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Characterization of polyphenol oxidase in coffee

Paulo Mazzafera [a], Simon P. Robinson [b,*]

[a] *Departamento de Fisiologia Vegetal, Instituto de Biologia, CP 6109, Universidade Estadual de Campinas, 13087-970, Campinas, SP, Brazil*
[b] *CSIRO Plant Industry, Horticulture Unit, PO Box 350, Glen Osmond, SA 5064, Australia*

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Abstract

Polyphenol oxidase (PPO) was characterized in partially purified extracts of leaves (PPO-L) and fruit endosperm (PPO-E) of coffee (*Coffea arabica* L.). PPO activity was higher in early developmental stages of both leaves and endosperm of fruits. Wounding or exposure of coffee leaves to methyl jasmonate increased PPO activity 1.5–4-fold. PPO was not latent and was not activated by protease treatment. PPO activity was stimulated 10–15% with sodium dodecyl sulphate (SDS) at 0.35–1.75 mM, but at higher concentrations activities were similar to the control samples, without detergent. Prolonged incubation of extracts with trypsin or proteinase K inhibited PPO activity but pepsin had no effect. Inhibition of PPO with proteinase K was increased in the presence of SDS. PPO activity from both tissues was optimal at pH 6–7 and at an assay temperature of 30°C. Activity was highest with chlorogenic acid as substrate with a K_m of 0.882 mM (PPO-L) and 2.27 mM (PPO-E). Hexadecyl trimethyl-ammonium bromide, polyvinylpyrrolidone 40, cinnamic acid and salicylhydroxamic acid inhibited PPO from both tissues. Both enzymes were inactivated by heat but the activity in endosperm extracts was more heat labile than that from leaves. The apparent M_r determined by gel filtration was 46 (PPO-L) and 50 kDa (PPO-E). Activity-stained SDS–polyacrylamide gel electrophoresis (PAGE) gels and western blots probed with PPO antibodies suggested the existence of a 67 kDa PPO which is susceptible to proteolytic cleavage that generates a 45 kDa active form.

Keywords: *Coffea arabica*; Rubiaceae; Coffee; Endosperm; Leaf; Methyl jasmonate; Polyphenol oxidase; Catechol oxidase; Protease; Wounding

1. Introduction

Polyphenol oxidase (PPO; EC 1.10.3.2) is a copper-containing enzyme responsible for hydroxylation of monophenols to *o*-diphenols and oxidation of *o*-diphenols to *o*-diquinones. PPO is widely distributed in higher plants but it still has no defined biological function, although many possible roles have been proposed (Hind et al., 1995; Lax and Vaughn, 1991; Spruce et al., 1987; Steffens et al., 1994; Trebst and Depka, 1995; Vaughn and Duke, 1984; Vaughn et al., 1988). At present, the most likely functions for PPO are its involvement in plant resistance against diseases (Bashan et al., 1985; Goy et al., 1992; Ray and Hammerschmidt, 1998) and against insect herbivory (Felton et al., 1989, 1992). While in the first case, an increase in PPO activity has been observed in incompatible interactions, in the second, the enzyme seems to induce an antinutritive defence. Upon wounding, the quinones formed by PPO induced oxidation of phenols can modify the plant proteins, reducing their nutritive value to herbivores.

For most plant tissues PPO is compartmentalized in plastids, whereas its phenolic substrates are located in the vacuoles (Vaughn and Duke, 1984; Vaughn et al., 1988). Action of PPO only occurs when this compartmentation is disrupted after tissue wounding, as observed in diseased tissues or those damaged by insect attack. However, an increase in PPO mRNA has been detected in artificially wounded tissues (Boss et al., 1995; Thipyapong et al., 1995). In some plants the increase in PPO activity and PPO mRNA have been related to production of compounds (jasmonic acid and methyl jasmonate) of the octadecanoid signal transduction pathway (Constabel et al., 1995; Czapski and Saniewski, 1988), but this does not appear to be universal, as several plants had little or no induction of PPO by wounding or treatment with methyl jasmonate (Constabel and Ryan, 1998).

Due to their high reactivity, the quinone molecules can react with each other as well as with other substances in the wounded tissue, forming a variety of

* Corresponding author. Fax: +618-83038601.
E-mail address: simon.robinson@pi.csiro.au (S.P. Robinson).

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brown or black compounds, which are not the direct result of PPO activity (Mayer and Harel, 1991). This makes PPO a very important enzyme in the food industry since during the processing of fruits and vegetables any wounding may cause cell disruption and lead to quinone formation. The appearance of the product may be affected, but also the taste and its nutritional value, often decreasing the quality of the product (Martinez and Whitaker, 1995; Whitaker, 1995).

In coffee the relationship between coffee quality and PPO activity is not well known, although it is well established that low quality coffee has low PPO activity (Amorim and Silva, 1968; Amorim and Amorim, 1977; Amorim and Melo, 1991). Many factors associated with reduced beverage quality, such as cultural aspects, method of bean processing and time and storage conditions, have been associated with low PPO (Clifford, 1985a). Upon storage in inadequate conditions, there is deterioration of coffee quality, which is followed by a decolouration of the beans (green to yellowish) and decrease of PPO activity (Melo et al., 1980).

The decrease of PPO in coffee during quality deterioration has been attributed to the exhaustion of substrates, mainly chlorogenic acids, the major phenolics in coffee seeds (Clifford, 1985b). According to Clifford (1985a), the sequence of events leading to low PPO are membrane damage, enzyme activation, the oxidation of chlorogenic acids to quinones, and enzyme inhibition by the quinones. It has been observed that humidity lower than 10% leads to marked disruption in membranes of coffee seeds (Begnami et al., 1999). Low quality coffee beans have marked alterations at the ultra-structural level (Dentan and Illy, 1985). Therefore, the low beverage quality may be related to membrane damage but not with PPO per se (Clifford, 1985a). However, since quinones are formed during the process, it cannot be ruled out that they play some role in the loss of beverage quality. Indeed, it has been shown that soluble protein contents decline during coffee storage (Clifford, 1985a), and this could be related to quinone complexation. To our knowledge, the contribution of the oxidation and polymerization products to coffee quality have not yet been determined.

