of water added to the dough. Dry yeast (1.5 g) was dispersed in the 90 mL of water.

### Enzyme Assays

**Lipoxygenase (EC 1.13.11.12).** Flour (4 g) or dough (6 g) was homogenized with 10 mL of phosphate buffer (100 mM, pH 7.5) in an ice bath using an Ultra Turrax homogenizer for 15 sec followed by a 30-sec rest period and another 15-sec treatment. Homogenates were immediately centrifuged at 30,000 × g for 20 min at 4°C. The supernatant containing lipoxygenase can be stored at 4°C for at least 24 hr without loss of activity. The lipoxygenase activity of the supernatant was determined polarographically using linoleic acid (5 mM) dispersed in a phosphate buffer solution (100 mM, pH 6.5) containing Tween 20 (0.125%) and saturated by air at 30°C as described by Nicolas et al (1982). Activity is expressed in nkat (nmol of oxygen consumed per sec in the assay conditions).

**Peroxidase (EC 1.11.1.7).** Flour (1 g) or dough (1.5 g) was homogenized in 20 mL of acetate buffer (100 mM, pH 4.2) under the conditions described for lipoxygenase extraction. Then, the homogenate was gently agitated for 15 min at 4°C and centrifuged

at 30,000 × g for 20 min at 4°C. The supernatant containing peroxidase can be stored at 4°C for at least three days without loss of activity. The peroxidase activity of the supernatant was determined spectrophotometrically at 465 nm and at 30°C as described by Iori et al (1995) with minor modifications. The assay mixture contained guaiacol (40 mM), hydrogen peroxide (10 mM), CaCl<sub>2</sub> (20 mM) in acetate buffer (100 mM, pH 4.2). The activity is determined by the slope from the linear increase in absorbance at 465 nm. One unit of activity is defined as a change of one absorbance unit per sec for a total volume of 3 mL in the assay conditions.

**Catalase (EC 1.11.1.6).** Flour (1 g) or dough (1.5 g) was homogenized in 20 mL of phosphate buffer (100 mM, pH 7.5) under the conditions described for lipoxygenase extraction. Then, the homogenate was gently agitated for 15 min at 4°C and centrifuged at 30,000 × g for 20 min at 4°C. The supernatant was left at least for 3 hr at 4°C before the assay of activity. It can be stored for at least two days at 4°C without loss of activity. The catalase activity of the supernatant was determined polarographically using hydrogen peroxide (1M) in a phosphate buffer solution (100 mM, pH 7) saturated by air at 30°C. Activity is expressed in  $\mu$ kat ( $\mu$ mol of oxygen formed per sec in the assay conditions).

### Experimental Design

The process variables (independent variables) investigated were amount of water added to the dough (52, 60, and 68%), mixing speed (50, 75, and 100 rpm) and mixer temperature (18, 25, and 32°C). The dependent variables measured were lipoxygenase, peroxidase, and catalase activities of dough samples obtained after 2, 5, and 20 min of mixing, and 20 min of mixing followed by a rest period of 30 min. A 2<sup>3</sup> factorial design was used with two replicates collected for each of the 12 combinations (including the four center points).

## RESULTS

### Preliminary Results

Preliminary experiments were conducted to optimize both the extraction procedure from flour and from dough and the assay conditions for the three enzymes tested.

For lipoxygenase extracted from flour, use of phosphate buffer at pH 7.5 instead of acetate buffer at pH 4.5 (Nicolas et al 1981) or pH 5.5 (Kieffer et al 1982) allows a 25% increase of activity. Moreover, the suspension obtained after homogenization was immediately centrifuged because a 30-min period of mechanical stirring resulted in a 60% loss of activity. Under these conditions, the activity measured on a flour extract is equivalent to the one obtained with the same amount of flour directly added in the polarographic cell. A similar procedure was applied to dough samples, and we found again that the activity measured on freeze-

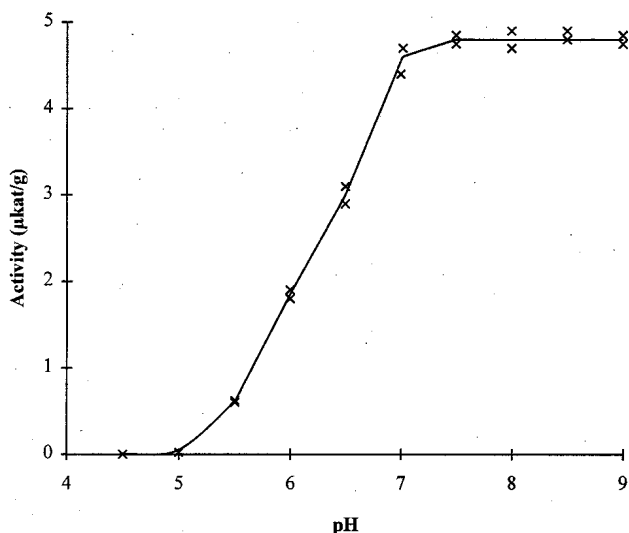


Fig. 1. Effect of the pH of extracting buffer solution (acetate pH 4.5–6, phosphate pH 6–7.5, and borate pH 7.5–9) on flour catalase activity.

