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Characterization of 'Biuti' Peach Polyphenoloxidase

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Abstract In Brazil canned 'Biuti' peach is a very popular form of this sub-tropical fruit. This production represents an important economic agro-activity in Minas Gerais, Brazil during the summer period, in preparation for the Christmas celebrations. The aim of this work was to characterize the 'Biuti' peach polyphenoloxidase (PPO), since peach products show enzymatic oxidation of the polyphenols by oxidative enzymes, which affects the products during their shelf life. Two different hypothesis for the browning problem in processed peaches were studied: the inadequacy of the blanching treatment and the presence of a latent phenolase in the peaches. The PPO was characterized: pH optimum (5.5) and stability (5.5-6.5); optimum temperature at 20°C and 80% of the activity retained after 30 min at 15-40°C. The test for the presence of latent PPO in the processed and canned peaches was negative. Ascorbic acid, ß-mercaptoethanol, sodium metabisulfite, and cysteine were efficient in inhibiting the PPO.

Keywords: polyphenoloxidase, peach 'Biuti', biochemical characterization, enzymatic browning

Introduction

Peach is a fruit highly appreciated throughout the world due its flavor, attractive color, and high economic value in the productive chain. In Brazil, the annual production of peaches is increasing more than 15% per year, and the knowledge about the processing behaviour is a determinant factor for its increasing production. One of these new regional varieties peaches is the 'Biuti' peach, produced mainly in Minas Gerais and São Paulo, Brazil (1). The annual peach production in Brazil is last year 2008 was 130,000 ton, more than 90% of this production is destinated to processing products for Brazilian market. Today this production represents already 100% of total consume in Brazil, and products from Greece and Chile has decreased due the improvement of our internal production.

The 'Biuti' peach is an excellent fruit for both *in natura* consumption and industrialization. These peaches are typically large and spherical, with very yellow, acid-sweet tasting pulp, which is easy to separate from the stone (2). These characteristics have resulted in the 'Biuti' peach having an increasing share of the industrialized peach market in recent years.

Despite all the potential for development in the food industry, peach products suffer from some serious technological problems. In particular, enzymatic oxidation of the polyphenols by endogenous oxidative enzymes affects the products during their shelf life. The browning problem is frequent and affects canned products (3).

This study focused on two different hypothesis to understand the browning phenomena in the processed peaches. The first and most common one was an inadequacy of the blanching process. In order to test this hypothesis, the polyphenoloxidase (PPO), present in the 'Biuti' was isolated, characterized, and measured its activity after several combinations of heat treatment and chemical inhibition.

The second idea concerned the presence of a latent endogenous phenolase. The literature describes the unusual characteristic of some enzymes to remain in an inactive or latent state (4-7). The PPO can be released from latency or activated, by a variety of treatments or agents, including acid shock (8), urea (9), anionic detergents such as sodium dodecyl sulfate (SDS) (10), proteases (11), and fatty acids (12). The use of SDS as an activating agent is particularly interesting because few enzymes are known to be activated by SDS (13). Swain *et al*. (9) showed, for the first time in 1966, that this enzymatic activation process was related to a limited conformational change in the latent enzyme.

With respect to the production of regional peach varieties that only grow in Brazil the majority of the research carried out is focused on genetic improvement of the plants, on tillage and on storage of the fruits. The increasing size of the fruit processing industry in Brazil, and the lack of information about the technological problems the industry is facing, has created a demand for research data on the oxidative enzymes in these fruits and their characteristics. The objective of this work was to provide experimental data on the oxidative enzymes present in the 'Biuti' peach, which shows an increasing participation in the national industry.

Materials and Methods

Fresh fruits The 'Biuti' peaches were obtained from a commercial cultivar of these peaches in São Sebastião do Paraíso, MG, Brazil. They were picked at commercial harvest maturity, ready to be sold to the industry or used for direct consumption. The fruits were refrigerated until processed the next day.

