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Oxidation of Ferulic Acid or Arabinose-Esterified Ferulic Acid by Wheat Germ Peroxidase

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The oxidation of ferulic acid (FA) or 5-O-(*trans*-feruloyl)-L-arabinose (EFA) by a purified wheat germ peroxidase was followed by UV spectrophotometry and high-performance liquid chromatography using an electrochemical detection. Wheat peroxidase (POD) exhibits a ping-pong bireactant mechanism forming phenoxy radicals more rapidly from FA than from EFA in routine assay conditions. When both the free and the esterified forms of FA are present, the reverse was found. This result could be due to a nonenzymatic cooxidation of FA by the phenoxy radicals of EFA leading to the formation of phenoxy radicals of FA and the EFA regeneration. Addition of ascorbic acid (AA) provokes a delay of FA consumption. AA reduced very rapidly the phenoxy radicals formed by POD back to initial phenol avoiding the formation of ferulate dimers until it was completely oxidized in dehydroascorbic acid. Conversely, cysteine addition slowed but did not delay the FA consumption. The thiol reduced a fraction of the phenoxy radicals produced by wheat POD and was oxidized into cystine, while the other part of phenoxy radicals formed ferulate dimers. These results could be of interest to understand the POD effect on the wheat dough rheological properties.

KEYWORDS: Ferulic acid; peroxidase; wheat; ascorbic acid; cysteine

INTRODUCTION

Peroxidase (POD, EC 1.11.1.7) is able to catalyze different types of reactions including peroxidatic reactions, oxidatic reactions, and hydroxylations (*1*, *2*). Peroxidatic reaction occurs when phenolic compounds are the hydrogen donors (AH_2) and when hydrogen peroxide (H_2O_2) is present. According to Whitaker (*1*), the oxidatic and hydroxylation reactions, which are much slower than the peroxidatic reactions, are a result of the formation of the phenoxy radicals AH* in the medium in the presence of oxygen (oxidation) and monophenols (hydroxylation). The kinetics of peroxidation is of Michaelian type with a ping-pong mechanism (*3*, *4*). The effects of POD in breadmaking have been described by several authors. Gélinas et al. (*5*) showed that supplementation of wheat flour with POD and linoleic acid or with POD and lipase led to the bleaching of dough. They demonstrated that in liquid media containing β-carotene, addition of POD and linoleic acid increased oxidation of this pigment. Similarly, Kieffer et al. (*6*) reported that wheat flours with poor baking properties can be improved by addition of horseradish peroxidase (HRP), which results in bread with increased volume. This supplementation led also to a bleaching of the crumb and a modification of the bread flavor. According to Nicolas and Drapron (*7*), this may be due to the enhancemnt of lipid oxidation either by its hematinic group or by consuming H_2O_2, which is an inhibitor of lipoxygenase. By this way, similar to that of lipoxygenase, POD enhances the polymerization of proteins and then modifies the dough properties. Moreover, according to studies in model systems, it has been proposed that POD can directly promote in dough the oxidative gelation of pentosans (*8*−*10*) and the polymerization of proteins (*11*−*14*). In these cases, the sulfhydryl and the tyrosyl groups of the proteins as well as the ferulic acid (FA) present in the pentosans can be used as substrates by POD. The mechanism by which POD can have these effects remains unclear because the occurrence of H_2O_2 has never been proven in dough although according to Liao et al. (*15*), H_2O_2 can be formed by yeast during fermentation. Conversely, H_2O_2 is undoubtedly formed during mixing after addition of glucose oxidase (GOX) to the dough. This enzyme catalyzes the oxidation of glucose in the presence of molecular oxygen and produces H_2O_2 (*1*). During breadmaking, GOX supplementation led to changes in the structural properties of the dough affecting its consistency and enhancing the bread volume (*16*−*20*). The improving effect of the addition of GOX to wheat flour can be explained by activation of endogenous POD by the H_2O_2 produced, which could lead to the oxidation of the protein SH into disulfide bridges and/or the gelative oxidation of pentosans. In recent papers, we reported that the wheat flour POD activity remained remarkably stable during mixing, even in the presence of GOX or its substrates (*21*, *22*). According to Icard-Vernière

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and Feillet (*23*), this stability could be related to its well-known thermoresistance.

Wheat POD has been studied and purified by different authors. Several isoenzymes were purified from durum wheat (*24*, *25*) and from wheat germ (*26*, *27*). Billaud et al. (*27*) purified both cationic and neutral/anionic enzymatic fractions. They reported that the cationic isoenzymes represent more than 95% of the PODs from wheat germ (*Triticum aestivum* L.). However, their kinetic characterization has been made with guaiacol, which is not present in wheat flour.

Among the phenolic compounds found in wheat flour, FA is largely predominant (*28*). Although present in a free form, it is mainly esterified to arabinose in the pentosan fraction in the range of 0.3−0.5 μmol/g of flour (*29*, *30*). It is also esterified to low molecular weight compounds such as stanol and sterol (*31*) and glucose (*32*, *33*) but to a much lesser extent. Obviously, when oxidized by POD, FA is involved in the oxidative gelation of pentosans (*34*, *35*). In addition, it has been suggested that FA either free or esterified to low molecular weight compounds could react with protein sulhydryl groups (*36*, *37*) or increase the rate of protein sulfhydryl−disulfide interchange (*38*) responsible for the dough breakdown phenomenon.