TABLE I  
Lipoxygenase, Peroxidase, and Catalase Activities of Flour and Dough<sup>a</sup>

| Sample <sup>b</sup>       | Lipoxygenase <sup>c</sup> | Peroxidase <sup>d</sup> | Catalase <sup>e</sup> |
|---------------------------|---------------------------|-------------------------|-----------------------|
| Flour                     | 33 ± 1.7                  | 22 ± 1                  | 4.8 ± 0.1             |
| Fresh dough               | 11 ± 1.1                  | 22.5 ± 1.2              | 3.1 ± 0.09            |
| Frozen dough <sup>f</sup> | 10.8 ± 1.2                | 21.9 ± 0.9              | 3.3 ± 0.12            |
| Lyophilized dough         | 7.8 ± 1.4                 | 22.2 ± 1                | 2.4 ± 0.08            |

<sup>a</sup> Mean ± standard deviation obtained from three extractions and two assays per extract.

<sup>b</sup> Dough samples obtained after mixing 150 g of flour and 90 mL of water for 5 min at 25°C and 75 rpm.

<sup>c</sup> Activity is expressed in nkat (nmol of oxygen consumed per sec in the assay conditions).

<sup>d</sup> One unit of activity is a change of one absorbance unit per sec per gram.

<sup>e</sup> Activity is expressed in  $\mu$ kat ( $\mu$ mol of oxygen formed per sec).

<sup>f</sup> Samples of frozen doughs gave similar activities when measured immediately after freezing or after three months of storage at –20 °C.

dried dough extracts was equivalent to the one obtained with a direct addition of the same amount of dry dough in the polarographic cell. Therefore, the lipoxygenase present is fully solubilized from flour and from dough in our extracting conditions.

Concerning peroxidase, the amount of extracted activity either from flour or from dough was not affected by the pH of the buffer solution between pH 4.2 and pH 7.5 (acetate and phosphate buffers). Moreover, the 15-min period of mechanical stirring that follows the homogenization step allowed a 10–15% increase of peroxidase activity of the extract. A further increase of the duration of stirring (from 15 to 60 min) did not change the extract activity (*results not shown*). Extraction with a more acidic buffer resulted in a 10% increase of activity, but this activity is less stable. Therefore, the buffer solution at pH 4.2 was selected as the extracting solution. In another way, we confirm that the calcium ions are activators of the wheat peroxidase system because addition of  $\text{Ca}^{2+}$  (20 mM) promotes a 50% increase of activity in our assay conditions. These last results are in agreement with previous findings on purified durum wheat peroxidase (Jeanjean et al 1975, Iori et al 1995).

The catalase extracted from flour is highly sensitive to the pH of the extracting buffer solution (Fig. 1). Thus, the catalase activity of the extract is almost 0 for  $\text{pH} < 5$ , it increases steadily  $\text{pH} \leq 7$ , and then reaches a plateau for higher pH values. A similar effect is found for dough samples (*results not shown*). Moreover, the amount of extract activity from both flour and dough is not modified by a mechanical stirring phase (from 0 to 60 min) after the homogenization. When stored at 4°C, the catalase activity of the extract increases steadily during the first 3 hr and then remains constant for at least three days at 4°C. For flour samples, a 30–35% increase was observed between the activities measured immediately after centrifugation and 3 hr later. Moreover, the catalase activity of flour (or dough) extracts measured under these conditions was always significantly higher than the one obtained with the same amount of flour (or dry dough) directly added to the polarographic cell. The presence of a labile inhibitor could explain these findings. However, no experimental proof is available to support this hypothesis. Nevertheless, the catalase activity of flour and dough samples was always assayed after at least 3 hr of storage at 4°C. Moreover, the catalase present appears fully solubilized from flour and dough under our extracting conditions. In earlier studies on wheat catalase, the assay conditions were between 6.8 and 7.3 for the pH and between 0.006 and 0.2M for the hydrogen peroxide concentration (Blish and Bode 1935, Irvine et al 1954, Kruger 1976). We found an optimum at pH 6.8 (the catalase activity was almost constant between pH 6.5 and 7.5) and a Michaelis-Menten constant ( $K_m$ ) value of 0.13M at pH 6.8 (*results not shown*). Consequently, a pH of 6.8 and a substrate concentration of 1M was selected for the routine assay of catalase by polarography.

The activities of the initial flour for lipoxygenase, peroxidase, and catalase extracted and assayed under the above conditions are given in Table I.

Finally, we checked the effect of deep-freezing and lyophilization treatments on the lipoxygenase, peroxidase, and catalase activities of dough samples (Table I). It appears that the activities for the fresh doughs and the deep-frozen doughs were similar, even after a three months storage at -20°C for the frozen doughs, whereas a loss close to 30% was found for the lipoxygenase and the catalase activities in the lyophilized dough samples. In consequence, dough activities were always determined on samples which were either fresh or frozen.

### Effect of Mixing Time and Rest Period

The amount of extractable activity as a function of mixing time varies greatly from one enzyme to another (Fig. 2). Thus, the lipoxygenase activity is most affected by mixing, because about two thirds of activity are lost during the first 5 min of mixing, then the activity slightly declines until 20 min of mixing and is not

affected by the rest period of 30 min. Losses in catalase activity are less perceptible. The decline in activity is close to 30% after 5 min of mixing, but additional losses are observed after 20 min of mixing and during the rest period. Conversely, the peroxidase activity is remarkably stable during mixing and rest because all the dough samples show an activity similar to that of the initial flour.

