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Characterization of Polyphenol Oxidase (PPO) from Blackberry Thorny Wild Rubus Fruticosus and its Inhibition using Natural Extracts

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Abstract

The Polyphenol oxidase (PPO) leads to the enzymatic browning of fruits and vegetables and needs to be prevented in food browning and quality. The present study aimed to investigate the use of natural extracts and chemical inhibitors to prevent browning of the PPO of blackberries. Purification, characterization, and kinetics of PPO of blackberry parameters for five substrates, namely, pyrocatechol, 4-methylcatechol, Pyrogallol, Gallic acid, and tyrosine, were described. The results showed that the DEAE-Sepharose and Superdex G-200 purification methods, which achieved electrophoretic purity, increased PPO activity by 556 fold. Purification with Sephadex GE-200 and SDS-PAGE reveals two PPO isoenzymes with an apparent molecular weight of 22 kD and 70 kD. The optimum pH and temperature values indicated were 6.6 and 25°C, respectively. The PPO showed variable affinity towards o-dihydroxy phenolic substrates with catecholase activity but without any activity observed with phenol, a monohydroxy substrate, and it was very effective towards pyrocatechol, pyrogallol, and 4-methyl catechol. The results revealed that inhibition of the PPO using both synthetic inhibitors and natural extracts was the most effective method. Quercetin and ascorbic acid showed higher inhibition with the lowest Ki values. Fresh onion (Allium cepa) and wild Arbutus unedo extract were able to inhibit the blackberry PPO activity up to 50% and 60%,

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respectively. Therefore, the use of natural extracts from Arbutus unedo L as anti-browning agents on the blackberry PPO may provide new insight to overcome the enzymatic browning.

Introduction

The thorny wild blackberry (*Rubus fruticosus*) has a short post-harvest lifespan, therefore, it loses its quality due to its extremely fragile structure and breathing high rate. It cannot be kept in the air, even for a short period of two or three days.[1] The consumption of this fruit is still considered very low[2,3] despite being abundant in nutritious components, such as, vitamin C, bioactive chemicals, and functional molecules with biological activities. Blackberry is rich in antioxidants that play an important role in our health as it contains polyphenol, which is one of the most important antioxidants. This phenolic is highly oxidized by polyphenol oxidase (PPO) (monophenol, dihydroxy phenylalanine) (oxygen oxidoreductase; E.C. 1.14.18.1.), to its corresponding quinone. The quinone produced is a highly reactive substance that normally reacts further with other quinones, amino acids or proteins to produce coloured compounds responsible for food quality deterioration.[4,5] The organoleptic quality may change adversely due to the enzymatic oxidation of phenolic compounds. PPO has been studied in a wide variety of fruits. In the presence of oxygen, PPO catalyzes two types of reactions: hydroxylation of monophenols to o-diphenols and oxidation of o-diphenols to o-quinones, representing monophenolase (cresolase) and diphenolase (catecholase) activity, respectively.[6,7,8] PPO is a major contributor to enzymatic browning or color degradation in fruits and vegetables during postharvest processing operations due to these reactions.[9,10]

The PPO is vital for wound healing, pathogen defense, and many cellular processes, such as oxygen level control in chloroplasts.[6,11] As reported by Yoruk,[5] browning reaction affected the sensory quality with diminution marketing of fruit by lowering nutritional quality. Quieroz *et al.*[12] also suggested that the enzymatic browning leads not only to color alteration and antioxidant degradation but also organoleptic and nutritional losses because of the quinones' condensation with other compounds such as amino acids, proteins, phenols, and sugar. In food industry, the inhibition of PPO enzyme and prevention of browning is a big challenge for fruit and vegetable.[6] Most of the anti-browning systems, both physical as well as chemical methods, have been used to inhibit PPO activity and thereby prevent browning.[13] Thermal technologies are the most commonly used physical-based preservation systems for processing juice.[14]

However, one of the study reported a negative impact of heat processing on the organoleptic and nutritional qualities of final products.[15] Chemical-based methods to control enzymatic browning in postharvest are related to the application of PPO inhibitors such as sulphur, cysteine ascorbic acid, citric acid cyclodextrins, and oxalic acid.[16] However, those synthetic additives have a potential hazard on human health.[17,18]

Browning effect can occur within a short time leading to deterioration of nutritional value and appearance of Blackberry.[19] The characterization studies of PPO of the blackberry were reported in a previous study[19] without inhibition and purification. These data are very important during the processing of the wild blackberry, rich in antioxidants and anthocyanin. Natural extract has been studied for PPO inhibitors for potato,[20] pear[21] and banana. [22] However, the study of anti-browning agents' effects on blackberry needs to be established. In this research, we try to reveal more information on the PPO purification, chemical and natural anti-browning agents in blackberry, which is yet to be established. There is no scientific information on how the natural and chemical inhibitors may influence the PPO activity of blackberries. Thus, the inhibitory effects of chemical and natural inhibitors (onion and *Arbutus unedo* extracts) with their kinetics on blackberry PPO were thus investigated in this work.

The objective was to report all the properties that have never been mentioned before, regarding the purification of PPO, effects of pH and temperature on PPO activity and substrate specificity, performed with pyrocatechol, 4-methylcatechol, pyrogallol, gallic

acid, and tyrosine. Inhibition study of the blackberry thorny wild PPO using natural and chemical inhibitors was investigated to show the inhibitory effects on our enzyme. These data may be used in testing effective methods to inhibit discoloration naturally without adversely affecting our health, while avoiding the breakdown of anthocyanins in blackberry juice and other processed products.

Materials and Methods

Plant Material and Natural Inhibitors

Blackberry thorny wild (*Rubus fruticosus*) originated from local coast (Slookia (35°47'16.3"N 5°54'31.3"W) Tangier, Morocco, was used in this study. Red onion (*Allium cepa*) and Wild *Arbutus unedo* L, were local products also. All chemicals used in this study were analytical grade.

