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Purification and Partial Characterization of Polyphenol Oxidase from Sapodilla Plum (*Achras sapota*)

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ABSTRACT

The browning of fruits can be considered as an enzymatic oxidation which is believed to be one of the main causes of quality loss during post-harvest handling. The enzymes responsible for this are the oxidoreductases; the polyphenol oxidase (PPO) (monophenol, *o*-diphenol, oxygen oxidoreductase; EC 1.14.18.1) belongs to this group. This enzyme, which is found in the sapodilla plum (*Achras sapota*), was purified using a phenylsepharose and a SephacrylS-200 columns. The molecular weight of the purified enzyme was estimated to be about 66 kDa by gel filtration and 29 kDa by SDS-PAGE. A single protein band was found using the latter system (SDS-PAGE), which shows that the PPO of the pulp of the sapodilla plum may be composed of two protein subunits with similar molecular weight. The optimum pH was 7.0 and the optimum temperature 60˚C. The most effective inhibitors tested were ascorbic acid, sodium metabisulfite and acetic acid.

Keywords: Enzymatic System; Oxidation; Polyphenol Oxidase; Browning Process; Nutritional Value

1. Introduction

For nearly a century, fruits and vegetables have been recognized as a good source of vitamins, minerals and fiber. They are considered so important for our nutrition that five daily rations are suggested. Compared with people who consume a diet with only small amounts of fruits and vegetables, those who eat generous amounts of them as part of a healthful diet are likely to have reduced risk of chronic diseases, including stroke and perhaps other cardiovascular diseases, as well as certain cancers.

The sapodilla plum (*Achras sapota*) is a tree from the tropical regions of the American continent, mainly from the South of Mexico and Central America. The fruit is climacteric, reaches commercial maturity around 6 - 8 days after harvest and enters senescence two days later; it is, therefore, a perishable fruit.

The browning process is one of the main causes of the loss of quality and nutritional value in fruit and vegetable preservation [1]. This phenomenon can be considered as an enzymatic oxidation. Polyphenol oxidases (PPO) are responsible for the browning of fruits and vegetables by catalyzing the oxidation of phenolic compounds; these enzymes can be inactivated by heat or by the elimination of oxygen [2,3].

The sapodilla plum is a perishable fruit. Enzymatic browning, which is mainly caused by polyphenol oxidase (monophenol, *o*-diphenol, oxygen oxidoreductase; EC 1.14.18.1), makes its conservation and commercialization a difficult thing [4]. This enzyme is widely distributed in microorganisms, animals, and plants, being responsible not only for browning in plants but also for melanisation in animals. Previous studies have reported the purification and characterization of PPO from different sources: the enzyme from cabbage showed a molecular weight of 40 kDa, an optimal pH of 7.6 and a residual enzymatic activity of 25% after the protein was subjected to a 10 min thermal treatment at 100˚C [5]; the polyphenol oxidase from avocado was isolated and partially purified in the presence of TX-114, showing monophenolase/diphenolase activity with an optimum pH of 7 for both [6]; the purification and characterization of polyphenol oxidase from banana pulp [7], purification and characterization of the polyphenol oxidase in beets [8]. The purification of PPO from sapodilla plum has not been reported

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yet.

The commercial potential of the Sapodilla plummight be expanded if the enzymatic browning process is controlled. Therefore, our objective was to purify and partially characterize the polyphenol oxidase from sapodilla plum. This study could help understand the role of polyphenol oxidase on the quality deterioration of the sapodilla plum, which could be useful in the search of effecttive methods for inhibiting browning during storage.

2.Materials and Methods

2.1. Materials

Sapodilla plum (*Achras sapota*), harvested at commercial maturity in the February-May season from an orchard in the Cansahcab town of the state of Yucatan, Mexico, stored at 4˚C.

2.2. Extraction Procedure

Unless otherwise stated, all extraction procedures were carried out at 4˚C in order to reduce enzyme activity. Ripe fresh fruit (50 g after peeling and coring) were blended for 1 min with 100 ml of 0.1 M phosphate buffer, pH 7, and 0.2 g of ascorbic acid, different concentrations of polyvinylpolypyrrolidone (PVPP) and 0.1% of TX100 at 4˚C.

