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Polyphenol oxidase activity of oregano at different stages

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Abstract

The effects of pH and temperature on polyphenol oxidase (PPO) activity of organs such as root, stem and leaf, of *Origanum vulgare* ssp. *hirtum*, collected at different stages (vegetative and generative) and from various localities around Balikesir, Turkey, were investigated using catechol as a substrate. PPO obtained from organs of *Origanum* was partially purified by $(NH_4)_2SO_4$ precipitation, followed by dialysis. The PPO activities of organs of *Origanum* varied among the different localities and between the different stages, and leaf had the highest PPO activity, followed by stem and root, in both stages. Optimum pH of *Origanum* in the transition from the vegetative stage to the generative stage decreased by 0.4 of a unit, approximately, whereas optimum pHs of *Origanum*-PPO collected from various localities were closely similar. Optimum temperature decreased very little in the transition from the vegetative stage to the generative stage. Activation energy values were calculated from the Arrhenius equation.

Keywords: *Origanum vulgare* ssp. *hirtum*; pH; Temperature; Catechol; Activation energy

1. Introduction

In Europe and in general, all over the world, the most commonly found oregano species belong to the botanical genus *Origanum*. Forty-nine taxa, divided into 10 sections, belong to this genus, most of them having a very local distribution around the Mediterranean. *Origanum vulgare* ssp. *hirtum* is a very polymorphic taxon as it possesses by far the largest distribution of all *Origanum* species (Jerković, Mastelić, & Miloš, 2001). In medicinal treatments, Oregano is used as an antiseptic, antibacterial, antispasmodic, antiasthmatic agent, and as an expectorant and as a fungicide due to its volatile oils and terpenic materials. Oregano is an economically important culinary herb and tea for food industry application. Therefore, it plays a primary role among temperate culinary herbs in world trade as it is exported in large quantities. Oregano is dried and stored for future use in Turkey and in other countries.

Another important property of Oregano is that it contains an enzyme called polyphenol oxidase (PPO), widely distributed in plants. PPO catalyses the oxidation of *o*-dihydroxyphenols to *o*-quinones (Doğan & Doğan, 2004). The production of dark tea is dependent on the oxidative changes that polyphenols undergo during processing. Such changes are particularly important for the development of colour.

The objective of this study was to investigate the distribution of PPO in organs such as root, stem and leaf of *Origanum vulgare* ssp. *hirtum* collected from various localites in the vegetative and the generative stages around Balikesir, Turkey, and to determine the most appropriate stage and organ for dried preparations such as culinary herbs or tea of *Origanum*. The effects of pH and temperature on PPO activity were studied. The values of activation energy were calculated from enzyme

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activities at various temperatures, using the Arrhenius equation. Furthermore, the relationships between both the PPO activities among the different stages and those among different organs were studied using ANOVA to interpret the results.

2. Materials and methods

2.1. Materials

Origanum used in this study was freshly collected in the vegetative and the generative stages around Balıkesir in 2001, and kept for 2 days in the refrigerator at 4 °C before extracting PPO. All chemicals used in this study were of analytical grade.

2.2. Enzyme extraction

Origanum was cleaned to remove visible soil and washed with tap water and bidistilled water several times. The *Origanum* was subsequently separated into organs such as root, stem and leaf in the vegetative and the generative stages. All organs of *Origanum* were washed again with bidistilled water. For preparing extract, 10 g of *Origanum* was placed in a Dewar flask under liquid nitrogen which decomposes cell membranes, transferred to a stainless steel Waring blender, and ground to a powder under liquid nitrogen. The powder was transferred to a small beaker. The frozen plant powder was added to the extraction solution (100 ml of 0.1 M phosphate buffer containing 5% poly(ethyleneglycol) at pH 6.5 and 10 mM ascorbic acid) and mixed with a magnetic stirrer for 4 min at 4 °C. The crude extract was filtered, and the filtrate centrifuged at 20,000*g* for 30 min at 4 °C. The supernatant was brought to 80% saturation with solid $(NH_4)_2SO_4$. The precipitated PPO was separated by centrifugation at 20,000*g* for 30 min. The precipitate was dissolved in a small amount of 0.05 M phosphate buffer (pH 7.0) and dialysed at 4 °C in the same buffer for 2 days with three changes of buffer during dialysis. The dialysed extract was used as the PPO enzyme source in the following experiments (Wesche-Ebeling & Montgomery, 1990).

