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A N I N T E R N A T I O N A L J O U R N A L

Ionically Bound Peroxidase from Peach Fruit

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ABSTRACT

Soluble, ionically bound peroxidase (POD) and polyphenoloxidase (PPO) were extracted from the pulp of peach fruit during ripening at 20°C. Ionically bound form was purified 6.1-fold by DEAE-cellulose and Sephadex G-100 chromatography. The purified enzyme showed only one peak of activity on Sephadex G-100 and PAGE revealed that the enzyme was purified by the procedures adopted. The purified enzyme showed a molecular weight of 29000 Da, maximum activity at pH 5.0 and at 40°C. The calculated apparent activation energy (Ea) for the reaction was10.04 kcal/mol. The enzyme was heat-labile in the temperature range of 60 to 75°C with a fast inactivation at 75°C. Measurement of residual activity showed a stabilizing effect of sucrose at various temperature/sugar concentrations (0, 10, 20 %, w/w), with an activation energy (Ea) for inactivation increasing with sucrose concentration from 0 to 20% (w/w). The Km and V_{max} values were 9.35 and 15.38 mM for 0-dianisidine and H_2O_2, respectively. The bound enzyme was inhibited competitively by ferulic, caffeic and protocatechuic acids with different values of Ki,. L-cysteine, p-coumaric and indolacetic acid and Fe^{++} also inhibited the enzyme but at a lower grade. N-ethylmaleimide and p-CMB were not effective to inhibit the enzyme demonstrating the non-essentiality of SH groups.

Key words: Peach peroxidases, ripening, purification, kinetics, heat stability

INTRODUCTION

Peroxidase (E.C. 1.11.1.7) (POD) has been implicated in a variety of physiological process such as ethylene biogenesis, cell development, membrane integrity, response to injury, disease resistence (Abeles & Biles, 1991, Gillikin & Graham , 1991, Haard, 1977, Hammerschmidt *et al.*, 1984, Vàmos-Vigyàzo, 1981). The properties of peroxidase and its physiological role in postharvest fruits and vegetables have been reviewed by several authors (Haard, 1977, Gorin & Heidema, 1976, Miesle *et al.*, 1991, Rhotan & Nicolas, 1989). The enzyme has been also involved in deteriorative changes in flavor, texture and color in raw and processed fruits and vegetables (Haard, 1973, Burnette, 1977, Clemente & Pastore, 1998, Vàmos-Vigyàzo, 1981). Peroxidase activity has been investigated in a range of fruits including grape (Robinson *et al.*, 1989), mangoes (Khan & Robinson, 1993-a), papaya (Silva *et al.*, 1990), pears (Moulding *et al.*, 1989), oranges (Clemente, 1996), peaches (Lourenço & Neves, 1997, Neves & Lourenço, 1998) and apples (Moulding *et al.*, 1987). The enzyme occurs in multiple molecular forms and is present in most fruits and vegetables in a soluble and bound (ionically and covalently) forms (Moulding *et al.*, 1987, 1989, Robinson *et al.*, 1989, Silva *et al.*, 1990, Thomaz *et al.*, 1981, Wang & Lu, 1983), whose catalytic properties are influenced by variety or/and cultivar, growth and physiological stages. A correlation between peroxidase activity and fruit ripening has been shown in a number of cases (Flurkey & Jen, 1978, Rhotan & Nicolas, 1989, Silva *et al.*, 1990,

Thomaz *et al.*, 1981). In mango (Khan & Robinson, 1993) apples (Moulding *et al.*, 1987) and papaya (Silva *et al.*, 1990) peroxidase activity increases with ripening. The activity of bound and soluble enzymes isolated from the pulp of ripening banana fruit were increased at the onset of the respiration climateric (Haard, 1973). In contrast, the soluble peroxidase form from tomato (Kokkinakis & Brooks, 1979) and papaya fruit (Silva *et al*, 1990) reach a maximum peak followed by a marked decrease at the initial levels.