Figueroa-Espinoza (*10*, *39*, *40*) has shown in model systems that HRP (in the presence of H_2O_2), fungal laccase, or manganese POD are able to oxidize FA either free or esterified to pentosans. In the latter case, these enzymes promote the oxidative gelation of these macromolecules. These authors observed that cysteine (CSH) and glutathione hindered the oxidative gelation (*39*). Recently, Labat et al. (*38*, *41*) have shown that the oxidation of FA either free or esterified is accelerated by laccase and manganese POD during mixing of wheat flour dough or of a mix of wheat gluten and pentosans. Although several authors have recently studied the effect of plant PODs on FA and on the structure of the diferulate (*42*−*47*), to our knowledge, the effect of a POD extract from wheat flour has never been studied with this substrate. Therefore, the aim of this paper is to study the oxidation of FA and 5-O-(*trans*-feruloyl)-L-arabinose (EFA) by the H_2O_2/wheat POD system. FA esterified by 1 mol of arabinose does not exist in the flour, but it can be considered as a good intermediate between FA and wheat arabinoxylans. For this purpose, the main cationic wheat POD has been purified from the wheat germ fraction. Our first aim was to develop a simple UV spectrophotometric method for the assay of wheat POD activity on FA since it is the most abundant hydroxycinnamic acid in wheat flour (*28*). In a second step, a high-performance liquid chromatography (HPLC) method was developped in order to quantify the evolution of FA (as well as that of EFA and other reactants) during the POD reaction. Relations between spectrophotometric measurements and evolution of substrate have been established and applied to the study of the effects of reductant (thiol and ascorbic acid (AA)) on the oxidation of FA catalyzed by wheat POD.

MATERIALS AND METHODS

Chemicals. H_2O_2 30% (w/w), *m*- and *o*-phosphoric acids were purchased from Prolabo (Paris, France). L-*threo* AA, FA, L-CSH, and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St. Louis). EFA and a mixture of dimers of FA were obtained from maize bran (*48*) and kindly supplied by L. Saulnier (INRA, Laboratoire de Biochimie et Technologie des Glucides, Nantes, France). Peroxidase (FC_1) was purified from wheat germ according to Billaud et al. (*27*).

Plant Material. Industrial wheat germ provided by les Moulins Soufflet (Nogent-sur-Seine, France) was defatted by cold acetone according to Nicolas et al. (*49*).

POD Assay. In routine conditions, the POD activity was measured by a spectrophotometric method based on the decrease in absorbance at 310 nm for FA and 324 nm for EFA using a diode array spectrophotometer (Hewlett-Packard, 8452A). The reaction mixture contained FA or EFA (100 μM) and H_2O_2 (500 μM with FA and 250 μM with EFA) in sodium acetate buffer (0.1 M, pH 5.6). The reaction was started by addition of 80 μL of a FC_1 solution conveniently diluted. The activity was determined by the initial slope from the linear decrease in absorbance at 310 or 324 nm. One unit of activity is defined as a change of 1 absorbance unit (AU)/s for a total volume of 3 mL in the assay conditions. For kinetic studies, the FA or EFA concentrations were varied between 30 and 120 μM and those of H_2O_2 between 35 and 500 μM. All assays were performed at 25 °C in duplicate. Kinetic parameters were determined using nonlinear regression data analysis software (*50*).

Effect of pH and Ca^{2+}. The optimum pH of FC_1 was determined with H_2O_2 (500 μM) and FA (100 μM) in the absence or in the presence of calcium chloride (20 mM) with 0.05 M acetate buffer (pH 4−5), 0.05 M citrate/0.1 M disodium phosphate buffer (pH 5−6), and 0.05 M Tris maleate buffer (pH 6.5−8.5). The reaction was started by addition of 80 μL of a FC_1 solution conveniently diluted. The effect of Ca^{2+} ions upon the POD activity was studied for concentrations ranging from 0 to 40 mM.

Simultaneous Spectrophotometric Measurement and HPLC Analysis. When spectrophotometric measurement and HPLC analysis were carried out simultaneously, the reaction mixtures were prepared as follows: To 2.59 μL of 0.1 M sodium acetate, pH 5.6, buffer solution were added 30 μL of FA (10 mM) and 300 μL of H_2O_2 (5 mM). The reaction was started by addition of 80 μL of a FC_1 solution conveniently diluted. When the effects of AA or CSH were tested, buffer solution contained AA or CSH in concentrations ranging from 0 to 500 μM or 0 to 300 μM, respectively. The evolution of FA or EFA was followed spectrophotometrically and samples (0.1 or 0.2 mL) were periodically withdrawn for analysis by HPLC using an electrochemical detector (ECD). Before injection, these samples were added to 0.5 mL of a stopping solution containing the mobile phase acidified at pH 2.3 with *m*-phosphoric acid (20%).

HPLC-ECD Analysis. A modification of the HPLC-ECD method of Kaïd et al. (*51*) was used to quantify CSH and AA. H_2O_2, FA, and EFA were also quantified by HPLC-ECD. These compounds are simultaneously measured by HPLC using a 9012 pump driven by a 9020 workstation (Varian), a Valco C6W injector with a 10 μL sample loop, and an ECD equipped with a dual glassy carbon working electrode and an Ag/AgC1 reference electrode (Eldec 201 model from Chromatofield, France). Separation was achieved using a 5 μm YMC ODS AQ C_{18} column (250 mm × 4 mm i.d.) (AIT, France). The mobile phase was a mixture of ammonium dihydrogenophosphate (0.1 M) adjusted to a final pH of 2.8 with *o*-phosphoric acid and of acetonitrile (70/30). Samples were run isocratically at a flow rate of 1 mL min^{-1}. The potential of the first working electrode was fixed in oxidation mode at +0.80 V (vs Ag/AgCl reference electrode) for the quantification of FA and AA. It was fixed at +0.90 V for the quantification of EFA. The potential of the second working electrode used for the quantification of CSH and H_2O_2 was fixed in oxidation mode at +1.02 V. The detector response was verified by injection of pure standard solutions containing AA, CSH, H_2O_2, FA, and EFA (30, 20, 50, 20, and 30 μM, respectively) every four samples in order to overcome electrode passivation.

The detectable quantities were 5, 1, 25, 20, and 20 pmol for AA, CSH, H_2O_2, FA, and EFA, respectively.

