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Purification and Biochemical Characterization of Polyphenol Oxidases from Embryogenic and Nonembryogenic Cotton (*Gossypium hirsutum* L.) Cells

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Abstract Polyphenol oxidases (PPOs) were isolated from cell suspensions of two cultivars of cotton (*Gossypium hirsutum* L.), and their biochemical characteristics were studied. PPO from Coker 312, an embryogenic cultivar, showed a highest affinity to catechol 20 mM, and PPO from R405-2000, a nonembryogenic cultivar, showed a highest affinity to 4-methylcatechol 20 mM. The optimal pH for PPO activity was 7.0 and 6.0 for Coker 312 and R405-2000, respectively. The enzyme had an optimal temperature of 25 °C and was relatively stable at 20–30 °C. Reducing sodium metabisulfite, ascorbic acid, dithiothreitol, $SnCl_2$, and $FeCl_3$ markedly inhibited PPO activity, whereas its activity was highly enhanced by Mg^{2+}, Ca^{2+}, and Mn^{2+} and was moderately inhibited by Ba^{2+}, Cu^{2+}, and Zn^{2+}. The analysis revealed a single band on the sodium dodecyl sulfate polyacrylamide gel electrophoresis which corresponded to a molecular weight of 55 kDa for Coker 312 and 42 kDa for R405-2000.

Keywords Cell suspension · Characterization · Cotton · *Gossypium hirsutum* L. · Polyphenol oxidase · Purification

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Introduction

Polyphenol oxidase (PPO; monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, EC 1.14.18.1), which is also known as tyrosinase, phenolase, catechol oxidase, catecholase, monophenol oxidase, *o*-diphenol oxidase, and orthophenolase according to its substrate specificity [36, 52], is widely distributed in the plant kingdom. It plays an important role in browning reaction in fruits and vegetables [42].

Enzymatic activity occurs in plants via phenolic compounds which are oxidized to *o*-quinones in the presence of molecular oxygen by PPO. The *o*-quinones are then polymerized to pigments [15]. PPO has monophenolase and diphenolase activities. Monophenolase activity is the hydroxylation of monophenols to *o*-diphenols, whereas diphenolase activity is the oxidation of *o*-diphenols to quinines [7, 35].

PPO has been studied in several plant tissues, such as bananas [64], plums [47], and tea leaves [22]. Several authors suggested that it might be associated with many important functions of plants such as defense, growth, cell differentiation, and somatic embryogenesis [5, 21].

So far, there have been very few reports on the PPO of cotton. Cotton as a main cash crop is widely cultivated in many countries and is one of the most important fiber crops in the world [67]. *Gossypium hirsutum* L. is the principal species currently cultivated on the world market. Genetic improvement of cotton through conventional breeding is limited by several factors such as lack of incompatibility barriers and the long time periods that are required [39]. Although plant biotechnology is an attractive means for improving cotton, its use requires an effective in vitro culture system from somatic plant tissues. Plant regeneration through somatic embryogenesis is an ideal system of the process of cell differentiation since it offers appropriate material for genetic transformation [14]. Somatic embryogenesis in cells is genetically dependent [19]. Genotypic variations in plants are expressed by different forms of metabolic expression in these plants. Relationships have been established between somatic embryogenesis and the activity of glucose metabolism enzymes [53], protein content [49], and phenolic compounds [31]. While Kishor et al. [27] and Kouakou [29] have found that PPO activity significantly changed during somatic embryogenesis, there have been no further studies on its isolation and characterization.

In the present study, PPO was extracted from cotton cell suspensions, and some of its characteristics were examined. In addition, enzyme inhibition was investigated by using some ions and inhibitors.

Materials and Methods

Plant Material and In Vitro Seed Germination

Two cultivars of cotton (*G. hirsutum* L.) were used. Coker 312 seeds were obtained from Centre de Coopération Internationale en Recherche Agronomique pour le Développement, France, and R405-2000 seeds from Centre National de Recherche Agronomique, Ivory Coast (West Africa). The germination conditions were those described previously [31]. Briefly, seeds were pre-treated for 1 min using 70% ethanol, surface-sterilized in a 2.5% aqueous solution of sodium hypochlorite for 20 min, and rinsed three times with sterile double-distilled water. They were then sown without coats in tubes containing autoclaved half-strength Murashige and Skoog (MS) [40] salts supplemented with vitamin B5, 30 g/l sucrose, and 0.75 g/l $MgCl_2$ and solidified with 2.5 g/l gelrite. They were then

placed in culture tubes and incubated in the dark at 28±2 °C for 3 days to initiate germination, and seedlings were obtained after 4 days with a 16/8 h photoperiod.

Callus and Cell Suspension Cultures

Hypocotyls of 7-day-old sterile seedlings were cultivated in a 250-ml Pyrex flask containing MS medium including B5 vitamins [17], 30 g/l glucose, 0.5 mg/l kinetin, 0.1 mg/l 2,4-D, and solidified with 2.5 g/l gelrite and 0.75 g/l $MgCl_2$ Murashige and Skoog medium for calli culture (MSC). Calli were maintained and stabilized through monthly subcultures on the same medium. Friable and well-grown calli were used to initiate cell suspensions.

