Phytochemistry 69 (2008) 2297–2302

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

A wounding-induced PPO from cowpea (*Vigna unguiculata*) seedlings

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ARTICLE INFO

Article history:
Received 2 November 2007
Received in revised form 30 May 2008
Available online 31 July 2008

Keywords:
Vigna unguiculata
Leguminosae
Cowpea
Kinetic parameters
Wounding
Systemically-induced responses
Polyphenol oxidase

ABSTRACT

Polyphenol oxidases (PPO) are induced in cowpea plants by wounding. The highest activity levels were detected 48 h after this stimulus in both wounded and neighbor-to-wounded unifoliates of cowpea seedlings; the increase of activity was in the order of 13 to 15-fold, respectively, in comparison to control unifoliates. Multiple molecular forms of active PPO (Mrs 58, 73 and ≅220 kDa) were detected by partially denaturing SDS–PAGE. Wounding-induced cowpea PPO were extracted and purified through $(NH_4)_2SO_4$ precipitation and ion-exchange chromatography. The effects of substrate specificity, pH, thermal stability and sensitivity to various inhibitors – resorcinol, EDTA, sodium azide and tropolone – of partially purified soluble PPO were investigated. Purified wounding-induced cowpea PPO (wicPPO) showed the highest activities towards 4-methylcatechol (K_m = 9.86 mM, V_{max} = 24.66 EU [$\Delta A\ min^{-1}$]) and catechol (K_m = 3.44 mM, V_{max} = 6.64 EU [$\Delta A\ min^{-1}$]); no activity was observed towards L-tyrosine, under the assay conditions used. The optimum pH for wound-induced cowpea PPO was 6.0 with 4-methylcatechol as substrate. The enzyme was optimally activated by 10 mM SDS and was highly stable even after 5 min at 80 °C. The most effective inhibitor was tropolone, whereas addition of 10 mM of resorcinol, EDTA and sodium azide were able to reduce PPO activities by 40%, 15% and 100%, respectively.

1. Introduction

Polyphenol oxidases [PPO (EC 1.14.18.1 or EC 1.10.3.2)] are enzymes that catalyze the O_2-dependent oxidation of monophenols (e.g. tyrosinases) or *o*-diphenols (e.g. tyrosinases and laccases) to *o*-diquinones (Mayer and Harel, 1979). Several plant PPO genes encode mature proteins of 52–62 kDa, and transit peptides of 8–12 kDa which are responsible for the transport of the enzyme into the thylakoid lumen of chloroplasts (Demeke and Morris, 2002). Although PPOs were first found in plants almost one century ago (Bertrand, 1896), a complete knowledge of their role in such organisms is yet unresolved. Distinct functions have been attributed to PPO, such as: tissue browning (Boonsiri et al., 2007; Valentines et al., 2005), action in the Mehler reaction, electron cycling and/or oxygen regulation (Thipyapong et al., 2004), in plant protection against plagues, as well as against pathogens (Constabel and Ryan, 1998). However, a full comprehension of PPO function in plants is far from complete (Mayer, 2006). Reports on the presence of latent PPOs in several plant species have been published (Sellés-Marchart et al., 2007; Laveda et al., 2001; Chazarra et al., 1997; Moore and Flurkey, 1988). Plant tissue injury was also noted to trigger proteolytic activation of some latent PPOs, providing evidence for a protective function for these proteins during predator attacks (Mazzafera and Robinson, 2000; Laveda et al., 2001). The defensive role for foliar PPO against leaf-eating insects has been proposed in particular and well documented (Felton et al., 1989; Duffey and Felton, 1991). PPOs are defense-related proteins whose activities are most intensively induced in tomato plants submitted to either wounding and/or to methyl jasmonate (MeJa) vapour treatment (Constabel et al., 1995). A strong induction of PPO by wounding was also observed in tobacco (*Nicotiana tabacum*) and hybrid poplar (*Populus trichocarpaxdeltoides*) (Constabel and Ryan, 1998).

