

Effect of Adding Exogenous Oxidative Enzymes on the Activity of Three Endogenous Oxidoreductases During Mixing of Wheat Flour Dough

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ABSTRACT

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The behavior of different exogenous enzymes (soybean lipoxygenase [SLOX], horseradish peroxidase [HPOD], catalase from bovine liver [BCAT], and glucose oxidase [GOX] from *Aspergillus niger*) added to dough was studied during mixing. The effect of adding these exogenous oxidoreductases on the activity of three oxidative enzymes present in wheat flour (lipoxygenase [WLOX], peroxidase [WPOD], and catalase [WCAT]) was examined. Proper assay conditions were established to differentiate between added WLOX, WPOD, and WCAT and the corresponding activities present in wheat flour. For doughs with added SLOX, an immediate loss of extractable SLOX (≈40%) was observed which remained constant during further mixing. When compared with the control dough, addition of SLOX decreased the losses in WLOX and WCAT activities, whereas WPOD activity was unaffected. With doughs supplemented by HPOD, an immediate loss of 20% in the HPOD activity was observed which did not change after 20 min of mixing. Compared with control dough, addition of HPOD did not affect the behavior

of WLOX and WPOD, whereas a slight decrease in the WCAT losses was observed. Addition of BCAT to the dough did not change the behavior of WLOX and WPOD, whereas the losses in WCAT were less rapid. Half of the extractable activity of BCAT was lost at the beginning of mixing with no change during further mixing. For doughs supplemented with GOX, 25% of the GOX activity was lost in the first 5 min of mixing and an additional loss of 20% was observed after 20 min of mixing. Compared with dough without GOX, addition of GOX decreased the losses in WLOX, whereas losses in WCAT and WPOD increased. Glucose and ferulic acid were also added to doughs supplemented with GOX. Added glucose decreased the losses in GOX and WLOX and did not change the behavior of WPOD and WCAT during mixing. Addition of ferulic acid promoted a slight increase of the losses in WLOX and WCAT and almost no change for GOX and WPOD.

Lipoxygenase (EC 1.13.11.12), peroxidase (EC 1.11.1.7), and catalase (EC 1.11.1.6) are naturally present in wheat flour, where they catalyze oxidative reactions of great importance to the rheological properties of dough (Nicolas and Drapron 1983, Grosch 1986, Van Dam and Hille 1992). Previous work (Delcros et al 1998) showed that these oxidative enzymes exhibit different behaviors during mixing. Thus, extractable wheat peroxidase (WPOD) activity remained almost unaffected by mixing, whereas losses in wheat lipoxygenase (WLOX) and catalase (WCAT) activities progressively increased during mixing. A self-destruction mechanism was postulated for WLOX because the highest losses were found when the mixing conditions were favorable to the lipoxygenase activity (i.e., high mixing speed, increasing the mixing temperature from 20 to 32°C, addition of linoleic acid, and absence of yeast). For WCAT, a nonreversible denaturation due to the acid environment of the dough was postulated. However, in both cases, the relative importance of the two mechanisms, and therefore the residual activities of these enzymes in dough, were largely modulated by mixing conditions.

Adding exogenous enzymes to bread formulations is becoming more and more popular. Lipoxygenase from soybean flour (SLOX) and from horse bean flour (in France) has been used for decades as an improver in breadmaking (Frazier et al 1973, Drapron et al 1974, Hosney et al 1980, Kieffer and Grosch 1980, Cumbee et al 1997). Similarly, horseradish peroxidase (HPOD) has been proposed to improve wheat flours with poor baking properties (Kieffer et al 1981). Currently, the baking industry is deeply involved in research for alternatives to chemical improvers such as potassium bromate due to the potential hazards (Dupuis 1997). Among the different oxidative enzymes proposed, glucose oxidase (GOX) (EC 1.1.3.4) is often cited for commercial use (Luther and Baldwin 1957, Haarisalta et al 1991). In the presence of oxygen, GOX catalyzes the oxidation of β-D-glucose to δ-D-gluconolactone, and produces hydrogen peroxide. The gluconolactone is then slowly hydrolyzed to gluconic acid by a nonenzymatic mechanism. The hydrogen peroxide produced

is able to oxidize thiol groups in disulfide bonds, which explains its improving effect (Haarisalta and Pullinen 1992). However, according to Vemulapalli et al (1998), the H₂O₂ effect (produced by GOX) may be due to its ability to form a gel from the water-soluble pentosans in the presence of peroxidase. During breadmaking, GOX supplementation leads to a change in the textural properties of the dough, affecting its consistency and enhancing the bread volume (Martinez-Anaya and Jimenez 1998, Wikström and Eliasson 1998, Vemulapalli et al 1998). Recently, hexose oxidase (EC 1.1.3.5) isolated from the red algae *Chondrus crispus*, which acted on a number of mono- and oligosaccharides (including glucose and maltose), has proven to be a more efficient enzyme than GOX (Poulsen and Bak Hostrup 1998).