Disulfide Determination. Cystine (CSSC) produced after reaction with H_2O_2/POD system and FA or EFA was determined as the difference between the total CSH after CSSC reduction and the CSH contents without reduction. The reduction method was adapted from Kaïd et al. (*51*) To 0.5 mL of the sample was added 0.75 mL of DTT solution at 7 mM prepared in 0.2 M Tris buffer (pH 8.2). After 30 min at 0 °C, DTT in excess was extracted three times with 2.5 mL of ethyl acetate. A 0.2 mL amount of the mobile phase was added to 1 mL of the aqueous phase before analysis by HPLC.

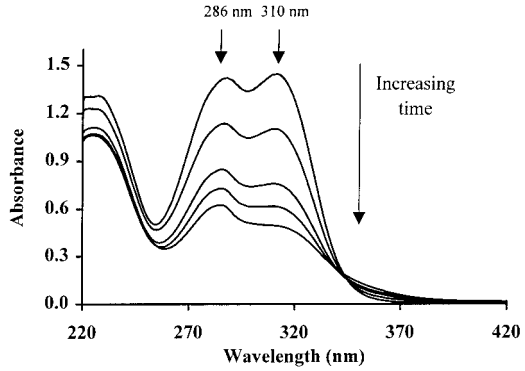

Figure 1. Oxidation of FA catalyzed by FC_1 in the presence of H_2O_2.

RESULTS AND DISCUSSION

In agreement with the results of Billaud et al. (*27*), we found that the four cationic isoforms, FC_1, FC_2, FC_3, and FC_4, represent more than 95% of the total POD activity of the wheat germ. Among these four isoforms, fraction FC_1 accounts for more than half of the total cationic activity. Therefore, the POD FC_1 was retained for the following experiments.

POD Assay with Free and Arabinose-Esterified FAs. The working wavelength was selected after the following of the UV spectrum of FA during its oxidation by wheat POD in the presence of H_2O_2 using a diode array spectrophotometer. The UV spectrum of FA in sodium acetate buffer at pH 5.6 shows two maxima at 286 and 310 m (**Figure 1**). FA oxidation resulted in a general decrease of absorbance in the UV region and a small increase at 360 and 510 nm, but the most important variation is observed at 310 nm. Consequently, for the subsequent measurements, the FA oxidation by the H_2O_2/POD system was followed at 310 nm.

Effect of Ca^{2+} and pH. When $CaCl_2$ was added to the reaction medium, the FA oxidation rate by FC_1 was largely accelerated. Thus, a nearly 25-fold increase in FC_1 activity was observed when the $CaCl_2$ concentration was increased from 0 to 10 mM. Further increase to 30 mM did not change the FC_1 activity.

In the absence of calcium, FC_1 exhibited a maximum of activity between pH 5.5 and pH 6.5 whereas half of the maximum activity was observed at pH values close to 4.9 and 7.8. When $CaCl_2$ (20 mM) was present, the pH activity curve was almost not modified between pH 5.5 and pH 8.5 whereas the activity remained maximum from pH 7 to pH 4. According to Vamos-Vigyazo (*52*) and Robinson (*2*), most plant PODs are activated by calcium ions and exhibited a broad domain of maximum activity between pH 4 and pH 7. More precisely, Billaud et al. (*27*) found a similar effect of Ca^{2+} and pH on the FC_1 activity using guaiacol as hydrogen donor. These results show that FC_1 is potentially fully active in wheat flour dough where the pH is between 5.5 and 6.2 (*6*).

Kinetic Properties of FC_1 toward FA. Kinetic analysis was performed at pH 5.6 with calcium chloride (20 mM). The double reciprocal plots gave a series of parallel lines characteristic of a ping-pong bireactant mechanism (not shown). This behavior is frequent for plant PODs (*3*, *4*). According to Segel (*53*), a secondary plot of $1/V_{mapp}$ axis intercept and $1/K_{mapp}$ vs $1/[H_2O_2]$ obtained for the different constant concentrations of FA allows us to determine the kinetic parameters V_m, $K_{mH_2O_2}$, and K_{mFA} (**Figure 2**). When the same study was performed without calcium chloride, the FA oxidation rates were largely decreased but the pattern of parallel lines was still observed. Therefore, if the kinetic constants are largely modified by the presence of

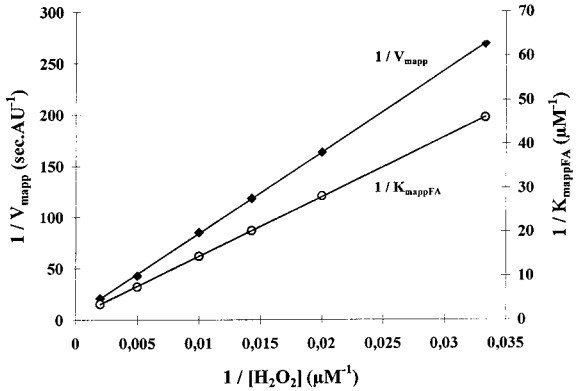

Figure 2. $1/V_{mapp}$ and $1/K_{mappFA}$ replot vs $1/[H_2O_2]$.

Table 1. Effect of Calcium Chloride on the Kinetic Constants of the Wheat POD FC_1 at pH 5.6

	$[CaCl_2] = 0$ mM		$[CaCl_2] = 20$ mM	
	FA	EFA	FA	EFA
K_{mH2O2} (μM)	730	730	1200	1200
K_{mFA} (μM)	580		1500	
K_{mEFA} (μM)		250		500
V_m (AU s^{-1} mL^{-1})	0.48[a]	0.4[b]	13.3[a]	9.9[b]
V_m (nkat mL^{-1})	170[c]	110[d]	4700[c]	2750[d]

[a] Measured at 310 nm and calculated for 1 mL of the FC_1 solution. [b] Measured at 324 nm and calculated for 1 mL of the FC_1 solution. [c] Calculated from V_m expressed in AU s^{-1} mL^{-1} using a $\Delta\epsilon_{310} = 8400$ M^{-1} cm^{-1} (see text). [d] Calculated from V_m expressed in AU s^{-1} mL^{-1} using a $\Delta\epsilon_{324} = 10\,800$ M^{-1} cm^{-1} (see text).