The activity of the ionically bound peroxidase form from papaya was greatly increased with ripening but gradually fell as the fruit turned from ripe to over-ripe stage (Silva *et al.*, 1990). It is generally accepted that peroxidase is the most heat-stable enzyme in vegetables and that under conditions of limited treatment, it can regain its activity during storage of processed foods (Burnette, 1977, Chang *et al.*, 1988, Khan & Robinson, 1993b, Clemente, 1996, Neves & Lourenço, 1998). Polyhydric alcohols and sugars have been utilized as anti-denaturant agents against freeze and heat-induced denaturation in proteins (Arakawa & Timashef, 1982, Lee & Timashef, 1981, Chang *et al.*, 1988) and in the fruit processing most of these compounds could be used. In contrast, the effects of these additives on thermal behaviour of the heat resistant enzymes related to off-flavors generation, as peroxidase and polyphenoloxidase, need to have better understanding. To our knowledge, the ionically bound peroxidase from peach fruit has not been adequately investigated. The present investigation was designed to study the extraction of soluble, ionically bound peroxidase and polyphenoloxidase from peach fruit and the changes in the enzyme activities during ripening. Procedures for purifying the ionically bound peroxidase and some physicochemical properties are also described.

MATERIALS AND METHODS

Plant material and chemicals: Peach fruit (*Prunus persica* L.) cv. Rei da Conserva, grown under standard conditions in the state of São Paulo, were picked at the ripe stage, washed, peeled, cut into small pieces, stored at -20°C and used for enzymes extraction. All chemicals were reagent grade. DEAE-cellulose, Sephadex G-100, polyethylene glycol 20000, L-cysteine, bovine serum albumin, soybean trypsin inhibitor, ovalbumin, lactate dehydrogenase, cytochromo C, caffeic, ferulic, protocatechuic, p-coumaric acids, o-dianisidine, N-ethylmaleimide, p-chloromercuribenzoate (p-CMB) were obtained from Sigma Chemical Co (St Louis, MO, USA).

Storing at 20°C: Peach fruit were picked at mature-green stage, washed and stored at 20°C in a chamber with relative humidity control. The evolution of the ripening was observed by color/texture alterations and aliquots of three fruits were select at different times, peeled, cut into small pieces and used for enzymes extraction.

Enzymes extraction: Twenty-five g of tissue was homogenized in 0.1 M potassium phosphate buffer, pH 6.5 containing 1 mM L-cysteine with addition of Polyclar aT (0.1 g/10 g of tissue) as phenolic scavenger (Neves & Lourenço, 1985). The suspension was filtered through four layers of cheescloth and centrifuged at 25000 g for 30 min. The supernatant was collected (crude extract) and used to assay for soluble POD and PPO (polyphenoloxidase). The sediment fraction was resuspended in 40 ml of the above buffer containing 1.0 M NaCl, stirred mechanically for 12 hr (overnight), and centrifuged as above. The supernatant was used as a source of ionically bound peroxidase. In some experiments, NaCl was replaced with $CaCl_2$ or $MgCl_2$. All procedures were carried out at 4°C.

Enzyme assay: Soluble and ionically bound peroxidase activities were determined by change in absorbance at 460 nm due to o-dianisidine oxidation in the presence of hydrogen peroxide and the enzyme (Neves & Lourenço, 1998). The reaction mixture consisted of 0.2 ml 15 mM O-dianisidine; 0.2 ml 10 mM hydrogen peroxide, 0.1 M potassium phosphate-citrate buffer, pH 5.0 in a total volume of 3.0 ml. One enzyme unit is defined as the amount of enzyme producing a 0.00l absorbance change per min under the assay conditions used. The reaction mixture for PPO assay contained 6 mM of 4-methylcatechol, citrate-phosphate buffer, pH 5.0 and enzyme solution in a total volume of 3 ml. The reaction was initiated by the addition of the enzyme and the absorbances were automatically recorded at 420 nm and 30°C. The enzyme unit was defined as cited above for POD.

