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Analysis and Modeling of the Ferulic Acid Oxidation by a Glucose Oxidase−Peroxidase Association. Comparison with a Hexose Oxidase−Peroxidase Association

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A commercial glucose oxidase (GOX) from *Aspergillus niger* was partially characterized. The enzyme exhibited a two-step transfer mechanism, and the kinetic constants toward glucose and oxygen were determined. Under conditions similar to dough making (glucose concentration and pH), GOX does not exhibit maximum activity. A hexose oxidase (HOX) from *Chondrus crispus* was partially characterized as well. The HOX activity is not far from the optimum in the kneading conditions (pH and glucose concentration). A peroxidase (POD) purified from wheat germ was used to oxidize ferulic acid in the presence of GOX or HOX. Hydrogen peroxide produced during the glucose oxidation activates the wheat germ POD. Ferulic acid oxidation in solutions containing different ratios of POD + GOX or HOX + POD was followed by UV spectrophotometry. For the same dosage, the HOX−POD system is the most efficient for peroxidase activation. Using absorbance data and kinetic constants of GOX and POD, a mathematical model describing the release or consumption of the different reactants (hydrogen peroxide, oxygen, and ferulic acid) in the medium was developed, and experimental data correlated well with calculated values. The results obtained will be applied to investigate the effect of GOX and HOX activities on the rheological properties of dough.

KEYWORDS: Ferulic acid; peroxidase; bread making; glucose oxidase

INTRODUCTION

Potassium bromate has been used extensively, as an oxidant, to improve the quality of flour during dough making. It enhances the baking performance and aids the development of a strong relaxed gluten matrix (*1*). In the last two decades, several toxicological studies have shown that potassium bromate causes tumors in rats (*2*), and a few cases of poisoning have been reported (*3*). As a consequence, in 1987, the International Cancer Research Agency classified potassium bromate as a carcinogen (*4*). Baking industry researchers have endeavored to identify substitutes for potassium bromate, and one of the possibilities is the use of enzymes. Additions of glucose oxidase (GOX) (EC 1.1.3.4), hexose oxidase (HOX) (EC 1.1.3.5.) or phytase (*4*−*7*) have been suggested during dough mixing. The use of HOX (*5*) or GOX, alone (*8*, *9*), or in combination with hemicellulase (*10*) or α-amylase (*11*) has also been proposed to improve baking performance.

GOX, in the presence of molecular oxygen, catalyzes the oxidation of glucose to gluconolactone and hydrogen peroxide (*12*). GOX kinetics are Michaelian type with a Ping-Pong mechanism. *Aspergillus niger* GOX has been the most studied (*5*, *13*, *14*), and its effect on bread making have been extensively reported. Faisy and Neyreneuf (*10*) proposed the use of a GOX/hemicellulase association as a substitute of potassium bromate. They reported that the addition of both enzymes increased bread volume. Vemulapalli et al. (*15*, *16*) compared the use of chemical oxidants such as calcium peroxide or potassium bromate with GOX in bread making and found that doughs where GOX is added are strong and dry. Miller and Hoseney (*17*) reported similar results. Ameille et al. (*18*) also suggested an activation of endogenous peroxidase (POD) (EC 1.11.1.7) by GOX addition in dough. An oxidative gelation of water-soluble pentosans catalyzed by POD, and causing a limitation in water mobility could explain these effects. The ability of POD, in the presence of hydrogen peroxide, to promote gelation of pentosans (via ferulic acid) is well documented (*19*−*23*). Figueroa-Espinoza and Rouau (*24*) and later Figueroa-Espinoza et al. (*25*) confirmed that the cross-linking of pentosans by a fungal laccase, a horseradish peroxidase (HRP), or manganese peroxidase takes place through the coupling of their esterified ferulic acid. In a previous communication, we described the oxidation of ferulic acid and 5-*O*-(*trans*-feruloyl)-L-arabinose by a wheat germ POD (*26*). It was shown that ferulic acid dimers can be formed enzymatically and assumed that the enzyme can use the ferulic acid esterified to arabinoxylans as substrate, leading to the formation of a gel. These results are consistent with those of Figueroa-Espinoza et al. (*25*).

HOX, unlike GOX can use several monosaccharides and oligosaccharides as substrates and it catalyses the conversion

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of the latters into corresponding lactones with the formation of hydrogen peroxide. Poulsen and Bak Hostrup (*5*) compared the effects of HOX and GOX in dough and bread. They showed that HOX caused dough strength and increased bread volume more efficiently than GOX in the same dosage.

The effect of GOX on sulfhydryl (SH) groups has also been investigated (*5*, *15*). SH groups of gluten protein are oxidized to disulfide bridges (S−S) when GOX is added, resulting in a dough strengthening and a better gas retention during fermentation (leading to bread with increased volume). Furthermore, it was confirmed that GOX is able to activate endogenous POD. Kieffer et al. (*27*) reported that the addition of HRP, H_2O_2, and catechol improved the rheological properties of wheat dough. This effect was attributed to the oxidation of protein-bound cysteine to cystine. The addition of soya POD had the same effect (*28*, *29*). Labat et al. (*30*) investigated the effect of laccase and ferulic acid addition on wheat flour doughs and showed that the addition of both compounds caused an increase in the oxidation of SH groups and the rate of protein depolymerization during mixing. A coupling reaction involving enzymatically (laccase) generated feruloyl radicals and thiol radicals generated through the mechanical breakdown of interchain disulfide bounds could explain their findings.

