

## EXTRACTION, PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF A PEROXIDASE FROM *Copaifera langsdorffii* LEAVES

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Recebido em 3/1/06; aceito em 12/1/07; publicado na web em 24/7/07

EXTRACTION, PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF A PEROXIDASE FROM *Copaifera langsdorffii* LEAVES. The aim of this work is to obtain, purify and characterize biochemically a peroxidase from *Copaifera langsdorffii* leaves (COP). COP was obtained by acetone precipitation followed by ion-exchange chromatography. Purification yielded 3.5% of peroxidase with the purification factor of 46.86. The COP optimum pH is 6.0 and the temperature is 35 °C. COP was stable in the pH range of 4.5 to 9.3 and at temperatures below 50.0 °C. The apparent Michaelis-Menten constants (Km) for guaiacol and H<sub>2</sub>O<sub>2</sub> were 0.04 mM and 0.39 mM respectively. Enzyme turnover was 0.075 s<sup>-1</sup> for guaiacol and 0.28 s<sup>-1</sup> for hydrogen peroxide. *Copaifera langsdorffii* leaves showed to be a rich source of active peroxidase (COP) during the whole year. COP could replace HRP, the most used peroxidase, in analytical determinations and treatment of industrial effluents at low cost.

Keywords: *Copaifera langsdorffii*; peroxidase; vegetal extract.

### INTRODUCTION

Peroxidases are heme containing enzymes that oxidize a variety of proton donor compounds such as diphenols, polyphenols aminophenols, among others, in the presence of hydrogen peroxide<sup>1</sup>. Peroxidases represent a group of enzymes widely distributed in the plant kingdom<sup>2</sup> and has been extracted from several plants including horseradish (*Armoracia rusticana*), peach (*Prunus persica*), yam (*Alocasia macrorrhiza*), manioc (*Manihot utilissima*), artichoke (*Cynara scolymus L.*), sweet potato (*Ipomoea batatas ex L., Lam.*), turnip (*Brassica campestris rapifera*), zucchini (*Cucurbita pepo*) and others<sup>3-6</sup>. For vegetables and fruits the various types of isoperoxidases, including the anionic and cationic types with low and high isoelectric point values respectively, can be extracted as both soluble and membrane bound isoforms<sup>7</sup>.

Plant peroxidases are known to be comprised of a range of isoenzymes differing in number from one vegetable source to another and have been observed to differ with respect to thermal stability, optimum pH, substrate specificity, amino acid composition and their physiological roles in the plant tissues<sup>8,9</sup>. The catalytic characteristics and thermal properties of the individual isoenzymes have received considerable attention due to their thermostability and ability to recover their enzymatic activity after partial heat-inactivation and hence their potential effect on food quality<sup>10</sup>. Peroxidases from a number of sources lose activity on heating following biphasic-linear first-order kinetics. Such behavior has been attributed either to isoenzymes of different heat stabilities<sup>11</sup>, or the conversion of the native enzyme to new isoforms with enhanced stability<sup>12,13</sup>.

In biotechnology and associated research areas (enzymology, biochemistry, medicine, genetics, physiology, histo- and cytochemistry),

peroxidases have conquered a prominent position<sup>14</sup> and it remains the major enzyme used to evaluate the heat processing of vegetables<sup>7</sup>. The investigation of this enzyme may be of interest, not only for its negative effects on color and flavor, degradations of pigments and nutritional value, but also for its positive effects in food<sup>15,16</sup>.

Peroxidase is used for developing reliable methods of the determination of hydrogen peroxide, which is of great importance in both the biological and industrial fields<sup>17</sup>. This enzyme can still be employed in the determination of lipid peroxidation in cell membranes<sup>18</sup> and in meat food products<sup>19-21</sup>. The application of plant and microorganism peroxidase to catalyze the polymerization and precipitation of aqueous phenols and decolorization of bleach plant effluent by hydrogen peroxide is potentially promising, because those peroxidases are less expensive than horseradish peroxidase, which has been the focus of most wastewater research<sup>22-24</sup>. The use of peroxidases in the construction of biosensors and other enzymatic procedures, immobilized in various matrixes or peroxidase rich microorganisms in modern organic synthesis are important emerging applications of this enzyme<sup>3,14,25-33</sup>.

