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Peroxidase activity in roots of arracacha affected by pH and temperature

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ABSTRACT. In this paper, roots of arracacha (*Arracacia xanthorrhyza* Bancroft) were stored at 5ºC to induce chilling injury symptoms and stress-related peroxidase activity. Later, peroxidase kinetic activity was determined in different pH and temperature conditions. For this, soluble crude extract was sequentially saturated with ammonium sulfate, obtaining a semi-purified enzyme solution used for the analysis. Activity of peroxidase induced by the chilling at 5ºC was determined from pH 2.5 to 9.0 and at temperature ranging from 10 to 80ºC. The peroxidase had higher activity when the reaction occurred between pH 5.5 and 6.0 and at temperature of 30ºC. Complete inactivation of the activity was observed in pH 2.5 after 60 minutes of pre-incubation or at 60ºC for 10 minutes or alternatively at 70ºC after 5 minutes of pre-incubation. The enzyme is more susceptible to inactivation in acid than alkaline pHs or alternatively using heat treatment.

Keywords: *Arracacia xanthorrhyza* Bancroft, oxidative enzyme, inactivation.

RESUMO. Atividade da peroxidase em raízes de batata-baroa afetada pelo pH e temperatura. Neste trabalho, raízes de batata-baroa (*Arracacia xanthorrhiza* Bancroft) foram armazenadas a 5ºC para induzir injúria por frio e expressar atividade da peroxidase de estresse. Posteriormente, a cinética de atividade foi determinada em diferentes condições de pHs e temperatura. Para isto, extrato solúvel da raiz foi sequencialmente saturado com sulfato de amônio, obtendo-se uma preparação semi-purificada para a análise enzimática. Atividade peroxidativa induzida pela temperatura de armazenamento de 5ºC foi determinada em pHs de 2,5 a 9,0 e a temperaturas de 10 a 80ºC. A atividade da peroxidase foi maior quando a reação foi realizada nos pHs de 5,5 e 6,0 e temperatura de 30ºC. A inativação completa da enzima ocorreu em pH de 2,5 após 60 min. de pré-incubação ou a 60ºC por 10 min., e alternativamente a 70ºC após 5 min. de pré-incubação. A enzima foi mais susceptível à inativação em pH ácido do que alcalino, podendo também ser inativada pelo tratamento de calor.

Palavras-chave: *Arracacia xanthorrhyza* Bancroft, enzima oxidativa, inativação.

Introduction

Arracacha (*Arracacia xanthorrhiza* Bancroft) is a native root crop from the Andes region of South America and the cultivation is usually located in regions of mild climate, in altitudes usually between 1500 and 2000 m and optimum growth in temperatures of 15 to 20ºC (SANTOS; CARMO, 1998). In Brazil, arracacha is cultivated mainly in the states of Paraná and Minas Gerais, in regions where the climatic conditions are quite similar to the center of origin (SANTOS, 2000).

Arracacha postharvest storage capacity is compromised by several factors of abiotic and biotic nature, and among them, the susceptibility of the tuberous root to chilling injury is one of the most important (RIBEIRO et al., 2007). In this root, the symptoms caused by storage at 5ºC are primarily shown at surface, developing irregular pit lesions in the whole surface, followed by intense internal discoloration (RIBEIRO et al., 2005).

Peroxidases (EC 1.11.1.7) are oxidoreductases that have important role on the mechanism of defense in plants, caused by abiotic or biotic stresses. In works done with pineapple and mandarin (EL-HILARI et al., 2003; GONÇALVES et al., 2002), an increase in the activity of peroxidase was observed when the development of chilling occurs. Peroxidases act on the removal of hydrogen atoms most usually from the alcohol groups, which are combined with hydrogen peroxide in order to form molecules of water and oxidized phenolic

compounds, acting as detoxifying enzymes and as a cell wall crossing linked enzyme during wounding stress (PASSARDI et al., 2005). Miller et al. (1989) has also observed that the enzymatic darkening in fresh cucumber fruits might has been linked to the peroxidase enzymatic activity and by a form of polyphenoloxidase present mainly in the skin. In cut jicama roots, regardless of storage temperature, peroxidase also was involved in tissue darkening due to lignin formation induced by the wounding (AQUINO-BOLAÑOS; MERCADO-SILVA, 2004).

