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CONTROLLING OF POLYPHENOL OXIDASE-BASED BROWNING IN SELECTED FRUITS AND VEGETABLES

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ABSTRACT

The activity of polyphenol oxidase (PPO) lowers the nutritive qualities, and hence lower the cost of the fruits and vegetables. The present study was designed to use different physical and chemical treatments in fruits and vegetables for inactivation of PPO. The crude PPO was extracted from selected fruits (Apple, Banana, Mango, Grapes, Peach) and vegetables (Potato, Eggplant, and Lettuce) using standard protocols and PPO activity was recorded with spectrophotometer using catechol as substrate. Physical treatments were performed at 4ºC, 45ºC and 5 ºC for 20 minutes. The chemical treatments were performed using EDTA (1mg/L), ascorbic acid (0.5%), sodium chloride (1g/L), citric acid (0.75%) and honey (10% and 50%). Results indicated that PPO inhibition was maximum in banana (20.699%) at 55ºC for 20 minutes. The PPO extract from mango showed maximum inhibition (75.930%) with 50% honey. In vegetables, the PPO extract of potato showed (38.731%) inhibition at 4ºC for 20 minutes and (72.874%) with 50% honey. The results indicated that storage at 4ºC and treatment with citric acid (0.75%), ascorbic acid (0.5%) and honey (10% and 50%) were most effective for reduction of enzymatic browning in plant products. Results of this study provide bases for development of dual control strategies to prevent browning of fruits, vegetables and other food products.

Keywords: Enzymatic Browning, Polyphenol Oxidase, Peroxidase, Antibrowning

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1. INTRODUCTION

Plant products especially fruits and vegetables are vital, owing to the presence of fiber, antioxidant compounds and vitamins. Changes occur during the process of collection, preparation (fresh-cut fruits) and packing of fruits and vegetables, that lead to reduction of biological and nutritional properties of plant products (Lindley 1998). Consequently, in the food industry safety of food products from oxidation has become a growing priority. Reduction of unwanted loss due to spoilage and brown color production has more attention of researchers because these changes decrease the biological and nutritious worth of fresh fruits and vegetables (Toivonen and Brummell 2008). After microbiological infection oxidation is one of the most noteworthy reason for the loss of food products. These oxidative reactions are catalyzed by the oxidoreductase enzymes, mainly the polyphenol oxidase (Ioannou and Ghoul 2013). The polyphenol oxidase (PPO) activity on polyphenols produces quinones that results browning of plant products (Constabel and Barbehenn 2008; Chang 2009).

The browning reaction of PPO occurs because of oxidation and dehydrogenation of polyphenolic compounds that are colorless. PPOs known with other names are phenol oxidase, phenolase, monophenol oxidase, diphenol oxidase and tyrosinase. PPO results in oxidative browning reaction in several fruits, vegetables and food products (Chi et al. 2014). Two reactions are catalyzed by polyphenol oxidase, a hydroxylation of monophenols to diphenols that is a slow process and leads to the formation of colorless products. Another reaction is the oxidation of diphenols to quinines that is a rapid reaction and forms colored products. There are many techniques for the inactivation of polyphenol oxidase activity (Amado et al. 2014). EDTA inhibits PPO activity either by binding to the active PPO copper site, or by providing the enzyme with copper (Ioannou and Ghoul 2013). Acidulants are commonly used for reducing browning in food processing. Sodium chloride is commonly used to prevent browning at concentration of 2-4 percent in food processing industries (Allende et al. 2009). Today most reliable method for browning control is thermal processing. It has the capacity to destroy microorganisms and also inactivate enzymes. PPO was destroyed at 80ºC but the internal portion of the fruit or vegetables requires sufficient time (Vishwasrao et al. 2017). Adding bisulfate, reduced glutathione and thiol-compounds as well as L-cystine will delay the PPO causing food browning

