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Purification of polyphenoloxidase from coffee fruits

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Abstract

Polyphenoloxidase (PPO) was purified from coffee beans at the pinhead stage. Purification was carried out by precipitation of contaminating proteins with 30% $(NH_4)_2SO_4$, dialysis of the supernatant and chromatography on phenyl sepharose and DEAE columns. A purification factor of 364 was obtained. Two bands (29 and 64 kDa) of the purified PPO were detected after SDS-PAGE which presented activity in partially denaturing SDS-PAGE and gave a positive response when probed in Western blots with an apple 27 kDa PPO antibody. It is suggested that the 29 kDa band was a cleavage product of the 64 kDa PPO. The N-terminal sequencing of the 29 kDa protein did not show similarity with others PPOs. Other reports have shown that the 64 kDa is a pre-protein which is converted to a 45 kDa mature form by action of a chloroplastid protease. The 45 kDa was not detected in pinhead fruits but usually detected in the leaves and endosperm of coffee. It is suggested that the appearence of the 45 kDa form might be controlled by the developmental stage of the fruit. Chlorogenic acid was the preferred substrate for the purified PPO.
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1. Introduction

Polyphenoloxidase (PPO) is a cupric enzyme that catalyzes the hydroxylation of monophenols to *o*-diphenols, and the oxidation of *o*-diphenols to *o*-quinones (Robinson & Eskin, 1991). Quinones are very reactive compounds which strongly interact with other molecules, leading to a large variety of dark colored compounds (Eskin, 1990; Mayer & Harel, 1991).

Because of the importance of this reaction in the food industry, PPO has been intensively studied in several plants (Chevalier, Rigal, Mbéguié-A-Béguié, Gauillard, Richard-Forget, & Fils-Lycaon, 1999). PPO has been found to differ in isoforms, latency, catalytic behavior, molecular weight, isoeletric point, specificity and hydrophobicity (Ho, 1999) and recent reports have shown that in some plants the protein is coded by a multigene family with a tissue-specific expression pattern (Thygesen, Dry, & Robinson, 1995; Ho, 1999).

Studies with partially purified extracts of leaves and the endosperm of coffee beans showed two PPO bands of approximately 45 and 64 kDa (Mazzafera & Robinson, 2000). It was suggested that the 64 kDa protein was a pre-protein of the 45 kDa protein, which was released after hydrolysis of a transit peptide by a plastid peptidase, as demonstrated by Koussevitzky, Neeman, Sommer, Steffens, and Harel (1998).

Considering the importance of coffee as a worldwide beverage and the increasing demand of the international coffee market for quality, it becomes important to understand the factors affecting product quality. In addition to the traditional cup sensorial test, PPO activity has been proposed as an additional tool for beverage classification (Carvalho, Chagas, Chalfoun, Botrel, & Juste Júnior, 1994). This motivated the present study on the purification of a PPO from coffee fruits.

2. Materials and methods

2.1. Materials

Coffee fruits were harvested at the pinhead stage from a coffee tree (*Coffea arabica* cv. Catuaí Vermelho), growing outdoors in Lavras-MG, Brazil. Sephadex G-25;

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phenyl sepharose column (8×2.5 cm); HiPrep 16/10 DEAE-FF column; protein markers were obtained from Amersham Pharmacia Biotech, São Paulo, Brazil. All other chemicals used were of analytical grade.