PPO Extraction

The extraction procedure of PPO from the blackberry thorny wild was performed according to Yagar.[23] 200 g of the blackberry thorny wild was homogenised with 200 mL of 0.1 M phosphate buffer, pH 6.8, containing 10 mM ascorbic acid, 0.1% polyvinylpyrrolidone and 0.5% Triton X-100 using a industrial blender (Waring Laboratory ScienceTM LB20EG, French) at 5,000 rpm for 3 min. The homogenate was filtered through filter paper and the filtrate was centrifuged at 160000 xg for 30 min at 4 °C by an ultracentrifuge (Centrifuge Universal 320R, Hettich Zentrifugen, Tuttlingen, Germany). The supernatant was used as the crude enzyme extract. Then it was brought to 30-90% $(NH_4)_2SO_4$ saturation, pH 6.8. The fractions were solubilized in 30 mL of phosphate buffer (pH 6.8, 0.1 M) and dialyzed, using a dialysis cassette of 3,5 K cut off the membrane in 2000 mL of phosphate buffer (pH 6.8, 0.1M) at 4 °C, with three buffer changes at 6 and 8 hour intervals for salt removal.

Superdex Gel Filtration Chromatography

The dialysed enzyme extract was chromatographed on a Sephadex 200 GE column according to Mishra.[10] The protein was loaded onto a column and the elution was carried out in Tris-HCL buffer (pH 8.0, 2 mM) with 150 mM of NaCl. The flow rate was maintained at 1 mL/min and the detection was carried out using an UV detector set at 280 nm. The PPO activity and protein estimation were also performed for different fractions collected at 30 s intervals.

DEAE Anion-Exchange Chromatography

The final step of purification was conducted using DEAE. Sepharose column material (40 ml) was prepared according to the method of Mishra *et al.*,[10] with some modifications. Initially, washing was done with distilled water (150 mL) and then with Tris-HCl buffer (pH 8.10 mM). The fractions containing activity were pooled and subjected to anion exchange chromatography and mixed with DEAE column material, kept at slow stirring for 30 min at 4 °C. This slurry was then washed twice with 250 mL of Tris-HCL buffer (pH 8.0, 2 mM) and loaded onto the column. The elution was carried out using an increasing gradient of NaCl from 0 to 0.5 M in 150 mL of tris-HCl buffer (pH 8.0, 2 mM). The flow rate was 1 mL/min and fractions of 1 mL were collected. PPO activity was determined for each fraction.

Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis under reducing and denaturing conditions (SDS–PAGE) (10%) was carried out in the same way as described by Davis and Laemmli.[24] The protein samples were prepared by mixing 2× (double strength) gel loading buffer (0.1M Tris-C1, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and β-mercaptoethanol (0.2 M), mixed in a proportion of 1:1, boiled for 5 min, centrifuged (10,000g) for 2 min, and then loaded onto the gel, along with the molecular weight marker mix. We adjusted the electrophoresis to 25–40 mA with a duration of 4 h at room temperature. The quantity of the proteins deposited in the well was 20 µg. Following electrophoresis, the gel was fixed in acidified methanol water (40% methanol and 10% acetic acid) for 1 hour before being colored in 0.1% Coomassie brilliant for 3 hours on a rocker (Neolab, Mumbai, India). Later, the gel was de-stained with the same acidified methanol–water until the protein bands became distinguishable and prominent. The substrate staining of the gel was performed with pyrocatechol (0.05 M) substrate in phosphate buffer (pH 6.6, 0.2 mM) for up to 1 h, until the bands became prominent.

Assay of Protein and Polyphenol Oxidase (PPO) Activity

The enzyme activity was assayed according to the method of Gonzalez[19] with some modification. The reaction mixture consisted of 2.5 mL of 0.05 M

pyrocatechol in 0.2 M phosphate buffer (pH 6.5) and 0.5 mL of PPO extract. The increase in absorbance at 420 nm was monitored at 30 s intervals for 3 min using a spectrophotometer (Model UV-3100), and the average change in absorbance per min was calculated. The reaction velocity (V) was calculated from the linear part of the plot of absorbance (A) against time (t). The unit of PPO activity was defined as the change in the absorbance of 0.001 min (DA 420 nm/min) due to the oxidation of the substrate. The PPO activity was expressed as U/g of blackberry weight. The specific activity was determined by expressing PPO activity/mg protein. The protein content of blackberry extract was determined by the Bradford method,[25] using bovine serum albumin (BSA, Sigma Chemical, St. Louis, USA) as a standard. The total enzyme molecule concentration was determined by dividing the estimated total protein by the molecular weight of PPO. The catalytic efficiency (vmax/km) was calculated by dividing Vmax by Km.

Determination of Substrate Specificity

In order to determine the Michaelis constant (K_m) and maximum velocity (V_m), PPO activities were measured using pyrocatechol, 4-methyl-catechol, pyrogallol, acid gallic and tyrosine. All compounds were prepared in a 0.2 M sodium phosphate buffer, pH 6.5. The changes in absorbance for substrates 4-methycatechol and pyrocatechol were monitored at 420 nm. The absorbances for pyrogallol, tyrosine, and gallic acid were monitored at 334, 472, and 350 nm, respectively. The absorbance was recorded at 30 s intervals for 3 min, and the average change in absorbance per min was calculated and termed as activity. The K_m and V_{max} values were calculated by plotting one/activity (1/V) versus one/substrate (1/[S]) using Lineweaver and Burk's direct linear plot method.[26] The relative activities of these substrates were expressed as percentages with respect to those of the pyrocatechol substrate (considered 100%).

pH and Temperature Profile of PPO

The optimum pH of the partially purified PPO was investigated by measuring its activity at room temperature (25 °C) at pH ranging from 5 to 8. The tests were carried out using 0.2 M sodium phosphate buffer. The effect of temperature on PPO activity was carried out by assaying the reaction mixture at various temperatures ranging from 10 °C, 20 °C, 30 °C, 40 °C, 50 °C, 60 °C, and 70 °C.

