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Purification and Biochemical Characterization of Polyphenol Oxidase from Alanya Banana (*Musa carevendishi*)

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Polyphenol oxidase was isolated from Alanya banana (*Musa carevendishi*) fruit grown in Southern Turkey with ammonium sulfate precipitation and dialysis method. One protein fraction was obtained with gel filtration chromatography. Optimum temperature for banana polyphenol oxidase was 25 °C with catechol substrate. Polyphenol oxidase showed maximum activity at pH 7.0 with catechol, caffeic acid, pyrogallol, 7.2 with 4-methylcatechol, 7.4 with L-tyrosine, 7.8 with *p*-cresol, 8.0 with gallic acid. K_m and V_{max} values in the case of 20 mM catechol as substrate were 15.95 mM and 1776 $\Delta A\ min^{-1}$, respectively. Six different inhibitor were tested in this study and the most effective inhibitors for banana polyphenol oxidase were found to be L-cysteine, β-mercaptoethanol and sodium diethyldithiocarbamate. Inhibition constants (K_i) were calculated for every inhibitor at optimum pH of polyphenol oxidase activity. Polyphenol oxidase isoforms were determined by using the partially purified banana polyphenol oxidase. Polyphenol oxidase isoforms migrated as 4 active bands with catechol substrate, 4 bands with Coomassie Brillant R-250 during native polyacrylamide gel electrophoresis (N-PAGE). On the SDS-PAGE, banana polyphenol oxidase produced 5 bands of *ca.* 105 kDa, 52 kDa, 26 kDa, 18 kDa and 15 kDa molecular weights. The activation energy, inactivation rate constant and half-lives ($t_{1/2}$) of polyphenol oxidase were also calculated.

Key Words: Banana, Enzymatic browning, *Musa carevendishi*, Polyacrylamide gel electrophoresis, Polyphenol oxidase.

INTRODUCTION

Browning reactions in fruits and vegetables are recognized as a serious problem in the food industry. Polyphenol oxidases (PPO) are responsible for the enzymatic browning reactions occuring during the handling, storage and processing of fruits and vegetables. This problem is of considerable importance to the food industry as it affects nutritional quality and appearance, reduces consumer acceptability and

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therefore causes significant economic impact, both to primary producers and the food processing industry[1-3]. The damage caused by harvesting and transport can make the fruit unfit for sale and the use of banana in part processed fresh foods like fruit salad and in processed foods, such as cakes and gateaux, could become viable if the fruit itself remained pale yellow and attractive to the consumer after being peeled and sliced.

Polyphenol oxidases (PPO) are very important enzymes in food industry for their involvement in the enzymatic browning of edible plants. Plant polyphenol oxidases (PPO) are responsible for the enzymatic browning reactions occurring during the handling, storage and processing of fruits and vegetables. In plant tissues, the browning pigments lead to organoleptic and nutritional modifications, thus depreciating the food product and the quality and economics of the product also lowers the nutritional value. The banana belongs to the genus *Musa* of the family Musaceae. Banana is a tropical fruit of commercial significance and undergoes textural, colour transformations as they pass through the ripening process. Biochemical characterization of bananas have been important because the chemical changes in the banana fruit during various stages of development. In addition, the degree of browning in banana, after cutting, was correlated with polyphenol oxidase activity and the concentration of free phenolic substrates[4].

Polyphenol oxidase (PPO; EC 1.14.18.1) is widely distributed in the plant and animal kingdoms, is a copper-containing enzyme and is responsible for the enzymatic browning reaction occurring in many plants and vegetables[4]. PPO catalysis two distinct reactions involving phenolic compounds and molecular oxygen, namely: (a) hydroxylation of monophenol to *o*-diphenol or cresolase activity, (b) oxidation of *o*-diphenol to *o*-diquinones or catecholase activity. PPO is widely distributed in higher plants but it still has no defined biological function, although many possible roles have been proposed[5-9]. Today, its most likely functions of PPO are its involvement of plant resistance against diseases[10,11].