2.2. Enzyme extraction and purification

The coffee fruits were frozen in liquid nitrogen immediately after harvesting and freeze-dried. Ten grams of fruits were extracted according to Mazzafera and Robinson (2000). PPO was extracted with 0.1 M sodium-phosphate buffer, pH 7.0, containing 2% ascorbic acid and 1g PVPP and the crude extract centrifuged at 36.000 *g* (20 min at 4 °C). The supernatant was applied to a Sephadex G-25 column equlibrated and eluted with 0.1 M sodium-phosphate buffer, pH 7.0. Purification was carried out according to Chevalier et al. (1999), as follows. Contaminating proteins were removed by precipitation with $(NH_4)_2SO_4$ at 30% saturation and the resulting supernatant obtained after centrifugation dialyzed overnight against 50 mM sodium-phosphate buffer, pH 6.5, containing 0.6 M KCl and 0.6 M $(NH_4)_2SO_4$. The dialyzed extract was then loaded on to a phenyl sepharose column (8×2.5 cm), previously equilibrated with the dialysis buffer. The column was washed with three volumes of 50 mM sodium-phosphate buffer, pH 6.5, containing 0.6 M KCl and 0.6 M $(NH_4)_2SO_4$ in order to eliminate contaminating proteins and PPO eluted with 50 mM sodium-phosphate buffer, pH 6.5, containing 0.1 M KCl and 0.1 M $(NH_4)_2SO_4$ at a flow rate of 1.5 ml/min. Aliquots of 7 ml were collected and the absorbance at 280 nm and PPO activity determined. The fractions showing high PPO activity were combined, dialyzed overnight with 10 mM phosphate buffer (pH 5.8) and applied to a DEAE sepharose column previously equilibrated with the same buffer. The column was washed with three times its volume of the initial buffer and PPO eluted by a linear gradient formed by 0–0.25 M $(NH_4)_2SO_4$ in 10 mM Sodium-phosphate buffer, pH 5.8. The flow rate was maintained at 1 ml/min and the absorbance at 280 nm and PPO activity were determined for the 5 ml aliquots.

2.3. Assay of PPO activity

The PPO reaction mixture consisted of 0.25 M sodium-phosphate buffer, pH 7, 1 mM of chlorogenic acid (5-caffeoylquinic acid—5CQA) and 70 μl of protein extract, in a final volume of 1 ml. The reaction was initiated with the addition of 5CQA and the absorbance at 420 nm recorded after 1 min.

2.4. Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli (1970), using a Bio Rad MiniGel System. The protein samples were fully denatured by boiling with -mercaptoethanol and SDS and separated in a 12.5% polyacrylamide gel. When PPO activity was to be detected in the gels (Mazzafera & Robinson, 2000), the protein extract was not denatured and the electrophoresis conditions were the same for denatured samples.

2.5. Protein blotting and Western blotting

Purified PPO was blotted on PVDF membranes (Fraignier, Marqués, Fleuriet, & Macheix, 1995) using a TE-22 Transfer Unit (Armersham Pharmacia). Immobilized proteins on PVDF membranes were visualized by Coomassie Blue staining. For the Western analysis the blotted proteins were probed with a antibody raised against a 27 kDa PPO (kindly donated by Dr. Laurence Marquès, Université des Sciences et Techniques du Languedoc, Montepellier, France). The second antibody reaction was carried out with a anti-rabbit IgG conjugate with alkaline phosphatase.

2.6. N-terminal sequencing

Protein sequencing was performed by Edman degradation using a Procise automatic sequencer (Applied Biosystems).

2.7. Kinetic properties

Kms were determined for the substrates DOPA, catechol, chlorogenic acid and pyrogallic acid using the purified extracts. The reaction mixture contained 70 l of enzyme, 880 μl of 25 mM sodium-phosphate buffer, pH 7.0, and substrate which ranged from 0.1 to 10 mM. The final reaction volume was 1 ml and the enzyme activity was recorded at 420 nm after incubation for 1 min. Km and errors were calculated by a linear regression analysis using a double reciprocal plot. Curve Expert v.1.3™ was used for curve fitting.

3. Results and discussion

Although coffee tissues are rich in phenols (Clifford, 1985) addition of ascorbic acid and PVPP to the extraction buffer prevented oxidation and yielded clear protein extracts. A summary of the purification of PPO is given in Table 1.

A broad peak of PPO activity was obtained following phenyl sepharose chromatography (Fig. 1a) with a purification factor of 25 (Table 1). Fractions 11–16 from this column were pooled, dialyzed and loaded on to DEAE column. PPO eluted as a single peak from this column (Fig. 1b) with a purification factor of 364 (Table 1). SDS-PAGE of the purified extract showed two bands with apparent molecular masses of approximately 64 and 29 kDa (Fig. 2a) and both gave positive

Table 1
Summary of polyphenoloxidase purification in young coffee fruits

Purification step	Volume (ml)	Total activity (uni)	Protein (mg)	Specific activity (uni/min mg protein)	Purification factor
Crude extract	20	26.37	52.83	0.499	1.0
30% $(NH_4)_2SO_4$	20	20.01	14.74	1.36	2.73
Phenyl Sepharose	7	21.85	1.72	12.7	25.45
DEAE	5	15.29	0.084	181.97	364.6

reations on probing with an antiboy raised against an 27 kDa PPO from apple (Fig. 2b).

