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Extraction, Purification and Characterization of Polyphenol Oxidase from Tobacco Grown in Northern Iran

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Polyphenol oxidase plays a key role in plant defense systems. Screening of tobacco (*Nicotiana tobacum*) leaves grown in Northern Iran demonstrated that there was a polyphenol oxidase with some similarities with polyphenol oxidases from other sources. Using the fresh leaves of the plant as a source, the polyphenol oxidase content was isolated, purified and characterized. The specific activity of polyphenol oxidase was increased at various stages of purification process. Its optimum pH and temperature were similar to those reported for polyphenol oxidase from citrus grown in other parts of the world.

Key words: Polyphenol oxidase, *Nicotiana tobacum*, tobacco, enzyme extraction, purification.

Polyphenol oxidases (PPOs, E.C. 1.14.18.1) are a group of copper containing proteins widely distributed in bacteria, fungi, higher plants and mammals[1]. They are important members of '*Oxidase*' super-family occurring in various isoforms such as immature, mature latent and active forms. PPOs catalyse the oxidation of hydroxyphenols to their quinine derivatives, which then spontaneously polymerize. There are three types of proteins related to PPOs including catecholase, laccase and cresolase[2]. In general, PPOs catalyze the hydroxylation of monophenols to o-diphenols and the oxidation of o-diphenols to o-quinones that eventually form melanin pigments after other stages of oxidation and decarboxylation (Fig. 1). Melanin formation is the most important function of PPOs in plants and animals. Melanin pigments cause undesirable darkening of the skin in animals. On the other hand, enzymatic browning in cut vegetables and flowers and raw fruits is also the result of PPOs action[3]. Due to their ability for oxidation of phenols, PPOs are efficient reagents used for cleaning wastewater that contain polyphenols[4,5].

The importance of PPOs in tobacco and tea industry is due to their effect on taste and color of the leaves by oxidizing polyphenols present in the plant parts. The aim of the present work was the design of an effective method for separation and purification of PPO from fresh leaves of

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tobacco (*Nicotiana tobacum*). Some characteristics of the enzyme including optimum pH and temperature as well as molecular weight were obtained and compared with some other PPOs reported in literature[6-8].

MATERIAL AND METHODS

Material

Fresh and healthy leaf samples were harvested directly from tobacco plants grown at tobacco field of the centre for research on tobacco and control its commercial standards located in Rasht at the North of Iran 40 km from Caspian Sea. Mushroom PPO was purchased from Sigma, Sephadex G-75 obtained from Amersham Pharmacia Biotech Corporation. Electrophoresis reagents and other chemicals such as 3-methyl-2-benzothiazolinon hydrazone (MBTH), dimethyl foramide (DMF) and ammonium sulphate were purchased either from Sigma or from Merck and were of analytical grade.

Selection of tobacco leaf samples

Fresh, young and healthy (undamaged) leaves of tobacco plant were harvested and delivered to the laboratory within three to four hours. They were washed and stored at 4°C for at least 24 hours.

Preparation of leaf extract

Five grams leaves of tobacco plant were homogenized in a porcelain mortar using liquid nitrogen. 10 ml of 0.05 M phosphate buffer solution (pH 6.8) was added and mixed thoroughly. The resulting solution was centrifuged at 6500 rpm at 4°C for 20 minutes. The pellet was discarded and the supernatant containing crude enzyme was used for further purification. The biological activity of PPO was measured in the presence of its substrate, dopamine hydrochloride.

Enzyme assay

Polyphenol oxidase activity was determined spectrophotometrically using dopamine hydrochloride as its substrate. 10 ml of enzyme solution was added to 990 ml of 0.5 M phosphate buffer containing the substrate (55mM), MBTH (5 mM), DMF (2% v/v) and phosphoric acid (0.08% v/v). The absorbance change at 505 nm was then measured at 25°C.

One unit of activity (U) is defined as the amount of polyphenol oxidase oxidizing 1 mmole of substrate per minute under standard conditions. Specific activity is expressed as units of activity per mg of protein.