Except for the works on beverage coffee quality (comprehensively reviewed in Amorim and Amorim, 1977; Amorim and Melo, 1991; Clifford, 1985a) there is little information about PPO in this crop. PPO was investigated in compatible and incompatible interactions of coffee with the causal agent of coffee leaf rust (Maxemiuc-Nacache and Dietrich, 1985) and with nematodes (Mazzafera et al., 1989). PPO was partially characterised in coffee beans (Draetta and Lima, 1976) but there has been no detailed study of PPO in coffee. We report here a detailed study in which PPO activity has been characterized in leaves and endosperm of coffee fruits.

2. Results and discussion

2.1. Developmental changes

PPO was investigated in leaves and fruit endosperms of coffee at different developmental stages (Fig. 1). As observed in other plants (Dry and Robinson, 1994; Flurkey and Jen, 1978; Rathjen and Robinson, 1992), young tissues showed the highest activities on a fresh weight basis in leaves of coffee plants (Fig. 1A). PPO activity (PPO-L) was high in the youngest leaves and declined with increasing leaf length, both on a fresh weight and per mg protein basis (Fig. 1A). This indicates that PPO is only synthesized very early in leaf development and the enzyme is reasonably stable, resulting in the decrease in enzyme activity at later stages of leaf development as the leaf expands.

In coffee fruits, the first cellular divisions of the endosperm start 20–30 days after anthesis and pollination (Mendes, 1941). As the endosperm grows, it occupies the inner fruit space, replacing the perisperm, until it reaches full size, at approx. 140–150 days after pollination. At 160 days, the embryo is differentiated, the seed is completely formed, and the ripening process takes place without any further change in size of the

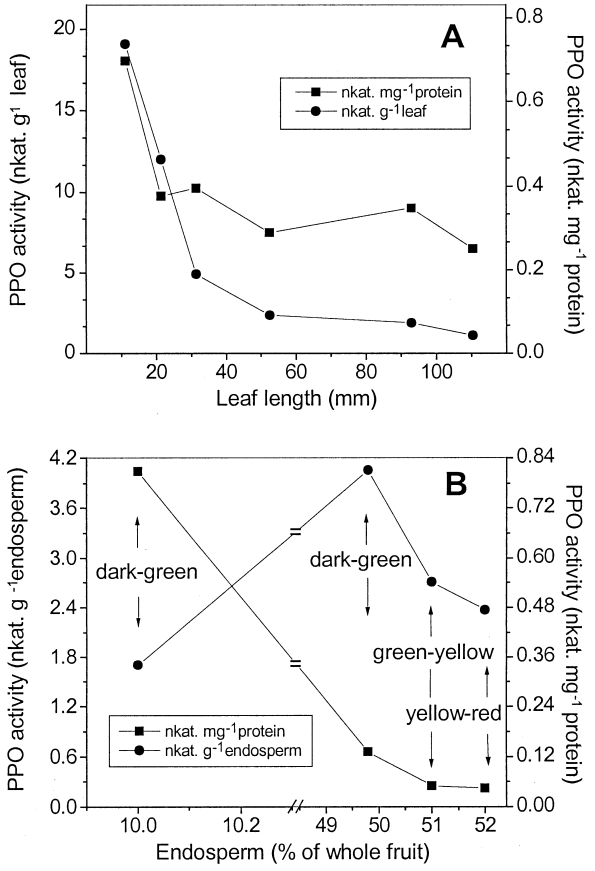

Fig. 1. PPO activity in (A) coffee leaves and (B) endosperm of fruit at different developmental stages.

endosperm. The endosperm starts to lose water and become hard and maturation is completed 34–36 weeks after pollination. Since total fruit size and weight do not change significantly during these later stages of development, these are not useful parameters to denote fruit developmental stage and a better indication is given by plotting the percentage of the whole fruit occupied by the endosperm. PPO activity in endosperm was lowest on a fresh weight basis at the earliest developmental stage collected, when the fruits were dark green and the endosperm comprised only 10% of the total fruit weight (Fig. 1B). Activity increased in later stages of fruit development and this may reflect synthesis of PPO or the decreasing water content of the endosperm. When PPO was calculated as specific activity (per mg of protein), activity was highest in the endosperm of young fruits, and decreased with fruit development. This presumably reflects that increase in storage protein levels during endosperm development (Rogers et al., 1999). As with the leaves, PPO appears to be synthesized in the early stages of endosperm development in coffee.

2.2. Wounding and methyl jasmonate induction of PPO

Coffee leaves wounded by crushing with a haemostat showed an increase of PPO activity compared to control leaves on opposite branches (Table 1). The control leaves had similar PPO activities to untreated plants, suggesting that PPO is induced by wounding but that this induction is not systemic in coffee, at least among leaves of different branches.

Table 1
Activation of coffee leaf PPO by wounding and methyl jasmonate

PPO activity (ηkat mg^{-1} protein)[a]			
Wounding		Methyl jasmonate	
Plant 1		Plant 1	
Treated leaf	0.465	Treated leaf	1.205
Control leaf	0.215	Control leaf	0.291
Plant 2		Plant 2	
Treated leaf	0.442	Treated leaf	0.361
Control leaf	0.294	Control leaf	0.243
Plant 3		Plant 3	
Treated leaf	0.921	Treated leaf	0.461
Control leaf	0.386	Control leaf	0.420
Absolute controls		Plant 4	
1	0.271	Treated leaf	0.636
2	0.360	Control leaf	0.466
		Absolute controls	
		1	0.633
		2	0.576
		3	0.411
		4	0.388

[a] Treated leaf indicates the leaf that was wounded or treated with jasmonate; control leaf indicates the leaf of the opposite (untreated) branch in the same plant; absolute control indicates the leaf of an untreated plant.

Although we selected plants with leaves of similar length to be used in the experiments, the variation in PPO activity was considerable among plants, as demonstrated by the controls and the treated plants themselves. The coffee plants used in this study were maintained in a glasshouse and received the same treatments (watering, fertilization, etc.), however, these variations may reflect the previous history of the plants, since they were obtained from a commercial nursery and transplanted to new pots. It has been shown that PPO can respond to fertilizers and stresses (Bower and van Lelyveld, 1985; Mayer and Harel, 1991). Constabel and Ryan (1998), who analysed PPO in several plant species also observed large variations among repetitions.

