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Effects of Metals and Anti-browning Agents on Polyphenol Oxidase Activity from Sorrel (*Rumex acetosa*)

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Abstract

Enzymatic browning is a chemical process which occurs in fruits and vegetables by the enzyme polyphenol oxidase (PPO). Ascorbic acid, honey, cysteine can act as anti-browning agents by inhibiting PPO. Heavy metals are toxic for live organisms and inhibit enzyme activities. In this work, the effect of some metals, anti-browning agents and metal-anti-browning agent complexes were investigated on sorrel PPO enzyme. According to the results. Cu(II) and Fe(II) metals increased the PPO activity however, Sn(II) had the maximum inhibitory effect. Glutathione (GSH) was the most potent anti-browning agent of the PPO activity among others. In order to reduce the toxic effect of heavy metals on PPO enzyme, the effects of metal-anti-browning agent complexes were investigated on sorrel PPO enzyme activity. EDTA and metal complexes had no significant effect. However, GSH–metal complexes had the best inhibitory effect except GSH-Fe complex on sorrel PPO enzyme activity.

Keywords: *Anti-browning, metal, sorrel, Rumex acetosa, Polyphenol oxidase.*

1. Introduction

Polyphenol oxidase (E.C. 1.14.18.1) is a copper-containing enzyme, widely distributed in plants and microorganisms. The enzyme is responsible for the enzymatic browning reaction during the handling, storage and processing of fruit and vegetables. In the presence of molecular oxygen, plyphenol oxidase (PPO) catalyzes the o-hydroxylation of monofenols to o-diphenols (monophenolase activity) and oxidation of the o-diphenols to o-quinones (diphenolase activity) [14]. The quinones are then polymerized to brown or black pigments which lead to organoleptic and nutritional modifications and reduce food quality. This has been a serious problem in the food industry. Several studies have focused on the inhibition of enzymatic browning by ascorbic acid. Ascorbic acid can reduce o-quinones, produced by PPO-catalyzed oxidation of polyphenols, back to dihydroxy polyphenols and has been widely used as an anti-browning agent for processing of fruits and vegetables. However, the effect of ascorbic acid is temporary since once it is added, it is completely oxidized and o-quinones could accumulate, leading to browning pigment formation [7, 23]. Sulphydryl (thiol) compounds L-cysteine and reduced glutathione (GSH) are also excellent inhibitors of browning of potato powder [14].

Extensive studies have been carried out on the purification, characterization and inhibitors of PPO enzyme. Polyphenol oxidases have been purified and characterized from various plant sources such as peppermint [19], artichoke [3], broccoli [16], butter lettuce [17], iceberg lettuce [10], mulberry [2] and spiderflower [15]. Some of the natural agents proposed to have an inhibitory effect on PPO are honey, natural aliphatic alcohols, L-ascorbic acid (vitamin C), L-cysteine. It has been suggested that the amino acid, cysteine, can form a stable complex with copper, thus retarding enzymatic browning [24]. Ascorbic acid also acts as an oxygen scavenger for the removal of molecular oxygen in polyphenol oxidase reactions. It has been used to reduce enzymatic browning during food process [14]. EDTA is generally used in combination with other chemical treatments for the prevention of enzymatic browning in foods. It is not very effective as an inhibitor of peach polyphenol oxidase [27]. These anti-browning agents inhibit PPO enzyme and lower the browning reaction during the process. Some heavy metals in environment also inhibit PPO activity and killed the enzyme during the growth of plants.

Heavy metal contamination of agricultural soils is a major environmental problem that can reduce both the productivity of plants and the safety of plant products as foods and feeds [5, 6]. Heavy metals like copper (Cu), nickel (Ni) and zinc (Zn) are essential micronutrients for plants, but in excess all these metals are harmful to humans, animals and plants; as are the non-essential metals Pb, Sn and Hg [7]. These heavy metals effect the enzymes at the cellular level by altering enzyme activities, resulting in enzymes activation or inhibition [11]. Several investigations were carried out to assess the effects of different metals (singly or in combination) on various plants in relation to their biochemical response [9, 18].

In the recent years, many studies regarding interactions between chemicals, metals and various substances with different parameters of enzymes have gained considerable interest [6]. Although there are studies regarding characterization and purification of PPO from various plants, no reports have been found on purification and characterization of PPO enzyme from sorrel (Rumex acetosa). Therefore, the objective of our study was to isolate, purify and characterize the PPO from the sorrel (Rumex acetosa). In addition, the enzyme activity was investigated by using heavy metals, anti-browning agents and both of their complexes.