$CaCl_2$, the mechanism of FA oxidation remains the same. The initial rate of the reaction catalyzed by FC_1 can then be calculated using the equation of a ping-pong bireactant mechanism (eq 1):

$$v = V_m \frac{[H_2O_2][FA]}{K_{mFA}[H_2O_2] + K_{mH_2O_2}[FA] + [FA][H_2O_2]} \quad \text{(eq 1)}$$

The values of the different kinetic constants are given in **Table 1**. The V_m value is largely increased by $CaCl_2$ (20 mM), illustrating its activation effect. However, both $K_{mH_2O_2}$ and K_{mFA} are increased when $CaCl_2$ is present, meaning that the affinity of FC_1 for its two substrates decreases in the presence of calcium.

Effect on EFA. A similar study has been carried out with EFA as substrate. The EFA spectrum is similar to that of FA with a bathochromic shift for the two maximas of UV absorbance, which are located at 295 and 324 nm. EFA oxidation also resulted in an overall decrease of absorbance with the most important variation observed at 324 nm wavelength, which was selected for the subsequent experiments performed with EFA.

The addition of increasing amounts of $CaCl_2$ also resulted in a large activation of the EFA oxidation rate by FC_1. However, the plateau was reached at 20 mM of Ca^{2+} instead of 10 mM of Ca^{2+} for FA.

A ping-pong bireactant mechanism was also observed for FC_1 in the presence of EFA and H_2O_2, both in the presence and in the absence of calcium chloride. The kinetic constants are given in **Table 1**. The addition of $CaCl_2$ greatly increases the V_{mEFA} value, which again illustrates its activation effect on the FC_1 activity. Similarly, it decreases the affinity of FC_1 for EFA as shown by the increase of the K_{mEFA} value. When the kinetic constants of FA and EFA are compared, the K_{mFA} values were

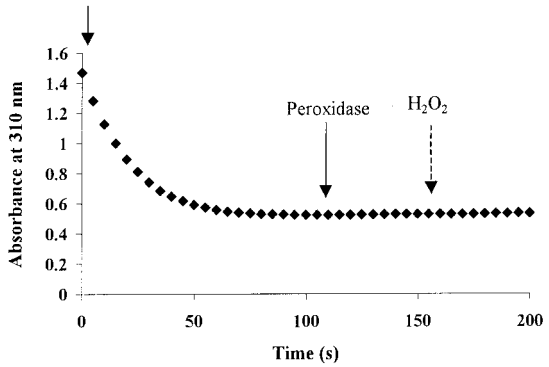

Figure 3. FA oxidation (100 μM) catalyzed by FC_1 in the presence of H_2O_2 (500 μM) and $CaCl_2$ (20 mM). Arrows indicate the FC_1 or H_2O_2 additions.

Table 2. Retention Times of CSH, AA, H_2O_2, FA, and EFA in HPLC-ECD (See Text for the Conditions)

compd	CSH	AA	H_2O_2	FA	EFA
retention time (min)	2.6	2.9	3.2	8.1	4.5

higher than the K_{mEFA} values, both in the presence and in the absence of $CaCl_2$, indicating that FC_1 has a better affinity for the FA esterified by arabinose than for the free acid. A valuable comparison of the V_m data needs a transformation of the absorbance decrease into the mole number of FA (or EFA) consumed. This is one of the purposes of the experiments described in the following paragraphs. The comparison of the V_m values expressed in nkat mL^{-1} shows that the addition of $CaCl_2$ (20 mM) leads to a close to 25-fold activation for both FA and EFA. In addition, the V_m values associated with FA are close to 1.5 and 1.7 times higher for those associated with EFA, in the absence and in the presence of calcium, respectively.

In conclusion, the routine conditions for the spectrophotometric assay of POD activity using FA (or EFA) are the following: FA or EFA (100 μM), H_2O_2 (500 μM for FA and 250 μM for EFA), and $CaCl_2$ (20 mM) in a solution of sodium acetate buffer (0.1 M at pH 5.6). The FA (or EFA) initial concentration, far from the saturation, is dictated by its high absorbance value at 310 (or 324 nm). H_2O_2 concentrations were chosen in order to avoid inhibition by substrate excess. In these conditions, the slope of the absorbance decrease vs time remained proportional to the FC_1 amount until an activity of 25 mUA s^{-1} in a total reaction volume of 3 mL.

HPLC-ECD Determination of Free and Arabinose-Esterified FAs. The HPLC coupled with an electrochemical detection was chosen for the simultaneous quantitation of FA, EFA, H_2O_2, CSH, and AA. In the original method of Kaïd et al. (*51*), CSH, GSH, and AA were separated using ammonium dihydrogenophosphate (0.1 M) adjusted to a final pH of 2.8 as mobile phase. In these conditions, the FA and EFA retention times were higher than 30 min, which is unsuitable for a rapid assay. Addition of acetonitrile in the mobile phase allowed us to accelerate the elution of FA and EFA. The retention times of the compounds of interest, corresponding to the best conditions obtained with 30% of acetonitrile in the mobile phase, are given in **Table 2**. These conditions are convenient provided CSH and AA are not present at the same time in the sample.

Oxidation of Free FA by FC_1 POD. FA oxidation was followed both by spectrophotometry at 310 nm (**Figure 3**) and by HPLC-ECD. As soon as FC_1 was added, absorbance decreased very fast, then decreased more slowly, and reached a plateau different from zero. The absorbance at the plateau was not affected by the initial amount of FC_1 but was proportional to the FA initial amount. Neither addition of POD nor addition of H_2O_2 at the plateau allows the reaction to restart. Therefore, it can be assumed that all of the FA has been consumed at the plateau and that FA oxidation products absorb at 310 nm. This has been confirmed by the HPLC-ECD analyses performed during FA oxidation. At the plateau, all FA was consumed and four peaks of oxidation products were apparent. Among them, the peak corresponding to the 8-5′-benzofurane was predominant. This ferulate dimer was also found to be predominant in wheat flours by Bonnin et al. (*54*) as well as when esters of FA were oxidized by horseradish POD (*10*, *44*, *54*).