The peroxidases belong to a family of glycoproteins containing iron atoms as a prosthetic group and different quantities of carbohydrate residues (VAN HUYSTEE, 1987). They are located mainly in the cell wall and in the cells vacuoles of plants; their location varies according to age, species and developmental stage (GASPAR et al., 1982). The optimum pH for the enzyme activity *in vitro* varies from 5.5 to 7.5 according to the substrate and the vegetal tissue used. The most adequate temperature to evaluate the peroxidase activity *in vitro* is variable according to the species, also the activity of this enzyme can regenerate after it has been inactivated by high temperature, and it may need severe thermal treatments aiming to avoid regeneration of the catalytic activity (GASPAR et al., 1982). In arracacha roots, no kinetic properties of peroxidase are known, or which treatment could best be used to inactivate the activity.

Ribeiro et al. (2005) had shown that tap roots of arracacha are very sensitive to developing severe chilling injury symptoms when stored at temperatures below 10ºC. Later on, Menolli et al. (2008) verified that the storage of arracacha at 5ºC induced an increase in the activities of peroxidase and polyphenoloxidase, which were related to the appearance of dark discoloration symptoms induced by the cold treatment. However, under this storage condition, the peroxidase was the main enzyme induced by the stress of low temperature. Thus, the present paper had as purpose to partially purify and to determine the peroxidase activity in wide range of pH and temperature conditions, in order to develop postharvest techniques to diminish the enzymatic darkening.

Material and methods

The work was carried out in laboratory conditions, using arracacha roots (*Arracacia xanthorrhiza* Bancroft) from the cultivar Amarela de Carandaí acquired in a retail store, located in the city of Viçosa, State of Minas Gerais, Brazil. After 24 hours at room temperature, the roots were selected according to the good visual aspect for commercialization and stored in cold chamber at 5ºC for 28 days to induce injury or at 10ºC as control (RIBEIRO et al., 2005; MENOLLI et al., 2008). Afterwards, 40 g of roots, previously macerated in liquid nitrogen, were homogenized in 100 mL of extraction buffer containing 0.1 M phosphate buffer pH 6.5, 0.1% sodium bisulfite and 0.15 M sodium chloride at 4ºC filtered through four layers of cheesecloth (LAGRIMINI et al., 1997). The filtered solution was centrifuged at 17000 x *g* at 4ºC for 30 minutes. The supernatant was saved and the pellet discarded. The supernatant remains was sequentially saturated with solid ammonium sulfate [$(NH_4)_2SO_4$)] up to 80%, with the following saturation salt fractions, from 0-20, 20-40, 40-60 and 60-80% stirring for 30 minutes in ice bath and each fraction was collected by centrifugation at 17000 x *g* for 30 minutes at 4ºC. The resulting pellet from each salt saturated fraction with ammonium sulfate was resuspended in 2 mL of buffer phosphate dialysis buffer containing 10 mM pH 6.5 and 0.1% sodium bisulfate, followed by dialysis at the same buffer for 12 hours in dialysis bags at 4ºC. After that, the dialyzed extracts were centrifuged at 17000 x *g* for 5 minutes at 4ºC to clear the supernatant.

Peroxidase activity of crude extract and ammonium sulfate fractions were added to the reaction medium constituted of 0.2 M phosphate buffer pH 6.5, 0.3% hydrogen peroxide and 0.28% guaiacol. The reactions were followed in a spectrophotometer, by the absorbability variation in the wavelength of 470 nm over three minutes at 25ºC, immediately after adding the enzymatic extract to the reaction mixture, and the activity was expressed in absorbability unites (AU) $min.^{-1}$ mg^{-1} protein (LAGRIMINI et al., 1997).

The protein concentrations of the enzymatic preparations were determined as described by Bradford (1976), using bovine serum albumin (BSA) as pattern.

In order to determine the pH effect on peroxidase activity, reaction buffer solutions of 0.2 M citric acid (pH 2.5 to 4.0), 0.2 M phosphate buffer (pH 4.5 to 7.5) and 0.2 M boric acid (pH 8.0 to 9.0) using 1 N NaOH or HCl to adjust the pHs used. The pH in which the enzyme showed biggest activity for the partially ammonium sulfate purified extract was used in the later assays.

In the acid and alkaline pHs in which the enzyme showed the smallest activities were investigated. For this, the enzymatic extract were mixed to the buffer-solutions (1:1; v v^{-1}) and pre-incubated from 0 to 120 minutes at pH 2.5 and 9.0.