PPO was also studied in coffee leaves and fruit endosperm and two bands of 45 and 67 kDa were observed (Mazzafera & Robinson, 2000) leading to the suggestion that the 67 kDa protein is converted to a mature 45 kDa protein after proteinase cleavage of a transit peptide. This is supported by the results of Robinson and Dry (1992), who obtained a 42 kDa PPO by in vitro hydrolysis of a purified 60 kDa PPO from broad bean leaves. Mazzafera and Robinson (2000) also observed other PPO activity bands in partially denaturing SDS-PAGE and suggested that proteolysis may also occur in vitro during purification, what might explain multiple bands reported in several other PPO studies. Multiple PPO isoforms observed in activity staining electrophoresis gels have also been attributed to polymerization, glycosylation, proteolysis and disulfide bond formation (Ho, 1999). King and Flurke (1987) detected several PPO activity bands on polyacrylamide gels after treating plant extracts with proteases. Studies on PPO folding and unfolding led Mari et al. (1998) to suggest a link between PPO capacity to resist proteolysis and resistance of plants to herbivore insect. Insect proteases might inactivate the enzyme, inhibiting quinone production and consequently the decrease of the protein digestibility (Felton, Donato, Broadway, & Duffey, 1992). Depending on the denaturing conditions Mari et al. (1998) observed shifts between 27 and 41 kDa, and 41 and 42 kDa PPOs. Marquès, Fleuriet, Cleyet-Marel, and Macheix (1994) also observed a 27 kDa band during apple PPO purification, attributing the appearance of this band to involuntary proteolysis.

Since most of the studies on PPO purification have focused on the heaviest PPO forms, we decided to sequence the 29 kDa protein. The N-terminal amino

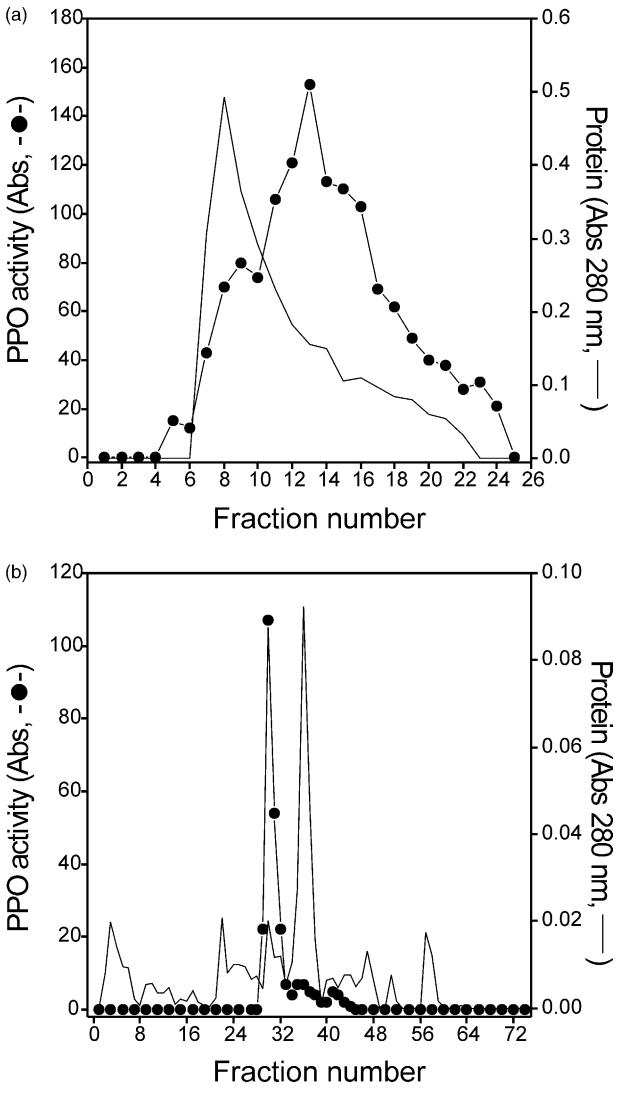

Fig. 1. Elution profile of proteins and PPO activity from phenyl sepharose (a) and DEAE sepharose (b) columns.