GOX supplementation leads to changes in the structural properties of the dough affecting its consistency and enhancing the bread volume (*5*, *9*, *16*, *31*).

Addition of GOX or HOX may also modify the balance between the different enzymatic systems present in dough. Hydrogen peroxide production could activate catalase and peroxidase systems and oxygen consumption could reduce lipoxygenase activity. Rakotozafy et al. (*32*) reported that the loss in lipoxygenase activity, usually observed during mixing, is reduced when GOX is added. According to these authors, the decrease in oxygen available could limit the lipoxygenase activity and therefore its catalytic denaturation during mixing.

In this paper, the oxidation of ferulic acid by a GOX−POD association or by a HOX−POD association (hydrogen peroxide enzymatically supplied) is investigated to understand the effects of GOX and HOX in bread making. GOX and HOX were kinetically characterized, to define the optimum conditions for the enzyme activity. The effect of altering GOX/POD relative concentrations was analyzed by spectrophotometry. A mathematical model, which describes the release or consumption of the different reactants in the medium, was developed and a comparison was made with the experimental data.

HOX was partially characterized and the ability of the HOX−POD system to oxidize ferulic acid was compared with the ability of the GOX−POD system to do so.

MATERIALS AND METHODS

Plant Material. Industrial germ was provided by Les Moulins Soufflet (Nogent-sur-Seine, France) and defatted by cold acetone according to Nicolas et al. (*33*).

Enzymes. Wheat germ POD was purified according to Billaud et al. (*34*) and the major cationic fraction was used for the study. GOX was extracted from *Aspergillus niger*, its activity was 760 nkat/mg in the standard assay conditions. HOX, extracted and purified from the red algae *Chondrus crispus*, was a gift from Danisco Ingredients (Brabrand, Denmark), its activity was 33 nkat/mL in the standard assay conditions.

Chemicals. Hydrogen peroxide 30% (v/v), *m*- and *o*- phosphoric acids, D-glucose, calcium chloride, and ascorbic acid were purchased from VWR (Paris, France). Ferulic acid (FA) was purchased from Sigma Chemical (St Louis).

Table 1. Apparent Kinetic Constant Values of GOX for $[O_2] = 220$ μM

	pH 5.0	pH 5.6	pH 6.0
K^{GOX}_{mappG} (mM)	57.6	22	16
V^{GOX}_{mappG} (nkat/mg)	1020	1130	580
$V^{GOX}_{mappG}/K^{GOX}_{mappG}$	17.7	51.4	36.3

Peroxidase Assay. The POD activity was determined as previously described (*26*), and the spectophotometric data were converted into residual ferulic acid concentrations ($[FA]_{residual}$) using the relationship formerly established (*26*)

$$[FA]_{residual}(\mu M) = [FA]_{t0} + 18.7(Abs_{t0} - Abs_t)^2 - 119(Abs_{t0} - Abs_t)$$

where $[FA]_{t0}$ and $(Abs_{t0} - Abs_t)$ represent the initial ferulic acid concentration (μM) and the decrease in absorbance value at 310 nm during the reaction time *t*, respectively. POD activity is expressed in nanokatals (nmol of FA consumed per second). In these assay conditions, the purified wheat germ POD used in this study had an activity of 0.26 μkat/mL and the slope of the absorbance decrease remained proportional to the enzyme amount until an activity of 10 nkat in the reaction solution.

Glucose Oxidase Assay. The GOX activity was determined polarographically using glucose (220 mM) dissolved in a 100 mM acetate buffer pH 5.6 saturated by air at 30 °C according to Rakotozafy et al. (*32*). Activity is expressed in nanokatals (nmol of oxygen consumed per second).

Hexose Oxidase Assay. The HOX activity was determined polarographically using glucose (50 mM) dissolved in a 100 mM acetate buffer solution at pH 5.6 saturated with air at 30 °C. Activity is expressed in nkat (nmol of oxygen consumed per second in the assay conditions).

GOX−POD Mixtures. Evolution of FA in GOX-POD mixtures was analyzed by UV-spectrophotometry (310 nm) according to Garcia et al. (*26*). GOX solution used in these experiments was prepared at a concentration of 0.27 mg/mL. Wheat POD used in these experiments had a V^{POD}_m value of 5.4 μkat/mL of enzymatic solution (corresponding to the 0.26 μkat/mL determined in the standard assay conditions).

The composition of mixtures containing a fixed GOX amount was as follows: 6.7 μg of GOX (5.1 nkat), 90 μM FA, 50 mM D-glucose, 10−100 μL of POD (2.6−26 nkat), and 20 mM $CaCl_2$ in 100 mM acetate buffer pH 5.6 (3 mL final volume).

