PARTIAL PURIFICATION AND CHARACTERISATION OF POLYPHENOL OXIDAZE FROM SWEET BELL PEPPER (CAPSICUM ANNUUM) SEEDS

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Abstract: Polyphenol oxidase (PPO) from sweet bell pepper seeds was extracted and partially purified through $(NH_4)_2SO_4$ precipitation, dialysis and gel filtration chromatography. The optimum temperature and pH values were 40 and 30 ˚C, 5.0 and 7.0 for 4-methyl catechol and catechol, respectively. Thermal inactivation of PPO was investigated at 55, 65, 75, 85 ˚C. Kinetic parametres, *Km* and *Vmax* were calculated from Lineweaver-Burk graph. The PPO activity was inhibited by SDS, sodyum azide and askorbic acid. The enzymatic properties of PPO in this study may lead to practical application for inhibition of the PPO activity and thus preventing enzymatic browning in the process of picking and storage of pepper seeds.
Keywords: Characterisation; Enzyme inhibition; Pepper seeds; Polyphenol oxidase; Purification.

Introduction

Polyphenol oxidases (PPO), (EC 1.14.18.1) belong to a set of copper containing metalloenzymes that are members of oxido reductases, which catalyze the oxidation of a wide range of phenolic compounds by utilizing molecular oxygen (Queiroz et al 2008).

Polyphenol oxidases can be found in almost all living organisms including plants, animals, fungi and bacteria. PPO is of importance because it is responsible for melanization of eye, skin, hair, and fruits and vegetables browning (Marín-Zamora et al., 2005). It is well known that the browning causes nutrient loss in tropical fruits by more than fifty per cent, resulting in reduction in the food quality, favour and taste of vegetables and fruits (Mayer, 2006).

PPO has been characterised in a wide variety of plants including lotus seed (Cai et al., 2015), African mango seeds (Sanni, 2016), grapes (Nunez-Delicado et al., 2007), potato (Lourenço et al., 1992), apricot (Arslan et al., 1998), banana (Ünal, 2007), broccoli (Gawlik-Dziki et al., 2007), mango (Palma-Orozco et al., 2013), eggplant (Mishra et al., 2012), corn tassel (Gul Guven et al., 2016), Ataulfo mango (Cheema and Sommerhalter, 2015), parsley (Gul Guven et al, 2017).

The bell pepper (*Capsicum annum* L.) is a species belong to the Solanaceae family. Pepper is widely cultivated in Asia, Mediterranean countries and Africa (Silva et al., 2013). The bell pepper comprises of various bioactive compounds along with significant amounts of beta-carotene and other similar compounds (Sun et al., 2007).

To our best knowledge, there is no any study on the PPO purification and characterisation from pepper seeds to understand and prevent the browning caused by the enzyme in food technology.

Material and Mehods

Materials and Reagents

The pepper used in this study was obtained from a local supermarket in Diyarbakir City, Turkey. It was frozen at -25 °C until used. The substrate catechol was a product of Merck (Darmstadt, Germany). Ammonium sulphate, polyethylene glycol (PEG), 4-methylcatechol, and all chemicals for electrophoresis studies were purchased from Sigma Chem. Co. All chemicals used in this study were of analytical grade.

Enzyme purification

16 grams of pepper seeds were homogenized using a blender for 10 min in the extraction solution (0.1 M phosphate buffer at pH 6.5 containing 4% PEG and 10 mM ascorbic acid). Double layered filter paper was used to filter the homogenate. The centrifugation of extract samples was carried out at 15000 g for 20 min at 4ºC. 60 % Ammonium sulphate, $(NH_4)_2SO_4$ saturation was obtained by adding solid ammonium sulphate slowly to the supernatant under cold conditions. The centrifugation was carried out at 15000 g for 30 min to separate the precipitated proteins. Enzyme extract was then re-dissolved in a small volume of buffer (0.05 M phosphate, pH

6.5) and finally dialysed at 4ºC in the same buffer overnight with three changes of buffer during whole process. The samples as enzyme source were kept at 4ºC until use in the following experiments.

