SUBMISSION

OF

MINOR RESEARCH PROJECT

File No: 47-637/08(WRO)

Comparative Study & Characterization of Poly Phenol Oxidase (PPO) From Fruits of Rosaceae Family

Submitted to

UGC WESTERN REGIONAL OFFICE, PUNE

BY

SHAZIA SHADAB
MAULANA AZAD COLLEGE OF ARTS, SCIENCE AND COMMERCE, AURANGABAD

Comparative Study & Characterization of Poly Phenol Oxidase (PPO) From Fruits of Rosaceae Family

Through ammonium sulphate precipitation, dialysis, and gel filtering, three isoenzymes of polyphenol oxidase (PPO) were recovered from Apple. The partly purified enzyme was characterised using the dialysis sample that was collected following ammonium sulphate precipitation. Pyrogallol's optimal pH value was 8.2, 4-methylcatechol's was 7.2, catechol's was 7.0, D-was tyrosine's 5.6, p-was cresol's 5.0, and L-was dopa's 4.8. PPO performed best at a temperature of 35 °C when 4-methylcatechol was present.

Although 4-methylcatechol, chlorogenic acid, and caffeic acid were also effective substrates, catechol was oxidised more quickly than the other substrates. In this paper, the impact of 6 different inhibitors on PPO activity was examined. The most efficient inhibitors were L-ascorbic acid, L-cystein, and sodium diethyldithiocarbamate. For catechol substrate, the enzyme's Km and Vmax values were calculated to be 5.55 mM and 344.5 IU/mL min, respectively. The apparent activation energy was 0.19 cal/mol according to thermal inactivation data. By using activity analysis in nondenaturing polyacrylamide gel electrophoresis, three isoenzymes of Apple PPO were found. By using sodium dodecyl sulfate-PAGE, their molecular weights were found to be 60, 40, and 28 kDa, respectively.

INTRODUCTION

The enzyme polyphenol oxidase, also known as monophenol dihydroxyphenylalanine:oxidoreductase (PPO; E.C. 1.14.18.1), is abundantly present in both plants and microbes. Browning and degeneration occur in apples. These factors have caused this fruit's market value to decrease significantly. The primary cause of skin browning is the enzymatic catalysis of PPO, which results in the oxidation of phenolic substances by molecular oxygen. 1−7 Fruit contains both PPO and phenolic chemicals. This is especially important for apples because they contain a lot of polyphenols and are prone to enzymatic browning.

EXPERIMENTAL WORK

Apples were used as the research material. Smaller and fleshier fruits were selected as they are preferred in the manufacture of jam and marmalade and also for fresh consumption.

Enzyme Extraction and Purification.

Unless otherwise noted, enzyme extraction was done at 4 degrees Celsius. 170 g of apple tissue were swiftly divided into thin slices after being quickly sliced in half, cored, and partially cleansed. The tissue was then homogenised in a Waring blender for 3 minutes with 300 mL of cooled 0.2 M phosphate buffer (pH 6.8) containing 0.05 M ascorbic acid and 0.5% polyvinylpolypyrolidone (PVP). The homogenate was passed through a cheesecloth filter, and the filtrate was then centrifuged at 30.000 x g for 30 min. at 4 °C. The supernatant's proteins were gathered. The enzyme solution was fractionated with solid ammonium sulphate, and a precipitate with a saturation level of 50–80% was obtained by centrifuging the mixture at 15.000 x g for 20 minutes. The pellet was redissolved in 10 mL of homogenization buffer (0.2 M phosphate buffer, pH 6.8) and dialyzed at 4 °C in cellulose dialysis tubing against the same buffer (mol. wt. cut off 12,000-14,000 Da).

At 8-hour intervals, the dialysis buffer was replaced three times. The dialyzed samples were stored at -20 °C in stoppered test tubes. A small amount of 0.2 M phosphate buffer was used to re-dissolve the dialyzed solution before applying it to a Sephadex G-100 column. The Lowry et al.27 method was used to determine the protein concentration of the samples at various stages of purification.