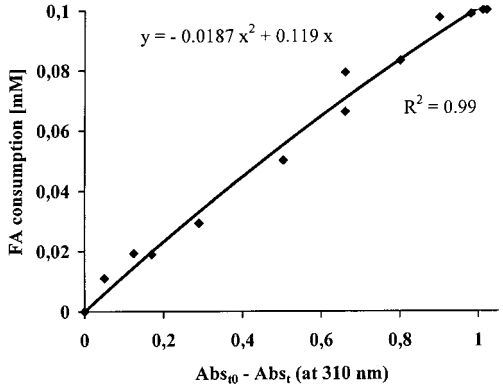

Figure 4. Relation between spectrophotometric and HPLC-ECD measurements.

Several coupled experiments, spectrophotometry, and HPLC-ECD have been carried out with initial FA concentrations ranging from 20 to 100 μM in order to establish the relationship between the absorbance variation at 310 nm and the FA consumption (**Figure 4**). A parabolic curve was obtained, the equation of which is

$$[FA_{cons}] = -0.0187\,(Abs_{t0} - Abs_t)^2 + 0.119\,(Abs_{t0} - Abs_t) \quad R^2 = 0.99 \quad \text{(eq 2)}$$

where $[FA_{cons}]$ and $(Abs_{t0} - Abs_t)$ represent the decreases in FA concentration (mM) and in absorbance at 310 nm during the reaction time t, respectively.

This equation indicates first that in the beginning of the reaction, the absorbance variation associated with a decrease of FA concentration of 1 mM is close to 1/0.119, i.e., that $\Delta\epsilon_{310} = 8400\ M^{-1}\ cm^{-1}$. Recently, Oudgenoeg et al. (*56*) have used a $\Delta\epsilon$ of 700 M^{-1} cm^{-1} at 318 nm to calculate the FA consumption at pH 7.4 by HRP. Then, the initial FA oxidation rates as well as the V_m values expressed in ΔA_{310} s^{-1} can be calculated in nkat (nmol of substrate consumed s^{-1}). Second, it also indicates that for the same absorbance variation, the amount of FA consumed was higher at the beginning than at the end of the reaction. This probably means that as the reaction proceeds, secondary reactions take place, which modify the proportionality between the absorbance variation at 310 nm and the amount of FA consumed.

Different kinetics was performed in the presence of FA (100 μM) and various initial H_2O_2 concentrations (from 50 to 500 μM) in order to determine the stoichiometry (FA_{cons}/H_2O_{2cons}) of the reaction. Both FA and H_2O_2 consumption rates increased as the initial H_2O_2 concentration was increased from 50 to 250 μM (**Figure 5**). A similar result was observed for FA initial concentrations of 20 and 50 μM. Last, almost no change in the

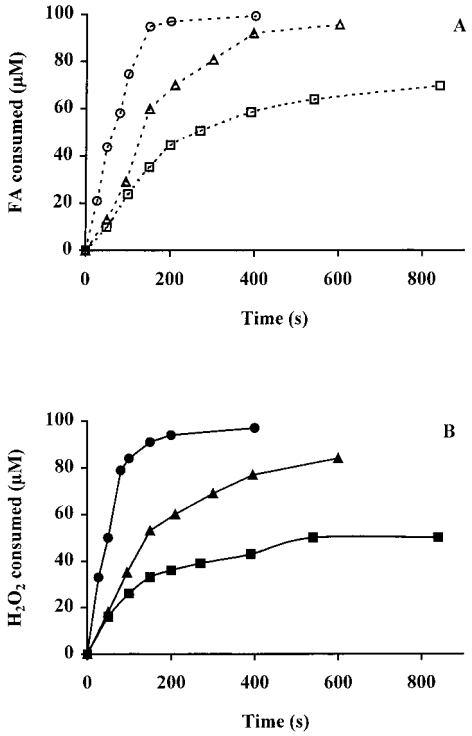

Figure 5. FA and H_2O_2 consumptions by FC_1 POD at different H_2O_2 initial concentrations. (**A**) FA consumed (square $H_2O_2 = 50$ μM; triangle $H_2O_2 = 100$ μM; circle $H_2O_2 = 250$ μM). (**B**) H_2O_2 consumed (square $H_2O_2 = 50$ μM; triangle $H_2O_2 = 100$ μM; circle $H_2O_2 = 250$ μM).

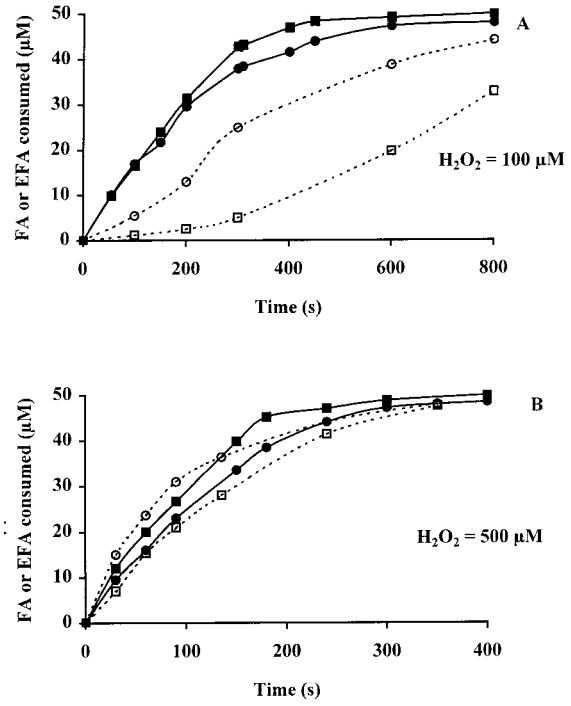

Figure 6. Oxidation of FA and EFA either alone or together catalyzed by FC_1 POD. (**A**) $H_2O_2 = 100$ μM; each phenol = 50 μM. (**B**) $H_2O_2 = 500$ μM; each phenol = 50 μM. Full symbols: FA (circle) or EFA (square) alone. Open symbols: FA (circle) and EFA (square) both present.

oxidation rates was observed when the initial H_2O_2 concentration was increased from 250 to 500 μM.

