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Purification and Characterization of Cell Suspensions Peroxidase from Cotton (*Gossypium hirsutum* L.)

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Abstract Two peroxidases, cPOD-I and rPOD-II, have been isolated and purified from cotton cell suspension and their biochemical characteristics studied. rPOD-II from R405-2000, a non-embryogenic cultivar, has higher activity than cPOD-I derived from Coker 312, which developed an embryogenic structure. The cPOD-I and rPOD-II had molecular mass of 39.1 and 64 kDa respectively, as determined by SDS-PAGE. Both enzymes showed high efficiency of interaction with the guaiacol at 25 mM. The optimal pH for cPOD-I and rPOD-II activity was 5.0 and 6.0, respectively. The enzyme had an optimum temperature of 25 °C and was relatively stable at 20–30 °C. The isoenzymes were highly inhibited by ascorbic acid, dithiothreitol, sodium metabisulfite, and β-mercaptoethanol. Their activities were highly enhanced by Al^{3+}, Fe^{3+}, Ca^{2+}, and Ni^{2+}, but they were moderately inhibited by Mn^{2+} and K^{+}. The enzyme lost 50% to 62% of its activity in the presence of Zn^{2+} and Hg^{2+}.

Keywords *Gossypium hirsutum* L. · Cell suspension · Peroxidase · Purification · Characterization

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Introduction

Peroxidase (POD; EC 1.11.1.7) is an oxidoreductase widely present in the nature. It catalyzes the oxidative coupling of phenolic compounds using H_2O_2 as the oxidizing agent [1]. The reaction is a three-step cycling in which the enzyme (POD) is oxidized by H_2O_2 and then reduced in two sequential one-electron transfer steps from reducing substrate, typically a small phenol derivative molecules [2]. Higher plants usually contain a large number of peroxidase isoenzymes classified as acidic, neutral, or basic based on their isoelectric points. POD has been isolated and characterized from a large number of sources: palm leaf [3], sweet potato tubers [2], broccoli [4], and tomato [5]. Plant peroxidases can oxidize various phenolics, lignin precursors, and various secondary metabolites as a hydrogen donor. It is known to be multi-functional, participating in a broad range of physiological processes such as auxin metabolism, lignin and suberin formation, cross-linking of cell wall components, defence against pathogens, cell growth, and cells differentiation or somatic embryogenesis [6–10]. POD is of great interest and has been widely used as an important component of ELISAs and enzyme immunoassay kits [2, 3].

Cotton (*Gossypium hirsutum*) is the tropical plant, the most cultivated for its fibers, which constitute the principal raw material for textile industries [11]. Once gossypol is removed and flour is obtained, oil cake of seeds, rich in proteins, is used for human and animal feeding [12]. Genetic improvement of cotton through conventional breeding is limited by several factors such as incompatibility barriers and the duration required. Although plant biotechnology is an attractive means for improvement of cotton, its use requires an effective in vitro culture system from somatic tissues of the plants. Plant regeneration through somatic embryogenesis is an ideal system for the process of cell differentiation. This method offers appropriate material for genetic transformation [13, 14]. Somatic embryogenesis in cells is genetically dependent [15]. Genotypic variations in plants are expressed by different metabolic expression in these plants. Several studies reported a relation between somatic embryogenesis and the activity of glucose metabolism enzymes, protein content, and phenolic compounds [16–19]. Recent reports showed that PDO activity significantly changed during somatic embryogenesis process [20, 21].

Presently, few articles focused on cotton POD during somatic embryogenesis, and there were no deep studies on its isolation and characterization.

The aim of this study was to purify and characterize the POD from embryogenic and non-embryogenic cell suspension of cotton. POD was characterized with respect to molecular mass, kinetic parameters, optimum conditions of pH and temperature, thermal stability, effect of some chemicals, and metal ions.

Materials and Methods

Plant Material and Culture Conditions

Cotton seeds were obtained from CIRAD, France (cultivar Coker 312) and CNRA, Ivory Coast, West Africa (cultivar R405-2000). The conditions of seed germination were those described previously [22].

Hypocotyls of 7-day-old sterile seedlings were cultivated in a 250-ml Pyrex flask containing MS medium [23], including B5 vitamins [24], 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 $MgCl_2$ (MC). Calli were maintained and stabilized through monthly subcultures on the same medium (MC).

Cotton suspension cultures were established by transferring approximately 2 g of friable callus into a 250-ml Erlenmeyer flask containing 50 ml of the aforementioned medium (MC) devoid of gelling agent (ML1). Suspensions were incubated on an orbital shaker at 110 rpm during 4 weeks. The resulting cell suspension was filtered under partial vacuum through a 250-μm mesh sieve, and the filtrate was harvested (primary culture). Then, cell suspensions were subcultured three times at 4-week intervals on MS liquid without growth regulators; glucose was replaced by 40 g/l sucrose. This medium was supplemented with 1.9 g/l KNO_3 and 0.5 mg/l casein hydrolysate (ML2). Approximately 2 g (fresh weight) of the fraction collected at the end of each subculture were resuspended in 50 ml of ML2 as described above into 250-ml Erlenmeyer flasks. At the end of each subculture, cell suspensions were respectively filtered through 150 μm then 100 μm mesh sieves, and samples were examined with a stereomicroscope to observe the evolution of cell suspensions concerning somatic embryogenesis. Cells of each culture were harvested, weighed, and frozen for POD analysis. The pH of all media was adjusted to 5.8 before autoclaving at 121 °C for 30 min. All cultures were kept in a room at 28±2 °C under 24-h photoperiod (16-h light/8-h dark). Illumination was supplied by cool white fluorescent lamps at approximately 2,000 lux light intensity.

