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ORIGINAL PAPER

Partial characterization of polyphenoloxidase from a hybridized wheat (*Triticum aestivum* L.)

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Abstract Polyphenol oxidase was extracted and partially purified from wheat leaves by a procedure that included ammonium sulfate fractionation followed by dialysis and gel filtration chromatography. These procedures led to 35.21-fold purification with 17.65% recovery. Optimum pH, temperature, and ionic strength were determined with six substrates. Some kinetic properties of the enzyme such as $V_{max,}$ K_M, and k_{cat} were calculated for the substrates. The k_{cat}/K_M values of the PPO for catechol, catechin, pyrogallol, L-dopa, dopamine, and 4-methyl catechol were 31408, 31167, 28404, 15378, 4865, and 4967 mM/min, respectively. The best substrate of wheat PPO was found to be catechol. The native molecular weight of the PPO was estimated to be 243 kDa based on its mobility in gel filtration column. The inhibitory effects of glutathione, sodium azide, ascorbic acid, oxalic acid, L-cysteine, and thiourea on the reaction catalyzed by the enzyme were tested, and I_{50} values were estimated to be 8.0 mM, 10.12 mM, 11.18 mM, 77.33 mM, 183 mM, and 413 mM, and K_i constants were also calculated as 0.416 ± 0.244 mM, 0.317 ± 0.208 mM, 0.820 ± 0.111 mM, 13.80 ± 1.179 mM, 14.10 ± 5.069 mM, and 130 ± 62.45 mM, respectively, by means of Lineweaver–Burk graphs. The most effective inhibitor was glutathione. Glutathione, sodium azide, oxalic acid, and thiourea were competitive inhibitors, whereas ascorbic acid and L-cysteine were also noncompetitive inhibitors.

Keywords Wheat · *Triticum aestivum* L. · Polyphenol oxidase · Kinetics · Inhibition

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Introduction

Hybridization and introgression are natural biological processes that can occur among closely related species. It has long been recognized that cultivated plants, such as wheat, maize, rice, bean, and sugarcane, naturally hybridize with their wild relatives [1]. Until recently, there has been little interest in gene transfer from crops to their wild relatives. However, with the advent of genetically modified crops, there has been renewed interest in understanding the implications of gene transfer from a cultivated crop to a wild relative [2]. The objective of wheat hybridization processes is to obtain productive and qualified yields. This process is important for wheat, because it is the most widely grown crop in the world for covering the food need of increasing human population.

The enzymatic browning often occurs in fruits and vegetables and is the cause of a decrease in their sensory properties and nutritional value, but may be useful for preparation of dark tea [3]. Polyphenol oxidase (PPO) is a common copper-containing enzyme, which is responsible for browning in plants. The enzyme catalyzes two distinct reactions involving molecular oxygen: the o-hydroxylation of monophenols to o-diphenols (cresolase activity; E.C. 1.14.18.1.) and the oxidation of o-diphenols to o-quinones (catecholase activity; E.C. 1.10.3.2.) [4]. Quinones are highly reactive electrophilic molecules that can polymerize, leading to the formation of brown or black pigments.

The physiological role of PPO in plants is not yet clear. It is suggested that it may be involved in immunity reactions and in biosynthesis of plant components and it also may play the role of a scavenger of free radicals in photosynthesizing tissues [5].

Because of its involvement in this adverse browning effect, PPO has received much attention from researchers in the fields of plant physiology and food science. Purification and characterization of plant PPO has been focused on many fruits, vegetables, and crops because of the significance of enzymatic browning in post-harvest physiology and food technology [3].

Enzymatic browning caused by PPO can be controlled in different ways: by removal of one of its necessary components such as, oxygen, enzyme, copper and substrate [6] or inhibition of the enzyme with a wide range of chemicals [7].

The objective of this study was to determine kinetic and characteristic properties of a wheat PPO extracted from leaf tissue, including affinity for common PPO substrates and the effect of inhibitors on activity.

Materials and methods

Materials

Wheat (*Triticum aestivum* L.), a hexaploid hybrid ($2n = 42$), used in this study was harvested in June from a field cultivated by East Anatolia Agricultural Research Institute near Erzurum in Turkey. Sephadex G-100 was used as gel filtration column and other chemicals were purchased from Sigma Chem. Co. All chemicals used in this study were the best grade available. Substrates and inhibitor stock solutions were prepared freshly in distilled water.