The composition of mixtures containing a fixed POD amount was as follows: 25 μL of POD (6.5 nkat), 90 μM FA, 50 mM D-glucose, 2.7−27 μg GOX (2.05−20.5 nkat), and 20 mM $CaCl_2$ in 100 mM acetate buffer pH 5.6 (3 mL final volume).

The composition of mixtures for the analysis of the ascorbic acid effect was as follows: 80 μL of POD (21 nkat), 27 μg of GOX (20.5 nkat), 90 μM FA, 20 mM $CaCl_2$, 50 mM D-glucose, and 100 mM acetate buffer pH 5.6 containing from 0 to 500 μM ascorbic acid (3 mL final volume). Evolution of FA in GOX−POD mixtures containing ascorbic acid was followed both by UV-spectrophotometry and ECD-HPLC, according to Garcia et al. (*26*).

Comparison of GOX−POD and HOX−POD Mixtures. Evolution of FA in the mixtures was followed by spectrophotometry according to Garcia et al. (*26*). The composition of the mixtures is as follows: 25 μL of POD (6.5 nkat), 90 μM FA, D-glucose (from 2 to 50 mM), and 0.42 to 2.6 nkat of GOX or HOX in 100 mM acetate buffer pH 5.6 (3 mL final volume).

RESULTS AND DISCUSSION

Glucose Oxidase Characterization. The effect of glucose concentration on the enzyme activity was determined at three different pHs, at one oxygen concentration (220 μM). The enzyme affinity toward glucose increases with pH. Conversely, V^{GOX}_{mappG} values decrease when pH increases (**Table 1**). The best efficiency ($V^{GOX}_{mappG}/K^{GOX}_{mappG}$) of the enzyme was found at pH 5.6.

Consequently, experiments were carried out at this pH, hereafter. As glucose concentration in dough is around 4 mM (*35*) and dough pH is between 5 and 6.2 (*32*), it can be adduced that the conditions in dough (regarding the reducing substrate concentration) are not optimal for GOX activity.

Then, the effect of oxygen concentration on the GOX activity was determined. GOX kinetics are Michaelian type with a ping-pong mechanism in agreement with Whitaker (*12*), so the velocity of the reaction is

$$v_G = V_m^{GOX} \frac{[O_2][G]}{K_{mO}^{GOX}[G] + K_{mG}^{GOX}[O_2] + [G][O_2]} \quad (1)$$

where $[O_2]$ = oxygen concentration, $[G]$ = glucose concentration, V_m^{GOX} = maximal velocity, K_{mO}^{GOX} = Michaelian constant toward oxygen, and K_{mG}^{GOX} = Michaelian constant toward glucose.

For a constant oxygen concentration, "apparent" kinetic constants V_{mappG}^{GOX} and K_{mappG}^{GOX} are

$$V_{mappG}^{GOX} = \left(V_m^{GOX} / \left(\frac{K_{mO}^{GOX}}{[O_2]} + 1 \right) \right) \quad (2)$$

$$V_{mappG}^{GOX} = \left(K_{mG}^{GOX} / \left(\frac{K_{mO}^{GOX}}{[O_2]} + 1 \right) \right) \quad (3)$$

Values previously determined are apparent kinetic constants for an oxygen concentration of 220 μM (**Table 1**).

Likewise, for a constant glucose concentration, apparent kinetic constants V_{mappO}^{GOX} and K_{mappO}^{GOX} are

$$V_{mappO}^{GOX} = \left(V_m^{GOX} / \left(\frac{K_{mG}^{GOX}}{[G]} + 1 \right) \right) \quad (4)$$

$$K_{mappO}^{GOX} = \left(K_{mO}^{GOX} / \left(\frac{K_{mG}^{GOX}}{[G]} + 1 \right) \right) \quad (5)$$

These values were determined by polarography for high glucose concentration. As the stoichiometry of the reaction is one mole of oxygen for one mole of glucose, the maximal consumption of glucose is 220 μM in an air-saturated solution at 30 °C. For high initial glucose concentrations (e.g., 50 mM in our experiments) this consumption is negligible. Therefore, the equation governing the rate of the reaction is only dependent on oxygen concentration variation

$$v_G = V_{mappO}^{GOX} \frac{[O_2]}{K_{mappO}^{GOX} + [O_2]} \quad (6)$$

To determine the values of apparent kinetic constants, oxygen consumption was analyzed at two enzyme concentrations (78 and 156 μg/mL) (**Figure 1**). The initial rates of the reaction were determined and apparent K_{mappO}^{GOX} and V_{mappO}^{GOX} average values (**Table 2**) were 0.20 ± 0.01 mM and 1.41 ± 0.02 μkat/mg, respectively.

Using results reported in **Tables 1** and **2**, the K_m (using eqs 3 and 5) and the V_m^{GOX} (using eqs 2 and 4) values toward glucose and oxygen can be calculated. The K_{mO}^{GOX} value is 0.48 mM (**Table 3**), which is close to those found by Gibson et al. (*13*) and Vanstroebizen et al. (*36*), namely 0.6 and 0.8 mM. The K_{mG}^{GOX} value is 70 mM and V_m^{GOX} = 3.5 ± 0.1 μkat/mg (**Table 3**).

