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Peroxidase activity in *Spondias dulcis*

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ABSTRACT. In this study, the best conditions to obtain crude extracts showing Peroxidase activity from *Spondia dulcis* (caja-mango) were evaluated. Fresh fruits (25 g) were blended in different sodium phosphate buffer (0.05 to 0.2 M) with a pH varying from 3.0 to 9.0. The muddy material was centrifuged for 20 minutes. In order to improve POD activity, the crude extract was submitted to precipitation with ammonium sulfate at 90% saturation. This precipitated was re-suspended in sodium phosphate buffer 0.2 M pH 6.5 and then, optimum pH for activity assay (pH varying from 5.0 to 9.0) and thermal stability (exposure to different temperatures varying from 30 to 75ºC for periods between 0 to 15 minutes) were determined. The best conditions for activity assay were in phosphate buffer 0.2 M at pH7.0. The results obtained for thermal inactivation study suggest that the heating at 75ºC for 15 minutes inactivated 95% of initial POD activity.

Key words: *Spondias dulcis*, peroxidase, extraction, heat-inactivation.

RESUMO. Atividade da peroxidase em *Spondias dulcis*. Foram avaliadas, neste trabalho, algumas condições para a obtenção de extratos brutos com atividade peroxidase de *Spondias dulcis* (cajá-manga). Frutas frescas (25 g) foram trituradas com tampão fosfato de sódio (0,05 a 0,2 M) em pHs diferentes (3,0 a 9,0). O material obtido foi centrifugado por 20 min. O extrato bruto foi submetido à precipitação com sulfato de amônio até 90% de saturação. Este precipitado foi ressuspenso em tampão fosfato de sódio 0,2 M pH 6,5 e, assim, o pH ótimo para o ensaio de atividade (pH que varia de 5,0 a 9,0) e a estabilidade térmica (exposição a temperaturas de 30, 60, 65, 70 e 75ºC por um período de 0 a 15 min.) deste foram determinados. As melhores condições encontradas para o ensaio de atividade foram em tampão fosfato 0,2 M pH 7,0. Os resultados para a inativação térmica sugerem que o aquecimento a 75ºC por 15 mininativa 95% da atividade de POD inicial.

Palavras-chave: *Spondias dulcis*, peroxidase, extração, inativação térmica.

Introduction

Spondias dulcis (caja-mango) is a small fruit cultivated in the northeast region of Brazil, mainly during the rainy season. As with most regional fruits, caja-mango is available during a short period of the year, and rarely found in other regions of the country, and it is not found in foreign countries (HAMANO; MERCADANTE, 2001).

Spondias spp. are genera members of the family *Anacardiaceae*. This genus is widespread in tropical regions of the world. Tropical fruits have found a firmly growing acceptance in the markets of Europe and United States. This increasing interest may be related to the public's nutritional awareness and search for a healthier lifestyle, as well as for their exotic flavors (CEVA-ANTUNES et al., 2003; CEVA-ANTUNES et al., 2006; LIRA JÚNIOR et al., 2005; NARAIN et al., 2004).

Brazil has a natural abundance of tropical fruits with distinctive flavors appealing to the foreign consumer (FRANCO; SHIBAMOTO, 2000). In some regions of the country exploitation of natives fruits is responsible for a large parcel of employment (CEVA-ANTUNES et al., 2006).

In general, vegetables are very susceptible to undesirable alterations as a consequence of injuries suffered during storage, handling and processing. The most usual alterations are changes in texture, nutritive qualities, color and flavor, which decrease the market value of the product (ESCRIBANO et al., 2000; VALDERRAMA et al., 2001). An important cause of color alterations is due to either formation or degradation of pigmented compounds usually present in the product. This process of deterioration is mediated by endogenous enzymatic activities such as polyphenol oxidase (PPO) and peroxidase (POD), which leads to the formation of dark polymers of a quinoidal nature (ESCRIBANO

et al., 2000; FRICKS et al., 2006; PÉREZ-TELLO et al., 2001; VALENTINES et al., 2005; ZHOUA et al., 2003).

Peroxidases (EC 1.11.1.7.) are members of a large group of enzymes widely distributed in nature, the oxidoreductases, which are involved in the oxidation of different substrates at the expense of hydrogen peroxide (KHAN; ROBINSON, 1993a; SANTOS et al., 2003, 2004). Generally, plant peroxidases have a specific requirement for hydrogen peroxide and may be considered as peroxide scavengers (KHAN; ROBINSON, 1993b). These enzymes have been implicated in metabolic process such as ethylene biogenesis, cell development and membrane integrity (SILVA et al., 1990).