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(Khalil and Adam 2016). Sodium chlorite (SC) has the dual purpose of being both an antibrowning and antimicrobial agent. The SC successfully deactivates fresh-cut apple PPO and microbial growth (Hengphum et al. 2015). Sulfites are operative for PPOs inhibition, but these were limited by the Food and Drug Administration (FDA) because of their health hazards in asthmatic patients (Eissa and Salama 2015). Chemical pretreatments in *Solanum surattense* are recognized as good in the shelf life (Dharmabandu et al. 2007). 1-Methylcyclopropene treatments have been used to minimize respiratory and browning levels and maintain quality in pineapple processed for 12 days at 4.5 pH (Budu and Joyce 2003). However, browning is helpful as it improves the consistency of beverages by shaping flavored goods, e.g. in the manufacturing of tea, coffee (Amorim and Melo 1991), and cocoa (Lopez and Dimick 1991).

This work was carried out to determine the impact of chemical and physical pretreatments that were widely used to prevent browning of fruits and vegetables due to PPO activity. These treatments were used to reduce browning of food products so that economic loss can be minimized by improving quality of fruits and vegetables for marketing and storage. Enzymatic browning caused by PPO have been experimentally tested using physical treatments at high temperatures 55ºC and 45ºC by incubation in water bath and keeping at low temperature 4ºC. The chemical treatments were performed using EDTA (1mg/L), ascorbic acid (0.5%), sodium chloride (1g/L), citric acid (0.75%) and honey (10% and 50%). All chemicals used showed PPO inhibition but in different food products citric acid treatment, sodium chloride with ascorbic acid and 50% honey showed more better inhibition of PPO than other chemical treatments.

2. MATERIALS AND METHODS

This study was conducted to reduce polyphenol oxidase activity on selected fruits and vegetables. These fruits and vegetables are affected more due to polyphenol oxidase activity and show maximum browning, so selected for experimental work. The experimental work was performed in the Plant Biochemistry Laboratory of PMAS-Arid Agriculture University Rawalpindi. All the chemicals and reagents were systematically graded purchased from university suppliers (Shalimar scientific stores, Adam gee road Massey gate, Saddar Rawalpindi).

2.1. Sample Collection

The fresh sample of selected fruits (Grapes, Mango, Peach, Apple, Banana) and vegetables (Eggplant, Potato, Lettuce) were collected from commercial market, Rawalpindi during almost ripening stage (Table 1). The fruits and vegetables were of middle size and with soft pulp.

Table 1: Sample collection of different fruits and vegetables at different physiological stages

Sr. No	Sample	Botanical Name	Physiological State
Fruits			
1	Grapes	*Vitis vinifera*	Ripe, Green
2	Mango	*Mangifera indica*	Ripe, Soft Pulp
3	Peach	*Prunus persica*	Yellow & Red in color
4	Apple	*Malus domestica*	Red and Light Yellow
5	Banana	*Musa acuminate*	Yellow color peel with some black spots
Vegetables			
1	Eggplant	*Solanum melongena*	Purple in color, soft
2	Potato	*Solanum tuberosum*	Brown, small in size
3	Lettuce	*Lactuca sativa*	Green

2.2. Extraction of Polyphenol Oxidase

The fruits were sliced into equal pieces using a sharp knife. Sliced fruit samples were homogenized using grinder in sodium phosphate buffer (1mL pH 6.5 to pH 7) and then filtered. The filtrate was centrifuged at 4000 rpm for 30 minutes and supernatant was collected and put off in cold acetone (1.5mL) and stirred for 30 minutes. The supernatant was centrifuged again at 4000 rpm for 30 minutes at 4ºC. After that precipitates containing PPO were collected and dissolved in sodium phosphate buffer 2mL (Khalil and Adam 2016). The extracted crude enzyme was used for further studies.