The observed stoichiometry (FA_{cons}/H_2O_{2cons}) is not constant during these experiments. The calculated values were clearly less than one at the beginning of the reaction and increased to one at the end. Theoretically, the stoichiometry should be two since two phenoxy radicals are needed to react with 1 mol of H_2O_2 to form 1 mol of dimer and 1 mol of H_2O (*55*, *57*). These variations could be explained by secondary reactions involving FA, phenoxy radicals (generated by POD), and O_2 from the one hand or involving dimers and H_2O_2 on the other hand (*1*, *55*). The latter reactions are probable. Thus, for high H_2O_2 concentrations, we have observed that the dimers accumulated rapidly until FA was totally consumed and then decreased concomitantly with a slow consumption of H_2O_2 (not shown).

Oxidation of EFA by FC_1 POD. Similar coupled experiments (spectrophotometry at 324 nm and HPLC-ECD) were carried out with EFA for initial concentrations ranging from 20 to 100 μM. Again, the final absorbance value did not reach zero whereas all EFA was consumed. The relationship established between the absorbance variation at 324 nm and the EFA consumption is also parabolic and the equation is

$$[EFA_{cons}] = -0.0042\,(Abs_{t0} - Abs_t)^2 + 0.0926\,(Abs_{t0} - Abs_t) \quad R^2 = 0.99 \quad \text{(eq 3)}$$

where $[EFA_{cons}]$ and $(Abs_{t0} - Abs_t)$ represent the decreases in EFA concentration (mM) and in absorbance at 324 nm during the reaction time t, respectively. Similarly to FA, eq 3 indicates that in the beginning of the reaction, the absorbance variation associated with a decrease of the EFA concentration of 1 mM is close to 1/0.0926, i.e., that $\Delta\epsilon_{324} = 10\,800$ M^{-1} cm^{-1}. Then, the initial EFA oxidation rates as well as the V_m values expressed in ΔA_{324} s^{-1} can be calculated in nkat (nmol of substrate consumed s^{-1}). Second, it also indicates that for the same absorbance variation, the amount of EFA consumed was higher at the beginning than at the end of the reaction.

Oxidation of FA and EFA by FC_1 POD. The oxidation of FA and EFA, when both compounds are present in the medium at the same concentration (50 μM), has been followed for two H_2O_2 concentrations, 100 μM (**Figure 6a**) and 500 μM (**Figure 6b**). Control experiments with each phenolic (50 μM) taken separately were also carried out in the same conditions. When H_2O_2 was limitant (100 μM), the oxidation rates of FA and EFA taken separately were almost the same. When the H_2O_2 initial concentration was increased to 500 μM, the oxidation rates were largely increased, +44% for FA and +80% for EFA. This result can be explained with a theoretical calculation of oxidation rates using eq 1. Thus, for a constant FA (or EFA) concentration, eq 1 can be transformed into

$$v = V_{mapp} \times \frac{[H_2O_2]}{K_{mH_2O_2app} + [H_2O_2]} \quad \text{(eq 4)}$$

where

$$V_{mapp} = \frac{V_m}{\left(\frac{K_{mFA}}{[FA]} + 1\right)} \text{ and } K_{mH_2O_2app} = \frac{K_{mH_2O_2}}{\left(\frac{K_{mFA}}{[FA]} + 1\right)}$$

Using the K_m and V_m values given in **Table 1** for FA, EFA, and H_2O_2 and eq 4, the ≪ apparent ≫ K_m and V_m and the oxidation rates were calculated for both FA and EFA (**Table 3**). Oxidation rates were almost identical when H_2O_2 was limitant (100 μM), and they increased when H_2O_2 was in excess (500 μM). The increases found by calculation correspond to those obtained experimentally.

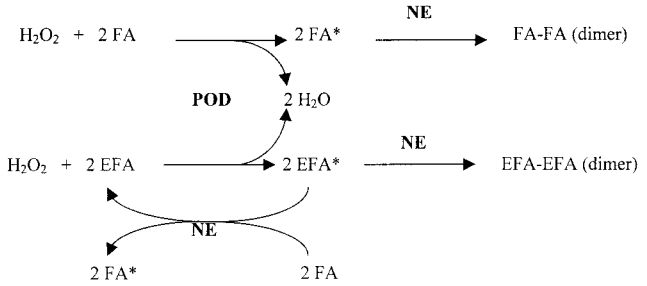

Figure 7. Hypothetical scheme of oxidation of FA and EFA by POD. FA* and EFA* represent the corresponding phenoxy radicals produced by POD. NE = Nonenzymatic reactions.

Table 3. Apparent Kinetic Constants of FC_1 Calculated for a Constant Concentration of FA and EFA (50 μM) and Theoretical Oxidation Rates for Two H_2O_2 Concentrations (100 and 500 μM)

	FA (50 μM)	EFA (50 μM)
$K_{mappH2O2}$ (μM)	58	122
V_{mapp} (nkat mL^{-1})	13.5	18.5
oxidation rate at H_2O_2 = 100 μM (nkat mL^{-1})	8.55	8.35
oxidation rate at H_2O_2 = 500 μM (nkat mL^{-1})	12.1	14.9
increase of oxidation rate between 100 and 500 μM (%)	+42%	+78.4%

When the two forms of FA are in the medium, FC_1 oxidizes more rapidly FA than EFA (**Figure 6**). When H_2O_2 is limitant (100 μM), the two forms are consumed more slowly in the mixture than when they are present separately. The decrease in oxidation rate was more marked for EFA than for FA. This is obviously due to the K_{mapp} for H_2O_2, which is higher for EFA than for FA (**Table 3**).

