

Research Article

## Purification and characterization of polyphenol oxidase enzyme from Iğdır apricot (*Prunus armeniaca* L.)

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### Abstract

In this study, polyphenol oxidase enzyme obtained from Iğdır apricot was purified with method of affinity chromatography. The apricot cultivar "şalak" was provided from Iğdır region. To purify polyphenol oxidase enzyme, obtained from Iğdır apricot, phosphate buffer at 7.3 pH was used and the homogenate was prepared. The homogenate was applied to activate sepharose 4B-tyrosine-p-aminobenzoic acid affinity column. For quantitative protein analyses, fractions obtained from column, and showing activity, was performed at 595 nm with coomassie blue method. In addition, optimum pH of enzyme, its optimum temperature, ionic strength effect and inhibition kinetics of some drugs and chemicals on enzyme were investigated. Optimum pH of enzyme and its optimum temperature were found to be 6 and 30°C, respectively. Furthermore, the study carried out on the ionic strength revealed that the highest activity was observed in concentration of 0.16 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>.

**Keywords:** Affinity chromatography, Iğdır apricot, polyphenol oxidase, purification, *P. armeniaca*

### 1. Introduction

Polyphenol oxidase (E.C. 1.14.18.1; PPO) is an oligomeric enzyme, formed of more than one subclasses belonging to the oxidoreductase group, which is the cause of plant browning. The number of subclasses within PPO changes based on the source and substrate at which the enzyme is isolated (Vamos-Vigyazo 1981).

The most commonly used substrates of the PPO enzyme are chlorogenic acid and caffeic acid (3,4-dihydroxy cinnamic acid), derivatives of cinnamic acids. Catechol (o-dihydroxyphenol) is used as a model substrate in enzymatic oxidation studies. Dopa and tyrosine are amino acids found in nearly all plant tissues and the substrates of the PPO (Mathew & Parpia 1971).

Polyphenol oxidase catalyses the hydroxylation reaction, which catalyses o-hydroxylation of monophenols to o-dihydroxyphenols (cresolase activity), and the oxidation reaction, which catalyses the oxidation of o-dihydroxyphenols to o-quinones (catecholase activity) (Valero et al. 1988). Polyphenol oxidase can be inhibited by metal chelate agents, such as cyanide, carbon monoxide, sodium diethyldithiocarbamate (DIECA), mercaptobenzothiazole, dimercaptoproponate, azide or potassium methylxantate, as it is a copper-containing metalloprotein cofactor (Wildanger & Herrmann 1973).

The purpose of this study was to investigate certain properties of the polyphenol oxidase enzyme purified from the Iğdır apricot.

### 2. Material and Method

#### 2.1. Reagents

(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, catechol, triton X-100, PEG (8000), p-aminobenzoic acid, NaCl, KH<sub>2</sub>PO<sub>4</sub>, Na<sub>2</sub>HPO<sub>4</sub>, PVPP, glacial acetic acid, hydrochloric acid (37% v/v), activated - CNBr -

sepharose 4-B, mercaptoethanol, ascorbic acid and sodium metabisulfite were obtained from Sigma Chemical Co (St. Louis, USA), potassium cyanide, coomassie blue-250, ammonium sulphate and L-tyrosine were purchased from Merck (Germany). Other reagents were analytical grade.

#### 2.2. Preparation of the crude extract

The apricot used in this study was obtained from the region of Iğdır. The fruit was maintained in a deep-freezer (-70°C) until the experimental works. 50 grams of fruit per 100 mL (0.5% polyethylene glycol) was homogenised with a 0.5 M phosphate tampon (pH 7.3) using a household blender for two minutes in order to prepare the crude extract.

The homogenate was filtered through double layered filter paper. 20000 x g (filtrate was centrifuged for an hour at +5°C in order to eliminate the precipitate containing the plant walls and cellulosic fibre part. The obtained supernatant was used as the crude extract (Yerlitürk 2003). The dialysis procedure was conducted on a magnetic stirrer in a refrigerator (+4°C) (Cuatrecasas 1970).