Leaf PPO was also induced by methyl jasmonate but activities in leaves of the untreated opposite branches were similar to control leaves, again suggesting that there was not a systemic response among leaves of different branches (Table 1). PPO increased 2.2, 1.5 and 2.4-fold following wounding in the three plants used here. In a survey for plants in which PPO was induced by methyl jasmonate, Constabel and Ryan (1998) detected increases up to 24-fold in tobacco and 46-fold in tomato following wounding. However, several plants did not show significant increases and among those which did respond to the treatment, most had a limited increase in PPO activity of 2 to 4-fold. Considering the data in Table 1, coffee could be grouped with those plants which have relatively high constitutive levels of PPO activity, but limited induction by wounding or methyl jasmonate.

2.3. Characterization of PPO activity

Several works have shown that in some plants PPO is found in a latent state, even after cell disruption, when it is released from plastids and interacts with vacuolar phenolics (Steffens et al., 1994). In some cases PPO continues to be latent even after extraction from the tissue and activation is observed after treatment with proteases, detergents, $(NH_4)_2SO_4$, etc. One explanation for this activation has been that PPO has a proenzyme form, which is bound to membranes, and needs activators (Whitaker, 1995). Activation of PPO by SDS was observed by several researchers (King and Flurkey, 1987; Robinson et al., 1993; Sánchez-Ferrer et al., 1989a,b; Sojo et al., 1998), although significant variation may be seen depending on the plant source (Flurkey, 1986). In general, activation is observed at low concentrations of SDS, and higher concentrations may inhibit activity (Sánchez-Ferrer et al., 1989b; Sojo et al., 1998). Interestingly, coffee PPO was slightly activated by SDS only between 0.35 and 1.75 mM and higher concentrations did not have a major inhibitory effect

(Fig. 2A). Since the activities at higher concentrations were comparable to the controls, this would indicate that the activation at low SDS would not be a consequence of membrane solubilization or protein unfolding. All subsequent experiments were carried out with 3.5 mM SDS in the reaction buffer.

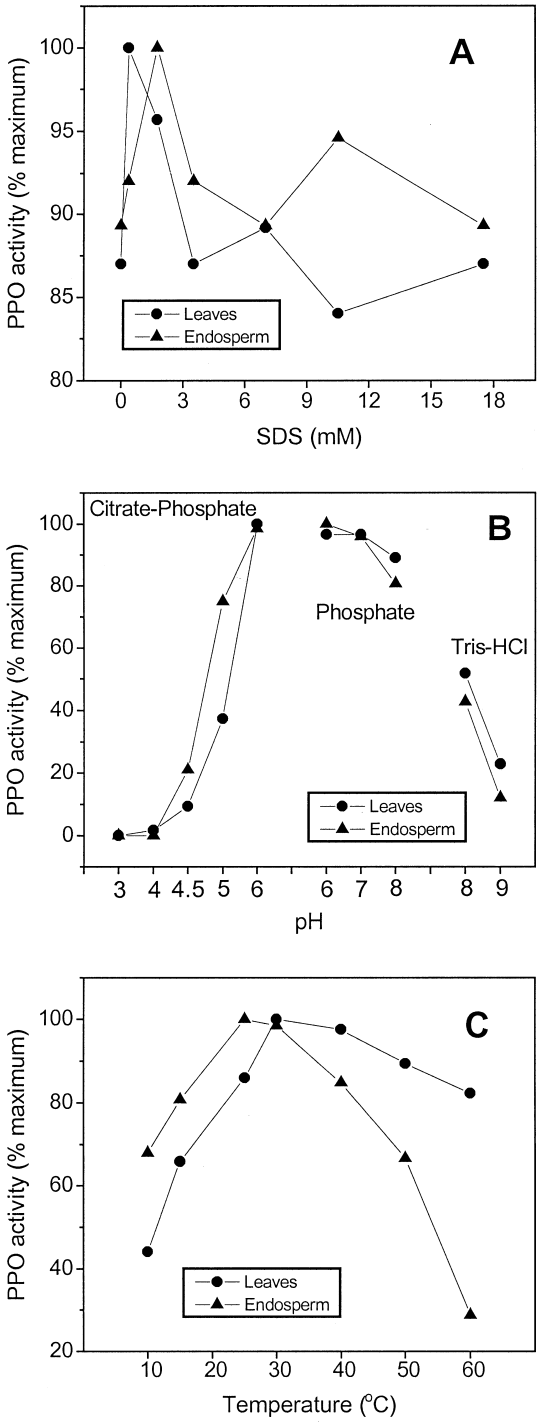

Fig. 2. (A) Effect of SDS on PPO activity and (B) determination of optimum pH and (C) optimum reaction temperature of the enzyme from coffee leaves and endosperm. The optimum pH was investigated with the buffers citrate–Pi (pH 3, 4, 4.5, 5 and 6), Na–Pi (pH 6, 7 and 8) and Tris–HCl (pH 8 and 9).

Optimum PPO activity was observed between pH 6 and 7 (Fig. 2B) and the optimum temperature was between 25 and 30°C for both leaf and endosperm PPO (Fig. 2C). This is in agreement with the optima of pH 6.0 and 30°C determined by Draetta and Lima (1976) for mature coffee beans. It is interesting to note that at pH 8, PPO activity was significantly lower in Tris-HCl than in Na–Pi buffer (Fig. 2B). Galeazzi et al. (1981) studied the effect of the extracting and dialysing media on the banana PPO activity, and observed an activation by Na–Pi buffer. The authors suggested that Pi would stabilize the enzyme and this may explain the higher activity in Na–Pi buffer. Alternatively, coffee PPO might be inhibited by Cl^- as has been reported for some other tissues (Robert et al., 1998). PPO-E was more sensitive to higher temperature than PPO-L (Fig. 2C).

The best substrate for both coffee leaf and endosperm PPO was 5-caffeoylquinic acid (5CQA; chlorogenic acid), followed by 4-methylcatechol (Table 2). However, PPO-E showed lower activity with 4-methylcatechol than PPO-L. In addition, L-3,4-dihydroxyphenylalanine (DOPA) was only oxidized by PPO-E. Since there was not activity towards hydroquinone, a *para*-diphenol, and syringaldazine, substrates for laccases, both of the coffee PPO enzymes appear to be of the catechol-oxidase type.

