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Purification and Characterization of Polyphenol Oxidase Enzyme from Igdır Apple and Inhibition Effects of Some Chemicals

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Abstract: ***Polyphenol oxidase enzyme (PPO, E.C. 1.14.18.1) is a member of the class of oxidoreductase which causes blackening in plants. In this study, the enzyme Igdir apple was extracted and purified using Sepharose 4B-L-tyrosine-p-amino benzoic acid affinity gel. The sample obtained by affinity chromatography was used to investigate the characterization of the enzyme PPO and the effect of some inhibitors. For this purpose, optimum pH, temperatureand optimum ionic strenght values were determined by using different substrates. The best substrate of PPO was 4-methyl catechol. The optimum pH, temperature and ionic intensity values for this substrate were determined as 6.0 and 30 ^{o}C and 0.16 M, respectively. In this study ten inhibitors were studied and the IC_{50} values were calculated separately for the molecules which were inhibited by these methods. While tyrosine, 2-mercapto ethanol, copper sulfate, citric acid, sodium bisphosphate, chemicals have no effective inhibition; Inhibition from sodium azide, benzoic acid, ascorbic acid, sulfosalicylic acid, p-amino benzoic acid compounds was observed and the best inhibition molecule was found to be ascorbic acid.***

Keywords: characterization, purification, Igdır apple, polyphenol oxidase, inhibition

1. Introductıon

Polyphenol oxidase enzyme was first found in 1856 by Schoenbein in edible fungi [1]. It is an oxidoreductase group enzyme found in the active center of copper, which causes the formation of brown melanin pigments that catalyze the oxidation of phenolic compounds commonly found in fruits and vegetables, browning them to o-quinones and browning them as a result of their polymerization [2]. This darkening of the color and browning in plants reduce the value of vegetables and fruits and this situation causes a significant problem for both the consumer and the producer[3,4].These reactions, called enzymatic blackening, are not desirable for the food industry, although they are partly desired in fruit and vegetables such as tea, eggplant and black plums. Decomposition in damaged plant tissues is a chemical reaction with PPO enzyme catalysis and can be expressed as the oxidation reaction of phenolic compounds to quinones in the presence of oxygen [5-8].PPO enzyme, as well as many plants, lobster, shrimp, crab like shellfish, microorganisms and fungi are also widely available [9-10].Although the PPO enzyme differs from that in plant cells, it is usually found in plastids carrying the color substances in the cell. Therefore, phenolic substrates take place in vacuoles [11].In Igdır province, apple production has increased in West Iğdır Plain where irrigation facilities are available.In Igdır province, 62.77% of the total apple production was Golden (15265 tons), 31.28% Starking (7606 tons), 5.75% Granny Smith (1399 tons) and the rest 0.20% other apple (48 tons) species [12].

The characterization of the polyphenol oxidase enzyme responsible for the enzymatic browning, which is responsible for the determination of transport, storage and storage conditions for marketing of this fruit, which is called as the red apple (local al apple) in our country and used as the main source of livelihood for many people determination of inhibition studies is important. In this study, polyphenol oxidase enzyme from Iğdır red apple was purified by affinity chromatography and its biochemical properties such as characterization and effect of inhibitors were investigated.

2. Methodology

Materiel

Apple was used as the enzyme source. The chemicals used in the studies were obtained from sigma and merck companies. UV-VIS Spectrophotometer device (LKB-Biochrom, Ultrosec-II model 4050) was used for activity measurements.

Preparation of raw extract

Igdır Apple used in the research was obtained from Igdır region. The fruit was kept in the freezer (-70 °C) until used in the study. For the preparation of the crude extract, 50 g of fruit was homogenized with a 100 mL (0.5% polyethylene glycol) 0.5 M phosphate buffer (pH 7.30) with a home blender for 2 minutes. The homogenate was filtered through two layers of filter paper. The filtrate was centrifuged for 1 hour at 20000xg and +5 °C. The precipitate containing the plant walls and the cellulosic fibrous portion was discarded. The obtained supernatant was used as a crude extract [13].

Purification of PPO by Affinity Chromatography

Preparation of Affinity Gel

The affinity gel was prepared on Sepharose-4B matrix. CNBr method has been widely used in the modification of free hydroxyl groups of Sepharose-4B [14].The same method has been pre-activated by the manufacturer. L-tyrosine was ligated to activated Sepharose-4B. Binding of tyrosine diazotated p-aminobenzoic acid in the reactive derivative was performed. Here, the tyrosine constitutes the specifically binding portion of the affinity gel, the p-

aminobenzoic acid is a specific inhibitor of the polyphenol oxidase enzyme, which has been successfully used in the purification of the enzyme by entering the structure of the affinity gel. The affinity gel was prepared according to the following procedure, p-aminobenzoic acid is the enzymatically binding portion (ligand) of the column.