Industrial samples Peaches in the form of fruit in syrup were processed and 400 g (wet weight) aliquots canned

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and stored at room temperature. Five-hundred g aliquots of the peach pulp were filled into glass jars and blanched at 95°C for 30 min. They were then stored at room temperature.

Sample preparation Nearly 10 kg of fresh peaches from 2 different cultivars were peeled, cut in half and the stone removed using stainless steel knives. The fruits were then cut into 1 cm cubes, and 500 g aliquots stored in plastic bags. The bags were frozen in liquid nitrogen and stored at –80°C.

Extraction of the oxireductases The enzymes were extracted according to Flurkey *et al*. (10). The fruits and their derivatives were homogenized in citrate-phosphate buffer (0.1 M, pH 5.5) at 4°C in the proportion of 1:1. A 1 1/2 volumes (as compared to the weight of the fruit) of acetone at 4ºC and 5% of the weight of the fruit in polyethylene glycol 6000 were added to the fruit and mixed. The mixture was filtered using a vacuum pump, and the procedure repeated twice more with the residue.

For the analyses of polyphenoloxidase (PPO) and peroxidase (POD), the enzyme extract was diluted in phosphate buffer (0.05 M, pH 6.2) containing 1 M KCl, in the proportion of 1:100 and agitated for 30 min. It was then centrifuged for 15 min, the solution filtered through cotton cloth and the enzymatic activity measured in the supernatant.

Polyphenoloxidase assay The PPO activity was assayed according to Oktay *et al*. (14), using catechol as the substrate. One unit of enzyme activity was defined as that causing an increase of 0.001 units of absorbance/min/mL of sample.

Peroxidase assay POD activity was determined according to Khan and Robinson (15) and Holschuch (16), using guaiacol as the substrate. One unit of enzyme activity was defined as that causing an increase of 0.001 units of absorbance/min/mL of the sample.

Biochemical characterization of the 'Biuti' PPO The effect of pH on the PPO was determined using a reaction system containing 0.1 mL of the enzyme extract; 1.7 mL of the various buffers; 1.2 mL of the substrate catechol solution (15 mM) in phosphate buffer (0.05 M, pH 6.0). The total volume of each tube was 3.0 mL. The blank was composed of 3.0 mL of the substrate solution in buffer.

The various buffers used were: citrate-phosphate buffer (0.1 M) with pH values from 3.0 to 5.5; phosphate buffer (0.025 M) with pH values from 6.0 to 8.0; and Tris-HCl buffer (0.1 M) with pH values from 8.5 to 9.0. The enzyme activity was determined as described above.

To check for pH stability, the enzyme was pre-incubated in the same buffers for 20 hr at the optimum temperature, as described by Fujita and Tono (17) and Fujita *et al*. (18). The reaction mixture was the same as described above, and the enzyme activity was determined as described above.

The effect of temperature on the PPO was determined using a reaction system containing 0.1 mL of the enzyme extract; 1.7 mL of phosphate buffer (0.05 M, pH 6.0); and 1.2 mL of the catechol (substrate) solution (15 mM) in phosphate buffer (0.05 M, pH 6.0). The total volume of each tube was 3.0 mL. The blank was composed of 3.0 mL of the substrate solution in buffer. The enzyme reaction was tested at different temperatures (from 10 to 80°C) for 5 min incubation, and the enzyme activity determined as described above.

Heat stability of the PPO was tested by pre-incubating 2 mL of the enzyme extract at different temperatures (from 10 to 80°C) for 30 min. The samples were then placed on ice and the enzyme activity determined as described above. To study the effect of various additives on PPO activity, 1, 5, and 10 mM of the additives tested were added to the enzyme reaction system described above. The reaction mixtures were incubated at the optimum temperature for 5 min according to Sidiqq *et al*. (19) and the enzyme activity determined as described above.