Results from previous work with PPO from mature coffee beans differ considerably with our results, regarding the substrate affinity. Draetta and Lima (1976) observed in spectrophotometric assays that catechol was the best substrate for PPO (100%), followed by DOPA (39%), pyrogaloll (3.7%), 5CQA (0.5%), guaiacol (0.45%) and tyrosine (0.2%). However, Draetta and Lima (1976) used a spectrophotometric assay in which

Table 2
Substrate affinity of PPO from leaves and endospems of coffee[a]

	Relative PPO activity[b]	
Substrates	Leaves	Endosperm
Chlorogenic acid	100	100
4-Methylcatechol	87.9	74
Caffeic acid	60.6	67.5
Catechol	18.2	15.6
DOPA	0	3.9
Protocatechuic acid	0	0
Tyrosine	0	0
Hydroquinone	0	0
p-Coumaric acid	0	0
Syringaldazine	0	0

[a] PPO activity was determined as oxygen uptake with each of the substrates at a final concentration of 1 mM.

[b] Activity is expressed relative to chlorogenic acid which was 0.23 ηkat mg^{-1} protein in leaves and 0.25 ηkat mg^{-1} protein in endosperms.

the extracts were incubated for 30 min. Although several reports in the literature used spectrophotometric assays to measure PPO activity, this method has several disadvantages. The presence of reducing agents in the assay can cause a delay in colour development, underestimating enzyme activity, and polymerization of the quinone products or reaction with other components of the extract can also result in incorrect estimates of activity (Mayer and Harel, 1991). In both cases, the colour formation may not be linear with time and, therefore, initial rates of reaction must be measured. We repeated the measurement of PPO activity in mature coffee endosperm extracts using spectrophotometric assays and measuring the initial rate of reaction. We found that 5CQA was a better substrate (12.9 absorbance units min^{-1} μg $protein^{-1}$) than pyrogallol (1.9), DOPA (1.65) and guaiacol (0) when the initial activity in the first minute was used to calculate the activities (data not shown), confirming the results obtained by measuring oxygen uptake.

These observations are consistent with those made by measuring PPO activity as oxygen uptake (Table 2) and confirm that 5CQA is the preferred substrate for coffee PPO. The relative activity of PPO with different substrates varies considerably from plant to plant. For example, 5CQA was the best substrate for potato tubers (Patil and Zucker, 1965), sugarcane (Bucheli and Robinson, 1994) and apple (Murata et al., 1992). On the other hand, Robinson et al. (1993) observed that PPO activity in the skin and sap of mango fruits measured with 5CQA as substrate was only 20 and 76%, respectively, of the activity detected with 4-methylcatechol.

In general, PPO has low affinity for its substrates and the reported K_m values are often in the 1–10 mM range (Shomer et al., 1979; Golbeck and Cammarata 1981; Sánchez-Ferrer et al., 1989b) although K_m's as low as 0.02–0.12 mM were reported for PPO from apple (Murata et al., 1992) and potato tuber (Patil and Zucker, 1965). When the concentration of 5CQA was varied in the normal assay for coffee extracts, the calculated K_m for 5CQA was 0.88 mM for PPO-L and 2.27 mM for PPO-E (data not shown). Thus both of the coffee PPOs appear to have a relatively low affinity for their substrates.

Chlorogenic acids are the main phenolics in *Coffea arabica* (5–7% on a dry weight basis), and 5CQA represents approximately 70% of the total phenolics (Clifford, 1985b). Estimates of 5CQA in leaves of the same species also showed significant contents, ranging from 0.45% (Baumann et al., 1991) to 0.56% (Sartor and Mazzafera, 2000). Other coffee species showed 1.7% of this monoisomer (Sartor and Mazzafera, 2000). Therefore, it is very likely that chlorogenic acids are the major substrates of

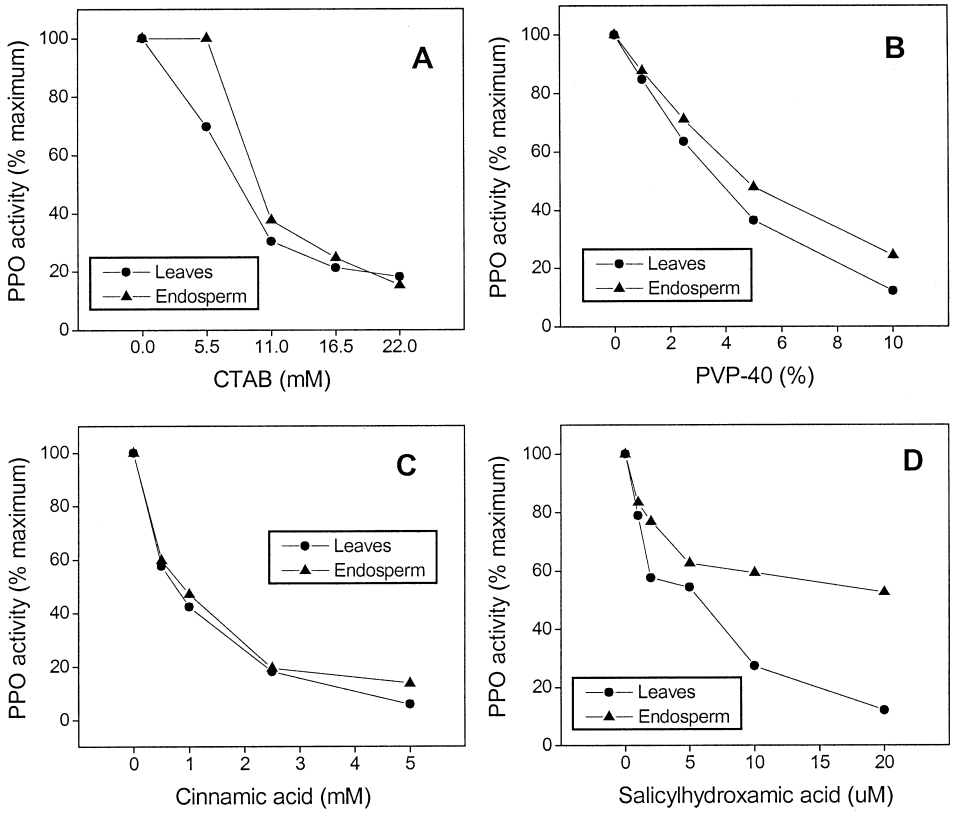

Fig. 3. Effect of (A) CTAB, (B) PVP-40, (C) cinnamic acid and (D) salicylhydroxamic acid on PPO activity from coffee leaves and endosperm of fruit.

coffee PPO in vivo, and this is consistent with the observed affinities for the different substrates in vitro (Table 2).