The raw enzymatic extract (PPO) was obtained from the sapodilla plum using Triton TX100 detergent from Sigma; this detergent has been used for the extraction and solubilisation of polyphenol oxidase in several fruits [6-8]; ascorbic acid (Sigma) was used as reducer agent of endogenous phenolic compounds found in the sapodilla plum; finally, PVPP from Sigma was used to prevent quinone formation, as it reacts with the proteins that are present in the enzymatic extract.

2.3. Monitoring of Polyvinylpolypyrrolidone (PVPP) Activity in Collected Fractions, and Purification Procedure

The presence of excessive quantities of impurities may interfere with bioassays. Phenolic compounds, which are widespread in plants and frequently occur in high concentrations, seem to be a major source of impurities and to have possible inhibitory activity. Polyvinylpolypyrrolidone (PVPP) has been shown to be reasonably specific in separating a phenolic fraction from plant tissue extracts by hydrogen bond formation [9].

In order to evaluate the effect of PVPP on the process of obtaining the enzymatic extract, several PVPP concentrations were analysed: 0%, 1%, 2%, 3%, 4%, and 5% in fresh fruit weight. We used 50 g of ripe fresh fruit, which were suspended in 100 ml of 0.1 M phosphate buffer, pH 7, for each of the above-mentioned PVPP concentrations, 0.2 g of ascorbic acid and 0.1% of TX100 at 4˚C.

This mixture was homogenised for 1 min and filtered through 6 gauze-layers right after it was filtered through a N.1 filter-paper; it was then centrifuged at 10,000 g for 20 min at 4˚C. The sapodilla plums filtrate was treated with solid ammonium sulphate with a saturation of 20% - 80%. The precipitate was collected by centrifugation at 1200 × g for 40 min and redissolved in buffer (0.01 M phosphate buffer, pH 7.0). The fraction obtained through precipitate with ammonium sulphate (Sigma) with the highest activity of PPO was dialyzed overnight against 4 changes (4 × 1 L) of the same buffer, centrifuged and later applied to a phenylsepharose column. The (6 cm × 1 cm) column, with a 4 ml packed volume, was equilibrated with a 0.01 M phosphate buffer, pH 7, and eluted with a decreasing ammonium sulphate gradient (0.3% to 0%). The first eluted fraction that maintained the enzymatic activity was introduced into another sephacryl S-200 column, and the protein amount (280 nm) as well as the PPO enzymatic activity (420 nm) were determined.

2.4. Protein Determination

Protein content was determined by the Bradford method [10], using immunoglobulin G (Sigma) as standard. Bradford reagent (1.5 ml) was added to 0.05 ml of sample. Absorbance at 595 nm was determined [11]. The enrichment process of the PPO fraction was determined according to the protein concentration method [12].

2.5. Molecular Mass Determination by Size-Exclusion Chromatography

The molecular weight of the native enzyme was determined by gel filtration with a Sephacryl S-200 (30 cm × 1 cm) column, which was equilibrated with a 0.01 M phosphate buffer, pH 7. The column was pre-calibrated using molecular weight markers: cythocrome c (12.5 kDa), carbonic anhydrase (29 kDa), bovine albumin (66 kDa), alcohol dehydrogenase (150 kDa), alpha-amylase (200 kDa), apoferritin (443 kDa), thyroglobuline (669 kDa) and blue dextran (2000 kDa). Filtration was carried out following Andrews's method [13].

2.6. SDS-PAGE

Electrophoresis was carried out in accordance with the Laemmli method [14] with the following characteristics: a 10% acrylamide resolution gel and a 6 cm high and 1.5 mm thick buffer Tris, pH 8.8; for the sample we used the Laemmli buffer 2×, and the run or electrode buffer was Tris-glycine, pH 6.8, with sodium dodecyl sulphate (SDS) from Sigma. Equal amounts of protein samples (10 mg) were loaded onto each lane. Protein bands were visualized by Coomassie blue staining according to the manu-

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facturer's protocol (Sigma).