Purification: The supernatant containing the ionically bound POD after extraction was dialysed against solid PEG-20000 to concentrate the protein. After concentration, the extract was applied to a column of DEAE-cellulose (1.5 x 20 cm), previously equilibrated and washed with 5 mM phosphate buffer, pH 7.0. Protein was eluted with a linear gradient of KCl (0-0.4 M) in 5 mM potassium phosphate buffer, pH 7.0 and 5 ml fractions were collected. The active fractions were pooled and applied to a column of Sephadex G-100 (2 x 50 cm) equilibrated and washed with the same buffer. The eluted fractions containing peroxidase activity were pooled and used for the studies.

Protein determination: Protein concentration of the various extracts and solutions was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard. Absorbance at 280 nm was used to monitor protein in the column eluates**.**

Effect of temperature/thermal stability: Reactions were carried out under standard assay conditions as described above at 10 to 60°C. The Ea (*apparent activation energy*) for the reaction was determined by measuring the reaction rate constant at different temperatures. Thermal stability of Sephadex G-100 purified ionically bound POD as a function of temperature was determined over the range of 60 to 75°C using 0.1 M potassium phosphate buffer, pH 7.0. The enzyme was placed in a test tube into a water bath, pre-set at the appropriate temperatures. Aliquots of purified enzyme solutions were withdrawn at timed intervals, rapidly cooled in an ice bath and assayed for the remaining activity under the assay conditions described above. Percent residual activity was calculated as percent of the original activity in the unheated preparation.

Effect of sucrose: To study the effect of sucrose on enzyme heat stability, purified POD was incubated at the temperatures in the presence of various concentrations of the sugar and the residual activity determined as cited above. The apparent activation energy (Ea) for inactivation was determined by measuring the reaction rate constant for inactivation at different temperatures in the presence and absence of sugar.

pH optimum: Enzyme activity as a function of pH was determined using o-dianisidine, citrate-phosphate buffer (pH 3.0-6.5) and tris-HCl buffer (pH 7.0-9.0). POD activity was assayed under standard conditions as described above.

Kinetic studies: The apparent K_m and $V_{max.}$ were determined from Lineweaver-Burk plots at optimum pH and temperature conditions .

Inhibitor studies: The enzyme was incubated with varying substrate concentrations at two fixed inhibitor concentrations. The values of inhibition constant (Ki) and the type were determined by Dixon plot (1953). All assays were performed in triplicates.

Molecular weight determination: Molecular weight was determined by gel filtration on a Sephadex G-100 column. The column was calibrated for molecular weight with standard protein; cytochrome C (12400), soybean trypsin inhibitor (21500), ovalbumin (43000), bovine serum albumin (67000) and lactate dehydrogenase (130000). The Mr was estimated using a plot of Ve/Vo vs log Mr of standard proteins according to the method of Whitaker (1963).

Polyacrylamide gel electrophoresis: The PAGE was performed by the method of Davis (1964) using 7% polyacrylamide gel in tris-glycine buffer, pH 8.3, at 4°C with 2.5 mA per tube. After the run the gels were incubated in a solution containing 0.2% (w/v) o-dianisidine at pH 6.0, followed by addition 30 mM H_2O_2. Staining bands indicating POD activity appeared within 20 min at 37°C and the gels were rinsed in distilled water. Relative mobility was determined by the migration of the bromophenol marker dye.

Effect of divalent cations and other compounds: The effect of cations, phenolic compounds and sulphydryl-blocking reagents were verified by the addition of the compounds at different concentrations at the enzyme assay as described above for inhibitors.

RESULTS AND DISCUSSION

Activities of soluble, ionically bound peroxidase and polyphenoloxidase during ripening at 20°C are shown in Figure 1. The fruit took about 6 to 12 days to reach the ripe (edible) stage from the mature-green stage. The activity of soluble POD showed a peak followed by a decrease, returning to the level found in mature-green fruit. The

ionically bound POD was not altered during the period, while polyphenoloxidase activity greatly increased with ripening with a little drop between 8 to 12 days. The PPO activity was 2.4-fold higher than the initial values, in contrast to the observed evolution of the browning rate of the tissue with ripening.