POD Extraction and Purification

The cells of each suspension cultures of both cotton cultivars (primary culture stage, first, second, and third subculture stage) were frozen immediately after harvesting, freeze-dried, and powdered. Two grams of cells were extracted according to Rompel et al. [25], with some modifications. POD was extracted in 0.1 M sodium phosphate buffer (pH 6.0), with 1% Triton X-100, 0.5% PVP, 0.25% sodium thiosulfate, and 0.5 mM EDTA (ethylenediamine-tetraacetic acid). The crude extract samples were centrifuged at 32,000×*g* for 20 min at 4 °C, and solid $(NH_4)_2SO_4$ was added to the supernatant to 35% saturation. After 30 min, the precipitate was removed by centrifugation (32,000×*g*, 4 °C, 20 min), and $(NH_4)_2SO_4$ was added to the supernatant to 85% saturation. The precipitate was collected by centrifugation (32,000×*g*, 4 °C, 20 min) and redissolved in 5 ml of 0.1 M sodium phosphate buffer (pH 6.0). This solution was loaded on a Sephadex G-200 column (5×100 cm, flow rate 5 ml/min) equilibrated with 0.1 M sodium phosphate buffer (pH 6.0). Fractions were collected and assayed for POD activity. Aliquots showing POD activity were pooled, lyophilized, and dissolved in small volume of 0.1 M sodium phosphate buffer (pH 6.0). After overnight dialysis against the same buffer, the dialysate collected was referred to as POD extract and used for further studies.

POD Activity Determination

POD activity was determined by monitoring the time course of the change in absorbance at 470 nm upon catalyzation by guaiacol oxidation. The reaction mixture contained 1.8 ml of 0.1 M sodium phosphate buffer, pH 6.0, 1.0 ml of 25 mM guaiacol (2-methoxyphenol) as a substrate and 0.1 ml of enzyme solution. The reaction was initiated by adding 0.1 ml of 1 mM H_2O_2 in 0.1 M sodium phosphate buffer, pH 6.0 and sopped after 3 min. POD activity was expressed in nanokatal per gram dry weight (nanomolar substrate converted per second gram dry weight). For each cotton cultivar, the stage of cell suspension cultures where POD activity was at the highest level was used in the following experiments.

SDS-PAGE Electrophoresis

SDS-PAGE electrophoresis enzyme purity and molecular weight were analyzed by SDS-PAGE in a Mini Protean III Electrophoresis Cell (Bio-Rad), with 12% resolving gel and 4% stacking gel. SDS-PAGE was performed according to the method of Laemmli [26]. Standard protein markers were bovine serum albumin (66.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), chemotrypsin (25.0 kDa), and lactoglogulin (14.0 kDa). Proteins were stained using the Coomasie Blue staining technique, and molecular weight was estimated by comparison to molecular weight markers (Bio-Rad Precision Plus Protein Standard 161-0373).

Substrate Specificity

Purified POD activity was measured using guaiacol as the substrate at a final concentration varying from 0 to 40 mM under optimal (pH 6.0 and 25 °C) conditions. The best concentration of substrate was used to investigate specificity of substrate. Ten commercial grade substrates were selected as follows: monophenols (guaiacol, methoxycinnamic acid, benzoic acid, and *p*-coumaric acid), diphenols (chlorogenic acid, catechin, catechol, and 4-methylcatechol), triphenols [pyrogallol and tetramethylbenzidine (TMBZ)]. The reaction of each substrate was measured at the absorption maximum of the corresponding quinone products, following the procedure suggested by Zhou et al. [27]. The relative maximum activities were calculated by considering activity with guaiacol as 100%.

pH Activity Profile and pH Stability

The POD activity profile was analyzed for 10 min in the pH range of 2–10 using 0.1 M sodium phosphate buffer. POD activity was performed according to the method described above and expressed as a percentage of the maximum activity. Percentage of enzyme activity was estimated, considering 100% as the highest activity detected in the assay. The purified enzyme was analyzed for pH stability ranging from 4.0 to 8.0 for 60 min at 25 °C prior to the assay for residual activity at fixed pH 6.0.

Temperature Activity Profile and Temperature Stability

The temperature optimum of POD was screened at various temperatures ranging from 4 °C to 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 enzymatic activity was measured. Thermal stability of the purified enzyme was determined by measuring residual activity after incubating the enzyme for 10 min in a circulating water bath at temperatures between 10 °C and 80 °C during 10 min at optimum pH. Heated samples were cooled in ice water, and the residual enzymatic activity was determined as described before.

Effect of Some Chemicals on Enzyme Activity

The purified POD was incubated for 10 min at 4 °C in the presence of the following compounds (1 mM): ascorbic acid, cysteine, dithiotreitol, β-mercaptoethanol, sodium metabisulphite, tween 80, EDTA, KI, and NaCl, previously dissolved in 0.1 M sodium phosphate buffer (pH 6.0). The percentage inhibition was expressed as relative to enzyme activity without inhibitor.

Effect of Metal Ions

The effects of various metal ions were determined by pre-incubating each isoenzyme of POD with the individual ions (1 mM) in 0.1 M sodium phosphate buffer at pH 6.0 for 10 min. Metal ions tested were $AlCl_3$, $CaCl_2$, $CuCl_2$, $FeCl_3$, $HgCl_2$, KCl, $MgCl_2$, $MnCl_2$, $NiCl_2$, and $ZnCl_2$. The residual enzymatic activity was then determined, under standard assay conditions, in the presence and absence of metal ions.