Activation of Sepharose-4B and Tyrosine Binding

2.86 g dry activated CNBr Sepharose 4-B gel was weighed for 10 ml bed volume. The reaction was continued until the pH did not change (10-15 minutes). A large amount of ice was added to the suspension and the mixture was transferred to a buchner funnel. It was then washed with 250 mL of cold 0.1 $NaHCO_3$ buffer (pH 10.0). A cold solution of the same buffer containing 15 mg of tyrosine in 20 mL was added and stirred for 90 minutes. The suspension was then allowed to stand at 4 ° C for 16 hours. At the end of this period, the wash water was washed with plenty of water until no absorbance was achieved at 420 nM. Thus, unreacted tyrosine was completely removed. The wash was repeated with 100 mL of 0.2 M $NaHCO_3$ buffer (pH 8.8). Tyrosine-modified Sepharose-4B was included in 40 mL of the same buffer [15].

Linking p-aminobenzoic Acid (Ligand Binding)

25 mg p-aminobenzoic acid was dissolved in 10 mL of 1 M HCl. A solution of 5 mL of 75 mg $NaNO_2$ was poured dropwise into the solution of p-aminobenzoic acid. After 10 minutes of reaction, the diazonated p-aminobenzoic acid was added to a suspension of 40 mL of sepharose-4B-tyrosine. The pH was kept constant by increasing to 9.5 and stirred at room temperature for 3 hours. It was then washed with 1L of distilled water followed by 200 mL of a 0.01 M Na_2HPO_4 (pH 6.0) buffer and maintained in the same buffer [15]

Application of Enzyme Solution to Affinity Column and Elimination of Enzyme

The prepared affinity gel was packaged on a 1x10 cm column and equilibrated with 0.05 M phosphate (pH 5.0). The enzyme solution obtained after dialysis was applied to the column and washed again with 0.05 M phosphate buffer (pH 6.0). Thus, most of the polyphenol oxidase cling to the affinity gel and the other impurities were removed. It was then carried out with 0.05 M Na_2HPO_4 / 1M NaCl buffer (pH 8.00) in the enzyme elution and 3 mL portions. Protein was collected at 595 nM and enzyme activity was determined at 420 nm in the collected tubes. Qualitative protein was determined by the Bradford method in the eluates, qualitative protein in 595 nm and enzyme activity determination at 420 nM [15].

Protein Determination

Protein determination was performed according to the Bradford method [16]. Bovine serum albumin (BSA) was used as the Protein Standard. The absorbances at 595 nm were read and the protein concentration was calculated by plotting the calibration graph.

Effect of pH

In order to determine the optimum pH value of PPO enzyme, the reaction rates were determined separately by using catechol as substrate at different pH values (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0). Each measurement was repeated three times and the averaged values were averaged. Activities were calculated by using the obtained values. Thus, the optimum pH value for the PPO enzyme was determined.

Effect of temperature

To determine the optimum temperature value of the PPO enzyme, the reaction rates were determined separately by using catechol at different temperatures (20, 30, 40, 50, 60, 70, 80, 90 °C) as substrate. Each measurement was repeated three times and the averaged values were averaged. Activities were calculated by using the obtained values. Thus, the optimum temperature for PPO enzyme was determined.

Effect of ionic strenght

To determine the effect of ionic strength on enzyme activity at constant temperature (at 37 °C) and at constant pH (7.4). Activity determinations were made by adding 0.02 M, 0.04 M, 0.06 M, 0.08 M, 0.10 M 0.12 M, 0.14 M, 0.16 M, 0.18 M 0.20 M, concentrations to ammonium sulfate $(NH_4)_2SO_4$ reaction medium.

Effect of Inhibitors

In this study tyrosine as inhibitor, 2-mercapto ethanol, copper sulfate, sodium azide, benzoic acid, citric acid, ascorbic acid and sulfosalicylic acid, p-amino benzoic acid and sodium bisphosphate (0.1, 0.2, 0.3, 0.4 and 0.5 ml). The activity before substance addition was accepted as 100%. In optimal conditions, the inhibitor concentration corresponding to the value at which 50% activity was retained was determined as the IC_{50} value from the plot drawn from the inhibitor concentration versus the percentage activity remaining after catechol inhibition [17].