The total peroxidase activity has been shown to increase with ripening banana (Haard, 1977), peach (Flurkey & Jen, 1978), apple (Gorin & Heidema, 1976), tomato (Kokkinakis & Brooks, 1979) and papaya (Silva *et al.*, 1990), and gradually fell as the fruits turned from ripe to senescence stage. To solubilize of ionically bound POD the addition of various salts to the buffer were needed to release the enzyme from cell components. The effect of $CaCl_2$ and $MgCl_2$ on bound POD extraction were decreased with the increase in the salts concentration up to 1.0 M, however, NaCl demonstrated to be more effective on extraction condition at concentrations above 0.5 M.

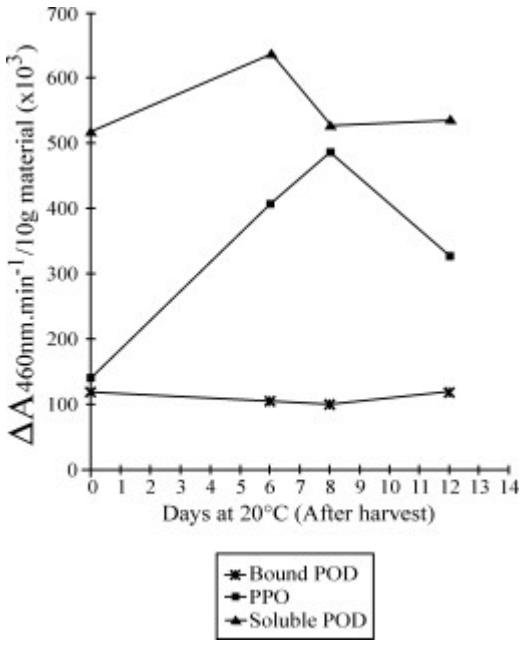

Figure 1 – Soluble, ionically bound peroxidase and polyphenoloxidase activities during the ripening of peach fruit.

Purification of ionically bound POD: Dialysis of the crude extracts against solid polyethylene glycol (PEG-20000) was most effective to concentrate. On DEAE-cellulose column (1.5 x 20 cm), the enzyme was eluted in a single activity peak (fraction 37-44) with the wash buffer, similar to observed with the bound POD from papaya fruit by Silva *et al*. (1990). However, this step brought about only 2.0-fold purification. Further purification of bound peroxidase involved gel filtration on Sephadex G-100 (2.5 x 50 cm), which resulted in 6.1-fold purification (Table 1). The enzyme activity was eluted between fractions 56-75, indicating the presence of only one enzyme species or more than one species of somewhat similar molecular weight (Figure 3). PAGE of this enzyme preparation revealed the presence of two bands stained for activity in the Sephadex G-100 peak (Figure 2).

Properties of Sephadex G-100 purified enzyme: Bound peroxidase activity as a function of pH showed a broad profile throughout the pH 4.5—6.0 range with an optimum at pH 5.0. The optimum pH of peroxidases from various vegetables sources studied occurred in the acid side of pH (Vàmos-Vigyàzo, 1981). The effect of temperature on bound peroxidase activity was studied under standard conditions. The optimum temperature was 40°C and 40% of the maximum activity was registered at 55°C indicating a heat labile enzyme. The apparent activation energy (*Ea*) value for product formation was found to be 10.04 kcal/mol, in contrast the temperature coeficient (Q_{10}) calculated from this Ea value was 1.78 in the range of 20-30°C, a similar value found for other peroxidases (Bruemmer *et al.*, 1976, Joffe & Ball, 1962, Neves & Lourenço, 1998, Lourenço *et al.*, 1995).

Thermal inactivation: Figure 4 shows the time course inactivation of purified peach ionically bound POD from 60 to 75°C. The enzyme was reasonably stable at 60°C and when exposed to 65°C, there was a 45% residual activity for 60 sec, in contrast to 30 and 15 sec at 70 and 75 °C, respectively. At 65°C, only 20% of the residual activity was detected after 4 min exposure. At 70°C, approximately 95% of the activity was lost after 3 min and 1 min at 75°C.