It has been reported that salicylhydroxamic acid, PVP-40 and cinnamic acid inhibit plant catechol oxidases (Allan and Walker, 1988; Walker and McCallion, 1980). Both coffee PPOs were inhibited by hexadecyl trimethyl-ammonium bromide (CTAB), PVP-40, salicylhydroxamic acid and cinnamic acid (Fig. 3A–D). PPO-E was less sensitive for the first three inhibitors. Therefore, in addition to substrate affinities observed here (see Table 1), our data indicate coffee PPO as a catechol oxidase type. However, it has been also reported that CTAB inhibits laccases but not catechol oxidases (Walker and McCallion, 1980). This cationic detergent did not activate PPO from the mango skin (Robinson et al., 1993), but activated PPO from grapes (Sánchez-Ferrer et al., 1989a), broad bean (Jiménez and García-Carmona, 1996), banana (Sojo et al., 1998) and potato leaf (Sánchez-Ferrer et al., 1993). On the other hand, this is not a general rule, since spinach PPO (Sánchez-Ferrer et al., 1989b) was strongly inhibited at similar concentrations to those used in this study. Coffee PPOs were inhibited by 30–40% with 11 mM CTAB (Fig. 3A).

In the assays of coffee PPO with SDS and CTAB, the enzyme preparations were exposed to these detergents a few seconds before addition of the substrate. In both cases small volumes of the protein extracts (5–10 μl) were mixed in the O_2 electrode chamber with buffer (950 μl) containing the detergents at the desired concentrations. Just after that, 50 μl of a 20 mM 5CQA stock solution was added and the initial rate of reaction was determined. Under these conditions, inhibition of PPO activity by CTAB was much higher than with SDS (see Fig. 2A), although SDS inhibition was also observed with prolonged exposure (see below).

The thermal stabilities of the coffee PPOs were studied by incubating the protein extracts at different temperatures for various periods of time and then determining PPO activity in the standard assay at 25°C (Fig. 4). As observed in the determination of optimum reaction temperature (Fig. 2C), PPO-E was more sensitive to high temperatures than PPO-L (Fig. 4B). While almost no alteration of the activity was observed for PPO-L at 50°C after 30 min, there was a decrease of 20% in activity with PPO-E, although this was observed in the first minutes of incubation and the activities remained stable after that (Fig. 4B). At 80°C, PPO-L lost 50% of activity after 5 min, while this took less than 2.5 min for PPO-E. Both enzymes lost 50% of activity at 90°C in less than 1 min incubation. Comparatively, the coffee PPOs are more stable to high temperature than apple (Murata et al., 1992) and sugarcane PPOs (Bucheli and Robinson, 1994), but less than mango skin PPO (Robinson et al., 1993).

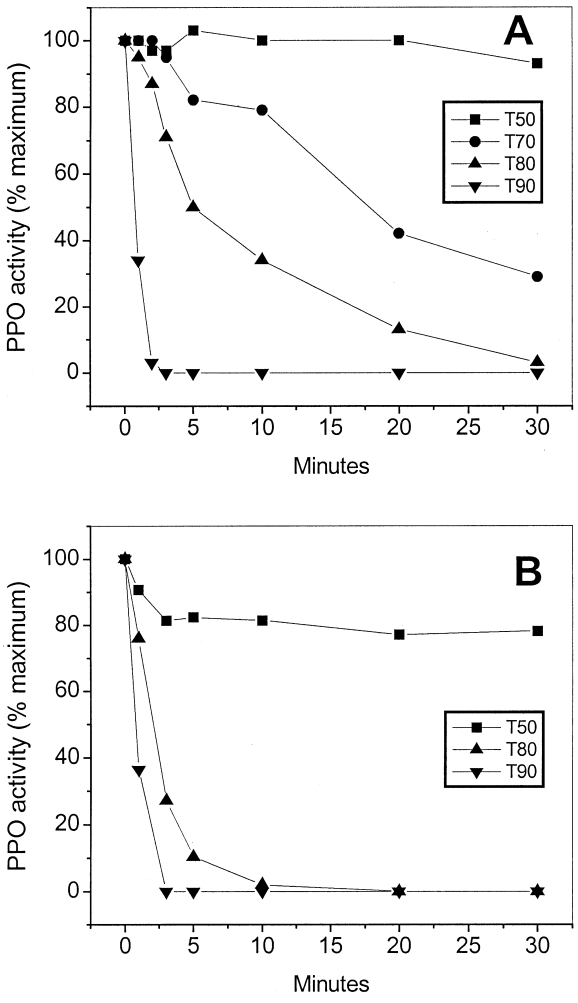

Fig. 4. Heat stability of PPO from (A) coffee leaves and (B) endosperm of fruits. The heat stability of PPO was investigated by incubating aliquots of the extracts at 50, 70, 80 and 90°C for different intervals. After incubation, the extracts were chilled on ice and PPO activity was determined in the standard assay at 25°C.

2.4. Protease treatment

Depending on the plant source, PPO can be present in a latent form and may then be activated in vitro by salt treatment, detergents and acid pH (Steffens et al., 1994). There are several reports of activation of PPO in vitro by proteases (King and Flurkey, 1987; Sánchez-Ferrer et al., 1989b; Steffens et al., 1994; Tolbert, 1973). To investigate activation of PPO in coffee, extracts were incubated with proteases (1:2, wt/wt, total protein in the extract/protease) for 3 h at room temperature then assayed for activity using the standard assay, with 0.1% SDS in the Na–Pi buffer. Trypsin, proteinase K and pepsin did not activate the enzyme, conversely, the first two led to a decrease of activity, proteinase K being more effective, while pepsin did not alter PPO activity in extracts from leaves or endosperms under these conditions (data not shown). These results are consistent with

the small increase in activity observed with SDS (Fig. 2A) and suggest that PPO activity was not latent in either of the coffee extracts.

