Purification and Characterization of Polyphenol Oxidase Enzyme from Damson Plum (*Prunus insititia*) by Spectrophotometrically

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Abstract

Polyphenol oxidase enzyme, performing browning reactions in fruits and vegetables, was purified from damson plum (*Prunus insititia*) which has a high antioxidant activity. Firstly, partially purified polyphenol oxidase was treated by 0-80% ammonium sulfate precipitation and dialysis, respectively. Characterization studies were carried out by using catechol, 4-methyl catechol, pyrogallol and caffeic acid as 0.05M/ pH:7.2/ 25°C; 0.2M/ pH:4.5/ 10°C; 0.01M/ pH:6.8/ 5°C and 0.2M/ pH:8.5/ 10°C, respectively. The kinetic constants of V_{max} and K_M were calculated for the same substrates as 17219.97 U/(mL*min) and 11.67mM; 7309.72 U/(mL*min) and 5mM; 12580.12 U/(mL*min) and 3.74mM; 12100.41 U/(mL*min) and 6.25 mM, respectively. Catechol gave the highest V_{max} value when compared to others. In the second step, purification was performed by using Sepharose 4B-L-Tyrosine-p-amino benzoic acid and Sepharose 6B-L-Tyrosine-p-amino benzoic acid affinity gels. A single band of approximately as 50-55 kDa was observed in SDS-PAGE and Native-PAGE. 90 and 10.2 purification folds were obtained for *Prunus insititia* PPO by the reference Sepharose-4B-L-Tyrosine-p-aminobenzoic acid and original Sepharose-6B-L-Tyrosine-p-aminobenzoic acid gels, respectively. PPO enzyme purification from *Prunus insititia* by affinity chromatography has not been investigated in literature yet.

Highlights

- *Prunus insititia* PPO enzyme was purified by affinity chromatography in UV spectrophotometer.
- Characterization studies were carried out as investigated optimum concentrations, pH and temperatures for different substrates.
- The kinetic measurements were taken for different substrates of the enzyme.
- The storage stability of the enzyme was proven by statistical analysis.
- IR analysis were performed in FT-IR spectrophotometer for the original affinity gel.

Introduction

Polyphenol oxidases (PPOs), which are fairly distributed over plants, fungi, bacteria and animals, having copper-containing enzymes catalyse the enzymatic browning reaction in fruits, vegetables and beverages as a result of hitting, crushing or harvesting that may occur during the storage process [1,2]. It occurs by the way of interaction with its phenolic substrates which are included into the same enzyme material [1]. Sufficient consumed amounts of nutritions including these phenolic compounds which have antimicrobial, antioxidant and anticancer activities are very important due to the functions of being removed of free radicals from the body. On the other hand, radical formation can be prevented by the removal of molecular oxygen from biological systems. Because cell enzymes form their products as predominantly effective by using molecular oxygen [3]. One of these enzymes is PPO enzyme that it breaks down molecular oxygen for its catalytic reaction in two steps as hydroxylation of monophenols to o-diphenol (monophenolase activity) and oxidation of o-diphenol to quinone (diphenolase activity), respectively [4]. Due to its phenol oxidizing properties, PPO enzyme is preferred in lots of industrial areas that environmental, nutritional, pharmaceutical and biomedical applications such as food, textile, wastewater treatment, paper, personal care, cosmetic, production of biofuel cells, biological fluids analysis and biosensors to establish pesticide residues etc. [4]. In addition, the role of PPO enzymes in plant defense against pathogens are highlighted with the increasing amount of PPO as a result of the enhancement in pathogen resistance among plant varieties [5-7].

PPO enzyme was purified from damson plum (*Prunus insititia*) in this study. *Prunus* family has high antioxidant activity to prevent free radical formation and damson plum includes high amount of phenolic compounds such as

malic acid, chlorogenic acid, anthocyanin, quercetin and catechin which have defender effects against cancer, cardio- and cerebrovascular diseases [8,9] as well as having soluble sugar [10]. *Prunus insititia* is used in jams as well as its protector role in social life of northwestern Spain with a growing feature even after collection and has a critical economical value [11]. Also Turkey is located in the forefronts of worldwide in the production of different types of plums such as *Prunus domestica*, *Prunus cerasifera*, *Prunus insititia*, *Prunus spinosa*, *Prunus divericata* and *Prunus salicina* [12]. On the other hand, Reig et. al. mentioned that the rootstock of *Prunus insititia* plum is an alternative choice in apricot industry [13]. The main reason is fairly effective to be preferred of damson plum (*Prunus insititia*) as a PPO enzyme source that there has been no any study in literature related to the purification of PPO enzyme by affinity chromatography from this type of plum which has high antioxidant and anticancer properties [8,9], additionally with the light of all these given informations.