2.3. Quantification of protein

2.3.1. Biuret Assay: Biuret reagent was prepared by dissolving 3g copper sulphate ($CuSO_4.5H_2O$) and 9g of sodium potassium tartrate in 500 mL of 0.2 mol/liter sodium hydroxide and then 5g of potassium iodide was added and volume was made up to 1 liter by sodium hydroxide 0.2 mol / liter. In test tubes, pipetted 0.0, 0.2, 0.4, 0.6, 0.8

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and 1-mL working standard. 1 mL of the given sample was pipette in other test tubes and taken up the volume of all test tubes to 1 liter. Distilled water was added in 1mL test tube taken as blank. Biuret reagent 3mLwas added to all test tubes including "blank" and "unknown" test tubes. Test tube contents were mixed by shaking the tubes and warm up for 10 min at 37ºC, cooled the contents to room temperature and absorbance was recorded against blank at 540 nm.

2.3.2. Quantification of Crude Polyphenol Oxidase Extract: The crude PPO was extracted and quantified using biuret assay method as described above.

2.3.3. Assay of Polyphenol Oxidase Activity: The activity of polyphenol oxidase was calculated by measuring the absorbance at λ420 nm with the spectrophotometer (CECIL 2021/ USA) using catechol as a substrate. The catechol (50mM) was prepared using a buffer with sodium phosphate. The sample cuvette contained 2.0mL of catechol ,0.9mL of 0.2M sodium acetate buffer pH 6.5 and 0.1mL of enzyme solution of fruits and vegetables (Apple, Banana, Mango, Peach, Grapes, Potato, Eggplant, Lettuce). Each sample was assayed in triplicate. Reference cuvette (blank) contained 2.0mL of the same substrate solution (Catechol) and 1.0mL of 0.2M sodium acetate buffer (Mehmood 2009). The effect of different chemical and physical treatments were tested using the catechol as substrate and the control enzyme reaction were compared under optimum conditions with no inhibitor. Percentage inhibition was calculated using the following equation.

Inhibition (%) = $(A_0 - A_i/A_0) \times 100\%$

where A0 was initial PPO activity (without the inhibitor), and Ai was PPO activity with the inhibitor (Sikora et al. 2019).

2.3.4. Physical Treatments: PPO behavior was estimated at temperatures of 45ºC to 55ºC for 20 minutes while incubated in the water bath and extracted crude enzyme was stored at 4ºC in Dawlance (9150) refrigerator for 20 minutes and after that enzyme activity was checked from spectrophotometer immediately.

2.3.5. Chemical Treatment: Various chemical treatments were used to inhibit the enzymatic browning of the fruits and vegetables. These treatments differ by their action depending on the chemical agents. The chemical treatments used in this study were acidulants, chelating agent, enzyme inhibitors, reduction agent, and honey.

2.3.6. Chelating Agents: These are chemicals that bind metal ions from their site of action and expel them. EDTA (ethylenediaminetetraacetic acid) is a natural preservative appropriate for use in food processing. EDTA 1mg/ liter water was used to inhibit PPO.

2.3.7. Reducing Agent: Ascorbic acid is a good antioxidant and reduction agent that eliminates oxygen in polyphenol oxidase reactions. In action, approximately 0.1-0.5% of ascorbic acid may have a protective effect against enzymatic browning (Garcia and Barrett 2002). 0.5% Ascorbic acid+ 1mg / liter of sodium chloride for 5 minutes was used to inactivate PPO; this was most effective treatment for delaying browning.

2.3.8. Acidulants: Enzymes that cause loss of catalytic function may be denatured by extreme pH. A pH greater than 3 effectively inhibits PPO activity. Citric acid is an acidulant commonly used as anti-browning agent, as it is readily available and cost-effective (Garcia and Barrett 2002). Citric acid in the concentration of 0.75% was used to inhibit the PPO activity.

2.3.9. Honey: In this research work 10% honey and 50% honey was used to inhibit PPO in fruits (apple, banana, mango, grapes) and vegetables (potato, lettuce, eggplant).

3. RESULTS

This study was designed to control the enzymatic browning in selected fruits and vegetables by different methods.