An additional experiment was carried out to determine the combined effect of proteases and SDS during prolonged incubation periods. Protein extracts subjected to digestion with proteinase K and trypsin (1:2, wt/wt, total protein/protease) were incubated for 24 h with or without detergent, and the PPO activity determined at different intervals using the standard activity assay (Fig. 5). After 24 h at room temperature in the absence of SDS, PPO activity was only decreased by 30–50%, indicating that the partially purified preparations were relatively stable, and there was little effect of trypsin in either leaf or endosperm extracts (Fig. 5A and B). In contrast, proteinase K significantly reduced PPO activity in both extracts (Fig. 5C and D). PPO activity was inhibited when the extracts were incubated with SDS to a greater extent than the inhibition observed with proteinase K. Trypsin did not cause any additional inhibition in the presence of SDS (Fig. 5), whereas the combination of proteinase K and SDS gave a greater inhibition than with each alone.

PPO activity can be detected in plant extracts following separation by partially denaturing SDS–PAGE (Robinson and Dry, 1992). Extracts were separated by SDS–PAGE and stained for PPO activity using 5CQA. To investigate the effects of proteolysis, extracts of coffee leaves or endosperms were prepared in the presence and absence of a cocktail of protease inhibitors. The extracts prepared with protease inhibitors were partially purified by $(NH_4)_2SO_4$ precipitation and desalting in mini-columns of Sephadex G25, then digested with proteases (1:1, wt/wt protease:protein extract) for 3 h at room temperature. A different banding pattern was observed for leaf and endosperm (Fig. 6A, compare lanes 1 and 6). The leaf gel showed prominent bands of activity at apparent M_r of 38, 56 and 85 kDa, while endosperm extracts had several active bands between 35 and 150 kDa. When extracted in the presence of protease inhibitors the banding patterns were altered. In the leaf extract, the band at 38 kDa was less intense and the bands at 53–56 kDa were increased in intensity compared to extracts without protease inhibitors (Fig. 6A, compare lanes 1 and 2). The band at 56 kDa was also more intense in the endosperm extracts prepared with protease inhibitors (Fig. 6A, compare lanes 6 and 7). Following treatment of the extracts with proteases, the band at 56 kDa was no longer detected in either leaf (Fig. 6A, lanes 3–5) or endosperm extracts (Fig. 6A,

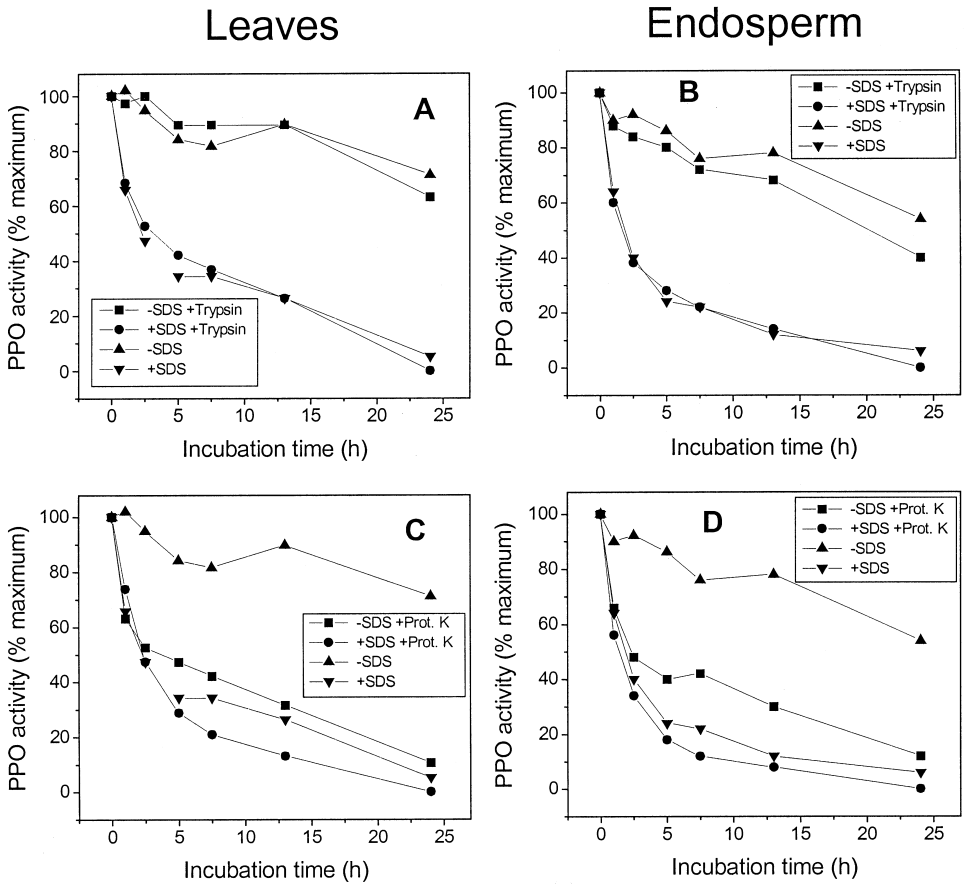

Fig. 5. Combined effect of (A, B) trypsin and (C, D) proteinase K with SDS on PPO activity from (A, C) coffee leaves and (B, D) endosperm of fruits.