PPO enzyme was purified by different biochemical methods such as ion exchange chromatography by using diethyl aminoethyl-sepharose or diethyl aminoethyl-A50 sephadex, gel filtration chromatography by using sephadex G-150 [14-17], Sephacryl S-200 [18] and three-phase partitioning [19,20]. Among these purification methods, affinity purification methods have been widely used in the separation of various carrier proteins which are used in phosphorylation, methylation, acetylation and ubiquitination [21] and unlike these such conventional chromatography methods [14-20], affinity chromatography is the most preferred one to warrant a high isolation of any enzyme from its initial material by using a specific ligand of an enzyme which is bounded covalently to a cross-linked support matrix. Separation of an enzyme from a solid matrix was generally accomplished by changing of ionic intensity such as pH or salt concentration [22]. Different matrices were used in affinity chromatography such as silica, glass, polystyrene, cellulose and sepharose. When compared to these materials, sepharose has the most commonly used solid support with the use of 60.2 percent [23]. Sepharose, an agarose cross-linked dextran, was also used as the solid matrix in this study. Sepharose activation with cyanogen bromide enables to bind a specific molecule by using its unprotonated amino groups [22] and covalent immobilization of the enzyme ligand to the matrix by the diazotisation reaction. Immobilization of proteins through their functional groups such as heterocycles or different types of phenols by diazotisation increases the bonding specificity between a purified biomolecule and activated matrix [24]. These immobilized matrices are commonly used in purification steps of affinity chromatography [21]. On the other hand, affinity ligands have high selectivity and affinity to their target enzymes or proteins under relatively moderate conditions as well as their increasing resolution properties to bind target protein and conjugated to surfaces of matrices. Type of ligands can be metal ions, amino acids, dyes, hormones, small organic molecules or acids [25]. p-aminobenzoic acid, which is a carboxylic acid derivative, is the known reversible potent inhibitor of PPO enzyme used as an affinity ligand in literature [26-31]. Carboxylic acid derivatives are the most comprehensive PPO inhibitors among organic acids in plants was also reported [1]. This type of inhibitor ligands prevent undesired browning in nutritions for industrial and agricultural applications. They also prevent hyperpigmentation in skin for therapeutic targets and used as a treatment agent for some cancer types such as lung and breast [2]. In this study, p-aminobenzoic acid was used as the affinity ligand for the synthesized of Sepharose-4B-L-Tyrosine-p-aminobenzoic acid and Sepharose-6B-L-Tyrosine-p-aminobenzoic acid affinity gels in affinity column. Moreover, column has the mostly preferred material in affinity purification at the percent range of 92.3 when compared to other material types such as capillary, monolith, perfusion, expanded bed or fiber membrane etc. In addition, enzymatic studies have been performed at a rate of 5.1% in the literature in recent times while the percentages of studies onto immunoglobulin-binding proteins, lectins and antibodies has been 13.5%, 9.7% and 59.9%, respectively [23]. So purification of PPO enzyme from damson plum (*Prunus insititia*) which is a new search of enzyme material by affinity chromatography was investigated in this study.

Materials And Methods

Material

HCl, NaOH, Sepharose 4B and 6B, CNBr, NaHCO₃, L-Tyrosine, p-aminobenzoic acid, NaNO₂, Na₂HPO₄*2H₂O, K₂HPO₄, polyethylene glycol, ascorbic acid, (NH₄)₂SO₄, NaCl, catechol, 4-methyl catechol, pyrogallol, caffeic acid, bovine serum albumin (BSA), Tris base, acrylamide/bis 30%, sodium dodecyl sulfate, Tetramethyl ethylene diamine, ammonium persulfate, glycerol, b-mercapto ethanol, bromophenol blue, coomassie brilliant blue (CBB) G250, CBB R250, acetic acid, methanol were purchased from Sigma-Aldrich Co and Merck. Multicolor broad range and pre-stained protein marker (10 to 260 kDa) were purchased from thermos scientific. Electrophoresis system was supplied from Bio-Rad. Dialysis membrane (D-9777) and 18mL liquid chromatography column as 1.5 cm*10 cm were purchased from Sigma-Aldrich Co. Damson plums (*Prunus insititia*) was supplied from Kocaeli. Spectrophotometric UV analyses were performed in Shimadzu UV-VIS Spectrophotometer and IR analyses in Thermo-Scientific FT-IR spectrophotometer.

Methods

Purification of PPO enzyme from damson plum (*Prunus insititia*) was carried out in two main steps as the partial purification of the enzyme and purification by affinity chromatography. For this purpose, the following methods were taken into consideration, respectively.

Partial Purification of Crude Polyphenol Oxidase Extract (CPE) from Damson Plum (*Prunus insititia*)

The extraction procedure was adopted from the study of Dogan et. al. [32]. In order to obtain the crude polyphenol oxidase extract (CPE), 150 g / 300 mL damson plum was homogenized with extraction buffer at pH: 7.3 for 2 minutes. Extraction buffer solution includes 0.5 M potassium hydrogen phosphate (pH: 7.3) containing polyethylene glycol (PEG) 0.5% (w/v) and 10mM ascorbic acid. The homogenate was filtered through 2 layers of cheesecloth and following centrifuged at 9000 rpm, 4°C for 50 min. to separate cellulose fibers which were included in the cell walls of damson plum from the supernatant by centrifugation. This supernatant was used as CPE enzyme. *Prunus insititia* CPE enzyme was purified by applying the partial purification processes including 0-80% ammonium sulfate precipitation in which the enzyme can be obtained with maximum efficiency and dialysis, respectively.

Application of Ammonium Sulfate Precipitation to CPE Enzyme

Ammonium sulfate allows the precipitation of proteins according to certain saturation degrees by not causing the formation of complex particles. Because it is a neutral salt with +2 valence and good solubility. In addition, its properties of being safe and economic allows it to be used frequently in precipitation of proteins [33]. The amount of ammonium sulfate required for the salt precipitation interval was calculated by the formula given in below:

$$g (NH_4)_2SO_4 = \frac{1.77 \times V \times (S_2 - S_1)}{3.54 - S_2} \quad (a)$$

V= Süpernatant volume

S₂: The last percent amount of (NH₄)₂SO₄ saturation

S₁: The initial percent amount of (NH₄)₂SO₄ saturation

In our study, the determined amount of $(\text{NH}_4)_2\text{SO}_4$ salt was added slowly to the CPE enzyme supernatant in the precipitation range of 0-80% in terms of the above formula. CPE supernatant saturated with $(\text{NH}_4)_2\text{SO}_4$ was precipitated at 9000 rpm, 4°C for 70 min. The CPE precipitate was dissolved with a minimum volume of 5mM K_2HPO_4 buffer solution and the sample was made ready for dialysis.

Dialysis of CPE Enzyme Treated with Ammonium Sulfate Precipitation

The CPE enzyme precipitate obtained in the previous step was dissolved in a minimum volume of dialysis buffer (5mM, 1L K_2HPO_4 , pH:6.3) and dialyzed at 4°C between 16-24 hours at 8-hour intervals in the same buffer type. So the partially purified polyphenol oxidase enzyme (PPE) was obtained with the procedure applied until this step. In the last experimental part, PPO enzyme was obtained by applying the PPE enzyme to the affinity column.