Enzyme purification

The tobacco leaves (300 grams) were homogenized in a porcelain mortar using liquid nitrogen. 200 ml of 0.05 M phosphate buffer solution (pH 6.8) was added and mixed thoroughly. The resulting solution was centrifuged at 6500 rpm at 4C for 20 minutes. The pellet was discarded and the supernatant containing crude enzyme was extract fractionated by adding ammonium sulphate, $(NH_4)_2SO_4$ to 85%. The temperature was kept at 4°C and the pH controlled using 1N ammonia solution. To precipitate all proteins, the sample was mixed for another 30 minutes followed by centrifugation at 5000 rpm at 5°C for 30 minutes. 2 ml of 0.2 M phosphate buffer was then added to the precipitate and mixed well. The mixture was centrifuged again under the same conditions for 10 minutes in order to eliminate ammonium salts. 2 ml of 10 mM phosphate buffer was added to the resulting precipitate.

The dialysis bags with pores less than 12000 daltons were boiled for 5 minutes in a solution containing 100 mM sodium bicarbonate (25 ml) and 10 mM EDTA (25 ml) prior to dialysis process. The bags were then transferred to deionized water and boiled for another 5 minutes. The crude enzyme solution was dialyzed 3 times against 500 ml phosphate buffer (pH 6.5) for 8 hours. The resulting polyphenol oxidase solution was applied to a Sephadex G-75 column (1 50 cm) equilibrated with the same buffer. The protein samples were eluted using the phosphate buffer in fractions about two times of the sample solution. The protein fractions were collected and tested for total protein concentration using UV absorbance at 280 nm. The polyphenol oxidase activity was also measured in all fractions and PPO active fractions with were kept at -20°C for further purification process. In order to achieve a pure form of the enzyme, gel filtration chromatography was followed by anion exchange chromatography.

Anionic chromatography

BioRad Fast Protein Liquid Chromatography (FPLC) equipment with UNO-

Q1 Q-Sepharose column was used for anion exchange chromatography of polyphenol oxidase. The protein solution obtained from gel filtration was centrifuged for 5 minutes at 12000 rpm. 100ml of the sample was applied to the column and the program was set. Polyphenol oxidase activity was measured in the elution fractions and the active fractions were stored at -20°C for electrophoresis.

Thermal stability

Thermal stability of purified polyphenol oxidase was measured at temperature range of 15-55 °C. 10 ml of enzyme solution, 990 ml of 0.5 M phosphate buffer containing the substrate, MBTH, DMF and phosphoric acid were incubated in the desired temperature and the absorbance change at 505 nm was measured.

Optimum pH

To measure the optimum pH, a range of citrate buffers with pH ranging from 3 to 12 were prepared (Table 1). 200 ml of each buffer were mixed with 750 ml substrate and any change in the pH corrected using sodium hydroxide or HCl. 50 ml of the enzyme solution was added and the absorption at 505 nm was recorded instantly.

Analytical methods

The purity and molecular weight of the extracted enzyme was determined by sodium dodecyl sulphate polyacryl amide gel electrophoresis (SDS-PAGE). Electrophoresis was performed under denaturing conditions as reported before[9]. 20 ml of the purified enzyme solution was mixed with 5 ml of sample buffer and boiled in water bath for 5 minutes. 20 ml of this processed solution was applied to each well and electrophoresis run continued for about one hour at constant voltage of 100V. The gel was stained with Comassie Brilliant Blue G-250 and de-stained in a solution containing 20 ml acetic acid, 20 ml ethanol and 160 ml distilled water.

RESULTS AND DISCUSSION

The enzyme extracts from tobacco fresh leaves were purified to homogeneity using the procedure summarized in Table 2. In the second step of purification process, the enzyme was precipitated by $(NH_4)_2SO_4$. In this conditions, specific activity was increased many folds and simultaneously some colored compounds and waxes were removed from the extract. Following ammonium sulphate precipitation, the dialyzed enzyme extract was applied to a Sephadex G-75 column. Finally, the anion exchange chromatography was carried out on UNO-Q1 Q-Sepharose column. The relative activity of PPO was about 40 times that of the crude extract, and specific activity of purified enzyme was 14.21 mmol/min mg.