Methods

Enzyme extraction and purification

Ten grams of wheat leaves was cleaned and prepared for extraction. These leaf samples were immersed in liquid nitrogen, in a Dewar flask to disrupt cell membranes, and were homogenized in 50 ml of 0.1 M potassium phosphate buffer pH 6.5, containing 10 mM ascorbic acid and 0.5% polyvinylpyrrolidone (PVP). The crude extract samples were centrifuged at 15,000 g for 30 min. The process was conducted at 4 °C. The supernatant was tested at 0–20, 20–40, 40–60 and 60–80% to find proper saturation point with solid $(NH_4)_2SO_4$. The proper saturation of PPO was determined to be in 20–60% $(NH_4)_2SO_4$ fraction. The precipitate was dissolved in a small amount of 10 mM phosphate buffer (pH 6.5) and dialyzed at 4 °C in the same buffer for 8 h with two changes of the buffer during dialysis. In order to conduct further purification, the dialysate was transferred to a column (2.5–70 cm) filled with Sephadex G-100 gel and equilibrated with 50 mM phosphate buffer pH 6.5 containing 50 mM KCl. The enzyme was eluted with the equilibration buffer by means of a peristaltic pump. The flow rate was adjusted to 20 mL/h [8].

The eluates were collected in test tubes as 2 mL volume and elution continued until zero absorbance was obtained at 280 nm. Each eluate fraction that showed absorbance at 280 nm was assayed for PPO activity. A_{280} and PPO activity were plotted against the tube number. The fractions having PPO activity were collected and degree of purification was determined by measuring specific activity before and after purification. Specific activity was determined from PPO activity and quantitative protein determination using the Bradford protein dye-binding method [9] with bovine serum protein as a standard.

PPO activity assay

PPO activity was determined using six different substrates by measuring the increase in absorbance at 420 nm for catechol and 4-methylcatechol [8], 380 nm for catechin, 475 nm for L-dopa, and 470 nm for dopamine [10], and 320 nm for pyrogallol [6] substrates with a Shimadzu Spectrophotometer UV-(1208). Reference cuvettes contained all of the components except the enzyme, with a final volume of 3 mL. One enzyme unit (U) was defined as a change in absorbance of 0.001 in 1 min under the conditions of the assay.

Determination of the optimal activity conditions

Effect of pH

PPO activity was determined with six different substrates at a concentration of 5 mM for catechol, 10 mM for L-dopa, dopamine, catechin, and pyrogallol, and 15 mM for 4-methylcatechol. Appropriate buffers (citrate for pH 4.0–5.5, phosphate for pH 5.5–7.0, and Tris–HCl for pH 7.0–9.0) were used for the determination of optimum pH of PPO. The optimum pH values obtained from this assay were used in all subsequent experiments.

Effect of temperature

PPO activity was measured at different temperatures in the range from 5 to 60 °C using the 6 different substrates to determine the optimum temperature of the enzyme. The desired temperature conditions were provided by using a Polyscience bath (Model 9105).

Effect of ionic strength

Ionic strength effect on the enzyme was studied with proper concentration of substrates using 0.05, 0.10, 0.20, 0.3, 0.4, 0.50, 1.00, 2.00, and 3.00 M concentrations of citrate/phosphate buffer at optimum pH of the substrates.

Effect of substrate concentrations

PPO activities were measured with six different substrates (catechol, catechin, pyrogallol, L-dopa, dopamine, and 4-methyl catechol) at varying concentrations to determine the optimum substrate concentration for the best activity of the enzyme under optimum conditions of pH, ionic strength, and temperature.

Michaelis constant (K_M) and maximum velocity (V_{max}) values of the enzyme for each substrate were calculated from a plot of 1/V against 1/[S] by the method of Lineweaver and Burk [11]. K_{cat} values were calculated from equation of $V_{max} = k_{cat}\ [E]_t$ was also determined to be 8.23×10^{-4} μmol. The catalytic power of the enzyme was determined from the relationship k_{cat}/K_M.

pH stability

To find pH stability, equal volumes of the buffers (citrate at pH of 4.0, 4.5, 5.0, and 5.5, phosphate at pH 6.0, 6.5, 7.0, and 7.5, and Tris–HCl at 8.0 and 8.5) and purified enzyme were mixed and kept in a refrigerator (+4 °C). The enzyme activity was assayed at the beginning and after each 1 day using catechol as substrate under optimum activity conditions.

