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Original Research

Purification and characterization of polyphenol oxidase from corn tassel

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Abstract: In this study, polyphenol oxidase (PPO) from corn tassel was extracted and partially purified through $(NH_4)_2SO_4$ precipitation and gel filtration chromatography. Optimal temperatures for subsrates catechol and 4-methyl catechol were 40 °C and 30 °C, respectively. The optimal pH values were 8.0 for catechol and 6.0 for 4-methyl catechol. Catechol was the most suitible substrate (*Km*: 3.48 mM, *Vmax*: 1.0 Abs./ min.). The moleculer mass of PPO was determined as 158 kDa. In this work, sodium azide, ethylenediaminetetraacetic acid (EDTA) and sodium dodecyl sulfate (SDS) were found to inhibit the enzyme activity as 26.6 %, 22.2 % and 12.2 % ratio, respectively. Besides, the effects of carbohydrates such as sucrose, fructose, ribose and glucose on PPO activity were investigated. The enzyme was found to be activated 17 % by fructose and ribose, 16 % by glucose and 4 % by sucrose.

Key words: Corn tassel , polyphenol oxidase,characterisation, substrate specifity, inhibition.

Introduction

Polyphenol oxidases (PPO), belong to a set of copper containing metalloenzymes that are members of oxidoreductases that catalyze the oxidation of a wide range of phenolic compounds by utilizing molecular oxygen (1). Polyphenol oxidase is widely distributed among higher plants, which is an enzyme responsible for hydroxylation of monophenols to form o-diphenols and their further oxidation to colored and highly reactive o-quinones. PPO is well known to be the principal enzyme involved in enzymatic browning. Particularly, when a plant gets a bruise, cut or damage, the enzyme leads to the oxidation of typical phenolic compounds to form a polymer structure, resulting in protection of the plant against microorganisms or insects (2). This process causes enzymatic browning of fruits and vegetables which is undesirable in food technology as it results in loss of quality (3, 4, 5).

Corn (Zea mays L.) is one of the most widely planted crops over the world and the world production was estimated to be 471 million tons (6). Corn tassels are a waste part of the plant, which can be processed to produce valuable products such as volatile oils (7), the flavonol glycosides of kaempferol, isorhamnetin and quercetin (8) and lipids (9). Corn tassel (CT) is mainly seen as waste by corn processing industry. Since it contains a variety of active ingredients, such as flavonoid, alkaloid, allantoin, pentosan, inositol, polysaccharide, vitamin K, vitamin C, several kinds of organic acids, and sterols, CT may well serve as an inexpensive herbal drug (10). Therfore, it is of importance to characterise the enzymes such as PPO leading to loss of quality in corn tassel.

PPO has been isolated and purified from various sources, such as Turkish tea leaf (11), strawberry (12) ,ispir sugar bean (13), persimmon (14), and Jackfruit (15), Ataulfo mango (16) and its properties have been extensively studied. To our best knowledge, there is no data in literature on the purification and characterization of PPO from corn tassel. The main objectives of the present study are to characterize PPO purified from corn tassel, to determine its kinetic and characteristic properties, as well as investigating the effect of some activators and inhibitors on the enzyme activity to elucidate the mechanism of its inhibition by selected chemical compounds.

Materials and Methods

Materials and reagents

The corn tassel was obtained from a local market Diyarbakir City, Turkey and stored in 4°C until used. Catechol was purchased from Merck (Darmstadt, Germany). Ammonium sulphate, 4-methylcatechol, Sephadex G-100 gel filtration resin, polyethylene glycol (PEG), citric acid and all chemicals for electrophoresis studies were purchased from Sigma Chem. Co. All chemicals used in this study were of analytical grade.