2.7. Enzyme ActivityAssay

Enzymatic activity was measured spectrophotometrically by the method described by Oktay [15] using pyrocatechol (J.T. Baker) as substrate. One unit of enzyme activeity was taken as the increase in absorbance at 420 nm at 30˚C. The standard reaction mixture consisted of 0.1 ml of enzyme solution and 2.5 ml of pyrocatechol (0.06 M) in 0.1 M phosphate buffer (pH 7). Activity measurements were carried out in triplicate. In order to determine enzyme activity in the presence of inhibitors, the enzyme was preincubated for about 15 min with these compounds.

The kinetic characterization of the sapodilla plum's PPO was carried out using a 0.1 M phosphate buffer, pH 7, at an incubation temperature of 30˚C, a 200 rpm shaking speed, 20 min enzyme-substrate reaction time, and, as substrate, pyrocatechol at several concentration levels (0.02, 0.04, 0.06, 0.08, 0.1 and 0.12 M).

2.8. Optimum pH

PPO activity as a function of pH was determined within a pH range of 4.0 - 6.0 in 0.1 M acetate buffer and of 6.0 - 8.0 in 0.1 M phosphate buffer adjusted with 0.1 MNaOH and HNO_3 [16]. PPO activity was assayed using the standard reaction mixture at 30˚C, but changing the buffer. PPO activity was calculated as percent residual activity at the optimum pH.

2.9. Effect of Temperature

In order to determine the optimum temperature values of the enzyme, PPO activity was measured at different temperatures in the range of 30˚C - 70˚C using pyrocatechol as substrate. The effect of temperature on the activity of PPO was tested by heating the standard reaction solutions (buffer and substrate) to the appropriate temperatures before introducing the enzyme. Once temperature equilibrium was reached, the enzyme was added and the reaction followed spectrophotometrically at a constant temperature, given time intervals and a pH of 7.

The standard reaction mixture consisted of 0.1 ml of enzyme solution and 2.5 ml of pyrocatechol in 0.1 M phosphate buffer (pH 7) [17].

2.10. Monitoring the PPO Inhibitory Activity

The inhibitors examined were ascorbic acid (Sigma), sodium metabisulfite (Merk), sodium azide (Merk), acetic acid (Fermont), citric acid (Sigma), tartaric acid (Baker), oxalic acid (Baker) and honey. These inhibitors are reducing agents that play a role in preventing enzymatic browning either by reducing *o*-quinones to colorless diphenols or by reacting irreversibly with *o*-quinones to form stable colorless products [18]. The reaction mixture contained 2.8 ml of pyrocatechol at a final concentration of 0.06 M in 0.1 M phosphate buffer (pH 7.0) and 0.2 ml of the enzymatic solution, plus the inhibitors solution in several concentration levels. Percentage inhibition was calculated using the following equation: Inhibition (%) = $[(A_0 - A_i)/A_0)]100$, where A_0 is the initial PPO activity (without inhibitor) and A_i is the PPO activity with inhibitor [19].

3. Results and Discussion

3.1. Extraction of the Sapodilla Plum's PPO

It has been reported that some plant PPOs are membrane-bound. Therefore, using detergents is required in order to solubilise the enzyme [20]. Phenolic compounds interfere with the purification of proteins from plants. They cross-link proteins by hydrogen bonds and covalent interactions. Furthermore, the homogenization of the plant tissues initiates enzymatic browning, which results in the formation of quinones. Quinones may also form covalent linkages that may be irreversible. Phenol- absorbing polymers such as PVPP and reducing agents such as ascorbic acid are commonly used in order to overcome these problems [20]. During the extraction of the sapodilla plum's PPO we noticed (**Figure 1**) that when the polyvinylpolypyrrolidone (PVPP) concentration increased from 0% to 5%, PPO activity in the extract increased; this can be a result of the PVPP effect on the sapodilla plum's endogenous polyphenols; this means a greater enzymatic activity per mg protein: between 0.097 and 0.091 mg/ml in the analyzed conditions. It was also discovered that a PVPP concentration of 3% allowed to preserve a maximum activity of PPO. Values near 2.5%