Molecular weight determination

The molecular weight of the native enzyme was determined on the Sephadex G-100 gel filtration column according to the method of Andrews [12]. At first, to establish the void volume, Blue Dextran (2,000 kDa) was passed through the column; then, horse heart cytochrome-*c* (12.4 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), bovine serum albumin (BSA) (66 kDa), alcohol dehydrogenase (150 kDa), and sweet potato *β*-amylase (200 kDa) were used as standard proteins (MW-GF-200; Sigma). The enzyme sample from gel filtration chromatography was mixed with glycerol to form a mixture including 50 mM glycerol. The final sample was loaded onto the column and eluted with 50 mM phosphate buffer pH 6.5 containing 50 mM KCl and 50 mM glycerol. Eluted fractions were collected as 2 mL in tubes. In each fraction, absorbance at 280 nm and activities at 420 nm were determined with catechol as substrate. Molecular weight of the PPO was determined from the curve plotted between relative log molecular weight and K_{av} values obtained from standard proteins.

Inhibition studies

The inhibition kinetic analysis of the enzyme was determined for the following inhibitors: glutathione, sodium azide, ascorbic acid, oxalic acid, L-cysteine, and thiourea. The activity of the enzyme was determined without inhibitor and in the presence of the inhibitors at five different concentrations (0, 5, 7, 10, 13, and 15 mM for glutathione, 0, 1.5, 3.0, 6.3, 12.5, and 25.0 mM for sodium azide, 0, 3.1, 6.3, 8.3, 12.5, and 25 mM for ascorbic acid, 0, 16, 32, 50, 100, and 150 mM for oxalic acid, 0, 10, 20, 50, 200 mM for L-cysteine, and 0, 100, 200, 300, and 400 mM for thiourea) with 5 mM catechol substrate at pH 4.5. Percent activity graphs were plotted from these results to find I_{50} values.

Later, using five different concentrations of the substrates, PPO activities were measured at three different constant inhibitor concentrations with inhibitors indicated above. Lineweaver–Burk graphs of this data were used to determine K_i (dissociation constant) and inhibition type for each inhibitor.

Results and discussion

Extraction and purification of PPO

The PPO was extracted from wheat in 0.1 M phosphate buffer pH 6.5, containing 0.5% PVP and purified from its leaves using a combination of ammonium sulfate precipitation and Sephadex G-100 gel filtration chromatography. PPO activity was found to be the highest in the precipitate of the 20–60% $(NH_4)_2SO_4$ fraction and this saturation point was used for all the purification processes. Polyvinylpyrrolidone was used during extractions to bind the phenols that could inactivate the PPO. It is well documented that oxidation of phenolic compounds by PPO produces quinones that would inhibit PPO [13]. Therefore, ascorbic acid was used to reduce quinonic compounds back to the phenolic compounds during extraction. Polyvinylpyrrolidone and ascorbic acid were removed from enzyme sample by dialysis after ammonium sulfate fractionation. The PPO was purified 35.21 fold with a 17.65% total recovery of the activity by the procedure. Results of the purification of PPO are given in Table 1. When the purification steps were compared, there was 1.68 fold purification after ammonium sulfate precipitation and 35.21 fold purification after gel filtration chromatography. Specific activity was 2.13 U/mg proteins in crude extract and 75.00 U/mg proteins in partial purified enzyme. Specific activity values in crude extract

Table 1 Purification of polyphenol oxidase from wheat leaves

Purification steps	Total volume (mL)	Activity (U/mL)	Total activity (U)	Protein (mg/mL)	Total protein (mg)	Specific activity (U/mg of protein)	Yield (%)	Purification n-fold
Crude extract	200	0.34	68.0	0.16	32.0	2.13	100.0	1.00
$(NH_4)_2SO_4$ precipitation and dialysis	5	2.15	10.75	0.60	3.0	3.58	15.8	1.68
Gel filtration chromatography	8	1.50	12.0	0.02	0.16	75.00	17.65	35.21