Gel filteration:

For gel filtration chromatography, a column measuring 1.2 x 70 cm was constructed with Sephadex G-100 and calibrated using 0.1 M phosphate buffer (pH: 6.8) containing 10% glycerin. After loading the column with the dialyzed enzyme solution, the elution rate was changed to 0.5 mL/min. Using a fraction collector, the eluates were collected as 3 mL quantities in tubes. When there was no longer any 280 nm absorbance, the elution process was complete. Each fraction's

predicted protein and PPO activity. The fractions with PPO activity were gathered, and the degree of purification was assessed by measuring the specific activity.

PPO Activity Measurement.

The Traverso-Rueda and Singleton28 pyrocatechin technique was used to measure PPO activity. Using a spectrophotometer (Jenway 6105 UV/vis), the absorbance increase at 420 nm over the course of two minutes was measured to quantify the PPO activity spectrophotometrically. Dialyzed enzyme solution was diluted 1/10 (v/v) with phosphate buffer. 2.8 mL of substrate in phosphate buffer and 0.2 mL of enzyme solution were both present in the sample cuvette. Only 3 mL of substrate solution in phosphate buffer were included in the blank cuvette. The substrates were used in the reaction, which was conducted at varied pH levels and temperatures. On the basis of the linear part of the curve, enzyme activity was determined. The amount of enzyme that increased absorbance by 0.001 units per minute at room temperature is considered one unit of PPO activity. The experiments were carried out twice.

Characterization of PPO

Substrate Specificity. For the evaluation of the substrate specificity of the enzyme, nine different commercial-grade substrates (catechol, 4-methyl catechol, D-tyrosine, L-dopa, pyrogallol, chlorogenic acid, p-cresol, gallic acid, and caffeic acid) were utilised. Except for D-tyrosine and caffeic acid, which were employed at 0.002 M concentrations due to their low solubilities, other substrates were utilised at 0.02 M concentrations.

Effect of pH and temperature

Seven different substrates were used to investigate the impact of pH on PPO activity (catechol, 4-methyl catechol, L-dopa, pyrogallol, D-tyrosine, caffeic acid and p-cresol). The amounts of catechol, 4-methyl catechol, and pyrogallol were 10 mM, D-tyrosine was 2 mM, and caffeic acid, p-cresol, and L-dopa were 5 mM. The ideal pH of PPO was determined using appropriate buffers (0.1 M citrate pH 4.2-5.2; 0.2 M phosphate pH 5.2-7.0; and 0.05 M Tris-HCl pH 7.0-9.5). All further studies were conducted using the ideal pH values that were determined from this assay.

Using the 7 substrates mentioned above, the impact of temperature on PPO activity was measured within the range of 10-75 C.

Thermal Inactivation

PPO was subjected to heat treatments in a temperature-controlled waterbath at 60, 70, 75, 80, and 85 C for varied lengths of time. Ten millilitres of enzyme solution were added to a preheated tube, and at predetermined intervals, 0.5 millilitres of the sample parts were removed, chilled, and tested for residual activity. The enzyme's stability was measured in terms of remaining activity. An Arrhenius plot of the log reaction rate constants (ln k) vs. the reciprocal of the absolute temperature was used to calculate the activation energy for denaturation of the enzyme.

Enzyme Kinetics

PPO's first reaction rates were tested using seven different substrates at varying concentrations at ideal pH and temperature conditions (1, 1.25, 2.5, 4, 7.5 and 10 mM). From the Lineweaver-Burk plot, Kmand Vmax values for each substrate were calculated29. The test described in the "PPO Activity Measurement" section was used to estimate the first reaction rate.

Effect of Inhibitors

At 20°C and pH 7.0, the inhibitory effects of L-cysteine, L-ascorbic acid, sodium azide, sodium diethyl dithiocarbamate, -mercaptoethanol, and thiourea on the PPO activity at fixed concentrations were calculated (1, 1.25, 2.5, 4, 7.5, 10 mM). Lineweaver-Burk graphs were used to estimate the inhibition constants (Ki) of each inhibitor.

Electrophoresis

PPO isoenzymes of the Apples were separated using polyacrylamide gel electrophoresis in accordance with Laemmli's method30 under nondenaturing circumstances. Using a discontinuous buffer system and 4% stacking gel and 7.5% acrylamide separating gel, electrophoresis was carried out. The well spaces in the stacking gel were filled with the enzyme samples from the dialysis (20 L) procedure. The gel was run until the bromophenol blue marker

had reached the bottom at a continuous current of 30 mA at 4 °C. The gel was split into three pieces once the run was finished.

