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Kinetic mechanism and product characterization of the enzymatic peroxidation of pterostilbene as model of the detoxification process of stilbene-type phytoalexins

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ABSTRACT

The enzymatic peroxidation of pterostilbene, a strong antifungal belonging to the stilbene family, by peroxidase (POX), is reported for the first time as a model of phytoalexin detoxification carried out by the enzymatic pool of pathogens. Kinetic characterization of the pterostilbene oxidation reaction pointed to an optimum pH of 7.0, at which value the thermal stability of POX was studied. Moreover, the data showed that pterostilbene inhibits POX activity at high concentrations of substrate. Several kinetic parameters, including V_{max}, K_m and K_I, were calculated and values of 0.16 ΔAbs min^{-1}, 14.61 μM, and 31.41 μM were reported. To understand the possible physiological role of this reaction in the phytoalexin detoxification process, the products of pterostilbene oxidation were identified using HPLC-MS and a radical–radical coupling reaction mechanism was proposed. Three main products with a high molecular weight and pronounced hydrophobicity were identified: pterostilbene *cis* dehydromer, pterostilbene *trans* dehydromer and pterostilbene open dimer. The dimeric structures of these molecules indicate that the pterostilbene oxidation reaction took place at the 4′-OH position of the hydroxystilbenic moieties and the three above mentioned dimeric products were found, due to the ability of electron-delocalized radicals to couple at various sites. Finally, the capacity of cyclodextrins (CDs) as starch model molecules in plants to complex both the substrate and the products of the oxidation reaction was evaluated. The inhibition process of POX activity was modified at high pterostilbene concentrations due to sequestering of the substrate reaction and to the different affinity of the reaction products for CDs.

1. Introduction

In response to infection by agents such as fungus, some plants can synthesize different antifungal compounds belonging to the phytoalexins family like resveratrol, piceid or some viniferins (Wilkens et al., 2010). Although a great number of authors have focused on the study of these three compounds belonging to the stilbenes family, the most important phytoalexins, in recent years the importance of pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene), another stilbene-type phytoalexin, has been reported in different papers as a potent antifungal compound (e.g., Breuil et al., 1999). Pterostilbene, a naturally occurring phytoalexin belonging to a group of phenolic compounds known as stilbenes, has been identified in several plant species such as the heartwood of sandalwood (Sehadri, 1972), leaves of *Vitis vinifera* (Langcake et al., 1979), peanuts (Medina-Bolivar et al., 2007), infected grape berries of *var*. Chardonnay and Gamay (Adrian et al., 2000), healthy and immature berries of *var*. Pinot Noir and Gamay (Pezet and Pont, 1988) and berries of some *Vacciunium* species (Pezet and Pont, 1988). It also appears to be a constituent of the bark of *Guibourtia tessmanii*, a tree found in central Africa and is commonly used in folk medicine (Fuendjiep et al., 2002). In addition to its antifungal role (Pezet and Pont, 1990; Mazullo et al., 2000), pterostilbene presents other biological activities, including antihyperglycemic (Manickam et al., 1997), antioxidative (Rimando et al., 2002; Kathryn et al., 2006; Remsberg et al., 2008), anticancer (Rimando et al., 2005; Kathryn et al., 2006; Remsberg et al., 2008; Pan et al., 2007; Nanjoo et al., 2007; Ferrer et al., 2005), anti-inflammatory (Remsberg et al., 2008), anticholesterol (Mizuno et al., 2008; Mazullo et al., 2000), hypolipidemic (Mazullo et al., 2000) and analgesic (Mazullo et al., 2000) activities.

It has already been reported that the pathogenicity of fungi like *Botrytis cinerea* or *Trabetes pubescens* is strongly associated with their ability to degrade stilbene phytoalexins such as pterostilbene or resveratrol (Ponzoni et al., 2007). These observations suggest that the enzymes of these pathogens attack the phytoalexins which represent the first line of the defence of the plant. Indeed, this type of fungus has an enzymatic pool able, for example, to lyse cell walls or to oxidize polyphenols (Gil-ad et al., 2000). Subsequently, direct evidence was obtained that resveratrol and pterostilbene can undergo degradation by enzymes such as the laccase produced by *B. cinerea* (Sbaghi et al., 1996).

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Although most works published concerning the detoxification of phytoalexins by the enzymes present in *B. cinerea* only focus on the role of laccase-like stilbene oxidase produced by this fungus (Breuil et al., 1999; Ponzoni et al., 2007; Gil-ad et al., 2000), Gil-ad et al. (2000) showed that *B. cinerea* has a peroxidase (POX) (E.C. 1.11.1.7) enzymatic activity not reported previously. However, no work has reported, as is done for first time in this paper, on whether the enzymatic peroxidation of pterostilbene is involved in the detoxification of phytoalexins. Thus, although POX catalyzes the oxidation of a wide variety of substrates, the potential oxidation of pterostilbene by this oxidative enzyme as a model of phytoalexin degradation by *B. cinerea* has not been reported.

As occurs with the detoxification of other phytoalexins carried out by laccase (Ponzoni et al., 2007; Sbaghi et al., 1996; Pezet, 1998; Breuil et al., 1998, 1999), a knowledge of both the mechanism by which POX acts on pterostilbene and the nature of the oxidation reaction products, is essential for understanding the possible physiological role of this reaction in the phytoalexin detoxification process. Indeed, metabolism of phytoalexins by *B. cinerea* or *T. pubescens* may manifest itself as an insolubilization of the products formed when different substrates are used. The fungus could, therefore, avoid the action of phytoalexins such as pterostilbene by transforming these compounds into other products of increasing molecular weight and hydrophobicity. Moreover, in the case of another phytoalexin-type stilbenes such as resveratrol, Dercks and Creasy (1989) stated that e-viniferin, a dimeric form of resveratrol analogous to the dimer produced by *B. cinerea* (Breuil et al., 1998), is less stable (soluble) in water than resveratrol itself, its biological activity against *B. cinerea* being lower. Similar results were found when pterostilbene was incubated in the presence of laccase from *B. cinerea*. These observations demonstrate the interest of indentifying the reaction products and underline the involvement of the enzyme-mediated oxidation of stilbenes in the pathogen-plant interaction.