2.3. Assay for polyphenol oxidase activity

PPO activity was determined using a spectrophotometric method based on the initial rate of increase in absorbance at 420 nm. Unless otherwise stated, 2.3 ml of 0.1 M phosphate buffer (pH 6.5), 0.6 ml of 0.1 M catechol as a substrate, and 0.1 ml of the PPO extract were pipetted into a quartz cuvette of 3 ml volume and mixed. In each measurement, the final volume of solution in the quartz cuvette was 3 ml. Buffer concentration was chosen as 0.1 M to avoid the influence of enzymatic extract ionic strength on PPO activity described by Angleton and Flurkey (1984). A portion of the mixture was rapidly transferred into a 1.0-cm pathlength cuvette. Absorbance was recorded immediately at 10 s intervals, at 20 ± 1 °C with a Cary |1E|g UV–Vis Spectrophotometer (Varian). The temperature was maintained at 20 °C using a Beckmann Peltier temperature controller attached to the cell-holder of the spectrophotometer. The instrument was zeroed using the same mixture without PPO. The assay was repeated twice using the same stock of the PPO extract. Activity was calculated from the linear portion of the curve. One unit of PPO activity was equivalent to a change in absorbance of 0.001 per min at 420 nm.

2.4. Effect of pH

PPO activity was determined at pH values of 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0, respectively, using 0.1 M acetate (pH 4–6) and 0.1 M phosphate (pH 6–9) buffer adjusted with 0.1 M NaOH or 0.1 M HNO_3. The optimum pH values for PPO obtained from organs of *Origanum* was obtained using catechol as a substrate. The reaction mixture contained 0.6 ml of 0.1 M catechol, 2.3 ml of 0.1 M buffer solution and 0.1 ml of enzyme solution (Arslan, Temur, & Tozlu, 1997).

2.5. Effect of catechol concentration

PPO activity was assayed in a quartz cuvette of 3 ml volume at 420 nm by mixing 0.1 ml of PPO extract, and 0.1 M catechol substrate and 0.1 M phosphate solution at selected volumes. In each measurement, the final volume of solution in a quartz cuvette was 3 ml. Each assay mixture was repeated twice and the data plotted according to linear regression analysis (the method of Lineweaver–Burk). Michaelis constants, K_M and V_{max}, for *Origanum*-PPO were calculated from the plots of $1/V$ versus $1/[S]$ (Doğan, Arslan, & Doğan, 2002).

2.6. Effect of temperature

PPO activity was determined at 20, 30, 40, 50, 60 and 70 °C, respectively. The effect of temperature on the PPO activity was assayed by heating the standard reaction solutions (substrate and buffer solutions) to the appropriate temperature with circulating water-bath before introduction of the PPO. The temperature was maintained by a Beckmann Peltier temperature controller attached to the cell-holder of the spectrophotometer. Once temperature equilibrium was reached, PPO was added and the reaction was followed spectrophotometrically at constant temperature at given time intervals. The reaction mixture contained 0.6 ml of 0.1 M catechol, 2.3 ml of 0.1 M buffer solution and 0.1 ml of PPO solution. Each assay mixture was repeated twice (Doğan et al., 2002).

2.7. Activation energy

The activation energy was calculated from experimental results obtained for PPO reactions by using the Arrhenius equation:

$$\ln V = \ln Z - \frac{E_a}{RT}, \quad (1)$$

where V is the enzyme activity (EU ml^{-1} min^{-1}), Z is the frequency factor, E_a is the activation energy (kJ mol^{-1}) and T is the temperature (K). The graph of $\ln V$ versus $1/T$ will give a straight line. The parameter Z is obtained from the intercept point at $1/T = 0$. The activation energies were calculated from the slopes of lines (Doğan, Alkan, & Onganer, 2000; Doğan et al., 2002).