Peroxidases are recognized as being one the most heat-stable enzymes present in vegetables. Then, inactivation of peroxidase activity is usually used to indicate the adequacy of blanching treatments (AGÜERRO et al., 2005; KHAN; ROBINSON, 1993a; McLELLAN; ROBINSON, 1984; ZANATTA et al., 2006). The high thermal resistance of peroxidases and their ability to undergo reactivation call for a study of the thermal stability of such enzymes during the blanching of fruits and vegetables (PHILIPPON; ROUET-MAYER, 1984).

Therefore, the quality of the blanched and frozen products may be better if there is some activity of peroxidase left at the end of the blanching process (GARROTE et al., 2004). A residual activity of peroxidases of about 3-10% after blanching was recommended for preservation by freezing (GÜNES; BAYUNDIRH, 1993). Blanching time would be the time required to achieve the inactivation of peroxidase and it depends on such factors as heating method, heating temperature, size, shape and thermal conductivity of the product and type and concentration of the enzymes. This multiplicity of concurrent factors makes it usual that the blanching time is established empirically (AGÜERRO et al., 2005; DENG et al., 2003; LEMOS et al., 2000; SERVANT et al., 1986a; SERVANT et al., 1986b).

The aim of the present work was to determine the initial conditions for purification and characterization of *S. dulcis* peroxidase, and to estimate conditions for the inactivation of the POD.

Material and methods

Plant material. 500 g of *Spondias dulcis* fruits were obtained in the Rio de Janeiro city market in September 2006. The samples were stored in a freezer.

Enzyme extraction. 25 g of fresh pulp was blended in different sodium phosphate buffer ranging pH 3.0 to 9.0 (Table 1) and the extract was centrifuged at 23000 x g for 20 min. at 4ºC (twice). The supernatant (raw extract) was collected and assayed for soluble peroxidase activity (FRICKS et al., 2006).

Precipitation of proteins. The proteins were precipitated from supernatants by adding solid ammonium sulfate to reach up to 90% (w v^{-1}) of saturation. Next the solution was centrifuged at 2300 x g for 40 min. at 4ºC, the suspension was discarded. The precipitate was dissolved in a minimal volume of phosphate buffer 0.2 M (pH 6.5) (FRICKS et al., 2006).

Determination of the peroxidase activity. Enzyme activity was determined measuring the change in absorbance at 470 nm due to the formation of the oxidation product of guaiacol, described to be tetraguaiacol ($\varepsilon_{tetraguaiacol}$ = 2.66 x 10^4 M^{-1} cm^{-1}) (HIRATA et al., 1998), in the presence of hydrogen peroxide and the enzyme (GORANLL et al., 1949). One unit of enzyme (U) is defined as the quantity of enzyme able of forming 1 µmol of chemical product per minute.

Protein determination. Protein concentrations of the extracts were measured according to the Bradford (1976) method, measuring optical density (OD) at 595 nm, with bovine serum albumin as standard.

Effect of pH. A study was made on the effect of pH on guaiacol oxidation by *S. dulcis* peroxidase. Enzyme activity was determined in 0.2 M sodium phosphate buffer at different pH values, ranging from 5.0 to 8.0.

Heat-inactivation. Heat-inactivation studies of peroxidase activity were carried out on the proteins precipitated in the test tubes. Following heat inactivation, the tubes were immersed in cold water to minimize any further change in enzymatic activity. By this method, it is determined the residual activity of the enzyme present in treated solutions.

Results and discussion

Determination of suitable conditions for peroxidase extraction. Several pH and concentration of sodium phosphate buffer were employed to select the most suitable ones to extract peroxidase from *S. dulcis* tissues. The optimum extraction pH was measured by extracting the peroxidase enzyme from fruits in buffers ranging from pH 3.0 to 9.0 (Figure 1). Maximum peroxidase activity (61 U mg^{-1}) was detected at pH 6.5, with guaiacol as substrate. Moreover, pH values under 5.0 showed low enzyme activity. This effect has been attributed to the loss of the heme group at low pH as described by Pomar et al. (1997). However, at

this stage of the work all enzyme assays were undertaken at pH 6.0, due to the best solubility of the enzyme extract aliquots in the reaction mixture.

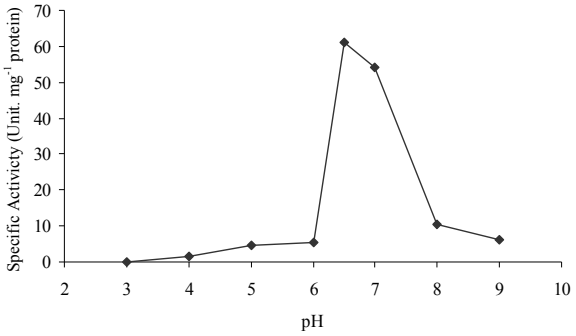

Figure 1. Effect of pH in the extraction of *S. dulcis* peroxidase.