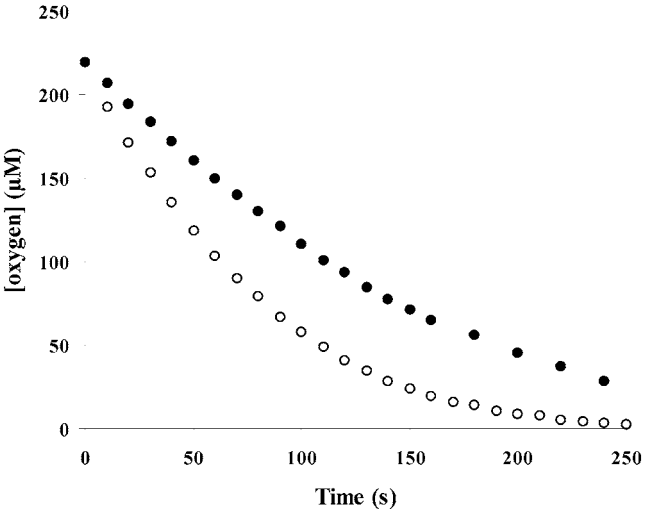

Figure 1. Oxygen consumption by two GOX solutions: 78 μg/mL (●) and 156 μg/mL (○), ([glucose] = 50 mM, total volume = 1 mL, 20 μL of enzyme).

Table 2. K_{mappO}^{GOX} and V_{mappO}^{GOX} for [glucose] = 50 mM and Two GOX Concentrations

	K_{mappO}^{GOX} (mM)	V_{mappO}^{GOX}
E_1 = 0.156 mg/mL	0.21	1.39 μkat/mg
E_2 = 0.078 mg/mL	0.19	1.43 μkat/mg

Table 3: Kinetic Constants of GOX toward Glucose and Oxygen

K_{mO}^{GOX}	K_{mG}^{GOX}	V_m^{GOX} (eq 2)	V_m^{GOX} (eq 4)
0.48 mM	70 mM	3.6 μkat/mg	3.4 μkat/mg

Table 4: Apparent Kinetic Values of HOX for $[O_2]$ = 220 μM

	pH 5.0	pH 5.6
K_{mappG}^{HOX} (mM)	0.6	1
V_{mappG}^{HOX} (nkat/mL)	24	34
$V_{mappG}^{HOX}/K_{mappG}^{HOX}$	40	34

As $CaCl_2$ is an activator of wheat germ POD (*26*), it was added in the GOX−POD mixtures. The addition of $CaCl_2$ from 0 to 30 mM at pH 5.6 did not have any effect on the consumption of oxygen by GOX (results not shown).

Hexose Oxidase Characterization. HOX exhibited a maximum of activity at pH 5.6, and few differences were detected between pH 5.0 and 5.6 (results not shown). The effect of glucose concentration was analyzed at pH 5.0 and 5.6 at one oxygen concentration (220 μM). Affinity toward glucose decreased with increasing pH, but conversely V_m^{HOX} values increased with pH (**Table 4**). The best efficiency was found at pH 5.0, and a loss of only 16% of this value was determined when pH was increased up to 5.6. According to these results, it can be assessed that conditions in dough are not far from optimum for HOX activity (regarding to the glucose concentration). Moreover, HOX can use other sugars as substrates in dough (*5*).

The addition of calcium chloride (from 0 to 30 mM) at pH 5.6 did not have any effect on the consumption of oxygen by HOX (not shown).

Glucose Oxidase−Peroxidase Association. Assuming that the endogenous glucose concentration in dough is approximately