When H_2O_2 is in excess (500 μM), EFA was still oxidized more slowly in the mixture than alone, but surprisingly enough, FA is oxidized more quickly in the mixture than alone. To explain this later result, a coupled oxidation mechanism can be proposed, similar to those observed for the enzymatic oxidation of a mixture of phenolic compounds by polyphenoloxidase from grape (*58*, *59*) and apple (*60*). EFA phenoxy radicals (EFA*), formed by FC_1, could react with FA in order to produce FA phenoxy radicals (FA*) and regenerate original EFA (**Figure 7**). In these conditions, oxidation rate of EFA decreases because of its regeneration. Conversely, oxidation rate of FA increases because this compound is consumed by two reactions (reactions 1 and 2 in **Figure 7**).

Effect of AA and CSH on the Oxidation of FA by the FC_1 POD. The effects of AA and CSH have been studied because these compounds are commonly used as additives in wheat flour doughs. Thus, AA is a worldwide improver added to improve the dough strength and consequently the bread volume (*61*, *62*). Similarly, CSH, which is naturally present in wheat flour at a concentration range from 8 to 22 nmol g^{-1} (*63*, *64*), is used to modify the rheological properties of biscuit and bread doughs (*65*−*67*).

As already found for other PODs (*42*, *68*), in the presence of AA, a lag period was apparent before any change in absorbance at 310 nm (**Figure 8a**) whereas the decrease of absorbance at 266 nm was immediate. In our conditions (H_2O_2 = 500 μM), the duration of the lag period was almost proportional to the AA concentation until 200 μM and then increased rapidly to become infinite as the AA concentration was increased to 500 μM. Similar results were found with EFA. Similarly to the effect

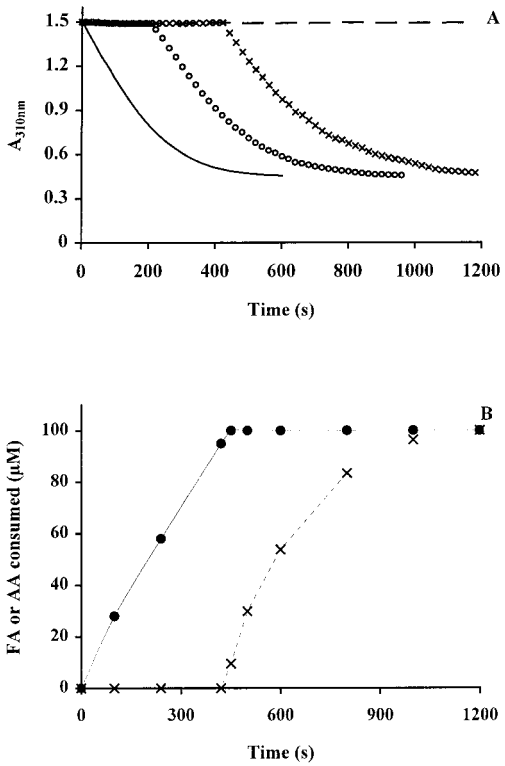

Figure 8. Effect of AA on the FA oxidation catalyzed by FC_1 POD. (**A**) Spectrophotometric measurement at 310 nm, without AA (full line), with 50 (circle), 100 (cross), or 500 μM (tiretted line). (**B**) HPLC-ECD measurement of FA (cross) and AA (circle) consumptions, with FA (100 μM), H_2O_2 (500 μM), and AA (100 μM).

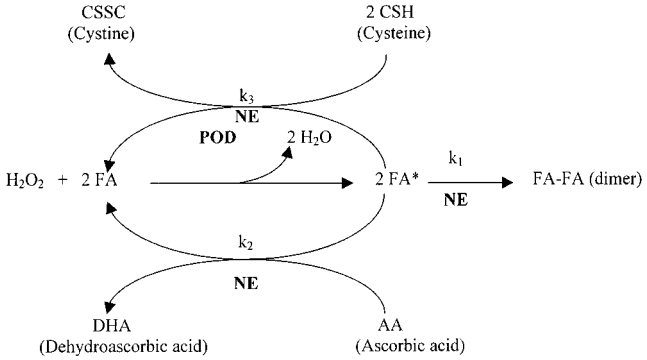

Figure 9. Hypothetical scheme of the effect of AA and CSH on the oxidation of FA catalyzed by POD. FA* represents the phenoxy radicals produced by POD. $k_2 \gg k_3$ and k_1. NE = Nonenzymatic reactions.

of AA on the *o*-quinones produced during the oxidation of *o*-diphenols by polyphenoloxidases in the presence of oxygen (*69*−*71*), this result can be explained by a coupled oxidation mechanism (**Figure 9**). AA is rapidly oxidized into dehydroascorbic acid (decrease of the absorbance at 266 nm) by the phenoxy radicals produced by FC_1 in the presence of H_2O_2 leading to an immediate regeneration of FA (no change in absorbance at 310 nm). HPLC-ECD analyses confirmed this explanation since, during the lag phase, both AA and H_2O_2 were consumed whereas FA remained unchanged (**Figure 8b**). The FA consumption began at the end of the lag period when all of the AA had disappeared.