The natural ability of PPO to oxidise either phenols or polyphenols reflects its mechanism of action as a defense protein, since the derivatives, the diquinones can covalently link to and precipitate proteins, and are frequently more toxic to plant predators than the original phenols. These derivatives also confer anti-nutritional characteristics to vegetal tissues, inhibiting, as an example, the digestion of chewing insects (Felton et al., 1992). The increase of cell wall resistance is another aspect of the PPO defensive actions, since they are involved with melanin formation (Valverde et al., 1996), which forms polymerised, insoluble complexes with PPO-oxidised phenols generating a resistant barrier against the entry and spread of pathogens (Johnson et al., 2003). Transgenic plants expressing anti-PPO gene constructs form the basis of novel potato varieties highly resistant to bruising and confirm the catalytic involvement of PPO enzymes in this phenomenon (Bachem et al., 1994; Krohn et al., 1998; Coetzer et al., 2001). The defensive roles for this protein class were further established by the regulation of PPO expression through the octadecanoid pathway and by the wound signal systemin (Pearce et al., 1991; Constabel and Ryan,

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0031-9422/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2008.06.003

1998). However, a direct effect of induced PPO on insect performance requires further mechanistic studies as no significant increase in phenolic oxidation by ingested PPO in the gut contents of lymantriid caterpillars was observed (Barbehenn et al., 2007). This led the authors to question the actual efficacy of PPO as a defense against this group of insects. Since the importance of PPO as an antimicrobial defense is less prone to question, the authors admit that one role of induced PPO could be to inhibit opportunistic pathogens that enter leaves at wounding sites.

Cowpea (*Vigna unguiculata* L. Walpers) is an important crop in many tropical countries, including Brazil. Their green tissues and seeds are heavily attacked both in the field and in storage by cowpea chewing weevils (*Chalchodermus bimaculatus* and *Callosobruchus maculatus*) and by a great number of pathogens. It is thought that this high susceptibility to predation is associated with low levels of either constitutive or induced defenses (Gomes and Xavier-Filho, 1994). This legume was already fairly well studied in respect to its constitutive defense proteins, with reports on the presence of variant vicilins (Macedo et al., 1993), serine proteinase inhibitors (Xavier-Filho et al., 1989), glucan hydrolases (Gomes et al., 1996), LTPs, defensins (Carvalho et al., 2001) and cystatins (Fernandes et al., 1993). Induced defenses, however, have received much less attention and literature reports are limited to the studies on the induction of cystatin and PPO activity in these plants, when submitted to drought-stress conditions (Diop et al., 2004; Manivannan et al., 2007).

The induction of PPO by wounding in cowpea unifoliates is reported, for the first time, in the present paper. The enzyme has been isolated and kinetically characterized, using 4-methylcatechol (**1**) as substrate and at a range of SDS concentrations, at pH 6.0. PPO was noted to be induced by wounding, both as a local as well as a systemic strategy, which lasts some days after the wounding stimulus. Different isoforms of the enzyme seem to be involved.

2. Results and discussion

Enzymatic assays established an increase in PPO enzymatic activity 24 h after wounding in both wounded and neighbor-to-wounded cowpea unifoliates. The maximum induction obtained was reached 48 h after treatment for both wounded unifoliates (13-fold) and neighbor-to-wounded unifoliates (15-fold). At 72 h, both wounded and neighbor-to-wounded unifoliates still maintained high PPO levels, when compared to control unifoliates (0 h) (Fig. 1). For *Lycopersicum esculentum*, such an induction in wounded tissues was also around 18-fold (Constabel et al., 1995). In contrast, the wounding of potato leaflets resulted in a smaller 1.7-fold increase, 48 h after the stimulus (Thipyapong et al., 1995). In both wounded and unwounded leaves of wounded tobacco plants, the PPO activity increased more than 10-fold for both leaves in 48 h. Similar results were obtained with two-month-old hybrid poplar plants (Constabel and Ryan, 1998). In the present paper, the levels of PPO activity significantly increased in the soluble fraction, whereas no detectable differences were noticed in the pellet fraction from either wounded or neighbor-to-wounded unifoliates. Soluble PPO, besides being the major induced form of the proteins, is also the only one susceptible to activation by SDS (Table 1). SDS-activated soluble PPO had an initial 0.86 EU level for the control unifoliates and this increased to 8.72 and 10.83 at wounded and neighbor to wounded unifoliates, respectively. Since more than 90% of the total PPO activity from unifoliates was concentrated in the insoluble fraction (Table 1), the method employed for analysis of PPO induction is quite relevant. The increase in PPO activity was restricted to a soluble protein fraction what raises a suspicion on regularly employed methods for analysis of PPO induction from previous published data, as for example the survey performed by Constabel and Ryan (1998), which uses 0.1% Triton X-100 for protein extraction. As PPO weakly adheres to membranes, it can be easily released using the above detergent and quantification of total activity (soluble plus membrane-associated PPO) may mask the level of variations of minority isoforms of the enzyme.