3. Results and discussion

In this study, we generally selected catechol as the most used substrate. Therefore, the kinetic parameters, V_{max} and K_M, for PPO activity of organs of *Origanum* collected from various localities at the vegetative and the generative stages were calculated using a linear regression analysis programme equipped with Lineweaver–Burk equation.

Michaelis constants (K_M) and maximum velocities (V_{max}) for PPO were calculated from a plot of $1/V$ versus $1/[S]$ using catechol as a substrate. The absorbance of the oxidation products was measured at 420 nm using a Cary |1E|g UV–Vis Spectrophotometer (Varian). V_{max} and K_M values calculated from the Lineweaver–Burk equation are given in Table 1. In Table 1, the V_{max}/K_M ratio is called the "catalytic power" (Rocha, Pilar Cano, Galeazzi, & Morais, 1998). The increase in the K_M value and the reduction in the V_{max}/K_M ratio indicate reduced affinity of the residual enzyme for its substrate. PPO activities for organs of *Origanum* varied among the five localities and between the vegetative and the generative stages. According to K_M and V_{max}/K_M values, the highest PPO activity occurred in leaves, followed by stems and roots, at both stages. The localisation of PPO in the plant cell depends on the species, age, and – in fruits or vegetables – on maturity. A considerable part of PPO activity is localised in chloroplasts. Chlorophyll gives to the plants its green colour. Leaves have the highest green colour within organs of *Origanum*. A cause of the most being in leaves of PPO may be chlorophylls. *Origanum*

Table 1
Kinetic parameters, activation energies, optimum pHs and temperatures calculated for *Origanum*

Stages	Organs	Localities	pH	T (°C)	E_a (kJ mol^{-1})	V_{max} (EU ml^{-1} min^{-1})	K_M (mM)	V_{max}/K_M (min^{-1})	R
Vegetative	Root	Ömerköy	7.5	30	−35.5	10,000	50	200	0.9987
		Karapürcek	7.5	30	−20.9	2500	10	250	0.9957
		Zeytinli	7.4	40	−32.5	3333	20	167	0.9992
		Ocaklar	8.0	30	−38.0	5000	25	200	0.9934
		Hisaralan	7.3	20	−4.67	5000	35	143	0.9984
	Stem	Ömerköy	8.0	30	−32.1	416	8	50	0.9976
		Karapürcek	7.6	30	−20.3	384	8	50	0.9983
		Zeytinli	7.5	30	−46.6	2500	15	167	0.9993
		Ocaklar	8.0	20	−21.9	1428	29	50	0.9996
		Hisaralan	8.0	20	−23.7	2000	20	100	0.9989
	Leaf	Ömerköy	7.0	30	−20.7	10,000	30	333	0.9984
		Karapürcek	7.2	30	−18.2	5000	10	500	0.9988
		Zeytinli	7.3	32	−14.0	50,000	100	500	0.9990
		Ocaklar	7.4	30	−14.5	11,111	22	500	0.9988
		Hisaralan	7.8	30	−11.4	50,000	200	250	0.9971
Generative	Root	Ömerköy	7.0	20	−45.3	5000	20	250	0.9972
		Karapürcek	7.0	20	−45.7	1666	12	143	0.9992
		Zeytinli	7.0	30	−55.2	5000	15	333	0.9835
		Ocaklar	6.5	20	−38.7	5000	25	200	0.9969
		Hisaralan	7.0	20	−54.4	10,000	20	500	0.9988
	Stem	Ömerköy	7.0	20	−51.0	5000	50	100	0.9994
		Karapürcek	7.0	20	−36.9	1250	13	100	0.9993
		Zeytinli	8.0	30	−45.0	2500	10	250	0.9946
		Ocaklar	7.0	20	−41.8	3333	23	143	0.9965
		Hisaralan	7.5	20	−55.3	10,000	30	333	0.9997
	Leaf	Ömerköy	7.0	30	−24.5	25,000	25	1000	0.9965
		Karapürcek	7.0	30	−18.4	16,666	17	1000	0.9965
		Zeytinli	7.0	30	−19.8	47,619	95	500	0.9983
		Ocaklar	7.0	30	−13.2	16,667	12	1429	0.9941
		Hisaralan	7.2	30	−15.2	33,333	33	1000	0.9987