Enzyme activity
PPO activity was determined using a spectrophotometric method based on the initial rate of increase in absorbance at 420 nm. PPO assay was carried out in 3 ml reaction mixture containing 0.1 ml of substrates, catechol or 4-methylcatechol (0.1 M) and 0.1 ml of enzyme sample in phosphate buffer (0.1 M, pH 6.5). The PPO activity was measured at 420 nm using a spectrophotometer. The blank consisted of 2.9 ml buffer and 0.1 ml substrate. One unit of enzymatic activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 per min.

Protein determination
Protein content was determined for every purification step by Bradford (1976) method using bovine serum albumin as the standart**.**

Effect of temperature and pH on PPO activity
Different temperatures in the range of 20-90°C were tested in order to determine the optimum temperature for PPO activity using catechol and 4-methylcatechol as substrate. The optimum pH was investigated using various buffers: 0.1 M sodium acetate buffer (pH 3-5) and 0.1 M sodium phosphate buffer (pH 6.0-10.0). The optimum pH which corresponds to the highest PPO activity was used for the study in order to determine the effect of inhibitors and temperature on enzyme activity.

The thermal stability of the PPO
The purified enzyme was examined at 55, 65, 75, 80 °C for various times (5,10 and 15 min) to determine the thermal stability of the PPO, after which the residual activity was measured under standard condition.

Substrate specificity
Michaelis-Menten constant (*Km*) and maximum velocity (V_{max}) values of PPO were calculated using the substrates catechol (1-15 mM) and 4-methylcatechol (1-18 mM) under the optimized pH and temperature conditions. A plot of 1/*V* versus 1/[S] by using Lineweaver and Burk (1934) method was utilised to obtain *K*m and *V*max values of PPO for each substrate.

Effect of inhibitors on enzyme activity
The effects of several inhibitors (ascorbic acid, sodyum azide, SDS and EDTA) on PPO activity were determined. PPO activities were measured at two constant inhibitor concentrations (0.5-2 mM) with 10 mM concentration of 4-methyl catechol as substrate.

Results and Discussion

Extraction and partial purification of PPO
Table 1 shows the purification steps of pepper seed PPO which include precipitation by ammonium sulphate, dialysis and size-exclusion chromatography. As can be seen from Table 1, PPO spesific activity increased at subsequent steps of purification. At the mean time, the protein content highly decreased at the final stage as expected. In this study, the purification level obtained was 2.23-fold with 10 % recovery of PPO activity (Table 1). In a previous study, purification fold of 10 with a recovery of 18.47 % for PPO in corn tassel was obtained (Gul Guven et al., 2016).

Effect of pH and temperature
As can be seen in Figures 1 and 2, the optimum pHs for catechol and 4-methyl catechol were found to be 7.0 and 5.0 for, respectively. Most of fruits and vegetables show maximum activity near neutral pH value (Sakiroglu et al., 2013). Several previous studies also reported that optimum pH values for PPO in various plants were 7.0 for lotus seed (Cai et al., 2015), Jackfruit (Tao et al., 2013), Amasya apple (Oktay et al., 1995), artichoke (Doğan et al., 2005), pH 7.2 for Barbados cherry (Kumar et al., 2008), pH 8.0 for corn tassel (Gul-Guven et al., 2016), using catechol as a substrate, whereas pH 5.4 for Ataulfo mango (Cheema and Sommerhalter, 2015), pH 4.5 for strawberry (Wesche-Ebeling and Montgomery, 1990) using 4-methyl catechol as a substrate.

Steps	Volume (ml)	Total Protein (mg)	Total Activity	Specific activity (unit/mg protein)	Purification (fold)	Recovery (%)
Crude enzyme	17,5	11.20	3344	298.5	1	100
$(NH_4)_2SO_4$ precipitation and dialysis	6	4.7	2270	483	1.61	67.8
Gel filtration chromatography	3	0.5	333	666	2.23	10

