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Research Article

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Purification and Determination of Kinetic Properties of the Polyphenol Oxidase from the Marfona Potato Cultivar

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This research aimed to purify the polyphenol oxidase (PPO) enzyme from the Marfona potato cultivar cultivated in the Ödemiş district in İzmir province of Türkiye and to determine its kinetic properties. For purification, ammonium sulfate precipitation at 0-80% saturation was applied first, followed by dialysis, and finally affinity chromatography by using Sepharose 4B-L-tyrosine-*p*-aminobenzoic acid. After affinity column purification, sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) was used to check the PPO enzyme's purity. For the catechol substrate, the optimum temperature and pH values were identified by measuring the PPO enzyme activity purified from the Marfona potato cultivar at different temperatures (15°C–45°C) and pHs (4.5–9). For catechol substrate, the optimum temperature value was found to be 25°C and the optimum pH value to be 7 for PPO enzyme purified from Marfona potato. The kinetic properties of the Marfona potato PPO enzyme were determined by using the equation of the Lineweaver-Burk plot under the determined optimum conditions. The *Km* and *Vmax* values of the PPO enzyme in the purified Marfona potato cultivar were found to be 6.7 mM and 3333.33 U/mLmin for the catechol substrate under optimum conditions, respectively.

Keywords affinity chromatography; purification; polyphenol oxidase; Marfona potato; characterization

1. Introduction

Polyphenol oxidase (PPO) enzymes, which are present in large amounts in all fruits and vegetables, are one of the most important enzymes in plants [1]. The PPO enzymes are copper-containing enzymes belonging to the family of oxidoreductases [2,3]. According to their substrate specificities and mechanisms of action, PPO enzymes is able to be classified into three main categories: catecholase (EC 1.10.3.1), tyrosinase (EC 1.14.18.1), and laccase (EC 1.10.3.2) [4]. The PPO enzyme causes melanization in animals and enzymatic browning in plants [5]. This enzymatic browning is a mechanism of defense that occurs as a result of mechanical and physical stresses during post-harvest processing and storage. Enzymatic browning is an undesirable phenomenon in the fruit and vegetable industry due to its detrimental effect on food quality. The PPO enzymes' extraction, purification, and characterization from vegetables and fruits have been focused on due to the importance of enzymatic browning in food technology and postharvest physiology. In several research studies, the PPO enzyme was purified by different methods including dialysis, ammonium sulfate precipitation, Triton X-100 extraction, Sephadex G-200 gel filtration chromatography, Phenyl-Sepharose hydrophobic chromatography, and affinity chromatography [6]. Chromatographic techniques can provide a high level of purification, and affinity chromatography is the most commonly used practical technique among these

chromatographic techniques. Affinity chromatography of 4-aminobenzoic acid (*p*-aminobenzoic acid) immobilized on Tyrosine-Sepharose is the more effective method [3].