has higher PPO activity at the generative stages than that at the vegetative stages. Most PPO was in the chloroplast and, furthermore, PPO depends on the maturity of the fruit and the development stage of the chloroplasts. Trypsin and light are necessary for PPO being active in chloroplast. PPO in chloroplast, during warming of air and developing of plant organs is more active in sunlight. Therefore, PPO activity increases in the transition from the vegetative stage to the generative stage.

The pH optimum for PPO activity of organs of *Origanum* using catechol as a substrate was measured by extracting the PPO in a pH range from 4.0 to 9.0 at the vegetative and the generative stages; pH optima for PPO varied between 4 and 8 in most cases. Optimum pH values for PPO of organs of *Origanum* collected from various localities at the vegetative and the generative stages are presented in Table 1. Furthermore, the changing of PPO activity with pH is illustrated in Fig. 1. In Table 1 or Fig. 1, the optimum pH values are 7.66 for root, 8.00 for stem and 7.60 for leaf at the generative stage; and 7.36 for root, 7.34 for stem and 7.32 for leaf at the generative stage, respectively. Mayer (1979) reported that PPO activity and optimum pH at different stages of the same plant were different. Table 1 shows that optimum pH in transition from the vegetative stage to the generative stage decreased by approximately 0.4 of a unit. This decrease may be a result of metabolic reactions occurring by the effect of sunlight. It can be concluded that optimum pHs for PPO of *Origanum* collected from various localities are approximately the same.

The optimum temperature for enzyme activity usually depends on experimental conditions. Generally, reaction rate decreases because of thermal denaturation when the temperature is increased. This situation, for most enzymes, is similar. The changing of PPO activity with temperature is presented in Fig. 2. From Table 1 or Fig. 2, optimum temperatures for PPO of organs of *Origanum* are in the range of 30.0 ± 7.07 °C for root, 26.0 ± 5.47 °C for stems and 30.4 ± 0.89 °C for leaves at the vegetative stage; and 22.0 ± 4.47 °C for roots, 22.0 ± 4.47 °C for stems and 30.0 ± 0.00 °C for leaves at the generative stage, respectively. The optimum temperature for PPO activities decreased a little in the transition from vegetative stage to generative stage. In Table 1, the PPO activity increased when the temperature was increased from 20 to 30 °C, and then enzyme activity decreased due to denaturation of enzyme with increasing temperature. We found previously that the temperature at which PPO enzyme showed the highest activity, was in the range of 25–30 °C, and it then decreased at temperatures above 40 °C (Doğan & Doğan, 2004; Doğan,

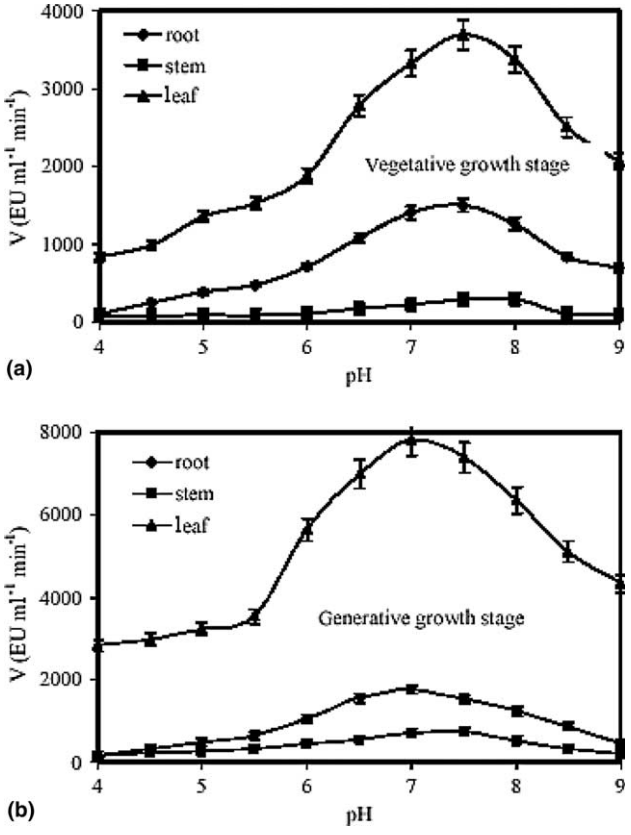

Fig. 1. The changing of PPO activity with pH.