Bearing the above in mind, and in order to study the possible role of POX in the detoxification of antifungal compounds and, consequently, in the pathogenesis of the organisms which contain POX activity, the four main objectives of this work were to: (i) to determine the enzymatic kinetic parameters of the oxidation of pterostilbene by POX, (ii) to identify the oxidation reaction products, (iii) to propose a reaction mechanism to justify the results obtained, (iv) to evaluate the use of cyclodextrins (CDs) as modulators of both the enzymatic activity and the oxidation products.

To perform the study, UV–VIS method which makes use of changes in the spectrophotometric parameters of pterostilbene in the presence of POX and an HPLC-MS tandem to identify the oxidation products in the absence or presence of CDs were used. Moreover, horseradish POX, the most studied POX type, was used as an enzyme model for oxidizing pterostilbene.

2. Results and discussion

2.1. Scanning spectrophotometric studies of the oxidation of pterostilbene by POX

Although many studies have focused on POX and its capacity to metabolize new substrates (O'Brien, 2000), no work has reported on the potential oxidation of the potent antifungal pterostilbene by this enzyme. For this reason, the first step in this investigation was to monitor the possible oxidation of pterostilbene by POX, observing the changes that occur in the UV spectrum of this phytoalexin with time.

The pterostilbene spectrum shows a single absorption band at around 300 nm with a bandwidth of 20 nm and two small maxima centred at 304 and 316 nm (Fig. 1). The changes in absorbance of a reaction medium when pterostilbene was incubated with POX and H_2O_2 pointed to maximal spectral changes in the 250–400 nm region, with a decrease in pterostilbene absorbance at 310 nm and an increase at 360 nm. Since the spectral changes observed were not detectable in the absence of H_2O_2, or in the absence of enzyme (data not shown), they were considered to be the result of POX activity. In addition, the dependence of these spectral changes on enzyme concentration confirms that the changes in the UV spectrum are a reliable measure of the enzymatic oxidation rate.

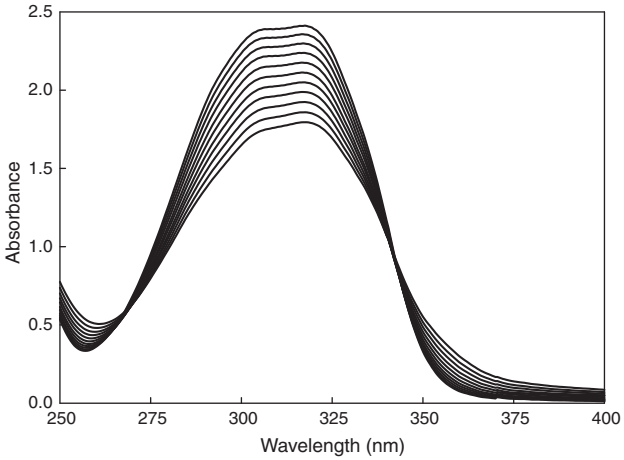

Fig. 1. Evolution of the spectrum of the peroxidation of pterostilbene by POX in the presence of H_2O_2. The reaction medium at 25 °C contained 20 μM of pterostilbene in the presence of 114 μM H_2O_2 at pH 7.0. The scans were carried out every 0.5 min.

Finally, the nature of the spectral changes of pterostilbene with time at 250–400 nm (Fig. 1) indicated that the formation of pterostilbene oxidation products was proportional to time during the first 11 min of the reaction. Moreover, the presence of two isosbestic points at 268 and 342 nm in the consecutive spectra of the reaction medium suggests two things: (i) the presence of one oxidation product formed during the course of pterostilbene oxidation by POX or (ii) the presence of several oxidation products with a constant stoichiometry during the time of the reaction. As will be demonstrated in following sections, the oxidation of pterostilbene by POX provides a mixture of products with a constant stoichiometry during the reaction.

2.2. Determination of the optimum physico-chemical conditions for studying the oxidation of pterostilbene by peroxidase

To ascertain the best physico-chemical conditions for kinetically characterizing the oxidation of pterostilbene by POX, the next step of this investigation was to study both the optimum pH and the thermal stability of the reaction. Reports in the literature describe how the optimum pH of any POX depends on different factors, such as the H-donor in the activity assay (Halpin et al., 1989), the substrate used (Chisari et al., 2007, 2008) or the POX source (Duarte-Vázquez et al., 2000). In the present study, the influence of the incubation medium pH on the rate of pterostilbene oxidation by POX is demonstrated (Fig. 2A). It was concluded that the rate of oxidation is strongly pH-dependent. As can be seen, the enzymatic activity showed an optimum at pH 7.0, above which it fell sharply. The pH profile depicted in Fig. 2A, with an optimum at pH 7.0 and a strong decrease in the region of basic pH, agrees with the observations of several authors (O'Brien, 2000). For these reasons, the optimum pH value selected for studying the oxidation of pterostilbene by POX was 7.0.

To continue the identification of the optimum physico-chemical parameters for studying the oxidation of pterostilbene by POX, the