Characterization Studies of Damson Plum PPE (*Prunus insititia*)

In order to determine the optimum conditions for *Prunus insititia* PPE enzyme, the optimum concentration was firstly determined by studying at 7 different concentrations of Na_2HPO_4 buffer solutions from 5mM to 300mM. In the second step, the optimum pH was determined by working with 14 different pH values (4-9) according to the optimum buffer concentration. In the third step, buffer solution prepared in the optimum buffer concentration and the optimum pH were used, and the optimum temperature was determined by using 10 different temperatures between 3°C-70°C. So the previous optimum value was taken into consideration for each parameter. Na_2HPO_4 buffer type was used as the activity buffer solution for the preparation of purification table and the measurements of enzyme storage for different time intervals.

Determination of Kinetic Constants (K_M , V_{max}) of *Prunus insititia* PPE Enzyme

Lineweaver-Burk plot was drawn by taking into account of the activity measurements of *Prunus insititia* PPE enzyme for different substrate concentrations, $1/[S]$ (M) versus enzyme activity, $1/V$ (U/min) at 420nm. V_{max} and K_M kinetic constants of *Prunus insititia* PPE enzyme were calculated from the graph for each substrate.

PPO Enzyme Purification by Affinity Chromatography

In this study, p-aminobenzoic acid, which is a known competitive inhibitor of PPO enzyme, was used as a ligand in the purification of *Prunus insititia* PPO enzyme by affinity chromatography. By using this ligand, Sepharose 4B-L-Tyrosine-p-aminobenzoic acid [34,35] and original Sepharose 6B-L-Tyrosine-p-aminobenzoic acid affinity gels were synthesized. In affinity gels, Sepharose 4B and 6B matrices were used as solid support materials, L-Tyrosine as the spacer arm and p-aminobenzoic acid as the ligand.

Preparation of Affinity Matrix for the Purification of PPO Enzyme

In our study, Sepharose 4B and 6B matrices were activated with CNBr. After the matrices were covalently bonded to the spacer arm and following the ligand active surfaces, separately [36]. The reference (4B) and original (6B) affinity gels were synthesized according to the methods used in literature [26-31, 34,35].

Sepharose 4B and 6B Activations for the Synthesis of Affinity Gels

Sepharose 4B and 6B suspensions were taken separately at a volume of 10mL and decanted with distilled water with an equal volume. Following, the free-OH groups in Sepharose 4B and 6B matrices were activated with 4.7 mM cyanogen bromide in cold environment for the attachment of the spacer arm to the matrix and immobilization of the

ligand, respectively. Since cyanogen bromide has an acidic character, NaOH was added until the pH value of the matrix mixture was quickly raised to 11 and stabilized at this value.

Covalent Bonding of L-Tyrosine to Sepharose 4B and 6B Matrices

CNBr activated Sepharose 4B and 6B were transferred to buchner funnel, washing with 250mL, 0.1M NaHCO₃ (pH: 10) cold buffer solution and unbound CNBr was removed from the medium. The gel suspensions were mixed for 90 min. at room temperature in order to achieve covalent bonding between 4.1 mM L-Tyrosine solved in 20mL 0.1M NaHCO₃ (pH: 10) solution as an spacer arm and the free hydroxyl group of the Sepharose matrices, separately. The gel mixtures were kept at 4°C.

Immobilization of p-aminobenzoic Acid to the Support Materials and FT-IR Analysis of the Gels by Spectrophotometrically

Sepharose-4B-L-Tyrosine and Sepharose-6B-L-Tyrosine were washed with 1L cold distilled water and 100mL, 0.2M pH: 8.8 NaHCO₃ buffer solution in buchner funnel, respectively. Following 18mM, 10mL p-aminobenzoic acid was dissolved in 1M HCl at 0°C. 5mL of sodium nitrite was added slowly to the cold p-aminobenzoic acid solution with an equilibrium concentration of 72mM at 15mL and stirred for 10 minutes to be prepared of ligand solution in briefly. After the ligand solution was added to the gel suspension immediately. NaOH was added to the reaction medium so that the pH was measured as 9.5 and following the gel suspension was stirred at room temperature for 3 hours to give a diazotisation reaction. Thus, the specific ligand of PPO enzyme was covalently linked to the support material (matrix- spacer arm) by diazotisation reaction (Scheme 1). In the last step, synthesized Sepharose-4B-L-Tyrosine-p-aminobenzoic acid and Sepharose-6B-L-Tyrosine-p-aminobenzoic acid affinity gels were washed with 1L distilled water and 200mL, 0.01M Na₂HPO₄ (pH:6) buffer solution, respectively to be removed of free spacer arms and ligands from the reaction medium (Scheme 1).

Following, the fourier transform infrared (FT-IR) spectra results of two affinity gels were taken as the graph between wavenumber (cm⁻¹) against transmittance % from a Thermo scientific model spectrophotometer. FT-IR spectra were obtained over a scan range of 450–4000 cm⁻¹ in this study. The gels were kept in 0.01M Na₂HPO₄ (pH:6) buffer until used.

Purification of Damson Plum PPO Enzyme (*Prunus insititia*) by Using Affinity Gel

In this step, equilibration buffer solution (0.05M Na₂HPO₄, pH:5) was used to pack and equilibrate of Sepharose-4B-L-Tyrosine-p-aminobenzoic acid and Sepharose-6B-L-Tyrosine-p-aminobenzoic acid gels into an 18 mL affinity column, separately. In the second step, PPE enzyme solution was loaded into the affinity column and washing with equilibration buffer solution. PPO enzyme was held in the column via the specific p-aminobenzoic acid ligand, while other foreign proteins in the PPE enzyme solution were removed from the column. In the third step, the enzyme molecules attached to the ligand were eluted as 2mL fragments with 0.05M Na₂HPO₄ pH:7 elution buffer solution containing 1M NaCl. At the last step, the absorbance of 2mL of eluents were measured at 280nm in the UV-Vis spectrophotometer. Following, the elution graphs which show the relation between the amount of enzyme at 280nm and PPO enzyme activity for each tube at 420 nm were drawn. In order to calculate the specific activity of the enzyme, the amount of PPO enzyme having higher activity in mg amount was determined by using Bradford method [37].