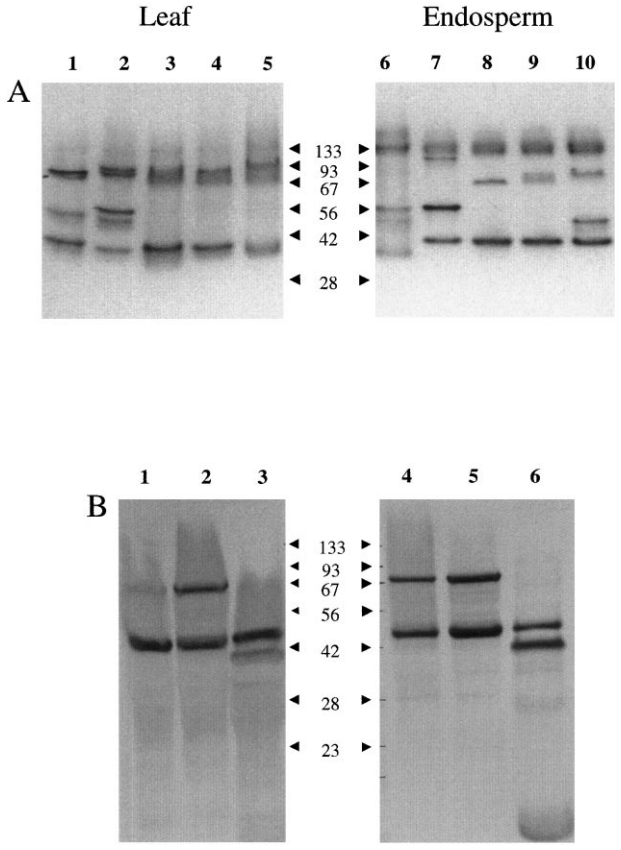

Fig. 6. Partially denaturating SDS–PAGE of protein extracts (A) of leaf (lanes 1–5) and endosperm of fruits (lanes 6–10) stained for polyphenol oxidase activity. Lanes 1 and 6 — extracts obtained without proteinase inhibitors; lanes 2 and 7 — extracts obtained with proteinase inhibitors; lanes 3 and 8, 4 and 9, and 5 and 10 — extracts obtained with proteinase inhibitors and incubated, respectively, with trypsin, pepsin and proteinase K. Western blot of fully denatured coffee leaf (lanes 1–3) and endosperm (lanes 4–6) extracts separated by SDS–PAGE and probed with grape antibodies (B). Lanes 1 and 4 — extracts obtained without proteinase inhibitors in the extraction buffer; lanes 2 and 5 — extracts obtained with proteinase inhibitors in the extraction buffer; lanes 3 and 6 — extracts obtained with proteinase inhibitors in the extraction buffer and incubated with trypsin.

lanes 8–10). In the leaf extracts, protease treatment resulted in more intense activity staining in the 38 kDa bands whereas in endosperm extracts there was also the appearance of new bands between 67 and 93 kDa, which varied according to which protease was used. An additional band at approx. 51 kDa was also observed following incubation of the endosperm extract with proteinase K.

Although activity stained gels can give valuable information on the presence of different isoforms of PPO, the results need to be interpreted with great caution. Because the extracts are not fully denatured, the mobilities only indicate apparent molecular weight, which can differ significantly from actual size (Robinson and Dry, 1992). When coffee extracts were analysed by gel filtration in Sephadex S300, single peaks of activity were observed at an apparent M_r of 46 kDa for leaf extracts and 50 kDa for endosperm extracts (data not shown). To further clarify the different forms of PPO in coffee extracts, fully denatured samples were separated by SDS–PAGE, blotted onto PVDF membranes and probed with antibodies to purified grape PPO (Fig. 6B). Grape antibodies revealed two main bands with M_r of approx. 67 and 45 kDa in the undigested extracts (Fig. 6B, lanes 1, 2, 4 and 5). In the leaf extract, the 67 kDa form was only significant when the extracts were prepared in the presence of proteinase inhibitors. In the extracts subjected to trypsin digestion, the 67 kDa band disappeared and an additional one of 41 kDa was observed (Fig. 6B, lanes 3 and 6).

PPO is synthesized as a pre-protein of 65–68 kDa which includes a plastid transit peptide of 10–12 kDa (Dry and Robinson, 1994). The transit peptide is removed during import of the PPO pre-protein into the plastid to yield a mature protein of approx. 60 kDa. Recently, it was demonstrated that a stromal peptidase is responsible for the cleavage of the transit peptide of PPO, during its import into the chloroplast (Koussevitzky et al., 1998). Robinson and Dry (1992) showed that purification of PPO from broad bean leaves in the presence of protease inhibitors yielded a 60 kDa protein, but in vitro proteolysis converted this into a 42 kDa protein, with no loss of enzyme activity. It appears that a region of the mature protein is sensitive to proteolysis, resulting in removal of a 16–18 kDa peptide from the C-terminal of the protein without diminishing its catalytic capacity (Robinson and Dry, 1992; Dry and Robinson, 1994). This proteolysis can occur in vitro during preparation, and this may explain numerous reports of purified PPO proteins of 40–45 kDa in plants (Golbeck and Cammarata, 1981; King and Flurkey, 1987; Marquès et al., 1994; Rathjen and Robinson, 1992; Dry and Robinson, 1994). It is not yet clear whether this proteolytic cleavage can also occur in vivo in some plants. The results in Figs. 5 and 6 demonstrate that coffee PPO can also undergo proteolytic cleavage, without significant loss of enzyme activity. The 67 kDa protein in coffee extracts was converted to a 45 kDa protein by trypsin treatment (Fig. 6B), indicating that the 45 kDa form in extracts may have arisen as a result of proteolysis during extraction. The presence of the 45 kDa form in extracts made in the presence of proteinase inhibitors suggests that inhibition of proteolysis during extraction was incomplete or that this proteolysis may also occur in vivo. It is clear that the coffee PPO proteins contain a proteolytically sensitive region outside of the catalytically active part of the protein, as has been observed with other plant PPO proteins. Proteolysis can occur during extraction, resulting in 40–45 kDa PPO proteins which retain full enzyme activity.

To our knowledge, there has never been a detailed comparative investigation of PPO from leaves and fruits

in the same plant. In addition, most of the reports in the literature are of fleshy fruits. In this study, leaf and endosperm PPO from coffee has been characterized in detail. The results suggest that both tissues contain a catechol oxidase type enzyme, similar to those found in other plants. The coffee leaf and endosperm PPOs have very similar properties and may represent the same or very similar proteins. In addition, it seems likely that, as in other plants, coffee PPO is synthesized as a precursor protein, which can be hydrolysed to a 45 kDa form while still retaining catalytic activity.

3. Experimental

3.1. Plant material

The plants used in this study were obtained from a commercial nursery and transplanted to pots (500 ml) containing a mixture of soil, sand and composted pulverized pine bark. They were maintained in a glasshouse and at the time leaves were collected, they were approx. 1-year-old. The leaves were classified according their length.

Immature fruits were obtained from a coffee (*C. arabica* L.) plantation in Brazil and transported to Australia refrigerated (8°C). At that developmental stage the endosperms represented approx. 10% of the fruit fresh weight and their appearance was ivory and milky. Immediately after arrival the fruits were cut in two halves, the endosperms removed with a spatula, and then frozen in liquid N_2 and kept at −80°C. While removing the endosperms care was taken to avoid perisperm contamination. Mature fruits were obtained from a coffee plantation (*C. arabica*) in Queensland, Australia. During the transport to Adelaide the material was kept refrigerated. They were cut in two halves and the endosperm removed with a spatula. Immature and mature fruits were classified according to their colour, being dark green, green and yellow-red, and the proportion between endosperm and total fruit fresh weight was determined.