Banana is an important crop worldwide and one of the most important food sources in the developing world. Alanya banana (*Musa carevendishi*) grows in southern Turkey and contains vitamin B_1, B_2, C, A and E and minerals like potassium, iron, calcium, phosphore, sodium and iodine as other banana species. It also contains 70 % water, carbohydrates at important amounts, protein and fat at small amount. Therefore it is useful for liver development, skin protection, bone development and renal and intestinal system[12].

In this work, PPO enzyme was isolated from Alanya banana fruit and partially purified. After the purification, the biochemical characterization of the enzyme was made with respect to kinetic properties, optimum temperature and pH, substrate specificity, thermal stability, inhibition studies. Native and SDS polyacrylamide gel electrophoresis were made for activity assay and molecular weight determination of the banana PPO enzyme, respectively.

EXPERIMENTAL

Banana (*Musa carevendishi*) fruits harvasted in Alanya in Turkey used in this study were obtained from the local market of Ankara and stored at 4 ºC until used. Polyvinyl pyrrolidone, ascorbic acid, *p*-cresole, L-DOPA, L-tyrosine, caffeic acid, L-cysteine, sodium diethyldithiocarbamate, β-mercapto ethanol, sephadex G-100, ammonium sulfate, monobasic and dibasic sodium phosphate were purchased from Sigma Chem. Co., 4-methyl catechol, sodium sitrate were purchased from Fluka, catechol, pyrogallol, gallic acid, sodium azid, potassium tartarate, sodium tungstate, sodium molybdate, copper sulfate, lithium sulfate, sodium hydroxide and hydrochloric acid were purchased from Merck. All chemicals used in this study were of analytical grade and were used without further purification. The water used was deionized.

Partially purified polyphenol oxidase preparation: For the extraction of the PPO enzyme from Alanya banana, 2 kg banana was stored at 4 ºC during 1 week. Then, 200 g bananas were peeled and homogenized with 100 mL of cold 0.2 M phosphate buffer (pH 7.2) containing 10 mM ascorbic acid and 0.5 % polyvinylpyrolidone (PVP) in a Waring blender for 2 min after washed in cold water. The homogenate was filtered through cheese cloth and the filtrate was centrifuged at 48.000 x g for 15 min at 5 ºC. The proteins in the supernatant were collected. The enzyme solution was fractionated with solid ammonium sulfate and the precipitate of 80 % saturation was collected by centrifugation at 48.000 × g for 0.5 h at 5 ºC. The pellet was redissolved in 45 mL homogenization buffer (0.2 M phosphate buffer, pH 7.2) and dialyzed at 4 ºC against the same buffer in cellulose dialysis tubing (m.w. cut off 12 000-14 000 Da). Dialysis buffer was changed 3 times with 8 h intervals. The dialyzed solution kept in stoppered test tubes at -20 ºC.

Gel filtration chromatography on Sephadex G-100: For gel filtration chromatography, the column (0.5 cm × 17.5 cm) was prepared by using Sephadex G-100. 2. 25 g Sephadex G-100 was dissolved in 40 mL 0.2 M phosphate buffer (pH 7.2) and kept 2 d. Then, Sephadex G-100 prepared was loaded the column and pre-equilibrated. The elution rate of this column was determined by using 0.2 M phosphate buffer (pH 7.2) after some glass-cotton and sand were added on the column. The eluats were collected from the column as 3 mL volumes in tubes by a fraction collector after the dialyzed enzyme solution was applied on the Sephadex G-100 column. Elution process was continued until no absorbance was observed at 280 nm. The protein and PPO activities of each fraction were determined. The fractions having PPO activity were collected and their purification degrees were determined by measuring specific activity.

Protein determination: Protein amounts of the samples at different purification stages were determined by Bradford method[13].

