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Artigo

Partial Purification and Thermal Stability of Two Peroxidases from *Pithecellobium dulce* (Roxb.) Benth. Aril

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Purificação Parcial e Estabilidade Térmica de Duas Peroxidases Extraídas de Arilos de *Pithecellobium dulce* (Roxb.) Benth.

Resumo: Duas formas de peroxidase (POX), denominadas PdPI e PdPII, foram parcialmente purificadas de arilos de *Pithecellobium dulce*, utilizando precipitação com sulfato de amônio e cromatografia de troca iônica. O zimograma das proteínas obtidas após precipitação com sulfato de amônio na faixa de 60 a 90% de saturação (fração F6090), mostrou duas bandas com atividade peroxidásica. Na coluna de celulose DE-52, a atividade peroxidásica foi detectada nos picos não adsorvido (PdPI) e adsorvido (PdPII) eluído com NaCl 0,2 mol L^{-1}, sugerindo que a PdPI (~114 KDa) e a PdPII (~77 KDa) são proteínas básicas e ácidas, respectivamente, sendo a PdPI estável a 80 ºC. Este é o primeiro relato de purificação parcial de POX obtida de arilos de *P. dulce*, indicando que esta espécie pode ser uma nova fonte de enzimas para utilização em biossensores e outras aplicações biotecnológicas.

Palavras-chave: Enzimas; Atividade peroxidásica; *P. dulce*; Zimograma.

Abstract

Two peroxidase (POX) forms, named PdPI and PdPII, were partially purified from the *Pithecellobium dulce* aril, using ammonium sulfate precipitation and anion exchange chromatography. Zymography of the proteins obtained after precipitation in the range of 60-90% ammonium sulfate (F6090 fraction) showed two bands with peroxidase activity. In DE-52 cellulose column, the peroxidase activity was detected in unadsorbed peak (PdPI) and adsorbed peak eluted with 0.2 mol L^{-1} NaCl (PdPII) suggesting that PdPI (~114 KDa) and PdPII (~77 KDa) are basic and acidic proteins, respectively, being PdPI stable at 80 ºC. This is the first report of partial purification of POX from *P. dulce* aril, indicating that this species may be a new source of enzymes for use in biosensors and other biotechnological applications.

Keywords: Enzymes; Peroxidases activity; *P. dulce*; Zymography.

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Partial Purification and Thermal Stability of Two Peroxidases from *Pithecellobium dulce* (Roxb.) Benth. Aril

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1. Introduction

Peroxidase (EC 1.11.1.7) is a heme-containing enzyme that utilizes hydrogen peroxide (H_2O_2) to oxidize a wide variety of organic and inorganic compounds,[1] but has also been shown to generate H_2O_2 under high pH conditions, particularly during biotic interactions.[2-4]

Plant peroxidases (POX) are usually found in roots,[4] latex,[5] flowers,[6] leaves,[5] seeds and fruits.[7,8] They are present in multiple ionic forms and their *in vivo* functions include cellular growth, the synthesis of cell walls, fruit growth and ripening, hormone regulation, lignification, senescence and defense mechanisms against abiotic and biotic stress.[2-4]

Various types of POX can be found in the same plant species and these may differ significantly in molecular weight, substrate specificity and thermal stability.[2] According to their isoelectric points (pIs), they are usually classified as acidic, neutral or basic.[9]

Peroxidases are important enzymes in analytical biochemistry. They are used in industrial applications due to their catalytic properties, thermal stability, and versatility, being useful for recognizing various substrates. Thus, peroxidases have potential applications in different areas, which increase the appeal of studying these enzymes. In addition, plants as sources of enzymes has become a promising field of study, because of the simplicity and ease of obtaining this

biological material.[4,5] Currently, horseradish peroxidase is one of the most widely used enzymes for the construction of organic biosensors and diagnostic test kits for glucose and cholesterol blood level sensors.[10-13] However, additional plant species should also be investigated in order to identify novel peroxidases with commercial value.

Pithecellobium dulce (Roxb.) Benth. (Fabaceae: Mimosoideae) is an evergreen tree from tropical and subtropical America that has now become a pan-tropical species.[14] In countries such as Mexico and India, seeds have been used as both a food source and livestock feed.[15,16] In alternative medicine, infusions of different parts of this species have been used to treat various diseases including intestinal disorders and ulcers.[16-18]

Although there is literature regarding research on the chemical constituents of seeds and leaves of the *P. dulce*,[19-21] there are still several aspects of the isolation and characterization of these compounds that remain unexplored.