Inhibition Studies

Effect of Chemical Anti-Browning Agents

The blackberry PPO was incubated in the presence of the following chemical compounds, reduced glutathione, ascorbic acid, L-cysteine, quercetin, sodium sulfate, citric acid, chloride acid, and sodium fluoride, to at 0.1, 1.0, and 10 mM.

Additionally, 50 mM pyrocathechol in 0.2 M phosphate buffer (pH 6.5) was added. The reaction mixture contained 2 mL of substrate, 0.5 mL of inhibitor solution, and 0.5 mL of enzyme. The percent inhibition was calculated using the following equation.

$$\text{Inhibition (\%)} = (1-(Ai/A0)) \times 100$$

Where A0 is the initial PPO activity (i, e. Without inhibitor), and Ai is the PPO activity with inhibitor.

The inhibitory concentration that reduced the enzyme activity by 50 % (IC50) was determined for the inhibitors with a lower percentage of PPO residual activity at a concentration of 0.1 mM using three different concentrations of inhibitor. Finally, PPO activities were measured at constant inhibitor concentrations (IC50) using five different substrate concentrations. The Lineweaver-Burk graphs were used to determine the type of inhibition.

Effect of Natural Anti-Browning Agents

Two natural sources, red onions and arboursier (*Arbutus unedo*), were prepared according to the Wong *et al.*[27] method. Thus, 100 g of each natural source was homogenized separately with 200 mL of 0.1 M phosphate buffer, pH 7.5, for 2 min using a waving blender (Waring Laboratory ScienceTM LB20EG, French) at maximum speed. The homogenized thus prepared from onion and arboursier mixture was centrifuged for 20 minutes at 4°C at 4,000 rpm. After centrifugation, the supernatant was filtered using filter paper, and the filtrate was collected.

The PPO activity was determined with and without the presence of natural anti-browning agents extracted from local fresh wild arboursier diluted

fruit (6%) and fresh red onion. The reaction mixture contained 0.5 mL of the enzyme, 0.5 mL of 0.1 M phosphate buffer, pH 7.5, and 0.5 mL of fresh extract as an inhibitor, incubated for 5 min at 20°C. Then, 1.5 ml of pyrocatechol (0.05 M to 0.01 M) was added to the reaction mixture. The control contained 0.5 mL of PPO and 2 mL of substrate solution in 0.5 mL of 0.1 M phosphate buffer. The total volume of the assay was 3 ml. The linear graph of the absorbance versus time (in seconds) curve was plotted to determine the initial rate of PPO activity. The PPO activity obtained was used to calculate the percentage inhibition as compared to the initial PPO (Ao) activity without inhibitor[27]

Statistical Analysis

All analytical values represent the means and standard deviations were calculated of triplicate measurements. One way ANOVA (Significance threshold: $P \leq 0.05$) was performed to ascertain the significance of the means. Pairwise comparison were performed based on Tukey HSD test. Statistical analyzes were performed using the statistical software SAS (Statistical Analysis System. Version 9.1. 2002).

Results and Discussion

Purification of PPO

Blackberry PPO was sequentially purified by ammonium sulfate with precipitation from 30% to 90% salt saturation, dialysis, chromatography to gel filtration, and anion exchange chromatography. The results are summarised in (Table 1). The purification resulted in about a 556 fold increase in enzyme activity, and the purified PPO had an overall activity yield of 3,13%, which is similar to the activity yield reported for PPO from banana, [28] but with a different specific PPO activity of 315757,6 U/mg. The specific activity in crude extract was 568.2 U/mg protein, which is similar to the specific activity reported for PPO from Barbados cherry[29] and higher than that reported for PPO from Loquat.[30]

Table 1: Purification of PPO from Blackberry

Purification step	Volume (mL)	Protein mg/mL	Proteintotal (mg)	Activity U/ml	Activity total (U)	Specific activity (U/mg)	Purification (fold)	Yield %
Crudeextract	200	0,440	88	250 ±1,44	50000	568,2	1	100%
(NH4)2SO4	70	0,960	67,2	348 ± 1,20	24360	362,5	0,64	48%
Dialysate	3	0, 300	0,9	460 ± 1,44	1380	1533,3	2,7	27,6%
(Sephadex 200 GE)	2,5	0,055	0,138	950 ± 2,88	2375	17272,7	30,4	4,8%
Ion exchange	1,5	0,003	0,005	1042 ±3,55	1563	315757,6	556	3,13%

During steps of purification, the PPO activity/mL increased and, as expected, the protein (PPO) content decreased. This resulted in an increase of specific activity of up to 556 fold after gel DEAE chromatography (Table 1). However, at each step of purification, a loss in total activity was noticed because only peak fractions displaying PPO activity were purposely collected. This significantly increased the purification-fold; however, the total yield was significantly reduced to about 3,13% (Table 1). PPO blackberry extract was dialyzed and chromatographed on Sephadex GE-200 (Figure 1 a). Fractions comprising two peaks are shown in (Figure 1a). The latter shows high activity of PPO towards pyrocatechol. The filtration gel separated our PPO according to their sizes, that is to say, into two different molecular weights The first eluent is the first peak, which corresponds to the large molecule of the PPO, and the second eluent corresponds to the smallest molecule of our PPO. Then these active fractions were pooled and subjected to anion-exchange chromatography (Figure 1b.) with Tris-HCL (2 mM, pH = 8) buffer. The bound enzyme was eluted with a linear gradient of NaCl (0–0.5 M). To test the activity, high protein fractions were collected.These fractions (peaks 20–26) corresponded to NaCl concentrations between 0.25–0.35M. The protein content of the elution was determined on a single peak (the second) using Coomassie Brilliant Blue and measured at 595 nm. Enzyme activity was determined using pyrocatechol and measured at 420 nm (Figure. 1 a).

Active fractions, with high enzyme activities, were collected and pooled. The resulting pooled fractions were concentrated by ammonium sulfate precipitation. Finally, the precipitated protein was re-dissolved in a small volume of 0.1 M phosphate buffer (pH 6.8) and dialyzed with the same buffer to use it in the SDS gel.