### Effect of Temperature, Mixing Speed, and Amount of Water Added to the Dough

A 2<sup>3</sup> factorial design was conducted to study simultaneously the effect of the mixer temperature (between 18 and 32°C), the mixer speed (between 50 and 100 rpm), and the amount of water added to the dough (between 52 and 68%). The results expressed in activity losses (percentage of the flour activity) are presented in Table II for the three enzymes and for different mixing times (2, 5, and 20 min) and after 30 min of rest following 20 min of mixing.

The experimental and theoretical values of enzymatic losses are given in Table III for the centerpoint of the experimental design. A greater variability was always observed for the activity measurements after 2 min of mixing (Fig. 2). This can probably be related to an heterogeneity of the dough which is not fully formed after this short time of mixing. Nevertheless, a good similarity is observed between experimental and theoretical values for the three enzymes tested at each time of mixing and after the rest period. Therefore, the first order equation given in Table II for enzymatic losses during mixing is valid for the domain studied in this experimental design.

Concerning lipoxygenase, it appears that the losses in activity are mainly sensitive to the mixer speed (denaturing effect) and to the water added to the dough (protecting effect) in the first 5 min of mixing. After this period, the protecting effect of the hydration level is less marked, whereas the denaturing effect of the mixer speed disappears. Conversely, the small but significant protecting effect of the mixer temperature increases during mixing and after the rest period. However, it remains always less important than the one observed for the hydration level. A positive interaction term water-temperature, almost equivalent to the main effect of temperature, is also apparent in the first 5 min of mixing. This means that in this period, the protecting effect of the amount of water added to the dough is more marked at a low temperature and

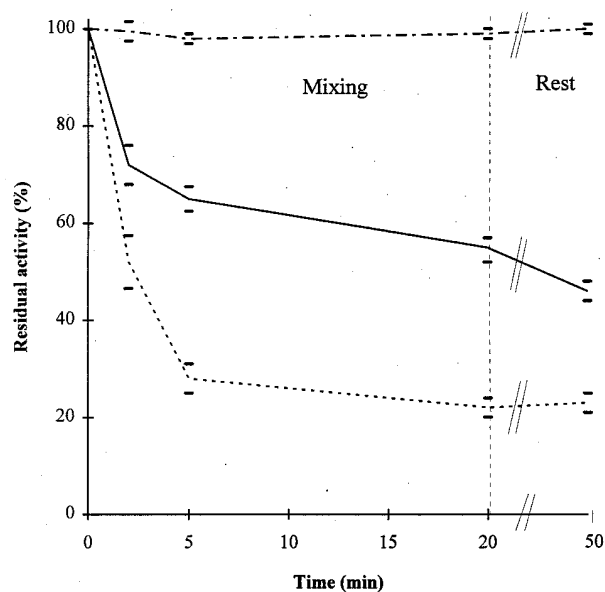


Fig. 2. Effect of mixing and rest periods on dough enzyme activities (--- peroxidase, - - - lipoxygenase, — catalase). Mixing conditions: 75 rpm, 25°C and 60% water. Bars represent standard deviation obtained from four replicates and two extractions for each replicate.

that the effect of temperature is inverted when the amount of water added to the dough increases from 52 to 68% as illustrated by Fig. 3.

The peroxidase activity of dough shows a remarkable stability in the experimental domain tested because the highest loss observed was only 8% (for 20 min of mixing at 100 rpm, 32°C and 52% of water added to the dough). In most of the experiments, the losses in peroxidase activity are <5%. The only constant main effect is observed with the amount of water added to the dough which exerts a protecting effect. In addition, a negative interaction term water-temperature is also apparent whatever the mixing time is, meaning that the protecting effect of the amount of water added to the dough is more marked at high temperature than at low temperature.

Concerning catalase, the losses in activity are almost insensitive to the sole amount of water added to the dough. The main effects are observed with the temperature and the speed of the mixer.

Increasing the two factors causes increasing losses in catalase activity, mainly at the end of the experiment (i.e., 20 min of mixing and 20 min of mixing followed by the 30-min rest period). Moreover, during the rest period, a negative interaction term mixer speed-water and a positive interaction term mixer temperature-water are observed. The first interaction term means that the denaturing effect of the speed is more marked for the high level of hydration, whereas the second interaction term means that the denaturing effect of the temperature is more marked for the low level of hydration as illustrated by Fig. 4A and B, respectively.

### Effect of pH

When the deionized water added to the dough is replaced with different buffer solutions at pH 3.6, 4.2, 5.6, and 7.5, the behavior of the three enzymes tested is variable (Fig. 5).