2. Material and Methods

2.1 Chemicals

Sorrel (*Rumex acetosa*) used in this study was obtained from Sakarya region and stored at –20°C until used. Polyviniylpolypyrolidone (PVP), Sephadex G-100, (NH4)2SO4 and other chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

2.2 Extraction and purification

15 g of Sorrel (*Rumex acetosa*) was obtained from local Sakarya region. After that samples were added to 10 ml 50mM sodium phosphate buffer (pH; 7.0), 0.3 g polyvinylpolypyrolidone (PVPP), and extraction was prepared. The mixture was homogenized with blender. After the filtrate was centrifuged at 14.000xg for 30 min and supernatant was collected. Extraction was fractionated with $(NH_4)_2SO_4$, solid $(NH_4)_2SO_4$ was added to the supernatant to obtain 80% saturation. The mixture was centrifuged at 14,000xg for 30 minutes and the precipitate was dissolved in a small amount of phosphate buffer and then dialyzed at 4°C in the same buffer for 24 h with three changes of the buffer during dialysis. The dialyzed enzyme extract was centrifuged and loaded onto Sephadex G-100 column previously equilibrated with extraction buffer, and washed with the same buffer to remove unbound proteins. The eluate was used as the PPO enzyme source in the following experiments. The amount of PPO was performed according to method of Bradford with bovine serum albumin as standard [8].

2.3 Enzyme assay

PPO activity was determined by measuring the initial rate of quinone formation as indicated by an increase in absorbance at 420 nm. PPO activity was assayed using catechol, 4-methyl catechol, caffeic acid and pyrogallol as substrates. The optimum pH, temperature obtained for all substrates was used for determining substrate specificites. For each substrate, the kinetic data were plotted as reciprocals of activities versus substrate concentrations. The Michaelis-Menten constant (Km) and maximum velocity (Vmax) were determined as the reciprocal absolute values of the intercepts on the x- and y-axes, respectively, of the linear regression curve. Substrate specificity (Vmax, Km) was calculated by using the data obtained on a Lineweaver-Burk plot [21].

2.4 Effect of pH and temperature

PPO activity, as a function of pH, was determined in a pH range of 4.5–5.5 in 50 mM acetate buffer, 6.5–7.5 in 50 mM phosphate buffer and 8.5–9.5 in 50 mM Tris–HCl and Tris-Base buffer. The optimum pH values obtained from this assay were used in all the other experiments. The effect of temperature on PPO activity obtained at different temperature values (4-60ºC) and the optimum temperature of sorrel PPO was determined.

2.5 Effect of various metals and anti-browning agents

Cu(II) ($CuSO_4$) , Fe(III) ($FeCl_3$), Zn(II) ($ZnSO_4$), Pb (II) ($PbCl_2$), Sn(II) ($SnCl_2$), Hg (II) ($HgCl_2$), as metals were used to determine their effects on sorrel PPO enzyme activities at their different concentrations (1 and 10 mM). L-cysteine, glutathione (GSH), ethylenediamine tetraacetic acid (EDTA) and L-ascorbic acid as anti-browning agents were used to determine their effects on the PPO enzyme activities at their different concentrations (1 and 10 mM).

2.6 Effect of metal and anti-browning agent complexes

Each metal and each anti-browning agent were pre-incubated separately to form metals-anti browning agent complexes for 30 minutes at 25 ºC. Then, the effects of complexes on the PPO enzyme activities were determined by kinetic methods using catechol as a substrate at pH 7.0. The remaining enzyme activities were calculated for all the metals and metal-anti-browning agent complexes.

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3. Results and Discussion

3.1 Purification of sorrel PPO

A sequential purification of sorrel PPO, starting from crude extract from sorrel leaves, precipitation with

(NH4)2SO4 from 10% to 80% salt saturation, dialysis, to gel filtration chromatography, gave purified sorrel PPO. Results for the purification of PPO were given in Table 1. Ammonium sulphate fractionation as the first step of sorrel PPO purification proved convenient and effective to remove large amounts of non-targeted proteins and brown pigments. When the purification steps were examined, there was 4.2 fold purification after ammonium sulfate precipitation. Further purification was followed by loading the dialyzed enzyme on to the Sephadex G-100 gel filtration column. After, the gel filtration chromatography, the most active fractions were collected and examined for characterization and purification. The purification procedure gave an overall purification of 14 fold, and the purified sorrel PPO had an overall activity yield of 12.7 %, with specific PPO activity of 8300 U/mg (Table 1).