Preparation and extraction of polyphenoloxidase (PPO) from corn tassel

Corn tassel (14.50 grams) obtained from the local market was homogenized in the extraction solution 100 ml of 0.1 M phosphate buffer containing 4 % PEG at pH 6.5 and 10 mM ascorbic acid by using a blender. The crude extract samples were centrifuged at 10.000 g for 20 min at 4 ºC. The homogenate was filtered through double layered filter paper. Solid ammonium sulphate $(NH_4)_2SO_4$ was slowly added to the supernatant to get 80 % $(NH_4)_2SO_4$ saturation under cold conditions. After 1 h, centrifugation was carried out at 15.000 g for 30 min to separate the precipitated proteins. The precipi-

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tate was then re-dissolved in a small volume of 0.05 M phosphate buffer (pH 6.5) and finally dialyzed at 4 ºC in the same buffer overnight with three changes of buffer during dialysis. The dialyzed sample is then applied to a stirred ultrafiltration cell (PBGC membrane, Millipore), after which the reduced volume was applied to a Sephadex G-100 column.

Gel filtration chromatography

The gel filtration chromatography was conducted by preparing a column (1.2 x 70 cm) using Sephadex G-100 and equilibrated with 0.1 M phosphate buffer(pH 7.0). Following the equilibration of column, the dialyzed and ultrafiltrated enzyme solution was loaded and the elution rate was adjusted to 1.5 mL/min. The eluates (3 mL) were then collected in tubes by a fraction collector. The elution process continued until there is no observed absorbance at 280 nm. The fractions collected were assayed for both their protein concentrations and for polyphenol oxidase activity. The active fractions were pooled, ultrafiltrated and then stored at -20 ºC until use in the further experiments. The samples were kept at 4ºC and then were used as the PPO enzyme source in the following experiments.

Enzyme activity assay

PPO activity was assayed by using a spectrophotometric method based on the initial rate of increase in absorbance at 420 nm. Enzyme activity was measured in 3 ml of reaction mixture consisting of 0.1 mL substrate (0.1 M catechol or 4-methylcatechol) and 0.1 mL enzyme preparation in 0.1 M phosphate buffer (pH=6.5). PPO activity was determined by measuring the absorbance at 420 nm using an GENESYS 10 S UV-Vis spectrometer (Thermo Fisher Scientific, U.S.A.) with a 1 cm light path quartz cuvette. The blank contained 2.9 mL buffer and 0.1 mL substrate. PPO activity was assayed in triplicate and one enzyme unit was defined as the amount of enzyme that produces a rise of 0.001 absorbance in one minute at 420 nm.

Protein determination

Protein analysis in all samples were carried out according to Bradford (17) method using bovine serum albumin (BSA) as the standart.

pH and temperature effect on PPO activity

The effect of pH on PPO activity was investigated using 0.1 mL of enzyme preparation, 0.1 mL of 0.1M catechol and finally topped-up to 3 mL with 0.1 M sodium acetate buffer (pH 3.0-5.0) or 0.1 M sodium phosphate buffer (pH 6.0-10). The optimum pH corresponding to the highest PPO activity was determined in order to study the effect of inhibitors and temperature on enzyme activity.

Thermal stability

To determine the temperature effect on PPO stability, 0.1 mL of crude enzyme solutions were transfered into eppendorff tubes and incubated at various temperatures (60,70,75 ºC) for different time(5,10,15 minutes) in a waterbath, and the tubes were cooled in an ice bath immediately. The samples were then transfered to cuvette containing 0.1 ml 0.1M catechol and 2.8 ml 0.1 M phosphate buffer. The percentage residual PPO activity was calculated in comparison with unheated enzyme.

Enzyme kinetics and substrate specificity

Michaelis-Menten constant (*Km*) and maximum velocity (*Vmax*) values for PPO were calculated using the substrates catechol (1-22 mM) and 4-methylcatechol (2-18 mM) under the optimized pH and temperature conditions. *K*m and *V*max values of PPO for each substrate were obtained from a plot of 1*/V* versus 1*/*(S) by the method of Lineweaver and Burk (18). Measurements were carried out in triplicate.