This means that in our conditions, the rate constant k_2 of the reaction between AA and the phenoxy radicals is much higher than the rate constant k_1 of the phenoxy radicals dimerization (**Figure 9**). This coupled oxidation mechanism explains the

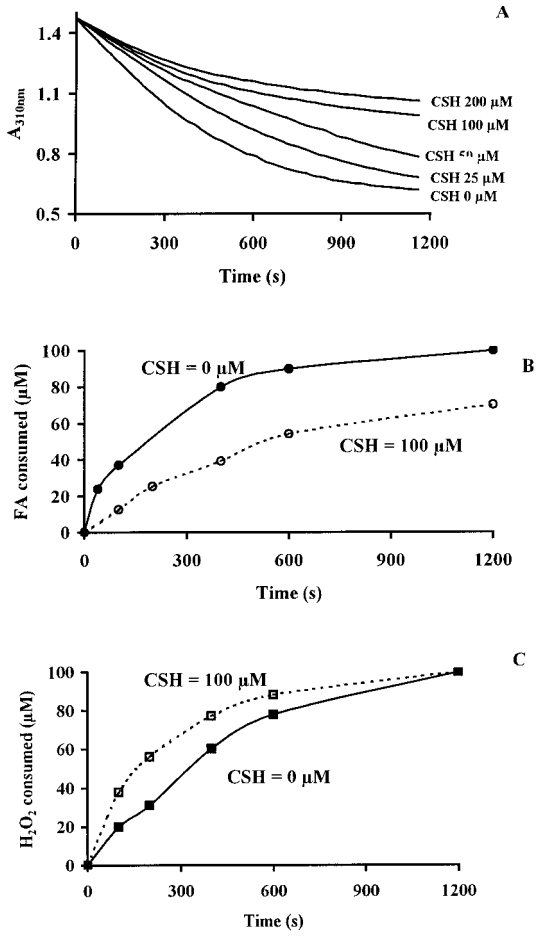

Figure 10. Effect of CSH on the ferulic oxidation catalyzed by FC_1 POD. (**A**) Spectrophotometric measurement at 310 nm. (**B**) HPLC-ECD measurement of FA consumption. (**C**) HPLC measurement of H_2O_2 consumption. FA (100 μM), H_2O_2 (100 μM), and the indicated concentration of CSH.

findings of Vinkx et al. (*72*) who observed that addition of AA delayed the oxidative gelation of rye arabinoxylans catalyzed by HRP in the presence of H_2O_2. To explain their results, these authors proposed that FA and AA compete for H_2O_2. With the FC_1 POD, the competition cannot be proposed since we have observed that this enzyme was unable to oxidize AA in the presence of H_2O_2. From our results, it can be also inferred that AA addition in wheat dough, if it provokes an increasing formation of disulfide bonds (*62*), may also delay the oxidative gelation of pentosanes.

Conversely to the effect of AA, addition of CSH did not provoke a lag time but resulted in a slow of the decrease in absorbance at 310 nm and an increase of the final absorbance value at 310 nm (**Figure 10a**). The higher the amount of added CSH was, the slower the decrease rate at 310 nm was.

HPLC-ECD measurements showed that in the presence of CSH, FA consumption began immediately together with the formation of dimers but less rapidly than in the control experiment without CSH (**Figure 10b**). Concomitantly, the CSH consumption increased with time (not shown) and as compared to the control experiment, H_2O_2 consumption is increased (**Figure 10c**). Whatever the reaction time was, when samples of the reaction medium were treated by DTT in order to reduce all of the disulfide bridges, CSH was totally recovered. In the absence of FA, control experiments have shown that the FC_1 POD did not oxidize CSH in the presence of H_2O_2.

These results are partly in agreement with the findings of Figueroa-Espinoza et al. (*39*) on the gelation of feruloylated water extractable pentosans catalyzed by laccase or the H_2O_2/HRP system. They indicated that the addition of CSH (or glutathione) provoked a lag time before thickening, proportional to its initial concentration, during which no FA was consumed. They proposed that FA oxidized by laccase or the H_2O_2/HRP into phenoxy radicals (semiquinones) was regenerated whereas CSH was oxidized into CSSC. This mechanism, different to the one proposed by Hoseney and Faubion (*73*), who indicated that CSH could add to the double bond of the FA, is qualitatively equivalent to effect of AA (**Figure 9**). With CSH, a part of phenoxy radicals, produced by POD, is able to form dimers, while the major part is regenerated into FA with the formation of CSSC. This means that the rate constant k_3 of the reaction between CSH and the phenoxy radicals is of the same order of magnitude than the rate constant k_1 of the phenoxy radicals dimerization and therefore much less than the rate constant k_2 of the reaction between AA and the phenoxy radicals (**Figure 9**). Thus, AA prevents the FA dimerization until it is present whereas CSH slows down but does not prevent this reaction. We have observed that glutathione exhibited a similar behavior than CSH, but the reaction between phenoxy radicals and glutathione was less rapid than with CSH (not shown). Last, the relative proportions of the different dimers were similar either in the presence of AA or CSH or in their absence, meaning that the dimerization reactions are not affected by these compounds.

CONCLUSION

The H_2O_2/FC_1 POD system is able to oxidize free FA as well as EFA. When the two forms of hydroxycinnamic acid are present, FC_1 POD preferentially oxidizes the free FA rather than the FA esterified by arabinose, probably because of the occurrence of coupled oxidation mechanism. These results confirmed those obtained by Figueroa-Espinoza and Rouau (*10*) who observed that when free FA was added to feruloylated pentosans, the free acid was preferentially oxidized by laccase or by the H_2O_2/HRP system. Two consequences can be inferred from the effect of AA and CSH on the FA dimerization catalyzed by wheat POD in the presence of H_2O_2. First, if one assumes that the beneficial effect of the addition of GOX in wheat flour dough is due to the formation of H_2O_2, which activates the POD system, the simultaneous addition of AA could be antagonist to this effect since AA hindered the FA dimerization. Second, although the wheat POD does not act directly on thiols, this enzyme, in the presence of H_2O_2 and phenolic compounds (e.g., FA either free or esterified), is able to promote the formation of disulfide bonds and therefore modifies the thiol/disulfide interchanges during the breadmaking process.

ABBREVIATIONS USED

AA, ascorbic acid; CSH, cysteine; CSSC, cystine; DTT, dithiothreitol; ECD, electrochemical detector; EFA, 5-O-(*trans*-feruloyl)-L-arabinose; FA, ferulic acid; FC_1, main cationic peroxidase isolated from wheat germ; GOX, glucose oxidase; HRP, horseradish peroxidase; POD, peroxidase.

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