It has been reported that the activity of some plant enzymes may depend on the season it is extracted from the plant tissue[10]. In many cases,

Table 1. Preparation of buffers used for optimum pH measurements

pH	Citric acid (ml)	Sodium monohydrogen phosphate (ml)	Deionized water (ml)
9.2	0	40	10
8.1	1	50	13
7.1	20	68	22
6.1	20	40	15
5.2	20	20	10
3.9	20	10	7.5
2.5	40	0	10

Table 2. Purification of peroxidase from leaves of *Nicotiana tobacum*.

Procedure	Volume (ml)	Activity (mmol/min)	Specific activity (mmol/minmg)	Yield (%)	Purification fold
Homogenate	6.370	-	-	100	1
$(NH_4)_2SO_4$	0.980	1.14	0.72	39	5
Sephadex G75	0.614	0.011	12.34	35	20
Sepharyl S100	0.01	0.008	24.5	18.2	40

however, the activity of other enzymes such as peroxidase does not depend on the age and height of the plant[11-13]. In this research, we measured the quality and activity of the polyphenol oxidase in fresh tobacco leaves during April-May and no detectable changes were observed (the results are not shown).

However, using various step by step purification processes, polyphenol oxidase was isolated almost specifically with a purification fold of about 40.

The molecular weight of tobacco polyphenol oxidase was estimated from its migration in SDS-electrophoresis as a single band. Comparing electrophoregram of the leaf extracts (not shown) before and after gel filtration showed that a number of bands have been omitted after gel filtration. However, 5 bands were still present, but the exact position of the band related to polyphenol oxidase was not known. On the other hand, it was suggested that anion exchange chromatography would be able to separate the polyphenol oxidase band specifically. Fig. 2 shows the single band of PPO at about 35 kDa after FPLC anion exchange chromatography. This confirms the molecular weight of 35kDa for this type of polyphenol oxidase. It has been reported that one type of *Nicotiana tobacum* grown in China contains 2 kinds of PPOs, designated PPO I and PPO II, respectively, according to the order of elution from Sephadex A-50 chromatography column[6]. In this study, we found only one kind of polyphenol oxidase from leaves of *Nicotiana tabacum* grown in north of Iran. A single electrophoretic band at 35 kDa (Fig. 2) shows that only one type of polyphenol oxidase was present.

