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Partial Purification and Characterization of Polyphenol Oxidase Enzyme from Common-Morning Glory (*Ipomoea purpurea*)

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ABSTRACT

This study aimed to purify and biochemically characterize polyphenol oxidase (PPO) enzyme from the plant *Ipomoea purpurea* (*I. purpurea*) for the first time. For this purpose, the crude extract sample obtained from the extraction of *in vitro* cultured plant leaves under optimum conditions (25 mgmL^{-1} Polyvinylpolypyrrolidone, pH 7.0) was subjected to three-phase partitioning, and the PPO enzyme was 10.5-fold purified with a 57% activity recovery. The optimum pH and temperature values were determined as 7.0 and 30°C, respectively. Laccase, peroxidase, and catechol oxidase activities were observed after activity staining of partially purified enzyme. From stability tests, it was noted that more than 75% and 65% of its original activity were maintained at temperatures 20℃-40℃ and pH 7.0-9.0, respectively.

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Gündüz Sefası (*Ipomoea purpurea*) Bitkisinden Polifenol Oksidaz Enziminin Kısmi Saflaştırılması ve Karakterizasyonu

ÖZET

Bu çalışmada, polifenol oksidaz (PPO) enziminin *Ipomoea purpurea* (*I. purpurea*) bitkisinden ilk kez saflaştırılması ve enzimin biyokimyasal özelliklerinin belirlenmesi amaçlandı. Buna göre, in vitro olarak üretilen bitkiye ait yapraklardan optimum koşullarda (25 mg ml-1 Polivinilpolipirrolidon, pH 7.0) gerçekleştirilen ekstraksiyon sonrası elde edilen ham ekstrakt örneğinden PPO enzimi üçlü faz ayırma tekniği ile yaklaşık %57 aktivite geri kazanımla 10,5 kat saflaştırıldı. Enzimin optimum pH ve sıcaklık değerleri sırasıyla 7.0 ve 30°C olarak belirlendi. Aktivite boyama sonrası kısmen saf enzimde lakkaz, peroksidaz ve katekol oksidaz aktiviteleri tespit edildi. Ayrıca, 20°C ile 40°C arasında enzim aktivitesinin ≥%75, pH 7.0 ile 9.0 arasında ≥%65 oranında korunduğu tespit edildi.

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INTRODUCTION

Polyphenol oxidases (PPOs) (E.C. 1.14.18.1, E.C. 1.10.3.1, or E.C. 1.10.3.2) are a group of copper proteins found in nearly all prokaryotic and eukaryotic cells. PPOs are capable of oxidizing a considerable amount of phenolic and non-phenolic aromatic compounds. In most cases, its physiological functions are either pigmentation or protection from the harmful effects of the environment. Despite the uncertainty in the classification of PPOs, they can be divided into three groups according to the literature: laccase (*p*-diphenol: oxygen-oxidoreductase, E.C. 1.10.3.2), catechol oxidase (*o*-diphenol: oxygen-oxidoreductase, E.C. 1.10.3.1) and tyrosinase (monophenol-monooxygenase, E.C. 1.14.18.1) (Kocabas et al., 2011; Panadare and Rathod, 2018).

PPO activity is vital to determine food products' quality and shelf life; therefore, a fast, reliable, and

precise method is desired to estimate PPO activity in agricultural and horticultural crops. Biosensors are perfect for this purpose. Industrially, PPOs have an extensive range of application areas, including medicine, pharmaceutical and chemical industries, analytical devices (biosensor preparation), and the food industry (Panadare and Rathod, 2018).

In countries where it is essential to produce economically significant amounts of fruits and vegetables, green plants are used as a source of PPO. There are many studies on plant PPOs especially from fruits and leaves, in the scientific literature (Kocabas et al., 2011).

Ipomoea purpurea (L.) Roth (Common-morning glory, also known as Tall Morning-glory) is an annual ornamental plant. *I. purpurea* naturally spread throughout Central America but has become naturalized in most parts of the world due to its ornamental value. It is also used medicinally. The stems, seeds, roots, and flowers of *I. purpurea* have been utilized as laxative and hallucinogenic agents. Also, several parts of the species have been used to treat rheumatism, fungal and urinary infections, acne, diarrhea and constipation, infertility, liver diseases, and syphilis (Srivastava, 2017; Muhammad et al., 2019). Although it has wide use, studies on *I. purpurea* are pretty limited. Kıran and Acemi (2019) investigated the effects of chitosan on photosynthetic pigment, protein, and dry matter contents of *I. purpurea* to determine chitosan's indirect impact on plant leaves. Atala et al. (2014) performed three separate greenhouse experiments to test the twining induced in *I. purpurea* by applying different levels of artificial damage (mechanical) and natural damage with snails. Park et al. (2007) showed that phytomelanins are mainly found in the outer epidermis and palisade layers in wild type *I. purpurea*.

This study aimed to purify and biochemically characterize the PPO enzyme from leaves obtained from *in vitro* cultured *I. purpurea* for the first time. *I. purpurea* was selected as a PPO source due to its broad ornamental and medicinal uses and the limited number of studies in the literature. We have used the three-phase partitioning (TPP) technique to extract PPO enzyme from plant extract since TPP is a cost-efficient and straightforward method that purifies protein of interest in a single step (Panadare and Rathod, 2018).

MATERIALS and METHODS

Materials

The seeds of Common-Morning Glory (*Ipomoea purpurea*) were purchased from Anadolu Tohum Production and Marketing Incorporated company. The chemicals used during the experiment with the highest purity grade were purchased from BioRad (California, USA), Biolife (Viale Monza, Milan, Italia), Duchefa Biochemie (RV Haarlem, The Netherlands) and Sigma-Aldrich (St. Louis, MO, USA).