**TABLE II**  
Enzymatic Losses as a Function of Mixer Speed ( $X_1$ ), Amount of Added Water ( $X_2$ ), and Mixer Temperature ( $X_3$ ) for 2, 5 and 20 min of Mixing and 20 min of Mixing + 30 min of Rest Time<sup>a,b</sup>

| Variables                   | Enzymes      | Mixing Time       |       |                 | Mixing (20 min) + Rest Time (30 min) |
|-----------------------------|--------------|-------------------|-------|-----------------|--------------------------------------|
|                             |              | 2 min             | 5 min | 20 min          |                                      |
| Center point                | Lipoxygenase | 60                | 75    | 81              | 80                                   |
|                             | Peroxidase   | 0                 | 1     | 2               | 1                                    |
|                             | Catalase     | 28                | 35    | 49              | 57                                   |
| Mixer speed                 | Lipoxygenase | 7.7               | 5.6   | ns <sup>c</sup> | ns                                   |
|                             | Peroxidase   | ns                | -1.3  | 2.6             | ns                                   |
|                             | Catalase     | -1                | 2.4   | 4.1             | 4.7                                  |
| Amount of water             | Lipoxygenase | -7.9              | -7.9  | -4.1            | -4.9                                 |
|                             | Peroxidase   | -1.3              | -1.3  | -2.5            | -3                                   |
|                             | Catalase     | weak <sup>d</sup> | ns    | ns              | weak                                 |
| Mixer temperature           | Lipoxygenase | -1.7              | -2.4  | -2.8            | -3.2                                 |
|                             | Peroxidase   | weak              | weak  | weak            | -2                                   |
|                             | Catalase     | weak              | ns    | 4.2             | 10                                   |
| Speed × water               | Lipoxygenase | weak              | weak  | weak            | ns                                   |
|                             | Peroxidase   | ns                | ns    | weak            | weak                                 |
|                             | Catalase     | -1.6              | ns    | -2              | -3                                   |
| Speed × temperature         | Lipoxygenase | weak              | ns    | ns              | ns                                   |
|                             | Peroxidase   | -1.6              | weak  | weak            | weak                                 |
|                             | Catalase     | 1.5               | 1.7   | 2.2             | 1.5                                  |
| Water × temperature         | Lipoxygenase | 2.3               | 2.4   | weak            | ns                                   |
|                             | Peroxidase   | -1.3              | -1    | -1.2            | -1.4                                 |
|                             | Catalase     | ns                | ns    | weak            | 2.3                                  |
| Speed × water × temperature | Lipoxygenase | ns                | weak  | ns              | ns                                   |
|                             | Peroxidase   | ns                | -1    | -1              | ns                                   |
|                             | Catalase     | ns                | -2    | weak            | -1.8                                 |

<sup>a</sup> Losses (%) =  $b_0 + b_1 X_1 + b_2 X_2 + b_3 X_3 + b_{12} X_1 X_2 + b_{13} X_1 X_3 + b_{23} X_2 X_3 + b_{123} X_1 X_2 X_3$ , where  $b_0$  = center point,  $b_1$  = mixer speed,  $b_2$  = amount of water,  $b_3$  = mixer temperature,  $b_{12}$  = speed × water,  $b_{13}$  = speed × temperature,  $b_{23}$  = water × temperature,  $b_{123}$  = speed × water × temperature.

<sup>b</sup> Only terms significant at  $P \leq 0.05$  are given.

<sup>c</sup> Not significant at  $P \leq 0.05$ .

<sup>d</sup> Weak effect <1 (or -1) but significant at  $P \leq 0.05$ .

**TABLE III**  
Comparison of Experimental and Theoretical Losses of Enzymatic Activities During Mixing at the Center Point of the Experimental Design<sup>a</sup>

| Enzyme       | Losses       | Mixing Time |         |         | Mixing (20 min) + Rest Time (30 min) |
|--------------|--------------|-------------|---------|---------|--------------------------------------|
|              |              | 2 min       | 5 min   | 20 min  |                                      |
| Lipoxygenase | Experimental | 52 ± 6      | 71 ± 4  | 78 ± 4  | 75 ± 4                               |
|              | Theoretical  | 60 ± 3      | 75 ± 1  | 81 ± 2  | 80 ± 2                               |
| Peroxidase   | Experimental | 1 ± 2       | 2 ± 1   | 1 ± 1   | 0 ± 1                                |
|              | Theoretical  | 0 ± 0.8     | 1 ± 0.5 | 2 ± 0.7 | 1 ± 0.4                              |
| Catalase     | Experimental | 29 ± 3      | 34 ± 2  | 45 ± 3  | 54 ± 2                               |
|              | Theoretical  | 28 ± 1      | 35 ± 1  | 49 ± 2  | 57 ± 2                               |

<sup>a</sup> Values are % of the initial flour activity ± standard deviation.

Mixing in acidic conditions causes increased losses of lipoxygenase activity which is higher at pH 3.6 than at pH 4.2, whereas almost no differential effect is observed when mixing is operated in pH conditions close to neutrality (Fig. 5A).

For catalase, an almost total loss of activity is observed when the dough is mixed with buffer solutions at pH 3.6 and 4.2 (Fig. 5B). With the buffer at pH 5.6, the activity losses are 5–20% higher than with deionized water, and the differences increase with the mixing time, whereas mixing in a buffer solution at pH 7.5 results in a large protecting effect because losses of catalase activity do not exceed 20%.

Finally, peroxidase remains unaffected by mixing because the extractable activities are always close to (pH 7.5) or 5–10% higher than (pH 3.6 and 4.2) those of the initial flour (*results not shown*). The effect of acid conditions on extractable activities could be related to the observation that extraction of peroxidase with buffer solution at pH <4.2 resulted in a 10% increase of extract activity (*results not shown*).