Then, a single gel fragment was submerged in a solution of 0.2 M phosphate buffer (pH 7.2), 20 mM catechol, and 0.05% o-phenylendiamine. The 20 mM 4-methyl catechol and 10 mM pyrogallol solution was used to submerge the remaining fragments. Each gel fragment was then washed in a 1 mM ascorbic acid solution for 4 min after the isoenzyme bands had grown for 100 min. The gel fragments were then preserved in 30% ethanol, exposed to catechol, 4-methyl catechol, and pyrogallol substrates, and photographed. Denaturation solution containing 2.5% glycerol, 0.05% SDS, 1.25% -mercaptoethanol, and 0.001% bromophenol blue in 0.06M Tris buffer was added to enzyme solutions in order to prepare them for SDS-PAGE. Following denaturation of the samples, electrophoresis was performed as previously mentioned. The gels were dyed with Coomassie brilliant blue following the electrophoretic run. The gels were then imaged after being destained in a 7% acetic acid solution. By comparing the relative migration distances of the enzyme and marker proteins at various molecular weights, the molecular weight of the PPO was determined.

Results and Discussion

Purification of PPO

Due to its affinity for phenolics and capacity to bind them, polyvinylpolypyrrolidone (PVP) was employed during the enzyme extraction to avoid phenol-protein interactions. Despite the fact that PVP inhibits PPO 31, its removal by centrifugation prevented this action. To determine the correct saturation point, many precipitations with solid ammonium sulphate contents ranging from 40 to 80% were evaluated. As a result, the precipitate with 80% (NH4)2SO4 saturation was discovered to have the maximum PPO activity, and all extraction operations used this saturation value. During extraction, PPO oxidises phenolics to create quinones. As a result, ascorbic acid was utilised to stop quinones from forming. Table 1 displays the outcomes of the PPO's purification from the Apple. Sephadex G-100 PPO elution profile showed 3 fractions (S1, S2, S3) (Figure 1). According to Table 1's statistics, gel filtering slightly boosted the specific activity of fractions S1 and S2. After gel filtration, the purification degrees of PPO were 13.3, 11.3 and 8.7-fold, indicating that the enzyme has three isoenzymes.

Table 1. Purification of PPO from Apple

Purification steps	Volume (mL)	Activity (EU/mL)	Total activity	Protein (mg/mL)	Total protein (mg)	Specific activity (EU/mg of protein)	Yield (%)	Purification-fold
Crude Extract	350	255.4	89401.4	0,882	308.7	289.57	100	0
Partially purified extract (40-80% $(NH_4)_2SO_4$ fractionation) (Redissolved and dialyzed)	190	462.5	87871.2	0.461	87.6	1003.25	98	3.5
Sephadex G-100 Fraction S_1	94	388.6	36530.2	0.101	9.5	3847.52	41	13.3
Fraction S_2	56	298.7	16727.2	0.091	5.09	3282.42	19	11.3
Fraction S_3	74	314.0	23236.0	0.124	9.18	2532.26	26	8.7

Characterization of Apple PPO

Using the dialyzed enzyme solution produced by ammonium sulphate fractionation, enzyme characterisation was carried out. The enzyme's specific activity and protein concentration were respectively 1003.25 U/mg and 0.461 mg/mL.

Substrate Specificity

Table 2 displays the findings of the PPO substrate specificity investigation. When compared to monophenols like p-cresol and D-tyrosine and tri-hydroxy phenols like pyrogallol and gallic acid, the PPO from the Apples shown a higher affinity for diphenolic substances.