Despite the variety of plant peroxidase sources, there is no previous work on peroxidase from *C. langsdorffii* Desf. leaves (Leguminosae). This plant is popularly known as "copafaba", and is a large tree that grows abundantly through out Brazil, mainly in the Central region and Minas Gerais State<sup>34</sup>. The present investigation reports the isolation, purification and biochemical characterization of anionic peroxidase (COP) from *Copaifera langsdorffii* leaves.

### EXPERIMENTAL

#### Plant material

Leaves of *Copaifera langsdorffii* were collected during the whole year from plants grown under native conditions at the

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Department of Botany of the State University of Campinas (UNICAMP), São Paulo, Brazil. The leaves were transported to the laboratory, washed and used for peroxidase extraction. The leaves were freshly used or stored at 8 °C up to 7 days. For the evaluation of peroxidase activity, the extract was immediately used or stored at -18 °C up to three months.

### Crude extract preparation

The leaves were homogenized in cold (4 °C) 50 mM phosphate buffer pH 6.0 (4 mL/g of leaves), in a domestic blender for 5 min. The homogenate was rapidly filtered through six layers of cheesecloth and centrifuged at 11,000 x *g* for 15 min at 4 °C. The supernatant was taken as the source of peroxidase, and the determination of activity was performed immediately<sup>35</sup>.

### Partial peroxidase purification

Semi-purified peroxidase was obtained from the crude extract, which was homogenized with 65% pre-cooled acetone (-18 °C). Using a burette the acetone was added slowly down the sides of the container held in an ice bath. After standing for 12 to 14 h at 4 °C, the homogenate was centrifuged at 11,000 x *g* for 15 min at 4 °C. The supernatant was discarded, and the acetone powder was dried at a temperature of 10-15 °C and used for the peroxidase assay and the estimation of protein content. The semi-purified peroxidase was stored at -18 °C until use.

### Purification of the anionic peroxidase (COP)

All purification steps were carried out at 15 °C. The enzymatic fraction was obtained by a three-step purification procedure. The first step consisted of the crude extract preparation, followed by partial purification and finally the purification of the enzyme. An adequate amount of partial purified peroxidase was dissolved in 50 mM sodium phosphate buffer pH 6.0. The enzyme was then loaded on a DEAE-Sepharose column (1mL) and eluted with a 0 to 0.5 M NaCl linear gradient in 50 mM sodium phosphate buffer pH 6.0, at a flow rate of 1.0 mL/min, in a FPLC System Pharmacia. The protein concentration of the fractions was routinely estimated by measuring the absorbance at 280 nm and the activity in each eluted fraction was determined in triplicate by measurement of the absorbance at 470 nm of tetraguaiacol ( $\epsilon = 6.65 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ )<sup>36</sup>.

Active fractions from the DEAE-Sepharose column were pooled and dialyzed against deionized water, then concentrated by freeze drying and stored at -20° C for use to the biochemical characterization.

### Protein electrophoresis

The purified peroxidase was used for PAGE or SDS-PAGE in order to estimate the enzyme activity, MW (molecular weight) and purity. The sample preparation for native PAGE was performed according to Davis<sup>37</sup>, after dialysis overnight. Enzyme-substrate staining of the native PAGE gels was carried out using guaiacol-hydrogen peroxide<sup>38</sup>. For SDS-PAGE the samples were boiled in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol and separated in a 12% gel according to Laemmli<sup>39</sup>. The proteins were stained with coomassie brilliant blue R-250. The standard molecular weight used were: phosphorylase b (94,000 Da), bovine serum albumin (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da), soybean trypsin inhibitor (20,100 Da), and  $\alpha$ -lactalbumin (14,400 Da).