Determination of Protein Amount of Damson Plum PPO (*Prunus insititia*) by Bradford Method

Bradford method is based on measuring of the complex formed by interacting between the positive charges of proteins and the negatively charged CBB G-250 reagent in a phosphoric acid environment at 595nm [37]. Bradford method is generally preferred among the protein quantification methods to show the maximum complex absorbance at 595nm and the disruptive factors are low during the reaction measurement. While determining the amount of protein by Bradford method, a series of standard protein solutions were prepared from BSA which was used as standard protein. For this reason, BSA solution (1mg / 1mL) was vortexed by adding 5mL of CBB G-250 reagent to each tube with a total volume of 5.1mL in the concentration range from 0.39 mg/mL - 19.61 µg/mL, separately. 100µL volume of distilled water with the addition of 5mL blue dye was used as a blank solution for standard curve and pre-purification steps. The same volume of elution buffer solution (0.05M Na₂HPO₄ pH:7) with the addition of 5mL CBB G-250 dye was used as a blank solution for affinity elution. After 10 minutes, absorbance measurements were taken at 595 nm for standard solutions against reagent blank solution and enzyme solution of unknown concentration. A standard line graph was created based on the standard concentrations corresponding to the standard solution absorbances, and the amount of protein corresponding to the absorbance read from the graph was found. The necessary dilutions were made for the concentrated pre-purified samples and these proteins amounts were calculated by taking the dilution factor into consideration.

Determination of Activity of Damson Plum PPO (*Prunus insititia*)

The activity of purified damson plum PPO (*Prunus insititia*) enzyme was determined spectrophotometrically by using 0.1M catechol, 4-methyl catechol, pyrogallol and caffeic acid, respectively. According to the modify method of Espin et. al.; activity buffer, substrate (0.1M) and enzyme solutions were added in the volumes of 2410mL/390mL/200mL to the measurement cuvette, respectively [38]. The change in enzyme absorbance against blank solution was detected by measuring the initial ration of quinone formation, as expressed by an increasement in the absorbance units for the first minutes at 420 nm. An increase in absorbance of 0.001 min⁻¹ for the equilibrium concentration was taken as 1 enzyme unit (1EU) [38]. All measurements were made in triplicate. In the enzyme extracts obtained as a result of all the procedures, Bradford and PPO enzyme activity methods were applied and the obtained results were used in the formation of purification tables. The PPO enzyme purity was checked by using sodium dodecyl sulfate (SDS-PAGE) and Native-PAGE gel electrophoresis, respectively.

Determination of Molecular Weight of *Prunus insititia* PPO Enzyme by SDS-PAGE Gel Electrophoresis

SDS-PAGE was applied for the control of enzyme purity and determination of molecular weight of *Prunus insititia* PPO enzyme after purification steps by affinity chromatography. For this purpose, separation 10% gel was prepared according to the volumes showed in Table 1 [39].

For this purpose, two glass plates were fixed on top of each other with a plastic strip preventing flow at the bottom and placed in the electrophoresis tank after the gel was loaded. Separation gel was prepared according to the amounts of the chemicals specified in Table 1. In the preparation phase of the gel, ammonium persulfate was added to the mixture and the gel was slowly mixed and then loaded quickly between glass plates. The polymerization of the loaded gel was checked by looking at the remaining gel mixture in the beaker. Once the separating gel was quickly loaded, electrophoresis comb was placed into the separation gel to form wells for loading samples into the running buffer after the polymerization stage. The comb was carefully removed when polymerization was completed and the gel system was placed in the electrophoresis tank. Approximately 250mL of running buffer (0.02M, 3g Tris base, 0.19M, 14.4g glycine and 3.5x10⁻³M, 1g SDS/1L distilled water) was added into the tank and gel system. The loaded samples were mixed with electrophoresis sample buffer prepared in terms of chemicals and quantities specified in Table 2 [39].

The PPO enzyme eluates were mixed with the sample buffer solution prepared according to Table.2 at a ratio of 1:1 in generally and boiled for 5 minutes. Protein denaturation was achieved by SDS contained in the sample buffer to eliminate the influence of the protein charges. So proteins are separated only based on their molecular weights. Because proteins charges are negligible besides the negative charge of SDS [39]. Enzyme samples loaded into electrophoresis wells were run in 80 volt and 150 volt, respectively. The running process was continued until there was 1cm from the end of the gel. The gel was carefully removed from the glass plates and waited for approximately 20 min into the colored solution (3.0mM, 264mL of CBB R-250 including 120mL of methanol and 24mL of acetic acid) with a stirring. Following, the gel was taken into the decolorization solution (7.5% acetic acid (v/v) and 5% (v/v) methanol in 1L) through overnight so that the colored protein bands can be observable in the solution.

Prunus insititia PPO Enzyme in Native-PAGE Gel Electrophoresis

Native-PAGE was applied to investigate the existing subunit of *Prunus insititia* PPO enzyme purified by affinity chromatography. Glass plates were cleaned and placed as stated in the method of SDS-PAGE. The separation gel of 15% T and 5% C_{bis} was prepared and loaded between the glass plate [40] (Table 3).

The process was carried out by adjusting the current to 80W for approximately the first 30 minutes and 150W during electrophoresis. In electrophoresis system, the electrophoresis tank buffer solution (63 mM Tris base, pH:7.5) and running buffer solution (37.6 mM Tris base, pH:8.9, including 40 mM glycine) were used as separately, which were different from SDS-PAGE buffers. Sample buffer solution (5g sucrose, including 0.01g/mL bromophenol blue in 10mL) was used for staining of samples and marker during electrophoresis [40]. For coloring, 1×10^{-1} M catechol solution was used under optimum conditions.