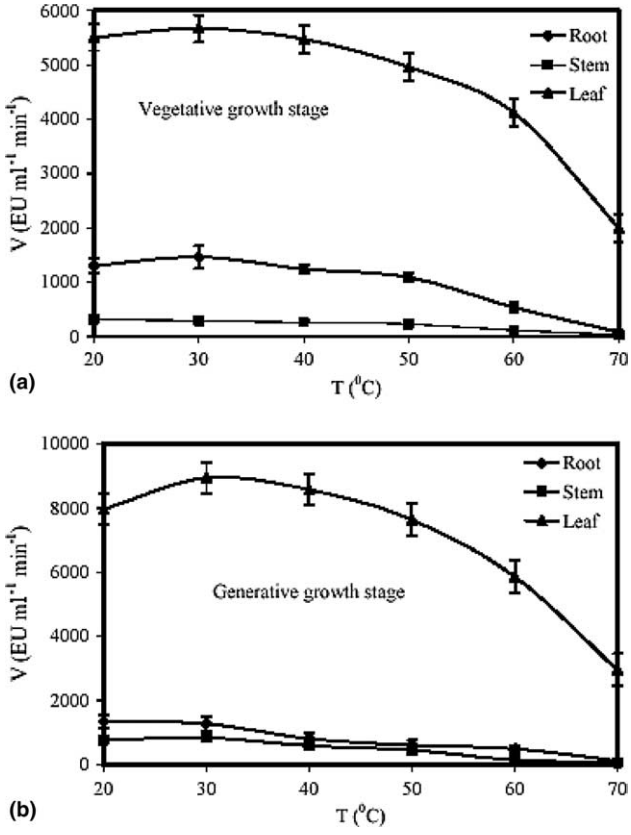

Fig. 2. The changing of PPO activity with temperature.

Table 2
Relationships among organs of *Origanum*

Stages	Relationship	*P* values
Vegetative	Root–stem	0.006
	Root–leaf	0.004
	Stem–leaf	0.001
Generative	Root–stem	0.060
	Root–leaf	0.002
	Stem–leaf	0.023
Vegetative–generative	Root–root	0.187
	Stem–stem	0.083
	Leaf–leaf	0.07

Doğan, & Arslan, 2003). This result is similar to our results obtained in this study.

Activation energy values calculated from plots of $\ln V$ versus $1/T$ for PPO of organs of *Origanum* collected from both different stages and various localities are presented in Table 1. From Table 1, the activation energy values are negative. This may be explained by inactivation of the PPO at high temperature. Activation energy values are generally not published; therefore, comparsions are not possible. There are only few data related to activation energy. Activation energy values are 7 kcal mol^{-1} for kiwifruit PPO with catechin as substrate (Park & Luh, 1985) and 21.4 kcal mol^{-1} for mango kernel with catechol as substrate (Arogba, Ajiboye, Ugboko, Essienette, & Afolabi, 1998).

P values calculated using the ANOVA statistical analysis programme are presented in Table 2. There is no important relationship between PPO activities of organs of *Origanum* at the vegetative and the generative stages. There is an important relationship among PPO activities of organs of *Origanum*.

4. Conclusions

PPO activities of organs of *Origanum* were different at both stages and localities. The vegetative stage is the most appropriate stage for dried preparations or herb use of *Origanum* because of the small PPO activity. Leaves exhibit the highest PPO activity. Therefore, *Origanum* leaves can be used in dark tea production due to their high PPO activity. PPO activity is also different at different growth stages. PPO activity at temperatures above 30 °C decreased, due to thermal denaturation.

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