In Brazil's semiarid regions, plants may present compounds of great biotechnological interest, which are traditionally used for medicinal and nutritional purposes.[22-24] *P. dulce* is well adapted to Brazilian semiarid environments and widely distributed throughout Ceará's State, where it is popularly known as "mata-fome".[25]

The fruit of this legume is a pod, 10 to 15 cm long, and dehiscent on both sides. Each pod contains 5-10 shiny black seeds, which are surrounded by a sweet white or pink aril.[14] The aril is a good source of antioxidants, hepatoprotective, and flavonoids compounds.[16,26,27] In some countries, the aril is consumed uncooked with salt, pepper and lemon or in fruit juices.[15,26] In Ceará's State, the aril is commonly eaten by the local population, mainly by children.

P. dulce is widespread and grows in abundance in northeastern Brazil, and no studies have reported the presence of peroxidase in the species. Thus, to obtain a new peroxidase with potential analytical applications, in this work we aimed to effect the extraction, isolation, and evaluation of the thermal stability of this enzyme from the *P. dulce* aril.

2. Experimental

P. dulce fruits were collected on campus of the Universidade Estadual Vale do Acaraú, Sobral-CE, Brazil, and identified at the Herbarium Prof. Francisco de Abreu Matos. To obtain soluble peroxidase, arils (10.00 g) were isolated manually from black seed and homogenized in a blender for 5 minutes in cold 34 mM phosphate buffer (pH 6.7), containing polyvinylpolypyrrolidone (1:4:0.1 w/v/w, respectively). Homogenate was filtered through cheesecloth and centrifuged at 10,000 x *g* for 30 min at 4 °C. Supernatant, denominated crude extract of arils (EaPd), was subjected to 0-30%, 30-60%, and 60-90% ammonium sulfate fractionation. Precipitate of each fraction named F030, F3060 and F6090 corresponding to each band saturation, respectively, was collected by centrifugation, dissolved and dialyzed against water, lyophilized and stored at -4 °C until use. Twenty milligram the F6090 was dissolved in 2.0 mL of tris/HCl 50 mM (pH 7.5) and applied to a DE-52 cellulose anionic-exchange column (10 x 1.5 cm) equilibrated with same buffer. Elution was carried out at a flow rate of 1.0 mL min^{-1}, using the equilibrium buffer. Retained peaks were eluted with NaCl solution at concentrations of 0.2 and 0.4 M in a step-wise manner. Peaks containing a peroxidase activity were dialyzed against phosphate buffer and used in the assays.

Protein concentration was determined by the Coomassie brilliant blue G-250 method, using bovine serum albumin as standard.[28] Absorbance at 280 nm was followed spectrophotometrically and used to monitor protein elution profiles during chromatographies.

Guaiacol (2-methoxyphenol) and

hydrogen peroxide were used as substrates for spectrophotometric quantification of peroxidase.[29] The reaction mixture containing 2.7 mL of 50 µM guaiacol, 100 µL of 13 mM H_2O_2 in 34 mM phosphate buffer (pH 6.7) and 200 µL of enzyme extracts or fractions was monitored for 10 minutes in a spectrophotometer at 470 nm with readings at every 30 seconds. One unit (U) of peroxide activity corresponded to the amount of enzyme which caused a change of 0.1 in absorbance at 470 nm. All assays were performed in triplicate, and the results were expressed as specific activity ($U.mg^{-1}$).

SDS polyacrylamide (12.5%) gel electrophoresis (SDS-PAGE) in the absence of β-mercaptoethanol was conducted as described by Laemmli at 4 °C.[30] Proteins were detected by staining with 0.1% Coomassie brilliant blue G-250. Molecular weight was calculated by linear regression using a standard marker. Gels were stained for peroxidases (zymography) as described by Caruso *et al.*[31] with some modifications: after SDS-PAGE, gel was washed for 2 times, 10 min each, with 0.2% Triton X-100. Next, gels were soaked for 30 min in 50 mM sodium acetate buffer (pH 5.2). Then, gel was transferred to a solution containing 0.03% H_2O_2 (v/v), 0.2% guaiacol (v/v) and 0.01% 3-amino-9-etil-carbazol (w/v) in the acetate buffer, until visible brown bands appeared. Subsequently, gel was washed with distilled water to stop the reaction.