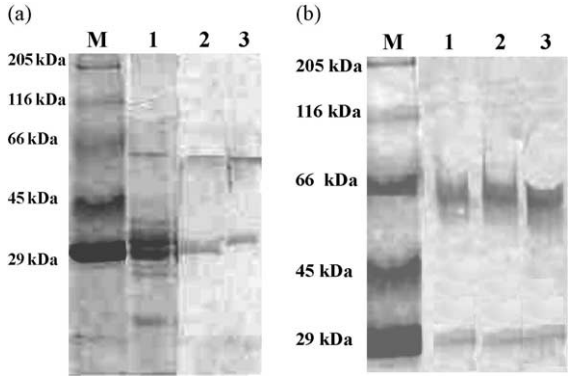

Fig. 2. Denaturing SDS-PAGE (15 μg of protein/lane) of protein fractions during PPO purification (a) and the Western blot of the same fractions probed with a 27 kDa PPO antibody (b). Lane 1—crude extract, lane 2—phenyl sepharose fraction 13, lane 3—DEAE fraction 30.

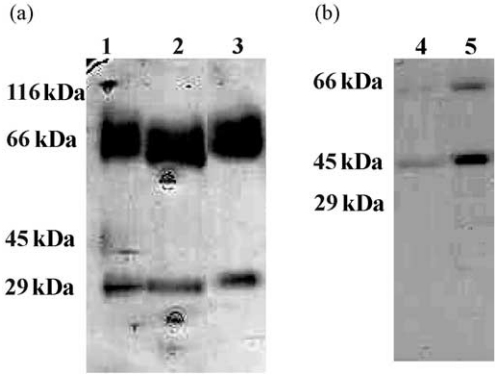

Fig. 3. Activity staining of PPO in partially denaturing SDS–PAGE (15 μg of protein/lane) of protein fractions during purification (a, lane 1—crude extract, lane 2—phenyl sepharose fraction 13, lane 3—DEAE fraction 30) and of crude extracts (b) from endosperm fruits (lane 4) and leaves (lane 5).

acid sequence obtained—AGIVRYXG—was not similar to any other protein deposited in databanks.

During the purification process, the 45 kDa PPO reported by Mazzafera and Robinson (2000) was not detected by activity staining following SDS-PAGE even when the polyacrylamide gel was overloaded with crude extract and purified fractions (Fig. 3a). However, crude extracts prepared from fruit endosperm and leaves showed this PPO form (Fig. 3b). With regard to genetic variability, *C. arabica* is highly homozygotic (Lashermes et al., 1999; Medina Filho, Carvalho, Söndahl, Fazuoli, & Costa, 1984; Orozco-Castilho, 1994) and consequently such variation would not be expected. Since the endosperm starts to develop in the coffee fruit approximately 50 days after anthesis (Mendes, 1941) and therefore absent in pinhead fruits, it is possible that the cleavage process of the 67 kDa protein is not fully active in tissues at this developmental stage. In addition, one cannot discard the possibility that different PPO isoforms may be expressed in these tissues. Tissue-specific differential expression of PPO has been observed in potato (Thygesen et al., 1995) and tomato (Thipyapong & Steffens, 1995).

The purified PPO showed higher affinity for chlorogenic acid ($K_m = 0.14 \pm 0.009$ mM), followed by DOPA (1.36 ± 0.46 mM), catechol (4.75 ± 0.26 mM) and pyrogallic acid (6.16 ± 0.80 mM). Chlorogenic acid is the major phenolic compound in *C. arabica* beans representing approximately up to 70% of total phenolics and amounting for 5–7% of the dry weight (Clifford, 1985). This phenol has also been shown to be the preferred substrate of PPO in a variety of plants such as potato (Patil & Zucker, 1965), sugar cane (Bucheli & Robinson, 1994), apple (Murata, Kurokami, & Homma, 1992) and coffee (Mazzafera & Robinson, 2000).

The present study reports for the first time the purification of PPO from coffee. The data show that the expression of the enzyme in fruits may be controlled by the developmental stage of the fruit or may be tissue specifically expressed, since activity of the mature form of the enzyme, the 45 kDa protein, was not detected. Further studies are necessary to elucidate this question, as well as to determine the role of specific proteases in the cleavage of the 67 kDa pre-protein in the different tissues of the fruit, with particular attention to the endosperm, in view of its economic importance.

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