Assay of polyphenol oxidase activity: Polyphenol oxidase enzyme activity was assayed with the spectrophotometric procedure. The increase in absorbance at 420 nm was recorded automatically for 3 min at 20 ºC temperature. with spectro-

photometer (Shimadzu UV-Vis-1601). Enzyme activity was calculated from the linear part of the curve of the absorbance at 420 nm *vs.* incubation time. The method was carried out at various temperatures and pH values with catechol substrate. One unit of PPO activity was defined as the amount of enzyme that cause to increase of 0.001 unit of absorbance per min at room temperature. Experiments were duplicated.

Some Properties of Alanya Banana PPO

Substrate specificity: Substrate specificity of the banana PPO enzyme was determined by using 7 different substrates (catechol, 4-methyl catechol, L-tyrosine, L-DOPA, pyrogallol, *p*-cresol, gallic acid, caffeic acid) at the concentration of 0.01 M (catechol, 4-methyl catechol, pyrogallol) and at 0.005 M (L-tyrosine, caffeic acid and *p*-cresol) due to the limited solubility with the 0.2 M phosphate buffer (pH 7.2).

pH optimum of banana polyphenol oxidase: For determination of the optimum pH of banana PPO for the substrates of catechol, 4-methyl catechol, cafeic acid, pyrogallol, L-tyrosine, gallic acid and *p*-cresol, the solutions of these substrates in the pH range between 4.2-9.0 were prepared by using the following appropriate buffers *i.e.*, 0.1 M citrate between pH 4.2-5.2; 0.2 M phosphate between pH 5.2-7.2 and 0.05 M *tris*-HCl between pH 7.4-9.0. The optimum pH values obtained from this assay were used in all the other experiments.

Thermal activity and stability: The banana PPO activity was determined at various temparatures controlled by a circulating water bath. The mixtures of each substrate solution were incubated for 5 min at various temperature over the range of 10-75 ºC at the optimum pH values of the catechol substrate (pH 7.2), prior to the addition of the enzyme solution. The relative activity of PPO at a specific temperature was determined spectrophotometrically by addition of the partially purified enzyme extract to the mixture as rapidly as possible.

The thermal stability of banana PPO was investigated by using catechol substrate (pH 7.2) at 9 different temperature between 10 and 75 ºC interval. The enzyme solution in the phosphate buffer (pH 7.0) within Eppendorf tubes was placed in a prewarmed tube at the adjusted temperature and 0.5 mL of the sample portions were withdrawn at various time intervals (incubation time of 15 min during 45 min), cooled and spectrophotometrically determined for residual activity assayed. Stability of the enzyme was expressed as remaining activity. The date obtained from the thermal stability profile have been used to analyse some thermodynamic parameters related to banana PPO activity in the partially purified enzyme. Thermal inactivation rate constants were calculated by comparing the activity changes upon heat treatment with the unheated enzyme extracts as reported[14]. The activation energy for denaturation of the enzyme was determined by means of an Arrhenius plot of log reaction rate constants (ln k) *vs.* the reciprocal of the absolute temperature.

Enzyme kinetic studies: Michael-Menten constant (K_m) and the maximum velocity (V_{max}) of the banana PPO enzyme were determined by measuring initial reaction rates of PPO at optimal pH and temperature values with catechol substrate at different concentrations (1, 1.25, 2.5, 4, 7.5 and 10 mM). K_m and V_{max} values were calculated by means of the Lineweaver-Burk plot[15].

Effect of inhibitors on PPO activity: The inhibitory effects of L-cysteine, L-ascorbic acid, sodium azide, sodium diethyldithiocarbamate, β-mercaptoethanol, thiourea on PPO activity at fixed concentrations were determined at different concentrations (1, 1.25, 2.5, 4, 7.5, 10 mM) at 20 ºC and pH 7.2 by using catechol as substrate. Each inhibitor was prepared in phosphate buffer (pH 7.2). Inhibition of PPO activity was investigated in a reaction mixture consisting of 1.4 mL catechol, 1.4 mL inhibitor and 200 µL enzyme solution. Inhibition constants (k_i) of each inhibitor were calculated by using Lineweaver-Burk graphs.