The purpose of this study was to: 1) determine proper assay conditions to differentiate between the activities of exogenous and endogenous enzymes in the same extract to compare their stability during dough mixing, and 2) study how the presence of exogenous oxidoreductases (SLOX, HPOD, bovine liver catalase [BCAT], and GOX) affect the activity of the endogenous oxidative enzymes in the dough.

MATERIALS AND METHODS

The wheat flour used in this study was an untreated, improver-free straight-grade flour commercially milled by les Moulins Soufflet (Nogent-sur-Seine, France). It contained 9.7% protein (N × 5.7) and 0.61% ash (db). The moisture content was 15.3%. The lipoxygenase, peroxidase, and catalase activities were 33 nkat/g of flour, 22 AU/sec/g of flour (AU = absorbance unit), and 4.8 μkat/g of flour, respectively, on a dry matter basis. Linoleic acid (>99%), Tween 20, guaiacol, ferulic acid, horseradish peroxidase (Type VI), catalase from bovine liver, and soybean lipoxygenase (Type I-S), were purchased from Sigma Chemical Co. (Saint. Louis, MO). Glucose oxidase from *Aspergillus niger* (Maxazyme GO) was obtained from Gist-brocades (Delft, Netherlands). All other chemicals were of reagent grade from Prolabo (Paris, France).

Mixing Conditions

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. Aliquots of dough were

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taken at 2, 5, 10, and 20 min of mixing (15–20 g), frozen in liquid nitrogen, and stored at -20°C until analysis. For the supplemented doughs, SLOX (3,350 nkat), HPOD (2,900 AU/sec), BCAT (600 μkat), GOX (780 nkat), glucose (0.22 mmol), and ferulic acid (0.05 mmol) were dissolved in the 90 mL of water added to the dough. The amount of each additive was selected to approximately double the initial amount present in wheat flour. Each experiment was at least duplicated, and two extractions were made from each dough sample.

Enzyme Assays

The conditions for the endogenous activities were those described by Delcros et al (1998) with minor modifications.

Lipoxygenase. 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 $38,000 \times g$ for 20 min at 4°C . Two assay conditions were used for the measurement of lipoxygenase activity in the supernatant. WLOX activity 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 with air at 30°C (Nicolas et al 1982). SLOX activity was determined using the same conditions, except that a borate buffer (100 mM, pH 9) was used instead of the phosphate buffer. Activity is expressed in nkat (nmol of oxygen consumed per sec under assay conditions).

Peroxidase. Flour (1 g) or dough (1.5 g) was homogenized with 20 mL of acetate buffer (100 mM, pH 4.2) under the conditions described for lipoxygenase extraction. The homogenate was then gently agitated for 15 min and centrifuged at $38,000 \times g$ for 20 min at 4°C . Two assay conditions were used for the measurement of peroxidase activity in the supernatant. WPOD activity was determined spectrophotometrically at 465 nm and at 30°C . The assay mixture contained guaiacol (40 mM), hydrogen peroxide (10 mM), and CaCl_2 (20 mM) in acetate buffer (100 mM, pH 4.2). HPOD activity was determined using the same conditions, except that the CaCl_2 was omitted in the substrate solution. Activity is determined by the slope from the linear increase in absorbance at 465 nm. One AU is defined as a change of 1 AU/sec for a total volume of 3 mL under the assay conditions.

Catalase. Flour (1 g) or dough (1.5 g) was homogenized with 20 mL of phosphate buffer (100 mM, pH 7.5) under the conditions described for peroxidase extraction. The catalase activity of the supernatant was determined polarographically using hydrogen peroxide (1M) in a phosphate buffer (100 mM, pH 7) saturated with air at 30°C . Activity is expressed in μkat (μmol of oxygen formed per sec in the assay conditions).

Glucose oxidase. Extraction conditions were the same as those used for lipoxygenase extraction. The GOX activity of the supernatant was determined polarographically using β -D-glucose (0.22M) in a citrate phosphate buffer (100 mM, pH 5.6) and saturated with air at 30°C . Activity is expressed in nkat (nmol of oxygen consumed per sec in the assay conditions).