The assays were then carried out with the reaction medium containing the buffer solution that promoted the biggest enzymatic activity at pH 6.0.

In order to determine the optimum temperature for the peroxidase activity, the enzymatic extract was pre-incubated in temperature ranging from 10 to 80ºC for 10 minutes, and them the reaction was carried out at the same pre-incubation temperature for a period of three minutes.

The determine the temperature versus time for enzyme inactivation and ability recovery its activity, the extract was pre-incubated at temperatures of 50, 60 and 70ºC for an interval of 0 to 120 minutes. After this, the samples were kept in ice bath at 4ºC for 30 minutes and then the enzymatic reaction done at temperature of 30ºC over three minutes.

The experiment was carried out in a totally randomized experimental design with five replications of individual chilled injured roots. The data were submitted to descriptive statistics.

Results and discussion

The roots exposed to temperature of 5ºC showed both internal and external symptoms of chilling. Externally, there were areas of pitting irregularly distributed all over the skin surface, after 14 days (Figure 1Aa) and 28 days (Figure 1Ab) of exposure. Internally, after 28 days an intense discoloration was present from the vascular ring towards the periderm (Figure 1Ac). At 10ºC, only slight symptoms of aging and chilling injury were detected up to 28 days of storage (Figure 1Bbc) compared to the freshly harvested roots (Figure 1Ba).

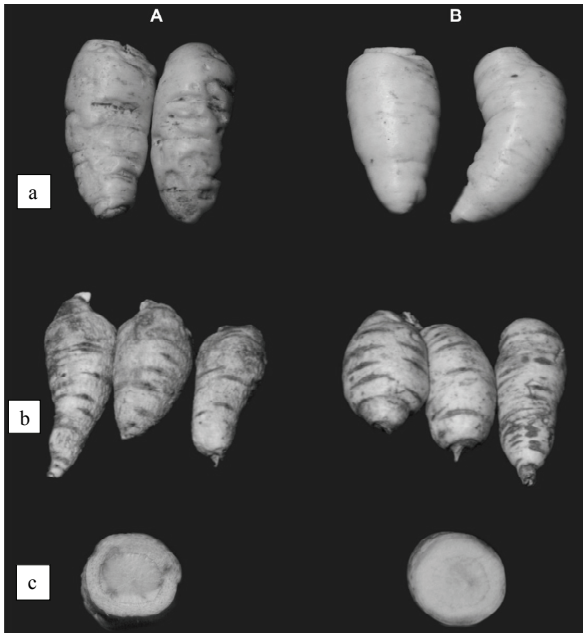

Figure 1. External an internal appearance of arracacha roots stored at 5ºC (column A) and a 10ºC (column B).

The partial purified peroxidase showed to be extremely soluble in aqueous buffer, and it was precipitated most in the ammonium sulfate fraction of 60-80% (Figure 2). In this fraction of salt saturation, there was an increase in the specific activity of 3.6-fold compared to initial extract, and the specific activity went from 25 to 90 AU $min.^{-1}$ mg^{-1} protein. In saturations below 60% ammonium sulfate there was little peroxidase precipitation, showing thus the elevated enzyme solubility (Figure 2).

With the partial purification peroxidase followed by dialysis, it was possible to analyze the effects pH and temperature on the enzyme behavior without any background by using the 60-80% fraction. The purification gain for arracacha was higher compared to the 2.3-fold increase in activity for the red pepper peroxidase saturated with 30-80% ammonium sulfate (SERRANO-MARTÍNEZ et al., 2008).

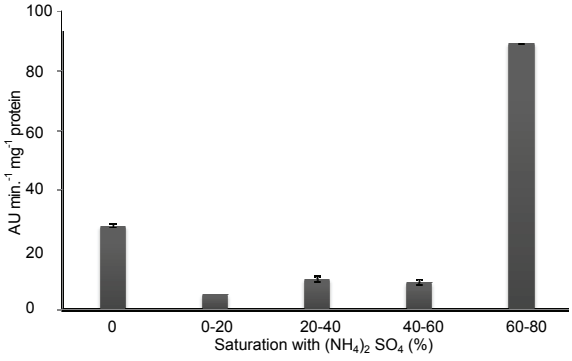

Figure 2. Fractionation of peroxidase with ammonium sulfate precipitation. The vertical bars represent the standard error from the average.