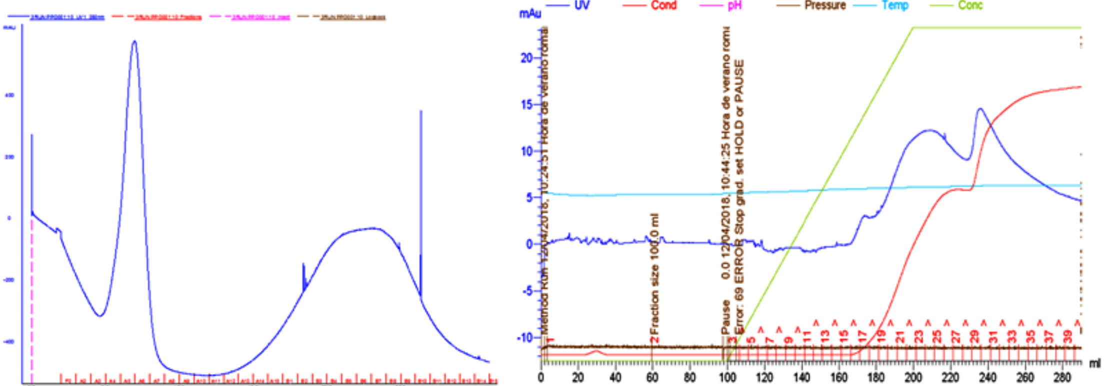


Fig. 1a: (a) Elution time (min) Gel filtration chromatography, Fig. 1b: Elution fraction no. DEAE ion exchange chromatography

PPO Molecular Weight

We have presented different purification steps on the SDS gel, and the amount of protein was minimal, which explains the appearance of thin strips. The crude extract showed us several bands which may be the bands of peroxidase and polyphenoloxidase, but during the following steps of purification of the PPO, two final bands clearly appear, one at 22 kD and the other at 70 kD at the level of the sephadex filtration gel, which proves the appearance of the two active peaks with different weights (Figure 2).

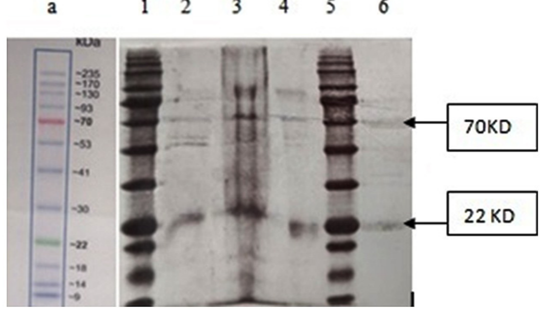


Fig. 2: SDS–PAGE of different steps of PPO purification. (1,5, a) molecular weight markers, (2) crude extract, (3) (NH4)2SO4 precipitation 90%, (4) dialyzed enzyme, and (6) purified sephadex GE200

During the purification steps, the concentration of the enzyme was significantly reduced by DEAE, which proves the absence of the gel band. These results are similar to that of Barbados cherry,[29] which also reveal two dominant bands but with molecular masses of 52 and38 kDa. Thus, this enzyme differs from PPO isolated from pear.[31] and Chinese cabbage,[32] and tomato PPO,[33] which were reported to be monomers.

PPO Substrate Specificity

Five substrates, namely pyrocatechol, 4-methyl-catechol, pyrogallol, gallic acid, and tyrosine, were tested and the PPO showed no activity using mono-hydroxyl phenol as substrates, low activity using tri-hydroxyl phenol, but displayed much greater activity with the o-dihydroxy phenol substrate (Table 2). Among the substrates tested, pyrocatechol had the maximum activity. The average enzymatic activity was observed even with the 4-methyl-catechol diphenolic substrate. On the other hand, the absence of the –CH3 group increased activity by 50%, as has been observed for pyrocatechol and is related to the role of the CH3 group and its location in the catechol structure, decreasing its specificity. These results are not similar to Bibhuti *et al*,[34] as they found that the CH3 group increased its specificity. Trihydroxyphenol substrates such as pyrogallol and

gallic acid exhibited low activity of the blackberry PPO. The monophenolic substrate, such as L-tyrosine, showed no activity against the blackberry PPO. These findings are similar to those reported for Yali pear peppermint, grapes Koshu PPO,[35] and lonicera japonica thunb.[36] These results revealed that the blackberry PPO was diphenolase, and there was no monophenolase or triphenolase activity.

Table 2:Substrate specificity of native PPO purified from Blackberry. ND: Relative Activity to pyrocatechol not detected.

Substrates	chemical structure of the substrates	Relative Activity to pyrocatechol (%)
Pyrocatechol		100±0.00[a]
4-Methylcatechol		52± 1.63[b]
Pyrogallol		30± 2.45[c]
Gallic acid		ND
Tyrosine		ND

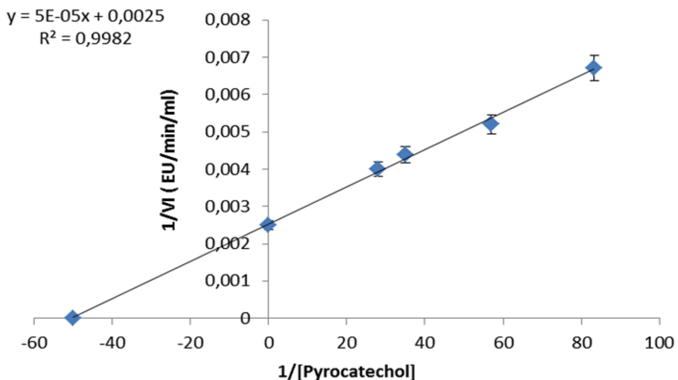


Fig. 3: Effect of substrate concentration (pyrocatechol) on blackberry PPO activity (Lineweaver-Burk plot)

Kinetic Characteristics of PPO

The Km and Vmax values were determined for substrates having higher activity (30%), using Lineweaver–Burk plots. Figures 3 to 5 show the Lineweaver-Burk curves of the mature PPO in the presence of the different substrates successively, pyrocatechol, 4-methylcatechol, and pyrogallol.