Potato (*Solanum tuberosum* L.) is the sixth most produced plant in the world after sugar cane, maize (corn), rice, wheat, and palm. Türkiye provides 1.36% of the potatoes produced in the world [7]. According to 2021 data, 5,200.000 tons of potatoes were produced in Türkiye. While Niğde province ranks 1st among potato-producing provinces with 679.653 tons of potato production, 13.07% of the potato production in Turkey, İzmir ranks 5th with 436.530 tons, 8.39% of this amount. Among the districts of İzmir, Ödemiş ranks first among the potato-producing districts of İzmir, supplying 90.60% of the province with 395.496 tons and 7.61% of Türkiye [8]. The potatoes grown in Ödemiş district are geographical indications, and their geographical limits are the towns, villages, and highlands of Ödemiş district (Gölcük, Bozdağ). Potato cultivars grown as Ödemiş potatoes are Ausonia, Agria, Jearla, Resy, Granola, Impala, Concerde, Marabel, and Marfona [9]. Potatoes are one of the most important foodstuffs that can be a solution to the increasing hunger problem in underdeveloped and malnourished countries, thanks to their high nutritional value and wide usage area. In particular, it is rich in vitamins, minerals, carbohydrates, and protein. A 100 gram potato tuber can provide 3% of the daily energy, 10% of the iron, a minimum of 7% of protein, 10% of vitamin B1, and 20–50% of vitamin C that a normal person needs. When these values are considered, the place and importance of potatoes in nutrition are clearly seen [10]. Enzymatic browning causes low nutritional value, limits food safety, and poor appearance, which are serious problems in the processing and consumption of potatoes [11]. Potato protein, which is produced as a byproduct of the production of starch, has grown in value as a result of the rising demand for alternative protein sources. Furthermore, the existence of active polyphenol oxidase enzyme in the potato protein fraction can have negative impacts on protein quality. The active polyphenol oxidase enzyme can change the side chains of fundamental amino acids, cross-link the proteins, and thus affect their nutritional value and functionality. To fully benefit from the potential of potato proteins, it is important to characterize potato PPO and find methods to decrease its negative effects while protecting the nutritional and functional quality of potato proteins [12].

In this study, the Marfona potato cultivar, which is one of the potato cultivars grown in the Ödemiş district of İzmir, was studied. The PPO enzyme was purified from a Marfona potato cultivar using affinity gel, and its kinetic properties were investigated.

2. Materials and Methods

In the study, Marfona potato cultivar obtained from Ödemiş district of İzmir, Türkiye, was used as material.

Preparation of crude extract

50 g of Marfona potato tuber were first peeled, and then, without waiting, it was homogenized with a hand blender in 100 mL of 0.5 M phosphate buffer with a pH value of 7.3, containing a mixture of 0.5% polyethylene glycol and 10 mM ascorbic acid. The homogenate that was obtained was first filtered by using 2 layers of cheesecloth, and afterwards the filtrate was centrifuged for 1 hour at +4 °C at 2000xg. The precipitate that occurred after centrifugation, which included the cellulosic fibrous and plant walls, was discarded, and the supernatant was taken and used as a crude extract [13].

Ammonium Sulfate Precipitation

The precipitation was performed by using solid ammonium sulfate at 0-80% saturation in the crude extract. After achieving 80% saturation by adding ammonium sulfate, the suspension was centrifuged for 1 hour at +4°C at 2000xg. After centrifugation, the supernatant was discarded, and the precipitate was solubilized with a minimum volume of 5 mM phosphate buffer (pH:6.3), which it could be dissolved in [13].

Dialysis

The sample obtained after precipitation by ammonium sulfate and dissolved in a small volume of buffer was taken into a dialysis bag and dialyzed for 24 hours by changing the buffer several times against 5 mM phosphate buffer (pH:6.3) [13].

Purification of the PPO enzyme by affinity chromatography

The enzyme solution, obtained after dialysis, was purified by affinity chromatography (Sepharose 4B-L-tyrosine-*p*-aminobenzoic acid) [13].

Purity control of the PPO enzyme by SDS-PAGE

The purity of the PPO enzyme that was purified from the Marfona potato cultivar by affinity chromatography was checked by discontinuous SDS-PAGE, which included stacking gel at 3% concentration and separating gel at 10% concentration [14].

Qualitative and quantitative protein assays

Qualitative protein determination was performed in each eluate that was obtained as a result of purification by affinity chromatography on the basis of UV light absorbance at 280 nm [15]. The quantitative protein determination in the crude extract and in the enzyme solutions obtained from each purification step was detected at 595 nm by using the Bradford method [16].

Determination of PPO enzyme activity

Activity determination of the PPO enzyme was performed spectrophotometrically. To determine the activity of the PPO enzyme, the enzyme solution was quickly added to the buffer + substrate (0.1 M catechol) solution, and the change in absorbance in one minute was measured against the blank solution using a spectrophotometer at 420 nm wavelength. One unit of polyphenol oxidase activity was identified as the amount of enzyme that causes an increase in absorbance of 0.001 $mL^{-1}min^{-1}$ [13].