In order to determine the thermal stability of the POX, 1.0 mL of the peaks with peroxidase activity from the column of DE-52 cellulose were incubated for 10 minutes in water bath at 25 (room temperature), 40, 50, 60, 70, and 80 ºC. At the end of the required time, the enzyme was cooled in an ice bath and brought to room temperature. The residual guaiacol-peroxidase activity was determined spectrophotometrically.[29] The residual percentage of the enzyme activity was calculated by comparison with unheated enzyme.

3. Results and Discussion

Currently, extensive investigations to characterize the occurrence, thermal and pH stability, substrate specificity and technological applications of enzymes presented in crude extract or purified fractions from plants are being carried out.[32,33] Chemical investigations of the different parts of the plant have resulted in the detection and isolation of a interesting enzymes, some of which having promising applications in the industrial and medical areas.[34-37]

In this study, we investigated the activity of POX in aqueous extracts of the *P. dulce* aril (EaPd) and two peroxidases were partially isolated using ammonium sulfate precipitation and ion-exchange chromatography. The EaPd showed 0.299 $mg.mL^{-1}$ of the soluble protein and peroxidase enzyme with a specific activity of 1,928 $U.mg^{-1}$. *P. dulce* aril is rich in tannins, flavonoids, antioxidants saponins, anthocyanin and although they have about 12% of protein there is no report of the bioactive enzymes identification, such as peroxidases, in *P. dulce* aril.[16,26] As is our knowledge, there is an account of only two proteins isolated from seeds of these species, namely, a lysozyme of 14.4 KDa with antifungal activity and a Kunitz-type protein inhibitor of 19.9 KDa.[19,38]

For the partial purification of POX enzyme, proteins of the EaPd were precipitated with ammonium sulfate, resulting in three fractions, F030, F3060, and F6090, respective for each band of saturation. The F030 no showed enzymatic activity. Fractions F3060 and F6090 showed activity for POX, and a greater specific activity was found in fraction F6090 (1,940 $U.mg^{-1}$) compared to F3060 (430 $U.mg^{-1}$). The presence of POX activity in both fractions suggests the existence of at least two forms of peroxidase in *P. dulce* aril.

Plants peroxidase can occurs in multiple molecular forms and being present in soluble

and bound forms (ionically and covalently bound) and each enzyme can shows variable amino acid sequences, diverse expression profiles, molecular weights and catalyze reactions with different substrates.[39-41] For example, this diversity of forms of peroxidases have been found at cucumber peelings, which showed two isoforms,[42] at tomato fruits, which showed four isoforms,[43] at broccoli stems, which showed three isoforms,[44] at horseradish root, which showing seven isoforms and at coconut endosperm, which showed two isoforms.[45,46] Peroxidase is a key enzyme in controlling plant differentiation and development, and one possible reason for the occurrence of multiple forms of POX is the diversity of biological functions and importance of POX to the growth and self-defense of plants.[39, 47, 48]

Aiming to separate the two forms of POX from F6090 fraction, which showed a greater specific activity, the fraction was passed through an anion exchange column (DE-52 cellulose). As observed in Figure 1, the peroxidase activity was observed in the unadsorbed proteins (fractions 2-3, peak I) and adsorbed peak eluted with 0.2 mol L^{-1} NaCl (factions 14-16, peak II), thus indicating that a unretained protein, named PdPI, has positive charges predominate at the surface of the enzyme. On the other hand, the adsorbed protein (PdPII) has a predominance of negative charge in their surface. This elution profile suggested that PdPI and PdPII are basic and acidic proteins, respectively. Similar elution characteristics were reported from POX from broccoli peroxidases when loaded onto DEAE-Sephacel, an anion exchange chromatography.[44]