### Effect of Mixer Atmosphere

When dough is mixed under pure nitrogen or oxygen, almost no effect is found upon extractable activities. The only effect is observed after the rest period under nitrogen, which results in a 5% increase of lipoxygenase loss and 8% decrease of catalase loss, whereas all other values fall within the values  $\pm$  standard deviation found when dough is mixed under air.

### Effect of Linoleic Acid, Hydrogen Peroxide, and Guaiacol

Three substrates of lipoxygenase, peroxidase, and catalase, namely linoleic acid, guaiacol, and hydrogen peroxide have been

added to the dough. Moreover, guaiacol and hydrogen peroxide were added at the same time to have a fully functional peroxidase. In this latter case, a pale brown coloration appears at the beginning of the mixing indicating that peroxidase is active in the dough. Further mixing results in a progressive bleaching of the dough.

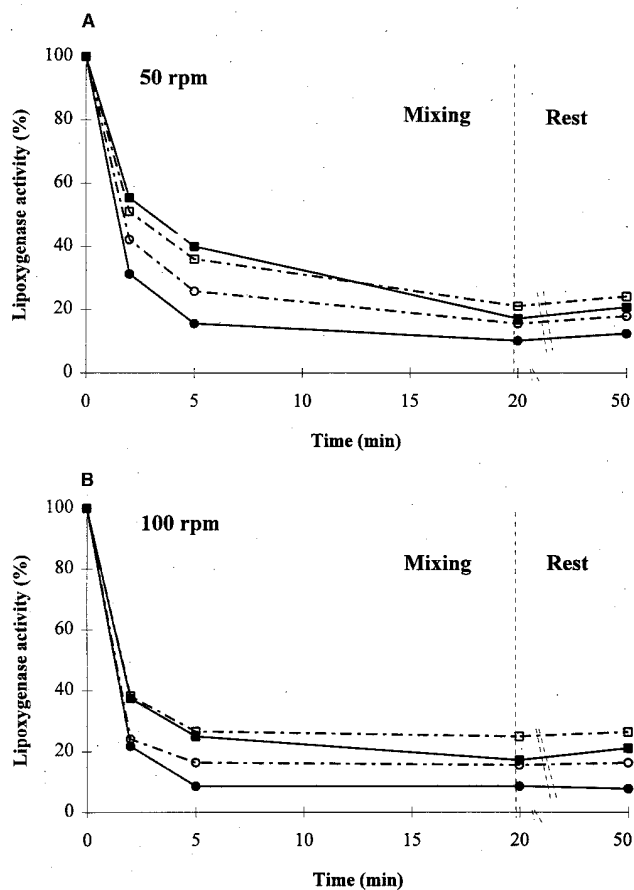
Whatever the compound added to the dough, no effect is found for peroxidase, the activity of which remains close to that of initial flour ( $100 \pm 2\%$ ).

For lipoxygenase, increasing losses of extractable activity are observed when each of these compounds is added to the dough (Fig. 6A). The highest loss is caused by hydrogen peroxide, followed by linoleic acid, and then by guaiacol. The loss due to the addition of both hydrogen peroxide and guaiacol is close to that observed with linoleic acid.

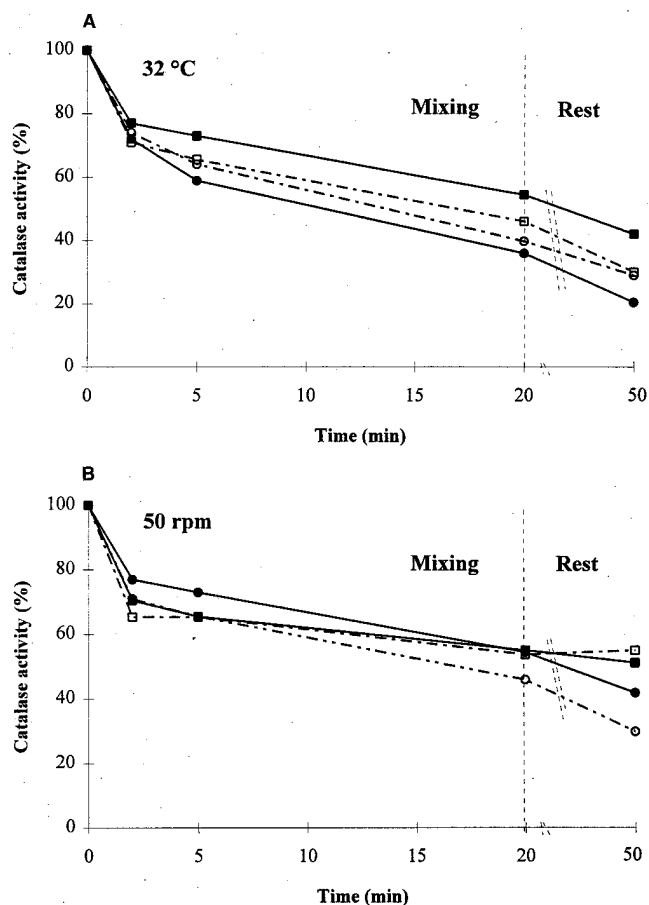
Similarly, increasing losses of activity are apparent for extractable catalase in doughs supplemented by these three compounds (Fig. 6B). However, the rank is different because the highest loss is obtained when both guaiacol and hydrogen peroxide are added followed by guaiacol alone, whereas addition of either linoleic acid or hydrogen peroxide results in a small increase ( $\approx 10\%$ ) of losses in extractable catalase activity.