Determination of optimum pH and optimum temperature values

Using catechol as substrate, to determine the optimum pH and optimum temperature values of polyphenol oxidase enzyme purified from Marfona potato cultivar, PPO enzyme activity was measured at different pH values (4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0) with 0.2 M Na_2HPO_4 buffer and at different temperatures (15°C, 20°C, 25°C, 30°C, 35°C, 40°C, and 45°C) [13].

Determination of *Km* and *Vmax* values at optimum pH and optimum temperature

The Michaelis–Menten constant (*Km*) and the maximum velocity (*Vmax*) values were detected to investigate the kinetic properties of the PPO enzyme purified from the Marfona potato cultivar. To determine *Km* and *Vmax* values, PPO enzyme activities were measured using different concentrations of catechol substrate at optimum pH and temperature. The assays were performed in triplicate. The Lineweaver-Burk plot was graphed with 1/[S]-1/V values obtained as a result of the measurement average. The *Km* and *Vmax* values of the polyphenol oxidase enzyme purified from the Marfona potato cultivar were detected using the Lineweaver-Burk plot [13].

3. Results

Extraction and purification of the enzyme

The polyphenol oxidase was successfully purified from the Marfona potato cultivar by using affinity gel (Sepharose 4B-L-tyrosine-*p*-aminobenzoic acid). The elution graph obtained from PPO enzyme purification is given in Figure 1. In the elution process from the affinity column, the highest PPO activity was determined in the 3rd and 4th tubes.

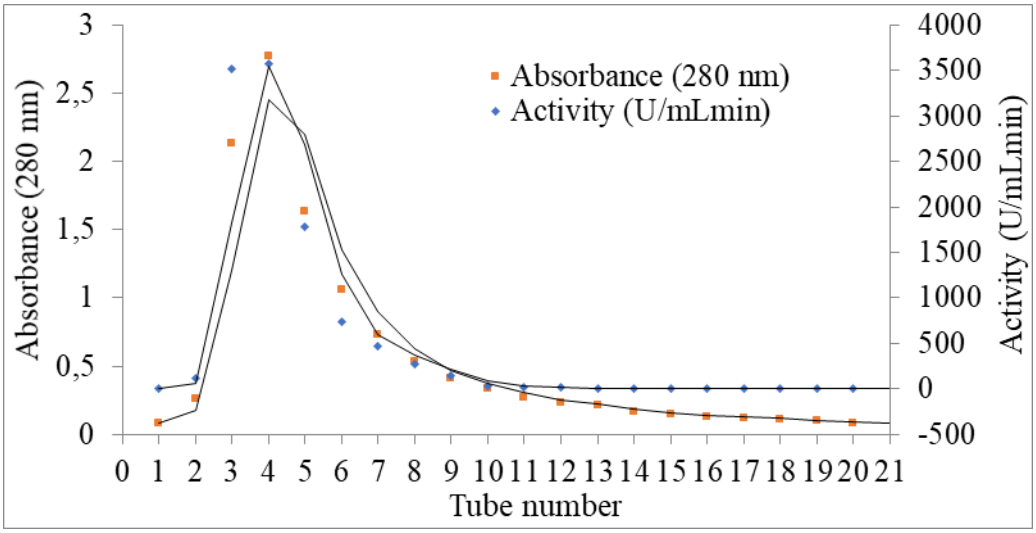

Figure 1: Sepharose 4B-L-tyrosine-tyrosine-p-aminobenzoic acid affinity column elution of polyphenol oxidase in the Marfona potato cultivar using 0.05 M Na_2HPO_4 (pH:7.0) buffer including 1 M NaCl.