The presence of a latent PPO in the enzyme extract obtained from the industrially processed and canned peaches was tested by adding 2 mM of SDS to the reaction system described above. A control test containing the same enzyme extract but without the SDS was also prepared and the enzyme activity determined as described above.

'Biuti' PPO inactivation study

***Effect of different inhibitors at different temperatures*:** The most effective inhibiters of the PPO, as determined in preliminary tests, were added at several different concentrations to the PPO reaction system and incubated at temperatures from 10 to 80°C for 30 min, this being the time used for the heat treatment by the industry. The treatments were selected according to their use by the fruits and vegetables industry. The enzyme activity was determined as described above.

***Chemical and heat treatment*:** The effect of the combination of heat treatment and chemical compounds on the inactivation of the PPO was studied by incubating samples with the enzyme extract and a chemical compound in a 1:1 proportion at the temperature indicated in Table 1. The enzyme activity was determined as described above.

Results and Discussion

Extraction of the oxireductases The enzymatic extracts of the different peaches samples (fresh frozen fruit, fruit in syrup, and peach pulp) were obtained as described, and the PPO and POD analyses were performed according to described on the methodology. On the fresh frozen fruit, the results were 2,557.0 (±8.6) U/mL of PPO and 1,175.0 (±7.8) U/mL of POD. On the other 2 industrialized samples, none activity of PPO or POD were detected.

The results of the fresh frozen peach showed that the PPO activity had a nearly 46% higher value than the POD activity. According to Brandelli and Lobes (3), the PPO is really the major responsible enzyme for fruits darkening. Toraless *et al*. (34) studied darkening events in 4 kinds of Brazilian peaches, and conclude that the PPO was responsible for the enzymatic darkening. Observing all these results, the rest of the study was dedicated to characterization and inhibition tests with only of the PPO present on the fresh frozen 'Biuti' peaches samples.

Characterization of 'Biuti' PPO

***Effect of pH on PPO stability*:** The pH optimum for the enzyme-catalyzed oxidation of catechol in phosphate

Table 1. Additives, time, and temperature applied in the inactivation of polyphenoloxidase (PPO)

Treatment	Chemical compound	Additive concentration	Heat treatment	
			Time (min)	Temperature (°C)
T1	None		none	none
T2	Ascorbic acid	0.1%	none	none
T3	Citric acid	0.1%	none	none
T4	Sodium metabisulfite	0.05%	none	none
T5	None		2	70
T6	Ascorbic acid+citric acid	0.1%	none	none
T7	Ascorbic acid+citric acid+sodium metabisulfite	0.1%+0.05%	none	none
T8	Ascorbic acid+EDTA	0.1%+30 ppm	none	none
T9	Ascorbic acid+sodium metabisulfite	0.1%+0.05%	none	none
T10	Ascorbic acid	0.1%	2	70
T11	Citric acid+sodium metabisulfite	0.1%+0.05%	none	none
T12	Citric acid+EDTA	0.1%+30 ppm	none	none
T13	Citric acid	0.1%	2	70
T14	Ascorbic acid+citric acid+EDTA	0.1%+30 ppm	none	none
T15	Ascorbic acid+citric acid+sodium metabisulfite	0.1%+0.05%	2	70
T16	EDTA	30 ppm	none	none
T17	None		5	90

buffer was found to occur between pH 5.0 and 6.0. The enzyme showed 80% of activity in an optimal pH range between 5.0 and 8.0. In the pH range between 4.0 and 4.5, 50% of PPO activity was observed. At a pH value above 8.0, the activity decreased very rapidly (Fig. 1A).

This result is in accordance with that of Gawlik *et al.* (21) for the phenol oxidase from broccoli (pH 5.7); that of Sidiqq *et al.* (19) for the phenol oxidase from pears cv. Red (pH 5.5) and with the results obtained by McCallum and Walker (22) for the phenol oxidase of wheat flour (pH 5.6). On the other hand, the phenol oxidase isolated from the peach cv. Halford showed maximum activity at pH 6.2 (23), that from aubergine at pH 4.0 (18), that from potato at 6.7 (24) and that from apples at 4.6 (25). According to Vamos-Vigyazo (26), the PPO system in fruits seems to be most active at a near neutral pH value.