3.1. Concentration of Crude PPO Extract

The exact amount of enzyme present in fruits and vegetables was determined using biuret assay of protein quantification and comparing with standard curve of BSA, concentration of protein was given (Table 2).

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Table 2: The protein concentration of crude PPO Extract

Samples	Protein contents (mg/ml)
Fruits	
Control	0±0.0
Grapes	3.1±0.1
Mango	2.1±0.15
Peach	1.9±0.2
Apple	2.5±0.1
Banana	3.1±0.15
Vegetables	
Egg plant	1.5±0.2
Potato	1.2±0.1
Lettuce	1.2±0.1

3.2. Impact of temperature on PPO activity in fruits

Activity of PPO in fruits was checked at temperatures from 45ºC to 55ºC. PPO inactivity was checked by taking readings from spectrophotometer using catechol as substrate. In grapes PPO showed percentage inhibition of 2.497% at 45ºC, 2.871 at 55ºC and 11.236% at 4ºC. The results of inactivity of PPO in mango were 3.131%, 10.959% and 3.327% at 45ºC, 55ºC and at 4ºC. PPO activity was reduced but not inhibited completely. At 55ºC activity was reduced but mango PPO showed less inhibition at this temperature. PPO activity was reduced very less in peach. At 55ºC more inhibition was recorded (5.780%). The results of inactivity of PPO in peach at 45ºC was 3.8954% and at 4ºC was 5.010%. Apple showed inhibition at 45ºC of 0.823%, at 55ºC was 6.580% and at 4ºC of 1.645%. In apple more inhibition was recorded at 4ºC. Banana PPO activity decreased at 55ºC (20.699%), 45ºC (10.842%) and at 4ºC (12.724%) activity, as shown in Table 3.

Table3: Physical treatments of fruits and vegetables at different temperatures

S.No.	Fruits/Vegetables Name	Inhibition (%) at different temperatures (℃) for 20 minutes		
		4	45	55
Fruits				
1	Grapes	11.236	2.497	2.871
2	Mango	3.327	3.131	10.959
3	Peach	5.010	3.854	5.780
4	Apple	1.645	0.823	6.580
5	Banana	12.724	10.842	20.699
Vegetables				
1	Eggplant	3.540	0.000	8.739
2	Potato	33.198	26.113	31.377
3	Lettuce	3.441	0.000	8.401

3.3. Impact of chemical treatments on PPO activity in fruits

PPO from fruits were treated with different chemicals citric acid, EDTA, sodium chloride+ ascorbic acid and honey (10% and 50%). Activity of PPO in grapes was reduced more with citric acid treatment (73.908%). The results with other treatments were as following EDTA (0.250%), ascorbic acid + NaCl (3.121%) and with 10% honey (0.250%), 50 % honey (0.499%). In mango 50 % honey treatment was found to be more effective and reduce browning to 75.930%. The results of other chemical treatments were citric acid (60.959%), EDTA (6.076%), ascorbic acid+ NaCl (1.174%) and with 10 % honey (40.802%). In peach more percentage inhibition was with citric acid (70.135%). Other chemicals showed following readings EDTA (16.763%), ascorbic acid + NaCl (21.965%),10% honey (1.7345) and with 50% honey (11.079%). Apple PPO showed more inhibition with the treatment of ascorbic acid + sodium chloride (7.051%) and less inhibition was recorded with the treatment of citric acid (0.588%). The results with other chemicals were as EDTA (4.935%), with 10 % honey (0.823%) and 50 % honey (1.058%). Banana PPO after treatment with citric acid reduced PPO activity to 51.613%. Citric acid was found more effective in PPO inhibition than other chemical treatments. Results with other treatment were EDTA (18.728%), ascorbic acid +NaCl (8.333%), and with 10% honey (4.211%) and 50% (30.645%), as shown in Table 4.