Quantitative protein determinations and activity determinations of each of the enzyme solutions obtained as a result of crude extract, ammonium sulfate, dialysis, and affinity gel purification were performed to determine their specific activities and the purification fold (Table 1). Using affinity gel, the polyphenol oxidase was successfully purified 10.66-fold from the Marfona potato cultivar.

Table 1: The purification of the polyphenol oxidase from the Marfona potato cultivar.

Purification steps	Volume (mL)	Activity (U/mLmin)	Total activity	Protein (mg/mL)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Crude extract	54.5	4650.00	253425.00	0.0145	0.7927	319695.74	100.00	1
Ammonium sulfate precipitation	7.5	12015.00	90112.50	0.0390	0.2926	307927.06	35.56	0.96
Dialysis	7.5	14150.00	106125.00	0.0206	0.1542	688432.12	41.88	2.15
Affinity chromatography	2	2385.00	4770.00	0.0007	0.0014	3407142.86	1.88	10.66

The SDS-PAGE was performed to control the purity of PPO purified from the Marfona potato cultivar by affinity gel, and the purified PPO enzyme was visualized by using Coomassie Brilliant Blue R-250 for staining (Figure 2). The data suggest that fractions of polyphenol oxidase separated by SDS-PAGE may have different degrees of aggregation of subunits of the same enzyme.

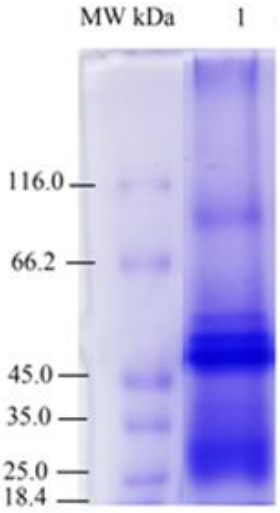

Figure. 2: SDS-PAGE: standard protein solution (marker) and 1) The polyphenol oxidase purified by using affinity gel from the Marfona potato cultivar.

Effects of temperature and pH on the PPO activity

In order to detect the optimum temperature and pH values of the PPO purified from the Marfona potato cultivar, PPO enzyme activity measurements were performed at different pH values (4.5–9.0) and different temperature values (15°C–45°C) by using catechol substrate. The PPO enzyme activity measurements were carried out using a 0.1 M concentration of catechol substrate solution. The determined optimum pH values of the polyphenol oxidase of the Marfona potato cultivar at each studied temperature using the catechol substrate are also presented in Table 2.

Table 2: Optimum pH values of PPO enzyme purified from Marfona potato cultivar by using catechol substrate at different temperatures.

Temperature (°C)	Optimum pH	Activity (U/mLmin)
15	6.5	299
20	7.0	378
25	**7.0**	**383**
30	7.0	140
35	6.5	328
40	6.5	260
45	5.5	205

Kinetic parameters

Using catechol substrate, the purified PPO of the Marfona potato cultivar enzyme activity values were determined at the different substrate concentrations under the optimum conditions (temperature: 25°C and pH: 7). The Lineweaver-Burk plot was plotted using the determined 1/[S]-1/V values (Figure 3). By using this plot, the *Km* and *Vmax* values of the Marfona potato cultivar purified PPO enzyme were detected (Table 3).