Table 1: Purification profile of sorrel PPO

Puricification steps	Enzyme activity (U/g) plant	Yield (%)	Total protein (%) mg/g plant	Specific activity (U/mg) protein	Fold Purification
Crude extract	32550	100	54.8	594	0
Ammonium sulfate	16560	51.1	6.5	2548	4.2
Sephadex G-100	4150	12.7	0.5	8300	14

3.2 Substrate specificity

Km and Vmax values of sorrel PPO were calculated from the Lineweaver–Burk graphs and the results are shown in Table 2. All the substrates, namely catechol, 4-methyl catechol, pyrogallol, and caffeic acid, were oxidized significantly by the enzyme, displaying simple Michaelis–Menton kinetics. Linear regression analysis of v versus S determined Vmax and Km values for each substrate (Table 2). The highest Km values were shown by caffeic acid, 4-methyl catechol and catechol and the lowest by pyrogallol. In order to evaluate the substrate specificity, Vmax/Km ratio was taken It appears that the substrate-binding site of sorrel PPO has a high affinity for small o-diphenols, such as catechol and 4-methyl catechol and less affinity for the larger o-diphenols, caffeic acid, and triphenol-pyrogallol. This result was consistent with the previous report on plant PPOs [20].

3.3 Effect of pH and temperature

Optimal pH and temperature of the PPO were estimated by using four different substrates (Table 2). Optimum pH value for sorrel PPO was found to be 7.0 with catechol and 4-methyl catechol, 8.0 with pyrogallol and 5.5 with caffeic acid. The pH optimum for enzyme-catalyzed oxidation of catechol in phosphate buffer was found to occur at pH 7.0 (Fig. 1). The pH optimum for sorrel PPO is within the range (pH4.0–7.0) in which most PPOs display optimum activity [13, 16, 22]. The low activity observed at more acidic and basic pH values may be due to enzyme instability at these pH values (Table 2).

The activity of PPO was also measured at different temperatures at optimum pH (Table 2). The enzyme showed the highest activity at 30°C with the substrate catechol and 4-methyl catechol, at 35°C with pyrogallol and 25°C with caffeic acid. This value was similar to those of peppermint PPO [19] and medlar PPO [13]. But the temperature optimum values were different from Barbados cherry PPO [20].

Table 2: Optimum activity and substrate specificities of sorrel PPO

Substrates	Km (mM)	Vmax (µM/min)	Vmax/Km (min^1)	Optimum pH	Optimum Temperature (°C)
Catechol	5.2	80.5	15.5	7.0	30
4-Methyl Catechol	3.7	71	19.2	7.0	30
Pyrogallol	1.35	9.1	6.7	8.0	35
Caffeic Acid	7.1	13.1	1.8	5.5	25

3.4 Effect of various metal Ions and anti-browning agents

Effects of various metals and other chemical reagents on the enzyme activity were studied by using catechol as the substrate (Table 3). It is clear that glutathione (GSH) was found to be the most potent anti-browning agent followed by L-ascorbic acid, L-cysteine and EDTA at 10 mM concentration (Table 3). Among the anti-browning agents, L-Cysteine was reported to be a strong inhibitor of apple PPO [20] and ascorbic acid is effective inhibitor for different PPOs [2, 7, 10]. Since cysteine and ascorbic acid are naturally occurring substances and non-toxic, they may be useful for preventing the enzymic browning of sorrel. L-cysteine and reduced glutathione (GSH) which are reactive thiol compounds are also excellent inhibitors of browning of plants PPO [13, 14]. One of the effective anti-browning agent is EDTA which is a well-known chelating agent permitted for use in the food industry as a chemical preservative. EDTA showed minimum inhibition to sorrel PPO activity like cherry PPO [20].
The effect of Cu(II), Fe(III), Zn(II), Sn(II), Hg (II), Pb (II) as metals were determined on sorrel PPO enzyme activity at different concentrations (1-10 mM) (Table 3). The results showed that Cu(II) and Fe(II) metals increased the enzyme activity at 10 mM. Similar activation of

artichoke heads PPO activity by Cu(II) and Fe(II) were reported by Aydemir in the same concentrations [3]. However, Sn(II) had the maximum inhibitory effect on the enzyme activity at 10 mM. Although heavy metals are toxic for all plant and human health, Zn(II), Hg (II) and Pb (II) had no significant effect on the enzyme activities, whereas the same metals had heavily inhibitor effect on the other plant PPOs [15].