Approximately 2 g of callus was placed in a 250-ml Erlenmeyer flask containing 50 ml of the above medium, without gelling agent (MSL0). The suspensions were placed on an orbital shaker at 110 rpm for 4 weeks (primary culture). The resulting cell suspension was sieved through a 250-μm mesh and the filtrate refreshed with MSL0 medium containing 40 g/l glucose, 1.9 g/l KNO_3, and 0.5 mg/l casein hydrolysate (MSL1). The second subculture was performed by sieving cells from the first subculture on a 150-μm mesh sieve. Cells collected were resuspended with MSL1 medium at the concentration of 40 mg/ml under the same culture conditions previously described. Cell fractions obtained after sieving on a 100 μm mesh were resuspended in MSL1 medium and incubated in the above-mentioned conditions to obtain the third subculture. Samples of each subculture were examined with a stereomicroscope to detect the formation of embryogenic structures. All cultures were kept in a room at 28±2 °C during 24 h photoperiod (16 h light/8 h dark). Illumination was supplied by cool white fluorescent tubes at approximately 2,000 lx intensity.

PPO Extraction and Purification

The cells of each suspension culture of both cotton cultivars (primary culture stage, first, second, and third subculture stages) were frozen immediately after harvesting, freeze-dried, and powdered. Enzyme extracts were prepared so that PPO activity was at the highest level (Table 1). Two grams of cells was extracted according to Mazzafera and Robinson [38] with some modifications. PPO was extracted with 0.1 M sodium phosphate buffer (pH 6.5), at 4 °C. The crude extract samples were centrifuged at 32,000×*g* for 20 min at 4 °C. Solid ammonium sulfate was added to the supernatant to obtain 80% saturation. The precipitated PPO was separated by centrifugation at 32,000×*g* for 20 min. The precipitate was dissolved in 0.1 M sodium phosphate buffer (pH 6.5). The enzyme extract was extensively dialyzed against the same buffer at 4 °C overnight. To conduct further purification of PPO, the dialyzed solution was lyophilized, dissolved again in a small volume of 0.1 M sodium phosphate buffer (pH 6.5), and applied to a Sephadex G-200 column balanced with 0.1 M sodium phosphate buffer (pH 6.5). The enzyme solution was eluted with the same buffer and fractions with the highest activity were pooled and lyophilized. After dissolution in a small volume of 0.1 M sodium phosphate buffer (pH 6.5) with a final concentration of 1.0 M sulfate ammonium added, the fraction was loaded onto a phenyl sepharose (fast flow) column equilibrated with buffer A (0.1 M sodium phosphate, 1.0 M ammonium sulfate, 1.0 M KCl, pH 6.5). The PPO was eluted with a gradient of 0% to 100% buffer A. The fraction was loaded on to a phenyl sepharose column showing PPO activity and was collected, lyophilized, and dissolved in a small volume of 0.1 M sodium phosphate buffer (pH 6.5). After overnight dialysis against the same buffer, the dialysate was collected and used as the PPO enzyme source.

Table 1 Influence of buffer type and molarity on the activity of PPO from cotton cell suspensions.

Buffer composition molarity (pH 6.5)	PPO activity (nkat/g dw)	
	Coker 312	R405-2000
Sodium phosphate		
0.05 M	9.94±0.39	4.28±0.35
0.1 M	10.86±0.41	5.22±0.23
0.2 M	9.52±0.50	4.15±0.26
0.3 M	7.75±0.33	3.94±0.21
0.5 M	6.81±0.27	3.37±0.19
Citrate phosphate		
0.05 M	8.11±0.44	3.57±0.21
0.1 M	9.48±0.41	3.71±0.25
0.2 M	10.02±0.36	4.30±0.30
0.3 M	8.15±0.50	3.20±0.20
0.5 M	5.73±0.35	2.47±0.11
Sodium acetate		
0.05 M	7.59±0.21	3.12±0.23
0.1 M	9.85±0.38	3.90±0.21
0.2 M	9.33±0.40	3.40±0.15
0.3 M	7.11± 0.21	2.91±0.19
0.5 M	5.94±0.34	2.28±0.24
Tris–HCl		
0.05 M	6.68±0.36	2.55±0.15
0.1 M	7.20±0.31	3.11±0.27
0.2 M	8.66±0.40	4.26±0.31
0.3 M	6.73±0.29	2.89±0.24
0.5 M	5.18±0.37	2.17±0.12

PPOs extracted with 0.1 M sodium phosphate buffer, pH 6.5 had the highest activity. Catechol was used as PPO substrate at 10 mM. Values represent the mean of three replicates. In a line and a column, values followed by the same letter are not significantly different (test of Newman Keuls at 5%).

Assay for PPO Activity

PPO activity was determined by measuring the initial rate of *o*-quinones formation as indicated by an increase in absorbance at 420 nm [8]. A Mitton Roy spectrophotometer (Spectronic 601) was used throughout the investigation. PPO activity was assayed in triplicate. The final reaction mixture contained 2.95 ml of 10 mM catechol solution in 0.1 M sodium phosphate buffer pH 6.5 and 50 μl of the enzyme solution. Molarity of the reaction buffer was selected as indicated in Table 2. The blank sample contained only 3 ml of substrate solution. The reaction was carried out at various temperatures and pH values with the substrates mentioned as follows. One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001/min [18]. The linear portion of the absorbance vs time curve was used to determine the initial rates [61]. PPO activity was expressed in nkat/g dw (nmol substrate converted/s/g dw).

For each cotton cultivar, the stage of cell suspension cultures where PPO activity was at the highest level was used in the following experiments.