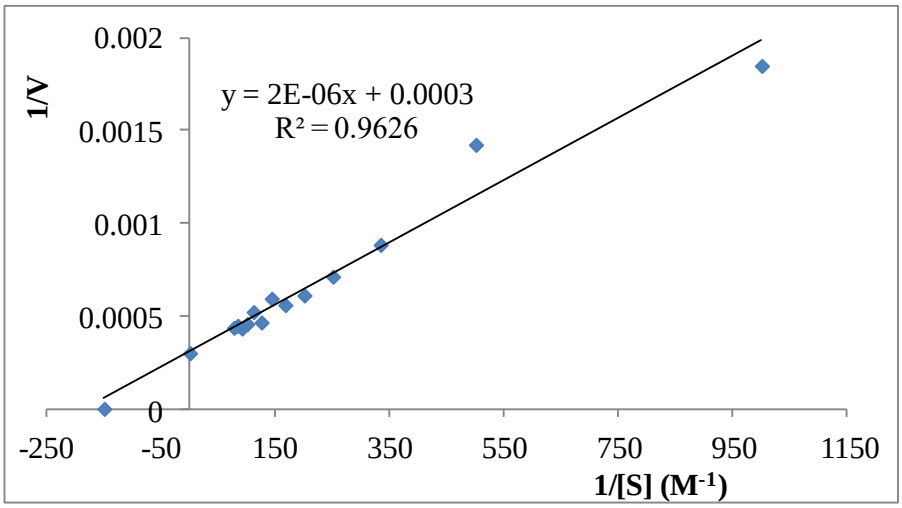

Figure 3: Lineweaver-Burk plot of PPO enzyme purified from the Marfona potato cultivar using catechol as substrate.

Table 3: The *Km* and *Vmax* values of polyphenol oxidase purified from the Marfona potato cultivar by using catechol substrate.

***Km* (mM)**	***Vmax* (U/mLmin)**	***Vmax*/*Km***
6.7	3333.33	500.00

4. Discussion

The aim of the present research was to purify and investigate the kinetic properties of the PPO enzyme from the Marfona potato cultivar, which is one of the potato cultivars grown in the Ödemiş district of İzmir, Türkiye.

The polyphenol oxidase from the Marfona potato cultivar was successfully purified 10.66-fold using the affinity gel (Sepharose 4B-L-tyrosine-*p*-aminobenzoic acid). Using the same affinity gel, in the other studies, the polyphenol oxidase was purified 15.73-fold from the Marabel potato cultivar [10], 52.25-fold from the Nevşehir potato [17], 7.87-fold from potato [18], and 5.14-fold from potato [6]. The polyphenol oxidase was purified 6.94-fold using Sepharose 4B-2-aminophenol-*p*-aminobenzoic acid (affinity gel using 2-aminophenol instead of L-tyrosine as the spacer arm) and 11.7-fold using Sepharose 4B-4-aminophenol-*p*-aminobenzoic acid (affinity gel using 4-aminophenol instead of L-tyrosine as the spacer arm) [6].

The results of the current study indicate that different degrees of aggregation of the same enzyme's subunits may exist in polyphenol oxidase fractions that were separated by SDS-PAGE. Using SDS-PAGE, it was determined in previous research that the potato PPO enzyme migrated as a single band with a molecular weight of 43 kDa. The native PPO enzyme was found to have a molecular weight of 86 kDa after gel filtration on the Sephadex G-150 column. The results of the study indicated that the potato polyphenol oxidase is a dimer composed of the same subunits [19]. The molecular weight of the potato PPO enzyme was determined to be 50 kDa, according to the SDS-PAGE and Native Polyacrylamide Gel Electrophoresis (Native-PAGE) results performed by [6]. Using SDS-PAGE revealed that soluble PPO and membrane-bound PPO enzymes purified from Favorita potato have a porminent singular protein band that is approximately 69 kDa in molecular weight [11].

The polyphenol oxidase enzyme purified from the Marfona potato cultivar has been determined to have optimum pH values of 7 and a temperature of 25°C. In other studies, the optimum pH and temperature values for the catechol substrate were determined to be 7 and 30–40°C, respectively for the PPO enzyme of some potato cultivars produced in Saudi Arabia [20]; to be 6.6 and 40°C, respectively for the PPO enzyme of potato [19]; to be 7.0 and 20°C for the PPO enzyme purified from Nevşehir potato [17]; to be 7 and 35ºC for the crude PPO enzymes of Atlantic, Desiree, and Granola potato cultivars [21]; to be 7 and 20°C for the PPO enzyme purified from the Marabel potato cultivar [10]; to be 6.5 and to be 20°C and 30°C, respectively for soluble PPO and membrane-bound PPO enzymes in Favorita potato [11]. In the present research, the optimum pH value is the same as in most of the other studies. Most of the studies indicate that the optimum pH and optimum temperature values of potato PPO enzymes are 7 and vary between 20°C and 40°C, respectively.