Statistical Analysis

Data were analyzed using Statistica software (release 7.5). Data were subjected to analysis of variance. The means of POD activities were tested for significant difference (Newman Keuls at 5%). Data are the mean of three replicates.

Results and Discussion

Cell Suspension Cultures

Cell suspensions of *G. hirsutum* were obtained from calli, which were derived from hypocotyl segments of plant and subcultured on MC medium for 1–4 months. We observed after 1 month of culture in liquid suspension the clusters of round cells with dense cytoplasm with Coker 312. These cells seem to be to be proembryos in the globular stage. Thus, somatic embryos were observed in different stages of somatic embryogenesis: codiforme, heart, and torpedo [28, 29]. On the other hand, the vacuolated large associated to round cells without cytoplasm observed with R405-2000 are characteristic of non-embryogenic cells. Davidonis and Hamilton [30] have reported that KNO_3 increased the number of embryogenic structures. Ragan et al. [31] suggested that casein hydrolysate was important to embryogenic structures development. We confirmed the beneficial effects of the removal of hormone and the addition of KNO_3 and casein hydrolysate on the induction of embryogenic. We observed that 1-month-old cell suspensions (primary culture) rarely contained embryogenic structures. However, when these cell suspensions are 2, 3, and 4 months old (first, second, and third subculture stage), they frequently contained numerous embryogenic structures. The formation of embryogenic structures was observed only in Coker 312 cell suspensions but not in R405-2000 ones (Fig. 1). The highest grow rate of cell suspensions obtained with Coker 312 could notify greater reactivity of the cells of this cultivar in medium culture [32, 33]. These results seem to show that somatic embryogenesis in cotton cell cultures is highly genotype dependent. That is in agreement with results reported by several authors [11, 34–37].

Enzyme Purification

Enzyme purification in plant extracts is a difficult process because of the presence of a large variety of secondary products that can bind tightly to the enzymes and change their characteristics [38]. To overcome this problem, different methods have been developed, such as the use of ammonium sulphate fractionation, salts, insoluble polymers, and detergents. This method has been used to separate hydrophobic proteins [39] and remove phenolic compounds [40, 41] and chlorophylls [42]. In this paper, cell suspension POD was purified using a combination of phase partitioning in compound A (triton X-100, PVP, EDTA, sodium thiosulfate) and ammonium sulphate fractionation in order to avoid changes

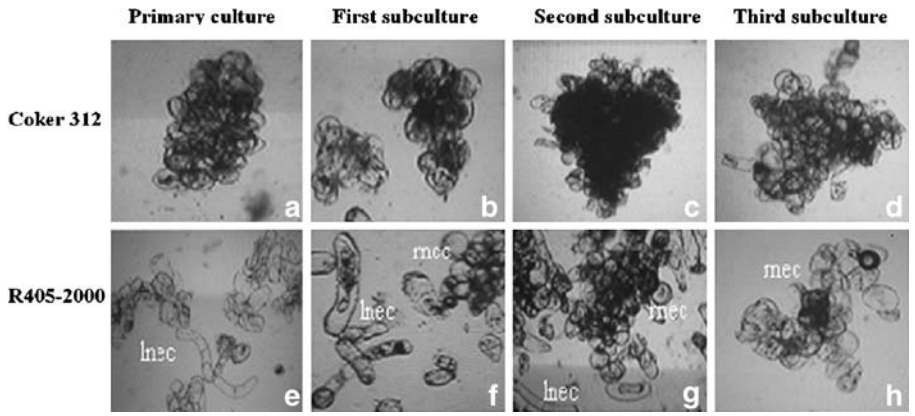

Fig. 1 Cell suspensions of cotton at different culture stages (×440). **a** Globular-staged embryo, **b** codiforme-staged embryo, **c** heart-shaped embryo, **d** torpedo-staged embryo were observed with Coker 312; in cell suspensions of R405-2000, we observed large vacuolated cells (**e**), large and round vacuolated cells (**f**), large vacuolated cells, and a pile of small rounded cells in intensive division (**g**), and round cells without cytoplasm (**h**). These cells are characteristic of non-embryogenic cells; *lnec* and *rnec*: large and round non-embryogenic cells

in the enzyme characteristics due to the binding of secondary products and to remove the pigments from the extract to obtain a clear solution as the enzyme source. The purified POD appeared as a single band in PAGE. This result contrasts with those described in the literature since multiple isoenzymatic bands have been described for POD extracted from different plant sources [43]. This may be due to the extraction method used in the present paper, allowing extraction of a unique isoenzymatic form and the removal of phenols avoiding the modification of POD isoenzymes.

POD Assay and Determination

Somatic embryogenesis is generally linked to biochemical processes. Shoemaker et al. [14] observed an accumulation of soluble proteins during embryo formation in cotton cell suspension. Cell protein content and enzyme activities are genetic processes of the cultivars. The evolution of these substances in the cells during embryogenesis may indicate the rule of enzyme substrate in somatic embryogenesis processes. Peroxidase activity seems tightly linked to the ability of cells to produce embryos. In fact, in primary culture, POD activity was identical with both cotton cultivars but increased in following subcultures. However, POD activity of non-embryogenic cell suspension of R405-2000 was higher than the one of embryogenic cell suspension of Coker 312 (Fig. 2). POD activity reaches a maximum value at the third subculture with R405-2000 at 16.77 nkat/g dw and with Coker 312 at 11.41 nkat/g dw. These results could indicate that low activity of POD in cotton cell suspensions would be linked to induction of embryogenic structures [20]. The present study suggests that the preferential substrate of POD cannot be regarded as a stimulating product of somatic embryogenesis.