Determination of Molecular Weight

The molecular weight of purified enzyme was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A Bio-Rad Miniprotean III Electrophoresis Cell was used for electrophoretic analysis. SDS-PAGE was performed according to the method of Laemmli [33]. Standard protein markers were bovine serum albumin (66 kDa),

Table 2 Influence of extraction buffer composition on the activity of PPO from cotton cell suspensions.

Buffer composition (0.1 M, pH 6.5)	PPO activity (nkat/g dw)	
	Coker 312	R405-2000
Sodium phosphate (control)	10.83±0.26 a	5.28±0.13 c
Sodium phosphate + PVP (0.5%)	11.94±0.44 ab	5.96±0.31 cd
Sodium phosphate + Triton X-100 (1%)	11.10±0.20 a	5.69±0.16 c
Sodium phosphate + sodium thiosulfate (0.25%)	12.10±0.39 ab	6.02±0.17 cd
Sodium phosphate + EDTA (1 mM)	11.68±0.33 ab	5.91±0.34 cd
Sodium phosphate + Triton X-100 (1%) + PVP (0.5%) + EDTA (1.0 mM) + sodium thiosulfate (0.25%)	13.91±0.48 b	6.86±0.27 e

PPOs were extracted with 0.1 M sodium phosphate buffer at pH 6.5. Addition of 1% Triton X-100, 0.5% PVP, 1.0 mM EDTA, and 0.25% sodium thiosulfate increased PPO activity to 30%. Catechol was used as PPO substrate at 10 mM. Values represent the mean of three replicates. In a line and a column, values followed by the same letter are not significantly different (test of Newman Keuls at 5%).

ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and lactoglobulin (14 kDa). PPO extract was mixed with bromophenol blue before being applied to 12.5% polyacrylamide gel. Proteins were stained with Coomasie Blue. The relative mobility of proteins was calculated, and molecular weight was estimated by comparison to molecular weight markers.

Enzyme Characterization

The specificity of cotton cell suspension PPO extracts was investigated for eleven commercial-grade substrates at 10 mM concentrations. The activity was determined using substrates by measuring the increase in absorbance at 420 nm for catechol [8, 26], 410 nm for 4-methylcatechol [24], 470 nm for L-dopamine [60], 380 nm for catechin [60], 334 nm for pyrogallol [24], 500 nm for gallic acid, 412 nm for tetramethylbenzidine, 472 nm for L-tyrosine [24], 470 nm for caffeic acid, 415 nm for ferulic acid, and 400 nm for *p*-coumaric acid [60]. To assess the effect of substrate concentration on PPO activity, PPO activity was measured using the substrate in which PPO activity was the highest. The final substrate concentration varied from 0 to 40 mM. The results obtained were used for the following experiments.

Effect of pH on Enzyme Activity and Stability

PPO activity as a function of pH was determined in a pH range of 2.0 to 10.0 in 0.1 mM sodium phosphate buffer. PPO activity was measured according to the method described above and expressed as a percentage of the maximum activity. The enzyme was analyzed for pH stability ranging 4.0 to 9.0 for 60 min at 25 °C. The pH value corresponding to the highest enzyme activity was taken as the optimal pH and used in all other studies.

Effect of Temperature on Enzyme Activity

The optimal PPO temperature was sought at various temperatures between 4 and 80 °C. The standard reaction mixture without the enzyme was heated to the appropriate temperature for 10 min. After equilibration of the reaction mixture at the selected temperature, the enzyme was added, and the enzyme activity was measured. The thermal stability was determined by heating the enzyme solution at various temperatures between

20 and 80 °C for 60 min at the optimal pH. The enzyme solution was rapidly cooled in ice, and the remaining activity was essayed in the above conditions. Residual PPO activity was expressed as relative to the maximal activity. The optimal temperature obtained was used in all subsequent experiments.

Effects of Inhibitors and Metal Ions on Enzyme Activity

The effects of several inhibitors (citric acid, ascorbic acid, NaCl, sodium metabisulfite, and dithiothreitol) and metal ions ($CaCl_2$, $MnCl_2$, $BaCl_2$, $ZnSO_4$, $CuSO_4$, $MgSO_4$, $FeCl_3$, and $SnCl_2$) on PPO activity were determined. PPO was preincubated for 10 min in buffer containing 1.0 and 10 mM of each inhibitor and metal ion. The residual enzymatic activity was measured under the above assay conditions in the presence and absence of inhibitors and metal ions.

Statistical Analysis

Data were analyzed using Statistica software (release 7.5). Differences in mean values were tested by analysis of variance, and significance levels were obtained with Newman Keuls's test. A significance level of <0.05 was used. Data are the means of three replicates.

Results and Discussion

Cell Suspensions

Cotton cell suspensions were obtained from calli derived from hypocotyl segments of plant and were subcultured on MSB1 medium for 1–4 months. Unlike 1-month-old cell suspension (primary culture stage in MSL1 medium) or 2- and 3-month-old cell suspensions in MSL2 medium (first and second subculture stage), which rarely contained any embryogenic structures, 4-month-old suspensions in MSL2 medium (third subculture stage) frequently contained numerous embryogenic structures. However, the development of embryogenic structures was observed only in Coker 312 cell suspensions but not in R405-2000 [30–32]. This result confirms the genotypic dependence of somatic embryogenesis in cotton cell cultures. Therefore, this confirmed the beneficial effects of the removal of hormones and the addition of KNO_3 and casein hydrolysate on the induction of embryogenic structures in Coker reported by others [54, 62].