Table 3: The effects of metal and Anti-browning agents on sorrel PPO enzyme activity

Metals	**Remaining Activity (%) (1 mM)**	**(10 mM)**
$CuSO_4$	110	130
$FeCl_3$	109	106
Zn SO_4	102	100
$PbCl_2$	109	104
$SnCl_2$	35	0
$HgCl_2$	106	100
Anti-Browning Agents		
EDTA	93	88
L- Ascorbic Acid	60	5
Glutathione (GSH)	10	0
L-Cysteine	70	54

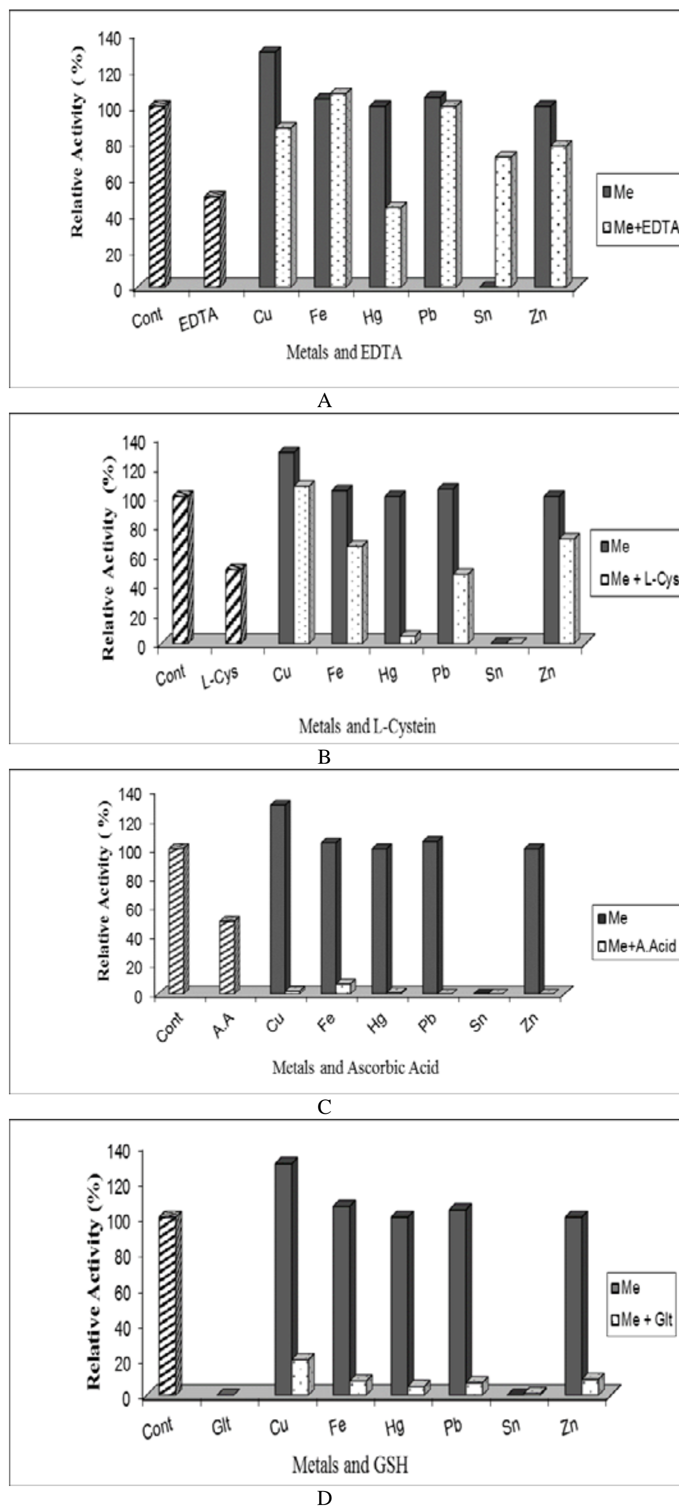

Fig. 1 Relative activity (%) profiles for sorrel PPO against metals and anti-browning agents. (A) Metals and Metal+ EDTA, (B) Metal and Metal+ L-Cystein, (C) Metal and Metal+ Ascorbic Acid, (D) Metal and Metal+ GSH.