Polyacrylamide gel electrophoresis and zymography

The PAGE to determine the purity and apparent molecular weight of the PPO in corn tassel was performed at 4 ºC under mild denaturing conditions using two parallel continuous 7 % gels in Tris-glycine buffer, pH=8.9 according to the method of Laemmli (19). The samples were prepared under partially denaturing conditions by mixing them with a certain voume of loading buffer 1 % SDS, 20 % v/v glycerol and 100 mM Tris, pH 6.8. The enzyme samples were not heated or reduced. Following electrophoresis, Coomassie Brilliant Blue (CBB) R-250 was used to detect the stained protein bands. After staining, the gels were destained in 7 % acetic acid solution and then photographed. The apparent molecular weight of the enzyme was estimated using prestained molecular weight markers (Sigma SDS7B2): triosephospate isomerase (26.6 kDa), lactic dehyrogenase (36.5 kDa), fumarase (48.5 kDa), pyruvate kinase (58 kDa), lactoferrin (90 kDa), β -galactosidase (116 kDa), α_2- macroglobulin (180 kDa).

For zymography of PPO, the gel was incubated in 0.1 M phosphate buffer (pH=8.0) containing 30 mM of catechol for 100 minutes at 40 ºC. Staining bands indicating PPO activity appeared within 5 min after adding 1mM ascorbic acid. The gel was rinsed in distilled water and stored in 30 % ethanol.

Effects of inhibitors

The effects of several inhibitors(EDTA, SDS, sodyum azide) on corn tassel PPO activity were studied by measuring PPO activity in the standard reaction medium in the presence or absence of the given inhibitor concentrations. PPO activities were measured at two constant inhibitor (0.5 and 2 mM) concentrations with substrate concentrations (4 mM). The percent inhibition was calculated using the equation:

Inhibition (%) = (1 - (*Ai/A0*))x 100, where *A0* is the initial PPO activity (without inhibitor), and *Ai* is the PPO activity with inhibitor.

Effects of carbohydrates

The effects of several carbohydrates (sucrose, ribose, fructose, glucose) on corn tassel PPO activity were studied at two constant carbohydrates (0.5 and 2 mM) concentrations with substrate concentrations (4 mM).

Results and Discussion

Purification of PPO

At subsequent steps of purification, PPO spesific

Table 1. The purification of PPO from corn tassel.

Steps	Volume (mL)	Total Protein (mg)	Total Activity (U)	Specific activity (U/ mg protein)	Purification (fold)	Recovery (%)
Crude enzyme	**16**	**12,43**	**3280**	**263,87**	**1**	**100**
$(NH_4)_2SO_4$precipitation,dialysis and ultrafiltration	1,5	3	**2311,5**	770,5	3	70,4
Sephadex G-100	0,75	0,232	**606**	2612,06	10	18,47

activity increased and as expected the protein content decreased to a great extent at the final stage. As can be seen in Table 1, the purification steps of corn tassel PPO include ammonium sulphate precipitation, dialysis and ultrafiltration, and finally gel filtration chromatography by a Sephadex G-100 column. The specific activity of the purified PPO was determined as 2612.06 U/mg, with a purification fold of 10 and a recovery of 18.47 %. The SDS –PAGE of the purified sample was shown in Figure 8. In a previous study, the specific activity of the purified PPO from atemoya fruit was determined as purification fold of 7.12 and a recovery of 3.20 % (20).

Temperature and pH effect on polyphenol oxidase activity

pH value is important as it can change charge state of substrate molecule and enzyme molecule, which in turn affect combination of enzyme and substrate (21). The pH optimum was found to be 8.0 for catechol and 6.0 for 4-methylcatechol as shown in Fig.1 and 2, respectively. This value is the same as the optima reported for PPO from kiwi at pH 8.0 (22). However, for brocolli florets, the optimal pH was 5.72 for both subsrates catechol and 4-methylcatechol (23).

The temperature profiles for catechol and 4-methylcatechol by the corn tassel PPO are shown in Fig.3 and 4, respectively. Optimum temperature of the activity is also affected by the substrate used in the assay. The

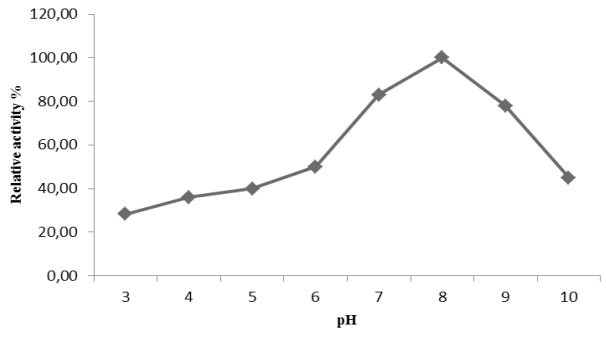

Figure 1. Effect of pH on PPO with catechol as substrate.