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

Molecular Masses of Cotton POD

The molecular weight and purity of the POD were also analyzed by SDS-PAGE. A single band was detected for both cotton cultivars corresponding to cPOD-I (Coker 312,

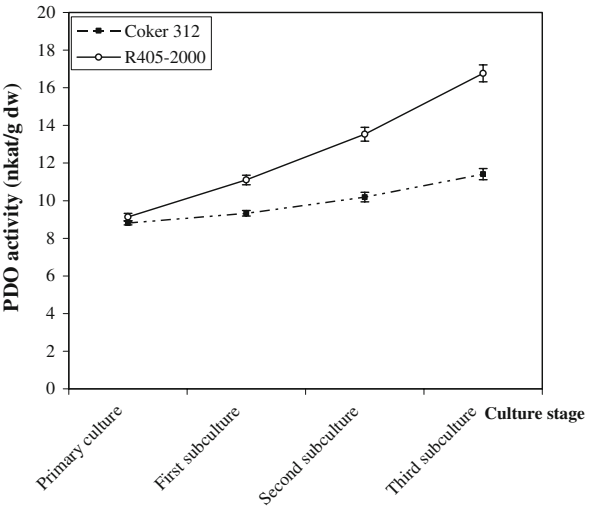

Fig. 2 Evolution of peroxidase activity during cotton cell suspension cultures. *dw* Dry weight. POD activity was assayed in 0.1 M sodium phosphate buffer (pH 6.0) with 25 mM guaiacol as substrate. pH of reaction mixture was 6.0. Coker 312 is an embryogenic cotton cultivar and R405-2000 a non-embryogenic cotton cultivar. POD activity of Coker 312 was stronger than that of R405-2000. Values are the mean of three replicates

embryogenic cultivar) and rPOD-II (R405-2000, non-embryogenic cultivar). cPOD-I and rPOD-II from cotton cell suspensions are monomeric, as indicated by the presence of single protein band (Fig. 3). These results are accordance with those of several authors working with different plants [3, 44, 45]. cPOD-I and rPOD-II molecular masses were 39.1 and 64 kDa, respectively. These peroxidase isoenzymes could possibly be genetically independent units, and they differ in their primary structures [46, 47]. The existence of multiple isoenzymes of POD with different molecular masses has been reported for broccoli (48 kDa) [4], palm leaf (48.0 kDa) [3], Korean radish at 45.0 and 44.0 kDa [48], peanut (40.0 and 42.0 kDa) [49], tomato (34.0 and 37.0 kDa) [5], and Brussels sprouts (32.0 and 26.0 kDa) [50]. The molecular masses of cotton cell suspension POD isoenzymes are higher than those reported for POD from wheat germ, with a molecular mass of 35.0 kDa [51]. The molecular mass of cPOD-I is similar to that of potato tuber sprout at 38.0 kDa

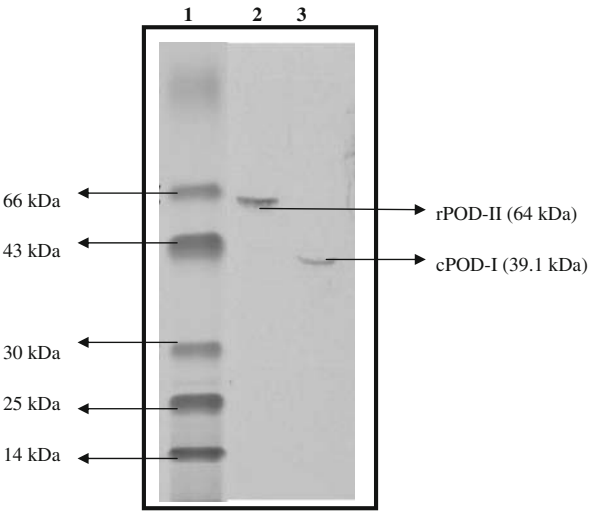

Fig. 3 SDS-PAGE of the peroxidase (*POD*) purified fraction from third subculture of cotton cell suspensions. *Lane 1*, protein standard; *lane 2*, POD from R405-2000; and *lane 3*, POD from Coker 312. Molecular weight of PDO from Coker 312 was 39.1 kDa (*cPOD-I*), whereas molecular weight of POD from R405-2000 was at 64 kDa (*rPOD-II*)

[52] and sweet potato at 39.5 kDa [53], whereas rPOD-II is similar to that of strawberry POD isoenzyme (65.5 kDa) [54]. Other POD showed higher molecular masses such as strawberry POD isoenzymes (65.5 kDa) [54] and *Araucaria araucana* (83.0 kDa) [55]. POD isoenzymes induction was dependent to plant type, genotype, and organ. Thongsook and Barrett [4] attributed these differences to the polypeptide chain, as well as the number and composition of glycan chains. This result seems to justify the behaving difference observed above between Coker 312, which develops embryogenic structures, and R405-2000, which cannot. It reinforces the thesis of genotype dependence of somatic embryogenesis in cotton described above.

Substrate Specificity

The effect of guaiacol concentration on POD activity was examined. The efficiency of 25 mM guaiacol was observed with both cotton cultivars because POD activity was the highest (Fig. 4). Our results indicate that cotton cell suspension POD substrate concentration is similar to that reported for palm date [21]. However, many authors reported the efficiency of guaiacol various concentration on POD in several plants such as cotton callus (10 mM) [56], white pine (15 mM) [57], and mint leaves (1.0 M) [58]. This indicates that the efficient concentration of guaiacol to obtain a high activity of POD was plant and organ dependent. The concentration of 25 mM was used to substrate specificity determination.