RESULTS

Preliminary Results

Preliminary experiments were performed to optimize the assay conditions for the exogenous enzymes and to differentiate them from the corresponding endogenous activities present in wheat. The differentiation of the two activities (endogenous and exogenous) allows us to calculate the proportion of each enzyme in the supplemented doughs and, thus, compare their behavior during mixing. In addition, the effect of enzyme supplementation on the endogenous enzymes can be evaluated by comparing their behavior in the presence or absence of exogenous enzymes.

For GOX from *Aspergillus niger*, we found optimum activity at pH 5.6, which is in agreement with the value given by Whitaker (1985) and a K_m value for glucose close to 20 mM. We have verified that these properties (optimum pH and K_m) were unmodified by mixing. We found an optimum range of pH 6.5–7.5, which is identical to the extraction from either flour or dough. Moreover, the activity measured for GOX-supplemented flour (or dough) extract was equivalent to that obtained with the same amount of flour directly added in the polarographic cell. These results indicate that in the extraction, GOX is fully solubilized from flour or dough.

Determination of two lipoxygenase activities in doughs supplemented with SLOX. In agreement with previous results (Irvine and Anderson 1953, Nicolas et al 1982, Whitaker 1985, Shiiba et al 1991), we found that the major isoform of SLOX (contained in the commercial preparation from Sigma) has optimum activity at pH 9, whereas WLOX is inactive at this pH level. Therefore, the measurement at pH 9 gives the amount of SLOX without any interference of WLOX. Moreover, when measured at pH 6.5 (the optimum pH level of WLOX), the activity of pure SLOX represents 75% of the value obtained at pH 9. Two successive measurements of activity at pH 6.5 and pH 9, on the same extract, differentiated the amounts of WLOX and SLOX. If A and B represent the activity values of the extract obtained at pH 6.5 [$A = (\text{WLOX} + \text{SLOX})_{\text{pH } 6.5}$] and pH 9 [$B = (\text{WLOX} + \text{SLOX})_{\text{pH } 9}$]:

$$\begin{aligned} (\text{WLOX})_{\text{pH } 9} &= 0 \\ (\text{SLOX})_{\text{pH } 6.5} &= 0.75 \times (\text{SLOX})_{\text{pH } 9} \end{aligned}$$

then

$$\begin{aligned} \text{WLOX} &= A - 0.75 \times B \\ \text{SLOX} &= 0.75 \times B \end{aligned}$$

where WLOX and SLOX represent the activities measured at pH 6.5 of the wheat and soybean enzymes, respectively, in the enzymatic extract.

Determination of activities of two peroxidase in doughs supplemented with HPOD. We have shown that calcium ions were activators of the wheat peroxidase system because addition of Ca^{2+} (20 mM) promotes a 50% increase of activity in our assay conditions (Delcros et al 1998). Under the same conditions, a slight inhibition (6%) was observed with a pure HPOD extract. Two successive measurements of activity with calcium (20 mM) and without calcium on the same extract, allowed us to differentiate the amounts of WPOD and HPOD. If A and B represent the activity values of the extract obtained with calcium [$A = (\text{WPOD} + \text{HPOD})_{\text{with Ca}}$] and without calcium [$B = (\text{WPOD} + \text{HPOD})_{\text{without Ca}}$], respectively:

$$\begin{aligned} (\text{WPOD})_{\text{without Ca}} &= (\text{WPOD})_{\text{with Ca}} / 1.5 \\ (\text{HPOD})_{\text{with Ca}} &= 0.94 \times (\text{HPOD})_{\text{without Ca}} \end{aligned}$$

then

$$\begin{aligned} \text{WPOD} &= 2.68 \times A - 2.52 \times B \\ \text{HPOD} &= 2.52 \times B - 1.68 \times A \end{aligned}$$

where WPOD and HPOD represent the activities measured in the presence of calcium of the wheat and horseradish enzymes, respectively, in the enzymatic extract.

Determination of two catalase activities in doughs supplemented with BCAT. We have observed that the catalase from wheat is more sensitive to acid pH than the catalase from bovine liver. Thus, an extract of wheat flour and a solution of catalase from bovine liver at pH 7.5 were acidified at pH 4.5 by addition of citric acid (0.55 mL of a citric acid solution [1M] for 10 mL of the extract). The wheat catalase activity was fully denatured, while the catalase from bovine liver kept 50% of its initial activity. Two successive measurements of activity at pH 7 (on the extract at pH 7.5,

before and after acidification at pH 4.5 with citric acid) allowed us to differentiate the amounts of WCAT and BCAT. If A and B represent the activity values of the extract obtained before [A = (WCAT + BCAT)_{without acid}] and after acidification [B = (WCAT + BCAT)_{with acid}], respectively:

$$\begin{aligned} (\text{WCAT})_{\text{with acid}} &= 0 \\ (\text{BCAT})_{\text{without acid}} &= 2 \times (\text{BCAT})_{\text{with acid}} \end{aligned}$$

then

$$\begin{aligned} \text{WCAT} &= A - 2 \times B \\ \text{BCAT} &= 2 \times B \end{aligned}$$

where WCAT and BCAT represent the activities (measured at pH 7) of the wheat and bovine liver enzymes, respectively, in the enzymatic extract.