### Effect of Yeast and Sodium Chloride

Yeast and sodium chloride are universal ingredients used in dough formulation. None of these additives, at the level used in this study, have an effect on peroxidase activity (*results not shown*).



**Fig. 3.** Effect of mixing and rest periods on dough lipoxygenase activity in different mixing conditions: 18°C and 68% water (■), 18°C and 52% water (●), 32°C and 68% water (□), 32°C and 52% water (○). A, 50 rpm. B, 100 rpm.



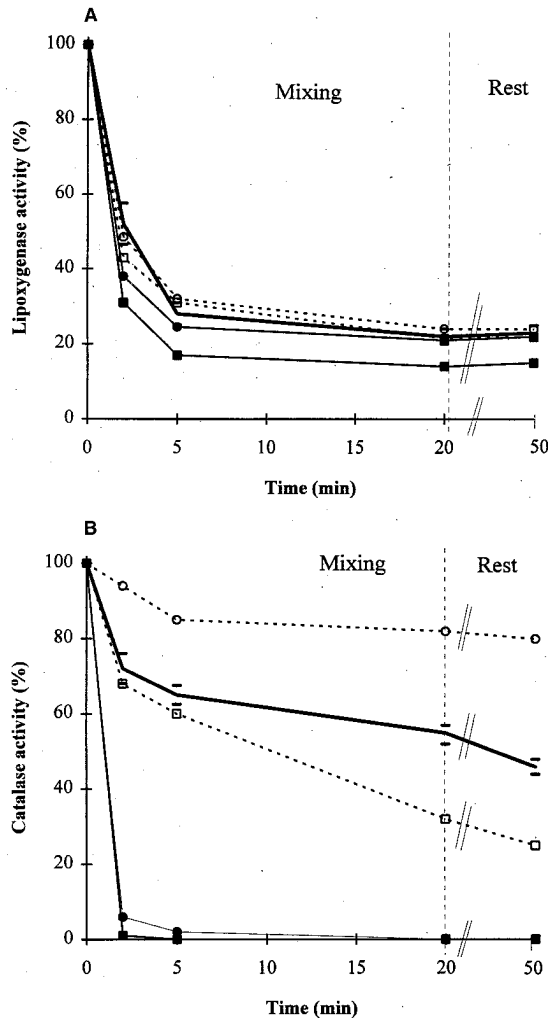
**Fig. 4.** Effect of mixing and rest periods on dough catalase activity in different mixing conditions. A, 32°C, 50 rpm, and 52% water (■), 32°C, 50 rpm, and 68% water (●), 32°C, 50 rpm, and 68% water (□), 32°C, 100 rpm, and 68% water (○). B, 50 rpm, 18°C, and 52% water (■), 50 rpm, 32°C, and 52% water (●), 50 rpm, 18°C, and 68% water (□), 50 rpm, 32°C, and 68% water (○).

## DISCUSSION

Conversely, both ingredients show a protecting effect for the two other enzymes (Fig. 7). Thus for lipoxygenase, addition of yeast causes a 15% increase in residual activity after 2 min of mixing. This protecting effect decreases as the mixing goes on. The protecting effect of NaCl is almost constant with a 5–10% increase in residual activity (Fig. 7A). For catalase, the protecting effect of NaCl is only apparent in the beginning of mixing (2 and 5 min) and is almost 0 after 20 min of mixing, whereas yeast causes a 15–20% increase of residual activity, whatever the mixing times (Fig. 7B).

### Effect of Ascorbic Acid and Cysteine

Ascorbic acid and cysteine are oxidoreducing compounds frequently added to the dough. None of these compounds, at the levels used in this study, exhibit an effect on peroxidase because extractable activity is always similar to that found in the initial flour. For the two other enzymes, an effect is only apparent with ascorbic acid in the first 5 min of mixing because after 20 min of mixing (and during the rest period), the values of residual activity for lipoxygenase and catalase are similar to those obtained with dough without additive. Thus, after 2 min of mixing, the addition of ascorbic acid causes a small increase of losses in lipoxygenase (10%) and catalase (14%) activities.