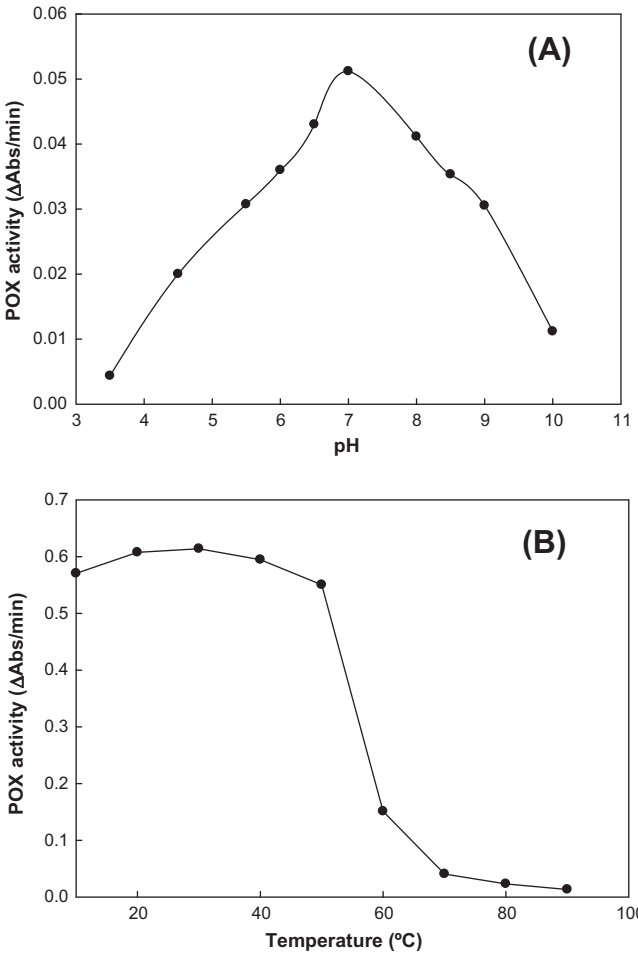

Fig. 2. (A) Effect of pH on the peroxidation of pterostilbene by POX. The reaction medium consisted of 8 μM pterostilbene, 114 μM H_2O_2 at different pH values at 25 °C. (B) Thermal stability of POX on pterostilbene at different temperatures for 40 min. The reaction medium consisted of 8 μM pterostilbene, 114 μM H_2O_2 at pH 7.0.

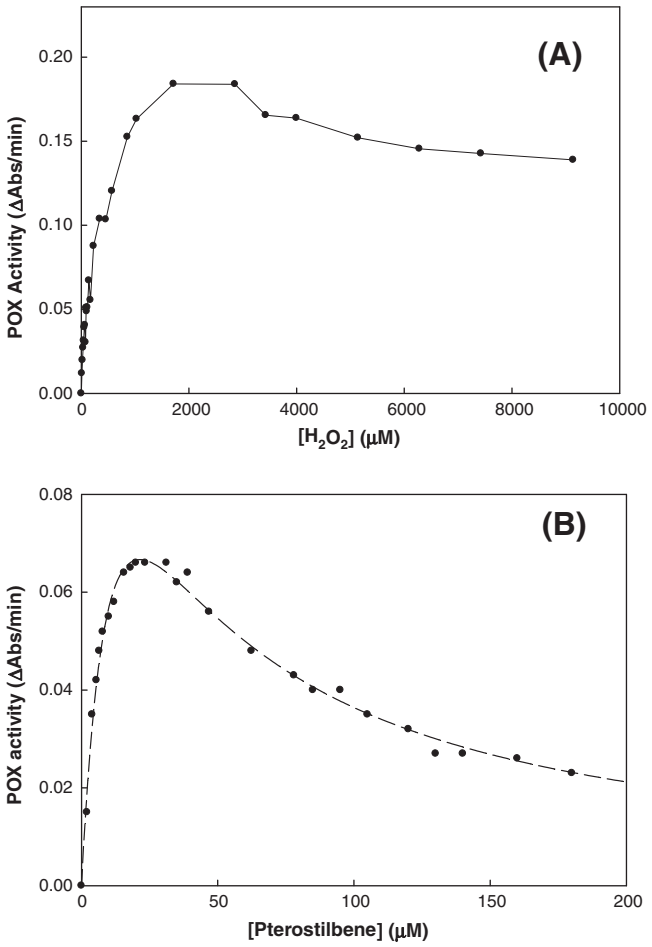

Fig. 3. (A) Effect of H_2O_2 concentration on the peroxidation of perostilbene by POX. The reaction medium at 25 °C contained 0.1 M phosphate buffer pH 7.0, 8 μM pterostilbene and increasing concentrations of H_2O_2 from 0 to 10 mM. (B) Effect of pterostilbene concentration on POX activity in the presence of 114 μM H_2O_2 at pH 7.0.

next step was to evaluate the thermal stability of POX at increasing temperatures from 15 to 90 °C. For this, the enzyme solutions were incubated at ten temperatures for 40 min and, after heating, the samples were assayed at 25 °C. As can be seen in Fig. 2B, a strong dependence of the oxidation rate of pterostilbene by POX on the incubation temperature was observed, being stable between 15 and 50 °C and strongly decreasing above 50 °C. As a consequence of these results, 25 °C was selected as the optimum temperature for the next sections.

Finally, as shown in Fig. 3A, pterostilbene oxidation was also seen to be dependent on the concentration of H_2O_2, being inhibited at high concentrations. These results are in good agreement with those described for asparagus and turnip peroxidases (Duarte-Vázquez et al., 2000; Rodrigo et al., 1996), and so a low H_2O_2 concentration of 114 μM was selected for the next steps of the investigation.

2.3. Study of the inhibition of POX activity at high pterostilbene concentrations

The results obtained in the previous sections show that the optimum reaction conditions for characterizing the oxidation of pterostilbene by POX are as follows: (i) pH: 7.0, (ii) temperature: 25 °C, and (iii) H_2O_2 concentration of 114 μM. These conditions were selected to calculate the main kinetic parameters of the enzyme catalyzed-reaction, evaluating the changes in the oxidation rate with changes in pterostilbene concentration.

As can see in Fig. 3B, the dependence of the pterostilbene oxidation rate on a variation in the pterostilbene concentration was complex. Thus, although this parameter showed a Michaelis–Menten type kinetic at pterostilbene concentrations of less than 27 μM, it showed strong inhibition at higher concentrations. For this reason, Eq. (1), which calculates the main kinetic parameters of an enzymatic reaction when an inhibition of the oxidation rate at high concentrations of substrate is observed, was used.

$$v = \frac{v_{max}[S]}{(K_m + [S] * (1 + [S]/K_I))} \quad (1)$$

where V_{max} is the maximum enzyme velocity, K_m is the Michaelis–Menten constant and K_I is the constant for substrate inhibition.

Fitting the experimental data to Eq. (1), the kinetic constants V_{max}, K_m and K_I were calculated and values of 0.16 ΔAbs min^{-1}, 14.61 μM, and 31.41 μM were obtained.