Pure solutions of WLOX, SLOX, WPOD, HPOD, WCAT, and BCAT were prepared, and the validity of these equations has been verified by measuring the activities of these solutions in different known proportions. In all cases, the differences between experimental and calculated values were <5%.

Doughs Supplemented with Exogenous Enzymes

For each exogenous enzyme, we first analyzed its behavior during mixing, and then we compared it with the behavior of the corresponding endogenous enzyme (when present in flour). Finally, we compared it with the behavior of endogenous enzymes in supplemented and control (without added enzyme) doughs. In all cases, the activity levels corresponded to those measured (or calculated) in the optimal assay conditions of the endogenous enzymes.

Addition of SLOX. The evolutions of the lipoxygenase activities in supplemented and control doughs are given in Fig. 1. For the exogenous enzyme, after an initial decrease of ≈40% in the first 2 min of mixing, the SLOX extractable activity remained constant during further mixing. Conversely, the WLOX extractable activity steadily declined to ≈40% of its original activity after 20 min of mixing. Thus, the drop of the total lipoxygenase activity at the beginning of mixing was mainly due to the losses in SLOX, whereas during further mixing, the losses in lipoxygenase activity corresponded to those in WLOX. In control dough, the decrease of WLOX was much more rapid. This was important because it was ≈50% in the first 2 min of mixing and reached 80% after 20 min of mixing. Thus, WLOX appeared less inactivated in the presence of SLOX than in the absence of SLOX. Finally, adding SLOX did not modify the behavior of WPOD during mixing, whereas a slight protecting effect was observed for WCAT (Table I).

Addition of HPOD. A loss of 10% of the total peroxidase activity was observed in the first 2 min of mixing (Fig. 2). Further mixing did not modify the amount of extractable peroxidase activity. This initial loss was due only to the HPOD behavior because a constant

decrease of 20% of its extractable activity was observed at any point during 2–20 min of mixing time. Moreover, the extractable WPOD activity of flour was unmodified by mixing in the HPOD-supplemented doughs or in the control dough. As for the behavior of the other endogenous enzymes (WLOX and WCAT), the evolution of WLOX was unmodified in the HPOD-supplemented doughs, whereas a protecting effect was observed for WCAT (Table I).

Addition of BCAT. A significant loss of the total extractable catalase activity (≈40%) was observed in the beginning of mixing, followed by a slow decline with further mixing (Fig. 3). At the end of the 20 min of mixing, half of the total extractable catalase activity was lost. In the first 2 min of mixing, the losses were mainly due to BCAT activity because a 50% decrease was observed for this activity, whereas the WCAT decrease was <30%. For mixing times >2 min, the BCAT extractable activity remained constant, whereas additional losses (≈15%) were apparent for the WCAT activity. However, the decrease in extractable WCAT activity was less rapid for the BCAT-supplemented dough than for the control dough. Adding BCAT to the dough did not modify the behavior of the other endogenous enzymes (WLOX and WPOD) during mixing (Table I).

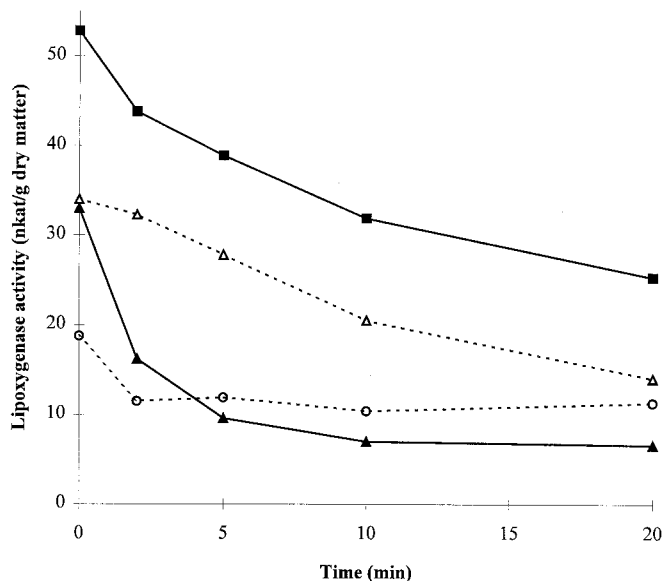


Fig. 1. Effect of mixing on lipoxygenase activity extracted from dough after addition of soybean lipoxygenase. Differentiation between endogenous (WLOX) and exogenous (SLOX) lipoxygenase activities. WLOX activity in control dough (▲); total lipoxygenase activity in supplemented dough (■); calculated WLOX activity in supplemented dough (△); calculated SLOX activity in supplemented dough (○). SLOX (3,350 nkat) added to 150 g of flour in the 90 mL of water added to the dough.