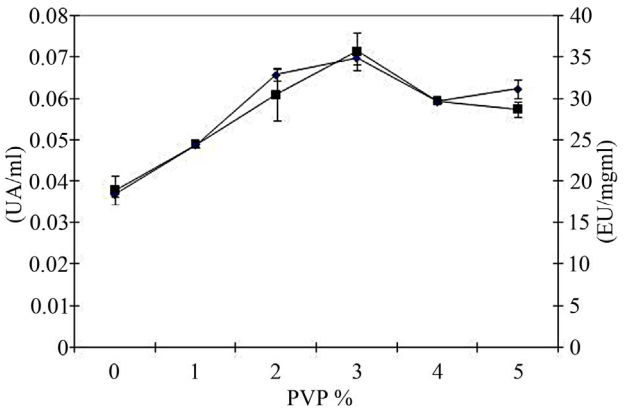

Figure 1. PVPP, the effect on polyphenol oxidase activity. PVPP concentrations of 0%, 1%, 2%, 3%, 4% and 5% of fresh fruit weight were analyzed. The reaction medium at 30˚C contained 0.1 M phosphate buffer, pH 7 for each PVPP concentration, 0.2 g of ascorbic acid and 0.1% of TX100. (■) Enzymatic activity; (●) specific activity.

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were found when PPO was extracted from apple tissue [21].

3.2. Enrichment of the Sapodilla Plum's PPO Enzymatic Extract

One of the most commonly used methods for protein enrichment and concentration is fractional precipitation with ammonium sulphate [22]. This method was applied to the sapodilla plum extract.

Table 1 presents a summary of the results of PPO extraction and purification from Sapodilla plums and shows that PPO activity increased in the precipitate with the highest saturation percentage of ammonium sulphate in the sapodilla plum extract. We can also observe that when there is an ammonium sulphate saturation concentration of 80% (**Table 1**), the sapodilla plum's PPO precipitation is almost total, due to the fact that under these conditions a purification factor of approximately 4.8 was obtained, and, therefore, a specific activity factor of 762.7 UE factor was found. On the other hand, a recovery factor of almost 3 was reported by Jharna R. [23] in the purification and characterization of the pineapple's PPO, and a similar value of 4.5 was obtained in the partial purification of persimmon by Nuñez, Sojo, García and Sánchez [24].

3.3. Purification of the PPO Enzyme with Phenylsepharose and Sephacryl S-200 Chromatography

The extract precipitate at 80% was resuspended in 1 ml of buffer; dialysed and passed through a phenylsepharose column. The first eluted fraction that maintained the enzymatic activity was introduced into another sephacryl S-200 column, and the protein amount as well as the PPO enzymatic activity was determined. Two major fractions were obtained from the column (16 and 17 fractions), and one peak with PPO activity was separated by sephacrylS-200 chromatography (**Figure 2**). The major peak corresponded to a 51-fold increase in specific activity over the crude extract and a recovery of 6.8%.

Hydrophobic interaction chromatography has been utilized in the purification of PPO from various fruits, including peaches [25], strawberries [26] and pineapples [27]. Purification factors, elution profiles, and the percentage of enzyme recovery varied between studies. Reported purification factors were 120-fold for PPO from Delicious cortex [28] and 13.8-fold for PPO from apricot [29]. Enzyme recoveries of 80% for PPO from apricot [29] and 40% for PPO from apple peel [30] have been reported.

SDS-PAGE was performed in order to check the homogeneity of the purified PPO. These results, according to the molecular weight patterns and the number of fractions 16 and 17, suggest that the native molecular weight of the sapodilla plum's PPO is approximately 66 kDa, reaching a purification factor of approximately 38 times for the phenylsepharose column, and 51 times for the sephacryl S-200 column. A molecular weigh of 57 kDa was found in the characterization and purification of the artichoke's polyphenoloxidase (*Cynarascolymus L.*) [31].