3.4 Effects of metal and anti-browning agent complexes

The effect of metal and anti-browning agent complexes were also tested on sorrel PPO enzyme activity. The results are in Fig. 1. The results showed that, EDTA and metal complexes had no significant effect on the enzyme activity. L-ascorbic acid and metal complexes decreased the enzyme activities but L-ascorbic acid-Cu(II)-complex had no effect on the enzyme activity. L-Cys-Pb, L-Cys-Hg and L-Cys-Sn had inhibitory effect respectively but the other L-Cys-metal complexes had no effect on the enzyme activities. GSH–metal complexes had the best inhibitory effect on the enzyme activities except GSH-Fe complex. The results suggested that the metal-anti browning agent complexes may increase the inhibitory effect of anti-browning agents for the enzymatic browning in plants and reduce the effect of heavy metals on the enzyme activity.

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4. Conclusions

There were two purpose of this work, one was to purify and characterize polyphenol oxidase (PPO) from a new plant source which is Sorrel (Rumex acetosa). Second was to reduce the effect of heavy metals and increase the effect of anti-browning agent on this new plant PPO enzyme. The results of this work showed that the PPO enzyme was partially purified and characterized from Sorrel. The data suggested that the metal-anti browning agent complexes may reduce the effect of toxic heavy metals on the enzyme activity and increase the inhibitory effect of anti-browning agents for the enzymatic browning in plants. Browning in fruits and vegetables is recognized as a serious problem in the food industry. Further studies are warranted to understand the PPO inhibitor in relation to the browning reaction of plant during storage and processing.

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References

[1] Arslan O., Doğan S., Inhibition of Polyphenol Oxidase Obtained from Various Sources by 2,3-Diaminopropionic Acid, Journal of the Science of Food and Agriculture, 85, 2005, pp. 1499–1504.

[2] Arslan O., Erzengin M., Sinan S., Ozensoy O., Purification of Mulberry (Morus alba L.) Polyphenol Oxidase by Affinity Chromatography and İnvestigation of ıts Kinetic and Electrophoretic Properties, Food Chemistry, 88, 2004, pp. 479–484.

[3] Aydemir T., Partial purification and characterization of polyphenol oxidase from artichoke (Cynara scolymus L.) heads, Food Chemistry, 87, 2004, pp. 59–67.

[4] Baker A. J. M., McGrath S. P., Reeves R. D., Smith J. A. C., Metal Hyperaccumulator Plants: A Review of the Ecology and Physiology of a Biological Resource for Phytoremediation of Metal-Polluted Soils, Phytoremediation of Contaminated Soil and Water, Lewis Publishers, Boca Raton, 2000.

[5] Balsberg-Pahlsson A. M., Toxicity of Heavy Metals (Zn, Cu, Cd, Pb) to Vascular Plants, Water Air Soil Pollut., 47, 1987, pp. 287–319.

[6] Barman S.C., Sahu R.K., Bhargava S.K., Chatterjee C., Distribution of Heavy Metals in Wheat, Mustard,and Weed Grown in Fields İrrigated with İndustrial Effluents, Bull Environ. Contam. Toxicol., 64, 2000, pp. 489–496.

[7] Beaulieu M., Be'liveau M., D'Aprano G., Lacroix M., Dose Rate Effect of Irradiation on Phenolic Compounds, Polyphenol Oxidase, and Browning of Mushrooms (Agaricus bisporus), Journal of Agricultural and Food Chemistry, 47, 1999, pp. 2537–2543.

[8] Bradford M. A., A rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-dye Binding, Analytical Biochemistry, 72, 1976, pp. 248–254.

[9] Chandra R., Bhargava R.N., Yadav S., Mohan D., Accumulation and Distribution of Toxic Metals in Wheat (Triticum aestivum L.) and Indian Mustard (Brassica campestris L.) İrrigated with Distillery and Tannery Effluents, J. Hazard. Mater, 162, 2009, pp. 1514–1521.