The pH stability profile for the enzyme showed that 100% of the PPO activity was retained between pH 5.5 and 6.5. In the pH range between pH 7.0 and 8.0, 70-90% of the PPO activity was retained. The enzyme was not stable below pH 5.0 or above 8.5 (Fig. 1B). This result corresponds well with the results obtained by Fujita *et al.* (18) for the phenol oxidase from lettuce (pH stability between 5.0 and 8.0, retaining 80-100% of the PPO activity) and for the phenol oxidase from eggplant (pH stability between 5.0 and 8.0).

The rapid inactivation of the enzyme above pH 8.0 may be due to conformational changes in the enzyme under the alkaline conditions and/or the enzyme may react rapidly with quinones via Maillard reactions and/or the Strecker degradation (27).

Effect of temperature on PPO stability: The enzyme showed the highest activity at 20°C with the substrate catechol, and showed 80% of activity between 15 and 30°C. At temperatures below 15°C, the activity decreased rapidly (Fig. 2A). The maximum activities for the PPOs obtained from grape cv. Emir (28), mango (29), and banana (30) were, respectively, 25, 30, and 30°C.

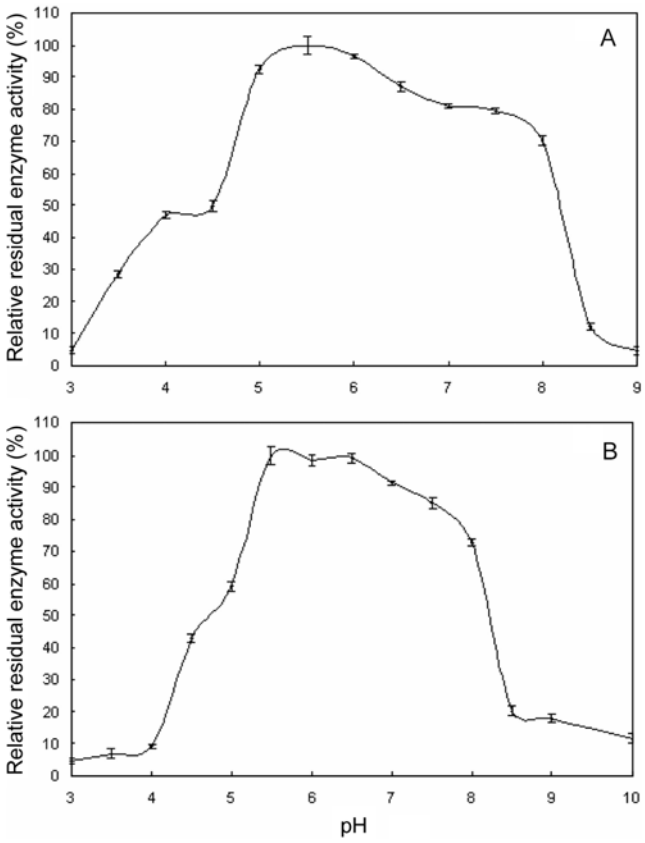

Fig. 1. (A) Activity of peach polyphenoloxidase (PPO) as a function of pH, (B) pH stability of peach PPO after a pre-incubation period of 20 hr at the pH indicated.

The enzyme was incubated at different temperatures for 30 min and the residual enzyme activity was measured.