3.4. Electrophoresis of the Purified Fractions (Phenylsepharose and Sephacryl S-200) in Polyacrylamide gel (PAGE)

A denaturing gel electrophoresis (**Figure 3**) was performedin order to examine the purification degree of the PPO protein in the phenylsepharose and sephacryl S-200 columns. **Figure 3** shows a typical molecular weight profile of fractions obtained at each purification step. A single band with the same molecular weight was noticed for each type of chromatography, suggesting that the fractions with the most activity and the most protein correspond to a single protein molecule. This protein band migrated, similarly to a protein with a molecular weight of 29 kDa, to a carbonic anhydrase molecular weight pattern. This suggests that the denaturalized molecular weight of the sapodilla plum's PPO is close to 29 kDa.

The results found by Jharna R. [23] in the purification and characterization of the Indian pineapple's PPO showed that the molecular weight was 105 kDa with a Sephadex G-150 column; however, the SDS-PAGE gel indicated a single 25 kDa polypeptide band, suggesting a tetramer of identical units. Applying this principle to the sapodilla plum's PPO, we think that, in this case, the polypeptide is a dimmer of identical units. The molecular weights of PPOs have been reported before: cabbage (39 kDa) [5], banana (41 kDa) [7]. Our results are in agreement with those reports in terms of the molecular weight of PPO. This purification process was repeated 3 times.

Table 1. Activity of the sapodilla plum's (*Achras sapota*) PPO, in precipitated fractions with ammonium sulphate.

Satura. (%)	Volume (ml)	Total Activity (units)	Total Prot. (mg/ml)	Specific activity (units/mg protein)	Purification factor (fold)	Recovery (%)
Crude extract	16	45.5	0.286	159.25	1	100
80% (NH_2SO_4)	13	104	0.137	762.7	4.8	4.8
Sephacryl S-200	2.5	159	0.0196	8112.24	51	6.8

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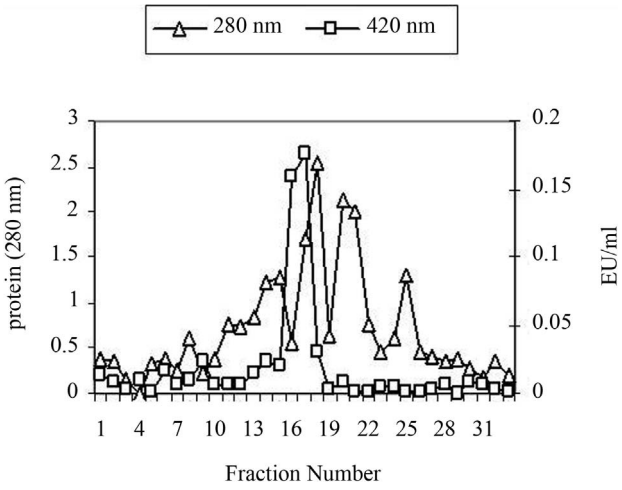

Figure 2. Elution profile of the sapodilla plum's (*Achras-sapota*) polyphenoloxidase in Sephacryl S-200 (Δ protein, □ activity).

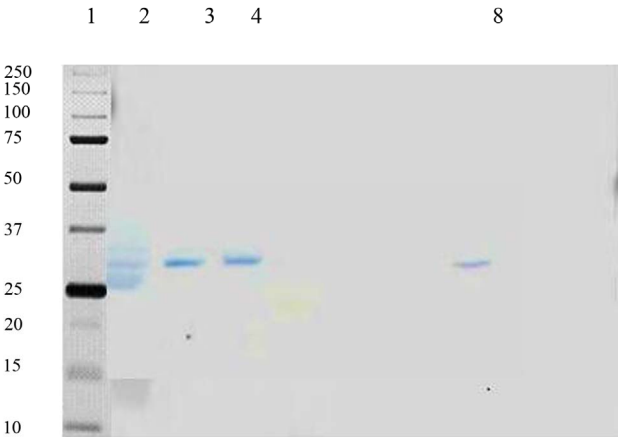

Figure 3. Denaturing SDS-PAGE (10% gel) from PPO fractions. Lane 1 shows prestained molecular mass markers (kDa); lane 2 shows proteins after 80% $(NH_4)_2SO_4$ fractionation; lane 3 and 4 show the PPO fraction from Phenyl-Sepharose, and lane 8 the PPO fraction from Sephacryl S-200.