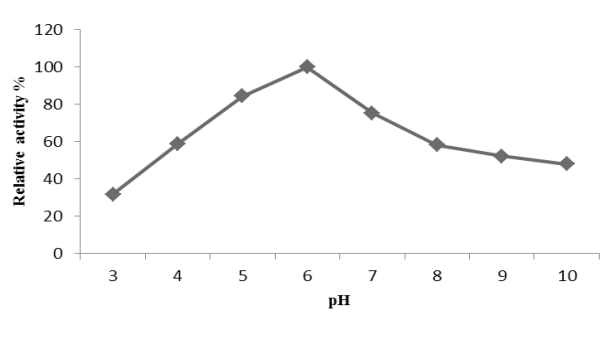

Figure 2. Effect of pH on PPO with 4-metilcathecol as substrate.

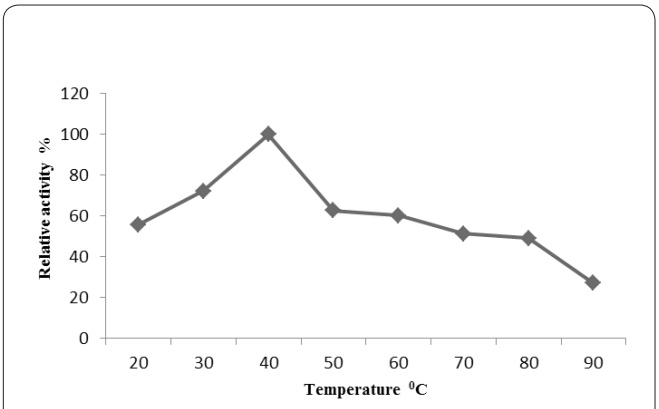

Figure 3. Effect of temperature on PPO with cathecol as substrate.

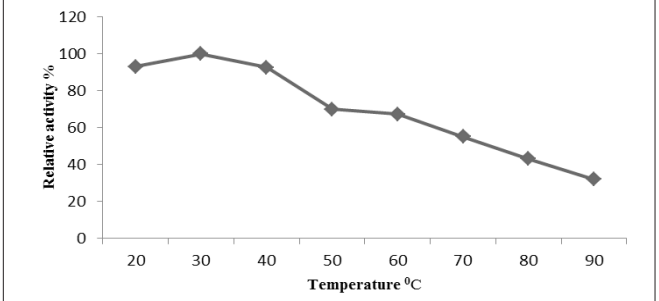

Figure 4. Effect of temperature on PPO with metilcathecol as substrate.

optimum temperature was found to be 40 °C for catechol and 30 °C for 4-methylcatechol. Similar reports for the optimal temperature and optimal pH have been presented. The previous studies also raported that optimum temperature values were 40 ºC for artichoke (24), Barbados cherry (25) , Cape gooseberry (26) and 45 ºC for chufa corns (27), using catechol as a substrate; 30 ºC for aubergine (28) , 56 ºC for Amaya apple (29) using 4-methylcatechol as a substrate.

Thermal stability

The thermal stability of the PPO obtained from corn tassel was investigated. Corn tassel PPO has thermal stability compared with other vegatable PPO. In water bath for 10 min at 60 ,70 and 75 °C, residual activities wereabout 60 %, 41 % and 34 %, respectively.When temperature was 75 °C, PPO residual activities was found as 30 % after 15 min (Fig. 5). It can clearly be seen that it is reduced rapidly when the tempureture was increased up to 75 °C. This result is similar to that of the PPO from lotus seed (30) and lemon balm (31).