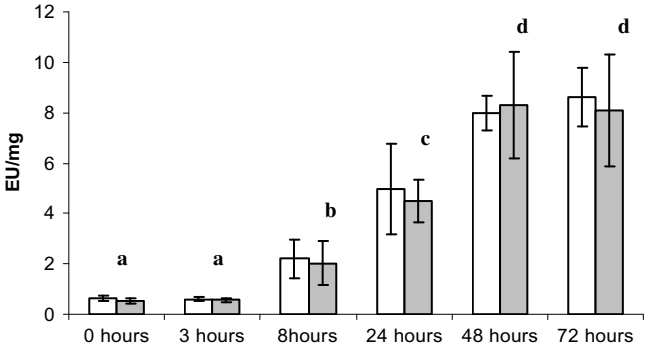

Fig. 1. Polyphenol oxidase activity of protein extracts from wounded unifoliates (white bars) and neighbor-to-wounded unifoliates (grey bars) from cowpea plants. One unit of enzyme activity (EU) was defined as an increase of 0.01 A units per min ($\Delta A\ min^{-1}$) (see Section 4). Each value represents the means (±SE), determined independently from samples of five wounded plants. Different letters indicate significantly different means at $P < 0.05$ according to the Student's test.

Table 1
PPO activities of soluble and insoluble PPO fractions in the presence and absence of SDS (EU = $\Delta A\ min^{-1}$)[a]

	EU/mg protein ± SE (% of total activity)		
	Control	Wounded	Neighbor to wounded
Without SDS			
Soluble PPO	0.46 ± 0.02 (4.8)	0.28 ± 0.07 (2.96)	0.66 ± 0.36 (7.51)
Insoluble PPO	9.22 ± 0.591 (95.20)	9.22 ± 0.88 (97.04)	8.14 ± 1.80 (92.49)
5 mM SDS			
Soluble PPO	0.86 ± 0.05 (10.64)	8.72 ± 1.04 (56.41)	10.83 ± 1.50 (60.96)
Insoluble PPO	7.26 ± 1.85 (89.36)	6.74 ± 0.22 (43.59)	6.93 ± 0.14 (39.04)

[a] PPO was assayed as described in Section 4, using 5 mM 4-methylcatechol (**1**).

Partially denaturing SDS–PAGE did not show any clear differences in the protein profiles of control, wounded, and neighbor-to-wounded unifoliates extracts (Fig. 2I). However, when the gel was incubated with catechol (**2**) for detection of PPO activity, an induction of protein bands with Mrs of 58, 73 and ≅220 kDa (Fig. 2IV) was readily observed in both wounded as well as in neighbor-to-wounded unifoliates of test plants (Fig. 2II; arrows). The 220 kDa form in the wounded sample seems to be more induced than in either neighbor-to-wounded or control samples. All bands were barely visible, however, when 1 mM tropolone (**9**) was added to the incubation medium (Fig. 2III). The isoforms of PPO from many plant sources were reported to range from 32 to over 200 kDa, mostly within the range of 35–70 kDa (Yoruk and Marshall, 2003). It is relevant to mention though that assignment of PPO multiplicity based on molecular weight estimates must be interpreted with caution. Interconversion of various forms of PPO determined under fully and partially denaturing electrophoretic conditions has largely been reported in the literature (Marques et al., 1995).