PPO Assay and Determination

Figure 1 shows the evolution of PPO activity in Coker 312 and R405-2000 cell suspensions. At the beginning, the activity was identical but then increased rapidly in Coker 312 to reach its high level at the third subculture (9.91 nkat/g dw). In R405-2000, it doubled from the primary (1.89 nkat/g dw) to the first subculture (3.69 nkat/g dw) but then remained almost constant until the third subculture (4.40 nkat/g dw). Therefore, the increase in PPO activity in cotton cell suspensions might be due to the induction of embryogenic structures. Kouadio et al. [28] also observed an increase in some enzyme activities during embryo formation in cotton cell suspensions and concluded that somatic embryogenesis is generally linked to biochemical processes.

For each cotton cultivar, the cell suspension resulting from the third subculture, where PPO activity was at its highest level, was used for the following experiments.

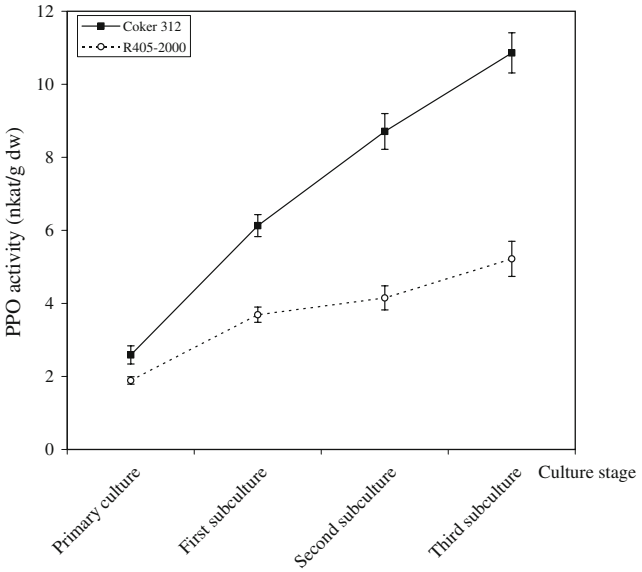

Fig. 1 Evolution of PPO activity during cotton cell suspension cultures. PPO activity was essayed in triplicate measurements with 10 mM catechol as substrate and 0.1 M sodium phosphate as buffer. pH of reaction mixture was 6.5. Coker 312 was an embryogenic cotton cultivar and R405-2000 a nonembryogenic cotton cultivar. PPO activity increased from primary culture to third subculture, and the highest PPO activity was obtained in third subculture. PPO activity of Coker 312 was strongest than that of R405-2000

Determination of Molecular Weight of Cotton PPO

SDS-PAGE analysis of the molecular weight and purity of the PPO (Fig. 2) revealed only one major protein band detected under the staining condition, which corresponds to a molecular weight of 55 kDa for Coker 312 (PPO-I) and 42 kDa for R405-2000 (PPO-II). Therefore, these isoenzymes were different, thus explaining the difference in behavior

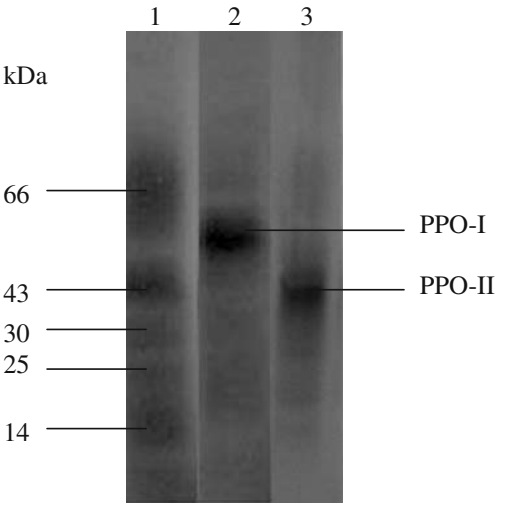

Fig. 2 SDS-PAGE of the purified PPO from third subculture of cotton cell suspensions. *Lane 1* Molecular mass of standard protein markers; *lane 2* Coker 312, and *lane 3* R405-2000. PPOs of Coker 312 and R405-2000 were purified. Molecular weight of PPO from Coker 312 was 55 kDa (*PPO-I*), whereas molecular weight of PPO from R405-2000 was revealed at 42 kDa (*PPO-II*)

observed above between Coker 312 and R405-2000. These data reinforce the findings of other teams indicating that the molecular weight of PPO ranges from 40 to 67 kDa [7, 9, 59]. It has been shown that PPO is synthesized as a 60–65 kDa protein, which can be converted to a 30–60 kDa form by proteolysis, as has been found in carrot and lettuce [7, 50].

Selection of Conditions for Enzyme Essay

Several buffer compositions were used to select the most suitable for extracting PPO from cotton cell suspensions (Table 1). PPOs extracted with sodium phosphate buffer at 0.1 M had the highest activity. While tanning reactions during enzyme extraction can cause partial inactivation of the enzyme, such reactions can be partially prevented in the presence of reducing agents or phenol-adsorbing agents like polyethylene glycol, polyamine, or polyvinyl pyrrolidone (PVP) [36]. Furthermore, the possible inhibiting action of certain metal ions can be avoided by adding to the extraction buffer a small quantity of ethylene diamine tetraacetic acid (EDTA). Lastly, sodium thiosulfate can be added to the mixture to protect the enzymatic sites [29]. In the present study, the use of each compound alone was not enough to maximize the PPO extraction, so the extracted PPO activity was low. However, addition of 0.5% PVP, together with 1% Triton X-100, 0.25% sodium thiosulfate, and 1 mM EDTA in sodium phosphate buffer (0.1 M, pH 6.5), considerably improved the extraction of PPO activity (Table 2), leading to a 30% increase in activity. An increase in enzyme activity by these compounds has been reported by several authors [29, 68]. Therefore, a 0.1 M sodium phosphate buffer containing 0.5% PVP, 1% Triton X-100, 0.25% sodium thiosulfate, and 1 mM EDTA was suitable to extract PPO from cotton cell suspensions and was therefore used for the following enzyme assays.