Table 1 - Purification of ionically bound peroxidase from peach.

Procedure	Volume (ml)	Total activity (units)	Specific activity (units/mg protein)	Purification (factor)
Crude extract	105	476.7×10^3	238.95	-
PEG-Dialysis	23	407.1×10^3	215.85	1
DEAE-cellulose	4.5	38.7×10^3	409.00	1.9
Sephadex G-100	4.0	10.56×10^3	1320.00	6.1

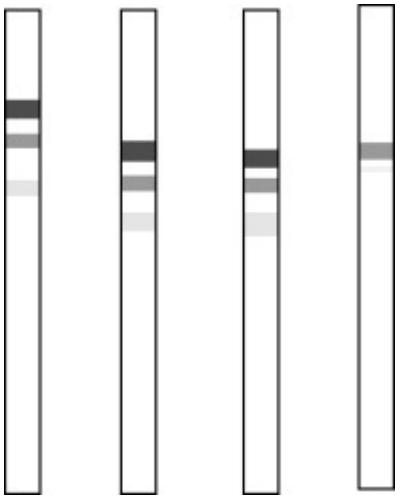

Figure 2 – Electrophoretic patterns of purified ionically bound peroxidase. (1) crude extract (10 µg), (2) PEG-concentrate (5 µg), (3) DEAE-cellulose (4 µg), (3) Sephadex G-100 (4 µg). Reactions on the gel were estimated accord to described.

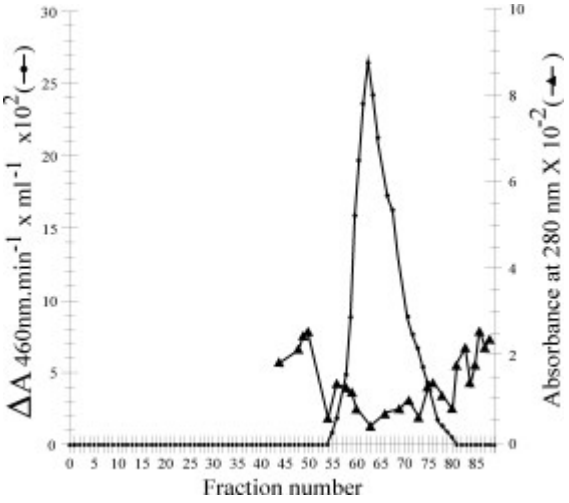

Figure 3 - Chromatography of purified ionically bound peroxidase on Sephadex G-100. Enzyme solution was applied to a column of Sephadex G-100 (2 x 50 cm) in K-Pi buffer, pH 7.5. Elution was conducted with the same buffer.

The peach bound enzyme showed a biphasic thermal inactivation behaviour only at 60°C. However several workers impute this character non-linear to the formation during heating of denaturated protein forms and groups of POD molecule that remain actives (Lu & Whitaker, 1974, Clemente, 1996, Khan & Robinson, 1993, Neves & Lourenço, 1998, Adams, J., 1997) or different conditions of the enzyme after heating (Adams, J., 1997, Clemente, 1996, McLellan & Robinson, 1984, Hemeda & Klein, 1991, Neves & Lourenço, 1998). The peach peroxidases, soluble and bound, showed distinct heat lability (Neves & Lourenço, 1998). This fact was also observed for isolated enzymes from apple (Moulding *et al.*, 1989), papaya (Silva *et al.*, 1990) and orange (Clemente, 1996).

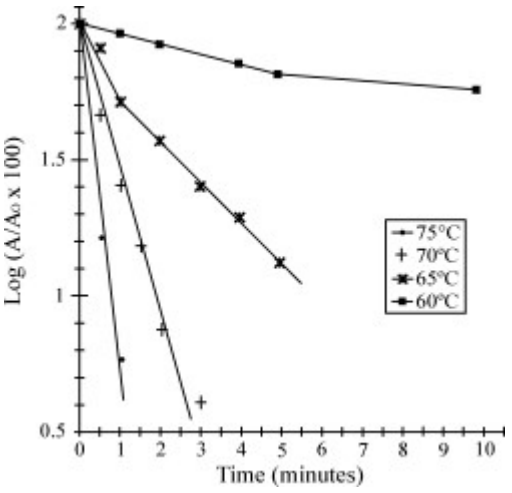

Figure 4 - Heat inactivation curves of Sephadex G-100 purified ionically bound peroxidase at 60, 65, 70 and 75°C. The residual activity was determined accord to described in Methods.