In order to isolate induced soluble PPO from the 48 h wounded unifoliates, an ethanol-clarified extract from this plant tissue (WUCE) was submitted to an ionic exchange chromatographic column. The major PPO activity was observed as an acidic protein, being retained to the matrix and eluted, using a stepwise gradient, with approximately 0.2 M NaCl (data not shown). Due to the high

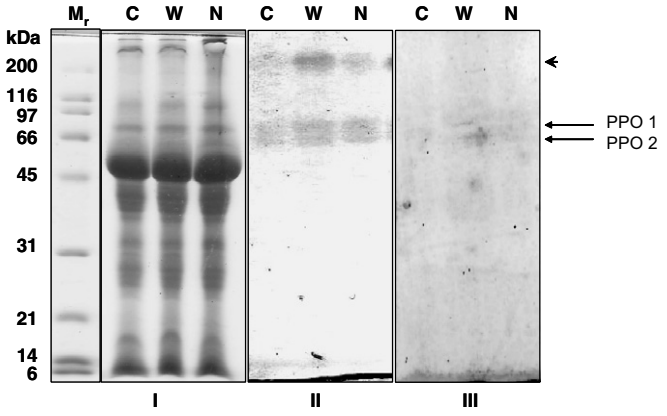

Fig. 2. SDS–PAGE (I) and PPO-activity gel in absence (II) or presence (III) of 1 mM tropolone (**9**) from unifoliates of control (C), wounded (W) and neighbor-to-wounded (N) cowpea plants. The arrows indicate two major PPO bands with M_r values of 58 and 73 kDa, determined through calculations based upon molecular weight markers (M_r) relative mobilities; arrowhead points to a high molecular mass activity band (ca. 220 kDa).

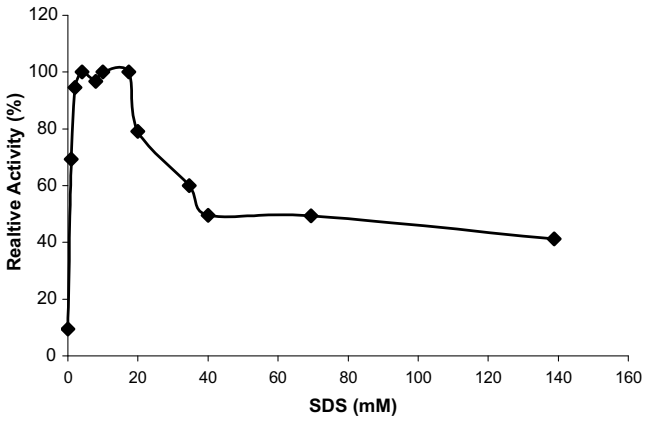

Fig. 3. Effect of SDS upon wicPPO activity, using 5 mM 4-methylcatechol (**1**) as substrate.

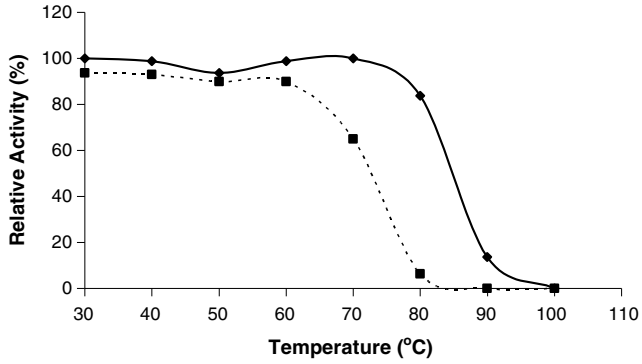

Fig. 4. Thermal stability of the wicPPO, in the presence (----) or absence (—) of 10 mM SDS.

homogeneity of this peak, it was further used for characterization of kinetic parameters and named as the wounding-induced cowpea PPO (wicPPO). As noted in Table 2, soluble PPO isolation procedures resulted in an 11.18-fold enrichment, going from a specific activity of 1.04 in the crude extract to 11.63 at the 0.2 M-salt eluted peak. A similar purification efficiency (11.59-fold purification) was reported by Gandia-Herrero et al. (2004) for a soluble beet root PPO.