2.4. Characterization of the reaction products of the oxidation of pterostilbene by peroxidase

Characterization of the pterostilbene metabolites allowed us to understand more about phytoalexin metabolism by different pathogens with POX activity and the relation with fungal pathogenicity. As

mentioned above, the production of phytoalexins is considered to form part of a general defence mechanism of plants. In this species, such a response includes the formation, mediated by laccase enzyme, of a range of biosynthetically related di- and oligomers of different stilbenes. For this reason, the next step of our investigation was to identify the products of pterostilbene oxidation by POX.

The products formed upon incubation of pterostilbene with POX at pH 7.0 were analyzed by HPLC-MS. As shown in Fig. 4, the HPLC chromatogram resulted in the presence of three reaction products (peaks A–C).

The mass analysis of a sample of this less polar product (Fig. 4A inset) showed a quasi-molecular ion of 511 Da and a molecular formula $C_{32}H_{30}O_6$. Identical results were reported for the oxidation of pterostilbene by *B. cinerea* laccase (Breuil et al., 1999) and for the incubation of pterostilbene with the *T. pubescens* laccase (Ponzoni et al., 2007). For this reason, peak A shown in Fig. 4 is consistent with the structure of a pterostilbene *trans* dehydromer presented in Fig. 5A. Moreover, mass analysis of peak B (Fig. 4) showed an identical quasi-molecular ion of 511 Da and a molecular formula $C_{32}H_{30}O_6$. According to the results reported by Breuil et al. (1999) for the oxidation of pterostilbene by laccase, this fact would be due to the existence of a photochemical isomerization of the product presented in Fig. 5B, which is a typical reaction for *trans*-stilbenes. For this reason, although pterostilbene degradation to a major compound that was in the *trans* ethylenic form (Fig. 5A), the HPLC chromatogram shown in Fig. 4 also presented (as secondary product) a pterostilbene *cis* dehydromer (Fig. 5B) with an identical molecular ion and molecular formula.

To confirm that the structures of peaks A and B correspond to pterostilbene *trans* dehydromer and pterostilbene *cis* dehydromer, respectively, the UV spectrum from 240 to 360 nm of these molecules are showed (Fig. 6). As can be seen in Fig. 6A, the UV spectrum of pterostilbene *trans* dehydromer presents a single absorption band around 300 nm with a bandwidth of 20 nm and two small maxima at 309 and 325 nm. This type of spectrum is

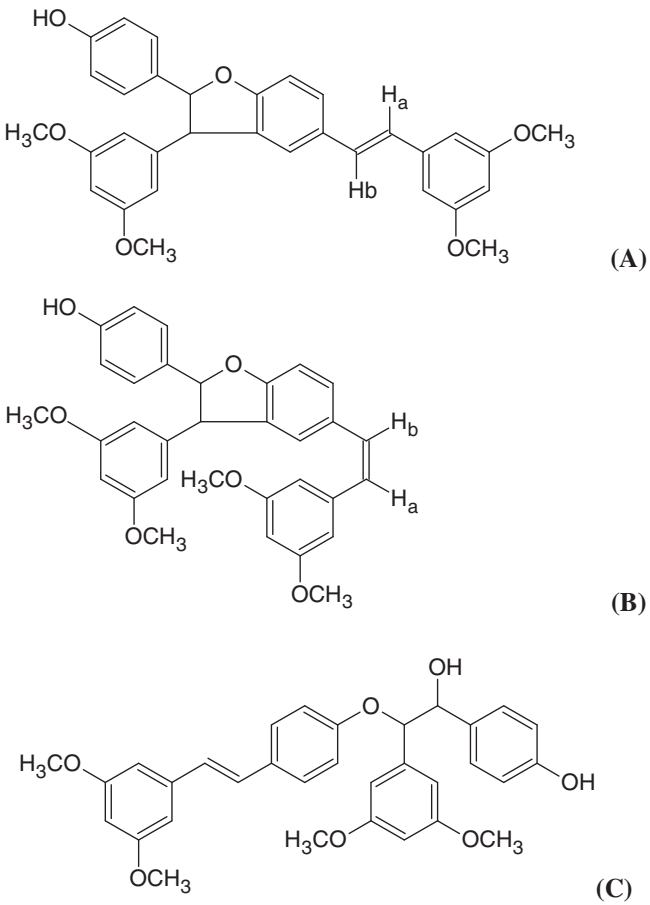

Fig. 5. Reation's products of the pterostilbene oxidation reaction products by POX. (A) pterostilbene *trans* dehydromer, (B) pterostilbene *cis* dehydromer and (C) pterostilbene open dimer.