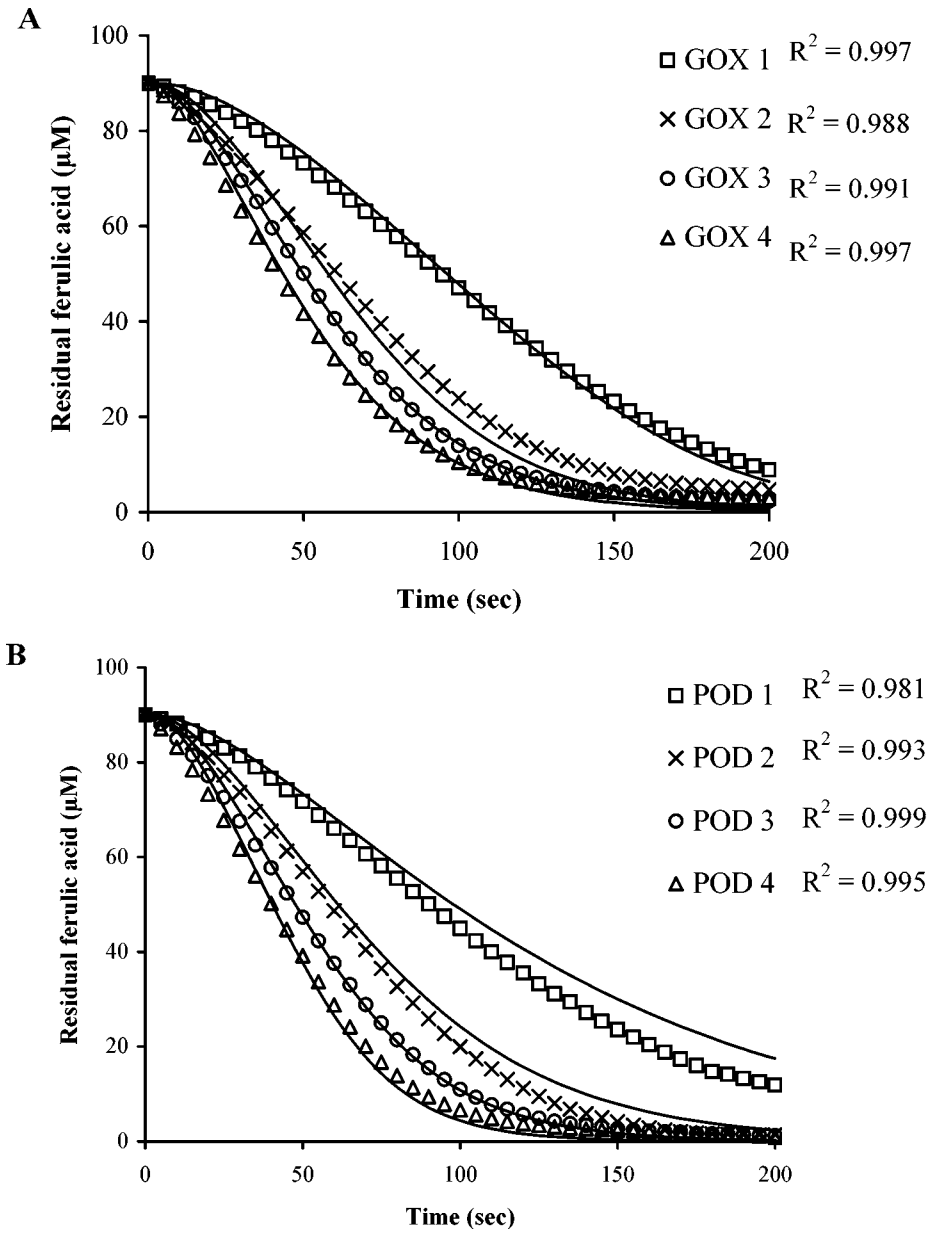

Figure 2. Comparison between experimental (points) and theoretical (lines) data for a GOX–POD association. (**A**) Case of a fixed POD amount (25 μL) and variable GOX amounts. GOX 1 = 2.7 μg; GOX 2 = 5.4 μg; GOX 3 = 8.1 μg; GOX 4 = 10.8 μg. (**B**) Case of a fixed GOX amount (6.7 μg) and variable POD amounts. POD 1 = 10 μL; POD 2 = 20 μL; POD 3 = 30 μL; POD 4 = 40 μL.

4 mM (*35*), the oxidation of FA in GOX−POD systems was analyzed at 2 and 5 mM of glucose. H_2O_2 production by GOX was monitored by ECD−HPLC according to Garcia et al. (*26*). At either glucose concentration, sufficient H_2O_2 was produced to activate POD and oxidize all the FA present in 600 (2 mM glucose) or 400 (5 mM glucose) seconds (data not shown). However, for the purpose of the modeling, glucose concentration was set at 50 mM to have a negligible variation of the reducing substrate concentration during the course of the reaction. FA consumption was analyzed for different GOX/POD ratios. FA consumption was followed by UV-spectrophotometry and confirmed by ECD-HPLC, according to Garcia et al. (*26*).

Incubations were made with FA (90 μM), a fixed POD amount (6.5 nkat) and increasing GOX amounts (**Figure 2A**). Then, the reciprocal experiment was made (**Figure 2B**). In all the experiments, FA consumption rates increased with increased enzyme concentrations and curves were all sigmoidal. This is due to the fact that, at the beginning of the reaction POD activity is equal to zero by a lack of hydrogen peroxide and during the course of the reaction, the rate increases as GOX produces H_2O_2. At the end of the reaction, the rate decreases, because the FA concentration becomes a limiting factor. Maximal rates were measured for each curve and values were plotted against GOX amount (**Figure 3A**) or POD amount (**Figure 3B**). In both cases, a plateau was reached. For a constant POD concentration and increasing amounts of GOX, the plateau value was lower than the control (same amount of POD in the presence of FA and 500 μM H_2O_2). The oxygen content of the medium obviously limited the production of H_2O_2 by GOX to 220 μM. For the same reason, when GOX concentration was constant, the rates were always slower than the rates in the control.