Substrate Specificity and Concentration

Extracted PPO from different sources has been shown to have varying substrate specificity, as reported by several authors [11, 57, 61]. The oxidizing ability of the PPO of both cotton cultivars was determined using monophenols, diphenols, and triphenols as substrates. PPO

Table 3 Substrate specificity on the activity of PPO from cotton cell suspensions.

Substrate (10 mM)	Activity of PPO (nkat/g dw)	
	Coker 312	R405-2000
Control	0±0.00 a	0±0.00 a
Monophenols		
p-Coumaric acid	0±0.00 a	0±0.00 a
L-Tyrosine	0±0.00 a	0±0.00 a
Ferulic acid	0.25±0.03 b	0.17±0.01 c
Caffeic acid	0.72±0.04 c	0.40±0.02 c
Gallic acid	1.40±0.05 d	0.91±0.02 e
Diphenols		
Catechol	13.85±0.29 e	6.91±0.12 g
4-Methylcatechol	10.10±0.11 f	8.07±0.18 g
Catechin	7.29±0.20 g	4.89±0.19 f
L-Dopamine	5.27±0.16 h	3.34±0.15 f
Triphenols		
Pyrogallol	2.73±0.14 i	1.15±0.06 h
Tetramethylbenzidine	2.17±0.10 i	1.10±0.03 h

PPOs were extracted with 0.1 M sodium phosphate buffer at pH 6.5 containing 1% Triton X-100, 0.5% PVP, 1.0 mM EDTA, and 0.25% sodium thiosulfate. Substrates were used at 10 mM, and catechol was found to be the best substrate of PPO from Coker 312 and 4-methylcatechol to be the best substrate of PPO from R405-2000. Values represent the mean of three replicates. In a line and a column, values followed by the same letter are not significantly different (test of Newman Keuls at 5%).

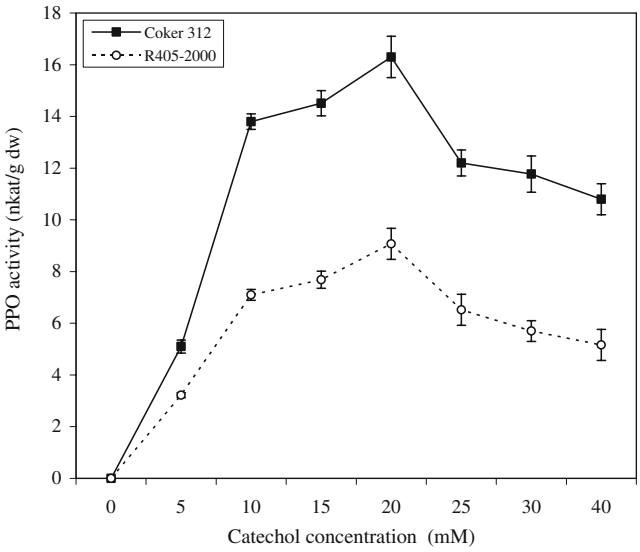

Fig. 3 Effects of catechol substrate concentration on PPO activity from cotton cell suspensions. PPOs were extracted with 0.1 M sodium phosphate buffer at pH 6.5 containing 1% Triton X-100, 0.5% PVP, 1.0 mM EDTA, and 0.25% sodium thiosulfate. Catechol at 20 mM was efficiency for PPO activity from Coker 312 and R405-2000. Values are the mean of three replicates

showed activity with the diphenol and triphenol substrates but none with the monophenol substrates used in this study (Table 3). The best PPO substrate from Coker 312 cell suspensions was found to be catechol. On the other hand, PPO activity was statistically identical with catechol and 4-methylcatechol for R405-2000. Hence, it is likely that there

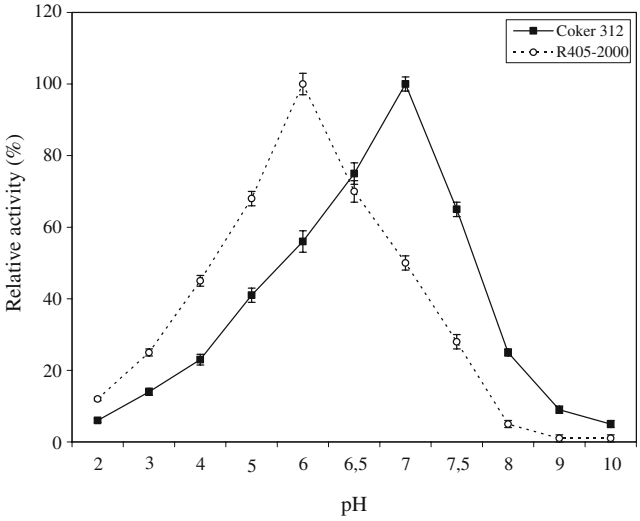

Fig. 4 Determination of optimum pH of cotton cell suspensions PPO activity. PPOs were extracted with 0.1 M sodium phosphate buffer containing 1% Triton X-100, 0.5% PVP, 1.0 mM EDTA, and 0.25% sodium thiosulfate. Catechol at 20 mM was used as PPO substrate. PPO showed optimum activity at pH 7.0 for Coker 312 and pH 6.0 for R405-2000. Values are the mean of three replicates