Kinetic characteristics of PPO using different substrates

For the determination of Michaelis-Menten constant (*Km*) and maximum velocity (*Vmax*) values of the en-

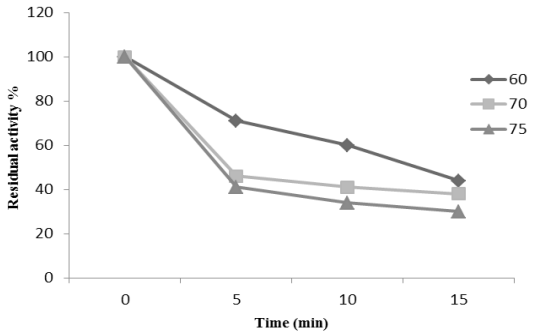

Figure 5. Thermal Stability of Corn Tassel Polyphenol Oxidase.

zyme, PPO activities were measured using the concentrations of catechol (1-22 mM) and 4-methylcatechol (2-18 mM) as substrates under optimized pH and temperature conditions. A plot of 1/*V* versus 1/(S) was drawn by the method of Lineweaver and Burk(18) to calculate *Km* and *Vmax* values of corn tassel PPO for each substrate. The data obtained on the Lineweaver-Burk plot was used to determine substrate specificity (*Vmax/Km*). The *Km* and *Vmax* values obtained from the plot analysis of PPO were found as 3.48 mM and 1.0 Abs./dak. for catechol and 8 mM and 1.04 Abs./min. for 4-methylcatechol, respectively (Fig. 6 and 7). When the *Vmax* and *Vmax/Km* values for two substrates were compared, the *Vmax* and *Vmax/Km* value for catechol was higher than 4-methylcatechol. Consequently, catechol was used as a substrate in the following kinetic studies.

There have been many studies reported on the kinetics of PPO in different plant species, using catechol and 4-methylcatechol as substrates. *Km* values were found to vary in Hemşin apple (32) and Chinese Toon (33) as 3.40 mM and 10.059 mM, respectively. When using 4-methylcatechol as the substrate for PPO, *Km* values were calculated as 3.14 mM in mango (34) and 18.2

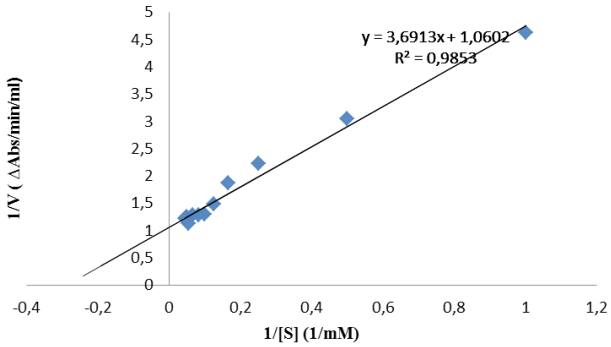

Figure 6. Lineweaver-Burk plots for PPO activity with catechol as subsrate.

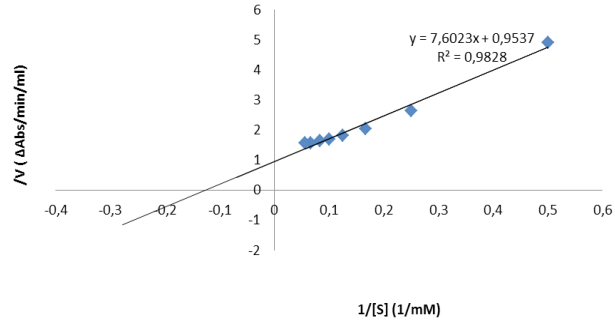

Figure 7. Lineweaver-Burk plots for PPO activity with metilcathecol as subsrate.

Table2. Effect of various agents on polyphenol oxidase activity.

Inhibitors	Concentration	Inhibitione (%)
EDTA	0.5 mM	16
	2 mM	22.2
SDS	0.5 mM	6.7
	2 mM	12.2
Sodium azide	0.5 mM	11.1
	2 mM	26.6

Table 3. Effect of various carbohydrates on polyphenol oxidase activity.

Carbohydrate	Concentration	Activation (%)
Glucose	0.5 mM	12
	2 mM	16
Sucrose	0.5 mM	4
	2 mM	4
Fructose	0.5 mM	4.5
	2 mM	17
Ribose	0.5 mM	12.6
	2 mM	17

mM in Jackfruit (15).