The wounding-induced cowpea PPO (wicPPO) fraction was used for investigation of the influence of SDS as an activator of the enzyme. Since 1957, SDS was reported as an important activator for PPO inactive forms (Kenten, 1957). The PPO reported in the present work is a latent enzyme which was seen to be optimally activated (11-fold) by SDS concentrations varying from 4 to 17 mM (Fig. 3). Fifty percent of maximum activity was kept even in concentrations as high as 140 mM. The stability and activation of the wicPPO by SDS was very similar to that observed for Napoleon grape PPO (Núñez-Delicado et al., 2007). The degree of the SDS activation varied greatly with the plant material, going from a 4-fold increase in potato leaf (Sanchez-Ferreer et al., 1993) to a 119-fold in broad bean (Jiménez and García-Carmona, 1996). However, higher detergent concentrations significantly inhibited this enzyme class activity (Robb et al., 1964; Sanchez-Ferrer et al., 1993), making the wicPPO stability under such conditions a less usual behavior.

The wicPPO showed also a high thermal stability. Fig. 4 shows a profile of its thermal stability, either in the presence or absence of SDS. PPO activity was highly conserved (84%), even after 5 min under 80 °C, decreasing to 13.4% of the initial activity when under 90 °C, in the absence of SDS. In the presence of this detergent, the enzyme thermal stability was diminished; loss of enzyme activity (31%) was noted at 70 °C and at 80 °C it was abolished. There are many works reporting on the energy of activation for denaturation of PPO and a wide range of thermal stability has been reviewed by Yoruk and Marshall (2003). Similar to the data obtained in respect to SDS activation and stability, the thermal stability of the wicPPO was comparable to that from Napoleon grape (Núñez-Delicado et al., 2007), although this latter PPO had a slightly lower thermal stability.

For optimal pH determinations, the PPO activities were measured in the presence of 10 mM SDS and 5 mM of 4-methylcatechol (**1**), using either citrate–phosphate buffer (pH range from 3 to 6) or potassium phosphate buffer (pH range from 7 to 8). WicPPO had no activity at pH 3; a significant activity increase was observed from pH 5.5, and a maximum activity was reached at pH 6.0; by contrast, from pH 7.0 to 8.0, the wicPPO activity decreased to ~60% of maximum activity (data not shown). WicPPO has shown a pH-dependent activity profile very similar to a latent PPO from beet root (Gandía-Herrero et al., 2004), to a loquat fruit PPO (Sellés-Marchart et al., 2006), and to a PPO from broccoli florets (Gawlik-Dziki et al., 2007).

The substrate specificity of wicPPO (Table 3) was analyzed using seven different substrates **1**–**7**. The highest oxidation activity was found towards 4-methylcatechol (**1**) and catechol (**2**), whereas the other substrates had a much lower rate of oxidation. 4-Methylcatechol (**1**) was the very best substrate found. No activity was

Table 2
Purification table of the wounding-induced cowpea PPO (wicPPO) (EU = ΔA min^{-1})

Isolation step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg protein)	Yield (%)	Purification (fold)
Crude extract	110	4.9	539.22	56.100	1.04	100	1
35–80% $(NH_4)_2SO_4$	7.5	1.57	11.80	4.050	3.43	7.22	3.3
P-0.2 DEAE-cellulose peak	4	0.62	2.48	2.880	11.63	5.4	11.18

Table 3
Values of specific activity of wicPPO using several distinct substrates[a] (EU = ΔA min^{-1})

Substrate	Wave length (nm)	Specific activity (EU/mg of protein)	Activity relative to 4-methylcatechol (%)
Di- or triphenols			
4-methylcatechol (**1**)	410	0.013	100
Catechol (**2**)	410	0.0113	87
Pyrogallol (**3**)	400	0.0012	9.24
Chlorogenic acid (**4**)	400	0.0008	6.15
L-Dopa (**5**)	475	0.00015	1.15
(−)-Catechin (**6**)	380	0.0001	0.77
Monophenol			
L-Tyrosine (**7**)	475	0	0