#### 2.3. Determination of the qualitative protein

The qualitative protein of every fraction, obtained from the affinity column, was determined. Qualitative protein determination is based on the maximum absorbance of amino acids tryptophane and tyrosine, found in the structure of protein, at 280 nm on the spectrophotometer (Segel 1968). The absorbance of fractions was measured against the obtuse, after they were placed in quartz basins.

#### 2.4. Quantitative protein analysis with the coomassie blue method

The quantitative protein of every fraction, obtained from the affinity column, was determined. This method is based on proteins forming a complex with the coomassie brilliant blue G-250 reactive in an o-phosphoric acid ambient, and the maximum absorbance of this complex at a wavelength of 595 nm.

The stain coheres with the protein extremely quickly (on average of 2 minutes). The accuracy of this method ranges between 1 µg and 100 µg (Bradford 1976). 10-90, and 100 µl of standard bovine serum albumin solution, containing 1 mg of protein in every 1 ml, was added to tubes in order to conduct protein determination procedures. A standard graph was obtained using the µg protein values corresponding to the recorded absorbance values. The amounts of protein in the enzyme solution were calculated with the help of this graph.

#### 2.5. PPO enzyme activation analysis

The activity of the polyphenol oxidase enzyme was determined using spectrophotometry. The change in absorbance for 420 nm was recorded in one minute. The 0.001 unit change in absorbance per minute for every 1 ml of enzyme solution was used as the activity unit (Coseteng & Lee 1978).

Among the purified enzyme solutions, those containing a high rate of protein were put in the dialysis bag. They were exposed to the dialysis first by changing the water once every three hours against to the distilled water for total 24 hours and then against to the phosphate buffer solution for 4 hours. The process of dialysis was performed on the magnetic mixer and in the refrigerator (+4°C) (Cuatrecasas 1970). In the process of purification of the enzyme, the affinity column, prepared for the homogenate column, was balanced first with 0.05 M Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 6.0).

The enzyme solution, prepared with centrifugation, was applied to the column. The column was washed with a 0.05 M Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 6.0). The elution was performed with 0.05 M Na<sub>2</sub>HPO<sub>4</sub> / 1 M NaCl buffer (pH 8.0). The 3 mL of eluates were put in the tubes. The process of elution was carried out until the absorbance at 280 nm became zero. By using the elution buffer as blank, the qualitative protein analysis and the activity analysis were performed for each tube at 280 nm and 420 nm respectively.

By conducting the quantitative protein analysis and the activity analysis with the Bradford method, the specific activities and purification rates were determined (Bradford 1976).

#### 2.6. Purifying the polyphenol oxidase enzyme using affinity chromatography

The first stage of the enzyme purifying procedure was counterbalancing the affinity column, prepared for the homogenate column, with a 0.05 M Na<sub>2</sub>HPO<sub>4</sub> tampon (pH 6.0). After counterbalancing the column, the gel on the tampon solution was reduced to gel level. The enzyme solution, prepared by being centrifuged, was applied to the column. The column was washed with the 0.05 M Na<sub>2</sub>HPO<sub>4</sub> tampon (pH 6.0). The elution procedure was conducted once the washing process was completed. The gel on the tampon solution was reduced to gel level prior to starting the elution procedure, after which elution was

achieved with a 0.05 M Na<sub>2</sub>HPO<sub>4</sub> / 1 M NaCl tampon (pH 8.0). 3 mL of eluates, taken from the column, were placed into tubes. The elution procedure was conducted until the absorbance at 280 nm was zero. The elution tampon was used as the obtuse to determine the qualitative protein at 280 nm, and the activity at 420 nm for every tube. In conclusion of affinity chromatography, tubes in which enzyme activity was observed were combined. Specific activities and purification rates were determined by using the Bradford method to determine the quantitative protein and activity for samples applied to the column and combined eluate solutions.

#### 2.7. The effect of pH on enzyme activity

Different activities were calculated using catechol substrate at different pH values (4.0-9.0) in order to determine the optimum pH value at which the polyphenol oxidase enzyme displays maximum activity; ultimately determining the optimum pH value of the polyphenol oxidase enzyme.