### Isoelectric point determination

Denaturing two-dimensional gel electrophoresis was used for determination of the isoelectric point of COP. Twenty  $\mu\text{g}$  of the purified protein were dissolved in 350  $\mu\text{L}$  of sample buffer containing 8 M urea, 4% (w/v) CHAPS ([3-(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate), 2% (v/v) carrier ampholytes pH 3-10, 70 mM DTT (dithiothreitol) and 0.001% (w/v) bromophenol blue. Except for CHAPS, which was from Sigma, all reagents were from Amersham Biosciences. The sample was then loaded on IPG (Immobilized pH Gradient) strips with a non-linear separation range of pH 3-10. After a 10 h rehydration, isoelectric focusing was carried out at 20 °C for 1 h at 500 V, for an additional hour at 1000 V and finally for 10 h at 8000 V in an IPGphor apparatus (Amersham Biosciences), maintaining a limiting current of 50  $\mu\text{A}$  per strip. After the run, the strip was soaked for 10 min in a solution containing 50  $\mu\text{M}$  Tris-HCl (pH 6.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 2% (w/v) DTT, and for an additional 10 min in the same solution containing 2.5% (w/v) iodoacetamide instead of DTT. The second dimension electrophoresis (SDS-PAGE) was performed on an SE-600 system connected to a Multitemp II refrigerating system (both from Amersham Biosciences). After laying the strip on top of a 12.5% polyacrylamide gel and sealing it with agarose, electrophoresis was carried out for 1 h at 90 V at a constant current (30 mA per gel) applied until the dye front reached the lower end of the gel. Proteins were detected using standard coomassie blue staining and the isoelectric point was determined using a calibrating curve supplied by the manufacturer.

### Molecular weight determination

Gel filtration chromatography of adequate quantities of COP was performed on a HiPrep 16/60 Sephacryl S-200 column (internal diameter of 16 mm, height of the packed gel bed of 60 cm, giving a bed volume of 120 mL), using blue dextran for the void volume determination and soybean trypsin inhibitor (20,100 Da); carbonic anhydrase (30,000 Da); ovalbumin (43,000 Da); bovine serum albumin (67,000 Da); phosphorylase b (94,000 Da) as standards. The molecular weight of the peroxidase was calculated from a regression curve, where log of the molecular weights of the standards were plotted against the ratio of the elution volumes of the standards and the void volume of the column.

### Peroxidase activity and total protein determinations

Peroxidase activity was determined in triplicate by measurement of the absorbance at 470 nm of tetraguaiacol ( $\epsilon = 6.65 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ )<sup>36</sup>. Briefly, we used 1.5 mL of 1% guaiacol and 0.4 mL of 0.1 M hydrogen peroxide in 50 mM phosphate buffer pH 6.0 at 35 °C and the reaction was started by adding 0.1 mL of peroxidase solution. The peroxidase activity was measured while the reaction was at the initial rate (1.5-3.0 min). One unit of activity was defined as the amount of enzyme that causes an increase of 0.001 absorbance per min<sup>36</sup>. Total protein concentration was determined in triplicate, using bovine serum albumin as standard, according to Lowry *et al.*<sup>40</sup>.

### Km and enzyme turnover determination

Michaelis-Menten constant ( $K_m$ ) and enzyme turnover ( $E_t$ ) for the COP substrates guaiacol and  $\text{H}_2\text{O}_2$  were determined by incubating 0.1 mL of purified peroxidase with different concentrations of guaiacol and a fixed saturated concentration of  $\text{H}_2\text{O}_2$  or vice-versa. The  $K_m$  and  $E_t$  values were determined from double reciprocal plots of enzyme activity and substrate

concentration, using Graft, Version 3.0, Erithacus Software<sup>41</sup>. The turnover number of an enzyme is a measure of the number of reaction processes, i.e. number of ES complexes that are converted to product, per enzyme molecule per unit time. This experiment was carried out at 35 °C.

### Analytical RP-HPLC of COP

The COP was analyzed for homogeneity by RP-HPLC, on the U-Bondapack C18 column, preparative scale, (0.78 cm x 30 cm). The peroxidase was dissolved in solution A, 0.1% TFA (trifluoroacetic acid) in water. After homogenization, the samples were clarified by high speed centrifugation at 11,000 x g for 2 min and applied to the chromatographic column. The proteins were eluted using a linear gradient of 0-70% acetonitrile in solution A, at 0.48 mL/min and freeze dried. The COP was eluted with a eluent volume of 11-17 mL.

### Amino acid analysis and N-terminal sequence determination

Amino acid analysis of COP was performed in duplicate with a PICO-TAG PTH amino acid (phenylthiohydantoin amino acid) analyzer. The COP was hydrolyzed in 6 N HCl in the presence of 1% phenol for 24 h (106 °C) and derivatize with PITC (phenyl isothiocyanate) before being injected in the column. Amino acids were eluted by using an acetonitrile linear concentration gradient in 50 mM sodium acetate buffer. The identification and quantification of each amino acid were carried out using the PTH amino acid standard.

N-terminal sequence analysis of COP was performed by automated Edman degradation using instrumentation protocols from Applied Biosystems. A model Procise of a gas-phase sequencer equipped with an online model 140 C Microgradient Delivery System and model 785A UV detector was used. Sample amino acid identification was done by comparison of its retention times to the standard PTH amino acids.