The best substrate for each enzyme depends on two factors: strong substrate binding or high affinity and high catalytic efficiency for a fixed enzyme concentration. The V_{max}/K_m ratio, referred to as "catalytic power", can identify the most effective substrate.

The lowest Km was observed for pyrocatechol (0.0199 M). The 4-methyl catechol and pyrogallol showed higher Km values compared to pyrocatechol. The catalytic efficiency (V_{max}/K_m) was also found to be maximal for pyrocatechol and less for pyrogallol (Table 3). The values of Km and Vmax show that pyrocatechol is the best substrate for PPO in wild blackberry. These results are similar to several studies of lonicera japonica thunb (36), blackberry (19), and blueberry (37).

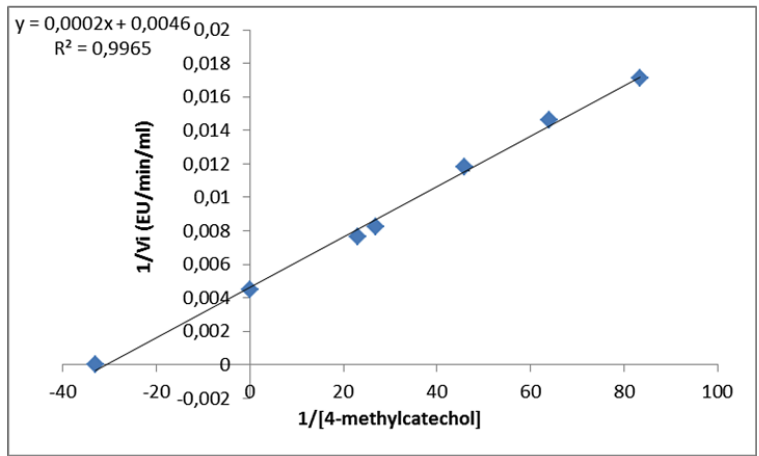


Fig. 4: Lineweaver-Burk plot of the Effect of substrate concentration 4-methylcatechol on blackberry PPO activity

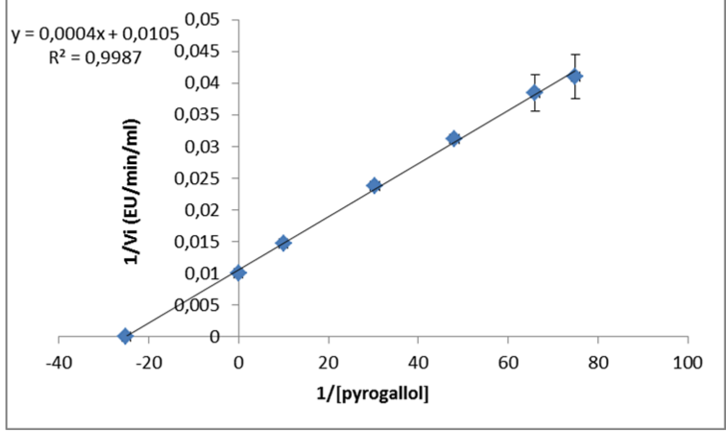


Fig. 5: Lineweaver-Burk plot of the Effect of substrate concentration pyrogallol on blackberry PPO activity.

Table 3: The kinetic characteristics of purified Blackberry PPO

Substrates	K_m (M)	V_{max}(EU/min/mL)	V_{max}/K_m (EU/min. mL^{-1}. M^{-1})
Pyrocatechol	0.0199± 0.00^b	500± 20^a	25010± 17^a
Pyrogallol	0.039± 0.00^a	99± 13^c	2530± 27^c
4-Methyl catechol	0.025± 0.00^b	250± 20^b	10014± 25
Tyrosine	ND	ND	ND
GallicAcid	ND	ND	ND

Km :Michaelis–Menten constant, V_{max} : maximum reaction velocity, V_{max}/K_m : catalytic efficiency.
The values are expressed asmeans ± SD of three parallel experiments. The values in column with different superscripts are significantly different (P ≤ 0.05). ND: Not detected.

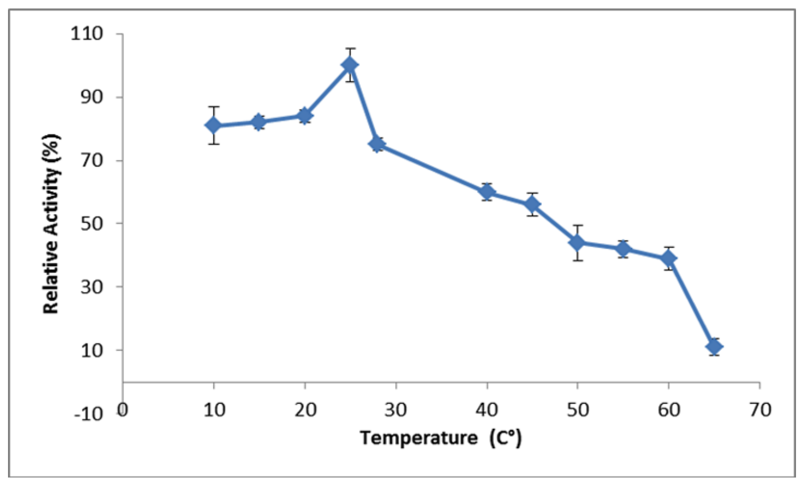


Fig. 6: Effect of temperature on the activity of Blackberry PPO.