#### 2.8. The effect of temperature on enzyme activity

Different activities were calculated using catechol substrates at different temperatures (20-90°C) in order to determine the optimum temperature at which the polyphenol oxidase enzyme displays maximum activity; ultimately determining the optimum temperature of the polyphenol oxidase enzyme.

#### 2.9. The effect of ionic strength on enzyme activity

Activity determinations were conducted by adding 0.02 M-0.50 M concentrated ammonium sulphate ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>) to the reaction environment at a constant temperature (37°C), and constant pH (7.4) in order to identify the effect ionic strength has on enzyme activity.

#### 2.10. Determining molecular weight of the enzyme

SDS-Polyacrylamide gel electrophoresis was used to check the purity of the polyphenol oxidase enzyme purified with affinity chromatography (Laemmli 1970).

### 3. Results and Discussion

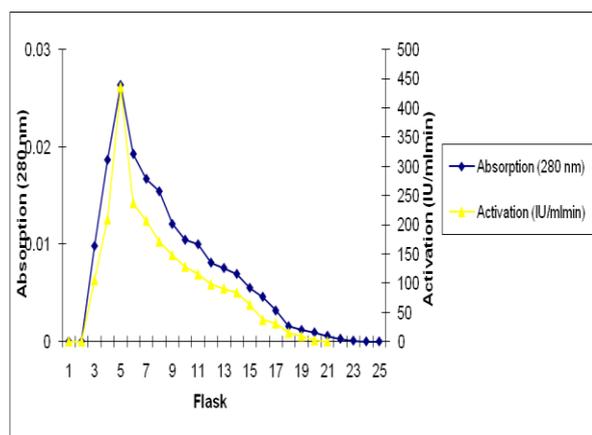
Polyphenol oxidase activity can be determined by measuring the rate at which the substrate disappears or the rate at which the product forms. The rate at which the product is formed is determined by measuring the optical density of coloured compounds formed in quinones using spectrophotometry. This method is used more for routine analyses, and preferred to the polarographic method as it can be used at long time intervals. In addition, it is a known fact that this method is as accurate and reliable as other methods (Vamos-Vigyazo 1981).

The purification coefficient of the polyphenol oxidase, extracted from the column counterbalanced by the sodium phosphate tampon (pH=6.0), was 373.39. The purification coefficient, determined with the help of affinity chromatography, was significantly high. There are two possible reasons as to why the purification coefficient identified in this study about Iğdır apricots is significantly high; purifying polyphenol oxidase using affinity chromatography, and the high amount of polyphenol oxidase within the şalak-cultivar Iğdır apricot (Table 1).

**Table 1.** The purification results of polyphenol oxidase enzyme purified from Iğdır apricot

Purification stages	volume (ml)	Enzyme activity (EU/ml)	Tot.Enzyme unit (EU)	Total protein (mg/ml)	Specific activity (EU/mg.pro)	Purification coefficient
Homogenate	60	567	34320	520.62	1.09	.....
Affinity column	45	1090	59950	2.677	407	373.39

The coomassie blue method was used to determine quantitative protein. A standard graph was first prepared in order to determine quantitative protein using the coomassie blue method. The protein amounts in the extract and the purified enzyme solution were calculated with the help of this graph. The quantitative protein of every fraction, extracted from the affinity column, was determined. This method is based on proteins forming a complex with the coomassie brilliant blue G-250 reactive in an o-phosphoric acid ambient, and the maximum absorbance of this complex at a wavelength of 595 nm. The stain coheres with the protein extremely quickly (on average 2 minutes). The accuracy of this method ranges between 1 µg and 100 µg (Bradford 1976). Figure 1 illustrates graph of enzyme activity and quantitative protein determination.