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Table 4: Chemical treatment of fruits and vegetables with different chemicals

S. No	Fruits/ Vegetables	Inhibition (%) with				
		Citric Acid	EDTA	Ascorbic Acid +NaCl	Honey (10%)	Honey (50%)
Fruits						
1	Grapes	73.908	0.250	3.121	0.250	0.499
2	Mango	60.959	6.067	1.174	40.802	75.930
3	Peach	70.135	16.763	21.965	1.734	11.079
4	Apple	0.588	4.935	7.051	0.823	1.058
5	Banana	51.613	18.728	8.333	4.211	30.645
Vegetables						
1	Eggplant	45.686	1.217	0.664	1.106	13.606
2	Potato	60.526	29.015	29.960	71.592	72.874
3	Lettuce	64.699	0.463	0.347	0.116	0.347

3.4. Impact of Temperature on PPO Activity in Vegetables

Activity of PPO in eggplant was checked at different temperatures, PPO showed no inhibition at 45ºC whereas at 55ºC (8.739%) and at 4ºC (3.540%) inhibition was recorded. Activity of PPO in potato was determined at 45ºC (26.113%), 55ºC (31.337%) and at 4ºC (33.198%). Activity of PPO in lettuce was at 55ºC (8.401%) and at 4ºC (3.411%) whereas at 45ºC no inhibition was recorded. More PPO inhibition was recorded at high temperature as shown in Table 3.

3.5. Impact of Chemical Treatments on PPO Activity

Eggplant PPO was treated with different chemicals was as citric acid, EDTA, sodium chloride+ ascorbic acid and honey (10% and 50%). Activity of PPO was reduced more with citric acid treatment (45.686%). Other treatments results were as following EDTA (1.217%), ascorbic acid + NaCl (0.664%) and with 10% honey (1.106%), 50 % honey (13.606%). Potato PPO showed more inhibition with the treatment of 50% honey (60.526%). The results with other treatments were EDTA (29.015%), ascorbic acid + NaCl (29.960%), with 10% honey (71.592%) and 50 % honey (72.874%). Lettuce PPO showed more percentage inhibition with the treatment of citric acid (64.699%). The results with other treatments were EDTA (0.463%), ascorbic acid + NaCl (0.347%), with 10% honey (0.116%) and 50 % honey (0.347%) as shown in (Table 4).

4. DISCUSSION

In the present study 08 extracts from different fruits (apple, banana, grapes, mango, and peach) and vegetables, (potato, eggplant and lettuce) were tested by physical and chemical methods to inhibit browning. Refrigeration was widely used in berries, vegetables and seafood to prevent browning (Schulbach et al. 2011). When grapes were treated physically at low temperature 4ºC for 20 minutes by refrigeration, more inhibition was recorded (11.223%) whereas less inhibition was recorded at 45ºC (2.497%), because freezing temperature decreases the amount of water that is necessary for enzyme activity. Chemical treatment of grapes showed maximum inhibition with citric acid (73.908%) and less inhibition was with EDTA and 10% honey (0.250%). Citric acid reduces browning by decrease in pH and complexation of copper which is essential for the enzyme to function. PPO from mango showed more inhibition at high temperature 55ºC up to (10.959%). The less inhibition was recorded at 45ºC (3.131%). PPO from mango showed more inhibition with 50% honey (75.930%). There are many reports of PPO inhibitors in natural sources, such as honey (Eissa and Salama 2015).

The mechanism of honey inhibiting PPO differs depending on the honey variety used, the source of PPO and the substrates used. Large concentrations of components such as phenolic acid, flavonoids and honey vitamins suggested their antioxidant properties (Lim and Wong 2018), less inhibition was with the treatment of ascorbic acid+ NaCl (1.174%) in mango. PPO from peach showed more inhibition at low temperature 4ºC (5.780%) due to unavailability of water for enzyme activity and less inhibition was recorded at 45ºC (3.854%). Chemical treatment of PPO from peach showed more inhibition with citric acid (70.135%). Citric acid reduces browning by decrease in pH and less inhibition was with 10% honey (1.734%). Acidity reduce the binding of the enzyme and intensifying the citric acid reaction with copper through chelation (Osuga et al. 1994). The literature includes many chelators, citric acid is commonly used for chelating (Rouf et al. 2018). Apple showed more inhibition at 55ºC (6.580%) as compared to 45ºC (0.823%). The increased temperature may change structural properties of enzyme causes the hindrance for substrate to find active sites (Kouassi et al. 2005). More inhibition was with ascorbic acid + sodium chloride (7.051%) treated apple PPO. These compounds inhibit the browning reaction by removing active reaction elements, i.e., enzyme, copper, or an intermediate reaction (Ferrar and Walker 1996). Ascorbic acid, depending on the concentration,