Mathematical Modeling of GOX−POD Association. In a first step, the equation describing the evolutions of oxygen and hydrogen peroxide relative to GOX activity was established. Oxygen consumption by GOX in a medium containing 50 mM glucose follows a Michaelian kinetic with one substrate, in

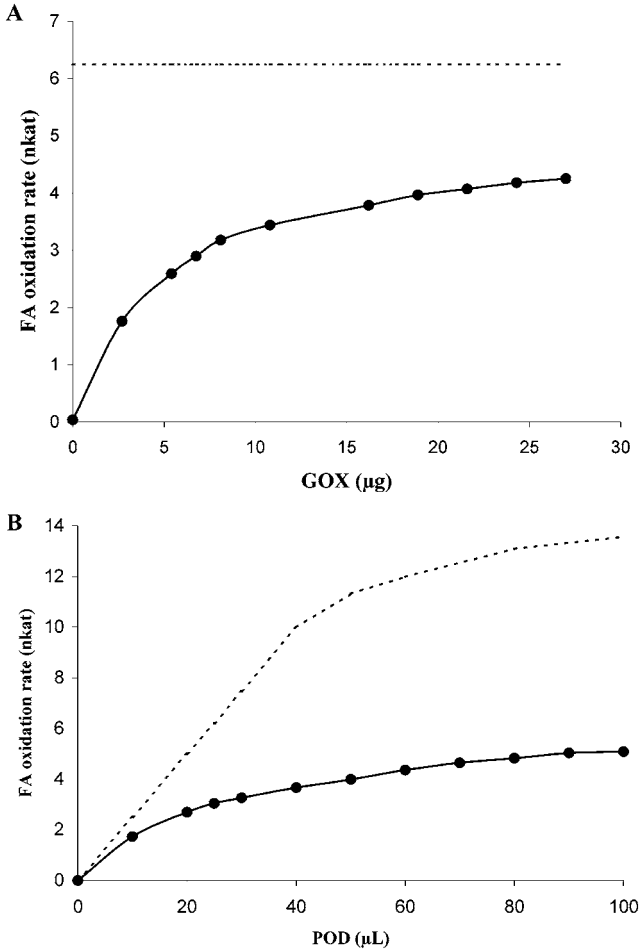

Figure 3. Evolution of FA oxidation rates in a GOX–POD mixture: - - -, control (500 μM H_2O_2 + POD), —●— GOX + POD. (**A**) For increasing GOX amounts (POD = 25 μL). (**B**) For increasing POD amounts (GOX = 6.7 μg).

which oxygen is limitant (see above). The equation is

$$v_t^{GOX} = -\frac{[O_2]_t - [O_2]_{t-1}}{dt} = V_{mappO}^{GOX}\frac{[O_2]_{t-1}}{[O_2]_{t-1} + K_{mappO}^{GOX}} \quad (7)$$

where v_t^{GOX} is the GOX oxygen consumption rate at time t.

The oxygen content in the medium at time t ($[O_2]_t$) can be calculated from the oxygen content at time $t-1$ ($[O_2]_{t-1}$), using the equation

$$[O_2]_t = [O_2]_{t-1} - V_{mappO}^{GOX}\frac{[O_2]_{t-1}}{[O_2]_{t-1} + K_{mappO}^{GOX}}dt = [O_2]_{t-1} - v_t^{GOX}dt \quad (8)$$

As the stoichiometry of the reaction is one mole of oxygen consumed for one mole of hydrogen peroxide formed, the hydrogen peroxide production is equivalent to oxygen consumption, so the H_2O_2 content in the medium at time t is

$$[H_2O_2]_t = [H_2O_2]_{t-1} + V_{mappO}^{GOX}\frac{[O_2]_{t-1}}{[O_2]_t + K_{mappO}^{GOX}}dt = [H_2O_2]_{t-1} + v_t^{GOX}dt \quad (9)$$

In a second step, the equation describing the evolution of substrates (FA and H_2O_2) relative to POD activity was established. POD kinetics are Michaelian type with a ping-pong mechanism, and kinetic constants were previously determined (*26*)

$$v_t^{POD} = \frac{[FA]_t - [FA]_{t-1}}{dt} = V_m^{POD}\frac{[H_2O_2]_{t-1}[FA]_{t-1}}{K_{mFA}[H_2O_2]_{t-1} + K_{mH_2O_2}[FA]_{t-1} + [H_2O_2]_{t-1}[FA]_{t-1}} \quad (10)$$

where v_t^{POD} is the FA consumption rate by POD at time t, and with $K_{mFA} = 1.5$ mM, $K_{mH_2O_2} = 1.2$ mM, and $V_m^{POD} = 5.4$ μkat/mL.

Ferulic acid content at time t ($[FA]_t$) can be determined using contents at time $t-1$ ($[FA]_{t-1}$ and $[H_2O_2]_{t-1}$) and according to the relationship

$$[FA]_t = [FA]_{t-1} - V_m^{POD}dt\frac{[H_2O_2]_{t-1}[FA]_{t-1}}{K_{mFA}[H_2O_2]_{t-1} + K_{mH_2O_2}[FA]_{t-1} + [H_2O_2]_{t-1}[FA]_{t-1}} = [FA]_{t-1} - v_t^{POD}dt \quad (11)$$

The stoichiometry is two moles FA for one mole H_2O_2 and it is assumed that there is no inhibition by any of the substrates. Likewise, peroxidase-catalyzed consumption of H_2O_2 can be calculated using content at time $t-1$ ($[H_2O_2]_{t-1}$) and the equation

$$[H_2O_2]_t = [H_2O_2]_{t-1} - \frac{1}{2}V_m^{POD}dt \times \frac{[H_2O_2]_{t-1}[FA]_{t-1}}{K_{mFA}[H_2O_2]_{t-1} + K_{mH_2O_2}[FA]_{t-1} + [H_2O_2]_{t-1}[FA]_{t-1}} = [H_2O_2]_{t-1} - \frac{1}{2}v_t^{POD}dt \quad (12)$$