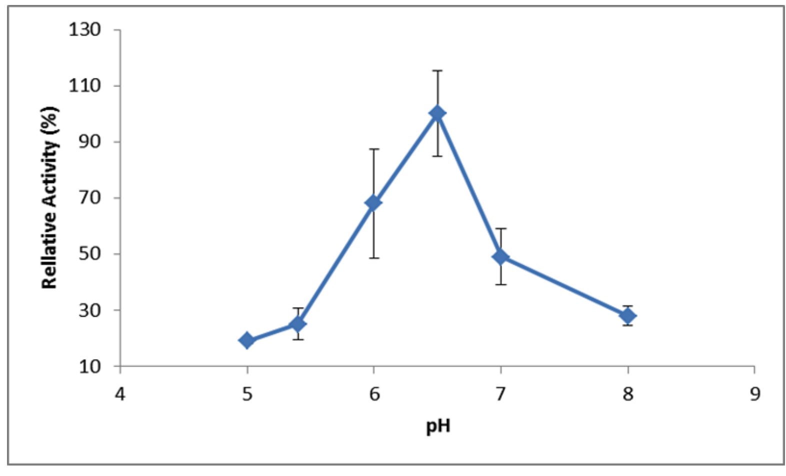


Fig. 7: Effect of pH on the activity of Blackberry PPO

Effect of Temperature on the PPO Activity

The blackberry PPO was active over a wide temperature range (10–70 °C), with the maximum activity being noted at 25 °C (Figure 6). Even at temperatures as high as 60 °C, the enzyme maintained 39% of its maximum activity; however, above this point, activity dropped dramatically to a minimum of 10% at 65 °C. The increase in the PPO activity as a function of temperature could be due to the fact that the increase in temperature enhances the kinetic energy, which leads to an acceleration of the reaction.[38] However, at temperatures higher than 65 °C, we observed a reduction in the PPO activity, probably due to thermal denaturation of the enzyme.

Effect of pH

Blackberry PPO presented (Figure 7) maximum activity in the range of pH 6–7. Although the activity decreases below and above 6.6, the enzyme was still active at pH 5.4 with a remaining activity of 60 ∆OD/min/g. At pH 8.0, the remaining activity of the enzyme was 68 ∆OD/min/g. Several authors reported that the optimal pH for PPO activity varied from about 4.0 to 7.0 depending on the extraction methods, substrates, and localization of the enzyme in the cell.[39]

Inhibitor Studies

Effect of Chemical Inhibitors on Enzyme Activity

The effect of various chemical antibrowning agents with different concentrations on the blackberry PPO was tested with pyrocatechol substrate (Table 4). The percentage inhibition increased with the increasing inhibitor concentrations. Ascorbic acid, reduced glutathione, and L-cysteine all strongly inhibited the enzyme, with PPO activity of 100%, 100%, and 93.33%, respectively, at a concentration of 10 mM, while quercitin could inhibit the enzyme at a low concentration of 0.1 mM with PPO activity of 89%. Furthermore, ascorbic acid, reduced glutathione, and quercetin have the lowest IC50 and Ki values among the other anti-browning agents, which were 0.18 mM, 0.2 mM, and 0.19 mM, respectively, except for L-cysteine, with 1.25 mM Ki. This further proves that L-cysteine, reduced glutathione, quercetin, and ascorbic acid have greater efficiency in inhibiting the blackberry PPO. However, less powerful are NaF, chloride acid, citric acid, and NaSO4, which exhibited the lowest percentage of inhibition. Lineweaver-Burk plots of the blackberry PPO in the presence of ascorbic acid, citric acid, L-cysteine, acid chlorhydric, quercetin, reduced glutathione, NaF, and NaSO4 respectively are represented in (Figure 8). Furthermore, it was found that the inhibitors exhibit different types of inhibition (Figure 8).

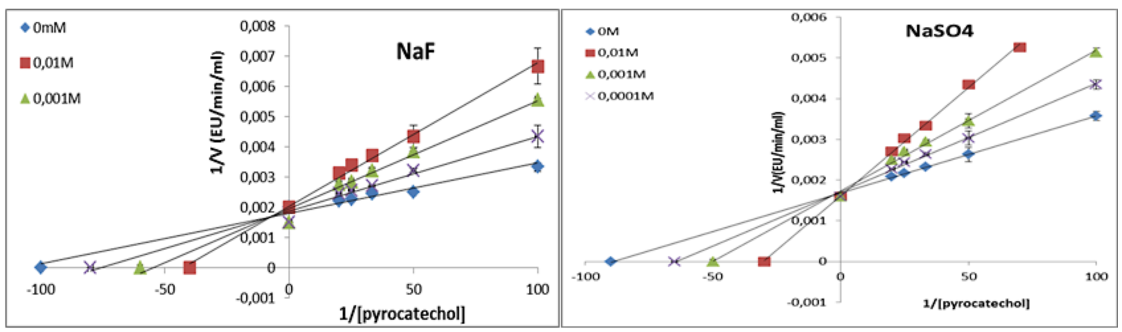
Ascorbic acid reduces quinones to hydroquinones without direct PPO inhibition. It can prevent enzymatic browning only as long as it is present in the reduced form. This prevents the formation of key intermediates and inhibits the activity of the oxidase[40] Ascorbic acid is the best competitive inhibitor, present at 0.1 mM with a 100% inhibitory effect on the PPO of the wild blackberry. This result is similar to that of blueberry, with a 99.7% inhibition effect at 2 mM.[37]

Glutathione does not appear to affect the enzyme directly, and oxygen uptake may be stimulated or inhibited depending on the particular phenol being oxidized.[41] Glutathione is a potent competitive inhibitor of the wild blackberry PPO. This result is identical to that of Parsley PPO.[42] In contrast to our results, some authors reported that the inhibition type of glutathione and ascorbic acid was a mixed-type inhibition for lettuce PPO.[41]

L-cysteine can readily bind to quinones to create complexes that prevent secondary oxidation and polymerization processes, which consume the substrate. It can also act as a reducing agent.[43] L-Cysteine is the best non-competitive inhibitor present at 0.1 mM, with a 93.3% inhibitory effect on the blackberry PPO. This result is similar to that of blueberry, with a 99.4% inhibitory effect at 2 mM,[37] and that of PPO parsley.[42] Quercetin was a potent inhibitor even at a low concentration of 0.1 mM, which exhibited an 89% inhibitory effect on pyrocatechol substrate with competitive inhibition. This result is identical to that of the cape gooseberry PPO.[44] with 71.7% but with a 4-methylcatechol substrate difference and uncompetitive inhibition. Sodium fluoride is a weak inhibitor of the wild blackberry PPO but is shown to be the best inhibitor of palm kernel PPO.[45] Citric acid and chloride acid are weak inhibitors of the blackberry. These results are different to those of the PPO for mamey (Pouteriasapota).[46] The enzyme kinetics indicated the presence of two isoenzymes.