Table 1 . The purification of PPO from pepper seeds

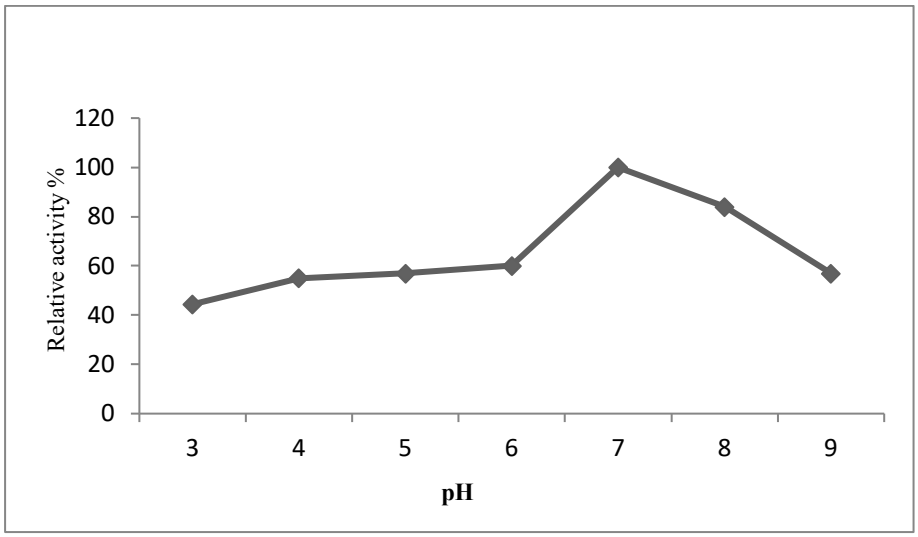

Figure 1. Effect of pH on PPO with catechol as substrate.

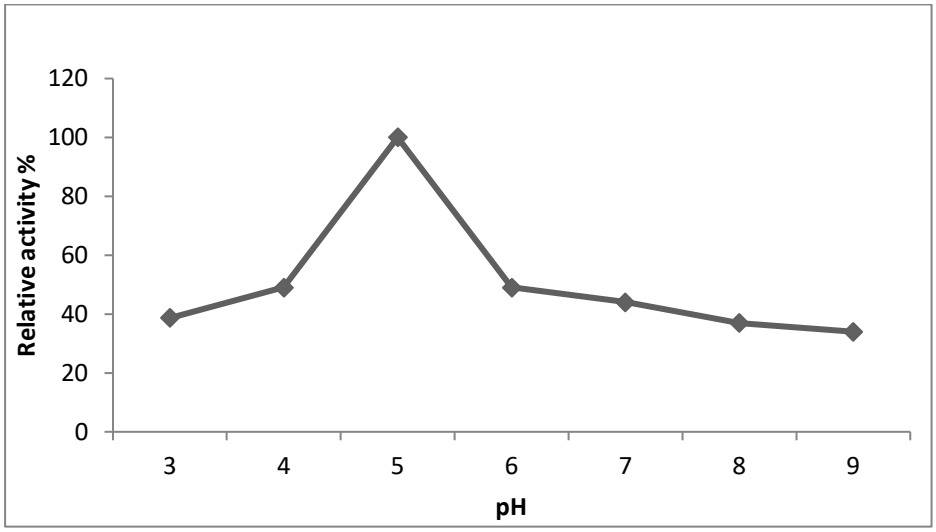

Figure 2. Effect of pH on PPO with 4-metilcathecol as substrate**.**

Optimum temperature activities were found as 30°C and 40°C for catechol and 4-methyl catechol as substrates, respectively (Figures 3 and 4). It has been previously reported that different plants exhibited different optimum temperatures. The examples are 40 ºC for artichoke (Doğan et al., 2005), 35 ºC for mamey (Palma-orozco et al., 2011) and 20 ºC for lotus seed (Cai et al., 2015) using catechol as the substrate, while 56 ºC for Amasya apple (Oktay et al., 1995) and 30 ºC for aubergine (Dogan et al., 2002), using 4-methyl catechol as the substrate.