The apparent activation energy (*Ea*) for enzyme inactivation estimated from an Arrhenius plot was found to be 40.88 kcal/mol. The enzyme was heated at 65, 70 and 75°C in the presence of sucrose at 0, 10 and 20% concentration in the incubation mixture. The increase in sucrose

concentration corresponded to an increase in enzyme stability regardless of the heating temperature, when compared to control without sugar (figures 5, 6 and 7), however the stabilizing effect was more pronounced at the lower temperature. The soluble peach POD presented higher heat stability considering the same sucrose concentrations and temperatures (Neves & Lourenço, 1998). The calculated values of the apparent activation energy (*Ea*) for enzyme inactivation in the absence and presence of sucrose at the temperature range studied revealed to increase with sugar concentration;

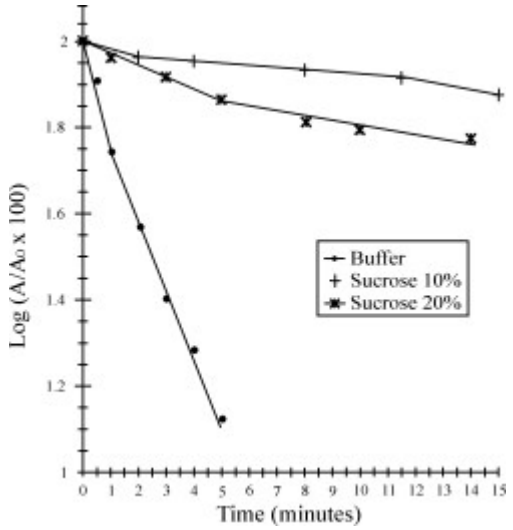

Figure 5 - Heat inactivation curves of Sephadex G-100 purified ionically bound peroxidase at 65°C in the presence of sucrose at 10 and 20% (w/v) concentrations. Residual activity was determined accord to described in Methods.

values of 40.88, 55.30 and 60.70 kcal/mol were found at 0, 10 and 20% sucrose, respectively. The protecting effect of poliols to thermal denaturation of proteins is a fact, however the mechanisms are still in discussion (Arakawa & Timashef, 1982, Gerisma, 1968, Frigon & Lee, 1972, Neucere e St Angelo, 1985). Several authors observed an increase of the activation energy (Ea) for denaturation with an increase in sucrose concentration for different enzymes, and they suggested that some physicochemical properties alterations of the sistem could to be occuring, specially related to the water structure (Arakawa & Timashef, 1982, Back *et al.*, 1979, Lee & Timashef, 1981). According to our results and those of other authors the enzymes from various sources behave distinctively in the presence of sugars on heating. Arakawa & Timashef (1982) and Lee & Timashef (1981) showed evidences of the protein-solvent relationship as a stabilizing factor of the protein structure, representing the preferential interaction protein-water as a function of the sucrose concentration in the system. To the bound POD in this study the presence of sucrose resulted in a higher stability of the protein structure whitout affecting the conformational form of the catalytic site as also