TABLE I

Residual Extractable Activities (%) of Endogenous Lipoxygenase (WLOX), Peroxidase (WPOD), and Catalase (WCAT) in Doughs Supplemented by Soybean Lipoxygenase (SLOX), Horseradish Peroxidase (HPOD) or Bovine Liver Catalase (BCAT) During Mixing^a

Mixing Time (min)	WLOX (%)			WPOD (%)			WCAT (%)		
	Control	Treated Dough		Control	Treated Dough		Control	Treated Dough	
		HPOD	BCAT		SLOX	BCAT		SLOX	HPOD
0 ^b	100	100	100	100	100	100	100	100	100
2	49.1a ^c	52.1a	48.5a	99.5a	99a	98.5a	75a	82.2ab	85b
5	29.1a	29.9a	30.5a	98a	101a	99.2a	67a	75b	77.1b
10	21.1a	20.5a	21.7a	99a	98.3a	100a	61.3a	67.8b	70b
20	19.9a	20.1a	20a	100a	100a	99a	48.2a	53b	51.3b

^a Mixing conditions at 25°C and 75 rpm: 150 g of flour and 90 mL of deionized water containing 3350 nkat of SLOX or 2,900 AU/sec of HPOD or 600 μkat of BCAT or no additive (control).

^b Initial activities in flour were 33 ± 1.7 nkat/g, 22 ± 1 AU/sec/g and 4.8 ± 0.1 μkat/g for lipoxygenase, peroxidase, and catalase, respectively.

^c For the same endogenous enzyme and the same mixing time, means followed by different letters are significantly different (*P* < 0.05).

DISCUSSION

Addition of GOX. We had verified that, in our assay conditions, the wheat flour did not exhibit any GOX activity. The extractable activity of GOX decreased 25% in the first 2 min of mixing (Fig. 4A) and then steadily declined during 2–20 min of mixing to reach a residual activity of 65% after 20 min of mixing. For the endogenous enzymes, the addition of GOX promoted a 10–15% loss of WPOD, whereas this activity was very stable in the control dough (Fig. 4C). Differences were also noticed for WLOX and WCAT. The losses in WLOX extractable activity were lower for the GOX-supplemented doughs when compared with that of the control dough (Fig. 4B), whereas the reverse was observed for WCAT (Fig. 4D). Thus, after 20 min of mixing, the residual activity was 36% for WLOX (instead of 20% in the control dough), 38% for WCAT (instead of 48% in the control dough), and 87% for WPOD (instead of 100% in the control dough).

Doughs with and without added GOX have also been supplemented with glucose (0.22 mmol for 150 g of flour) or ferulic acid (0.05 mmol for 150 g of flour). These amounts approximate the initial amounts of free glucose (Potus et al 1994) and esterified ferulic acid (Sosulski et al 1982, Andersson et al 1994) in wheat flour. For doughs supplemented with glucose or ferulic acid but without added GOX, there was no difference in the behavior of the three endogenous enzymes examined. The comparison of the behavior of GOX in doughs with or without added glucose showed that adding glucose had a protecting effect on the GOX activity during mixing (Fig. 4A). Thus, after 20 min of mixing in the presence of glucose, the losses in GOX extractable activity were \approx 20%, whereas in its absence, we observed a decrease of 36%. Conversely, addition of ferulic acid to GOX-supplemented doughs did not modify the behavior of GOX (Fig. 4A). For the endogenous enzyme activities, the protecting effect of GOX on the WLOX activity was enhanced in the presence of glucose, whereas addition of ferulic acid decreased this effect (Fig. 4B). For the WPOD and WCAT activities, addition of either glucose or ferulic acid to GOX-supplemented doughs did not modify the effect of GOX (Fig. 4C and 4D). Thus, when compared with the control dough, addition of GOX with or without glucose (or ferulic acid) resulted in a 10–13% decrease of the WPOD extractable activity and a 6–10% decrease of the WCAT extractable activity after 20 min of mixing.