[10] Chazarra S., Garcia-Carmona F., Cabanes J., Evidence for a Tetrameric form of Iceberg Lettuce (Lactuca sativa L.) Polyphenol Oxidase: Purification and Characterisation, Journal of Agricultural and Food Chemistry, 49, 2001, pp. 4870–4875.

[11] Dazy M., Masfaraud JF., Férard JF., Induction of Oxidative Stress Biomarkers Associated with Heavy Metal Stress in Fontinalis Antipyretica Hedw. Chemosphere, 75, 2009, pp. 297–302.

[12] Dincer B., Colak A., Aydin N., Kadioglu A., Guner S., Characterization of Polyphenol Oxidase from Medlar Fruits (Mespilus germanica L., Rosaceae), Food Chemistry, 77,2002, pp. 1–7.

[13] Dogru Y. Z. And Erat M., Demirkol A., Investigation of some kinetic properties of polyphenol oxidase from parsley (Petroselinum crispum, Apiaceae), Food Research International, 49, 2012, pp. 411–415.

[14] Friedman M., Food Browning and its Prevention: An Overview, J. Agric. Food Chem., 44, 1996, pp. 631-653.

[15] Gao Z. J., Liu J. B., Xiao X. G., Purification and Characterisation of Polyphenol Oxidase from Leaves of Cleome gynandra L. Purification and Characterisation of Polyphenol Oxidase from Leaves of Cleome gynandra L., Food Chemistry, 129, 2011, pp. 1012–1018.

[16] Gawlik-Dziki U., Szymanowska U., Baraniak B., Characterization of Polyphenol Oxidase from Broccoli (Brassica oleracea var. botrytis italica) Florets, Food Chem., 105, 2007, pp. 1047–1053.

[17] Gawlik-Dziki U., Złotek Z., S´wieca M., Characterisation of Polyphenol Oxidase from Butter Lettuce (Lactuca sativa var. capitata L.), Food Chemistry, 107, 2008, pp. 129–135.

[18] Gupta S., Satpati S., Nayek S., Gorai D., Effect of Wastewater İrrigation on Vegetables in Relation of Heavy Metals and Biochemical Changes, Environ Monit Assess, 165, 2010, pp. 169–177.

[19] Kavrayan D., Aydemir T., Partial Purification and Characterization of Polyphenol Oxidase from Peppermint (Mentha piperita), Food Chemistry, 74, 2001, pp. 147–154.

[20] Kumar V. B. A., Kishor Mohan T.C., Murugan K. , Purification and Kinetic Characterization of Polyphenol Oxidase from Barbados Cherry (Malpighia glabra L.), Food Chemistry, 110, 2008, pp. 328–333.

[21] Lineweaver H., Burk D., The Determination of Enzyme Dissociation Constants, Journal of the American Chemical Society, 56, 61934, pp. 58–666.

[22] Mdluli K. M., Partial purification and characterisation of polyphenol oxidase and peroxidase from marula fruit (Sclerocarya birrea subsp. Caffra), Food Chemistry, 92, 2005, pp. 311–323.

ISSN 2348 – 7968

[23] Ozoglu H., Bayindirli A., Inhibition of Enzymic Browning in Cloudy Apple Juice with Selected Antibrowning Agents, Food Control, 13(4),2002, pp. 213-221.

[24] Pongsakul N., Leelasart B., Rakariyatham N., Effect of L - cysteine, Potassium Metabisulfite, Ascorbic Acid and Citric Acid on Inhibition of Enzymatic Browning in Longan, Chiang Mai J. Sci., 33(1), 2006, pp. 137-141.

[25] Richard-Forget F. C., Goupy P. M., Nicolas J. J., Cysteine as an Inhibitor of Enzymatic Browning 2. Kinetic Studies, Journal of Agricultural and Food Chemistry, 40(11), 1992, pp. 2108–2113.

[26] Sanjeev K.D., Sarad K.M., Purification and Biochemıcal Characterızatıon of Ionically Unbound Polyphenol Oxidase from Musa paradisiaca Leaf, Preparative Biochemistry & Biotechnology, 41, 2011, pp. 187–200.

[27] Wong T.C., Luh B.S., Whitaker J.R., Isolation and Characterization of Polyphenol Oxidase Isozymes of Clingstone Peach, Plant Physiol., 48, 1971, pp. 19-23.