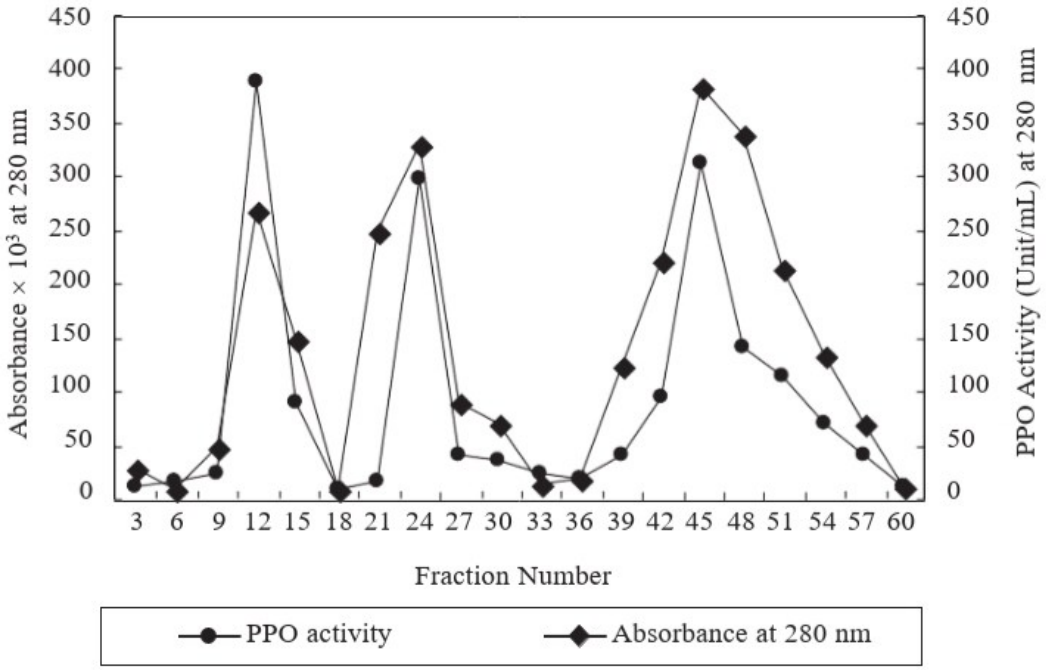

Figure 1. The gel filtration chromatography of the partially purified enzyme.

Table 2. Effect of phenolic substrates on PPO.

Substrate	Type of phenolic compound	Relative activity
Catechol	Dihydroxy	100
4-Methylcatechol	Dihydroxy	68.4
Chlorogenic acid	Dihydroxy	53.6
Caffeic acid*	Dihydroxy	50.0
L-Dopa	Dihydoxy	44.3
Gallic acid	Trihydroxy	20.1
Pyrogallol	Trihydroxy	12.5
p-Cresol	Monohydroxy	11.0
D-Tyrosine*	Monohydroxy	10.4

*Except for D-tyrosine and Caffeic acid (0.002 M) all substrates are 0.02 M.

Effect of pH and temperature.

Table 3 displays the findings from the estimation of the PPO's ideal pH and temperature values for the Apples using 7 different substrates. The PPO of the majority of fruits exhibits its highest

level of activity at pH levels that are neutral or nearly neutral. Although it was shown that the pH optimum for PPO from cherry, peaches, bananas, and lychees was between 6.2 and 7.2, the pH optimum for avocado PPO was between 4.7 and 4.8. Pear PPO's ideal pH can range from 5.8 to 6.4. Depending on the source of the material, the extraction technique, the fruit's age, and the substrate, the optimum pH of the PPO of various fruits may change. In this investigation, ripened apples were used. As a result, when an enzyme is isolated from fruits at various stages of maturity, its ideal pH can alter. Depending on the substrate, Apple PPO's ideal temperatures varied. Even at 75 C, the enzyme was not entirely inactivated above 45 C due to a reduction in activity as temperature rise.

Table 3. Optimum pH and temperature of the PPO from Apple.

Substrate	Optimum pH	Optimum temp (°C)
Pyragallol	8.2	30
4-Methylcatechol	7.2	35
Catechol	7.0	20
D-tyrosine	5.6	55
Caffeic acid	5.6	25
p-Cresol	5.0	40
L-Dopa	4.8	45

Thermal Inactivation Kinetics

Figure 1 displays the outcomes of the heat inactivation of Apple PPO at 60, 70, 75, 80, and 85 C. The enzyme displayed a significant degree of thermal stability. With increasing temperature, the rate of

The slope of the ln k - 1/T plot was used to determine the Arrhenius Ea, which was found to be 63.76 cal/mol (Figure 2b). The PPO's inactivation rate constants (ki) and half-lives (t1/2) were calculated (Table 4).