When both GOX and POD are in the medium, the H_2O_2 concentration at time t can be calculated from the value of H_2O_2 content at time $t-1$ ($[H_2O_2]_{t-1}$) and the sum of H_2O_2 produced by GOX (v_t^{GOX}) and H_2O_2 consumed by POD ($^1/_2$ v_t^{POD})

$$[H_2O_2]_t = [H_2O_2]_{t-1} + v_t^{GOX}dt - \frac{1}{2}v_t^{POD}dt \quad (13)$$

The different equations (11−13) allow the calculation of oxygen, H_2O_2, and FA concentrations during the course of the reaction.

Comparison Between Model and Experimental Data. Equations 11−13 were used for the modeling of FA oxidation under different experimental conditions.

Two sets of experiments were carried out. First, a fixed GOX amount (6.7 μg) was associated with increasing POD amounts (10−100 μL). Then, a fixed POD amount (25 μL) was associated with increasing GOX amounts (2.7−27 μg).

For each experiment, V_m values for GOX and POD needed to be calculated.

In the case of GOX, values needed for the calculation of V_{mapp}^{GOX} (in μkat for *1 liter of reaction solution*) were K_{mappO}^{GOX}, V_{mappO}^{GOX} (in μkat for *1 mg of enzyme powder*) (both in **Table 2**), the enzyme amount (in μg) and the glucose concentration (50 mM).

Table 5. POD and GOX Amounts and Corresponding V_m Values Used for Modeling the GOX–POD Association

POD amount		GOX amount		V_m^{POD}	V_{mapp}^{GOX}
μL	(nkat)	μg	(nkat)	(μkat/L of rxn soln)[a]	(μkat/L of rxn soln)[b]
25	6.5	2.7	2.05	45	1.3
		5.4	4.1	45	2.6
		7.1	6.15	45	3.9
		10.8	8.2	45	5.2
10	2.6	6.7	5.1	18	3.25
20	5.2			36	3.25
30	7.8			54	3.25
40	10.4			72	3.25

[a] Calculated using $V_m^{POD} = 5.4$ μkat/mL of enzyme [b] Calculated for $V_m^{GOX} = 3.5$ μkat/mg (**Table 3**) and using eq 4.

In the case of POD, values needed for the calculation of V_m^{POD} (in μkat for *1 liter of reaction solution*) were the volume of enzyme used (in μL) and V_m^{POD} (in μkat for *1 mL of enzyme solution*). Kinetic constants previously determined for POD (*26*) were used: $K_{mFA} = 1.5$ mM, $K_{mH_2O_2} = 1.2$ mM.

Calculated values of V_m^{POD} and V_{mapp}^{GOX} used for the modeling are recorded **Table 5**.

The modeling of FA oxidation for each GOX−POD association was compared with the experimental data (**Figure 2**, parts **A** and **B**). A good correlation was found for all the associations (R^2 average values are 0.992), but some gaps appear at the end of the reaction. An inhibition due to an excess of one of the substrates when the other one is at low concentration (as it often happens for peroxidase) may be responsible for this anomaly, and this has not been integrated in the mathematical model. Another possibility may be that stoichiometry variation occurs, due to the oxidation of other substrates (e.g., FA dimers) by POD during the course of the reaction (*37*). Finally, as our system is not totally hermetic, external oxygen supplies ingress, especially for high GOX amounts or for periods after 60 s. Under these conditions calculated curves under-estimate FA consumption because H_2O_2 production by GOX was underestimated. In effect, with external supplies, oxygen concentration available could be more important than the concentration used for the model.

Nevertheless, the modeling of FA evolution appears possible (using equations 11,−13) in a GOX−POD system, at least in the experimental field studied.

Ascorbic Acid Effect on GOX−POD Association. The effect of ascorbic acid (AA) was determined by UV-spectrophotometry and ECD-HPLC. This compound delays the oxidation of FA and HPLC data confirm that during the lag period, AA and H_2O_2 are the only species consumed (not shown). When all AA is consumed, FA consumption begins. This result can be explained by a coupled oxidation mechanism, as previously described in the case of FA oxidation by the system wheat POD/H_2O_2 (*26*). AA is rapidly oxidized into dehydroascorbic acid by the phenoxy radicals produced by POD in the presence of H_2O_2 leading to an immediate regeneration of FA.

This result has a consequence in baking technology. It is generally accepted that the improving effect of GOX is due to the H_2O_2 produced, which can then be used by POD to gel pentosans. It seems that the use of formulations containing both AA and GOX is not judicious, as AA will delay the effect of POD.

Hexose-Oxidase−Peroxidase Association. *Comparison with GOX−POD Association*. To compare the activation of POD by the H_2O_2 produced by GOX or HOX, the amounts of each enzyme necessary to obtain the three initial oxygen consumption velocities (2.6, 1.5, and 0.42 nkat) were determined for three glucose concentrations, namely 2, 5, and 50 mM (**Table 6**).