3. Result

Purification of PFO by Affinity Chromatography

In the purification of the enzyme, the affinity column prepared on the homogenate column was first equilibrated with 0.05 M Na_2HPO_4 buffer (pH 6.0). After the column stabilization was completed, the buffer solution on the gel was reduced to the gel level. The centrifuged enzyme solution was applied to the column. The column was washed with 0.05 M Na_2HPO_4 buffer (pH 6.0). After the washing process was completed, the elution process was started. Before starting the elution, the buffer solution on the gel was reduced to the gel level and the elution was carried out with 0.05 M Na_2HPO_4/1M NaCl buffer (pH 8.0). The eluates from the column were taken into 3 ml each. The elution was continued until the absorbance at 595 nm was zero. Qualitative protein determination at 595 nm and activity determination at 420 nm were performed using elution buffer as blunt. Tubes with enzyme activity at the end of affinity chromatography were combined. Quantitative protein and activity assays were determined by the Bradford method for sample and combined eluate solutions applied to the column and specific activities and purification rates were determined (Table 1).

Table 1: Purification results of polyphenol oxidase enzyme purified from Iğdır apple.

Purification steps	Volume	Enzyme activity (EU/ml)	Total enzyme unit(EU)	Totalm protein (mg/ml)	Spesific activity (EU/mg.p rotein)	Purification coefficient
Homogenates	50	334	16700	138.2	2.42	-
Affinity column	45	589	26505	2.21	266.52	110.580

Protein Determination

Protein was determined by the Bradford method. (Figure 1).

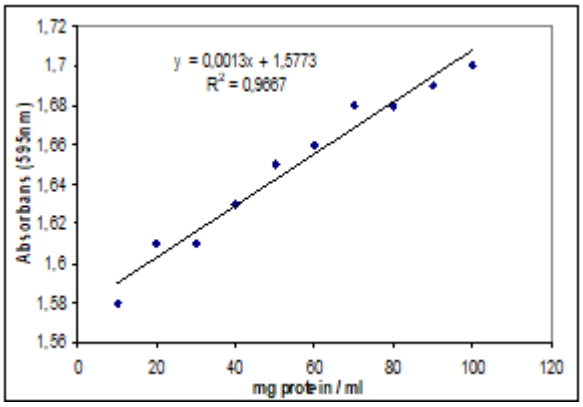

Figure 1: Protein standart chart

Effect of pH on Enzyme Activity

In order to determine the optimum pH values of PPO enzyme, reaction rates were determined by spectrophotometric method by using catechol substrate at different pH values (4, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0). In addition, the optimum pH of the enzyme was determined as 6.0. The results are given in Figure 2. In some literature studies, PPO obtained from different sources in the presence of catechol substrate, Lactarius salmonicolor optimum pH 7.5 [18], Izmir grape (Vitis vinifera L.) 's optimum pH 7.2 [19], Nevşehir potatoes (Solanum Tuberosum L.) optimum pH 7.0 [20], optimum pH of physalis peruviana L. (Bravo and Osorio, 2016), optimum pH of potatoes (solanum tuberosum) 6.0 [21].

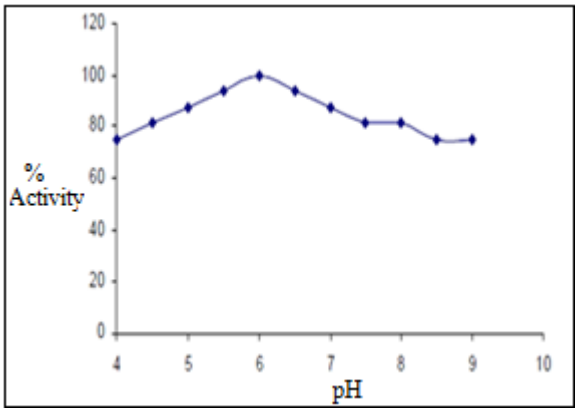

Figure 2: Effect of pH on the PPO activity

Effect of temperature

The effects of temperature on the speed of the catalyzed reactions of enzymes are known. The speeds of the enzymes catalyzed reactions generally increase in a direct proportion to the optimum temperature. At first, with the increase in temperature, the kinetic energy of the environment increases and in parallel increases in speed. After a certain temperature, a decrease in the activity of the enzymes in the functioning of the three-dimensional structure of a decrease occurs. In order to determine the optimum temperature values of PPO enzyme activity, the reaction rates were measured separately by spectrophotometric method at different temperature values (20, 30, 40, 50, 60, 70, 80, 90 °C) by using catechol substrate and the optimum temperature value was determined as 30 °C. . The results are given in figure 3. The optimum temperature values in the presence of catechol substrates of PPO obtained from different sources in some literature studies, Nevsehir potato (Solanum Tuberosum L.) 20 °C, Ocimum basilicum L. 40 °C, pear (pyrus elaegrifolia) was found to be 35 °C [15] and lactarius piperatus L. at 20 °C [22].