Calculation of Yield %, Specific Activity and Purification Fold of *Prunus insititia* PPO Enzyme

Specific activity is the enzyme activity corresponding to mg amount of an enzyme. For this reason, the specific activity, yield % and purification fold were determined by calculating the total volume, total protein amount and enzyme activity values of the enzyme extracts for each purification step. The ratio between the total activity of the enzyme extract and the total amount of protein for the relevant purification step was used to calculate the specific activity. Total activities for each step were proportioned according to first step to find yield as a percent, separately. When calculating the final purification folds, the specific activity values calculated for each step were divided by the specific activity value obtained for the first step.

Determination of Storage Stability of *Prunus insititia* PPO Enzyme and Statistical Evaluation of the Stability Results

The storage stability of *Prunus insititia* PPO enzyme was investigated under optimum conditions obtained from the characterization studies. A graph was drawn by calculating time-dependent activity and activity % values over the measurements taken during 3 months. The time dependent enzyme activity change, expressed as the storage stability of the enzyme, was statistically evaluated by using one-way ANOVA analysis method in excel.

Results And Discussion

Partial Purification of *Prunus insititia* CPE Enzyme

The supernatant volume of *Prunus insititia* CPE enzyme was measured as 313 mL after the first centrifugation. The amount of ammonium sulfate required for precipitation was determined as 161.75 g, according to 313 mL. Enzyme

treated with 0-80 % ammonium sulfate was centrifuged as the second time and 27 mL enzyme solution was loaded into dialysis by dissolving in dialysis solution. The volume after dialysis was determined as 36 mL. After separation of 2mL for purification table, 34 mL PPE enzyme solution was used for purifications from the both affinity gels as 17 mL for each one.

Characterization Studies of *Prunus insititia* PPE Enzyme

Buffer concentration, pH and temperature values at which the observation of optimum *Prunus insititia* PPE enzyme activity for catechol, 4-methyl catechol, pyrogallol and caffeic acid were investigated in spectrophotometrically (Fig. 1-3).

PPE enzyme activity for catechol, 4-methyl catechol, pyrogallol and caffeic acid were determined as 0.05M; pH:7.2; 25°C / 0.2M; pH:4.5; 10°C /0.01M; pH:6.8; 5°C and 0.2M; pH:8.5; 10°C, respectively (Fig. 1-3). In addition, catechol showed higher activity when compared to other substrates as 11236.5 U/(mL*min.) and 11875.5 U/(mL*min.) in Fig. 2-3. It was observed that PPE enzyme reaches its highest activity value at 25°C but it protected its activity up to about 36°C (Fig. 3).

Determination of Kinetic Constants (K_M , V_{max}) of *Prunus insititia* PPE Enzyme

The kinetic constants were determined for catechol, 4-methyl catechol, pyrogallol and caffeic acid substrates of *Prunus insititia* PPE enzyme under optimum conditions at 420 nm by using UV-Vis Spectrophotometer. Lineweaver-Burk graph was drawn and K_M , V_{max} and catalytic efficiency (V_{max}/K_M) values were calculated for each substrate from the intercept points of each graph in spectrophotometrically (Fig. 4-7).

The kinetic constants of V_{max} and K_M calculated for catechol, 4-methyl catechol, pyrogallol and caffeic acid as 17219.97 U/(mL*min) and 11.67mM; 7309.72 U/(mL*min) and 5mM; 12580.12 U/(mL*min) and 3.74mM; 12100.41 U/(mL*min) and 6.25 mM, respectively. The kinetic data obtained were summarized by calculating the catalytic efficiency ratio for each substrate (Table 4).

It was determined that PPE enzyme activity measured by using catechol substrate gave the highest V_{max} value when compared to other substrates (Table 4).

PPO Enzyme Purification by Affinity Chromatography

PPE dialysis sample (17mL) were loaded into the gels, separately. Since different studies about Sepharose 4B-L-Tyrosine-p-aminobenzoic acid gel exist in literature [26-31], only elution tubes were collected for *Prunus insititia* PPO enzyme in sepharose 4B after foreign proteins were removed from the column by washing. Higher protein contents and enzyme activities were observed between 3.-5. elution tubes compared to the others. Protein absorbances (280 nm) and activity values (420 nm) for the 3., 4. and 5. tubes were measured as 0.087/ 1803 U/(mL*min.), 0.128/ 3850.5 U/(mL*min.) and 0.092/ 1777.5 U/(mL*min.), respectively (Fig. 8).

On the other hand, affinity purification by Sepharose-6B-L-Tyrosine-p-aminobenzoic acid gel was done in terms of the method of gel preparation. *Prunus insititia* PPO enzyme activities were followed and graphed in both the washing and elution steps for the original Sepharose-6B-L-Tyrosine-p-aminobenzoic acid gel (Fig. 9).

To expression of the structure of Sepharose 6B-L-Tyrosine-p aminobenzoic acid in a more clear and healthy way, FT-IR spectra analyses of Sepharose 6B and Sepharose 4B affinity gels were compared. The FT-IR spectra of both synthesized affinity gels are shown in Fig. 10.