Nevertheless, increasing the molar concentration of sodium phosphate buffer the activation of peroxidase is affected (Table 1). It was determined that a 0.2 M concentration of sodium phosphate increases peroxidase activity.

Table 1. Effect of buffer concentration on *S. dulcis* peroxidase activity.

Buffer Composition (pH = 6.5)	Specific Activity (U mg^{-1} protein)
0.2 M sodium phosphate	50
0.1 M sodium phosphate	25
0.05 M sodium phosphate	24

pH optimization for activity assay. Using the optimized extraction conditions with a 0.2 M sodium phosphate buffer, pH 6.5, a study was performed to determine the optimal pH values for reaction of the enzyme (Figure 2). Maximum peroxidase activity was assigned at pH 7.0 (specific activity 106 U mg^{-1} protein).

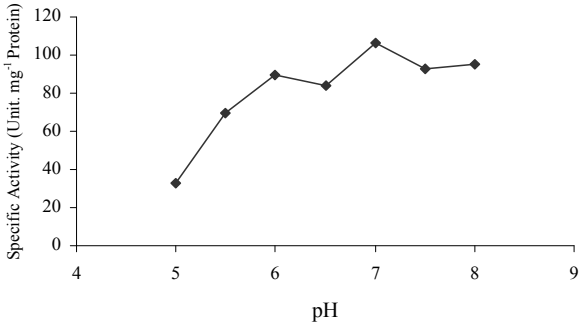

Figure 2. Effect of pH on the enzymatic activity of *S. dulcis* peroxidase.

Efficiency of precipitation of the raw extract protein. A summary of the activities of the peroxidase (Table 2) shows that the ammonium sulfate fractionation of the raw protein extracts of fruits was efficient, as shown by an increase (13%) of the specific activity and good recovery (66.6%) of the enzymatic activity.

Table 2. Peroxidase activity of the *S. dulcis*.

Parameter	Raw extract	$[NH_4(SO_4)_2]$ 90% saturation
Volume (mL)	43	8
Total Protein [mg]	7.3	4.1
Enzymatic activity [U mL^{-1}]	1.4	10
Specific activity [U mg^{-1}]	50	57
Total activity [U]	175	116
Recovery [%]	100	67

Heat-inactivation. Thermostability plot (Figure 3) shows the residual activities (in per cent) of peroxidase at the protein solution obtained after precipitation of raw extract.

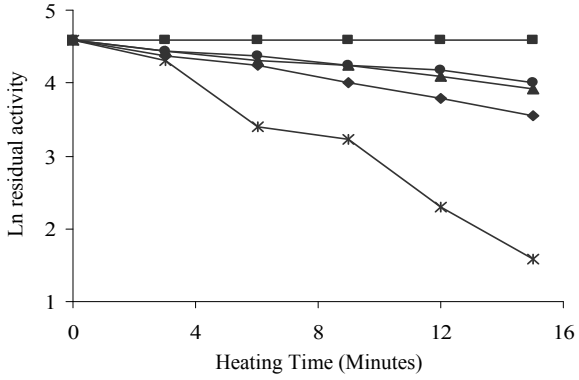

Figure 3. Residual activities after POD inactivation at different temperatures. (■ 30ºC, ● 60ºC, ▲65ºC, ♦70ºC, ★75ºC).

The inactivation of peroxidase was non-linear with heating time. Khan and Robinson (1993b) reported that the exact cause of the non-linear plots for heat-inactivation is not know, but inactivation is normally a function of pseudo first-order kinetic. At 60ºC the enzyme is stable and activity decreases by 40% after 15 min. But, is has been suggested that this behavior is likely to be due to the presence of a number of iso-peroxidases with different thermostabilities. The thermostability of peroxidases has been attributed to the presence of sugars at their structure (POMAR et al., 1997). The thermal treatment was efficient to total inactivation of peroxidases, enzyme activity extending up to 10 min. After 15 min. at 75ºC the *S. dulcis* peroxidase maintains 5% of the total activity. Partial regeneration of peroxidase activity was not observed after treatment at 75ºC for 15 min. If the parameter is blanching, Günes and Bayundirh (1993) reported that a residual activity of peroxidases about 3-10% after this treatment suggest efficiency. However,

total inactivation was not achieved which suggests the presence of thermo resistant isoenzymes (ZANATTA et al., 2006). Other studies must be performed with the purpose to clarify the influence of thermal treatment in enzymatic behavior in this fruit.

Conclusion

Maximum peroxidase activity was extracted at pH 6.5 in the sodium phosphate buffer 0.2 M. In this work the enzyme activity assays were conducted at pH 7.0, with guaiacol as substrate.

The results to thermal inactivation of partially purified caja-mango fruit peroxidase suggest that the heating at 75ºC for 15 min. is efficient to inactivate 95% of initial POD activity.

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