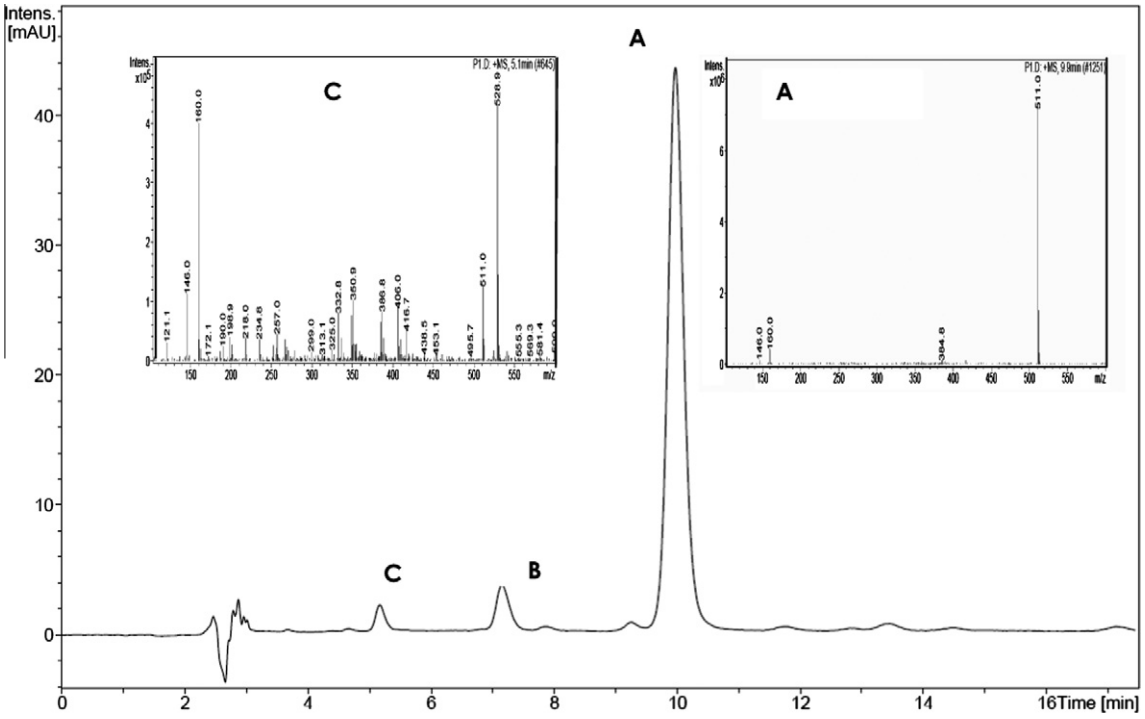

Fig. 4. Reversed-phase HPLC analysis of pterostilbene oxidation products by POX. (A) *trans* dehydromer pterostilbene; (B) *cis* dehydromer pterostilbene and (C) pterostilbene open dimer. The eluent was methanol/water (80:20 v/v). *Inset*: mass spectrum of A and C pterostilbene oxidation products.

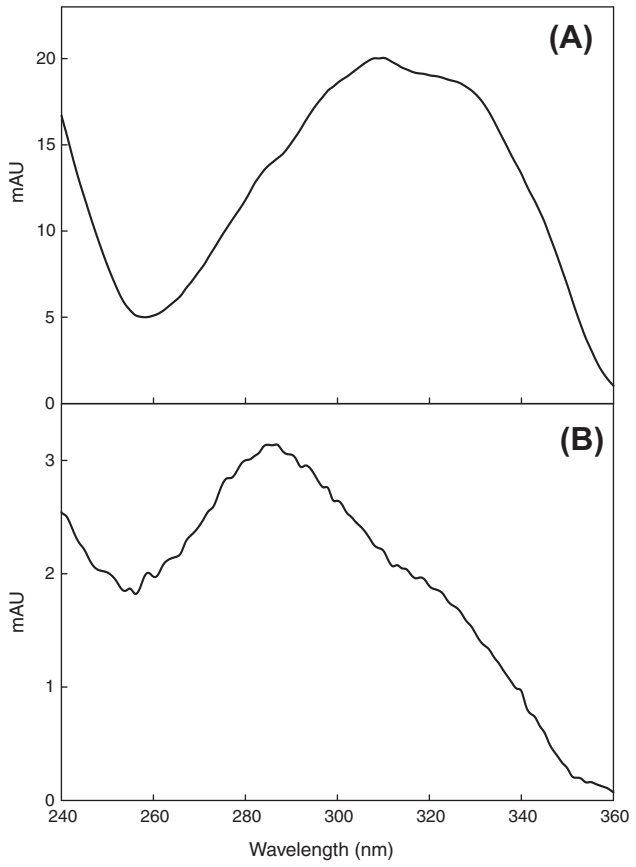

Fig. 6. (A) UV spectrum of pterostilbene *trans* dehydromer; (B) UV spectrum of pterostilbene *cis* dehydromer.

characteristic of the molecules belonging to the stilbene family which present a *trans* configuration as it is cited in different papers (Abert Vian et al., 2005; López-Nicolás et al., 2009a). However, the UV spectrum of molecules belonging to the stilbene family which present a *cis* configuration shows a single absorption band around 285 nm but with a bandwidth of few nm (Abert Vian et al., 2005) identical to the spectrum presented in Fig. 6B. These data confirm again the structures of pterostilbene *trans* dehydromer and pterostilbene *cis* dehydromer.

Finally, the HPLC study of the oxidation of pterostilbene by POX showed a third peak (C). The mass spectrum of the most polar product had a quasi-molecular ion of 529 Da (Fig. 4C inset), that is 18 amu more than the value obtained with the other products, and $C_{32}H_{31}O_7$ the formula molecular. This product was identified as an pterostilbene open dimer with two 3,5-dimethoxybenzoyl moieties (Fig. 5C) by Ponzoni et al. (2007) during the oxidation of pterostilbene by *T. pubescens* laccase.

2.5. Reaction mechanism of the oxidation of pterostilbene by peroxidase

On the basis of the results obtained, a mechanism for the synthesis of the different dimeric derivatives can be proposed. As stated above, phenols are among the most suitable POX substrates (O'Brien, 2000). Oxidations proceed via the formation of radical cations and the subsequent deprotonation of the phenolic hydroxy groups to give phenoxy radicals, which can undergo a broad variety of coupling reactions (Ponzoni et al., 2007). The main drawback of these biotransformations is the extensive polymerization that may occurs due to the radical mechanism of the oxidative process, which may frequently produce a complex mixture of polyphenolic oligomers.

As regards our investigation, the dimeric structures indicate that the pterostilbene oxidation reaction took place at the 4′-OH (4-OH) position of the hydroxystilbenic moieties. Due to the capability of electron-delocalized radicals to couple at various sites, different dimeric products were found, depending on the structural features of the starting substrates. In turn, the phenoxy radicals formed could delocalize as reported in Fig. 7.

Successively, the coupling of one radical "B" and one radical "C", followed by tautomeric rearrangement and intramolecular nucleophilic attack on the intermediate quinone, produced both *cis* and *trans* dihydrofuran pterostilbene dehydromers identified as the two least polar isomeric products in the HPLC chromatogram shown in Fig. 4 (peaks A and B).

In turn, the formation of the "open" hydroxylated (Fig. 4 peak C), the most polar of the reaction products, can be easily explained by the coupling of one radical "A" with one radical "C", followed by addition of a water molecule to the intermediate quinones.