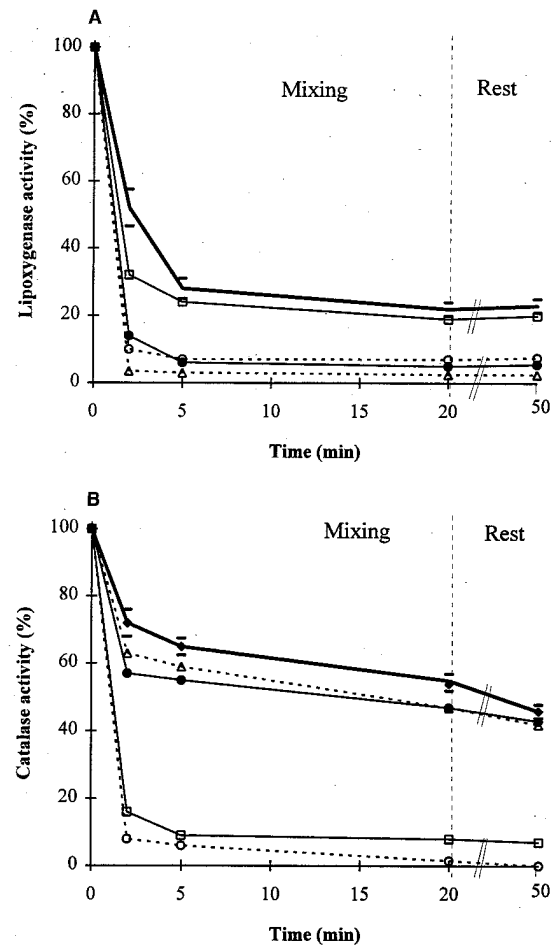


**Fig. 5.** Effect of buffer solution added to the dough on lipoxigenase and catalase activities extracted from dough. **A**, Lipoxigenase residual activity. **B**, Catalase residual activity. Mixing conditions: 75 rpm, 25°C, and 60% buffer solution. Acetate buffer, 0.1M, pH 3.6, (■); acetate buffer, 0.1M, pH 4.2 (●); phosphate buffer, 0.1M, pH 5.6 (□); phosphate buffer, 0.1M, pH 7.5 (○). Bars represent standard deviation obtained from four replicates and two extractions for each replicate.

The behavior of the three oxidoreducing enzymes during dough mixing is different because extractable peroxidase is almost insensitive to all the mixing conditions tested in this study, whereas losses in lipoxygenase and catalase are highly variable from one condition of mixing to another. For these two enzymes, our extracting conditions fully solubilize the activities present in dough. Therefore, the losses in extractable activity represent the decreases in enzyme activity during dough mixing for lipoxygenase and catalase. Under these conditions, two main reasons can explain the decrease in enzyme activity.

The first reason can be related to a self-destruction mechanism. In this case, the enzyme as it functions, synthesizes transitory (or secondary) products which are irreversibly detrimental to its activity. The second reason lies in the physicochemical environment which can denature irreversibly the enzyme protein.

A self-destruction mechanism has been already suggested for lipoxygenase in solution (Smith and Lands 1970, 1972; Cook and Lands 1975). Although there were some controversies with this mechanism because Gibian and Galaway (1976) indicated that loss of activity was due to physical adsorption of the enzyme on the glass vessel and to competitive inhibition by hydroperoxide, the transitory formation of highly reactive free radicals could be responsible, at least partly, for the enzyme denaturation during catalysis.

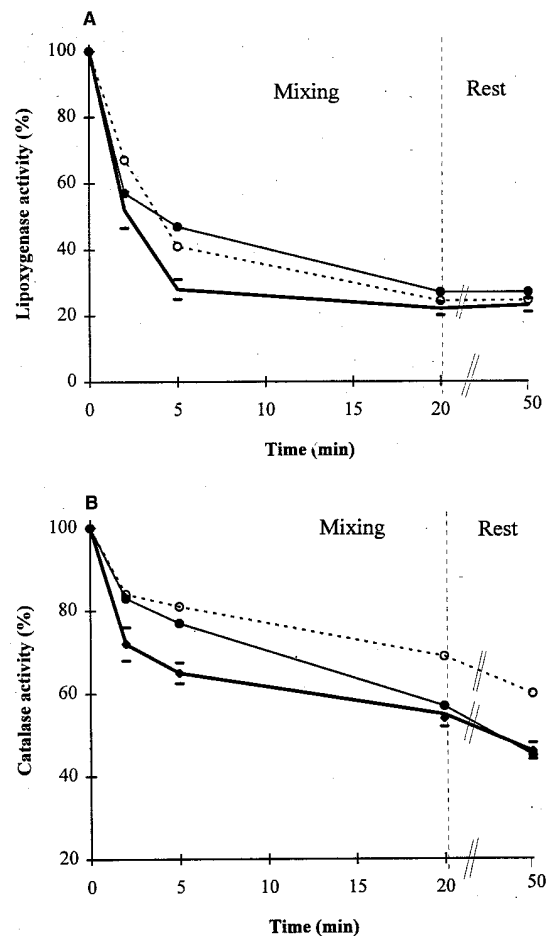


**Fig. 6.** Effect of mixing and rest periods on dough lipoxigenase and catalase activities after addition of linoleic acid, hydrogen peroxide, or guaïacol. **A**, Lipoxigenase residual activity. **B**, Catalase residual activity. No additive, standard mixing conditions, 75 rpm, 25°C, and 60% water (—); linoleic acid (●); hydrogen peroxide (Δ); guaïacol (□); guaïacol + hydrogen peroxide (○). Bars represent standard deviation obtained from four replicates and two extractions for each replicate.