Since the optimal conditions of

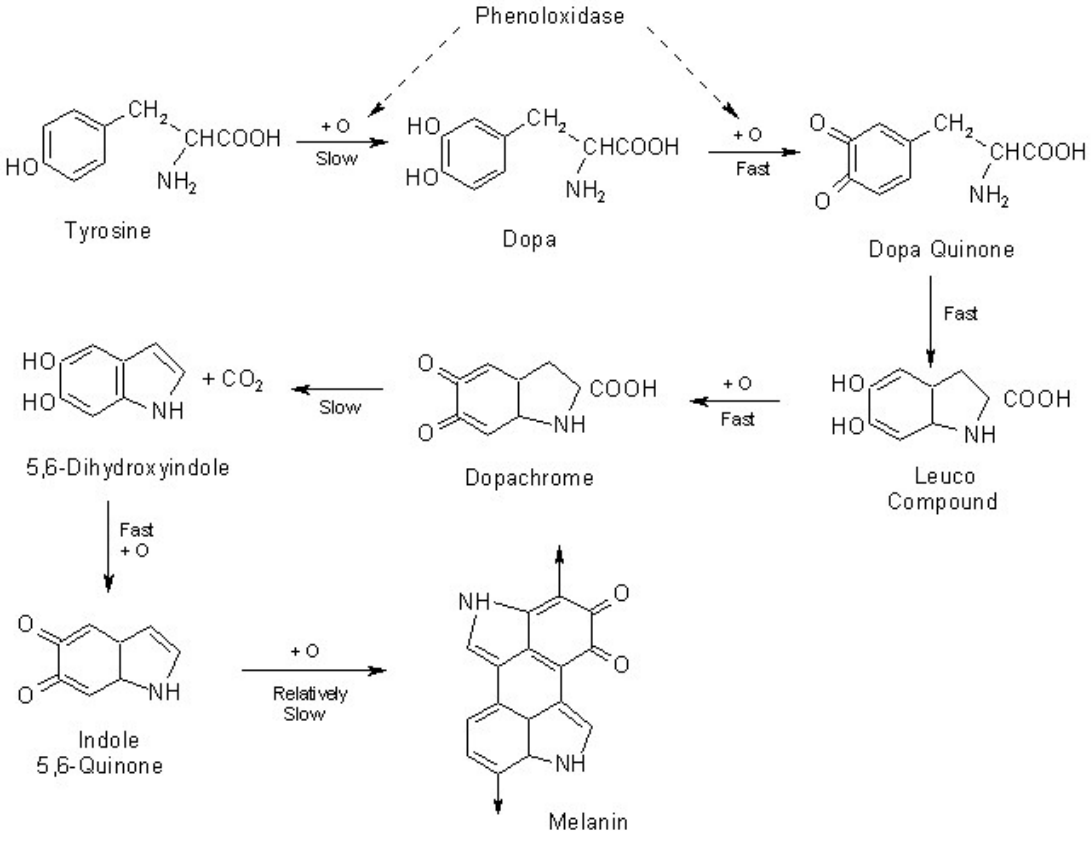

Fig. 1. Formation of melanin from amino acid, tyrosine. The monophenolase and diphenolase activity of polyphenol oxidase are pointed by doted arrows.

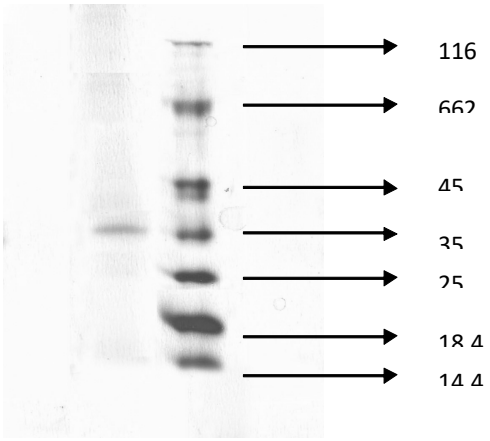

Fig. 2. Electrophoregram of purified polyphenol oxidase after anion exchange chromatography showing a single band at 35 kDa compared to the broad range protein molecular weight marker.

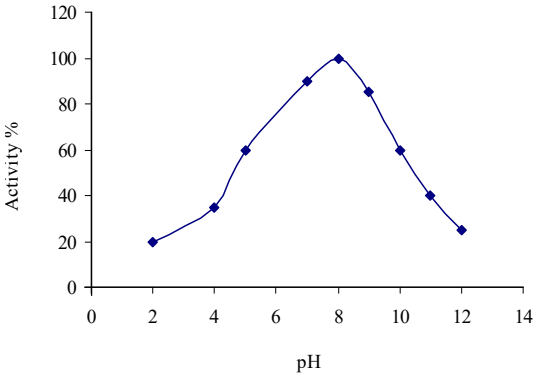

Fig. 3. The activity of tobacco polyphenol oxidase in various pH values.

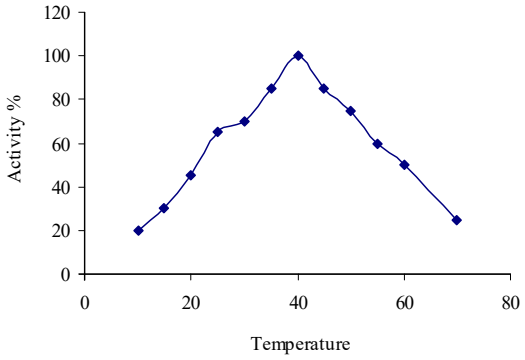

Fig. 4. Variations in the activity of tobacco polyphenol oxidase with temperature

polyphenol oxidase extracted from various plants may differ, the optimum pH and temperature of tobacco PPO were measured and compared to polyphenol oxidase reported in the literature [14]. It was found that tobacco PPO is most stable at pH 8.0 (Fig.3) that is similar to the optimum pH obtained for tomato and spinach 2, 14.. The optimum pH for other PPOs, for example loquat (pH 4.5) 4., highbush blueberry (pH 4.0 5. and Satsuma mandarin (pH 7.0) 15.. It is deducted from Fig. 3 that although optimum pH is 8.0, the enzyme is about 90% active at pH 7.0 and 9.0. However, at strong alkaline and acidic environment the activity decreases to about 20% of its original value.