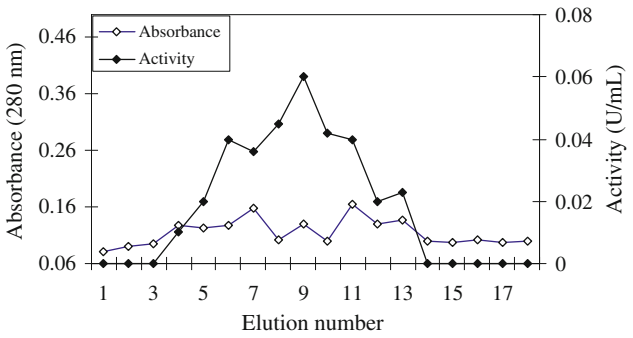

Fig. 1 Purification of polyphenol oxidase from wheat by gel filtration chromatography on Sephadex G-100

have been reported to be 1.8 and 1.6 U/mg proteins [4] and 0.117 U/mg proteins [14] in previous reports. This result shows that hybridized wheat has higher PPO activity than its parental lines. Plots of absorbance at 280 nm and PPO enzyme activity of eluated fractions from gel filtration chromatography are shown in Fig. 1. Fractions 4–12 were pooled for the determination of purification degree.

Determination of the optimal activity conditions

Effect of pH

Activity of purified enzyme was measured with 6 different substrates to determine optimum pH for each substrate. Optimum pH was found to be citrate/phosphate buffer, pH 4.5 with catechol and dopamine, and citrate/phosphate buffer, pH 5.0 with catechin, pyrogallol, L-dopa, and 4-methylcatechol. In general, some plants, vegetables and fruits show maximum activity at or near neutral pH values [15]. For example, it is reported that optimum pH values are 7.0 for Amasya apple [16], Yhymbra [6], aubergine [17], and *Ferula* sp. [8]. Different optimum pHs for PPO obtained from various sources are also reported to be 5.5 for strawberry [18], 6.0 for DeChaunac grape [19], 6.5 for Lychee [20], 7.5 for *Allium* sp. [21], 8.0 for lettuce [22], and 8.5 for dog-rose[23], using catechol as a substrate. However, when using 4-methylcatechol as a substrate, the pH optima is 4.5 for wheat [14] and strawberry [17], 5.0 for wheat bran [24] and Thymbra [6], 5.5 for starking apple [25], 6.0 for aubergine [17], 6.5 for lettuce [22], 8.5 for dog-rose [23] and 9.0 for Amasya apple [16] and 8.5 for dog-rose [23] using dopamine as a substrate and 7.0 for dog-rose [23] and 8.6 for Amasya apple [16], 7.2 for starking apple [25], 7.5 for lettuce [22], 8.0 for Thymbra [6], using pyrogallol as a substrate. PPO activity varies with the source of enzyme and substrate within a relatively wide range of pH. Although, in most cases, pH optima have been reported to be between 4.0 and 7.0, it should be noted that the optimum pH can also be affected by the type of buffer and the source of enzyme. Our result shows that optimum pH of hybridized wheat is similar to PPOs from lines [14, 24, 26].

Effect of temperature

The temperature effects on PPO activity of wheat were studied between 5 and 65 °C with each of the six substrates used in the experiments (Table 2). As seen in the table, optimum temperatures are substrate dependent. It was found that the optimum temperature is 30 °C for pyrogallol, 35 °C for catechin, 4-methylcatechol, and dopamine, 40 °C for catechol and L-dopa. It has been reported that optimum temperature for PPO is 10 °C for dill [13], 12 °C for *Ferula* sp. [8], 15 °C for Amasya apple [16], 20 °C for DeChaunac grape [19], 25 °C for dog-rose [23], 30 °C for aubergine [17], 35 °C for Lychee [20], and 40 °C for Chinese cabbage [27], Thymbra [6], and lettuce [22], using catechol as a substrate. When using 4-methylcatechol as a substrate, optimum temperature has been determined to be 20 °C for dog-rose [23], 25 °C for *Ferula* sp. [8], 30 °C for aubergine [17], Thymbra [6], and Lettuce [22], and 56 °C for Amasya apple [16]. For pyrogallol as a substrate, 15 °C for dog-rose [23], 30 °C for lettuce [22], 50 °C for Thymbra [6], and 70 °C for Amasya apple [16]. As seen that there are highly different optimum temperature values for PPO obtained from different sources in the reports.