Native polyacrylamide gel electrophoresis: Native polyacrylamide gel electrophoresis (N-PAGE) was performed according to the method of Laemmli[16] for separating Alanya banana PPO isoenzymes in nondenaturing conditions. Electrophoresis were performed with a discontinuous buffer system using 4 % stacking gel and 10 % acrylamide separating gel. The enzyme samples obtained from dialysis (35 µL), were applied to the space of wells in the stacking gel. The gel was run at the constant current of 42 mA at 4 ºC until the bromophenol blue marker had reached to the bottom. After completion of the run, the gel was cut into pieces. Gel piece was then immersed in 30 mM catechol in 0.1 M phosphate buffer (pH 7.0). The isoenzyme bands were developed in 110 min and gel piece then rinsed in 1 mM ascorbic acid solution for 4 min. Then it was stored in 30 % ethanol and photographed. The PPO active bands were also obtained from the staining with Comassie Brillant Blue by using same procedure.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for determination of the number of protein bands in the partially purified enzyme extract and to estimate the molecular weights of these bands according to the method of Laemmli[16]. Enzyme solution was denaturated by adding the solution containing 2.5 % glycerol, 0.05 % SDS, 1.25 % β-mercaptoethanol and 0.001 % bromophenol blue in 0.06 M *Tris* buffer. After the samples denaturated, the electrophoresis was carried out according to this procedure. SDS-PAGE was performed with a discontinuous buffer system by using 4 % stacking gel and 10 % acrylamide separating gel in the 0.1 % sodium dodecyl sulphate. After electrophoretic run, gels were stained with Coomassie Brillant Blue. Then the gels were destained in 7 % acetic acid solution and photographed. The molecular weight of the PPO was calculated by comparing the relative migration distances of enzyme and marker proteins at different molecular weight (Myosine of 200 kDa, β-galactosidase of 116.25 kDa, phosphorilase B of 97.4 kDa, bovine serum albumine of 66.2 kDa and ovalbumine of 45 kDa).

RESULTS AND DISCUSSION

Partially purification of polyphenol oxidase: In this study, PPO was partially purified from Alanya banana using ammonium sulfate precipitation and dialysis. After dialysis procedure, the partially purified banana PPO enzyme extract obtained have been used to characterize the polyphenol oxidase enzyme. Polyvinylpyrrolidone (PVP) was used during enzyme extraction due to its binding ability the phenolics in order to prevent the phenol-protein interactions. PVP is an inhibitor of PPO, but its removal by centrifugation avoided this effect[17]. PPO activity of the precipitate at 30-80 % $(NH_4)_2SO_4$ saturation was found the highest and this saturation point was used for all the extraction processes. Oxidation of phenolics by PPO produces quinones during extraction[16]. Therefore ascorbic acid was used to reduce the formation of quinones. After the PPO enzyme obtained from precipitating by using solid ammonium sulfate was dialyzed at 4 ºC, the molecules that smaller than 12 000 Dalton are out of the dialysis tube and PPO enzyme are located in the tube. After gel filtration chromatography obtained one fraction showing that Alanya banana PPO activity (Fig. 1).