The activity of peroxidase reached its highest level when the reaction was done with phosphate buffer with pH values of 5.5 and 6.0, with specific activity of 2.7 UA $min.^{-1}$ mg^{-1} protein in pH 6.0 (Figure 3). Most peroxidases have higher activity in acid pHs, ranging from 4.0 up to 7.0 (BATTISTUZZI et al., 2001; GAZARYAN; LAGRIMINI, 1996; TEICHMANN et al., 1997). These differences in optimum pH for peroxidase reflect the origin of the species, location of the enzyme in cell, as well as the expression of several isozymes that form the peroxidases family (LURIE, 2003). In bananas, the presence of at least three isoforms with different chemical properties during fruits ripening was already identified (CANO et al., 1990).

Peroxidase presented lowest activity at pHs 2.5 and 9.0, similar has been determined for peroxidases purified from other plant species, including corn (TEICHMANN et al., 1997) cucumber

(BATTISTUZZI et al., 2001) and red pepper (SERRANO-MARTÍNEZ et al., 2008). Comparing the values of peroxidase activity in pH 6.0 and in the pH 2.5 and 9.0, the activity at pH 2.5 was only 2.6% of the maximum activity and when the reaction was done in pH 9.0, the enzyme kept 6.2% of its activity (Figure 3). Contrarily to arracacha, the sweet potato peroxidase was easier inactivated at alkaline pHs, showing 59% of activity at pH 2.5 compared to maximum activities at pH 5.5 and 3.5, also using guaiacol as substrate (LEON et al., 2002). The results of our experiment suggest that postharvest acidic pH treatments would be more effective in knocking down most of peroxidative activity in roots of arracacha.

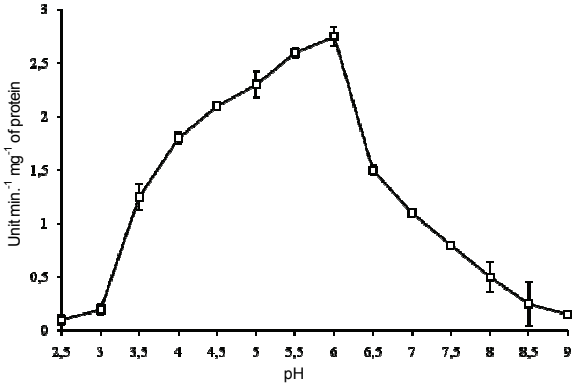

Figure 3. Influence of pH on peroxidase activity. The vertical bars represent the standard error from the average.

Pre-incubation of peroxidase extract in buffer with pH 2.5 for 5 minutes reduced enzyme activity by 96% in relation to the maximum activity, and after 60 minutes of pre-incubation, there was complete enzyme inactivation (Figure 4). Thus, the acid pH has a drastic affect in reducing enzyme activity, causing quick damage to the active site of the enzyme. The behavior of peroxidase in buffer with pH 9.0 was quite different from pH 2.5, since it was verified that in 5 minutes of exposition to pH 9.0 the enzyme activity was reduced in about 77%, keeping itself practically constant until the pre-incubation for 120 minutes (Figure 4). Thus, peroxidase activity was not inactivated in alkaline pH, contrary to the peroxidase behavior in acid pH. As arracacha root peroxidase is less stable in acid pH than in alkaline pH, treatments with acid substances would be more effective to inactivate the enzyme and reduce the tissues enzymatic darkening. Ducamp-Collin et al. (2008) have shown that the immersion of lychee fruits in solution of citric acid was efficient in delaying the darkening of the fruit skin due to the peroxidase and polyphenoloxidase activity.

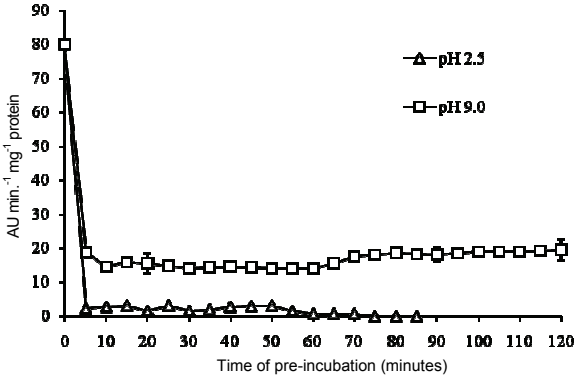

Figure 4. Influence of pre-incubation pH 2.5 and 9.0 on activity of peroxidase at pH 6.0 in tap roots of arracacha. The vertical bars represent the standard error from the average.