Effect of ionic strength

The optimum ionic strength of the enzyme was estimated to be 0.3 M for 4-methyl catechol, 0.4 M for catechol, L-dopa, dopamine, and pyrogallol, and 0.5 M for catechin

Table 2 Optimum activity conditions and substrate specificities of wheat polyphenol oxidase

Substrate	Optimum pH	Optimum temperature (°C)	Optimum ionic strength (M)	Optimum substrate concentration (mM)	K_M (mM)	V_{max} (U/mL min)	k_{cat} (min^{-1})	K_{cat}/K_M (mM/min)
Catechol	4.5	40	0.4	5	0.1243	3.2133	3,904	31,408
Catechin	5	35	0.5	10	0.1020	2.6160	3,179	31,167
Pyrogallol	5	30	0.4	10	0.1040	2.4307	2,954	28,404
L-Dopa	5	40	0.4	10	0.1629	2.0620	2,505	15,378
Dopamine	4.5	35	0.4	10	0.2335	0.9346	1,136	4,865
4-Methyl catechol	5	35	0.3	15	0.8861	3.0221	4,401	4,967

substrates. Each measurement was performed at optimum pH of the substrates with different concentrations of citrate/phosphate buffer.

Enzyme kinetics and substrate specificity

We selected six widely used substrates (catechol, 4-methylcatechol, L-dopa, and dopamine as diphenolic substrates, pyrogallol as a triphenolic substrate, and catechin as a pentaphenolic substrate) for kinetics studies. The Michaelis–Menten constant (K_M), maximum velocity of the reaction (V_{max}) and kinetic efficiency (k_{cat}/K_M) for the different substrates were determined by plotting the activity data at optimum pH, temperature, and ionic strength as a function of substrate concentration according to the method of Lineweaver–Burk. K_M and V_{max} values of PPO, for each substrate, were calculated from a plot of $1/V$ versus $1/[S]$ and shown in Table 2. As seen in Table 2, the PPO of wheat has the greatest efficiency toward catechol of the six substrates, taking from the highest V_{max} value (3.2133 U/mL min) and k_{cat}/K_M ratio into account (31408 mM/min). Consequently, catechol was used as a substrate in the other kinetics studies.

This observation was similar to that of the work on PPO from Amasya apple [16], *ferula* sp. [8], dill [13], and broccoli [28]. Substrates showing the best activity with the PPO from different sources in the literature, such as L-dopa [27], dopamine [10], pyrogallol [22], and 4-methylcatechol [23, 29], were selected. It has been seen that K_M value for PPO activity has varied with the type of substrate and plant sources. K_M values were determined to be 3.3 mM with 4-methylcatechol and 8.3 mM with L-dopa substrates by Kihara et al. [14]. Since the lower K_M means the higher affinity, the affinity of hybridized wheat PPO to the substrate is higher than that of other wheat.

pH stability

Stability of PPO activity was studied when pH value was ranging from 4.0 to 8.5 over a period of 3 days by using catechol as a substrate. It was found that PPO activity of wheat was the highest at pH 5.5. Fig. 2 suggests that the enzyme is very unstable at high pH, with no activity after 24 h at 8.5, and a significant reduction in activity at 8.0.

Molecular weight determination

The native molecular weight of the enzyme was estimated to be approximately 243 kDa by gel filtration chromatography on Sephadex G-100 column (Fig. 3). The molecular weight of the native enzyme estimated is slightly higher than that of reported for parental wheat [14], carrot cell culture [30], broccoli [28], latex of *Hevea brasiliensis* [10], and ostricina furnacalis [31]. SDS–PAGE analysis was not performed, so subunit of the enzyme could not be determined.

Effect of inhibitors

I_{50}, K_i values and inhibition modes for six inhibitors are given in Table 3. As seen from I_{50} values, glutathione was the most effective inhibitor for wheat PPO, followed by sodium azide, ascorbic acid, oxalic acid, L-cysteine, and thiourea, respectively. From the Lineweaver–Burk plots, it was concluded that the inhibition modes for four of the inhibitors (glutathione, sodium azide, oxalic acid, and

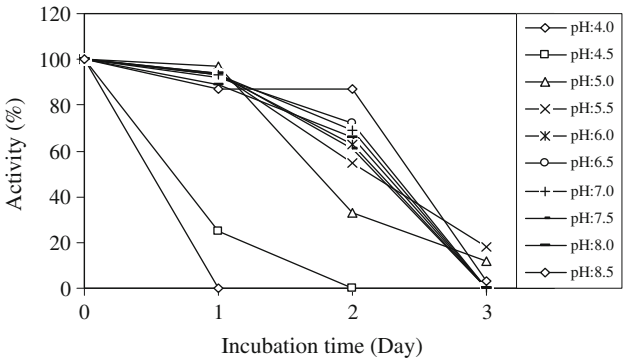

Fig. 2 Stability of PPO activity at various pH values using catechol as a substrate