### Stability and optimal temperature and pH determination

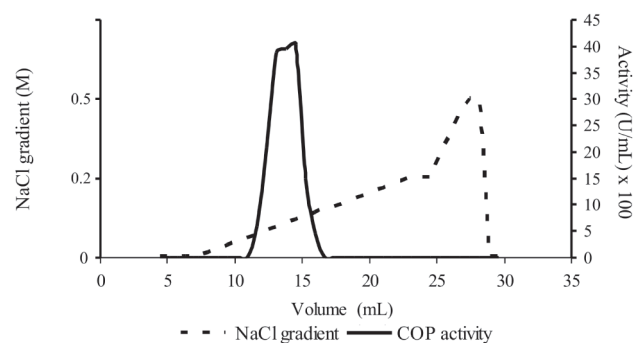
COP stability, optimal temperature and pH were determined using factorial design experiments. The impact of pH and temperature on COP activity was measured by varying the pH and temperature according to the intervals proposed by the factorial analysis and the design of the experimental levels. Experiments were performed using different buffer species to achieve optimum pH and temperature conditions. The COP was incubated in buffer solutions with intervals of pH (3.7-9.7) and temperature (7.7-92.3 °C), as proposed in the factorial analysis and design of experimental levels during 2, 4, 8, 16 and 24 h. After that, the COP was transferred to pH 6.0 at 35 °C, its activity was determined and compared to the activity of a same amount of enzyme that was not previously incubated at different pH and temperature. COP was considered stable when the activity of the incubated enzyme did not differ significantly ( $p < 0.05$ ) from the activity of the non-incubated enzyme. The results were analyzed using the software Statistica.

## RESULTS AND DISCUSSION

### Extraction and purification of the anionic peroxidase (COP)

The optimum extraction buffer was found to be 50 mM sodium phosphate buffer, pH 6.0. Other buffer systems as used by Jackson and Ricardo<sup>35</sup> resulted in lower peroxidase activity per gram of *C. langsdorffii* leaves. The results of the purification of COP are shown in Table 1. A 60.5% recovery of peroxidase activity was obtained by acetone precipitation.

Further purification was obtained by ion-exchange chromatography on a DEAE-Sepharose-FF column in FPLC, resulting in one peak eluted with a 0.2 M salt (NaCl) gradient (Figure 1). From this end-step in the purification, a final purification factor of 46.86 was obtained (Table 1), with a yield of 3.5%.



**Figure 1.** Anion exchange chromatography of COP on DEAE-Sepharose FF-FPLC. Peroxidase activity was detected by oxidation of guaiacol with  $H_2O_2$  at 470 nm

### Electrophoresis, isoelectric point and molecular weight determination

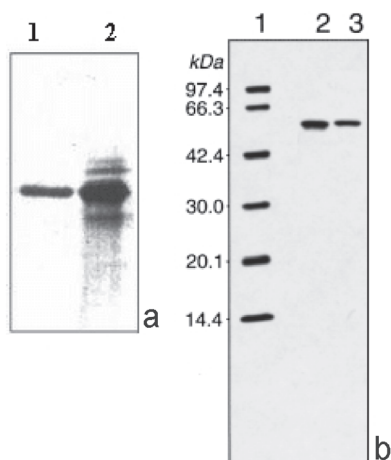
The soluble anionic fraction obtained from PAGE under non-denaturing conditions of the purified anionic peroxidase from *C. langsdorffii*, (COP) showed only one band detected *in situ* by a chromatogenic assay with guaiacol: hydrogen peroxide staining (Figure 2a) or with coomassie brilliant blue under denaturing conditions (Figure 2b).

The results indicate preservation of the enzyme activity, which is known to be adversely affected in purification procedures for the separation of peroxidase isoenzymes from plant extracts<sup>42</sup>. It is interesting to point out that the semi-purified enzyme showed a strong band in both electrophoresis systems, denaturing and non-denaturing, and high activity. Thus, the leaves of *C. langsdorffii* can be considered as a potential source of anionic peroxidase.