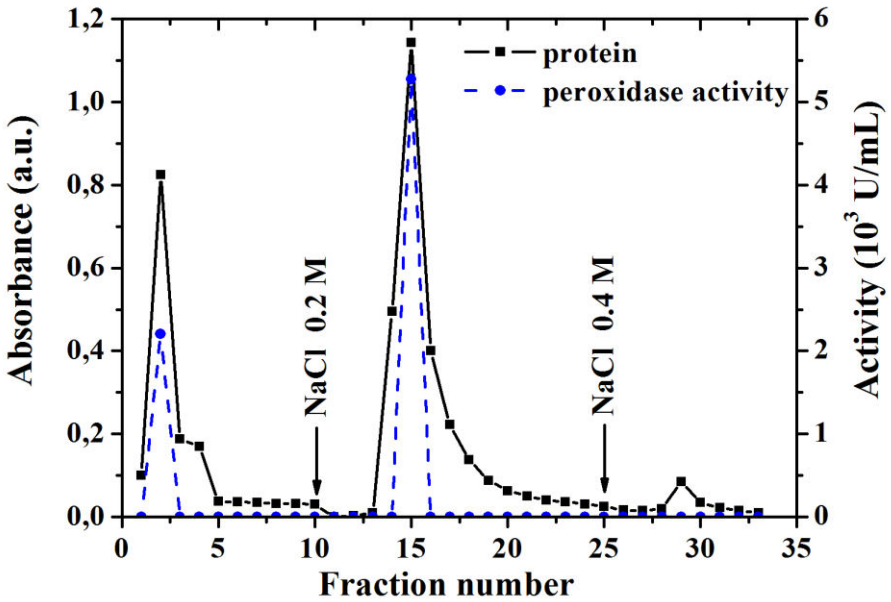

Figure 1. Elution profile from DE-52 cellulose anionic column of the F6090 fraction of the *P. dulce* arils. Protein concentration was monitored at 280 nm and peaks were assayed for peroxidase activity. All measurements were performed in triplicate

As shown in Table 1, the PdPI was characterized by a high specific activity (10,369 U.mg^{-1}) when compared to the PdPII (6,394 U.mg^{-1}). PdPI and PdPII was purified to 5.3 and 3.3 folds with overall recovery of 25% and 91%, respectively. PdPII total activities units were similar than those isolated from other plant sources, for example, *Copaifera langsdorffii* leaves, and broccoli stems.[5,44]

Table 1. Purification summary of peroxidase (POX) extracted from *Pithecellobium dulce* arils

Purification step	Total protein (mg)	Total activity units (U)	Specific activity ($U \cdot mg^{-1}$)	Purification (fold)	Yield (%)
Crude extract	35.9	69,213	1,928	1.0	100
F6090	20.6	39,964	1,940	1.0	57
PdPI[a]	1.7	17,628	10,369	5.3	25
PdPII[b]	9.9	63,300	6,394	3.3	91

[a]Unadsorbed (PdPI) and [b]adsorbed peroxidases (PdPII) in anionic exchange column (DE-52 cellulose); F6090, 60-90% ammonium sulfate fraction.

PdPI and PdPII was subjected to SDS-PAGE and zymography for peroxidase (Fig. 2A and 2B, respectively) and the electrophoretic profile of proteins showed that the anion exchange chromatography was effective in separating the two forms of POX. Partially isolated PdPI and PdPII has ~114 KDa and ~77 KDa molecular weight (MW), respectively, as estimated by linear regression using a standard marker.

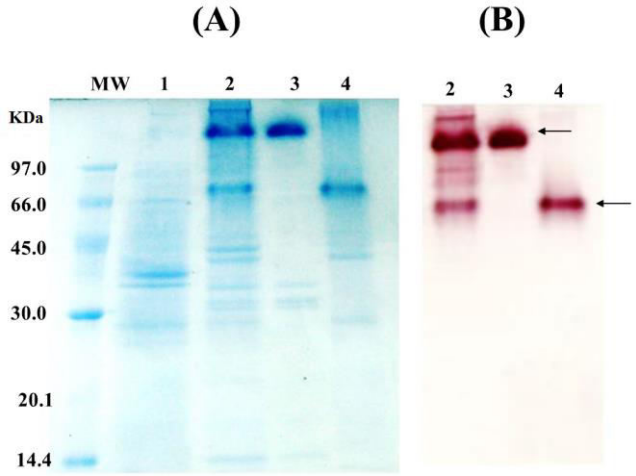

Figure 2. SDS-PAGE (A) and zymography pattern (B) of proteins from *P. dulce* aril: (1) crude extract (EaPd), (2) F6090 fraction, (3) PdPI (unadsorbed proteins in DE-52 column), (4) PdPII (adsorbed proteins in DE-52 column), (MW) molecular weight markers LMW-SDS (GE Healthcare). Fifty micrograms of protein was added to each well. The arrows indicate the bands with peroxidases activity

It is interesting to note that the molecular weight found for PdPI and PdPII is very higher than those reported for most peroxidases isolated from plant sources, namely, oil palm leaf (48 KDa),[49] sweet potato peel (37 KDa),[50] broccoli stems (KDa),[44] windmill palm leaf (50 KDa),[51] black gram's husk (35 KDa),[52] horseradish root (42 KDa),[45] coconut endosperm (~47 KDa).[46] These peroxidases are frequently found as monomeric proteins with molecular weights ranging from 35 KDa to 50 KDa.[42,52,53]

Although less frequent, there are reports of peroxidase with a molecular weight above at 100 KDa and this is due they be constituted as homo- or hetero- dimeric,

trimeric ou tetrameric proteins, such as those found in leucaena stem (200 KDa),[54] papaya fruit (~240 KDa) and bamboo shoots (111 KDa).[55,56] Thus, the high molecular weight found in PdPI and PdPII may suggest that this is multimeric enzymes but further studies are needed to confirm this speculation.