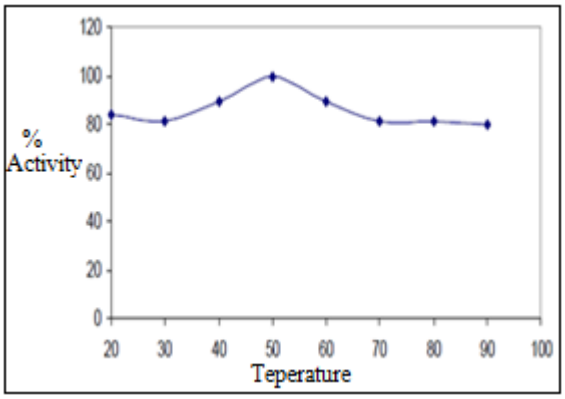

Figure 3: Effect of temperature on the PPO activity

Effect of ionic strenght

The behavior of the enzyme against ionic strenght is shown in Figure 4. Accordingly, the highest activity shows a concentration of 0.16 Molar $(NH_4)_2SO_4$. Since each protein is precipitated in different ionic severe environments, the proteins are separated from each other. Proteins retain their conformation during salting-out and can be reintroduced into solution phase in appropriate media. The enzymes show maximum activity at the most suitable salt concentration.

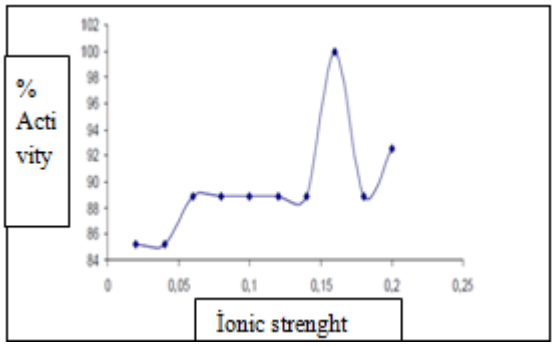

Figure 4: Graph showing the activity of PPO enzyme activity to ionic strenght

Inhibitory Effect

In this study, common inhibitors of PPO were used. From the curve obtained by plotting the residual activity of the enzyme corresponding to the concentration of each inhibitor, the inhibitory concentration corresponding to the value at

which the 50% activity of the enzyme was maintained was determined as the IC_{50} value. The IC_{50} values found are given in Table 2. Accordingly, tyrosine, 2-mercapto ethanol, copper sulphate, citric acid, sodium bisphosphate, sodium azide, benzoic acid, ascorbic acid, sulfosalicylic acid and p-amino benzoic acid were found to be the most effective of the ascorbic acid. IC_{50} values of sodium azide, benzoic acid, ascorbic acid, sulfosalicylic acid, p-amino benzoic acid in the presence of catechol subsutrate PPO of Igdir apple were found as 3,826 mM, 7,308 mM, 0,056 mM, 6,043 and 0,02, respectively, and the effective inhibitor ascorbic acid[22].

Table 2: Effect of some inhibitors on the PPO activity

Inhibitör	IC_{50}(mM)
Ascorbic acid	0.056
Sulfosalicylic acid	6.043
p-amino benzoic acid	0.02
Benzoic Acid	7.308
Sodium azide	3,826

4. Conclusion

In this study, some properties of the polyphenol oxidase enzyme purified from Iğdır Apple were also investigated and the inhibition effect of some chemicals was investigated. Because of the damage that occurs during the transportation or processing of fruits and vegetables or when the cut, sliced surfaces of these products are exposed to air or after freezing, dissolution leads to enzymatic dimming. Enzymatic decay reactions resulting from PFO enzyme catalysis are not desirable because they reduce the taste, appearance and nutritional value of the product. Polyphenol oxidase enzyme was purified and characterization studies such as optimum pH, optimum temperature and optimum ionic strength related to the enzyme were made in this study. In addition, the effect of some chemicals on enzyme activity was investigated. The IC_{50} values of the molecules which exhibit inhibition effect were calculated. The aim is to preserve the natural color and the content of the fruit.

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