[a] All assays were carried out as described Section 4.

detected when using L-tyrosine (**7**) as substrate under the assay conditions, suggesting the absence of any monophenolase activity. However, cases have been discussed in the literature where activation by *o*-diphenols of the monophenolase activity of PPO, as shown for *Dolichos lablab* PPO (Gowda and Paul, 2002), is required prior to detection. Also, a different substrate, such as 4-hydroxyanisole (Fan and Flurkey, 2004), was able to detect monophenolase activity when no activity towards L-tyrosine (**7**) was observed. The highest specificity towards 4-methylcatechol (**1**), catechol (**2**) and pyrogallol (**3**) indicates an improved affinity towards small substrate molecules. These preferential substrates of the wicPPO resemble those found for hybrid poplar PPO, which is also induced by wounding as well as by MeJa (Constabel et al., 2000; Wang and Constabel, 2003) and for a PPO from chloroplasts of *Vigna radiata* leaves (Shin et al. 1997).

K_m and V_{max} values were calculated from the Lineweaver–Burk plot, using the most efficiently catalyzed substrates 4-methylcatechol (**1**) and catechol (**2**). Although the K_m for catechol (**2**) was ~3.44 and towards 4-methylcatechol (**1**), ~9.86, a higher catalytic efficiency was attributed towards 4-methylcatechol (**1**) ($V_{max}/K_m = 2.5$). By contrast, the V_{max}/K_m ratio when using catechol (**2**) was ~1.93. The K_m value for 4-methylcatechol (**1**) differs from the 4 mM value found for *V. radiata* PPO (Shin et al., 1997). However, the relative activity towards 4-methylcatechol (**1**), in that case, was also higher than that of catechol (**2**), suggesting a similar chloroplastidic origin for the wicPPO, although the enzyme characterized by Shin et al. (1997) did not undergone activation by SDS, being fully active in native state.

The effects of a set of inhibitors on the activity of wicPPO were measured, using 5 mM of 4-methylcatechol (**1**) as substrate (Table 4). The most potent inhibitor found was tropolone (**9**), a competitor of Cu^{2+}, which is described as one of the most powerful specific PPO inhibitors (Khan and Andrawis, 1985). It reduced the wicPPO activity by 50%, when used at a low 0.01 mM concentration. Inhibition by thiol compounds is attributed to either binding to the active centre of PPO or due to the stable colorless products formed through additional reactions with *o*-quinones (Gawlik-Dziki et al., 2007). Resorcinol (**8**) was a competitive inhibitor resembling the structure of the substrates for PPO. Knapp (1965) showed that resorcinol competitively inhibits chlorogenic acid (**4**) oxidation by avocado PPO and acts directly on the active center of reaction. However, at the concentrations used, this inhibitor was only able to reduce to 40% the initial wicPPO activity. The chelating agents EDTA and sodium azide reduced the initial PPO activity by 15% and 100%, respectively, when used at maximum 10 mM concentration. Sodium azide toxicity towards a metal enzyme, especially in the case of a copper enzyme, is mainly due to its strong coordination ability with the metal within the active site, which provokes changes in the coordination number and conformation of the active site and depredates the active center metal (Dogan et al. 2005).

Table 4
Inhibition of the wicPPO by different inhibitors at increasing concentrations[a]

Inhibitors	PPO Inhibition (%)			
	0.01 m	0.1 mM	1 mM	10 mM
Resorcinol (**8**)	–	20	20	40
EDTA	–	15	15	15
Sodium azide	–	20	20	100
Tropolone (**9**)	50	70	77	–

[a] PPO was assayed as described in Section 4, using 5 mM 4-methylcatechol (**1**) and 5 mM of SDS.