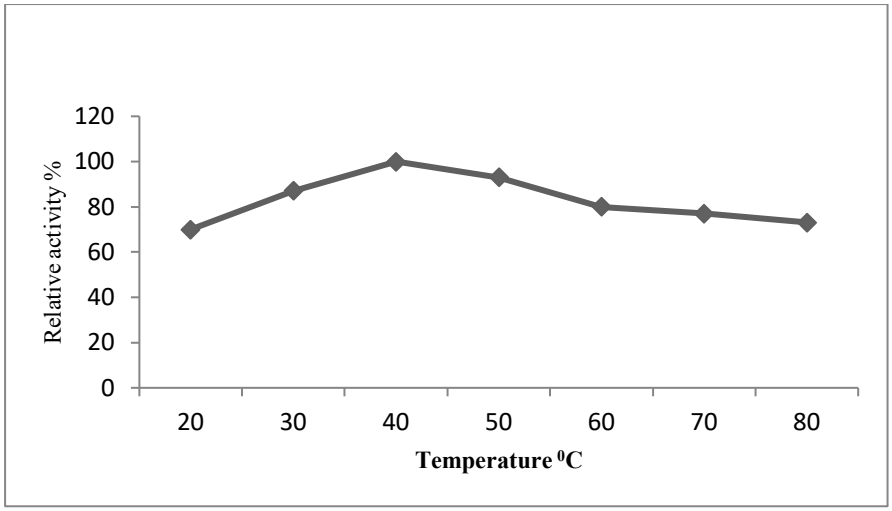

Figure 3. Effect of temperatureon PPO with cathecol as substrate**.**

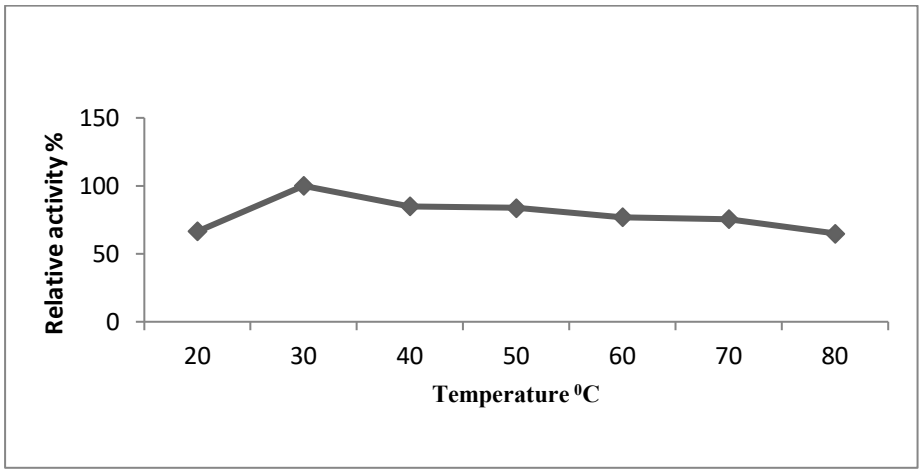

Figure 4. Effect of pH on PPO with 4-metilcathecol as substrate.

Kinetic characteristics of PPO using different substrates

For the determination of Michaelis-Menten constant (*Km*) and maximum velocity (*Vmax*) values of the enzyme, PPO activities were determined using the concentrations of the substrates catechol (1-15 mM) and 4-methylcatechol (1-18 mM) under optimized temperature and pH conditions. The values of Km and Vmax calculated from the plot analysis of polyphenol oxidase were 0.83 mM and 0.3317 abs/min for catechol, 0.732 mM and 0.5060 abs/min for 4-methylcatechol, respectively. 4-Methylcatechol seemed to be the best substrate as the comparison of the Vmax and *V*max/*K*m for two substrates tested showed a higher value for 4-methylcatechol, which was used at subsequent experiments.

Many studies have been carried out about PPO kinetics in different plants, using catechol and 4-methyl catechol as substrates. When using catechol as the substrate, Km values for PPO of Jackfruit (Tao et al., 2013), mamey (Palma- Orozco et al., 2014), Chinese Toon (Wang et al., 2013) were found as 8.2 mM, 44 mM and 10.059 mM, respectively. However, Km values were 3.14 mM in mango (Palma- Orozco et al., 2014), 18.2 mM in Jackfruit (Tao et al., 2013) and 24.6 mM in De Chaunac grape (Lee et al., 1983) when 4-methyl catechol used as the substrate.