Homogeneity of the sample of COP was confirmed by analytical reversed-phase HPLC (Figure 3). The isoelectric point of the enzyme was determined to be 4.0 by bidimensional, isoelectric focusing electrophoresis. The molecular weight (MW) of the anionic peroxidase (COP) was estimated to be 59 kDa by regression analysis. The results were in agreement with those reported by Civello *et al.*<sup>43</sup>, for isoperoxidases from strawberry which exhibited MW of 58.1 and 65.5 kDa. Isoperoxidases do not differ significantly

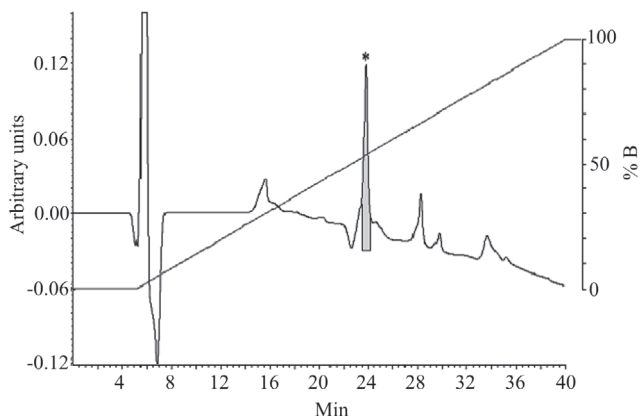
**Table 1.** Purification of peroxidase from *Copaifera langsdorffii* leaves (COP)

Fraction	Quantity	Total activity (U x 10 <sup>3</sup> )	Activity (U/mL)	Protein (µg/mL)	Specific Activity U/µg	Purification factor	Yield (%)
Crude extract	3900 mL	268476	68,840	23,826	2.89	1.00	100.0
Acetone powder	8 g	333	41,580	2,190	18.98	6.57	60.5
DEAE Sepharose	28 mL	67	2,400	18	135.44	46.86	3.5



**Figure 2.** Electrophoresis of a peroxidase (10  $\mu$ g) from *Copaifera langsdorffii* leaves (COP): a, Native PAGE: (1) Purified, (2) Semi purified COP; b, SDS-PAGE: (1) Molecular weight markers (kDa), (2) Semi-purified COP, (3) Purified COP

in size, with MW in the range from 40 to 60 kDa. Since the isoelectric points of the isoenzymes for a given species of plant vary considerably over a wide pH range, it is reasonable to expect that the isoenzymes differ substantially in amino acid content<sup>8</sup>.



**Figure 3.** COP RP-HPLC. The purification was carried out in a linear gradient of 0-70% acetonitrile in 0.1% trifluoroacetic acid (B) and the chromatography was monitored at 220 nm. The asterisk indicates the purified protein.

### COP activity, total protein, Km and $E_t$ determinations

The peroxidase activity in the samples decreased with purification, as well as the total protein content, but the specific activity increased (Table 1). Despite the decreased activity of COP, compared to the semi-purified enzyme, COP activity was higher than other sources of materials, for example, strawberry<sup>44</sup>, barley<sup>45</sup>, and leaves of *Vitis pseudoreticulata*<sup>46</sup>.

The kinetic parameters Km and  $E_t$  were estimated in the system of guaiacol: hydrogen peroxide at the optimal temperature found to the enzyme that was 35 °C. The Km of the COP for guaiacol and H<sub>2</sub>O<sub>2</sub> were 0.04 mM and 0.39 mM respectively.  $E_t$  values were 0.075 s<sup>-1</sup> for guaiacol and 0.28 s<sup>-1</sup> for hydrogen peroxide. The COP presented higher substrate affinity and  $E_t$  than other peroxidases previously reported. Holschuh<sup>47</sup> reported a Km of 20.28 mM and Vmax of 8,210 U/min for guaiacol, and for hydrogen peroxide a Km of 12.75 mM, and Vmax of 11,635 U/min for carambola anionic peroxidase.

### Amino acid analysis and N-terminal sequence determination

An N-terminal micro-sequencing of 15 amino acid residues was obtained for the COP (Table 2). The amino acid sequence of COP and peroxidase from *Arabidopsis thaliana*<sup>48</sup> and horseradish<sup>49</sup> showed just one invariant amino acid and COP showed no apparent similarities with other peroxidases, probably due to the presence of different genes for the peroxidase isoenzymes, which have been documented<sup>48,50</sup>. The amino acid composition of the purified COP is shown in Table 3.