3. Concluding remarks

In the present work, the increase of PPO activity was detected after wounding of cowpea unifoliates, both at the local of lesion (13-fold) as well as at neighboring sites (15-fold). The increased PPO levels were restricted to a soluble fraction extracted from these tissues, what suggests that induced isoforms refer to non-membrane bound chloroplastidic PPO. A soluble catecholase PPO activity was previously reported as being induced by chilling, but such an enzyme was fully activated in absence of SDS and represented the most active fraction from eggplant fruit when compared to the insoluble fraction (Concellón et al., 2004). In the present paper, the insoluble pellet fraction from cowpea unifoliates was noted to possess the majority of the total PPO activity, but this was not altered by a mechanical stimulus. Isolated wounding-induced cowpea PPO (wicPPO) was also observed to behave majorly as a catecholase, with its activity mainly towards *o*-diphenols. The enzyme was highly thermostable and activated in the presence of SDS. WicPPO was kinetically characterized using 4-methylcatechol (**1**) as its most specific substrate.

4. Experimental

4.1. General experimental procedures

PPO substrates, inhibitors and DEAE-cellulose were from Sigma Chemicals. EtoH used for extraction was of chemical grade and other reagents were of analytical grade.

4.2. Plant material and wounding treatment

Plants were grown under greenhouse conditions, with a dark period of 6 h at 25 °C, and a light period of 18 h at 30 °C. Light intensity during the light period was of 100 micromols photons m^{-2} s^{-1}. Cowpea (*V. unguiculata* (L.) Walp) seeds of the EPACE-10 cultivar were supplied by the Centro de Ciências Agrárias of the Universidade Federal do Ceará, Fortaleza, Brazil.

Seedlings (5 days-old) were wounded by using a hemostat in an unique unifoliate and kept at a constant 100 micromols photons m^{-2} s^{-1} light, as well as the non-wounded control plants. Wounded unifoliates, neighbor-to-wounded unifoliates as well as unifoliates from non-wounded control plants were collected 0, 3, 8, 24, 48 and 72 h after wounding treatment, and analyzed for PPO activity induction.

4.3. Protein extraction and PPO assays

Sample unifoliates were ground in 50 mM sodium phosphate buffer pH 5.3 (1:2 w/v ratio) containing 5% PVPP, 1 mM EDTA and 2 mM PMSF and submitted to centrifugation (20,000*g*,

20 min, 4 °C). The PPO enzymatic activity present in the supernatant was designated as being soluble protein (adapted from Sanchez-Ferrer et al., 1989). The 20,000*g* pellet was solubilized in 10 mM potassium phosphate buffer (pH 6.5) containing 1% Triton X-100. After centrifugation (60,000*g*) for 15 min at 4 °C, the supernatant was collected and designated as the insoluble PPO fraction (adapted from Sanchez-Ferrer et al., 1989). Soluble and insoluble PPO fractions were then analyzed in respect to total protein levels, as measured by the Bradford method (1976) as well as for PPO activity; the latter was determined following a protocol adapted from Shin et al. (1997). The reaction mixture (1 ml) contained enzyme fraction (20 μl) and 100 mM phosphate buffer (pH 6.0). Each sample was aerated for 2 min in a test tube followed by addition of 4-methylcatechol (**1**) as the substrate at a final concentration of 20 and 5 mM SDS as enzyme activator. SDS was either included or omitted when analyzing the specific activation of soluble and insoluble PPO fractions. Reactions were carried out at room temperature with absorbance readings taken immediately after substrate and activator addition. PPO activity was expressed as changes in absorbance (A) at 410 nm min^{-1} mg^{-1} of protein and one unit of enzyme activity (EU) was defined as a variation of 0.01 A units per min ($\Delta A\ min^{-1}$) (Shi et al., 2002). For statistical significance, Student's *t*-test values were calculated.

4.4. Active staining of polyphenol oxidase

After extraction, protein samples were prepared in Laemmli buffer (Laemmli, 1970) and were not boiled prior to loading. Separation of proteins was performed through partially denaturing SDS-PAGE, after running under cold conditions (6 °C). The PPO was detected by staining with 100 mM sodium phosphate buffer (pH 6.0) containing 5 mM 4-methylcatechol (**I**) on a rotary shaker. After 3 min incubation, dark bands indicative of PPO isozymes appeared in the gel (Mohammadi and Kazemi, 2002). Addition of 1 mM tropolone (**9**) to the incubation medium was alternatively used as control for specific prevention of the colored oxidative PPO reaction. For each gel activity, a similar gel, stained by Coomassie Blue, was made in order to determine molecular weight of active bands. The data for standard protein mobility was used to build a graph of the relationship between the molecular weight of standard proteins and their relative mobility.