Table 4. Inactivation rate constants (k_i) and half-lives ($t_{1/2}$) of the PPO.

Temperature (°C)	k_i	$t_{1/2}$
60	1.11	0.63
70	1.83	0.38
75	2.8	0.25

80	5.15	0.14
85	9.95	0.07

Enzyme Kinetics.

Table 4 displays the results of the calculation of the Km and Vmax values for Apple PPO using the Linewear-Burk graphs. The primary function of apple PPO is as an o-diphenol oxidase, with catechol and 4-methyl catechol exhibiting the highest levels of activity. For catechol and 4-methyl catechol, the apparent Km values for the substrate concentration were 5.5 and 7.82 mM, respectively. PPO's substrate affinity typically varies depending on the enzyme's source.

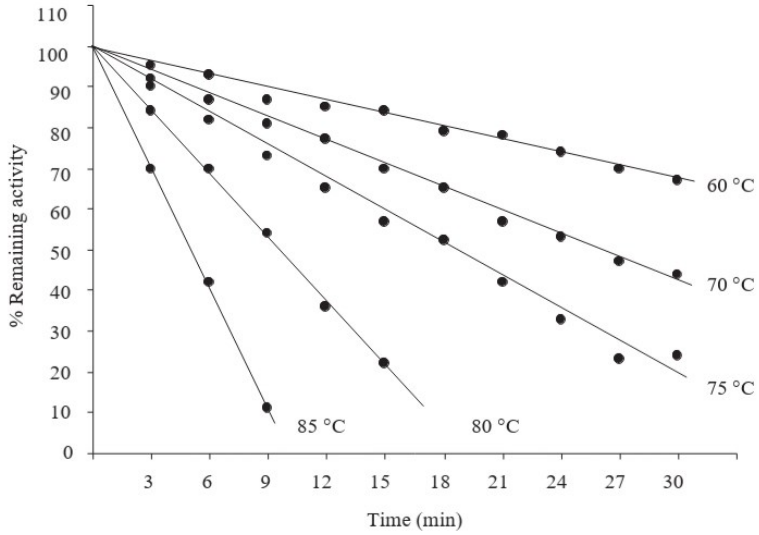

Figure 2a. Thermal inactivation of partially purified PPO at pH 7.2 in 10 mL 0.2 M phosphate buffer containing PPO enzyme.

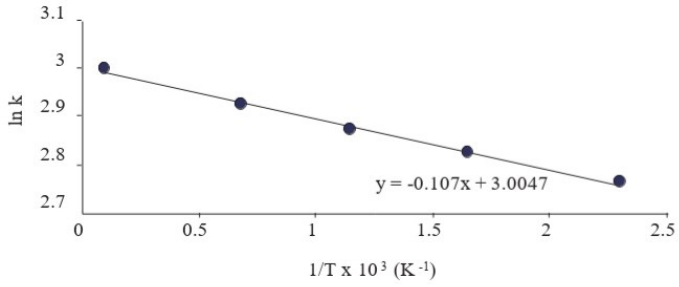

Figure 2b. Arrhenius plot for partially purified PPO at pH 7.2 in 10 mL 0.2 M phosphate buffer containing PPO enzyme.

Effect of Inhibitors.

Table 5 displays the degree of inhibition and Ki values discovered for seven distinct PPO inhibitors. The most effective inhibitor of Apple PPO was sodium diethyldithiocarbamate. According to reports, apple PPO is significantly inhibited by l-cystein. It has also been suggested that cystein can stop processed fruit items from turning brown. L-cystein and ascorbic acid may be helpful in avoiding the enzymatic browning of products because they are naturally occurring and harmless chemicals.

Table 5. K_m and V_{max} values of the PPO.

Substrate	$K_{m,}$(mM)	$V_{max,}$(IU/mL.min)
Pyragallol	29.00	112.78
4-Methylcatechol	7.82	41.67
Catechol	5.55	344.50
D-tyrosine	32.67	97.15
Caffeic acid	16.00	124.25
p-Cresol	45.50	142.68
L-Dopa	24.50	298.72

PPO is typically effectively inhibited by substances like sodium diethyldithiocarbamate (DETC) and thiourea, which interact with copper in the enzyme. The suppressed browning activities of these inhibitors, which are copper chelating agents, lend credence to notions that copper plays a direct role in the oxidation of phenolic chemicals.