**Table 2.** Comparison of the N-terminal sequence of peroxidases from plants

Peroxidase	N-terminal amino acid sequence														
COP <sup>a</sup>	F	G	S	N	L	L	Q	N	K	N	R	F	A	I	N
<i>Arabidopsis</i> <sup>b</sup>	M	Q	L	N	A	T	F	Y	S	G	T	C	P	N	A
Soybean <sup>b</sup>	Q	L	T	P	T	F	Y	R	E	T	C	P	N	L	F
Barley <sup>b</sup>	A	E	P	P	V	A	P	G	L	S	F	D	F	Y	W
HRP <sup>c</sup>	A	A	S	I	I	R	L	H	F	H	D	C	F		
HRP C1 <sup>d</sup>	E	L	T	P	T	F	J	D	N	S	C	P	M	V	S

<sup>a</sup>From this study. <sup>b</sup>PDB<sup>48</sup>. <sup>c</sup>Ref. 49. <sup>d</sup>Ref. 50

**Table 3.** Amino acid (AA) composition of peroxidase from *Copaifera langsdorffii* leaves (COP)

	AA	Mol%		AA	Mol%
1	Asp	7.20	10	Tyr	2.76
2	Glu	8.51	11	Val	7.14
3	Ser	9.75	12	Met	0.14
4	Gly	14.49	13	Cys	
5	His	0.72	14	Ile	3.65
6	Arg	3.85	15	Leu	4.92
7	Thr	3.78	16	Phe	17.62
8	Ala	8.99	17	Lys	5.01
9	Pro	1.18			

### Effect of pH and temperature on COP activity

The optimum reaction pH of the COP purified was between 5.0 and 7.0. The optimum reaction temperature was clearly between 30 and 40 °C. The maximum activity was obtained at pH 6.0 and 35 °C. Our results are in accordance with Catalá and Chamorro<sup>51</sup> and Wright and Nicell<sup>52</sup> for soybean peroxidase.

The stability of COP was evaluated after incubating the enzyme with different pH buffers, temperatures and incubation times. The results demonstrated that COP was stable over a wide range of pH and temperature values. The enzyme was stable between pH 4.5 and 9.3, in temperatures ranging from 7.7 to 50 °C, up to a period of 24 h. COP lost the activity after 6 min at 80 °C. Under acidic conditions, loss of activity is considered to be due mainly to heme splitting from the enzyme, whilst at neutrality, the heme bond to the protein is stable, and activity is lost through chemical changes to the heme and through protein denaturation<sup>7</sup>. The changes in the thermostability of peroxidase may be due to the splitting of covalently bound neutral carbohydrates, but this effect had not yet been proven<sup>53</sup>. The  $\beta$ -secondary structure is considered to be the most stable form as well as the most heat stable of the purified peroxidase preparations, since they contain a high content of hydroxyl amino acids<sup>39,54</sup>. COP has demonstrated catalytic ability similar to that of soybean peroxidase (SBP) over a wide range of temperatures and pH values<sup>52</sup>. SBP possesses a pH optimum at 6.4



and maximum activity range of from 5.7 - 7.0 and COP possesses a pH optimum at 6.0, with a maximum activity ranged from 5.0 to 7.0. Both peroxidases were very stable at 25 °C at neutral and alkaline pH values, but underwent rapid inactivation at acid pH (below 4.5)<sup>52</sup>. The purified COP showed similar stability and optimum pH value and temperature to those of the semi-purified enzyme (acetone powder). These results were confirmed by a statistical analysis using the Experimental Design.

In Brazil there are a great variety of plants that can be an inexhaustible source of enzymes which may be used in several areas, including analytical chemistry procedures, industry wastewater removal and as biosensors. There is a recent trend to use plant tissue or crude extract as an enzyme source. In this context, *Copaifera langsdorffii* seems to be an excellent source of stable peroxidase, which could be considered as an alternative to the use of HRP peroxidase.

## CONCLUSIONS

Anionic peroxidase from *Copaifera langsdorffii* was obtained from leaves and purified by anion-exchange chromatography, FPLC and size-exclusion chromatography. The purified peroxidase has very similar molecular and structural properties to plant peroxidase studied previously, but was more active. COP presented activity similar to HRP peroxidase, higher turnover value than other plant peroxidases. The COP peroxidase was stable at a wide range of pH and temperature, which may be advantageous to its application as a biosensor or for the industrial wastewater treatment at a lower cost than other peroxidases such as horseradish peroxidase.

## ACKNOWLEDGMENTS

We are grateful for the financial support from CAPES and from UNICAMP.

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