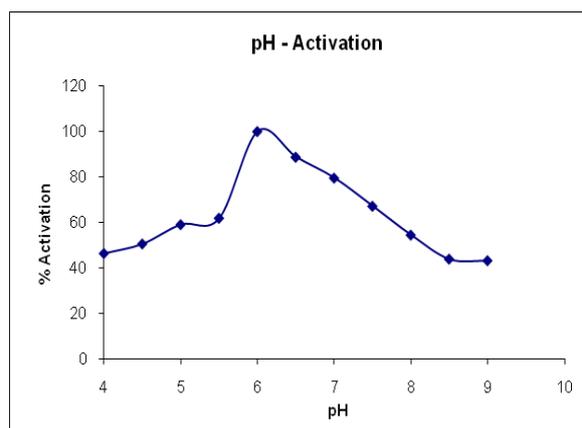
**Figure 1.** Enzyme activity- quantitative protein analysis graph

In a study (Erat et al. 2006), where the polyphenol oxidase enzyme was extracted from *Ferula* sp. (Apiaceae) using gel-filtration chromatography, the purification coefficient was 43.33 times. A study (Ziyan & Pekyardımcı 2004), conducted to purify and characterise the polyphenol oxidase enzyme in Ankara pears, concluded that the purification coefficient was 13.30 times. In another study (Jharna et al. 1997), polyphenol oxidase was purified from apples, and its purification coefficient was 25. In another polyphenol oxidase enzyme purifying and characterising study (Demir et al. 2006), conducted on apples grown in the region of Van (*Malus sylvestris* Miller cv. golden), the purification coefficient was 4.0 times. The purification coefficient of polyphenol oxidase extracted from the column, counterbalanced by a sodium phosphate tampon (pH=6.0), was 373.39 (Table 1). The purification coefficient, determined with the help of affinity chromatography, was significantly high.

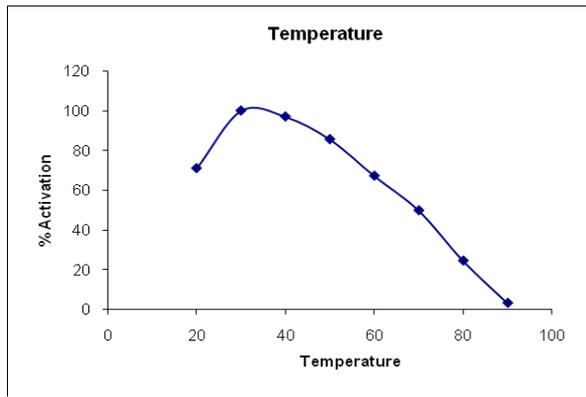
The optimum pH of the polyphenol oxidase enzyme extracted from the Iğdır apricot was 6.0. This is similar to

results of other studies referred to in literature (Jharna et al. 1997; Aydemir 2004; Ziyan & Pekyardımcı 2004; Erat et al. 2006; Unal 2007).

Various optimum pH values for polyphenol oxidase enzymes identified in other studies are 4-5 for green olives, 5 for potatoes, 7.0 for bananas and *Dioscorea bulbifera* (Palmer 1963; Anosike & Ojmelukwe 1982; Balasingam & Ferdinand 1997; Ben-Shalom et al. 1997). A study conducted in India (Jharna et al. 1997), extracted the polyphenol enzyme from apples, and then purified and characterised the polyphenol enzyme. The optimum pH was identified between 6 and 7, and compared to results of other studies (Jharna et al. 1997). In another polyphenol oxidase enzyme purifying and characterising study (Demir et al. 2006), conducted on apples grown in the region of Van, the optimum pH was 4.0, and the optimum temperature was 50°C. Figure 2 illustrates enzyme activity based on the pH.