The oxidizing ability of the POD of both cotton cultivars was determined using monophenols, diphenols, and triphenols as substrates. Comparative data on substrate specificity are reported in Table 1. In terms of catalytic efficiency, cPOD-I was found to follow the statistical order: TMBZ > guaiacol > pyrogallol ≈ methoxycinnamic acid ≈ chlorogenic acid ≈ catechol > 4-methylcatechol > catechin ≈ *p*-coumaric acid ≈ benzoic acid. With rPOD-II, the efficiencies for different phenols showed a slightly different order: TMBZ > guaiacol > chlorogenic acid ≈ methoxycinnamic acid ≈ pyrogallol > 4-methylcatechol > catechol > benzoic acid ≈ *p*-coumaric acid ≈ catechin. High efficiencies of TMBZ, guaiacol, pyrogallol, methoxycinnamic acid, and chlorogenic acid by POD were due to their high affinities for enzyme. This trend is similar to some other plant POD

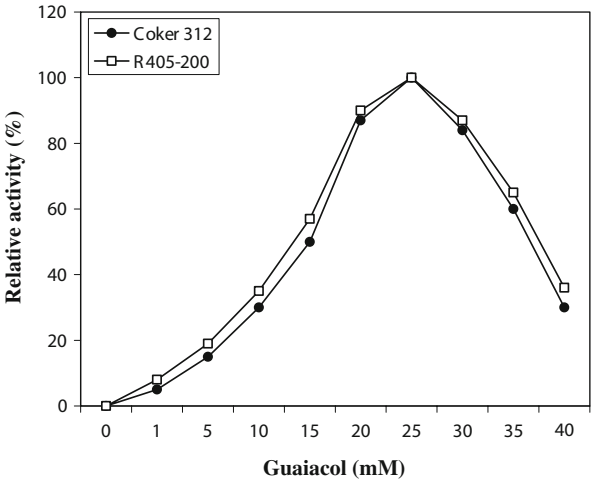

Fig. 4 Effect of guaiacol concentration on the activity of peroxidase from cotton cell suspensions. PODs were extracted with 0.1 M sodium phosphate buffer (pH 6.0). Guaiacol at 25 mM was efficient for POD activity in cotton cell suspension. Values are the mean of three replicates

Table 1 Substrate specificity on the activity of POD from cotton cell suspensions.

Substrate (25 mM)	Relative activity of PDO isoenzymes (%)	
	cPOD-I	rPOD-II
Control	0a	0a
Monophenols		
Guaiacol	100b	100b
Methoxycinnamic acid	85cf	90f
Benzoic acid	10d	19d
p-Coumaric acid	13d	15d
Diphenols		
Catechol	36e	30e
4-Methylcatechol	28e	47f
Catechin	19d	10d
Chlorogenic acid	80c	95f
Triphenols		
Pyrogallol	90f	84f
Tetramethylbenzidine	215g	206g

In a line and a column, values followed by the same letter are not statistically different (test of Newman Keuls at 5%). Each value represents the mean of 3 replicates, cPOD-I and rPOD-II, isoenzymes from Coker 312 and R405-2000, respectively.

enzymes, as reported for POD from pear [59] and royal palm leaves [58]. High interactions with TMBZ, guaiacol, pyrogallol, methoxycinnamic acid, and chlorogenic acid by POD isoenzymes confirmed that these compounds were good substrates for POD of cotton cell suspensions. POD showed a high relative activity to oxidize TMBZ followed by guaiacol. It should be emphasized that POD isoenzymes were highly reactive toward the aromatic amine; TMBZ was oxidized twice faster than guaiacol. The efficiency of TMBZ was unusually higher than the values calculated for the phenolic compounds tested. TMBZ could be considered as the best substrate for both POD isoenzymes, but it is not recommended for routine analysis because of its low solubility and carcinogenic effect [60]. Therefore, we can keep considering guaiacol as being the best POD substrate for cotton cell suspension. These results are in accordance with those of some authors, which reported that guaiacol is the best commonly used substrate for plant peroxidase activity assay [20, 21, 61–63].

pH Activity Profile and pH Stability

pH is a determining factor in the expression of enzymatic activity as it alters the ionization states of amino acid side chains or the ionization of the substrate [64]. The pH optima of cPOD-I and rPOD-II were 5.0 and 6.0, respectively (Fig. 5). This suggests that the enzyme may be found in an acidic and neutral environment, such as the vacuole [3]. These variations in the kinetic properties of POD isoenzymes suggest that they carry multiple genes, each one encoding different types of enzyme units. Generally, plant peroxidases are found in apoplasts or vacuoles, where the pH is lightly acidic. The pH optima cPOD-I and rPOD-II were therefore thought to be suitable for the environment in which they are localized. Our result was nearly similar to that reported for POD isoenzymes in Korean radish at pH 5.0 and 6.5 [48] and wheat germ at pH 5.3 and 6.3 [65] but slightly higher than POD in potato tuber sprouts at 4.0 [52], red pepper at 4.5 [66], marula fruit at 4.0 [67], and broccoli at pH 4.0 [4]. However, Lopez and Burgos [68] reported that the release of the heme group from the enzyme active site was pH dependent and occurred most rapidly below pH 5.0 and led to loss of POD activity. The active site on enzymes is frequently composed of ionized groups (prototropic groups) that must be in the proper ionic form in