are two different PPO isoenzymes. The high efficiencies of catechol oxidation by PPO from Coker 312 and R405-2000 could be due to their affinities for each isoenzyme. A similar trend was reported for PPO from grape [44], banana [64], and quince [63]. The inability of the PPO used in this study to oxidize monophenols suggests the absence of monophenolase (creolase) activity. These results are similar to those reported by Zhou and Feng [68] for yali pear PPO. Moreover, we analyzed the effect of the substrate concentration on enzyme activity. A 20 mM concentration was efficient because PPO activity was increased to 30% (Fig. 3). The affinity of PPO toward catechol was confirmed at this concentration. consequently, for the following experiments, catechol was used as substrate at 20 mM for PPO extracted from Coker 312 and R405-2000.

Effect of pH on Enzyme Activity

pH is a decisive factor in the expression of enzymatic activity as it alters the ionization states of amino acid side chains of the enzyme or the ionization states of the substrate [58]. The effect of pH on PPO activity is shown in Fig. 4. the PPO from R405-2000 and Coker

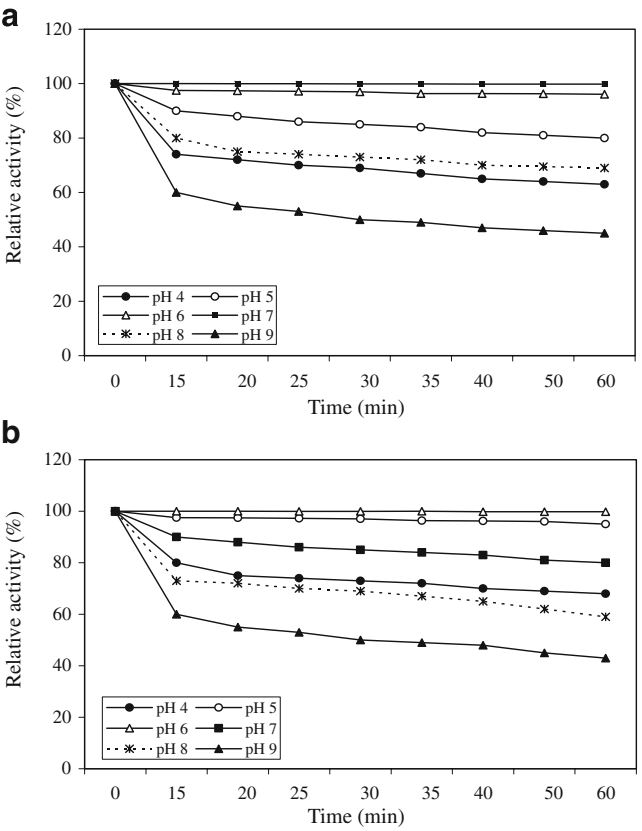

Fig. 5 The effect of pH stability on the activity of PPO from Coker 312 (**a**) and R405-2000 (**b**) cell suspensions. PPOs were extracted with 0.1 M sodium phosphate buffer containing 1% Triton X-100, 0.5% PVP, 1.0 mM EDTA, and 0.25% sodium thiosulfate. Catechol at 20 mM was used as PPO substrate. PPOs activities were stable with the range pH 5.0 to 6 for R405-2000 (**b**) and pH 6.0 to 7.0 for Coker 312 (**a**). Values are the mean of three replicates

312 showed maximum activities at pH 6.0 and 7.0, respectively. PPO activities decreased below and above of each optimal pH value. Differences in pH optima with various substrates have been reported for PPO from strawberries, and other sources [4, 12, 26, 36] reported that the optimum pH for maximum activity of PPO in plants varies from about 4.0 to 7.0, depending on the origin and genotype of plant material, extraction method, substrate used for assay, and the localization of the enzyme in the cell. Other authors reported that PPO which had an optimum pH near to 5.0 would come from chloroplasts and PPO with an optimum pH near to 7.0 from mitochrondria [36, 43, 46, 56, 64]. According to the optimal pH of the cotton cell suspension PPO observed (Fig. 4), PPO from R405-2000 seems to be mainly a chloroplastic enzyme, whereas PPO from Coker 312 seems to be mainly mitochondrial. The pH stability curve of PPO activity is shown in Fig. 5. The enzyme retained more than 95% of its initial activity within the pH range 5.0–6.0 for R405-2000 and 6.0–7.0 for Coker 312. However, a loss of activity was observed below pH 4 (PPO-II) and 5.0 (PPO-I) and above pH 6.0 (PPO-II) and 7.0 (PPO-I). The stability of the enzyme in basic media was lower than in acidic media. These results are similar to those reported by other authors who have found the optimum pH of certain plants to be near to 6.0 [13, 43, 51, 64].