The results of our experiments clearly supports our hypothesis that the reaction proceeded through radical–radical coupling and not through radical addition to the double bond of a non-radical substrate. These results are in accordance with most of the literature reports (Ponzoni et al., 2007) and not with the hypothesis suggested by Szewczuk et al. (2005), who proposed a mechanism in which the another stilbene-type molecules dimerization occurs via radical attack on a second molecule of stilbene.

2.6. Effect of the presence of cyclodextrins on the rate of pterostilbene oxidation by peroxidase

Different studies have demonstrated that cyclodextrins (CDs) can modify the enzymatic activity of an oxidorreductase enzyme, such as LOX, when a stilbene is used as substrate (López-Nicolás et al., 2009a). As regards the physiological role in plants, several works have reported that the oxidation of some antioxidant compounds forming inclusion complexes with CD by oxidative enzymes can be regarded as a model system of their oxidation in storage tissues complexed with starch (López-Nicolás et al., 1997). For these reasons, and in order to evaluate the effect of CDs on the structure and availability of this potent antifungal compound in the enzymatic reaction, the next step was to evaluate the response of POX to the presence of CDs in the reaction medium.

CDs are torus-shaped oligosaccharides made up of 6–8 glucopyranose units and originated by the enzymatic degradation of starch through the action of CD-glucano-transferase (Szente and Szejtli, 2004). Poorly water-soluble compounds and hydrophobic moieties of amphiphilic molecules interact non-covalently with the CD cavity to form so-called inclusion complexes, which are also highly water-soluble. Several publications have reported the aggregation behaviour of different enzymatic substrates such as fatty acids, phenols, and stilbenes, in the presence of CD, and evidence has been presented concerning the formation of guest/CD inclusion complexes with several stoichiometries (Bru et al., 1995; López-Nicolás et al., 1997, 2009a,b,c). Indeed, our group recently reported the formation of pterostilbene/CD complexes using fluorescence techniques (López-Nicolás et al., 2009b).

Fig. 8A shows the Hydroxy-propyl-β-CD dependence of POX at pH 7.0 when three pterostilbene concentrations were used. As can be seen, the addition of increasing concentrations of HP-β-CD produced a change in the POX enzymatic activity that depended on the pterostilbene concentration used. In the presence of the 24 μM pterostilbene in the reaction medium, the POX activity decreased when HP-β-CD concentration was increased (Fig. 8A filled circle). This typical behaviour is due to the ability of both natural and modified CDs to sequester part of the pterostilbene to form

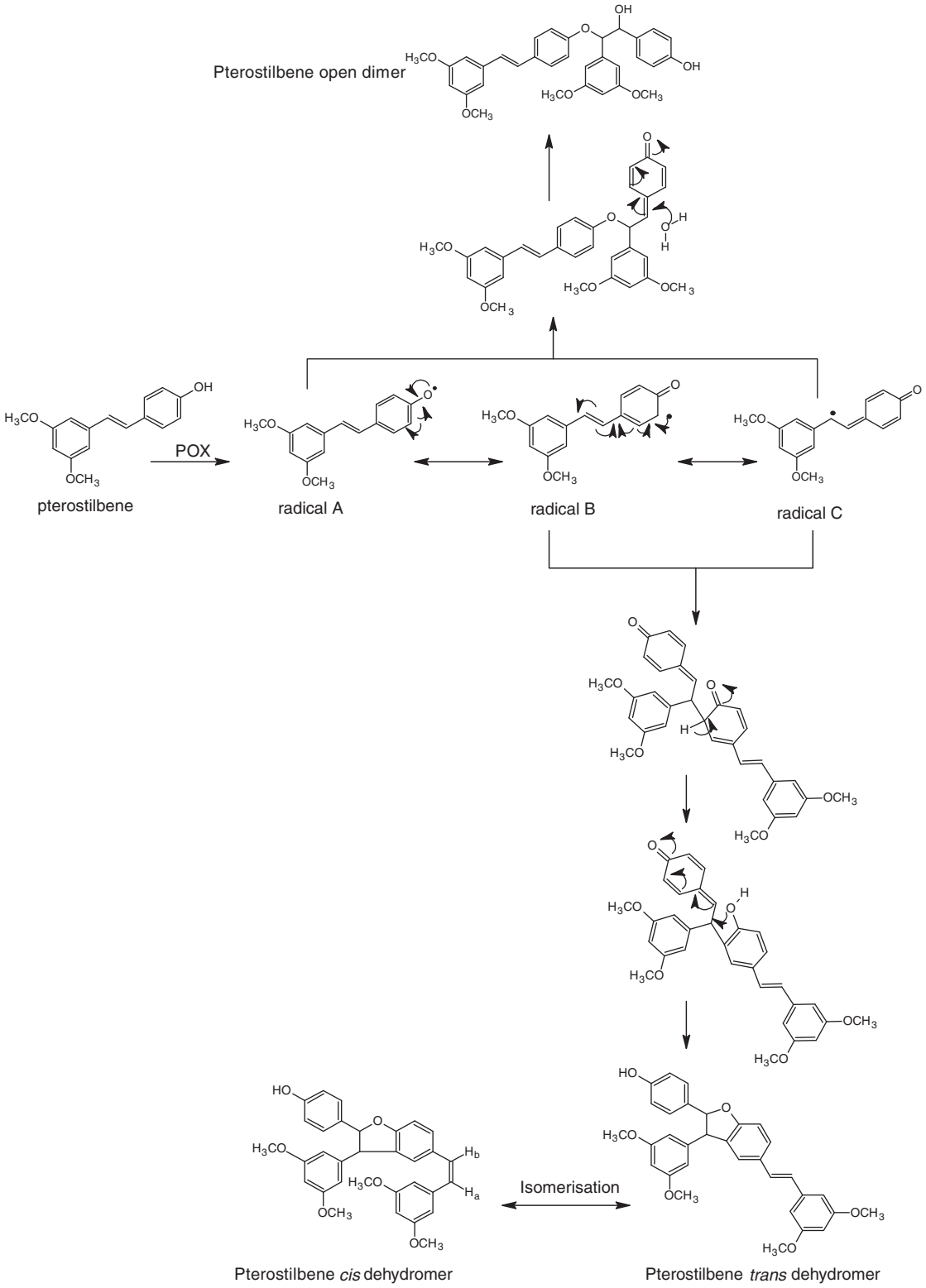

Fig. 7. Radical–radical coupling reaction mechanism proposed for the synthesis of pterostilbene *trans* dehydromer, pterostilbene *cis* dehydromer and pterostilbene open dimmer.

soluble inclusion complexes (López-Nicolás et al., 2009b), thereby reducing the concentration of the free pterostilbene. Thus, CDs act as substrate reservoir in a dose-dependent manner. Indeed, free pterostilbene is the only effective substrate and the oxidation of the complexed substrate requires the prior dissociation of the complex.