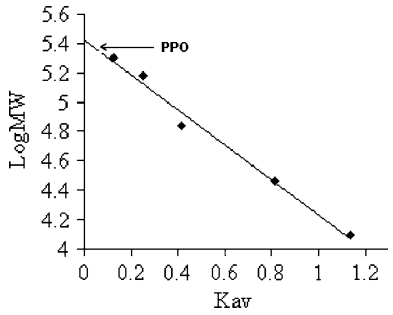

Fig. 3 Standard K_{av}-logMW graph of wheat PPO using gel filtration column

thiourea) are competitive, whereas for two of them (ascorbic acid and L-cysteine) are noncompetitive using catechol as a substrate.

The inhibition of enzymatic browning in plants can be the result of (1) inactivation of PPO, (2) elimination of one of the substrates (O_2, polyphenols) for the reaction, and (3) the action of inhibitors on reaction products of enzyme action to inhibit the formation of colored products in secondary reactions [22, 32].

There are a number of inhibitors for plant PPO, such as sodium metabisulphite [19, 23, 32], ascorbic acid [19, 23, 32, 33], glutathione [17, 19], sodium diethyldithiocarbamate [8, 19], L-cysteine, sodium azide, tannic acid, benzoic acid, and β-mercaptoethanol [23] used by researchers to prevent enzymatic browning.

Glutathione does not appear to affect the enzyme directly, and oxygen uptake may be stimulated or inhibited depending upon the particular phenol being oxidized [22]. Even though some authors have found a competitive inhibitory effect of L-cysteine on PPO, using 4 methylcatechol as a substrate, differences in type and degree of inhibition of various PPOs were reported [22]. L-cysteine can easily form complex with quinones and, thereby, inhibiting secondary oxidation and polymerization reactions. L-cysteine can also act as a reducing agent [18]. Ascorbic acid reduces quinones to hydroquinones and does not directly inhibit PPO. It prevents 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 [34]. In contrast to our results, some authors reported that the inhibition type of glutathione and ascorbic acid was mixed-type inhibition for lettuce PPO [22]. As seen earlier, the type of inhibition depends not only on the origin of the PPO studied but also on the substrate used.

Conclusions

The darkening and discoloration of wheat products are critical quality considerations of wheat breeding and improvement. PPO has a crucial role in these darkening and discoloration reactions. Although isolation and determination of some kinetic properties of wheat PPO have been studied, our understanding of the underlying biochemistry of discoloration is incomplete. Here, we report the isolation, some kinetic properties, and inhibition of

Table 3 I_{50} and K_i values and inhibition modes of six inhibitors for wheat PPO

Inhibitors	I_{50} (mM)	[I] (mM)	K_i constants (mM)	Mean K_i constants (mM)	Inhibition type
Glutathione (reduced)	8.0	6.0	0.63	0.416 ± 0.244	Competitive
		8.0	0.47		
		10.0	0.15		
Sodium azide	10.12	7.0	0.55	0.317 ± 0.208	Competitive
		10.0	0.25		
		13.0	0.15		
Ascorbic acid	11.18	8.0	0.92	0.820 ± 0.111	Noncompetitive
		10.0	0.84		
		12.0	0.70		
Oxalic acid	77.33	50.0	15.1	13.80 ± 1.179	Competitive
		70.0	13.5		
		90.0	12.8		
L-cysteine	183	100.0	18.1	14.10 ± 5.069	Noncompetitive
		180.0	15.8		
		260.0	8.4		
Thiourea	413	300	200	130.0 ± 62.45	Competitive
		500	110		
		700	80		

PPO from wheat. For the purpose, PPO was partially purified from wheat leaves. Optimal activity conditions and native molecular weight of the enzyme were determined. Different $K_{M,}$ V_{max}, and k_{cat} values were estimated for each of six substrates. I_{50}, K_i, and inhibition types of six inhibitors selected were determined. It was shown that hybridized wheat PPO exhibited different properties via substrate specificity, molecular weight, and inhibition kinetics from PPO present in the literature. As far as it is known, PPO enzyme is a very important protein for protecting wheat against environmental stress, such as dryness, saltiness, disease, virus, insect and herbivore damages by the high activity of PPO. Consequently, one of the reasons of productivity of hybridized wheat should be PPO presence with high activity.

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