Effect of Temperature on Enzyme Activity

Effect of temperature on PPO activity is shown in Fig. 6. Optimal temperatures were the same for Coker 312 and R405-2000 samples, i.e., 25 °C. This value is similar to that of grape [65] and plum [10]. The enzyme was incubated at different temperatures for 60 min at optimum pH, and after cooling, the residual enzyme activity was measured. Consequently, it was found that the enzyme was relatively stable at 20 and 30 °C for a 60 min preincubation period but unstable at temperatures above 30 °C. Figure 7 shows that temperatures above 30 °C resulted in a loss of enzyme activity. For instance, when the

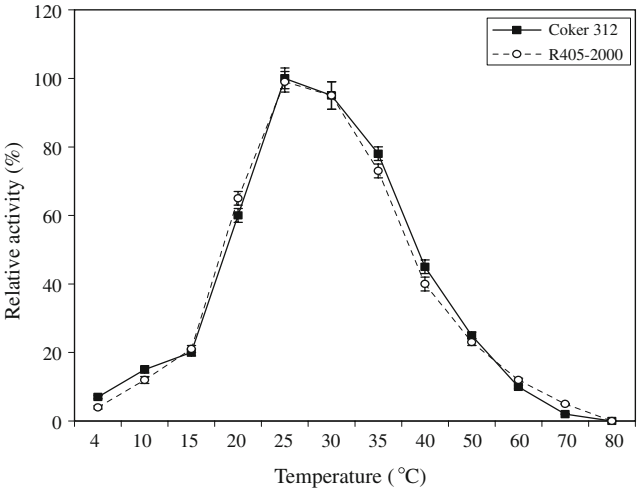

Fig. 6 Determination of optimum temperature of cotton cell suspension PPO activity. PPOs were extracted with 0.1 M sodium phosphate buffer containing 1% Triton X-100, 0.5% PVP, 1.0 mM EDTA, and 0.25% sodium thiosulfate at pH 6.0 for R405-2000 and pH 7.0 for Coker 312. Catechol at 20 mM was used as PPO substrate. PPO showed optimum activity at 25 °C for both cotton cultivars. Values are the mean of three replicates

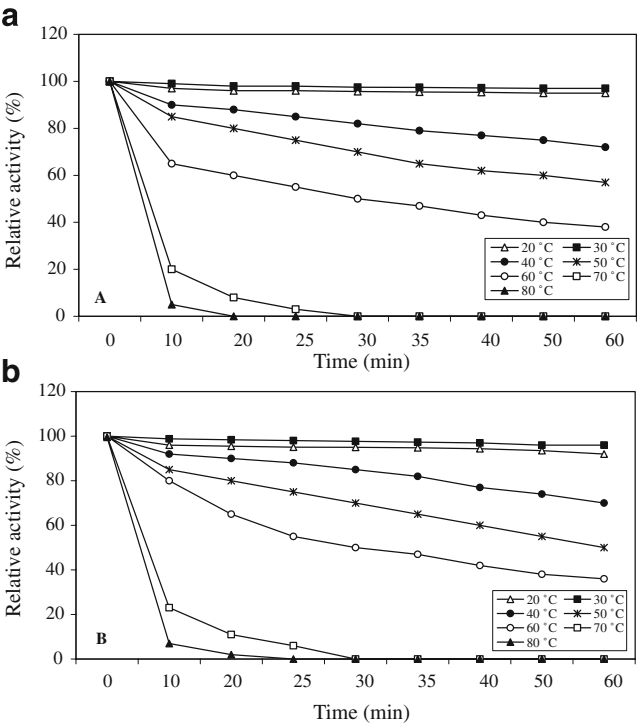

Fig. 7 Heat-inactivation of Coker 312 (**a**) and R405-2000 (**b**) cell suspension PPO at various temperatures. PPOs were extracted with 0.1 M sodium phosphate buffer at pH 6.5 containing 1% Triton X-100, 0.5% PVP, 1.0 mM EDTA, and 0.25% sodium thiosulfate at pH 6.0 for R405-2000 and pH 7.0 for Coker 312. Catechol at 20 mM was used as PPO substrate. PPO activities were stable between 20 and 30 °C. High temperature irreversibly denatured the enzyme. The time required for 50% inactivation of PPO activity at 60, 70, and 80 °C was found to be 30, 6, and 5 min, respectively. Values are the mean of three replicates

temperature was increased from 40 to 60 °C, the activity of PPO decreased from 62% to 28% and was inexistent when the temperature went above 60 °C after 10 min of incubation. This indicated that the enzyme was rapidly denatured at higher temperatures. The times required for 50% inactivation of PPO activity at 60, 70, and 80 °C were found to be 30, 6, and 5 min, respectively. The enzyme from medlar fruits was stable for 30 min at 60 °C [10]. In the case of mulberry PPO, the times required for 50% inactivation of activity at 50, 60, and 80 °C were about 50, 30, and 20 min, respectively [2]. Although PPO is generally considered to be an enzyme of low thermostability, onion PPO was stable at 40 °C for 30 min [3], strawberry PPO at 50 °C [45], Jerusalem artichoke PPO at 60 °C for 30 min [66], and Stanley plum and banana PPO at 70 °C were stable for 30 min [47, 64].

In addition, renaturation of the cotton cell suspension PPO was not observed by returning to ambient temperatures.

Effect of Inhibitors and Activators on Enzyme Activity

The effects of various inhibitors and activators at 1.0 and 10 mM on the purified cotton PPO are shown in Table 4. Several compounds have been reported to be PPO inhibitors [1, 11, 18, 41]. Of all the inhibitors used in this study, dithiothreitol was the most effective, followed by sodium metabisulfite and ascorbic acid. The inhibitory reaction mechanism

Table 4 Effects of inhibitors and activators on the activity of PPO from cotton cell suspensions.