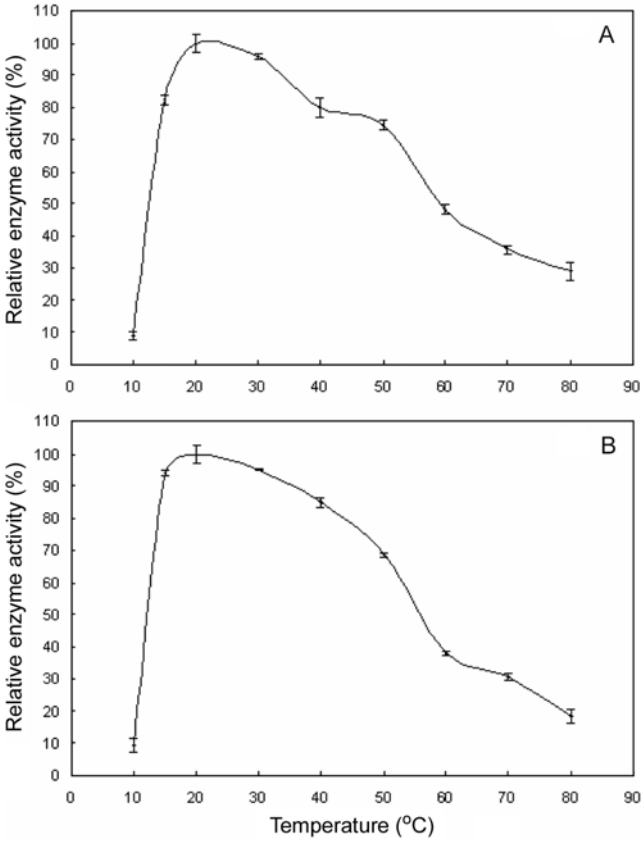

Fig. 2. (A) Activity of peach polyphenoloxidase (PPO) as a function of temperature, (B) temperature stability of peach PPO after a pre-incubation period of 30 min at the temperature indicated.

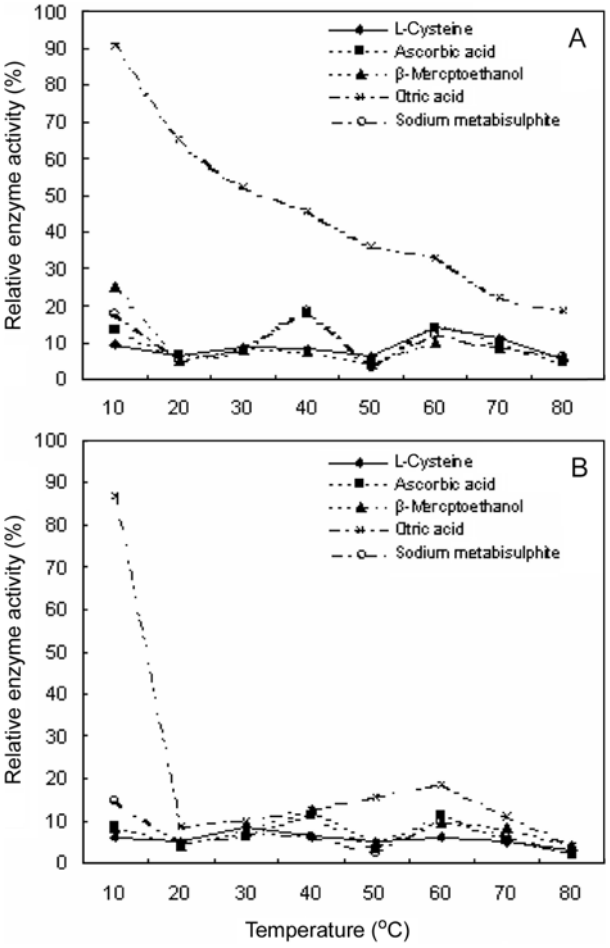

Fig. 3. Effects of the inhibitors at concentrations of 1 (A) and 5 mM (B) on the polyphenoloxidase (PPO) activity at different temperatures.