Table 4: Effect of inhibitors on the PPO activity and type of inhibition

Anti-Browning agents	[I] (mM)	IC50 (mM)	Ki (mM)	Inhibition%	Type of inhibition
Ascorbic acid	10	0.4±0.02[d]	0.18± 0.00[e]	100 ± 0.00[a]	Competitive
	1			78±0.02[d]	
	0,1			29±0.02[fg]	
Reduced glutathione	10	0.2±0.00[d]	0.2±0.00[e]	100± 0.00[a]	non competitive
	1			30±0.03[fg]	
	0,1			8±0.02[jk]	
Citric acid	10	1.2± 0.01[c]	0.6±0.00[d]	29±0.05[fg]	non competitive
	1			24±0.04[h]	
	0,1			17±0.03[i]	
L-Cysteine	10	1.25±0.04[c]	1.25±0.06[b]	93.3 ± 0.00[a]	non competitive mixte
	1			52±0.02[e]	
	0,1			27±0.02[hg]	
Chloride acid	10	5±0.40[a]	2.5±0.20[a]	30±0.02[fg]	Competitive
	1			10±0.02[j]	
	0,1			5±0.00[k]	
Quercetin	10	0.38±0.02[d]	0.19±0.01[e]	ND	Competitive
	1			ND	
	0,1			89±0.01[c]	
NaSo4	10	2.6±0.20[b]	1.3±0.10[b]	9±0.00[j]	Competitive
	1			8.7±0.01[j]	
	0,1			8.26±0.00[jk]	
NaF	10	1.2± 0.0[6]	1±0.006[c]	32±0.02[f]	Mixte
	1			11±0.04[j]	
	0,1			7.47±0.01[jk]	



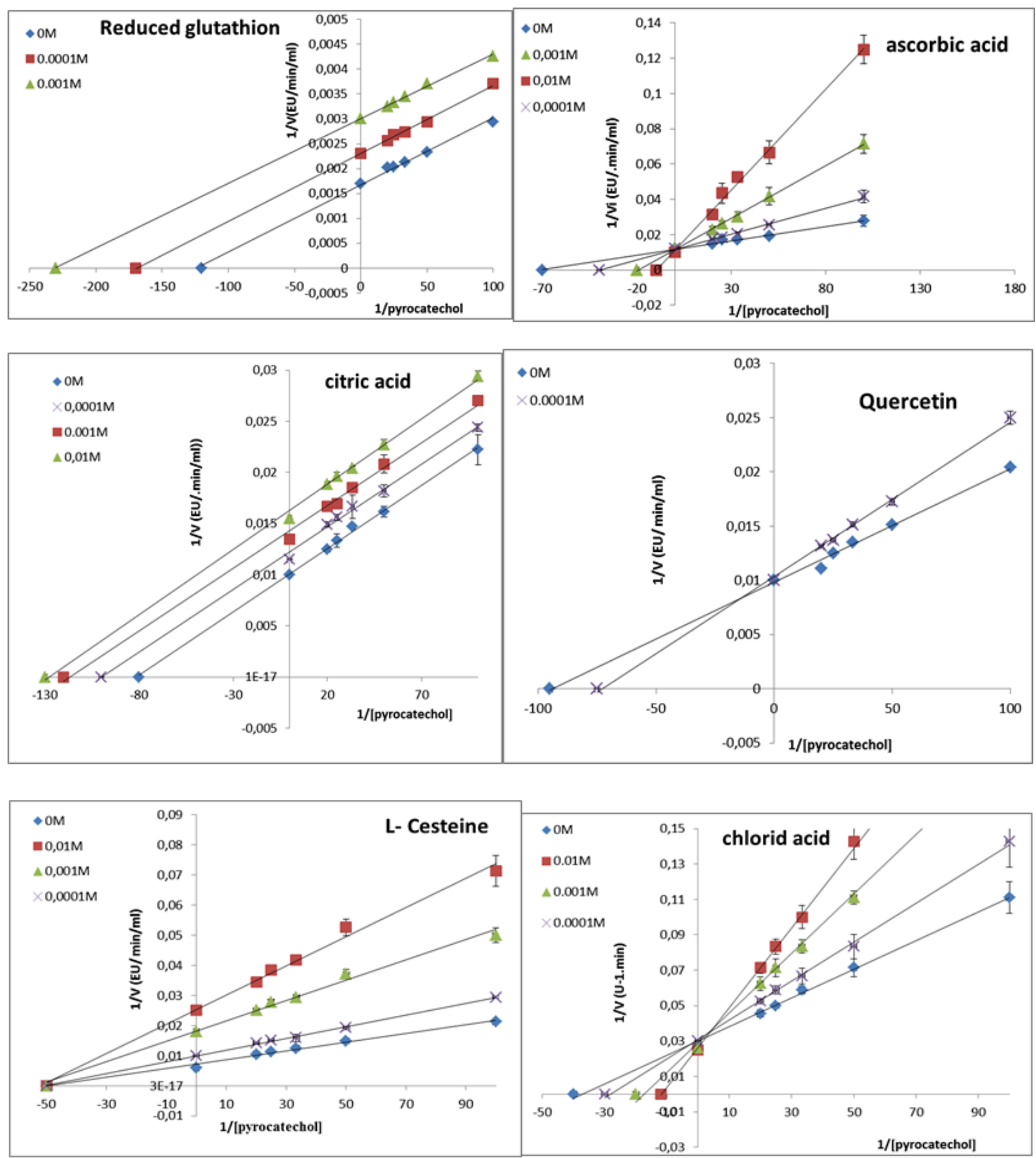


Fig. 8: Lineweaver–Burk graph with eight diferent substrate (pyrocatechol) concentrations and four diferent inhibitors concentrations for determination of Ki

Table 5: Effect of inhibitors naturals on the PPO activity and type of inhibition.