Thermal stability of the two partially isolated peroxidases was evaluated at pH 6.7 in room ambient temperatures and between 40 and 80 ºC, incubated for 10 minutes. The PdPII was heat-labile in the temperature range of 60 to 80 ºC with a fast inactivation at 60 ºC (Fig. 3). This loss of activity at temperatures above 50 °C also been observed for peroxidases of *Copaifera langsdorffii* leaves, coconut endosperm, and rocket leaves.[5,46,57]

On the other hand, the activity in PdPI remains almost constant in the temperature until 60 ºC. It showed mild decreases (~30%) in its activity at the temperature range of 70 to 80 ºC. Although peroxidase has been recognized as of the most heat-stable enzymes in plants,[58] the fact of PdPI retain approximately 70% of its activity after 10 min incubation at 80 ºC and pH 6.7, showed that PdPI was more resistant to heating than horseradish peroxidase, the only traditional commercial source of POX.[59] However, highly stable peroxidases can be found in *Moringa oleifera*, and *Phoenix dactylifera* L. leaves peroxidases which are among the most thermostable peroxidase described in the literature.[58,60]

It is noteworthy that the thermal stability of peroxidases may depend on the exposure time, the nature of the substrate employed, to the particular enzyme structure as the assayed conditions, especially the pH.[46,48,57] For example, the peroxidase from *Elaies guineensis* leaves (African oil palm tree) is a highly stable enzyme at ambient temperature over a broad range from pH 2.0 to 12.[49] The increasing temperature up to 70 ºC has no effect on their stability, after 1 hour of incubation, at pH 7.5. However, combination of the temperature at 70 ºC with acidic (pH 3.0) or alkaline (pH 12) conditions decreases dramatically their activity.[59]

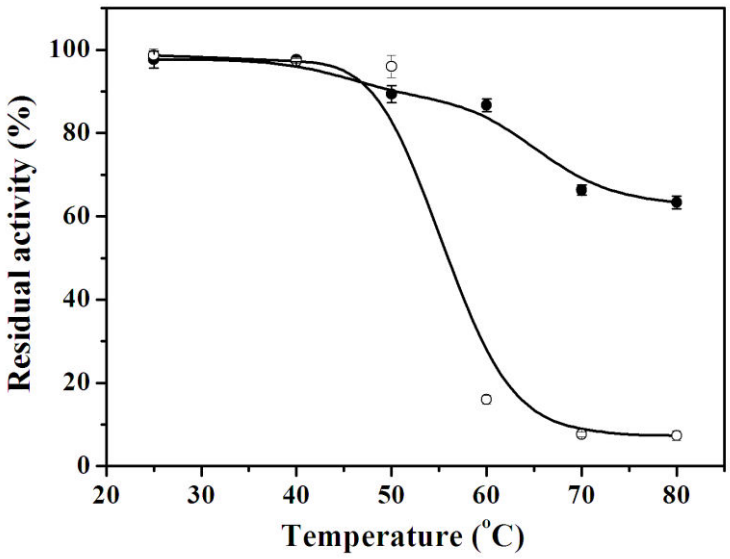

Figure 3. Residual activity of two peroxidases from *Pithecellobium dulce* aril after thermal treatment (40 to 80 °C for 10 min.). PdPI cationic peroxidase (-■-) and PdPII, anionic peroxidase (-o-). The percentage of the residual activity was calculated by comparison with the enzyme activity at room temperature (25 °C). The software Origin® 8.1 (OriginLab, Northampton, MA) was used for the calculation of the means and standard deviation

4. Conclusions

In conclusion, the *P. dulce* aril, a legume used as a food source, contains multiple forms of peroxidase, and the isolation and characterization of two of its forms was first reported in this study. That PdPI was stable a high temperature indicates that this POX may be a new source of enzyme suitable for use in biosensors and other biotechnological applications.

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