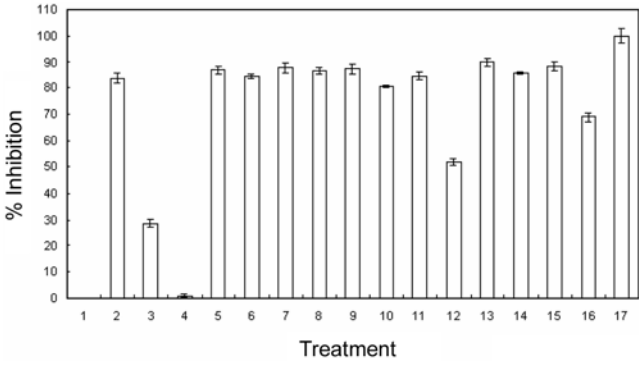

Fig. 4. % Inhibition of peach polyphenoloxidase (PPO) using different treatments.

After 30 min at 15 and 40°C, 80% of the PPO activity was retained. The activity decreased rapidly after 30 min at 80°C (Fig. 2B). It has been reported that cabbage PPO retained 40% of its activity after 10 min at 100°C (31), while eggplant PPO retained 50% of its activity after 5 min at 70°C. The PPO of artichoke cv. Jerusalem retained 80% of its activity after 60 min at 75°C (32).

Effect of inhibitors on PPO: The effects of various inhibitors on the PPO are shown in Table 2. EDTA, NaCl, and KCl showed minimal inhibition. The more efficient additives were: ascorbic acid, sodium metabisulfite, β-mercaptoethanol, and L-cysteine (approximately 100% inhibition of the PPO activities).

L-Cysteine was reported to be a strong inhibitor of apple PPO (14) and ascorbates are effective inhibitors of dog rose PPO (33).

The test for the presence of latent PPO in processed and canned peaches was negative.

Inactivation of 'Biuti' PPO

***Effect of the inhibitors at different temperatures*:** The PPO activity was approximately zero when the enzyme was incubated with 1 mM ascorbic acid, β-mercaptoethanol, sodium metabisulfite, and cysteine at 50°C. The same result was observed with all others inhibitors at 1 mM concentrations and at 80°C (Fig. 3A).

A concentration of 5 mM of ascorbic acid, β-mercaptoethanol, sodium metabisulfite, and cysteine was more efficient on the enzyme inhibition, than 1 mM at 50 and 80°C (Fig. 3A and 3B).

***Chemical compounds and heat treatment*:** The PPO showed zero activity when submitted to heat treatment alone for 5 min at 90°C (T17). An association of 0.1% citric acid with 2 min at 70°C resulted in 90% of PPO inhibition (T10).

Table 2. Effect of inhibitors on 'Biuti' polyphenoloxidase (PPO) activity

Inhibitors	Concentration (mM)	Residual activity (%)	Inhibition (%)
Control	Absent	100	0
EDTA	1.0	87.7	12.3
	5.0	84.6	15.4
	10.0	80.1	19.9
Ascorbic acid	1.0	5.3	94.7
	5.0	0	100
	10.0	0	100
Citric acid	1.0	80.0	20.0
	5.0	50.4	49.6
	10.0	6.9	93.1
NaCl	1.0	79.1	20.9
	5.0	78.2	21.8
	10.0	76.9	23.1
KCl	1.0	71.3	28.7
	5.0	67.3	32.7
	10.0	71.1	28.9
Sodium metabisulfite	1.0	0.8	99.2
	5.0	0.2	99.8
	10.0	0	100
β-Mercaptoethanol	1.0	0.5	99.5
	5.0	0	100
	10.0	0	100
L-Cysteine	1.0	2.1	97.9
	5.0	1.7	98.3
	10.0	1.0	99.0

The least efficient treatments were: T3 (0.1% citric acid; inhibition >30%), T5 (2 min at 70ºC; inhibition approximately 0%), T12 (0.1% citric acid+30 ppm EDTA; inhibition approximately 50%) and T16 (30 ppm EDTA; inhibition =60-70%).