3.2. Protein extraction and PPO assay

For the detection of PPO in leaves and endosperm of fruits at different developmental stages or to investigate the effect of wounding and methyl jasmonate, the samples were frozen in liquid N_2 and ground to a powder with a mortar and pestle. Endosperm from mature fruits was ground in a coffee grinder. Proteins were extracted with 100 mM Na–Pi, pH 7, containing 2% ascorbic acid, and 20% PVPP (w/w) and the extracts were kept at 4°C for 30 min with occasional agitation. After centrifugation (32,600 ***g***, 20 min, 4°C) the supernatants were recovered and desalted in PD-10 Sephadex G25 mini-columns (Pharmacia) using 50 mM Na–Pi, pH 6, for the elution. The protein concentration in the extracts was determined using the BioRad protein assay.

For PPO characterization leaves of 2–3 cm and endosperms from immature fruits were used. Protein extracts were obtained as above, however, before centrifugation they were passed through two layers of Miracloth. The supernatants recovered from the centrifugation were treated with $(NH_4)_2SO_4$ between 20 and 80% saturation, and the 80% pellet was solubilized in a small volume of 50 mM Na–Pi, pH 6. The extracts were desalted in a Sephadex G-25 column (160 ml bed volume, flow rate 1.3 ml min^{-1}, 50 mM Na–Pi, pH 6 as elution buffer), glycerol was added to 20% final concentration and aliquots of the extracts were frozen and stored at −30°C. The protein concentration was determined using the BioRad protein assay.

PPO activity was determined as oxygen uptake in an oxygen electrode (Hansatech), at 25°C. The reaction mixture (final volume = 1 ml) contained 1 mM 5-caffeoylquinic acid (5CQA), 3.5 mM SDS, and 50 mM Na–Pi, pH 6. The reaction was initiated with the addition of 5CQA and the rate of oxygen uptake over the first minute was used to calculate the activity. Experiments were carried out at least two times with separate plant extracts and typical results are presented.

3.3. PPO characterization

Activation of PPO by SDS was tested at a range of concentrations and in all subsequent activity measurements 3.5 mM SDS was included in the reaction mixture, with 1 mM 5CQA as substrate. Where present, inhibitors were dissolved in the reaction buffer (50 mM Na–Pi buffer, pH 6), at appropriate concentrations or added as concentrated solutions just before the addition of 5CQA.

For the determination of the optimum reaction temperature of coffee PPO, oxidation of 5CQA was followed spectrophotometrically at 420 nm (UV 2401PC-Shimadzu). In addition to the control of the cell cuvette temperature, the buffer was also previously chilled or heated. The initial 10–20 s interval was used to calculate the activity. All other PPO activity measurements were made with an oxygen electrode as described above.

The native M_r of coffee PPO was estimated with a S300-Sephacryl column (Pharmacia, 440 ml bed volume, flow rate of 2 ml min^{-1}) using the Sephadex G-25 desalted extracts stored at −30°C, as described above. Fractions (7 ml) were collected and assayed for protein concentration and PPO activity. The M_r markers were catalase (238 kDa), BSA (67 kDa), ovalbumin (44 kDa) and ribonuclease A (13.7 kDa).

PPO from leaves and coffee endosperms was incubated with proteinase K, trypsin and pepsin at room temperature (25°C). Proteases were added at 2:1 (wt/wt)

the total protein concentration in the extracts, i.e. 2 mg protease per mg extract protein. Incubations were carried out for 3 h or at different intervals, up to 24 h incubation, aliquots were taken and immediately assayed for the enzyme activity. When present, SDS was included in the incubation mixtures at a final concentration of 0.1%.

In another experiment, protein extracts were obtained with or without a cocktail of protease inhibitors (Complete, Boehringer Mannheim) and 1 mM phenylmethylsulfonyl fluoride (PMSF) in the extraction buffer. The proteins were pptd with $(NH_4)_2SO_4$ between 20 and 80% saturation, desalted in PD-10 columns and assayed for activity. Extracts obtained with protease inhibitors were incubated with proteases at room temperature (1:1, wt/wt, total protein in the extract/protease) for 3 h and together with the other extracts, they were used for activity-stained electrophoresis (partially denaturing, Robinson and Dry, 1992), using 12% polyacrylamide Tris–HCl Ready gels (BioRad) and a minigel system (Mini-Protean Cell, BioRad). The electrophoresis was carried out at 100 V, and 0.1% SDS was included in the tank buffer. For the activity staining, the gels were washed for 10 min with 50 mM Na–Pi buffer, pH 6.0, and the reaction carried out in the same buffer containing 2 mM 5CQA and 0.5 mM *p*-phenylenediamine. In addition, protein extracts obtained with and without protease inhibitors, as well as those incubated with trypsin, were fully denatured (5 min, 95°C), separated using the same electrophoretic conditions described before, then electrotransferred to PVDF membrane using a Mini Trans-Blot Transfer Cell (BioRad) and probed with grape antibodies (Rathjen and Robinson, 1992).

3.4. Wounding and methyl jasmonate induction

Both leaves of the second leaf pair (2–3 cm in length) of a branch with at least four leaf pairs were wounded by crushing with a haemostat (Constabel et al., 1995). The main vein was avoided and approx. 50% of the leaf blade was wounded. This was done in four plants and only these leaves were wounded in the plants. After 48 h in glasshouse conditions the leaves were collected for PPO measurement, using an oxygen electrode, as described above. As controls, leaves of the second pair of the opposite branch of the wounded branches were collected, as well as leaves of the second pair from unwounded plants. Care was taken to select branches with leaves of the second pair similar in length.

For methyl jasmonate treatment, a branch containing at least four leaf pairs (three plants) was enclosed with plastic bags, containing inside a small piece of cotton wool. Adhesive tape was used to seal the bag at the botton. A syringe was used to wet the cotton wool with 50 μl methyl jasmonate (Boehringer Mannheim) and the puncture made by the needle was immediately closed with adhesive tape. The plants were maintained under laboratory conditions and after 24 h a second 50 μl application was made. After a further 24 h the leaves of the first pair of the treated branches were collected for PPO activity determination with a oxygen electrode, as well as the first leaves of the opposite branches of the same plants and from untreated plants. Untreated branches were also enclosed in plastic bags.

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