Table 6. K_i values and inhibition modes inhibitors.

Inhibitors	Concentration (M)	Average values of K_i (M)	Type of inhibition
L-Ascorbic acid	2.0×10^{-4} 2.5×10^{-4}	7.5×10^{-11}	competitive
Sodium azide	5.0×10^{-2} 1.5×10^{-1}	3.47×10^{-9}	competitive
L-Cysteine	6.5×10^{-5} 2.0×10^{-4}	3.77×10^{-11}	competitive
Sodium diethyl dithiocarbamate	2.5×10^{-5}	1.11×10^{-11}	competitive
Mercaptoethanol β	6.5×10^{-4}	1.18×10^{-5}	noncompetitive
Thiourea	1.5×10^{-1}	2.42×10^{-2}	noncompetitive

Storage Stability of Apple PPO.

In a tiny Erlenmayer flask, partially purified PPO extracts were held for nine days at 4 and 20 degrees Celsius. Every day, samples were obtained to calculate the activity loss. The outcomes are displayed in Figure 2. The first two days at 20 C saw a significant decline in PPO activity. In the days that followed, the decline was gradual. At 4 C, activity losses were lower than they were at 20 C. This suggests that at low temperatures, the enzyme is more stable.

Electrophoresis.

By employing catechol and pyrogallol as substrates for polyacrylamide gel electrophoresis, three isoenzymes of Apple PPO were discovered. Also recovered via gel filtration chromatography were three isoenzymes. 3 PPO isoenzymes have reportedly been found in apples, pears, and cocoyam tubers, according to earlier studies. Figure 4 displays the nondenaturing-PAGE band patterns of Apple PPO isoenzymes. The nondenaturing electrophoretic pattern of Apple PPO using catechol and pyrogallol as substrates is shown in Figure 4(A and B).

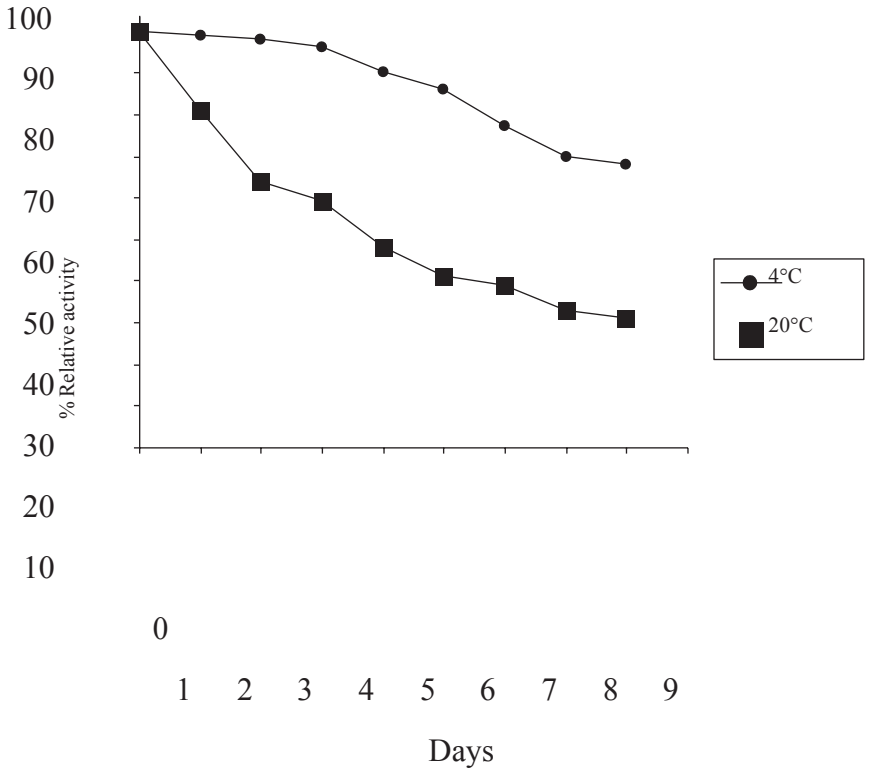

Figure 3. Stability of crude PPO enzyme during storage at pH 7.2 in 10 mL 0.2 M phosphate buffer containing PPO enzyme.