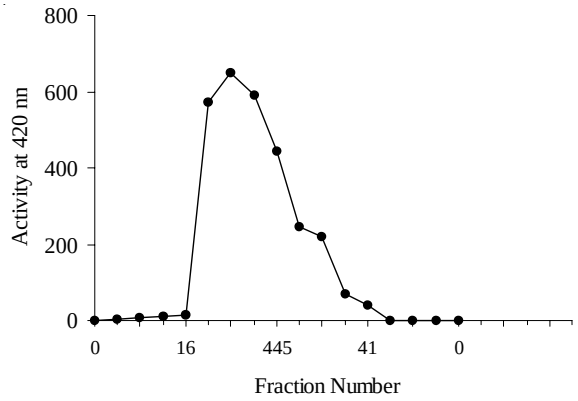

Fig. 1. Elution profile obtained from gel filtration chromatography

Polyphenol oxidase (PPO) isoforms were also determined by using the partially purified banana PPO. PPO isoforms migrated as 3 intense and 1 weak bands after activity staining with catechol substrate, while 4 bands stained with Coomassie Brillant R-250 during native polyacrylamide gel electrophoresis (N-PAGE) (Fig. 2). On the SDS-PAGE, banana PPO produced five bands of 105 kDa, 52 kDa, 26 kDa, 18 kDa and 15 kDa approximately molecular weights (Fig. 3).

Some properties of alanya banana polyphenol oxidase:

Substrate specificity: The relative activities were determined for each substrate related to catechol activity at 420 nm. In Table-1, the changings of PPO enzyme activity towards *o*-dihydroxy, trihydroxy and monohydroxy phenols are summarized in Table-1.

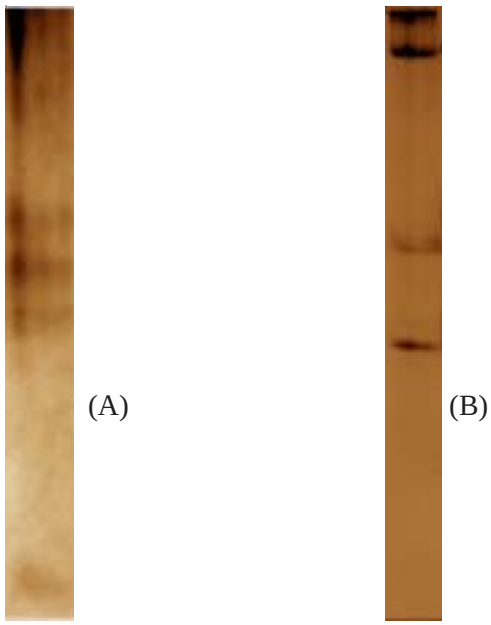

Fig. 2. NATIVE-PAGE electrophoretic pattern of partially purified Alanya banana PPO, (a) Activity staining by catechol (b) Activity staining by coomassie brillant blue

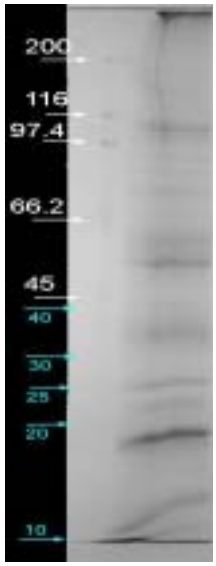

Fig. 3. SDS-PAGE gel electrophoresis of Alanya banana PPO; Line 1: The protein bands of partially purified PPO enzyme, Line 2: Molecular weight marker (Myosin 205 kDa, ß-Galactosidase 116 kDa, Phosphorylase 97,4 kDa, Bovin serum albumin 66 kDa, albumin egg 45 kda, Gliseraldehyde-3-phosphate dehydrogenase 30 kDa, Carbonic anhydrase 29 kDa, Trypsinogen 24 kDa, α-Lactalbumine 14.2 kDa)

TABLE-1
THE SUBSTRATE SPECIFICITY OF BANANA PPO TOWARDS SEVERAL PHENOLIC SUBSTRATES AT 420 nm

Substrate	Concentration (M)	Relative activity
Catechol	0.010	100.00
4-Methyl catechol	0.010	87.90
Caffeic acid	0.005	72.30
Pyrogallol	0.010	11.90
Gallic acid	0.005	10.10
L-Tyrosine	0.002	6.20
p-Cresol	0.002	3.05