Processed samples The processed samples were tested using the addition of SDS to the reaction system for PPO activity. The SDS addition showed no effect, there being no PPO activity with or without SDS only few days after the processing.

In this study, it was not possible to find any correlation between the level of browning and the inhibitors tested during the temperature process. The different mechanisms responsible for browning in different products must be taken into account. Probably the browning in fresh fruits the mainly cause is polyphenol enzymatic oxidation as observed in the Fig. 1-3. Peach in syrup browning, as a result of blanching and pasteurization processes, could be ascribed both to enzymatic reactions taking place before blanching and to non-enzymatic browning reactions occurring during pasteurization. The formation of pro-oxidant compounds (reactive radicals in the early stages of the Maillard reaction), as well as antioxidant compounds (free polyphenols as result of hydrolysis of glicosides, polyphenols in their intermediate state of oxidation and maillard reaction products) has been observed during plant food processing and affects antioxidant activity through different pathways (35).

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References

1. Madail JMC. Peach production aspects in Brazil and its relations to MERCOSUL. Embrapa: CPACT. p. 34 (1994)
2. Antunes LEC. The crops of peaches and plums in Minas Gerais State. Informe Agropecuário de Belorizonte (Agrobusiness report from Belo Horizonte). 189: 14-17 (1997)
3. Brandelli A, Lopes C. Polyphenol oxidase activity, browning potential, and phenolic content of peaches during postharvest ripening. J. Food Sci. 29: 624-637 (2005)
4. Mayer AM, Harel E. Review: Polyphenol oxidases in plants. Phytochemistry 18: 193-225 (1979)
5. Moore BM, Flurkey WH. Sodium dodecyl sulphate activation of a plant polyphenoloxidase. Effect of sodium dodecyl sulphate on enzymatic and chemical characteristics of purified broad bean polyphenoloxidase. J. Biol. Chem. 265: 4982-4988 (1990)
6. Sanchez-Ferrer A, Laveda F, García-Carmona F. Substrate-dependent activations of latent potato leaf polyphnol oxidase by anionic surfactants J. Agr. Food Chem. 41: 1583-1586 (1993)
7. Núñez-Delicado E, Bru R, Sánchez-Ferrwe A, Garcia-Carmona F. Triton X-114-aided purification of latent tyrosinase. J. Chromatogr. - Biomed. 680: 105-112 (1996)
8. Kenten RH. Latent phenolase in extracts of broad-bean (*Vicia faba* L.) leaves. 1. Activation by acid and alkali. Biochem. J. 67: 300-307 (1957)
9. Swain T, Maptson LW, Robb DA. Activation of *Vicia faba* (L.) tyrosinase as effected by denaturing agents. Phytochemistry 5: 469-482 (1966)
10. Flurkey WH. Peroxidase and polyphenol oxides activities in developing peaches. Plant Physiol. 81: 614-618 (1986)
11. King RS, Flurkey WH. Effects of limited proteolysis on broad bean polyphenoloxidase. J. Sci. Food Agr. 41: 231-240 (1987)
12. Golbeck JH, Cammarata KV. Spinach thylakoid polyphenol oxidase. Isolation, activation, and properties of the native chloroplast enzyme. Plant Physiol. 67: 977-984 (1981)
13. Laveda F, Núñez-Delicado E, Garcia-Carmona F, Sanchez-Ferrer A. Reversible sodium dodecyl sulphate activation of latent peach polyphenol oxidase by cyclodextrins. Arch. Biochem. Biophys. 379: 1-6 (2000)
14. Oktay M, Küfre Vioglu I, Kocacaliskani SH. Polyphenoloxidase from Amasya apple. J. Food Sci. 60: 494-505 (1995)
15. Khan AA, Robinson DS. The thermoestability of purified mango isoperoxidases. Food Chem. 47: 53-59 (1993)
16. Holschuh HJ. Isolation, purification, and biochemical characterization of peroxidase from carambola (*Averrhoa carambola* L.). PhD thesis, Food Science, Unicamp, Brazil (2000)
17. Fujita S, Tono T. Purification and some properties of polyphenol oxidase in eggplant (*Solanum melongena*). J. Sci. Food Agr. 46: 115-123 (1988)
18. Fujita S, Tono T, Kawahara H. Purification of polyphenol oxidase in head lettuce (*Lactuca sativa*). J. Food Sci. 55: 643-651 (1991)
19. Sidiqq M, Sinha NH, Cash JN. Characterization of polyphenoloxidase from Stanley plums. J. Food Sci. 57: 1177-1179 (1992)
20. Lima EDPA. Purification and biochemical characterization of polyphenoloxidase (PPO) from the fruit of *Annona squamosa* L. PhD thesis, Food Science, Unicamp, Brazil (1999)
21. Gawlik DU, Szymanowska U, Baraniak B. Characterization of polyphenol oxidase from broccoli (*Brassica oleracea* var. *botrytis italica*) florets. Food Chem. 105: 1047-1053 (2007)
22. McCallum JA, Walker JRL. *O*-Diphenol oxidase activity, phenolic content, and color of New Zealand wheats, flours, and milling