4.5. PPO isolation

The crude extract from wounded unifoliates was submitted to $(NH_4)_2SO_4$ precipitation, by adding the salt to 30% saturation. After overnight precipitation and centrifugation at 10,000*g* for 30 min, the pellet was discarded and the supernatant was re-precipitated to 80% saturation, according to Shi et al. (2002). The 80% saturated fraction was desalted by suspension in cold 30% EtOH, at a ratio of 3:1 (v/v) and centrifuged at 20,000*g* for 10 min at 4 °C. The pellet was dried at 8 °C for 24 h and suspended in a 100 mM sodium phosphate buffer pH 7.0, 1% Triton X-100, also employed as equilibration buffer for the next ion exchange chromatographic step. This wounded-unifoliate clarified extract (WUCE) was loaded onto a DEAE-cellulose column and the chromatography was performed stepwise, being eluted with 50 ml of each 0.1, 0.2 and 0.5 M NaCl. The major PPO activity peak (fractions 34, 35 and 36), identified by enzymatic assay as described at Section 4.3, was exhaustively dialysed against H_2O, lyophilized and stored at −20 °C. This was named as the cowpea wounding-induced PPO (wicPPO).

4.6. Kinetic parameters

All following kinetic determinations have employed a 20 μl volume of wicPPO, which corresponds to ca. 15 EU.

4.6.1. SDS activation and stability

The activator effect of SDS upon the wicPPO was tested by using 5 mM 4-methylcatechol (**1**) as substrate in 100 mM sodium phosphate buffer pH 6.0 and a range of SDS concentrations, which varied from 0 up to 140 mM. Soluble and insoluble forms of PPO from control, wounded and neighbor-to-wounded unifoliates were differentially analyzed for activation by SDS, being assayed in the absence or presence of a 5 mM solution of the detergent.

4.6.2. Thermal stability

The energy of activation for denaturation of the wicPPO was determined by measuring enzyme activity at various temperatures over a range of 30–100 with 10 °C increments, using a circulation water bath. After addition of substrate and buffer (as defined at Section 4.3), assay was incubated for 5 min at the diverse temperatures, being immediately cooled on ice after such time. The incubation was performed in the presence or absence of 10 mM SDS.

4.6.3. Optimum pH

The optimum pH for wicPPO activity was measured using 100 mM citrate-phosphate buffer at a range of pH from 3 up to 6.5 and 100 mM K–Pi buffer at a pH range varying from 7 up to 8. All assay conditions were the same as at 4.3.

4.6.4. Substrate specificity and K_m and V_{max} determination

The determination of wicPPO substrate specificities was made by using the substrates 4-methylcatechol (**1**), catechol (**2**), pyrogallol (**3**), chlorogenic acid (**4**), L-3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA) (**5**), (−)-catechin (**6**) and L-tyrosine (**7**). All determinations were made using 100 mM sodium phosphate buffer pH 6.0, 5 mM SDS and every substrate was assayed at the concentration of 5 mM. Measurements were taken at each min from the 1st to the 5th min of reaction. K_m and V_{max} were calculated from the data obtained with the substrates 4-methylcatechol (**1**) and catechol (**2**) at 1, 2, 4, 6, 8, 12, 16 mM increasing concentrations.

4.6.5. Inhibitory activity assays

Four inhibitors were used for inhibition tests towards wicPPO: EDTA, resorcinol (**8**), sodium azide and tropolone (**9**). The concentrations of inhibitors used in the assays were 0.01, 0.1, 1 and 10 mM. The conditions of assays were the same used for the assays described at 4.3.

Acknowledgements

This study is a part of the Doctorate degree thesis of M.S.T.P., carried out at the Universidade Estadual do Norte Fluminense Darcy Ribeiro. We acknowledge the financial support of the Brazilian agencies CNPq, FAPERJ and CAPES, and of the Universidade Estadual do Norte Fluminense supporting body FENORTE.

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