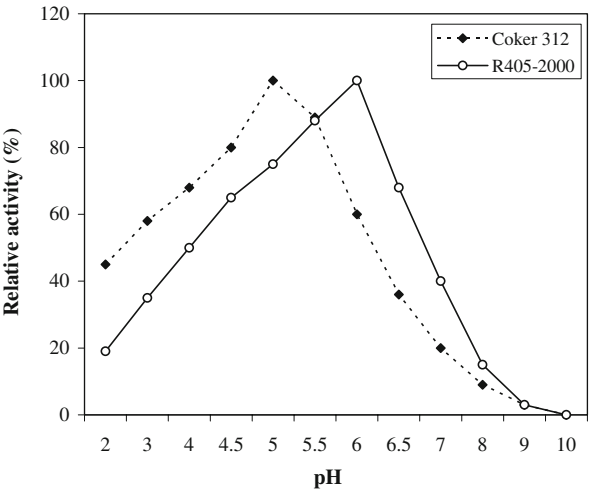

Fig. 5 Effect of pH on the activity of peroxidase from cotton cell suspensions. PODs were extracted with 0.1 M sodium phosphate buffer. Guaiacol at 25 mM was used as POD substrate. POD showed optimum activity at pH 5.0 for Coker 312 and pH 6.0 for R405-2000. Values are the mean of three replicates

order to maintain the conformation of the active site, enzyme-substrate binding, or reaction catalysis [56]. The behavior of the enzyme activity at different pH values could provide information concerning the identities of the phototropic groups at the active site once the p*K* values are known [56, 69]. The p*K* values of the essential ionizing groups involved in the catalytic process were determined for cPOD-I and rPOD-II isoenzymes, following the Dixon–Webb plot [70] (data not shown). rPOD-I shows p*K* value of 4.5. Whitaker [56] suggests that aspartate and cysteine could possibly be involved in the catalysis step. rPOD-II has one ionizable group involved in the catalysis process, with a p*K* value of 7.4. This neutral or alkaline group could possibly be due to histidine [56]. The ionization constant rate p*K* 4.0 was reported for horseradish POD [71]. However, it is difficult to assign an experimental p*K* value to the reactive group of an amino acid because it is based on approximation. These hypotheses could be confirmed by specific chemical modifications of the active site.

The pH stability of POD activity is shown in Fig. 6. The enzyme retained more than 90% of its initial activity within the pH range 5.0–6.0 for Coker 312 and 6.0–7.0 for R405-2000. The weakest activity of POD is observed at pH 8.0. Enzymatic activity was lowest after incubation for 60 min at pH 7.0 and 8.0 with cPOD-I and at pH 4.0 and 8.0 with rPOD-II. This situation was probably due to the detachment of the heme prosthetic group from the active site of the enzyme [3].

Temperature Activity Profile and Temperature Stability

The temperature effects on cotton cell suspension POD activity were studied between 4 °C and 80 °C (Fig. 7). As showed on the figure, optima temperatures were the same for Coker 312 and R405-2000 samples at 25 °C. Similar result was obtained by Castillo et al. [2] in investigations on POD from sweet potato tubers. This result contrasts with those described for others plants. Indeed, Murakami et al. [72] reported that optimum temperature of rye leaves POD was ranged from 45 °C to 50 °C, whereas Ofelia et al. [73] showed the optimum temperature of vanilla bean at 16 °C. This may be because the plant, the source of POD, the extraction, and the assay conditions of enzyme were different. Cotton cell suspension POD showed activity decrease at 40 °C (25% and 20% of Coker 312 and R405-

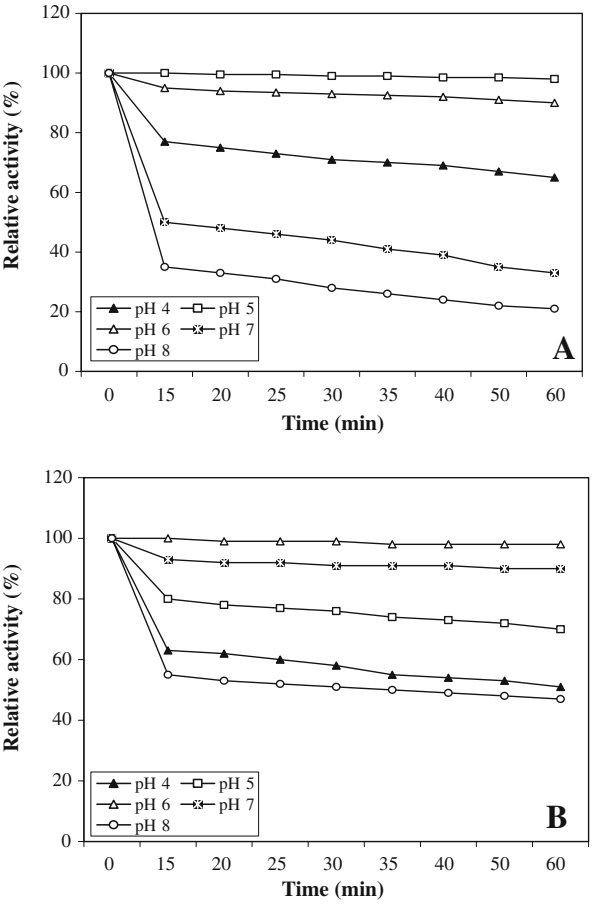

Fig. 6 Effect of pH stability on the activity of peroxidase from Coker 312 (**a**) and R405-2000 (**b**) cell suspensions. PODs were extracted with 0.1 M sodium phosphate buffer. Guaiacol at 25 mM was used as POD substrate. PODs activities were stable within the range pH 5 to 6 for Coker 312 (**a**) and pH 6.0 to 7.0 for R405-2000 (**b**). Values are the mean of three replicates

2000 activity, respectively), indicating possible thermal alteration in the catalytic mechanism of the enzyme. Usually, the loss of activity enzymes is severe at temperatures near 50 °C, which can be due to the unfolding of the enzyme or to an alteration in the interaction between the enzyme and the substrate, without a significant or irreversible modification of the three dimensional structure of the enzyme [74].