Alanya banana PPO has shown the highest activity towards dihydroxy phenolic compounds such as catechol, 4-methyl catechol. But those of tri-hydroxy phenols like pyrogallol and gallic acid and monohydroxy phenols like *p*-cresol and L-tyrosine didn't show any activity. Literature reports indicate that the PPO enzyme systems of most plants are specific for *o*-diphenolic substrates. Tate *et al.*[18] observed high activity against *o*-diphenolic substrates of Bartlett pear PPO. Siddiq *et al.*[19] also demonstrated that 4-methyl catechol was oxidized much more rapidly by PPO of stanley plums than that of other substrates. Cash *et al.*[20] found only catecholase activity with concord grape PPO and didn't show any activity when monohydroxy phenols were used as substrates. Catechol was found to be the best substrate of this enzyme. Similar results were also obtained for both PPO of pear and Jerusalem artichoke by Ziyan and Pekyardimci[21,22]. Some other researchers have reported only activity against *o*-diphenolic compounds in other fruits[17,22-24].

pH optimum of banana polyphenol oxidase: The pH optimum of banana PPO was determined by using seven different substrates and the results are shown Table-2.

TABLE-2
OPTIMUM pH VALUES OF PPO ENZYME OBTAINED FROM ALANYA BANANA AGAINST DIFFERENT SUBSTRATES

Substrate	Substrate concentration (M)	Optimum pH
Catechol	0.020	7.0
4-Methyl catechol	0.010	7.2
Caffeic acid	0.010	7.0
Pyrogallol	0.010	7.0
Gallic acid	0.010	8.0
L-Tyrosine	0.002	7.4
p-Cresol	0.002	7.8

In general, PPO of most fruits show maximum activity at neutral or near neutral pH values[19,21,22,24-28]. As seen in Table-2, the optimal pH values for catechol, 4-methyl catechol, pyrogallol, caffeic acid and L-tyrosine substrates are generally neutral or

near neutral, but those of gallic acid and *p*-cresol are basic. The optimum pH of the PPO of different fruits may vary as depending on the origin of material, extraction method, the maturity of the fruit and substrate[29]. The optimum pH values for the enzyme from different sources have been reported *i.e.*, 4.5 for green olive[30], 5.0 for potato[31] and 7.0 for peppermint[32] with catechol as substrate.

Thermal activity and stability: Thermal activity of partially purified PPO from Alanya banana is presented in Fig. 4. The optimum temperature for maximum activity of banana PPO with catechol substrate was at 25 ºC. Above 25 ºC the activity declined and remained of about 30 % activity until 45 ºC. The enzyme was completely denatured after 75 ºC. Wissemann and Lee[33] was also obtained 25 ºC of temperature optima for Ravat and Niagara grape PPOs. Ünal[34] is reported that optimum temperature for Anamur banana was 30 ºC.

The thermal stability profile of dialyzed banana PPO is presented in Fig. 5. It can be seen in Fig. 3, PPO enzyme activity didn't change between 20 and 30 ºC. The activity slightly changed at 40 and 45 ºC after 0.5 h, completely finished at the temperatures between 75 ºC after 45 min. PPO is generally considered to be an enzyme of low thermostability[4].