Under optimum conditions, that is, a pH value of 7 and a temperature value of 25°C, for the catechol substrate, the *Km* value of the PPO purified from the Marfona potato cultivar was determined to be 6.7 mM and the *Vmax* value to be 3333.33 U/mLmin. In a previous study, the *Km* value of the PPO enzyme purified from Nevşehir potato for the catechol substrate was found to be 5 mM, and the *Vmax* value was 5000 U/mLmin [17]. The *Km* and *Vmax* values of the polyphenol oxidase purified from the Marabel potato cultivar were determined to be 5.4 mM and 769.23 U/mLmin, respectively, for the catechol substrate [10]. With catechol as substrate, the *Km* and *Vmax* values of soluble PPO were 6.08 mM and 2161 U/S, whereas those of membrane-bound PPO were 2.95 mM and 2129.53 U/S, respectively, obtained from Favorita potato [11]. The PPO enzyme of Marfona potato's affinity for catechol substrate is close to the affinity of soluble PPO enzyme purified from Favorita potato and lower than the affinity of membrane-bound PPO enzyme purified from Favorita potato, PPO enzyme purified from Marabel potato cultivar, and PPO enzyme purified from Nevşehir potato.

5. Conclusion

In the present study, PPO enzyme was purified by using the affinity gel (Sepharose 4B-L-tyrosine-tyrosine-*p*-amino benzoic acid) from the Marfona variety potato grown in Ödemiş district of İzmir. Using the affinity column, the PPO enzyme was successfully purified, and following that, it was examined. The pure PPO enzyme was subjected to SDS-PAGE. The optimum pH and temperature values for the purified Marfona potato PPO by using catechol as the substrate were examined and determined to be 7 and 25°C, respectively. According to the results obtained from the study, the *Km* and *Vmax* values of the PPO enzyme purified from the Marfona potato cultivar were identified by using the equation of the Lineweaver-Burk plot. The *Km* and *Vmax* values of the PPO enzyme purified from the Marfona potato cultivar at optimum conditions were determined to be 6.7 mM and 3333.33 U/mLmin, respectively.

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References

[1]. Nixha, A. R., Ergun, A., Gencer, N., Arslan, O., & Arslan, M. (2019). Development of carbazole-bearing pyridopyrimidine-substituted urea/thiourea as polyphenol oxidase inhibitors: Synthesis, biochemistry, and theoretical studies. Archives of Physiology and Biochemistry, 125(3):263–269.