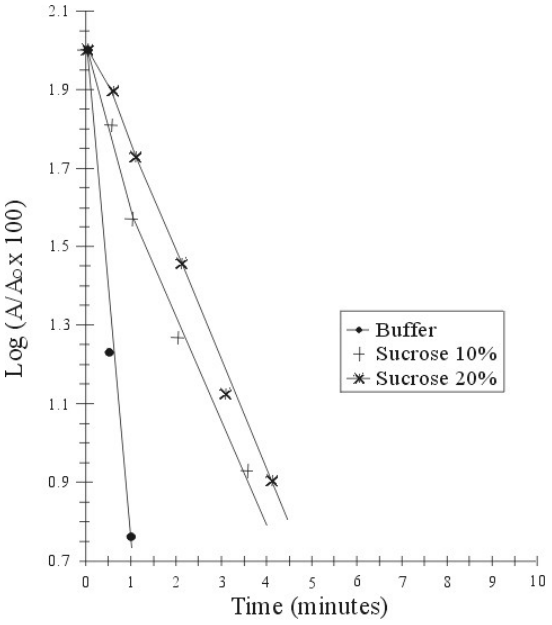

Figure 6 - Heat inactivation curves of Sephadex G-100 purified ionically bound peroxidase at 70°C in the presence of sucrose at 10 and 20% (w/v) concentrations. Residual activity was determined accord to described in Methods.

observed by Chang *et al* (1988) for horseradish peroxidase. Compared to the soluble peach POD (Neves & Lourenço, 1998), the bound enzyme showed a reduced thermal stability compared to the same range of sucrose concentrations and temperatures studied.

Kinetic studies and molecular weight: The effect of substrate concentration on bound peroxidase was investigated. The Km values for o-dianisidine and hydrogen peroxide were 9.35 and 15.38 mM, respectively. The V_{max} values were 1389 and 1052 un/ml. The ratios V_{max}/Km of 148.5 and 68.6 indicated a preferential action of the enzyme for o-dianisidine than hydrogen peroxide. The higher specificity for the donor substrate appeared a characteristic of peroxidases from different

sources (Silva *et al.*, 1990, Soda *et al.*, 1991, Vàmos- Vigyàzo, 1981). The molecular weight determination estimated by Sephadex gel filtration was calculated to be 29000 daltons, which was in agreement with the wide range of molecular weight (30-60000 Da) reported for peroxidase from various sources (Clemente, 1996, Floris *et al.*, 1984, Khan & Robinson, 1993, Lourenço & Neves, 1997, Silva *et al.*, 1990, Vàmos-Vigyàzo, 1981).

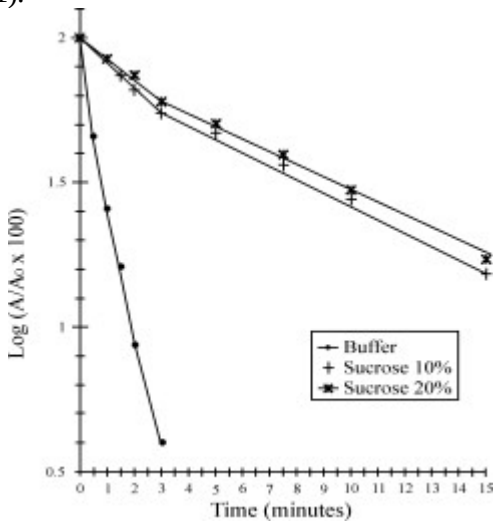

Figure 7 - Heat inactivation curves of Sephadex G-100 purified ionically bound peroxidase at 75°C in the presence of sucrose at 10 and 20% (w/v) concentrations. Residual activity was determined accord to described in Methods.

It is worth noting that the inhibitors had different effects on peroxidase. The following cations had no effect on the enzyme activity: Zn^{++}, Cu^{++}, Mg^{++}, Mn^{++}, $(NH_4)^{+}$, whereas Fe^{++} was more effective as an inhibitor (Tables 2 and 3).

Table 2 - Effect of salts on Sephadex G100 purified ionically bound peroxidase*.

Salt	Concentration in the assay (µmoles)	Inhibition (%)
Control	-	0
$ZnSO_4$	1.5	9.8
	6.0	9.8
$CuSO_4$	3.0	6.5
	6.0	6.5
$MgSO_4$	3.0	4.4
	6.0	4.4
$MnCl_2$	1,5	6.9
	6.0	8.0
$FeSO_4$	1.5	11.4
	3.0	59.40
	3.9	70.30

*assays as described in Methods.