Peroxidase presented maximum activity at temperatures of 30ºC, representing an increase of 58.8% in its activity compared the activity at 20ºC (Figure 5). In horseradish, the optimum temperature for activity ranged between 20 to 40ºC (TAMS; WELINDER, 1998), while in tomato the maximum activity was at 55ºC (LOUKILI et al., 1999). Arracacha peroxidase is less thermal resistant than the tomato peroxidase, being similar to the horseradish isoform.

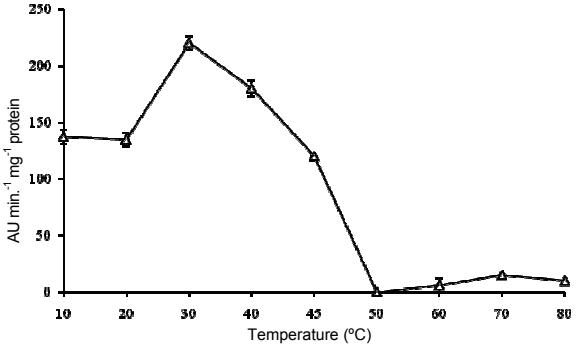

Figure 5. Influence of temperature on the activity of arracacha tap root. The vertical bars represent the standard error from the average.

Above 30ºC there was a decrease on the enzymatic activity, causing practically the complete inactivation of the enzyme at 50ºC (Figure 5). Therefore, the peroxidase in arracacha does not present elevated thermo stability, indicating that it can be inactivated with relatively low temperatures, reducing the cooking during blanching.

The likely inactivation necessary time was determined by pre-incubating the enzymatic extract at the temperatures of 50, 60 and 70ºC, followed by refolding period at 4ºC for 30 minutes. On the

extracts pre-incubated at 50ºC, a reduction of 62% on enzyme activity was observed after 5 minutes of exposition, and a 78% after 30 minutes of heat treatment compared to the maximum activity determined at 30ºC (Figure 6). Longer periods of heat treatment at 50ºC up to 120 minutes reduced on slightly the activity, showing the accumulated heat treatment was not able to irreversible damage the enzyme at this temperature.

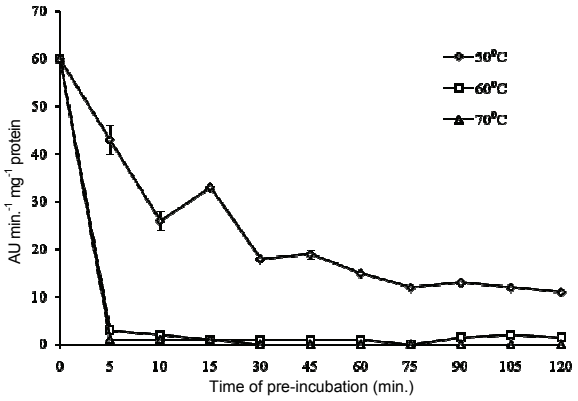

Figure 6. Activity of peroxidase after pre-incubation at 50, 60 and 70ºC in arracacha roots evaluated at pH 6.0 and 30ºC. The vertical bars represent the mean standard error from the average.

In the pre-incubated extracts at 60ºC, an almost complete enzyme inactivation was observed after 5 minutes of exposition, remaining only traces of activity (Figure 6). On the other hand, at temperature of 70ºC, this inactivation occurred 5 minutes after the incubation.

The enzymatic inactivation, through the exposition of the extract to high temperatures can be observed in works with pulp and apple peel as well, when Valderrama et al. (2001) verified that the peroxidase enzyme is very stable when exposed to temperatures of 60, 65, 70 and 75ºC during 10 minutes, obtaining only maximum inactivation of 85% of its activity after 5 minutes of treatment at 75ºC.

Conclusion

Low temperature induces severe symptoms of chilling in stored roots of arracacha. Partially purified peroxidase of arracacha roots has highest activity at acidic pHs and shows relatively low thermal tolerance. Its inactivation is better achieved at extremely at low than alkaline pHs. The enzyme can be completely inactivated by heat treatment in a relatively short period of time.

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