In this study, we added two types of enzymes to wheat dough: 1) enzymes (lipoxygenase, peroxidase, and catalase) that carry an activity already present in wheat, and 2) an enzyme (glucose oxidase) which is not detectable in wheat flour but which has a common substrate with endogenous enzyme (O_2 with WLOX) and which produces a substrate for endogenous enzymes (H_2O_2 for WPOD and WCAT). Our purpose was to compare their behavior with that of endogenous enzymes and to check the validity of our hypothesis on the mechanism of denaturation of wheat enzymes.

The activities of SLOX, HPOD, and BCAT were quite different from that of the corresponding endogenous activities. In each case, we observed an initial drop of the extractable activity during the first 2 min of mixing, with no modification of the residual extractable activity during 2–20 min of mixing (Figs. 1–3). This constant loss during mixing represents 40% of the SLOX activity (measured with a substrate at pH 6.5), 20% of the HPOD activity (measured with a substrate in the presence of calcium), and 50% of the BCAT activity. The loss may be due to isoforms that are denatured as soon as the dough is formed. For GOX, there is an initial 25% drop of the extractable activity in the first 2 min of mixing, but this is followed by a slow decline during 2–20 min of mixing (Fig. 4A). This behavior is similar to that of WCAT, although the rate of decrease was slightly lower. Because the losses in the GOX extractable activity are less important when the dough is supplemented with glucose, a self-destruction mechanism can be ruled out for this enzyme. For the same reason, the hydrogen peroxide formed by the enzyme cannot explain this result. Therefore, the losses in GOX during mixing may be attributed to a physicochemical denaturation by the dough environment, enzyme denaturation that is slowed down in the presence of its reducing substrate.

The mechanisms of the losses in WLOX and WCAT are confirmed by their behavior during mixing in the presence of exogenous enzymes. Thus, the decrease of the losses in WLOX observed in SLOX-supplemented doughs (Fig. 1) and GOX-supplemented doughs (Fig. 4B) may be explained by a competition for the available oxygen during mixing. Because part of the oxygen is consumed by SLOX or GOX, the self-destruction of WLOX is probably slowed

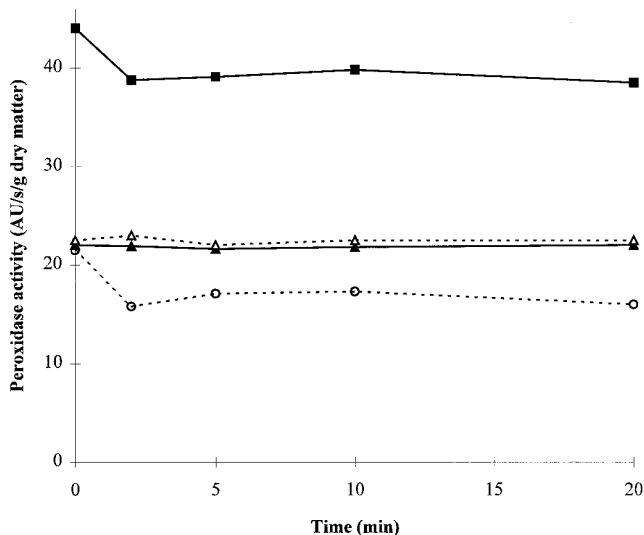


Fig. 2. Effect of mixing on peroxidase activity extracted from dough after addition of horseradish peroxidase. Differentiation between endogenous (WPOD) and exogenous (HPOD) peroxidase activities. WPOD activity in control dough (▲); total peroxidase activity in supplemented dough (■); calculated WPOD activity in supplemented dough (Δ); calculated HPOD activity in supplemented dough (O). HPOD (2900 AU/sec) added to 150 g of flour in the 90 mL of water added to the dough.

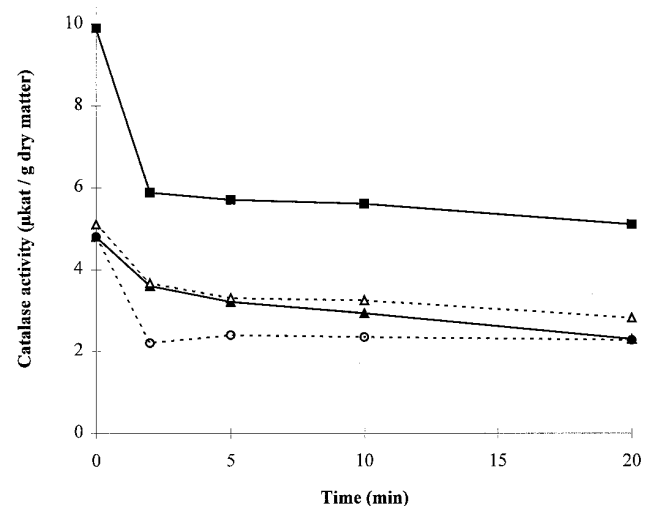


Fig. 3. Effect of mixing on catalase activity extracted from dough after addition of bovine liver catalase. Differentiation between endogenous (WCAT) and exogenous (BCAT) catalase activities. WCAT activity in control dough (▲); total catalase activity in supplemented dough (■); calculated WCAT activity in supplemented dough (Δ); calculated BCAT activity in supplemented dough (O). BCAT (600 μ kat) added to 150 g of flour in the 90 mL of water added to the dough.