L-cysteine was a more potent enzyme inhibitor when compared to sulphite (Table 3). A characteristic of the l-cysteine inhibition was to exhibit a crescent lag phase with the increase of the concentration. The effect of a number of phenolic compounds on bound POD activity are shown in Tables 3 and 4. The strongest inhibition was observed with caffeic acid, ferulic acid and protocatechuic acid followed by p-coumaric acid while indolacetic acid slightly inhibited the enzyme.

Table 3 - Effect of various compounds on Sephadex G-100 purified ionically bound peroxidase*.

Compound	Concentration in the assay (µmoles)	Inhibition (%)
Control	-	0
L-cysteine	0.18	20.83
	0.30	30.21
Na_2SO_3	0.6	0.0
	3.0	16.25
	6.0	50.00
$(NH_4)_2SO_4$	20	0.0
	120	1.0
Ferulic acid	0.050	85.0
	0.015	56.6
	0.005	43.3
Indolacetic	0.30	0.0
acid	0.75	10.0
	1.50	45.0
	4.50	61.7
p-Coumaric	0.30	8.30
acid	0.90	11.66
	1.5	50.00
	3.0	95.00

*assays as described in Methods

The inhibitory effects of caffeic, ferulic and protocatechuic acid were analyzed kinetically and the Dixon`s plot (1964) indicated that bound POD inhibition by these acids is a competitive type with differents Ki values (Table 4).

The sulphydril inhibitors p-chloromercuribenzoate and N-ethylmaleimide pratically were not effective inhibitors of bound peach peroxidase (Table 5), indicating that SH groups were not involved in ionically bound POD catalysis.

Table 4 - Inhibitors, type and inhibition constants (Ki) for Sephadex G-100 purified ionically bound peroxidase.

Inhibitor (acids)	Ki (mM)	Inhibition type
Caffeic	4×10^{-3}	competitive
Ferulic	$2,2 \times 10^{-2}$	competitive
Protocatechuic	$2,6 \times 10^{-1}$	competitive

*Inhibition constants (Ki) and type were obtained according to the method od Dixon (1964).

Table 5 - Effect of sulphydryl-blocking reagents on Sephadex G-100 purified ionically bound peroxidase.

Compound	Concentration in the assay (μmoles)	Inhibition (%)
Control	-	0
N-thylmaleimide	0.30	6.25
	1.50	7.50
	3.0	10.00
p-CMB*	0.30	0.0
	0.75	0.0
	1.50	2.5

* p-cloromercuribenzoate

Different workers have found the non-essentiality of SH groups for catalysis in peroxidases of distinct sources (Lourenço & Neves, 1997, Floris *et al*., 1984).

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RESUMO

As peroxidases solúvel, ionicamente ligada e a polifenoloxidase de pêsssego foram acompanhadas durante o armazenamento a 20°C. A forma ionicamente ligada foi purificada 6,1 vezes utilizando-se de cromatografias de DEAE-celulose e Sephadex G-100. A enzima purificada foi eluída em um único pico de atividade na cromatografia de Sephadex G-100 e a eletroforese em gel de poliacrilamida (PAGE) revelou uma enzima purificada pelos procedimentos adotados. A enzima purificada apresentou um peso molecular de 29000 Da , pH e temperatura ótimos de pH 5,0 e 40°C. O valor da energia de ativação da reação foi de 10,04 kcal/mol. A enzima apresentou-se termolábil na faixa entre 60-75°C com rápida inativação a 75°C. Medida da atividade residual mostrou um efeito estabilizante da sacarose a várias temperaturas e concentrações do açucar (0, 10 e 20%, p/v) com uma energia de ativação (Ea) para inativação crescente com a concentração de sacarose. As constantes cinéticas Km e Vmax foram calculadas para o-dianisidina e peróxido de hidrogenio. A enzima ligada foi inibida competitivamente por ácido ferúlico, cafeico e protocatéquico com valores distintos de Ki. L-cisteína, ácidos p-cumárico e indolacéico e Fe^{++}, inibiram a enzima porém em menor intensidade. N-etilmaleimida e p-CMB (p-cloromercuribenzoato) não foram eficientes na inibição enzimática.

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