Measuring thermal stability of polyphenol oxidase extracted from fresh tobacco leaves, showed that tobacco PPO is fairly active over a wide range of temperature i.e. 25-50°C (Fig. 4). The optimum temperature is around 40°C that is similar to the optimum temperature of mushroom tyrosinase[16]. It can be concluded from Fig. 4 that the enzyme is able to retain about 90% activity at ±10°C its optimum temperature, and the activity is almost lost at temperatures below 10 and above 70°C.

CONCLUSIONS

PPO was extracted and purified from *Nicotiana tobacum* using efficient chromatographic methods and ammonium sulphate. The method could overcome the polyphenol in tobacco that negatively affects the separation. It is suggested that PPO can play a significant role in defense against plant pathogens because of its reaction products and its wound inducibility[17, 18]. The purification process was efficient and improved the activity of the enzyme to about 40 times of the activity in the crude extract. The pH and temperature stability of this polyphenol oxidase was similar to mushroom tyrosinase. Most of industrial and commercial applications of PPO are related mushroom tyrosinase. However, the results obtained for specific activity, thermal stability and pH resistance of tobacco PPO suggest it as a replacement for mushroom tyrosinase, especially in this part of the country where tobacco is grown

legally and its wastes could be a good source of polyphenol oxidase.

Extraction and purification of PPO from tobacco also provides a way to study the molecular mechanism underlying the defense role of PPO in tobacco.

The molecular weight of tobacco PPO was estimated from its migration in SDS-electrophoresis as a single band. The molecular weight obtained in this way was 35 kDa.

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REFERENCES

1. D.A. Robb, in: R. Contie (Ed.), Copper protein and copper enzyme, CRC Press, Boca Raton, Florida, 1984, 207-241.
2. G. Yelena, Y. Sheptovitsky, G.W. Brudvig, 1999, *Biochemistry* 1999; **35**: 16255-16263.
3. A. Sanchez-Amat, F. Solano F, *Biochem. Biophys. Res. Comm*. 1997; **240**: 787-792.
4. C.K. Ding, K. Chachin, Y. Ueda, J. Agric. *Food Chem*. 1998; **46**: 4144-4149.
5. F. Kader, B. Rovel, M Girardin, M. Metche, *J. Sci. Food Agric*. 1997; **73**: 513-516.
6. P. Mazzafera, S.P. Robinson, *Phytochem*. 2000; **55**: 285-296.
7. C. Shi, Y. Dai, X. Xu, Y. Exi, Q. Lui, *Protein Expression Purification* 2002; **5**: 51-55.
8. K. Habaguchi, *Plant Cell Physiol*. 1979; **20**: 9-18.
9. Electrophoretic Theory, Hoffer Scientific Instruments, Electrophoresis Instruments, Techniques, and Exercises, Catalogue, 1992.
10. R. Sariri R, R.H. Sajedi, V. Jafarian, K.H. Khaje KH*, ournal of Molecular Liq*uids 2006; **124**: 137-142.
11. I. Yu. Sakharov, B.M.K. Vesga, I. Yu. Galaev, I.V. Sakharova, O. Yu. Pletjushkina, *Plant Science* 2001; **161**: 853-860.
12. I. Yu. Sakharov, J. A. Castellio, J. C. Areza, I. Yu. Galaev, *Bioseparation* 2000; **9**: 125-132.
13. I. G. Gazaryan, in: A.M. Egorov (Ed.), Biotechnology of peroxidases from plants and fungi, VNITI, Moscow, 1992, 4-28.
14. P. Thipyapong, J.C. Steffens, *Plant Physiol*. 1997; **100**: 1885-1890.
15. J.R. Das, S.G. Bhat, L.R. Gowada, *J. Agric. Food Chem*. 1997; **45**: 2031-2035.
16. H.W. Duckworth, J.E. Coleman, *J. Biol. Chem*. 1970; **245**: 1613-1625.
17. P. Thipyapong, D.M. Joel, J.C. Steffens, *Phytochem*. 1995; **40**: 673-676.
18. A.M. Mayer, E. Harel, *Phytochem*. 1997; **18**: 193-215.