FT-IR spectra of Sepharose 6B-L-Tyrosine-p-aminobenzoic acid and Sepharose 4B-L-Tyrosine-p-aminobenzoic acid were obtained from a Thermo Scientific spectrophotometer, over a scan range of 450–4000 cm^{-1} wavenumbers against transmittance %, respectively (Fig. 10). While the -OH stretch of the alcohol groups is expected to be between 3550 cm^{-1} and 3200 cm^{-1} in generally [41], the broad peaks were observed at 3354.40 cm^{-1} and 3354.85 cm^{-1} in IR spectra of Sepharose 6B-L-Tyrosine-p-aminobenzoic acid and Sepharose 4B-L-Tyrosine-p-aminobenzoic acid affinity gels taken from the FT-IR spectrophotometer, respectively. On the other hand, the peaks belonging to aromatic -OH groups are expected to be observed around 1600 cm^{-1} wavenumber [42], Sepharose 6B-L-Tyrosine-p-aminobenzoic acid and Sepharose 4B-L-Tyrosine-p-aminobenzoic acid gave 1632.33 cm^{-1} and 1634.42 cm^{-1} peaks in our study. While it is known that the peaks seen around 2200 cm^{-1} wavenumbers belong to ortho-, meta- and para-aromatic structures [42], IR peaks of 6B and 4B affinity gels gave 2161.37 cm^{-1} and 2195.36 cm^{-1} wavenumbers, respectively. In addition, the peaks observed at around 2000 cm^{-1} wavenumber can be explained by the presence of aromatic amines [42] as the functional groups used in the binding of the affinity ligand in the structure of both affinity gels. Moreover, the difference in the matrix structures of Sepharose 4B and Sepharose 6B is mainly due to the percentages of agarose they contain. While the amount of agarose in Sepharose 4B is 4% in the matrix structure, it is around 6% in Sepharose 6B [43]. So it can be explained that Sepharose 6B-L-Tyrosine-p-aminobenzoic acid affinity gel attached more proteins in the purification step by affinity chromatography depending on the percentage of its agarose amount while the amounts of two affinity gels synthesized in equal volumes (Fig. 9).

Determination of Protein Amount of Damson Plum PPO (*Prunus insititia*) by Bradford Method

The standard linear graph was drawn according to the absorbance values taken at 595nm in spectrophotometer against BSA concentrations (mg/mL) (Fig. 11).

Standard proteins and the enzyme amounts obtained from each purification step were calculated by using the $y=42.246x - 0.0127$ line equation (Fig. 11) for the formation of the purification table.

Determination of Activity of *Prunus insititia* PPO

PPO enzyme activity was determined as stated in the activity method. Enzyme activities after ammonium sulfate precipitation that is the first step of purification and secondly for dialysis step are 1896 U/(mL*min) and 10029 U/(mL*min), respectively. For Sepharose 6B-L-Tyrosine-p-aminobenzoic acid and Sepharose 4B-L-Tyrosine-p-aminobenzoic acid gels, the activity values are 459.0 U/(mL*min) and 3850.5 U/(mL*min), respectively. Considering the values, the increase in enzyme activity after dialysis shows that the enzyme interacts with its substrate faster with the removal of impurities.

Determination of Molecular Weight and Subunits of *Prunus insititia* PPO Enzyme by SDS-PAGE and Native-PAGE

Within the scope of characterization study, SDS-PAGE and Native-PAGE were applied to determine the molecular weight and subunits of purified *Prunus insititia* PPO enzyme, respectively (Fig. 12).

As a result of the experiments, it was determined that *Prunus insititia* PPO was a single subunit approximately around 50-55kDa in Fig. 12(a-b).

Calculation of Yield %, Specific Activity and Purification Fold for *Prunus insititia* PPO Enzyme

The values of yields %, specific activities and purification folds in each purification step for the enzyme were achieved in UV-spectrophotometer. The purification table belonging to *Prunus insititia* PPO enzyme which was

isolated from Sepharose 6B-L-Tyrosine-p-aminobenzoic acid are shown in Table 5.

According to Table 5, *Prunus insititia* PPO enzyme was purified by 10.2-fold in affinity chromatography performed with Sepharose 6B-L-Tyrosine-p-aminobenzoic acid. An increase in the specific activity was observed with decreasing in total protein amount in the last purification step (Table 5). In addition, the purification table prepared by using Sepharose 4B-L-Tyrosine-p-aminobenzoic acid was given in Table 6.

Prunus insititia PPO enzyme was purified by 90 fold in affinity chromatography performed with Sepharose 4B-L-Tyrosine-p-aminobenzoic acid (Table 6). Likewise, with the decrease in total protein amount during purification, an increase in specific activity was observed for each step. It was determined that *Prunus insititia* PPO was more selective to Sepharose 4B-L-Tyrosine-p-aminobenzoic acid affinity gel. The increase in yield from 100% to 333% in dialysis step can be explained by the increase in PPO enzyme activity with the removal of impurities and salts from the enzyme. Also different reasons are thought for the lower purification fold in Sepharose 6B-L-Tyrosine-p-aminobenzoic acid gel when compared to 4B that the enzyme conformation may have changed as a result of purification, and therefore it has lower activity than expected [44].

Determination of Storage Stability and Its Evaluation by Statistical Analysis

Prunus insititia PPE enzyme was used for storage measurements. Measurements related to the storage stability of *Prunus insititia* PPE enzyme were taken during 3 months. The graph was drawn between time-dependent activity and % activity (Fig. 13).

As seen in Fig. 13, it was determined that PPE enzyme protected its activity as 73.54 % over a 2-month period. In the last month, the enzyme continued to preserve its activity by 76.88 % compared to the first measured PPE activity and the enzyme activity remained constant that it was supported by statistical analysis data (Table 7).

For the purpose of expression of storage measurements; PPE enzyme activities were compared according to ANOVA one-way statistical data analysis in excel of triplicate storage measurements which were taken at 5 different times. No any significant difference was observed between the F values which were depend on the detection of more smaller $F_{\text{calculated}}$ value by otomatically according to F_{standard} for the comparison of rows in a single column and between different columns with a confidence level of $\alpha = 0.05$. Comply with the comparison of F values, it was also proofed as significance (P) > 0.05 from Table 7 to be comparised of the P value with the confidence level [45]. Therefore, it was concluded that *Prunus insititia* PPE enzyme stabilization was achieved by protecting the PPE activity (Table 7). Because PPO enzyme is quite important in terms of different industrial areas. Browning of fruits and vegetables which occurs as a result of the PPO enzymatic reaction shorten food shelf life and decrease their industrial quality. Following, the taste, odor and nutritional values of foods decrease significantly as a result of the condensation reaction of quinones as a PPO enzyme product together with proteins, sugar and phenols in foods. So this appearance is not accepted by the consumer and brings economic difficulties in food industry [1,46]. Because of this reason, ascorbic acid was used for pre-purification steps as an anti-browning agent in our study.