Effect of inhibitor

The present study is the first report on the inhibition of PPO activity in corn tassel. The effects of several inhibitors (EDTA, SDS and sodyum azide) on PPO activities were measured at two constant inhibitor concentrations with catechol concentrations (4 mM). In this work, sodium azide, EDTA and SDS were found to inhibit the enzyme activity as 26.6 %, 22.2 % and 12.2 %, respectively (Table 2). Similar results were also obtained for the PPO from sweet pepper (35). It is well known that the active site of polyphenol oxidase contains copper ions which are involved in catalytic activity and thus chelating compounds would inhibit polyphenol oxidase activity by removing copper ions (36). EDTA is a chemical preservative permitted for food industry use (37).

Effects of carbohydrates

The effects of several carbohydrates (sucrose, ribose, fructose and glucose) on corn tassel PPO activity were studied and found that the enzyme is activated by fructose, ribose, glucose and sucrose. The enzyme is activated at the rate of 17 %, 16 % and 4 % by glucose, fructose and ribose, respectively (Table 3).

Polyacrylamide gel electrophoresis

Partially denaturing SDS-PAGE showed that the molecular weight of the PPO from corn tassel was found to be around 158 kDa determined by Commassie staining and by zymogram analysis showing the presence of PPO activity (Fig.8). There are reports in the literature that PPO from various plants have moleculer weight varying from 31 kDa to144 kDa. (26,16,38).

Conclusions

In conclusion, PPO is a very important enzyme that is responsible for the enzymatic browning of vegetables and fruits, which is undesired process and need to be prevented in food technology. This work reports the

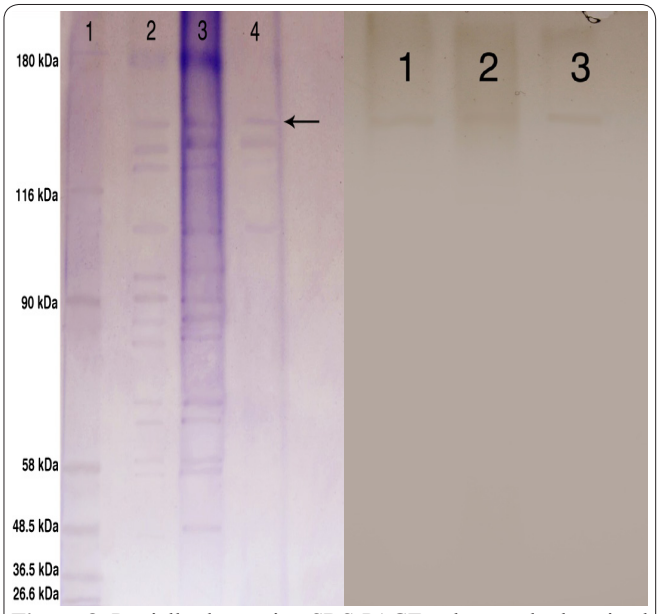

Figure 8. Partially denaturing SDS-PAGE gels were both stained with Coomassie Brilliant Blue(CBB) R-250 (Part a) and werestained for zymography of PPO activity using 30 mM of catechol (Part b). Lane 1 in Part a contains the prestained molecular weight markers (Sigma SDS7B2). Lanes 2, 3,4 in Part a and 1, 2 and 3 in Part b represent crude extract, ammoniumsulfate-dialysis and gel filtration samples loaded, respectively.

purification through $(NH_4)_2SO_4$ precipitation and gel filtration chromatography and characterization of PPO from corn tasselfor the first time. The pH and temperature optima were found to be 8.0 and 40 °C for catechol, 6.0 and 30 °C for 4-methylcatechol, respectively. The apparent molecular weight of the PPO from corn tassel was determined as high as 158 kDa by partially denaturing SDS-PAGE. In this work, sodium azide, EDTA and SDS were found to inhibit the enzyme activity by 26.6 %, 22.2 % and 12.2 %, respectively, but the enzyme is activated by fructose, ribose, glucose and sucrose. To prevent the enzymatic browning of corn tassel in industry, it might be advatageous to be held in some PPO inhibitors and some carbohydrates tested in this study.

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