Inhibitors	Concentration (mM)	Relative activity of PPO (%)	
		Coker 312	R405-2000
Control	0	100±0.1 a	100±0.13 a
NaCl	1.0	100±0.12 a	100±0.10 a
	10	100±0.15 a	100±0.10 a
Citric acid	1.0	95±1.1 a	98±0.96 a
	10	84±0.45 a	87±0.72 a
Ascorbic acid	1.0	13±0.63 a	15±0.40 a
	10	0±0.00 a	0±0.00 a
Sodium metabisulfite	1.0	8±0.60 a	10±0.48 a
	10	0±0.00 a	0±0.00 a
Dithiothreitol	1.0	0±0.00 a	0±0.00 a
	10	0±0.00 a	0±0.00 a
$MgSO_4$	1.0	130±1.20 a	127±1.60 a
	10	125±1.13 a	124±0.97 a
$CaCl_2$	1.0	127±1.05 a	126±1.10 a
	10	114±1.80 a	115±1.34 a
$MnCl_2$	1.0	122±1.15 a	120±0.85 a
	10	109±1.02 a	107±0.90 a
$BaCl_2$	1.0	105±0.77 a	103±1.33 a
	10	98±1.12 a	97±1.57 a
$CuSO_4$	1.0	101±1.44 a	99±1.91 a
	10	92±1.51 a	90±1.07 a
$ZnSO_4$	1.0	99±0.83 a	97±1.21 a
	10	89±1.06 a	90±1.30 a
$FeCl_3$	1.0	58±1.04 a	57±0.79 a
	10	20±0.66 a	18±0.21 a
	1.0	37±0.42 a	35±0.70 a
	10	12±0.18 a	11±0.23 a

Catechol and 4-methylcatechol were used as PPO substrate from Coker 312 and R405-2000, respectively. Dithiothreitol was most effective for inhibiting both cotton cultivars followed by sodium metabisulfite and ascorbic acid. On the contrary, metal ions Mg^{2+}, Ca^{2+}, and Mn^{2+} increased PPO activity to 20%. Values represent the mean of three replicates. In a line and a column, values followed by the same letter are not significantly different (test of Newman Keuls at 5%).

differs depending on the reducing agent employed. Inhibition by thiol compounds is attributed to either the stable colorless products formed through an additional reaction with *o*-quinones or to binding to the active center of PPO, like metabisulfite [23, 55]. Ascorbate reduces the initial quinone formed by the enzyme to the original diphenol before undergoing secondary reactions which lead to browning [34]. Ascorbic acid has also been reported to cause irreversible inhibition of PPO [20, 63]. The possible PPO inhibitory effects of two nontoxic compounds, citric acid and NaCl, have also been studied. NaCl was not able to inhibit cotton cell suspension PPO. This result is in agreement with those of several authors who reported that NaCl was the weakest PPO inhibitor in some plants [26, 41, 64]. Citric acid weakly inhibited cotton PPO activity at 10 mM. A similar inhibitory effect of citric acid was found in the browning of head lettuce [6].

In addition, the modulation of cotton PPO activity by metal ions has been studied. PPO activity was markedly inhibited by $SnCl_2$ and $FeCl_3$ but promoted by $MgSO_4$, $CaCl_2$, and $MnCl_2$. Jimenez and Garcia-Carmona [25] also reported that a latent activity of grape PPO can be activated in the presence of divalent cations such as Mg^{2+}, Ca^{2+}, and Mn^{2+}. Approximately, a decrease of 10% in enzyme activity was observed at 10 mM concentrations of $CuSO_4$ and $ZnSO_4$, respectively. However, metal ions (Cu^{2+} and Zn^{2+}) were poor inhibitors of PPO [16, 48]. Research has shown that ascorbic acid, sodium

metabisulfite, and dithiothreitol are the strongest inhibitors of cotton PPO because at 10 mM concentration they were able to nullify PPO activity. On the other hand, at low concentrations (1 mM), metal ions (Mg^{2+}, Ca^{2+}, and Mn^{2+}) improved cotton PPO activity by more than 20%. They are therefore suitable activators for cotton PPO extraction.

Conclusion

PPO isolated from cotton cells has an activity very similar to that of other plants Electrophoresis demonstrated that PPO was purified with a single band on SDS-PAGE. The molecular weight was 55 kDa for PPO (PPO-I) from Coker 312, which develops embryogenic structures, and was 42 kDa for PPO (PPO-II) from R405-2000 which cannot. PPO-I activities were higher than those of PPO-II which were nearly steady. Characterization of the enzyme showed that the optimal pH was 7.0 for PPO-I and 6.0 for PPO-II. The enzyme was also a catecholase, active toward diphenols, and has greatest substrate specificity toward catechol for PPO-I and 4-methylcatechol for PPO-II among the substrate tested, at concentration 20 mM. Both enzymes possessed the same optimal temperature (25 °C) and were stable from 20 to 30 °C. Furthermore, PPO-I and PPO-II were sensitive to some PPO inhibitors, particularly sodium metabisulfite, ascorbic acid, and dithiothreitol. On the contrary, Mg^{2+}, Ca^{2+} and Mn^{2+} greatly enhanced PPO activity and can be considered as the most potent PPO activators.

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