Effect of inhibitor

The effects of some inhibitors such as sodyum azide, ascorbic acid, SDS and EDTA on polyphenoloxidase activity were examined at two constant inhibitor concentrations using 4-methyl catechol as substrate. The PPO activity was inhibited by the concentrations of EDTA, SDS, sodyum azide and ascorbic acid tested. The most effective inhibitors were askorbic acid and sodyum azide.

The thermal stability of the PPO

PPO stability decreased with the temperature increase. All temperature treatments, except of 85 °C, could not completely inactivate the PPO activity. PPO from different sources exhibit different heat resistance. Sanni (2016) reported that enzyme from two species of African Mango was thermally stable at 40 ˚C and 50 ˚C.

Conclusions

The aim of this research is to refine and characterise the PPO enzyme in the seed of green pepper grown in The Southeastern Anatolia in Turkey. For this aim, the enzyme isolated from the seed of pepper was refined later with ammonium sulfate sedimentation, dialysis, ultrafiltration and gel filtration chromatography. Kinetic features of enzyme, the optimum pH and temperature, temperature inactivation and the effects of some inhibitors have been studied.

Polyphenol oxidase extracted from seed of green pepper was purified by 2.23 fold with 10 % protein yield. Optimum temperature activities were for 30°C and 40°C for catechol and 4-methyl catechol as substrates, respectively. The optimum pH values were 7.0 and 5.0 for catechol and 4-methyl catechol respectively. 4-Methylcatechol was more preferred by the enzyme, when compared to catechol, as shown by the Lineweaver-Burk analysis. The results also showed that, EDTA did not have any affect on PPO activity, wheras the enzyme inhibition was 64.4 % by sodium azide, 36.2 by % SDS and 65.7 % by asorbic acid. The PPO was found to be stable to heat up to 85 °C.

The enzymatic characteristics of PPO could provide practical application in inhibiting the PPO activity to understand and prevent the enzymatic browning in the process of picking and storage of pepper seeds. To prevent the enzymatic browning of seeds of green pepper in industry, it might be advatageous to treat the seeds with some PPO inhibitors defined in this study.

References

Arslan, O., Temur, A., Tozlu I. (1998). Polyphenol oxidase from Malatya apricot (Prunus armeniaca L.). J Agric Food Chem, 46, 1239-1241.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72, 248–253.

Cai, X.X., Hong, Y.X., Wang, S.Y., Zhao, L.Na., Rao, P.F. (2015). Purification and enzymatic characteristics of a novel polyphenol oxidase from lotus seed (*Nelumbo nucifera Gaertn*. International Journal of Food Science and Technology. 50, 1026–1032

Cheema, S., Sommerhalter, M. (2015) .Characterization of polyphenol oxidase activity in Ataulfo mango. Food Chemistry, 171, 382-387.

Dogan M.; Arslan O.; Dogan S. (2002). Substrat specifity, heat inactivation and inhibition of polyphenol oxidase from different aubergine cultivars. International Journal of Food Science and Technology; 37, 415–423

Dogan, S., Turan, Y., Erturk, H., Arslan, O. (2005). Characterization and purification of polyphenol oxidase from artichoke (Cynara scolymus L.). Journal of Agriculture and Food Chemistry; 53, 776–785.

Gawlik-Dziki, U., Szymanowska, U., Baraniak, B. (2007). Characterization of polyphenol oxidase from broccoli (Brassica oleracea var. botrytis italica) florets. Food Chem, 105: 1047-1053. 31.

Gul Guven, R., Aslan, N., Guven, K., Matpan Bekler, F., Acer, O. (2016). Purification and characterization of polyphenol oxidase from corn tassel. Cellular and Molecular Biology.62, 13, 6-11.

Kumar V.B., Mohan K., Murugan K. (2008). Purification and kinetic characterization of polyphenol oxidase from Barbados cherry (Malpighia glabra L.), Food Chemistry, 110, 328–333.

Lee, CY., Smith, NL., Pennesi, AP. (1983). Polyphenoloxidase from De Chaunac grapes. J Agric Food Chem 34, 987–991.

Lineweave H Burk., D. (1934). The determination of enzyme dissociation constants. J Am Chem Soc 56, 658–660.

Lourenço E.J., Neves V.A., Da Silva M.A. (1992). Polyphenol Oxidase from Sweet Potato: Purification and Properties. J Agric Food Chem, 40, 2369-2373.