Several experimental facts are in agreement with a self-destruction mechanism to explain the losses in lipoxygenase during mixing. First, the highest losses are observed at the beginning of mixing (when the substrates, oxygen, and polyunsaturated free fatty acids are present in the highest amounts), whereas their presence rapidly decreases as mixing time increases. Second, different parameters that can enhance enzyme activity cause increasing losses in lipoxygenase. Thus, the increase in mixing speed has a denaturing effect probably by increasing the enzyme-substrates (oxygen and polyunsaturated fatty acids) contacts and the incorporation of oxygen in the dough. Conversely, no additional loss is observed during the rest period. Similarly, the increase in the amount of water added to the dough has a protective effect. In this case, increase of water content from 52 to 68% corresponds approximately to a 25% dilution of the dry matter (25% dilution of the reactants in the water phase). This effect could slow down the lipoxygenase activity and, therefore, decrease its self destruction during mixing. Moreover, addition of free linoleic acid (to double the amount of available lipoxygenase substrate in the dough) results in increasing losses of lipoxygenase (Fig. 6A). Similarly, the protecting effect of yeast and salt may be due to a decrease in lipoxygenase functionality during mixing. Thus, yeast could reduce its activity by competing for the available oxygen. Likewise, different authors have shown that salt addition resulted in the decrease of lipoxygenase activity measured by the losses in either the polyunsaturated free fatty acids (Mann and Morrison 1975) or the carotenoid pigments (Nicolas 1978) during mixing. However, the self-destruction of lipoxygenase is probably not the sole mechanism that explains the lipoxygenase losses during mixing. Thus, additives such as hydrogen peroxide and guaiacol increase losses by other mechanisms. The former is a potent irreversible inhibitor of lipoxygenase (Mitsuda et al 1967, Egmond et al 1975) and phenolic compounds such as guaiacol, are well-known inhibitors of lipoxygenase activity (Richard-Forget et al 1995). Similarly, the results obtained on the effect of pH could be due to an irreversible denaturation of the enzyme during mixing in acidic conditions. Enzyme instability has already been observed in acid medium for lipoxygenase from other origins (Eskin et al 1977, Hugues et al 1994).

The probable lack of high amounts of hydrogen peroxide obviously rules out the self-destruction mechanism to explain losses in catalase activity during dough mixing. Therefore, the decrease of catalase activity is more likely related to the physicochemical dough environment of the enzyme. This assumption is in agreement with the observation that the catalase losses occur not only during mixing but also during the rest period. Moreover, it is during this phase that the main effects of temperature and mixer speed observed in the experimental design are the highest (Table II). In addition, catalase is obviously highly sensitive to an acid environment, as emphasized by the effect of pH during mixing (Fig. 5B) and on catalase extraction from flour (Fig. 1). The denaturing effect of acid pH has already been shown for catalase from other origins (Chan et al 1978, Esaka and Asahi 1982, Chandlee et al 1983) and is due to an irreversible dissociation of the prosthetic group from the apoprotein (Jones 1982). However, because the addition of hydrogen peroxide causes increasing losses of catalase, a self-destruction mechanism for this enzyme cannot be ruled out in this case. Moreover, the simultaneous addition of hydrogen peroxide and guaiacol results in a smaller loss than in the presence of hydrogen peroxide alone (Fig. 6b). This could be due to a protective effect of peroxidase which is able to rapidly consume this compound in the presence of guaiacol. A similar protective effect of peroxidase is also observed for lipoxygenase when guaiacol and hydrogen peroxide were both added compared to hydrogen peroxide alone (Fig. 6A). The two latter effects are illustrations of the interconnections among different oxidoreducing enzymes and their effectors during mixing (Nicolas and Drapron 1983).

In conclusion, the three oxidoreducing enzymes tested in this work exhibit different behavior during mixing. Peroxidase remains almost unaffected by mixing whereas losses in lipoxygenase and catalase activities are observed at different levels depending on the mixing conditions tested. For lipoxygenase, a self-destruction mechanism seems to be the main cause of enzyme denaturation during mixing, whereas losses in catalase seem mainly due to physicochemical denaturation occurring also during rest period of the dough. However, in both cases, the relative importance of these two mechanisms and therefore the residual activities in dough appear largely modified following the different mixing conditions. In consequence, it is likely that the impact of these oxidoreducing enzymes could be modulated by using appropriate additives or mixing conditions. Our results could be of importance owing to the fact that some oxidoreducing enzymes such as glucose oxidase and sulfhydryl oxidase have been proposed as improvers for the baking industry (Haarasilta et al 1991). The improving mechanism is probably due to the formation of hydrogen peroxide during catalysis which then can be used by peroxidase to promote crosslinking between the pentosan and protein chains (Hoseney and Faubion 1981). Catalase can compete for the use of peroxide and then could decrease the improving effect of peroxidase activity. Therefore, it would be of interest to know how the mixing conditions can affect the relative importance of these two endogenous enzymes to eventually optimize the different levels of peroxidase, catalase, glucose oxidase, and sulfhydryl oxidase activities.



**Fig. 7.** Effect of sodium chloride and yeast added to the dough on lipoxygenase and catalase activities extracted from dough. **A,** Lipoxygenase residual activity. **B,** Catalase residual activity. No additive, standard mixing conditions, 75 rpm, 25°C, and 60% water (—); NaCl (2%) (●); yeast (1%) (○). Bars represent standard deviation obtained from four replicates and two extractions for each replicate.

A future article will examine the behavior of exogenous enzymes (lipoxygenase, peroxidase, catalase, glucose oxidase, and sulfhydryl oxidase) added to the dough.

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