The thermal stability profile of cotton cell suspension POD, presented as residue activity after preincubation at the specified temperature, is shown in Fig. 8. The enzyme was relatively stable at 20 °C and 30 °C over a 60-min preincubation time period [66]. The range of temperatures required for the inactivation of cotton cell suspensions POD was lower, with significant inactivation at temperatures >40 °C. In some cases, inactivation is given as the *D* value, the time required to reduce the enzyme activity to 10% of its original value. The temperature required for a *D* value of 5 min was 40 °C, a lower value than for PODs from potato and carrot (83 °C and 80 °C, respectively) [75], indicating that cotton cell suspensions POD is less thermostable than those PODs. The time required to halve the activity at 60 °C was 7 min. This is much higher than the time for strawberry POD (4 min) [76], indicating again that cotton cell suspensions POD is highly thermostable than strawberry POD. The thermal inactivation study of this POD shows that it is a very thermolabile enzyme in comparison with PODs extracted from other plant sources.

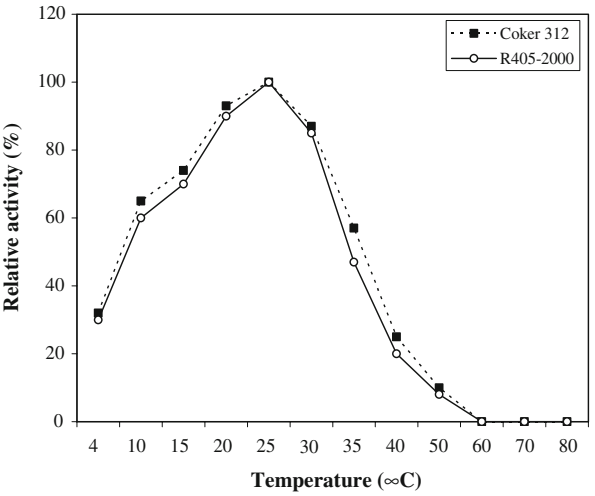

Fig. 7 Effect of temperature on the activity of peroxidase from cotton cell suspension. PODs were extracted with 0.1 M sodium phosphate buffer at pH 6.0. Guaiacol at 25 mM was used as POD substrate. POD showed optimum activity at 25 °C for both cotton cultivars. Values are the mean of three replicates

The times required for 50% inactivation of activity at 50 °C, 60 °C, and 70 °C were found to be about 5 min. A decrease in POD activity of 75%, 85%, and 90% was found after 10 min at 50 °C, 60 °C, and 70 °C, respectively. Moreover, the enzyme activity at 50 °C, 60 °C, and 70–80 °C was found to be completely exhausted in 40, 25, and 10 min, respectively. In addition, renaturation of the cotton cell suspension POD was also studied by lowering the temperatures to ambient. The renaturation feature was not observed for the enzyme. PODs are reported to be the most heat stable enzymes in plants, for example, a treatment at 121 °C for 6 min is needed to inactivate green peas POD [45]. However,

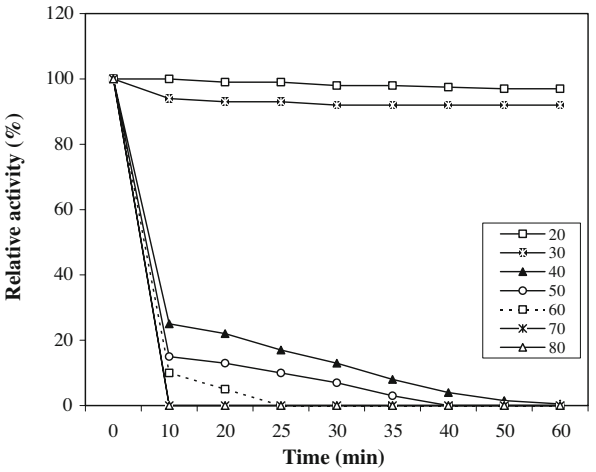

Fig. 8 Heat inactivation on the activity of peroxidase from both cotton cultivars cell suspensions at various temperatures. PODs were extracted with 0.1 M sodium phosphate buffer at pH 6.0. Guaiacol at 25 mM was used as POD substrate. POD activities were stable between 20 °C to 30 °C. High temperature irreversibly denatured the enzyme. The times required for 50% inactivation of POD activity at 50 °C, 60 °C, and 70 °C were found to be 5 min. A decrease in POD activity of 75%, 85%, and 90% was found at 50 °C, 60 °C, and 70 °C in 10 min, respectively. POD activity at 50 °C, 60 °C, an 70–80 °C was found to be completely exhausted in 40, 25, and 10 min, respectively. Values are the mean of three replicates

temperature enzyme activity resistance depends on the source of the enzyme, as well as on the assay conditions, especially pH and the type of substrate used. The variability in the heat stability of POD can be largely attributed to the particular structure of the enzyme. Non-covalent, electrostatic, and hydrophobic interactions of individual isoenzymes determine enzyme folding and stability, as well as extra ion pairs, hydrogen bonds, and the degree of glycosylation, which has also been found to play a role in enzyme stability [61]. It has also been shown that the thermal stability of POD is mainly due to the presence of a large number of cysteine residues in the polypeptide chain [3].