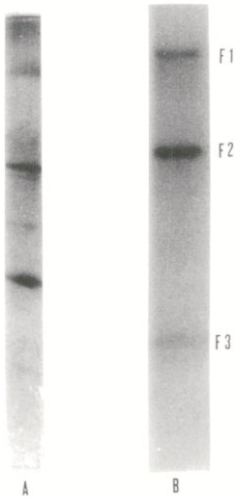

Figure 4. Nondenaturing electrophoretic pattern of Apple PPO A-Catechol substrate. B-Pyrogallol substrate.

The PAGE gels were stained with Coomassie brilliant blue R-250 to identify three isoenzymes. Figure 4 depicts the stained isoenzyme bands as F1, F2, and F3. For both substrates, the F1 and F2 bands appeared to be the most active, followed by the moderately active band F3.

Figure 5 shows the electrophoretic pattern of dialyzed Apple PPO enzyme extract under denaturing conditions. Three dominating bands with molecular weights of 60, 40, and 28 kD were found by SDS-PAGE (marked with arrows).

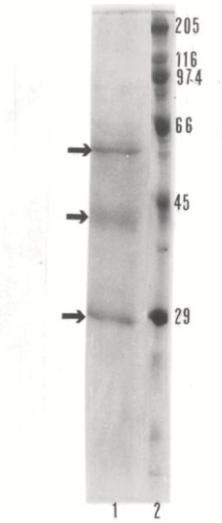

Figure 5. SDS-PAGE pattern of Apple PPO. **Line 1:** Dialyzed Apple PPO, **Line 2:** Line Molecular weight marker (Myosin 205 kDa, β-Galactosidase 116 kDa, Phosphorylase 97.4 kDa, Bovin Serum Albumin 66 kDa, Albumin Egg 45 kDa, Carbonic Anhydrase 29 kDa).

Conclusion:

Comparative Study & Characterization of Poly Phenol Oxidase (PPO) From Fruits of Rosaceae was successfully investigated and studied. Fruit browning was studied using different parameters and methods. Objective of the said study was successfully studied according to the proposed methodologies.

Acknowledgment

I hereby gratefully acknowledge the support funding of the UGC Grant (University Grants Commission)
Minor Research Fund [Project No: 47-637/08(WRO)]

References:

1. P.H. Ferrar and J.R.L. Walker, **J. Food Biochem. 20,** 15-30 (1996).
2. J. Zawistowski, C.G. Biliadeis and E.D. Murray, **J. Food Biochem. 12,** 1-22 (1987).
3. D.S. Robinson and N.A.M. Eskin (Eds.), **Eslevier Applied Science, London,** 20, 157 (1993).
4. J.R.L. Walker and P.H. Ferrar, **Biotechnology and Genetic Engineering Reviews, 15,** 457-498 (1998).
5. A.M. Mayer and E. Harel, **Phytochemistry 18,** 193 (1979).
6. A.M. Mayer, **Phytochemistry 26,** 11 (1987).
7. L. Vamos-Vigyazo, **CRC Crit. Rev. Food Sci. Nutr. 15,** 49 (1981).
8. G. Matheis, **Z. Lebensmittel Unters: Forsch**. **176,** 454 (1983).
9. S.S. Patil and M. Zucker, **J. Biol. Chem. 240**, 3938 (1965).
10. İ. Kocaçalışkan and O. Özbay, **Doğa Tur. Biyol. 11**, 124 (1987).
11. J.J. Jen and K.R. Kahler, **Hortic. Sci. 9,** 590 (1974).
12. W.N. Flurkey and J.J. Jen, **J. Food Biochem., 4,** 29 (1980).