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showed different behavior (Ali et. al 2015). Ascorbic acid showed effective inhibition of total PPOs when treated with other chemicals (Sikora et al. 2019).

Citric acid is usually used for the prevention of browning in fruits and vegetables (Mishra and Gautam 2016). When banana PPO kept at high temperature 55ºC (20.699%) more inhibition was measured. PPOs present in mango require more than 15 minutes at 80ºC for 50% loss of PPO activity (Taranto et al. 2017). Banana PPO showed more inhibition with citric acid (51.613%). Citric acid was found more effective in PPO inhibition than other chemical treatments. Because PPO show more activity in alkaline medium and in acidic medium PPO activity was decreased. Banana treated with ascorbic acid showed the low PPO activity, indicating less browning (Yildiz 2018). When eggplant was given physical treatment more inhibition of PPO was at 55ºC (8.739%). Chemical treatment of PPO from eggplant showed more inhibition with citric acid (45.686%) and less inhibition was with the treatment of acetic acid + sodium chloride (0.664%). Potato PPO showed more inhibition at low temperature 4ºC (33.198%) and less inhibition was at 45ºC (26.113%).

PPO from potato showed more inhibition with 50% honey (72.874%) and less inhibition was recorded with EDTA (29.015%). EDTA (ethylenediaminetetraacetic acid) is a chelator that inhibits PPO activity either by penetrating PPO's active Cu site, or by decreasing Cu accessibility to the enzyme (Du et al. 2012) Lettuce PPO showed more inhibition at 55ºC (8.401%) and no activity was recorded at 45ºC. Because in lettuce PPO inactivation require more temperature. More inhibition was recoded with the treatment of citric acid (64.699%) and less inhibition was with the treatment of ascorbic acid +NaCl treatment (0.463%). The polyphenols react with oxygen in the enzymatic browning reaction. If other compounds react with oxygen, there will be no enzymatic browning reaction. Such a chemical is called antioxidant. High temperature 55ºC and low temperature 4ºC was found to be effective. Chemicals treatment showed more PPO inhibition in grapes, mango, banana, and potato. Citric acid and 50% honey treatment was found to be more effective for inhibiting the enzyme activity. Other persuasive factors include the medium pH, temperature, availability of oxygen, quantity and type of phenolic compounds present (Othman 2012). The capacity of ascorbic acid to reduce quinone is dependent on its concentration in solution (Othman 2012). Research have shown that the degree of darkening varies significantly from one variety to another, depending on the availability and substrate levels of fruits and vegetables and their maturity.

Conclusion: Fruits and vegetables showed different results due to presence of different phenolic contents and ripening stage of fruits and vegetables. More inhibition was recorded in mango, banana and potato with physical treatment at 4ºC and 55ºC. Citric acid treatment was found to be more effective in grapes, peach, potato and lettuce. Honey 50% showed more inhibition in mango and potato. Chemical inhibitors are simple and quick to use. Many forms of enzymatic inhibitors have been identified and tested, and significant research is still needed in this area.

Author's Contribution: The experimental work has been performed in the Plant Biochemistry Laboratory of PMAS-Arid Agriculture University Rawalpindi by AK according to guidelines provided by supervisor MSA and technical support was provided by MEB and SK. AK prepared the draft of the manuscript. AK and AM edited the manuscript.

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