Natural anti-Browning agents	Inhibition %	V_{max} (EU/min/mL)	K_m (M)	Type of inhibition
Onion fresh	50%±1[b]	167±0.6[a]	0, 33±0.002[a]	Competitive
Arbutus 6% fresh	60%±2[a]	167±1[a]	0,17±0.002[b]	Competitive

Effect of Natural Extract Fresh Inhibitors on Enzyme Activity.

The results show that the antibrowning effect of the diluted by *A. Unedo* fruit fresh 6% is greater than that of the onion fresh. The values in percentages of the inhibition effect show that the arbutus has a value of 60% and that the fresh onion has a value of 50%. They have the same values of Vmax with different Km values. Figure 9 (a) and 9 (b) show the Lineweaver-Burk plots of the blackberry PPO in the presence of fresh *A. Unedo* fruit diluted 6% and fresh onion extract, respectively. It was found that two natural agents exhibit similar types of inhibition, which were competitive and irreversible.

These two natural sources have powerful antioxidant effects, anthocyanins, phenolic compounds, and vitamin C. The anti-browning effect of *A. Unedo* is stronger than that of the fresh onion, which may be due to its higher vitamin C content.[2] We conclude, therefore, that arbutus extract could be used as a natural food ingredient for the prevention of the browning caused by the blackberry PPO.

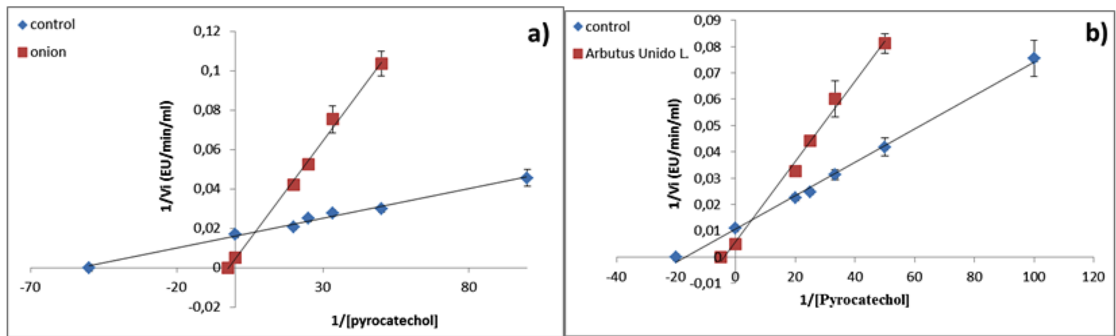


Fig. 9(a): Lineweaver -Burk representation of onion fresh extract competitive inhibition (b) Lineweaver -Burk representation of wild *A. Unido* fresh extract competitive inhibition.

Blackberry PPO showed maximum activity when observed with pyrocatechol, followed by with 4-methylcatechol. Our results were similar to blackberry,[19] blueberry,[37] and tea leaf (*Camellia sinensis*).[47] Quercetin is a stronger chemical inhibitor because it was able to inhibit 89% of our blackberry PPO at a concentration of 0.1 mM, followed by ascorbic acid, which was able to inhibit 29% at the same concentration. These two inhibitors are competitive in nature and exhibit a small ki. Onion competitively inhibits our PPO, which is similar to the yam PPO from Yapi *et al.*[48] Mayer claims that there are two categories of PPO inhibitors: those that bind to the copper side and those that interfere with the side of phenolic compounds. Competition-based inhibition is present in the category of interactions with copper, whereas non-competitive inhibition is present in the other category.[49]

Competitive inhibition was found for the tea leaf (*Camellia sinensis*) with sodium sulfate, ascorbic acid, glutathione, and dithioerythritol inhibitors using catechol as a substrate.[47]

Our natural extracts show a higher anti-browning effect than that provided by Lim,[50] who found 47.97%. These results outperform those provided by Lim.[50]

Fresh onion and wild *A. Unedo* extract were able to inhibit the blackberry PPO activity by up to 50% and 60%, respectively. These two natural inhibitors are competitive types therefore able to form chelate complexes with Cu in the active site of PPO, as cited by Mayer.[49] These results are identical for the onion as a natural inhibitor with the same type of competitive inhibition.[51] The use of chemical and natural inhibitors for controlling the blackberry PPO has not been investigated previously.

Conclusion

The polyphenol oxidase from the blackberry that leads to the enzymatic browning need to be prevented during storage and processing. PPO was purified, characterized and kinetic parameters for five substrates were pyrocatechol, 4-methyl-catechol Pyrogallol, gallic acid, and tyrosine were

determined. The optimum pH and temperature were pH 6.6 and 25°C, respectively. The wild blackberry PPO has two isoenzymes, one with 22 kD and 70 kD, respectively. The enzyme activity indicated variable affinity towards o-dihydroxy phenolic substrates and no activity was observed with phenol, a monohydroxy substrate, and was very effective towards pyrocatechol flowed by Pyrogallol and 4-Methyl catechol. Quercetin and Ascorbic acid have the lowest Ki values, and fresh onion and wild Arbutus extract were able to inhibit the blackberry PPO activity up to 50% and 60%, respectively. This may provide new insight into natural PPO inhibitors in the goal of replacing the chemical anti-browning agents such as sulphites with natural ones for protection human health against toxics and may contribute to increased knowledge of the natural inhibitor of PPO.

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Conflict o Interest
The authors declare no conflict of interest.

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