13. M. Oktay, O.I. Küfrevioğlu, I. Kocaçalışkan and H. Şakiroğlu, **J. Food Sci. 60**, 494 (1995).

14. A.H. Janovitz-Klapp, F.C. Richard, P.Goupy and J. Nicolas, **J. Agric. Food. Chem. 38,** 1437 (1990).

15. A.M. El-Tabey and W.V. Cruess, **Plant Physiol. 24**, 307 (1949).

16. J. Palmer, **Plant Physiol. 38,** 508 (1963).17. V.Kahn, **J. Food Sci. 50,** 11 (1985).

17. M.A. Galeazzi and C. Sgarbieriv, **J. Food Sci. 46,** 1404-1406 (1981).

18. J.N.Cash, W.A. Sistrunk and C.A. Stutte. **J. Food Sci. 41,** 1398 (1976).

19. E. Valero, R. Varon and F. Garcia-Carmona, **J. Food Sci. 53,** 1482 (1988).

20. N.J. Rivas and J.R. Whitaker, **Plant Physiol. 52**, 501 (1973).

21. G.M. Sapers and R.L. Miller, **J. Food Sci. 63(2),** 342-346 (1998).

22. Ben-Shalom, V. Kahn, E. Harel and A.M. Mayer, **Phytochemistry 16,** 1153 (1977).

23. W. Ebelling, M.W. Montgomery, **J. Food Sci. 55**, 1315 (1990).

24. M. Siddiq, N.K. Sinha, J:N. Cash, **J. Food Sci. 57,** 1177 (1992).26. E.Y. Park, B.S. Luh, **J. Food Sci**., **50,** 678 (1985).

25. Y.K. Park, H.H. Sato, T.D. Almeidia and R.H. Moretti, **J. Food. Sci. 45,** 1619 (1980).

26. O.H. Lowry, N.J. Rosebrough, A.I. Farr and R.J. Randall, **J. Biol. Chem. 193,** 265 (1951).

27. S. Traverso-Rueda and V.L. Singleton, **Am. J. Eol. Viticult., 24**,103-106 (1973).

28. H. Lineweaver, D. Burk, **J. Am. Chem. Soc., 56,** 658 (1934).31. U.K. Laemmli, **Nature, 227,** 680 (1970).

29. E. Harel and A.M. Mayer, **Phytochemistry, 10**, 17 (1971).

30. J.R.L. Walker, **Aust. J. Biol. Sci. 17**, 360 (1964).

31. W.N. Tate, B.S. Lahand and G.K. York, **J. Food Sci. 29,** 829 (1964).

32. N.D. Benjamin and M.W. Montgomery, **J. Food Sci. 38**, 799 (1973).

33. T.C. Wang, B.S. Luh and J.R. Whitaker, **Plant Physiol. 48,** 19 (1971).37. V. Kahn, **J. Food Sci. 42**, 38 (1977).

34. 38. J. Yue-Ming, **Food Chem. 66,** 75-79 (1999).

35. 39. K. Betrosian, M.P. Steinburg and A.I. Nelson, **Food Technol., 14**, 480 (1960).

36. 40. H.T. Chan and H.Y. Yang, **J. Food Sci. 35,** 169-173 (1971).

37. B.S. Luh and B. Phithalopol, **J. Food Sci. 37,** 264 (1972).

38. J.K. Palmer And J.B. Roberts, **Science 157,** 200 (1967).

39. Yue-Ming Jiang, **Food Chemistry 66,** 75 (1999).44. F.W. Knapp, **J. Food Sci.**

30, 930 (1965).

40. H. Şakiroğlu, O.I.Küfrevioğlu, I. Kocaçalışkan, M.Oktay and Y. Onganer, **J. Agric. Food Chem. 44**, 2982
41. (1996).
42. K. Nakamura, Y. Amano and M. Kagami, **Am. J. Enol Vitic. 34**, 122 (1983).

    A. Guadarrama and N. Rivas, **Rev. Fac. Agron. (Marakay) 16,** 65-86 (1995).

43. C.R. Santerre, J.N. Cash and D.J. Vannorman, **J. Food Sci. 53,** 1713-1716 (1988).
44. A.G. Mathew and H.A.B. Parpia, **J. Food Sci. 35**, 140-143 (1970).
45. K. Prabhakaran, E.B. Harris and W.F. Kırchheimer, **J. Bacteriol. 100,** 935 (1969).
46. D. Kertesz and R.Zito, **Nature (London) 179,** 1017 (1957).
47. E. Harel and A.M. Mayer, **Phytochemistry, 10,** 17 (1971).
48. D.H. Halim and M.W. Montgomery, **J. Food Sci. 43,** 603 (1978).
49. S.M. Constantinides and C.L. Bedford, **J. Food Sci. 32**, 446 (1967).