Moreover, when a pterostilbene concentration of 47 μM was used, the POX activity practically remained constant when HP-β-CD concentrations below of 0.5 mM were tested. However, when the HP-β-CD concentration was above 0.5 mM, the POX activity decreased, as occurred at the pterostilbene concentration of 24 μM (Fig. 8A filled squares).

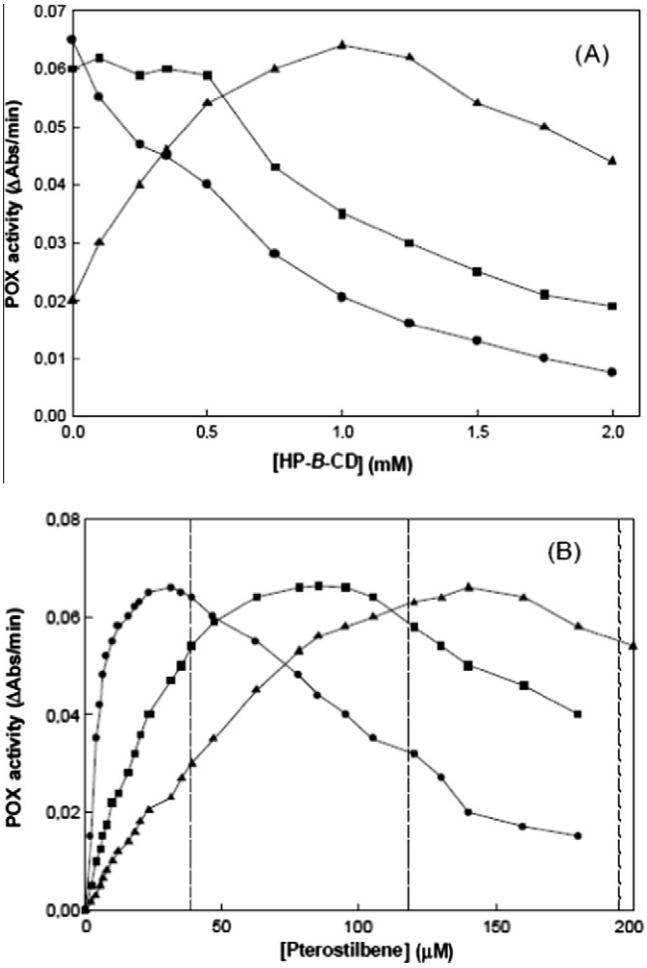

Fig. 8. (A) Effect of HP-β-CD concentrations on the reaction rate of POX-catalyzed pterostilbene oxidation at different substrate concentrations: (●) 24 μM, (■) 47 μM, (▲) 140 μM. The reaction medium at 25 °C contained increasing HP-β-CD concentrations, 114 μM H_2O_2 in 0.1 M sodium phosphate buffer pH 7.0. (B) Effect of the addition of different HP-β-CD concentrations on peroxidation of pterostilbene by POX: (●) no agent, (■) HP-β-CD 0.5 mM, (▲) HP-β-CD 1.0 mM. The reaction medium at 25 °C contained increasing pterostilbene concentrations, 114 μM H_2O_2 in 0.1 M sodium phosphate buffer pH 7.0.

Finally, Fig. 8A shows that when a high substrate concentration of 140 μM was used, increasing HP-β-CD concentrations up to 1 mM produced a sharp increase in POX activity, which decreased again when the concentration of the complexation agent exceeded 1 mM (Fig. 8A filled triangles).

Since of this different pterostilbene concentration behaviour of POX activity in the presence of HP-β-CD has not been previously reported, we studied the possible effect of CDs on the inhibition of POX activity by the high pterostilbene concentrations evaluated in previous sections.

In Fig. 8B it can be seen that when increasing HP-β-CD concentrations (0, 0.5 and 1 mM) were added to the reaction medium, the previously observed inhibition by substrate excess occurred at a higher pterostilbene concentration than observed in the absence of any agent. Thus, the concentration of pterostilbene at which the POX activity was inhibited in the absence of HP-β-CD increased in its presence, reflecting the formation of inclusion complexes and extending the range in which the pterostilbene concentration does not produce inhibition of the enzymatic activity. These results explain the different behaviour of POX activity dependent of pterostilbene concentration shown in Fig. 8A.

2.7. Effect of the presence of cyclodextrins on the stoichiometry of the pterostilbene oxidation products by POX

As shown in the previous sections, the oxidation of pterostilbene by POX produced three main products, which were identified as pterostilbene *cis* dehydromer, pterostilbene *trans* dehydromer and pterostilbene open dimer. Although CDs have usually been used for complexing the substrates of several enzymatic reactions, due to the importance of the pterostilbene oxidation products in the detoxification of phytoalexins, we studied the potential effect of the addition of CDs on the three main products of the oxidation of pterostilbene by POX.