Effect of Some Chemicals on Enzyme Activity

Until now, the inhibition of POD has been rarely reported because the focus of published research is a potential substitute for commercial horseradish peroxidase [43]. As it can be seen on Table 2, all reducing agents used in this study inhibited POD activity. Complete inhibition of cotton cell suspension PODs was achieved by using 1 mM of ascorbic acid and sodium metabisulphite. cPOD-I was also completely inhibited in the presence of dithiothreitol, but this inhibition of rPOD-II was only 98%. β-mercaptoethanol (94–96%) markedly inhibited POD activity, suggesting that POD has disulfide bonds in its structure. On the other band, moderate inhibition was observed in cysteine, which inhibited cPOD-I and rPOD-II by 73% and 78%, respectively. Poor inhibitory effect was observed when using halide ions, NaCl (44–49%) and KI (36–30%), and Tween 80 (38–45%). EDTA inhibited both isoenzymes moderately (25–28%). EDTA, as a metal chelator, was unable to fully combine with Fe^{3+} ions; consequently, the active site maintained its integrity. Debowska and Podstolski [77] also reported unsuccessful inhibition of POD by EDTA.

Effect of Metals Ions on Enzyme Activity

To further characterize POD, a study of its inhibition by metal ions was carried out. Table 3 shows the effect of some metals ions at 1 mM on POD. The activities of POD isoenzymes from cotton cell suspensions were variously affected by the presence of metal ions. Al^{3+}, Ca^{2+}, Fe^{3+}, and Ni^{2+} stimulated the activity of rPOD-II better than cPOD-I. On the other hand, Cu^{2+} and Mg^{2+} did not show significant stimulation of POD isoenzymes activity, whereas K^{+} and Mn^{2+} moderately inhibited the activity of both isoenzymes. rPOD-II was more resistant to the inhibition effect by Hg^{2+} and Zn^{2+} compared to cPOD-I. Fe^{3+} was able

Table 2 Effect of chemical compounds on enzyme activity on the activity of POD from cotton cell suspensions.

Chemical compounds (1 mM)	POD isoenzymes (% inhibition)	
	cPOD-I	rPOD-II
Control	0	0
Ascorbic acid	100a	100a
Sodium metabisulphite	100a	100a
Dithiothreitol	100a	98a
β-mercaptoethanol	96a	94a
Cysteine	73b	78b
NaCl	44c	49c
Tween 80	45c	38cd
KI	36d	30de
EDTA	24e	28e

In a line and a column, values followed by the same letter are not statistically different (test of Newman Keuls at 5%). Each value represents the mean of 3 replicates, cPOD-I and rPOD-II, isoenzymes from Coker 312 and R405-2000, respectively.

Table 3 Effect of metal ions on the activity of POD from cotton cell suspensions.

Metal ions (1 mM)	Relative activity of POD isoenzymes (%)	
	cPOD-I (Coker 312)	rPOD-II (R405-2000)
Control	100a	100a
$ZnCl_2$	38b	46b
$HgCl_2$	41b	50b
$MnCl_2$	73c	70c
KCl	77c	74c
$CuCl_2$	100a	101a
$MgCl_2$	100a	100a
$NiCl_2$	109a	123b
$CaCl_2$	140d	151d
$AlCl_3$	177e	208e
$FeCl_3$	293f	305f

In a line and a column, values followed by the same letter are not statistically different (test of Newman Keuls at 5%). Each value represents the mean of 3 replicates, cPOD-I and rPOD-II, isoenzymes from Coker 312 and R405-2000, respectively.

to greatly enhance the activity of rPOD-II (305%) and cPOD-I (293%). These results seem to indicate that the inhibitory effect at the same concentration on POD activity depends on the chemical structure of the metal ions used [78]. Little is known about the effect of metal ions on plant peroxidase activity. Hg^{2+} is potent inhibitor of enzymatic reactions by binding to SH groups present in the active site of enzyme, causing its irreversible inactivation [79]. Fe^{3+} is considered essential for the activity of most plant POD enzymes as it is involved in the binding of H_2O_2 and the formation of compound 'P' [56, 80]. The presence of Al^{3+} stimulates activity of rPOD-II better (208%) than cPOD-I (177%). Among the metal ions tested, Ca^{2+} is a cofactor that serves to maintain the conformational integrity of the enzyme's active site [47, 61]. Activation by Ca^{2+} ion was reported for avocado POD [81] and wheat germ POD [64].

Conclusion

This study describes the purification and characterization of POD from cotton cell suspensions. Molecular weight was 39.1 kDa for POD from Coker 312 (cPOD-I), which develops embryogenic structure, and 64 kDa for POD from R405-2000 (rPOD-II), which cannot. rPOD-II activity was higher than those of cPOD-II during cell suspension cultures. Moreover, cotton cell suspensions PODs have pH optima of 5.0 and 6.0, for cPOD-I and rPOD-II respectively, and they differ in prototropic groups at the active site. Both POD isoenzymes have optimum activity at 25 °C and were stable from 20 °C to 30 °C. Among the substrates tested, guaiacol has given the higher POD activity at 25 mM. The optimum conditions for enzyme activity permit measurement of POD in adequate reaction conditions. cPOD-I and rPOD-II were sensitive to some of general POD inhibitors, particularly to ascorbic acid, dithiothreitol, sodium metabisulfite, and β-mercaptoethanol, which markedly inhibited POD activity. On the other hand, Al^{3+}, Fe^{3+}, Ca^{2+}, and Ni^{2+} greatly increased POD activity and could be considered as the most potent POD activators. Owing to the high molecular masses for cPOD-I and rPOD-II, it would be interesting to further investigate the carbohydrate structure of POD isoenzymes and monosaccharide composition analysis. This will provide better understanding of the structure–function relationships of PODs.

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