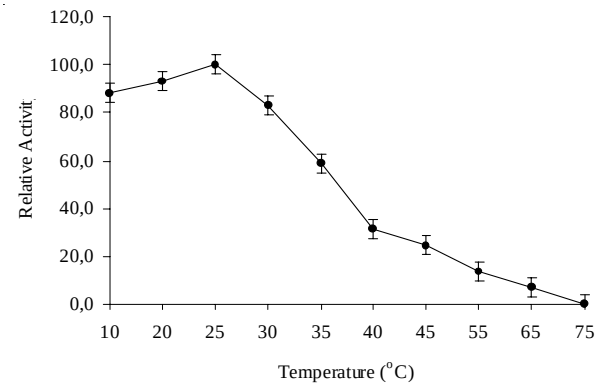

Fig. 4. Effect of temperature on banana PPO activity with catechol as substrate

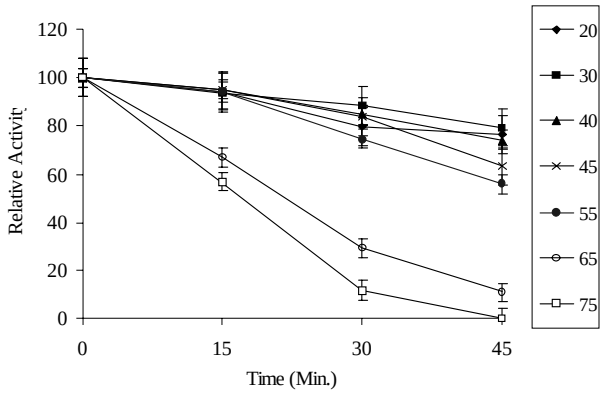

Fig. 5. Thermal stability profile for partially purified banana PPO

The rate constants for thermal inactivation k, for the first phase of thermal inactivation were calculated from the slope of the curve at each temperature. The temperature dependence of k was evaluated by using the Arrhenius equation: Ln k = C- ΔE/RT. Where C is a constant of integration, ΔE is activation energy (cal mol^{-1}), R (1.99 cal mol^{-1}) is the universal gas constant and T (K) is the absolute temperature. From a plot of ln k-1/T shown in Fig. 6, ΔE was calculated from the slope of straight line and found to 25.9 kcal mol^{-1}. The half-lives of banana PPO ($t_{1/2}$) were calculated from the following equation: $t_{1/2}$ = ln 2/k.

Inactivation rate constant and half-lives ($t_{1/2}$) of banana PPO is given in Table-3. If inactivation rate constant is higher, enzyme is less thermostable[35]. From this reason, it is seen in the table, the enzyme is less stable at higher temperature. The half-life ($t_{1/2}$) is another important parameter for the characterization and stability of banana PPO enzyme. It is seen in Table-3 that the increasing in the temperature causes the decrease in $t_{1/2}$ values. It is reported that the half-life of PPO was 18.8 min at 60 ºC for mango kernel PPO[36], 4.5 and 31.6 min at 75 ºC for Ravat and Niagara grapes[33], respectively.

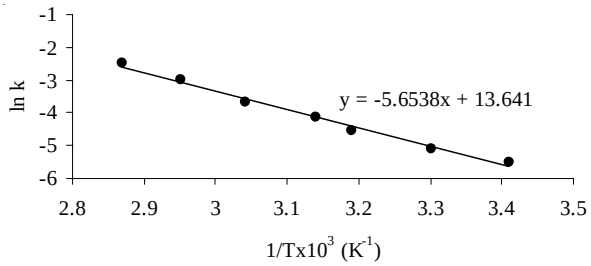

Fig. 6. Arrhenius plot for heat inactivation of partially purified banana PPO

TABLE-3
INACTIVATION RATE CONSTANT AND HALF-LIVES ($t_{1/2}$) OF BANANA PPO

Temperature (ºC)	k_i	$t_{1/2}$
20	0.0202	34.3
30	0.0345	20.1
40	0.0351	19.7
45	0.0369	18.8
55	0.0865	8.1
65	0.1090	6.4
75	0.1325	5.2

Enzyme kinetics: Michaelis constant (K_m) and maximum reaction velocity (V_{max}) values using catechol substrate under optimal conditions (pH and temperature) were determined by means of Lineweaver-Burk plot (Fig. 7). As seen in Fig. 7, K_m value was 15.95 mM for banana PPO with catechol as substrate. It was reported a Km of 52.6 mM for Victoria grape PPO[37], 19.81 mM for mulberry[38] and 67 mM for Concord

grape PPO[20] with catechol substrate. This value is lower than these PPO enzymes. The maximum reaction rate (V_{max}) value was 1776 ΔA min^{-1} for Alanya banana PPO with catechol as substrate (Fig. 7).