[2]. Sevindik, E. (2019). In silico analysis of putative polyphenol oxidases in olive using bioinformatics tools. Bangladesh Journal of Botany, 48(1):17-24.
[3]. Sarsenova, A., Demir, D., Çağlayan, K., Abiyev, S., Darbayeva, T., & Eken, C. (2023). Purification and properties of polyphenol oxidase of dried Volvariella bombycina. Biology, 12(1):53.
[4]. Demir, D., Eken, C., Çelik, E., & Alkan, N. (2021). Effect of Rhizoctonia spp. on polyphenol oxidase enzyme activity in alfalfa seedling. Research Journal of Biotechnology, 16(11):47–50.
[5]. Nixha, A. R., Arslan, M., Atalay, Y., Gençer, N., Ergün, A., & Arslan, O. (2013). Synthesis and theoretical calculations of carbazole substituted chalcone urea derivatives and studies their polyphenol oxidase enzyme activity. Journal of Enzyme Inhibition and Medicinal Chemistry, 28(4):808–815.
[6]. Aksoy, M. (2020). A new insight into purification of polyphenol oxidase and inhibition effect of curcumin and quercetin on potato polyphenol oxidase. Protein Expression and Purification, 171:105612.
[7]. FAO. (2021). Food and Agriculture Organization of the United Nations, Accessed: 14.09.2023. http://www.fao.org/faostat/en/#data/QCL.
[8]. TUİK. (2021). Türkiye İstatistik Kurumu, Accessed: 14.09.2023. https://biruni.tuik.gov.tr/medas/?locale=tr
[9]. Anonymous. (2002). T. C. Türk Patent Enstitüsü Çoğrafi işaret Tescil Belgesi, Accessed: 05. 08. 2019. https://www.turkpatent.gov.tr/TURKPATENT/resources/temp/504EC5AB-B0C8-45ED-B385-F86C115B0D53.pdf
[10]. Yavuz, T., & Demir, D. (2019). Determination of kinetic properties of polyphenol oxidase enzyme purified from Marabel potato variety. 2th International Congress of Agriculture, Environment and Health; 18-19 Ekim, Aydın, 1447- 1457.
[11]. Liu, H., Pan, M., Lu, Y., Wang, M., Huang, S., Li, J., Luo, K., Luo, L., Yao, M., Hua, D., & Wang, H. (2023). Purification and comparison of soluble and membrane-bound polyphenol oxidase from potato (Solanum tuberosum) tubers. Protein Expression and Purification, 202:106195.
[12]. Rasmussen, C. B., Enghild, J. J., & Scavenius, C. (2021). Identification of polyphenol oxidases in potato tuber (Solanum tuberosum) and purification and characterization of the major polyphenol oxidases. Food Chemistry, 365(15):130454.
[13]. Arslan, O., Erzengin, M., Sinan, S., & Ozensoy, O. (2004). Purification of mulberry (Morus alba L.) polyphenol oxidase by affinity chromatography and investigation of its kinetic and electrophoretic properties. Food Chemistry, 88(3):479-484.
[14]. Laemmli, U. K. (1970). Cleavege of structural ptoteins during in assembly of the head of bacteriophage T4. Nature, 227:680-685.
[15]. Jaenicke, L. (1984). Protein Bestimmung, Einführung in the Praxis des Biochemikers, Universität Köln, Köln.
[16]. Bradford, M. (1976). A rapid and sensitive method for the quantitaion of microgram quantites of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72(7):248.
[17]. Tozak, K. Ö. (2013). Nevşehir patatesinden (Solanum tuberosum L.) polifenol oksidaz enziminin afinite kromatografisi ile saflaştırılarak kinetik ve elektroforetik özelliklerinin incelenmesi. [Master dissertation]. [Aksaray]: Institute of Science and Technology, University of Aksaray. 72 p.
[18]. Bayrak, S., Öztürk, C., Demir, Y., Alım, Z., & Küfrevioglu, Ö. İ. (2020). Purification of polyphenol oxidase from potato and investigation of the inhibitory effects of phenolic acids on enzyme activity. Protein & Peptide Letters, 27(3):187-192.
[19]. Cho, Y.K., & Ahn, H. K. (1999). Purification and characterization of polyphenol oxidase from potato: I. purification and properties. Journal of Food Biochemistry, 23(6):577-592.
[20]. Alkhalifa, A. A., & Mahmoud, R. M. (1993). Polyphenol oxidase activities and properties in some potato (Solanum tuberosum) varieties produced in Saudi-Arabia. Arab Gulf Journal of Scientific Research, 11(3):377-390.

[21]. Wahyudi, A., Liliasari, S., & Supriyanti, T. (2019). Isolation and characterization of polyphenol oxidases (PPO) on potatoes (Solanum tuberosum) using age and environmental control. Journal of Engineering Sc*ience and Technology*, 14:1-9.