3.5. Some Characteristics of the Purified Enzyme

The isolated isoenzymes of superior plants and fruits can oxidize a wide range of monophenols and ortho-diphenols with highly variable kinetic parameters, including maximum velocity (Vm) and the Michaelis-Menten constant (Km). The Km is generally interpreted as a measure of the affinity of an enzyme for its substrate. The affinity of plant's PPO is generally low. This means that they have high Km values [32].

The sapodilla plum's Km value was 12.4 mM and its Vm was 69.49 UE/min·ml (results not shown); this is a high value for the sapodilla plum; meaning that the enzyme has little affinity for the substrate. Similar values have been reported by Vamos and Gazago [33] for a variety of Jonathan and Starking apples; they are within a range of 2 - 13 and 3 - 38 mM for 4-methylcathechol. Another similar value, of 12.52 mM, was reported by Halder, Tamuli and Bhaduri [34] for Indian tea leaves (CameliaSinensis) with catechol as substrate, although-much lower values have been reported, such as the one that was published by Ridgway and Tucker [35], who found a Km. of 3.6 mM for 4-methylcatechol.

3.6. Effects of Temperature on PPO Enzymatic Activity

The effects of temperature are very complex and depend on several factors; very high temperatures can alter the rupture speed of the enzyme-substrate complex, the pH values of functional groups taking part in the enzymatic reaction, the affinity of the enzyme towards inhibitors, activators and auxiliary systems, such as oxygen, which can be the reaction's substrate. In addition, it is possible that high temperatures can inactivate the enzyme.

Figure 4 shows that the sapodilla plum's PPO is active within a wide temperature range, due to the fact that it has an optimal activity at 60˚C. This can be due to the environmental conditions present in the season of peak production (April-May), when the temperature in the region is around 40˚C.

On the other hand, the enzyme's activity in high temperatures has been demonstrated by the studies of Aroba *et al*. [36]. This study found that the beginning of thermal inactivation started at 70˚C, reaching a 50% decrease at 18.8 minutes of thermal treatment. Also, Núñez, Serrano, Pérez and López [37] found that the activity range of the PPO of table grapes was between 30˚C and 60˚C, but a fast inactivation occurred after 70˚C.

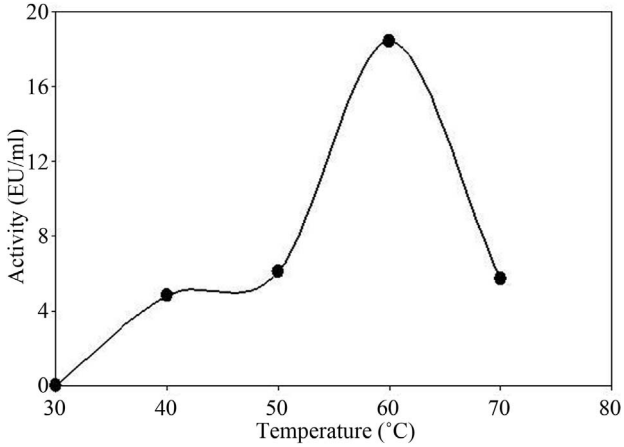

Figure 4. The optimum temperature of the enzyme was between 30˚C and 70˚C, as determined by an activity assay at 30˚C. The reaction medium contained 0.6 ml of the substrate, 2.3 ml of 0.1 M buffer solution, and 0.1 ml of enzyme solution. Standard deviations of all data were less than 10%.

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3.7. Optimal pH of the Sapodilla Plum's Polyphenol Oxidase Enzyme

There is an optimal pH at which enzymes are most active. The optimum pH for the activity of the sapodilla plum's PPO enzyme was determined using pyrocatechol as substrate; we can also see that there is a wide activity range at different pH values from 5 to 8. The optimum pH for the activity of PPO from sapodilla plum was found to be 7 (**Figure 5**). Optimal values of pH 6 - 7, with catechol as substrate, have also been reported for various fruits, including apple [10], pineapple [27], avocado, grape and pear [38]. However, at a pH below 4 the enzyme activity is low, suggesting that the enzyme can be controlled through the pH; similar values were found by Kavrayan *et al*. [39] in the purification and partial characterization of mint's (*Menthapiperita*) polyphenoloxidase, with an optimal value of pH 7, using cathechol as substrate; low activity was found at pH values below 5.