down. In the GOX-supplemented doughs, this effect is enhanced by the addition of glucose (Fig. 4B), which increases the oxygen consumption by GOX during mixing at the expense of WLOX. In addition, the SLOX enzyme also competes for the polyunsaturated fatty acids that could enhance its protecting effect on WLOX. Moreover, it produces hydroperoxides (and probably intermediary free radicals) that are different from those produced by WLOX (Nicolas and Drapron 1981). The slight protecting effect observed for WCAT in SLOX-supplemented doughs may be due to the formation of free radicals that are less harmful than those produced by WLOX.

Although Delcros et al (1998) found that the addition of H₂O₂ in dough resulted in important losses in WLOX extractable activity, we do not find this effect in the GOX-supplemented doughs (either alone or with glucose or ferulic acid). It is probable that in these cases, the hydrogen peroxide produced by GOX during mixing was immediately consumed by WPOD or WCAT. The higher losses in WCAT found in the GOX-supplemented doughs (Fig. 4D) may be related to the formation of δ -gluconolactone. This compound is

slowly hydrolyzed in gluconic acid, acidifying the environment. Thus, when comparing the GOX-supplemented doughs with the control dough, we found a slight decrease of 0.2 pH units after 20 min of mixing, which may explain the increase of the losses in WCAT. However, as emphasized by Delcros et al (1998), self-destruction cannot be ruled out for the WCAT activity because the addition of HPOD or BCAT enzymes that compete for H₂O₂ cause a decrease in the losses in WCAT. For WPOD extractable activity, the lack of effect in dough supplemented with GOX and ferulic acid, as compared with dough supplemented only with GOX, confirms the stability of this enzyme in the presence of its substrates during mixing. A similar result was found by Delcros et al (1998) in dough supplemented with H₂O₂ and guaiacol.

CONCLUSIONS

The four exogenous enzymes tested in this study exhibited different behavior during mixing. Three of them (SLOX, BCAT, and HPOD) are partly denatured at the very beginning of mixing and