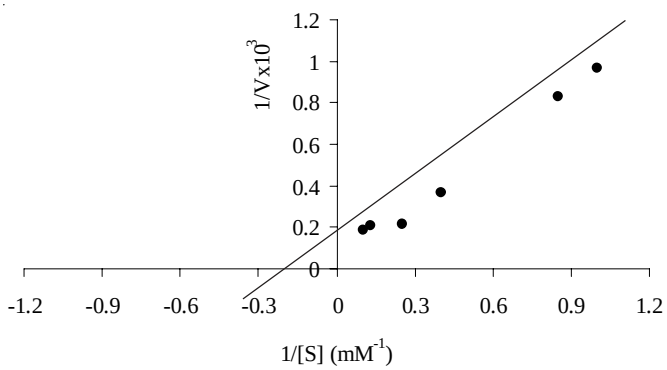

Fig. 7. Lineweaver-burk plot for partially purified polyphenol oxidase. Enzyme assay were carried out 20 mM phosphate buffer (pH 7.0)

Effect of inhibitors on PPO activity: The inhibition studies showed that the most potent inhibitors for banana PPO were thiourea, sodium diethyldithio carbamate and sodium azide because these compounds induced 100 % inhibition, even at the lowest concentration used (Table-4). Therefore, inhibition constants (K_i) of each inhibitor were calculated only for L-ascorbic acid, L-cysteine and β-mercaptoethanol at optimum pH of PPO activity. Inhibitors like sodium diethyldithiocarbamate (DETC) and thiourea which combine with copper in the enzyme, generally potent inhibitors of PPO[39,40]. L-cysteine and β-mercaptoethanol showed competitive inhibition and L-ascorbic acid showed non-competitive inhibition. It was reported

TABLE-4
THE INHIBITION TYPES OF PPO INHIBITORS AND AVERAGE VALUES OF k_i

Inhibitor	Concentration (mM)	Type of inhibition	Average k_i (M)
L-Ascorbic acid	0.20 0.50	Non-competitive	4.34×10^{-9}
L-Cystein	0.05 0.20	Competitive	3.30×10^{-16}
β-Mercapto ethanol	0.20 0.65	Non-competitive	3.04×10^{-16}
Thiourea	150 60	Non-competitive	3.66×10^{-10}
Sodium diethyldithiocarbamate	0.025 0.050	Non-competitive	4.73×10^{-14}
Sodium azide	50 150	Non-competitive	6.69×10^{-13}

that ascorbic acid showed nearly 100 % inhibition at some grape species[20,37,41]. In present study, L-ascorbic acid was also found to be very strong inhibitor of banana PPO. Since ascorbic acid are naturally occuring substances and non-toxic, they may be useful in preventing enzymatic browning of pear products.

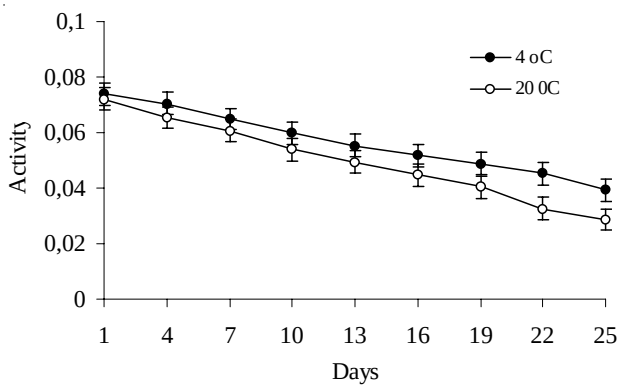

Fig. 8. Storage stability of banana PPO

Storage stability of PPO: In order to determine the storage stability of PPO, partially purified PPO extracts were kept in a small Erlenmayer flask at 4 and 20 ºC for 1 month. Activity measurements were carried out taking samples every day in order to determine the activity loss. Results are shown in Fig. 8. The PPO activity decreased slowly during the first 5 days at 20 ºC and continued to until day 25. Activity loss was losses at 4 ºC than that of 20 ºC. This indicates that PPO was more stable at cold temperatures.

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