streams. J. Cereal Sci. 12: 83-96 (1990)
23. Lu AT, Whitaker JR. Some factors affecting rates of heat inactivation and reactivation of horseradish peroxidase. J. Food Sci. 39: 1173-1178 (1974)
24. Cho YK, Ahn HK. Purification and characterization of polyphenol oxidase from potato: II. Inhibition and catalytic mechanism. J. Food Biochem. 23: 593-600 (1998)
25. Espin JC, Morales M, Varon R, Tudela J, Garcia-Carnovas F. Monophenolase activity of polyphenol oxidase from verdedoncella apple. J. Agr. Food Chem. 43: 2807-2812 (1995)
26. Vamos-Vigyazo L. Polyphenol oxidase and peroxidase in fruits and vegetables. CRC Crit. Rev. Food 15: 49-27 (1981)
27. Lee CY, Kagan V, Jaworski AW, Brown SK. Enzymatic browning in relation to phenolic compounds and polyphenoloxidase activity among various peach cultivares. J. Agr. Food Chem. 38: 99-102 (1990)
28. Ünai MU, Sener A. Determination of some biochemical properties of polyphenol oxidase from Emir grape (*Vitis vinifera* L. cv Emir). J. Food Sci. 86: 2374-2379 (2006)
29. Wang J, Jiang W, Baogang W, Lie S, Zhengli G, Luo Y. Partial properties of polyphenol oxidase in mango (*Mangifra indica* L. cv. Taiong) pulp. J. Food Biochem. 31: 45-55 (2006)
30. Ünai MU. Properties of polyphenol oxidase from Anamur banana (*Musa cavendishii*). Food Chem. 3: 909-913 (2007)
31. Fujita S, Saari N, Maegawa M, Ttsuda T, Hayashi N, Tono T. Purification and properties of polyphenol oxidase from cabbage (*Brassica oleracea* L.). J. Agr. Food Chem. 43: 1138-1142 (1995)
32. Ziyan E, Pekyardimci S. Characterization of polyphenol oxidase from Jerusalem artichoke (*Helianthus tuberosus*). Turk J. Chem. 27: 217-225 (2003)
33. Jiang YM. Purification and some properties of polyphenol oxidase of longan fruit. Food Chem. 66: 75-79 (1999)
34. Toraless RP, Vendruscolo JL, Haas LIR, Ferri NL, Del Pino FAB, Autunes PL. Partial characterization of enzimatic browning by polyphenoloxidase in Granada, Jade, Esmeralda, and Maciel peaches. Rev. Braz. Agr. 10: 241-244 (2004)
35. Nicoli MC, Anese M, Parpinel M. Influence of processing on the antioxidant properties of fruit and vegetables. Trend Food Sci. Tech. 10: 94-100 (1999)