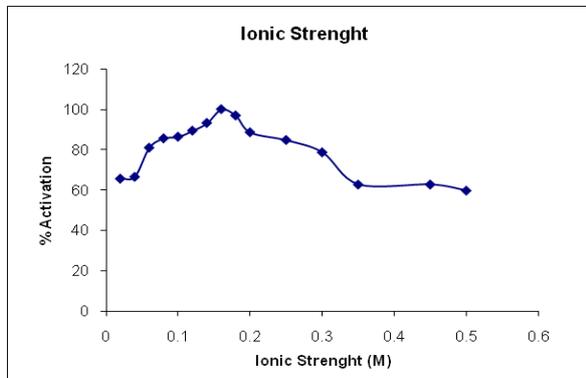
**Figure 2.** The graph of enzyme activity dependent on pH

In this study, the optimum temperature of the enzyme extracted from the Iğdır apricot was 30°C, an optimum temperature close to that of the polyphenol oxidase enzyme extracted from the Anamur banana (Ünal 2007). Optimum temperatures of polyphenol oxide identified by studies in literature state that the maximum activity for the catechol substrate of potatoes was at 22°C (Schaller 1972). In another study (Ünal 2007), the optimum temperature of the Anamur banana was 30°C, and its optimum pH was 7. In another study (Ziyan & Pekyardımcı 2004), the optimum pH, calculated using the catechol substrate, of the Ankara pear was 7.0; the optimum temperature was 20°C for the same study. In a study (Aydemir 2004), that extracted the polyphenol oxidase enzyme from *Cynara scolymus* L., the optimum pH was between 5.0 and 7.0; study results were discussed ambidextrously, and the optimum temperature was determined as 25°C. Figure 3 illustrates graph of enzyme activity based on the temperature.



**Figure 3.** The graph of enzyme activity dependent on temperature

When investigating the effect ionic strength had on the polyphenol oxidase enzyme, the highest activity was observed at 0.16 M  $(\text{NH}_4)_2\text{SO}_4$  concentration (Demir et al. 2006). Figure 4 illustrates graph of enzyme activity based on ionic strength.

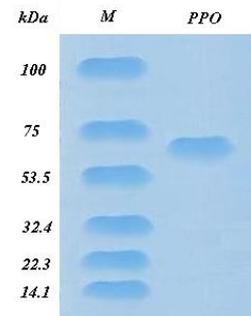


**Figure 4.** The graph showing the dependence of enzyme activity on ionic strength

SDS-polyacrylamide gel electrophoresis was used to check the purity of the polyphenol oxidase enzyme purified with affinity chromatography (Laemmli 1970). Only one band was observed after electrophoresis. The fact that only one band was observed after electrophoresis, proves that there was only one protein in the solution applied; that was the polyphenol oxidase enzyme. Figure 5 illustrates the SDS-PGE band of the PPO enzyme, obtained from Igdir apricots using affinity chromatography.

In general, studies to date have identified that the molecular weight of polyphenol oxidase isoenzymes is between 55.000 – 70.000 dalton. A subject-related study (Chevalier et al. 1999) defined that the molecular weight of a polyphenol oxidase enzyme, extracted from an apricot was 67.1 kDa, and had a single band, after electrophoresis. Studies conducted using various references identified the molecular weight of the polyphenol oxidase enzyme in apples as 25 kDa (Jharna et al. 1997). In another study (Doğan et al. 2005), the molecular weight of polyphenol oxidase extracted from an artichoke was 52 kDa, after electrophoresis. A study (Ziyan & Pekyardımcı 2004) conducted on the Ankara pear identified that the molecular weights of the polyphenol oxidase enzyme were 60 kDa, 40 kDa, and 28 kDa, respectively, as a result of electrophoresis conducted using a SDS-Page.

**Figure 5.** The SDS-PAGE band of the polyphenol oxidase enzyme purified from Igdir apricot with the affinity chromatography.



In this study, the molecular weight of the polyphenol oxidase enzyme extracted from the Igdir apricot was measured with electrophoresis using a SDS-Page; the molecular weight was 64.8 kDa. The polyphenol oxidase enzyme was identified by the single band observed as a result of electrophoresis, and its purity was proven. Results

of this study are parallel to those stated in literature (Chevalier et al. 1999; Demir 2004).

#### 4. Conclusion

In conclusion, a significant amount of polyphenol oxidase enzyme was extracted from the Igdir apricot, as a result of conducted studies. There are no other studies in which the purification rate is as high as the one in this study.

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