Zhou et. al. reported that the amount of benzoic acid required for PPO inhibition was used at values which were smaller than 10mM [1]. Also 12mM p-aminobenzoic acid was used for potential and reversible inhibitor as included in Sepharose-4B-L-Tyrosine-p-aminobenzoic acid and Sepharose-6B-L-Tyrosine-p-aminobenzoic acid affinity gels in our study. p-aminobenzoic acid is also a potent inhibitor for *Prunus insititia* PPO enzyme, according to the high purification fold we obtained from the Sepharose-4B based affinity gel when compared to the study of Zhou et. al.

Optimum conditions were also studied for *Prunus insititia* PPO in this study. Tinello and Lante were reported that 0.05M sodium phosphate buffer (pH=6.5) was used for PPO enzyme from *Prunus salicina* plum type for catechol substrate in 70mM under the enzyme assay conditions at 25°C and in 420nm [46]. Our optimization results showed that we achieved to perform the same optimum conditions approximately for *Prunus insititia* PPO enzyme as 0.05M sodium phosphate buffer (pH=7.2) at 25°C and 420nm as spectrophotometrically, even at a more smaller equilibrium concentrations of catechol as 13mM.

In literature, PPO enzyme were also purified by Sepharose-4B-L-Tyrosine-p-aminobenzoic acid affinity chromatography [47]. Aksoy showed that potato PPO enzyme was purified from crude extract by using Sepharose-4B matrix and p-aminobenzoic acid affinity ligand with different spacer arms as L-Tyrosine, 4-aminophenol and 2-aminophenol, separately. Different purification folds were determined for L-Tyrosine, 4-aminophenol and 2-aminophenol as 5.14, 11.7 and 6.94, respectively [47]. However, our study showed that 90 and 10.2 purification folds were determined according to *Prunus insititia* PPO enzyme homogenate treated with ammonium sulfate by Sepharose 4B-L-Tyrosine-p-aminobenzoic acid and Sepharose 6B-L-Tyrosine-p-aminobenzoic acid affinity gels, respectively. Arslan and Dogan reported that artichoke PPO and *Ocimum basilicum* L. PPO enzymes were purified from crude extract as 42.95 and 11.54 folds by Sepharose 4B-L-Tyrosine-p-aminobenzoic acid, respectively [30]. Faiz and Baltas reported that plum PPO was purified from crude *Diospyros lotus* L. (*Plum Persimmon*) as 15 fold by Sepharose-4B-L-Tyrosine-p-aminobenzoic acid [48]. On the other hand, Kumar et al. showed that PPO enzyme was obtained with a 60.0 purification fold from Sefadex-G100 and phenyl Sepharose, respectively [49]. Lonita et. al. studied that plum PPO was isolated from crude *Prunus domestica* extract as 32.81 purity fold by using hydrophobic interaction and ion exchange chromatography, respectively [50]. When compared to these results, *Prunus insititia* PPO was purified in a more higher purification fold as 90 times from CPE extract treated with ammonium sulfate by using Sepharose 4B-L-Tyrosine-p-aminobenzoic acid. At the same time, Lonita et. al. reported that V_{max} and K_M kinetic values of *P. domestica* PPO enzyme was calculated as 1193.4 EU/ 26.3 mM and 792.7 EU/ 15.5 mM for catechol and 4-methyl catechol substrates, respectively [50]. But in our study, V_{max} and K_M values for catechol and 4-methyl catechol were found 17219.97 U/mL*min/ 11.67mM and 7309.72 U/mL*min/ 5.00mM, respectively. When compared to the article of Lonita et. al., V_{max} values are higher and K_M values are smaller for both substrates, respectively. So *Prunus insititia* PPE enzyme shows diphenolase activity can be concluded. Moreover, broccoli PPO had 2000EU V_{max} /12.34 mM K_M , 28.20 EU V_{max} /21 mM K_M towards catechol and 4-methyl catechol, respectively [51]. Our results showed higher specificity as compared to study of Gawlik-Dziki et. al. The optimum pH for broccoli PPO was 5.7 with catechol and 4-methyl catechol as substrates [51], while pH:7.2 and 4.5 for catechol and 4-methyl catechol in our study, respectively.

The molecular weight of PPO ranges from 27 kDa to 144 kDa, depending on its source [52] while a single band was observed for *Prunus insititia* PPO at 50-55 kDa as a result of SDS-PAGE and native-PAGE electrophoresis performed in this study (Figure 12a-b). Comply with our results, molecular weight of PPO was detected in broccoli flower, apple, bean, potato, tomato (*Lycopersicon sp.*) and coffee as 51.3 kDa, 57 kDa, 58 kDa, 57-60 kDa, 57-62 kDa and 45-67 kDa, respectively [51, 53, 54].

Conclusion

In this study, PPO enzyme characterization was carried out with optimization measurements and PPO purified by affinity chromatography from *Prunus insititia* source as the first time with an original affinity gel was gained to the literature. Our study was also supported by kinetic data measurements. Biochemical and kinetic studies upon *Prunus insititia* PPO enzyme also enabled the clarification of its structural and functional properties by using its different

substrates. Enzyme stabilization was controlled by the storage stability measurements and the results were evaluated statistically. The fact that the stabilization of the enzyme maintained its activity over a period of 3 months and the observation of different affinities for different substrates has drawn attention to the industrial importance of the PPO enzyme. Therefore, this study provides an opportunity to know and control the effect of *Prunus insititia* PPO enzyme in critical immobilization, industrial and biosensor applications.

Declarations

Authors Contributions

S. Y.- analysis, software, resources, writing (original draft). **Ç. B.-** analysis, software, resources, term, conceptualization, methodology, writing, visualization (original draft, review and editing). **E.K.-** analysis, software, resources, term, conceptualization, methodology, writing (original draft, review and editing).

All authors read and approved the manuscript.

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Consent to Publish All authors consent to the submission and publication of this manuscript in Applied Biochemistry and Biotechnology.