Although in both the absence and presence of HP-β-CD, the sum of the three reaction products concentration remained constant (Fig. 9 filled diamonds), the presence of increasing concentrations of HP-β-CD had different effects on the concentration of the three reaction products determined by HPLC. For example, the area of the pterostilbene *trans* dehydromer decreased when increasing concentrations of HP-β-CD were added to the reaction medium (Fig. 9 filled circles), pointing to a significant interaction between this main reaction product and HP-β-CD. Moreover, the area of pterostilbene *cis* dehydromer increased (Fig. 9 filled squares) with increasing HP-β-CD concentrations due to the capacity of HP-β-CD to modify the isomerization equilibrium shown in Fig. 10. Thus, pterostilbene *cis* dehydromer presents higher affinity for HP-β-CD than the observed for pterostilbene *trans* dehydromer, and so a higher complexation constant ($K_{cis} > K_{trans}$). Finally, no significant variation in the area of pterostilbene *open* dimer was found when HP-β-CD was added to the reaction medium (Fig. 9 filled triangles), showing that this third pterostilbene oxidation product by POX cannot been complexed by HP-β-CD.

Although further investigations are necessary to determine the biological activity of the three pterostilbene oxidation products identified in this work, the fact that HP-β-CDs, complexant agents defined as starch model molecules in plants (López-Nicolás et al., 1997), can form inclusion complex with both pterostilbene *cis* dehydromer and pterostilbene *trans* dehydromer pterostilbene may be used in the detoxification process of stilbene-type phytoalexins. As mentioned above, an increase in the solubility of the formed products is related with the detoxification process. Thus, metabolism of phytoalexins by the enzymes present in pathogens may manifest itself as an insolubization of the products formed.

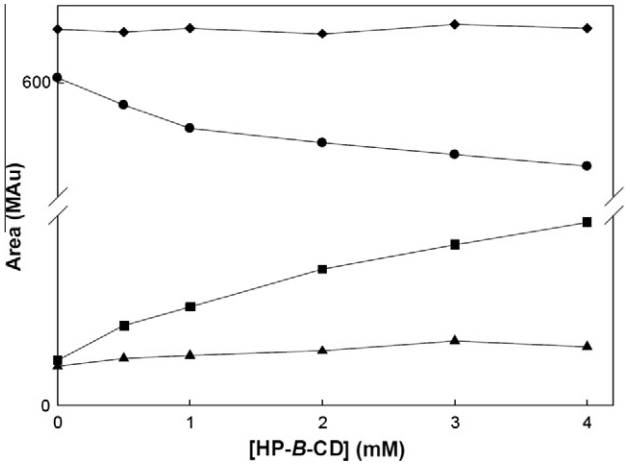

Fig. 9. Effect of HP-β-CD concentration on the area of the three main pterostilbene oxidation products by POX determined by RP-HPLC. (●) pterostilbene *trans* dimer, (■) pterostilbene *cis* dimer and (▲) pterostilbene open dimer, (♦) sum of the concentrations of the three products. The eluent system is methanol/water (80:20 v/v). Mau: Miliabsorbance units.

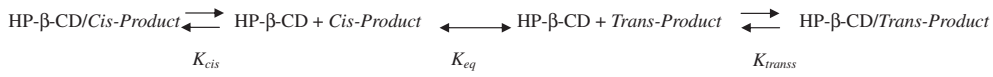

Fig. 10. Isomerization equilibrium between pterostilbene *trans* dehydromer and pterostilbene *cis* dehydromer in the presence of HP-β-CD.

Bearing in mind that one of the most important properties of CDs is it ability for increasing the solubility of the guest molecule complexed (Szente and Szejtli, 2004), the inclusion complexes formed by the interaction between HP-β-CD and both the pterostilbene *trans* dehydromer pterostilbene and *cis* dehydromer products obtained by the oxidation of pterostilbene by POX may show higher solubility than these molecules in the absence of HP-β-CD and slow down the detoxification process thus increasing the defence mechanisms of the plant against fungal attack.

3. Experimental

3.1. Materials

Biochemicals were purchased from Fluka (Madrid, Spain). Pterostilbene was from Sequoia Research Products Limited (Pangbourne, United Kingdom) and was used without further purification. HP-β-CD was purchased from Sigma (Madrid, Spain). Hydrogen peroxide and POX type II from horsedish were obtained from Sigma (Madrid, Spain). Pterostilbene is sensitive to light, and intense irradiation of solutions of the analyte induces the formation of a highly fluorescent compound if the irradiation is intense. Because of this, the samples were stored in darkness. The hydrogen peroxide, POX and pterostilbene were freshly prepared every day.

3.2. Enzyme assay

POX activity was followed spectrophotometrically in a Jasco V-650 spectrometer (Applied Photophysics Ltd.) at 25 °C equipped with thermostated cells at the absorption maximum of the oxidation product of pterostilbene. The reaction was started by adding the indicated volume of enzyme to a standard reaction medium which contained the indicated concentration of pterostilbene, H_2O_2 and CDs.

3.3. pH studies

To study the effect of pH on the oxidation of pterostilbene by POX, several buffers were used: 0.1 M sodium acetate from pH 4.0 to 5.5, 0.1 M sodium phosphate from pH 5.5 to 8.5 and 0.1 M sodium borate from pH 8.5 to 10.0.

3.4. Thermal stability

The enzyme solutions (in Eppendorf tubes) were incubated in a circulating water bath Julabo Shake Temp SW 22 at 10 temperatures (15, 20, 25, 30, 40, 50, 60, 70, 80 and 90 ± 1 °C) for 40 min. After heating, the samples were cooled in ice water and assayed immediately at 25 °C.

3.5. HPLC-MS analysis of pterostilbene oxidation products

For product analysis, 3 ml of a 100 μM solution of pterostilbene in 140 μM phosphate buffer (pH 7.0) was incubated with 100 μl enzyme extract at 4 °C and under constant aeration. After 30 min, the reaction was stopped by acidification to pH 4.0 with HCl, and the products were directly injected. These compounds were analyzed with an HPLC-MS AGILENT VL on a Kromasil C18 column (5 μm, 250 mm × 0.4) and detected at 306 nm. Products were eluted isocratically with methanol/water (80:20 v/v) at a flow rate of 0.7 ml/min. The ratio between the concentrations of the three reaction products was calculated from the peak areas. Parameters for mass spectrometric analysis: mass range mode: Std/normal; ion polarity: positive; ion source type: ESI; dry temperature: 350 °C; nebulizer: 60.00 psi; dry gas: 9.00 l/min. HR-ESI-MS technique was used for calculating the molecular formula of pterostilbene oxidation products.

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