3.8. Inhibition

The browning process of fruits and vegetables can be prevented by removing the reactants, such as oxygen and the internal phenolic compounds, or by using PPO inhibitors. The complete elimination of oxygen during fruit processing is very difficult, due to the fact that it is found in the environment.

Several compounds were studied in order to detect the inhibitive action against the sapodilla plum's PPO; **Table 2** shows the values that were obtained with several compounds used as inhibitors and pyrocathechol as substrate. Of the inhibitors used, ascorbic acid was found to be the most effective, followed by sodium metabisulfite, sodium azide, acetic acid and honey (**Table 2**). Honey was

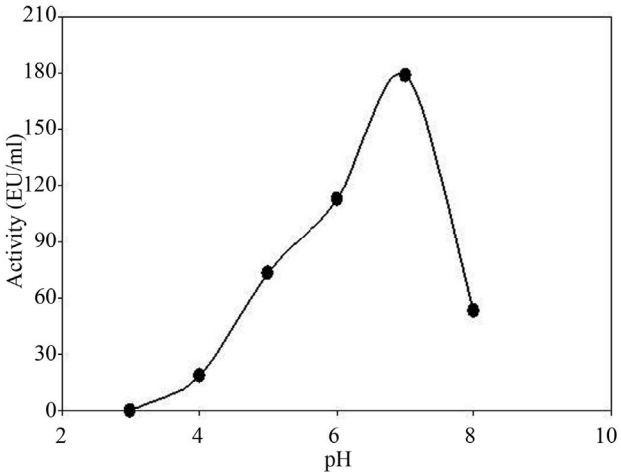

Figure 5. The optimum pH for polyphenoloxidase activity in the sapodilla plum. The buffers used were 0.1 M acetate (pH 4.0 - 6.0) and 0.1 M phosphate (pH 6.0 - 8.0) adjusted with 0.1 MNaOH and HNO_3. Standard deviations of all data were less than 10%.

Table 2. Effect of some inhibitors on the PPO activity from sapodilla plum (*Achras sapota*).

Compound	Concentration (%)	Inhibition (%)
Ascorbic acid	0.02	98
Sodium metabisulfite	0.050	97
Sodium azide	0.002	95
Acetic acid	0.2	93
Honey	0.2	56

the weakest inhibitor. Ascorbic acid and sodium metabisulfite have been shown to be strong inhibitors of PPO for Monroe apple peel and Stanley plum [30,40]. The citric, tartaric and oxalic acids did not show any inhibitive action against PPO in the sapodilla plum; however, ascorbic acid can be satisfactorily used to control the darkening of this fruit during processing as long as it is in its reduced form.

4. Conclusions

This work describes a method for the purification of PPO from sapodilla plum. Polyphenol Oxidase (PPO) was extracted from commercially ripe sapodilla plums that were gathered during the February-May season from an orchard in the Cansahcab town in the state of Yucatan, Mexico. This was the first report on the purification of PPO from sapodilla plum.

It can be concluded that the apparent molecular weight of the polyphenoloxidase from sapodilla plum pulp is approximately 60 kD, with a molecular weight (PAGE) of 29 kD; because of we can say that the polypeptide of the sapodilla plum's PPO is a dimer of identical units.

In this study, we achieved a purification factor of approximately 38 times with the Phenylsepharose column, and of 52 with the Sephacryl S-200 column; an optimal reaction pH of 7 and an optimal temperature of approximately 60˚C using pyrocatechol as substrate; the Km value for the sapodilla plum was 12.4 mM, and the H.S. was 69.49 U/min·ml. The PPO inhibitors, ascorbic acid, sodium metabisulfite, sodium azide, acetic acid and honey were particularly effective. Therefore, this study provides useful information about the sapodilla plum.

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