Table 6. HOX and GOX Amounts Necessary to Obtain the Same Initial Oxygen Consumption Velocities

[glucose] used for the assay (mM)	GOX amount (μg)	HOX volume (μL)	equivalent activity (nkat)
50	3.6	78	2.6
5	7.1	55	1.5
2	5	20	0.42

Table 7. Time Necessary to Consume 45 μM of FA ($t_{1/2}$) for Each GOX–POD or HOX–POD Association at Different Glucose Concentrations

	$[glucose]_{initial}$ (mM)	GOX amount (μg)	HOX volume (μL)	$t_{1/2}$ for GOX–POD (sec)	$t_{1/2}$ for HOX–POD (sec)
1	50	3.6	78	74	55
2	5	3.6	78	164	60
3	2	3.6	78	286	67
4	5	7.1	55	106	71
5	2	5	20	246	157

A decrease in glucose concentration and the initial oxygen uptake velocity has different effects on HOX and GOX: GOX amounts need to be increased, whereas HOX volumes need to decreased. This can be easily explained by the kinetic constants of each enzyme; HOX has much more affinity toward glucose than GOX has (**Table 1** and **Table 4**).

A set of experiments were then carried out to compare the GOX−POD and the HOX−POD associations. Several amounts of GOX or HOX were associated with a fixed amount of POD (6.5 nkat), and the oxidation of ferulic acid was followed by the absorbance decrease at 310 nm. **Table 7** shows the time ($t_{1/2}$) necessary to consume half of the initial amount of FA present in the mixtures (45 μM, equivalent to a decrease of 0.4 absorbance units at 310 nm) for different glucose concentrations.

The $t_{1/2}$ were always shorter for HOX than for GOX, indicating that HOX was the most efficient one. A decrease of glucose concentration from 50 to 2 mM had much more effect on GOX than on HOX activity (comparison of lines 1 and 3, **Table 7**): $t_{1/2}$ were lengthened 4 times for GOX−POD association versus 1.2 times for HOX−POD association. This could easily be explained by the fact that the H_2O_2 production rate was decreased 9 times for GOX, whereas it was only 1.5 times lower for HOX. Again, this effect can be attributed to the differences in affinity toward glucose for the two enzymes.

Finally, experiments were conducted with the same glucose concentration (2 or 5 mM) but with adapted amounts of GOX and HOX to obtain in both cases the same H_2O_2 production rate (lines 4 and 5, **Table 7**). HOX was still more efficient compared to GOX as $t_{1/2}$ values were always lower in the case of HOX−POD association.

A comparison of the oxygen uptake by GOX and HOX showed that if the initial rate is equivalent for both enzymes, the oxygen consumption slowed more rapidly with GOX than with HOX (**Figure 4**). This phenomenon is not due to an inhibition by hydrogen peroxide since both enzyme kinetics are not affected by H_2O_2 in the concentration range of this study (not shown); therefore, it can be assumed that the affinity for oxygen is higher for HOX than GOX.

Conclusions. Wheat peroxidase in association with GOX or HOX is able to oxidize FA. The hydrogen peroxide produced

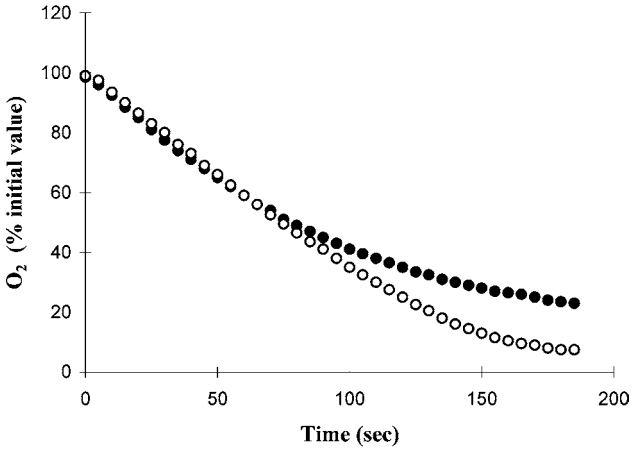

Figure 4. Comparison of the oxygen consumption by 2.6 nkat of GOX (●) or HOX (○), ([glucose] = 50 mM).

by GOX or HOX can activate POD (even for low glucose concentrations). Accordingly, part of the effect of GOX in bread making can be attributed to endogenous POD activation as suggested by Vemulapalli et al. (*16*) and Ameille et al. (*18*). Likewise, part of the effect of HOX in bread making could be attributed to POD-catalyzed pentosans gelation as proposed by Poulsen and Bak Hostrup (*5*).

A mathematical model for the GOX−POD association in oxidizing FA is proposed, which describes the evolution of the different reactants (H_2O_2, FA, and oxygen) during the course of the reaction. A good correlation with experimental data validated the model for the experimental field studied.

ABBREVIATIONS USED

AA, ascorbic acid; ECD, electrochemical detector; FA, ferulic acid; GOX, glucose oxidase; HOX, hexose oxidase; HRP, horseradish peroxidase; POD, peroxidase; SH, sulfydryl group; S−S, disulfide bridges

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