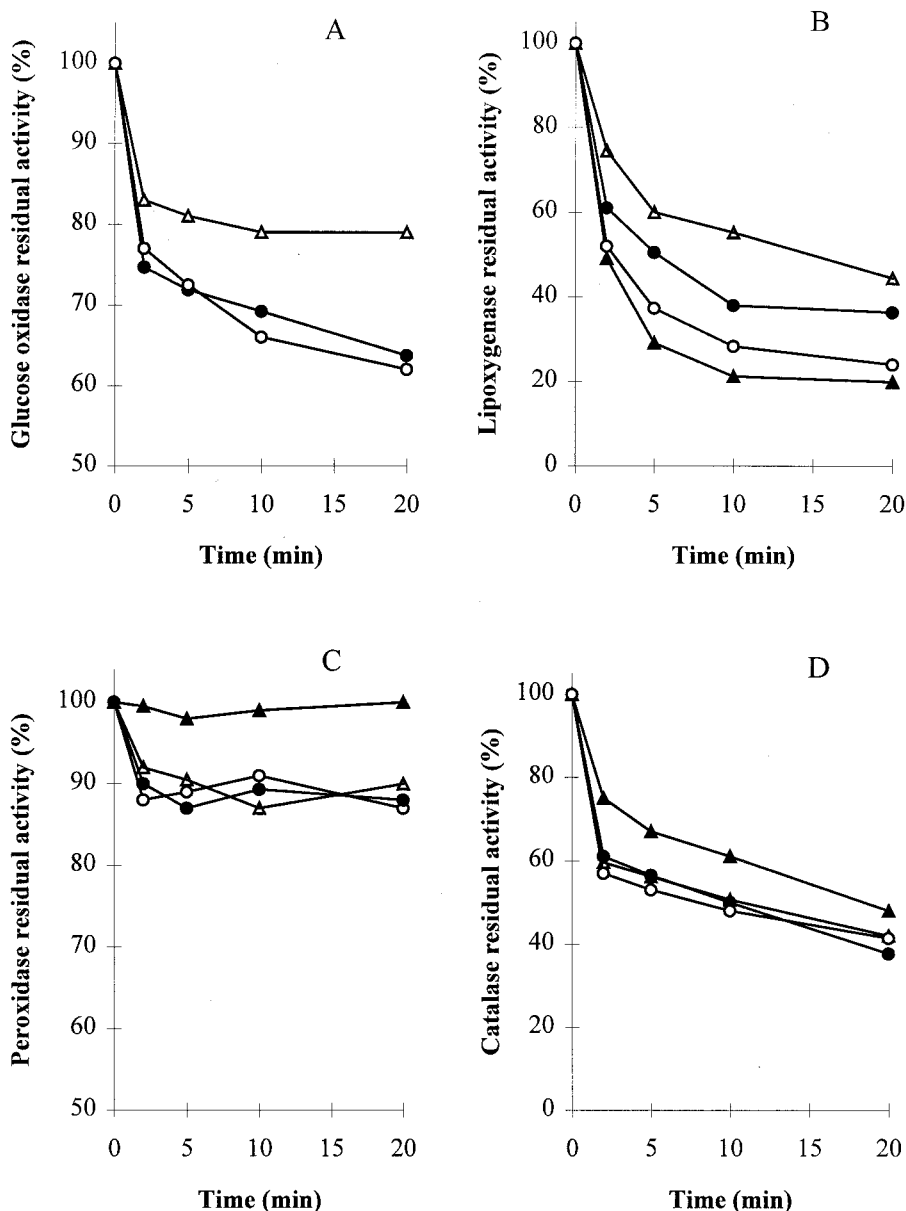


Fig. 4. Effect of mixing on glucose oxidase, lipoxygenase, peroxidase, and catalase activities extracted from dough after addition of *Aspergillus niger* glucose oxidase, glucose, or ferulic acid. **A**, GOX residual activity. **B**, WLOX residual activity. **C**, WPOD residual activity. **D**, WCAT residual activity. Control dough (▲); dough supplemented with: GOX (●); GOX and glucose (Δ); GOX and ferulic acid (○). GOX (780 nkat), glucose (0.22 mmol) and ferulic acid (0.05 mmol) were added to 150 g of flour in the 90 mL of water added to the supplemented doughs.

then their residual activity remains constant during further mixing, but at different levels. The fourth enzyme (GOX) also undergoes an initial drop, but further mixing induces a progressive decline of its extractable activity. Therefore, the behavior of exogenous enzymes cannot be predicted during mixing, and we must take in to account a possible partial denaturation during mixing in the formulation of bread recipes.

Moreover, adding exogenous enzymes leads to modifications in the behavior of endogenous enzymes. This is obviously true for exogenous enzymes that are added to enhance an endogenous activity already present in wheat flour (e.g., SLOX or HPOD). Our results clearly show that these additions result not only in an increase of the total activity present in the dough, but also in a protecting effect on the corresponding wheat enzyme during mixing. This is true also for an exogenous enzyme such as GOX, with activity absent (or undetectable) in wheat flour. However, in this case, no general rules can be discerned on their effect on the endogenous enzymes tested. Nevertheless, it seems that adding an activity that promotes acidification of the dough would result in increased losses in WCAT. In the same way, exogenous enzymes that reduce the WLOX activity would lead to decreased losses in this enzyme. This latter effect was confirmed by the addition of GOX and SLOX in this study, as well as by the addition of yeast in Delcros et al (1998). All these ingredients compete for the available oxygen during mixing, reduce the oxidation reaction of polyunsaturated fatty acids catalyzed by WLOX, and therefore decrease self-destruction. Conversely, addition of *Humicola lanuginosa* lipase increases losses in WLOX during mixing (unpublished data) because it increases the WLOX reaction by forming polyunsaturated free fatty acids. We confirmed the remarkable stability of the WPOD activity during mixing, as compared with WCAT and WLOX activities, because among the various exogenous enzymes used and the different mixing conditions tested, it was only after adding GOX that a loss of ≈10% was measured for WPOD.

Oxygen and hydrogen peroxide, when formed by yeast (Liao et al 1998) or by exogenous enzymes, play a key role in the oxidation of proteins and pentosans during mixing. Most of the reactions using these oxidizing compounds are under the control of enzyme activities in dough that were either initially present or were added. Further study is needed to clarify the relative importance of these reactions and to understand how the addition of exogenous enzymes can affect this balance. The answer to these questions will give the key for a successful replacement of chemical oxidants such as bromate and ascorbic acid with enzyme preparations.

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