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Tables

Table 1 Preparation of separation gel in SDS-PAGE

Composition of the gels	Separation gel 10%
Acrylamide/bisacrylamide solution (30%)	2.37 mL
Distilled water	2.87 mL
1.5M Tris-HCl (pH:8.8) buffer solution	1.80 mL
0.5M Tris-HCl (pH:6.8) buffer solution	-
SDS solution: 1g SDS/10 mL (10%)	0.07 mL
TEMED (N,N,N',N'- Tetramethyl ethylene diamine)	4.00 mL
Ammonium persulfate (APS),10 mL (10%)	110.00 mL

Table 2 Preparation of electrophoresis sample buffer solution

Composition of sample buffer	Amount
Bromophenol blue	5.00mg
0.5M Tris base (pH:6.8) buffer solution	1.25mL
SDS solution, 10% (w/v)	2.00mL
Glycerol	1.00mL
β- mercaptoethanol	0.50mL
Distilled water	0.25mL

Table 3 Composition of Native-PAGE separation gel and amounts

%15 T, %5 C _{bis} , Separation gel	Amount
Monomer solution (Acrylamide/bisacrylamide)	3.75 mL
Separation gel solution (0.95M Tris base and 0.29N HCl, pH:8.5)	2.50 mL
Distilled water	2.50 mL
TEMED	20.00 mL
Ammonium persulfate, 10%	250.00 mL

Table 4 Calculated K_M (mM), V_{max} U/(ml*min) and catalytic efficiency values for catechol, 4 methyl catechol, pyrogallol and caffeic acid substrates

Substrates	K_M (mM)	V_{max} U/(mL*min.)	V_{max} / K_M
Catechol	11.67	17219.97	1475.576
4 methyl catechol	5.00	7309.72	1461.945
Pyrogallol	3.74	12580.12	3355.119
Caffeic acid	6.25	12100.41	1936.065

Table 5 Purification tables of *Prunus insititia* PPO isolated from Sepharose 6B-L-Tyrosine-p-aminobenzoic acid

Purification step	Volume (mL)	Activity (U/mL*min)	Total Activity (U)	Protein (mg/mL)	Total Protein (mg)	Specific Activity (U/mg)*10 ⁵	Yield %	Purification Fold
CPE enzyme treated with (NH ₄) ₂ SO ₄	27	1896.0	51192	17.62	475.88	1.08	100.00	1.0
Dialysis	17	10029.0	170493	10.99	186.97	9.12	333.04	8.5
Sepharose 6B	2	459.0	918	0.41	0.84	10.95	1.79	10.2

Table 6 Purification tables of *Prunus insititia* PPO isolated from Sepharose 4B-L-Tyrosine-p-aminobenzoic acid

Purification step	Volume (mL)	Activity (U/mL*min)	Total Activity (U)	Protein (mg/mL)	Total Protein (mg)	Specific Activity (U/mg) *10 ⁵	Yield %	Purification Fold
CPE enzyme treated with (NH ₄) ₂ SO ₄	27	1896.0	51192	17.62	475.88	1.08	100.00	1.0
Dialysis	17	10029.0	170493	10.99	186.97	9.12	333.04	8.5
Sepharose 4B	2	3850.5	7701	0.39	0.79	96.83	15.04	90.0

Table 7 Statistical Analysis at 95 % Confidence Interval for the Evaluation of Storage Stability

Day / Measurement No	Day 64	Day 86	Day 88	Day 91	Day 95
Measurement 1	8785.50	10182.0	9924.00	8572.50	9328.50
Measurement 2	9630.00	9945.0	9385.50	10182.00	9924.00
Measurement 3	9207.75	10063.5	9654.75	9377.25	9626.25

SUMMARY					
Groups	Say	Total	Average	Variance	
Day 64	3	27623.25	9207.75	178295.10	
Day 86	3	30190.50	10063.50	14042.25	
Day 88	3	28964.25	9654.75	72495.56	
Day 91	3	28131.75	9377.25	647622.60	
Day 95	3	28878.75	9626.25	88655.06	

ANOVA						
Source of a variance	Sum of squares (SS)	Degrees of freedom (df)	Mean square (MS)	F _{calculated}	Significance (P value)	F _{standard}
Between groups	1263007	4	315751.8	1.577008	0.254364	3.47805
Within groups	2002221	10	200222.1			
Total	3265228	14				

Figures

Figure 1

Graph of optimum buffer concentration (M) against PPE enzyme activity [U/(mL*min)] for catechol, 4-methyl catechol, pyrogallol, and caffeic acid

Figure 2

Graph of optimum pH against *Prunus insititia* enzyme activity [U/(mL*min)] for catechol, 4-methyl catechol, pyrogallol, and caffeic acid

Figure 3

Graph of optimum T (°C) against *Prunus insititia* enzyme activity [U/(mL*min)] for catechol, 4-methyl catechol, pyrogallol, and caffeic acid

Figure 4

Lineweaver-Burk plot of catechol for *Prunus insititia* PPE enzyme

Figure 5

Lineweaver-Burk plot of 4 methyl catechol for *Prunus insititia* PPE enzyme

Figure 6

Lineweaver-Burk plot of pyrogallol for *Prunus insititia* PPE enzyme

Figure 7

Lineweaver-Burk plot of caffeic acid for *Prunus insititia* PPE enzyme

Figure 8

Elution graph of Prunus insititia PPO from Sepharose 4B-L-Tyrosine-p aminobenzoic acid by affinity chromatography

Figure 9

Washing and Elution graphs of *Prunus insititia* PPO from Sepharose 6B-L-Tyrosine-p aminobenzoic acid by affinity chromatography

Figure 10

FT-IR spectra of Sepharose-6B-L-tyrosine-p-amino benzoic acid and Sepharose-4B-L-tyrosine-p-amino benzoic acid affinity gels, respectively

Figure 11

Standard linear graph used in determination of protein amount

Figure 12

The appearances of *Prunus insititia* PPO enzyme by electrophoresis

Figure 13

Investigation of Storage Stability by Time-dependent Percent Activity

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