Marín-Zamora, M.E., Rojas-Melgarejo, F., García-Cánovas, F., García-Ruíz, P.A. (2005). Cinnamic ester of D-sorbitol for immobilization of mushroom tyrosinase. Journal of Chemical Technology and Biotechnology, 80,1356–1364.

Mayer, A. M. 2006. Polyphenol oxidases in plants and fungi: Going places? A review. Phytochemistry, 67, 2318-2331.

Mishra, B., Gautam, S., Arun, Sharma A. (2012). Purification and characterization of polyphenol oxidase (PPO) from eggplant (Solanum melongena). Food Chemistry, 134, 1855–1861.

Nunez-Delicado, E.; Serrano-Megias, M.; Perez-Lopez, A.J.; Lopez-Nicolas, J.M. (2007). Characterization of Polyphenol Oxidase from Napoleon Grape. Food Chemistry, 100, 108–114.

Oktay, M., Küfrevioğlu, I., Kocaçalişkan, I., Şakiroğlu, H. 1995. Polyphenoloxidase from Amasya apple. J Food Sci 60, 494–496.

Palma-Orozco, G., Marrufo-Hernández, NA., Sampedro, JG., Nájera, H. 2014. Purification and partial biochemical characterization of polyphenol oxidase from mango (Mangifera indica cv. Manila).J Agric Food Chem., 8, 62(40):9832-9840.

Palma-Orozco, G., Ortiz-Moreno, A., Dorantes-Alvarez ,L., Sampedro, JG., Najera, H.2011. Purification and partial biochemical characterization of polyphenol oxidase from mamey (Pouteria sapota). Phytochemistry, 72, 82–88.

Palma-Orozco, G., Marrufo-Hernández, NA.; Sampedro, JG.; Nájera, H. 2014.Purification and partial biochemical characterization of polyphenol oxidase from mango (Mangifera indica cv. Manila).J Agric Food Chem.; 8, 62 (40):9832-9840.

Queiroz, C.; Lopes, M. L. M.; Fialho, E.; Valente-Mesquita, V. L. 2008. Polyphenol oxidase: Characteristics and mechanisms of browning control. Food Reviews International. 24 (4), 361-375.

Guven, R.G., Guven, K., Bekler, F.M., Acer, O., Alkan, H., Dogru, M. 2017. Purification and characterization of polyphenol oxidase from purslane Food. Sci. Technol., 37(3), 356-362.

Sakiroglu, H., Yılmaz, E., Erat M., Öztürk, A.E. 2013. Selected properties of polyphenol oxidase obtained from ispir sugar bean .International Journal of Food Properties, 16, 1314–1321.

Sanni, D.M. 2016. Isolation, partial purification and characterization of polyphenoloxidase from two species of African Mango seeds (*Irvingia gabonensis* and *Irvingia wombolu*).Advances in Biochemistry. 4(4), 47-52.

Silva, LR., Azevedo, J., Pereira, MJ., Valentão, P., Andrade, PB. 2013. Chemical assessment and antioxidant capacity of pepper (Capsicum annuum L.) seeds. Food Chem Toxicol 53, 240-248.

Sun, T., Xu, Z., Wu, CT., Janes, M., Prinyawiwatkul, W., No, HK. 2007. Antioxidant activities of different colored sweet bell peppers (Capsicum annuum L.). J Food Sci, 72, S98- 102.

Tao, YM., Yao, LY., Qin, QY., Shen, W. 2013. Purification and characterization of polyphenol oxidase from jackfruit (Artocarpus heterophyllus) bulbs. J Agric Food Chem;; 61, 12662-9.

Unal, M.U. 2007. Properties of polyphenol oxidase from Anamur banana (Musa cavendishii). Food Chem, 100, 909-913.

Wang, C., Zhang, JF., Zhang, Y., Cheng., Bing. 2013. Characterization and Inhibitors of Polyphenol Oxidase from Chinese Toon. Food Biotechnology,27 (3), 261-278.

Wesche-Ebeling, P. & Montgomery M. W. 1990. Strawberry polyphenol oxidase: Purification and characterization J. Food Sci. 55, 1315-1319.