Preparation of Crude Extracts

The seeds of *I. purpurea* were germinated in LS (Linsmaier and Skoog, 1965) medium after surface disinfection treatments (Kıran Acemi and Acemi, 2019). The leaves from *in vitro*-raised individuals were collected after 30 d of the incubation period. They were used in experiments within a few hours after collection or stored at -80 °C until used. The leaves were thoroughly washed with dH_2O before use in the experiment. In the crude extract preparation, 30 g of leaf tissue was homogenized by thumping in 200 ml 0.1 M sodium phosphate buffer (pH 7.0) solution in a mortar at +4°C. Polyvinylpolypyrrolidone (PVPP) was added to a final concentration of 25 mg ml^{-1} (optimized in the present study) to remove the phenolic substances, and the mixture was filtered through cheesecloth and centrifuged at 10000×g for 30 min at +4°C (Kocabas et al., 2011). The resulting supernatant was collected and used as the crude enzyme extract for further purification.

PPO Assay and Protein Amount

PPO activity was measured by observing quinone production at 420 nm at room temperature on a spectrophotometer. In the calculation of the enzyme activity, the sample cuvette contained 500 µl substrate (pyrocatechol) at 100 mM concentration, 1 ml buffer solution of sodium phosphate (100 mM, pH 7.0), and appropriately-diluted 500 µl enzyme solution (Alici and Arabaci, 2016). The control (blank) cuvette contained the same chemicals but without the enzyme. One unit of the enzyme was the amount of enzyme producing 1 µmole of quinone per minute.

Protein concentration was performed according to the Bicinchoninic Acid (BCA) method. Bovine serum albumin (BSA) was used to draw the standard graph (Smith et al., 1985).

PPO Purification

The PPO enzyme was purified using a TPP system consisting of ammonium sulfate, t-butanol, and crude enzyme extract. The method is based on the appearance of three phases (bottom aqueous phase, protein-rich middle phase, and top t-butanol phase) after adding t-butanol to the crude enzyme extract saturated with ammonium sulfate (Dennison and Lovrien, 1997).

For TPP systems, different amounts of $(NH_4)_2SO_4$ (from 20% to 70% w/v adding in intervals of ten) were added to 2 ml samples of the crude enzyme (CE)

extract to bring different ammonium sulfate saturation, and the mixtures were vortexed gently until the ammonium sulfate was completely dissolved. Then, t-butanol was added at ratios of 1.0:0.5, 1.0:1.0, 1.0:1.5 and 1.0:2.0 (v/v) to CE extract. The mixture was vortexed gently on a magnetic stirrer for 1 min and kept at room temperature for 60 min for phase separation. After standing complete phase separation was attained by centrifugation at 4500×g for 10 min (Duman and Kaya, 2014; Alici and Arabaci, 2016). The t-butanol-containing upper phase, where the protein was not anticipated to be shown, was expelled by cautious pipetting. One ml of 0.1 M sodium phosphate buffer solution (pH 7.0) was used to dissolve the PPO enzyme-containing precipitate (middle phase). Afterward, activity and protein determination experiments were carried out at 420 nm using a spectrophotometer (Cary 60, Agilent) at room temperature on the middle and bottom phase samples. The parameters such as ammonium sulfate concentration, crude extract: t-butanol ratio, and system pH have crucial roles in the TPP system were also optimized to test their effects on the selective separation of biomolecules. CE extract activity for *in vitro*-cultured plant (6560 U) was taken as 100%. A blank system was set up, including $(NH_4)_2SO_4$, dH_2O, and t-butanol (excluding the CE extract).

Characterization of the PPO Enzyme

Activity staining

The activity staining procedure was carried out to identify the PPO activity through polyacrylamide gel electrophoresis (Rescigno et al., 1997). The SDS-free polyacrylamide gel contained 10% (w/v) separation gel and 4% (w/v) stacking gel. The electrophoresis was carried out at 150 V for 45 min at 4°C (Kaptan, 2004). After that, 20 ml of 0.1 M potassium phosphate buffer solution (pH 6.0) was used to wash the separation gel for 5 min. For the determination of laccase activity, the gel was treated with 40 mM 4-amino-N-N diethylaniline (ADA) prepared in 10 mM HCl solution. Then, 10 mM H_2O_2 for peroxidase activity determination was applied, and finally, it was exposed to 40 mM 4-tert-butyl catechol (tBC) prepared in 10 mM acetic acid solution for the determination of catechol oxidase activity. Before each treatment, the gel was washed with dH_2O. The existence of pink-red bands after ADA and H_2O_2 and dark blue bands after tBC were analyzed (Rescigno et al., 1997).

Effect of pH on enzyme activity and stability

The effect of different pH on *I. purpurea* PPO activity was determined by using 100 mM pyrocatechol as substrate at several pH values ranging from 4.0 to 9.0. The buffer solutions used in the experiment were 0.1 M citrate buffer for reactions between pH 4.0-5.0, 0.1 M phosphate buffer for pH 6.0-7.0, 0.1 M Tris buffer solution for pH 8.0, and 0.1 M glycine-sodium hydroxide buffer for pH 9.0 (Kavrayan and Aydemir, 2001; Gülçin et al., 2005; Kocabas et al., 2011). Percent relative activities were calculated by dividing the enzyme activity measured at each pH value by the maximum enzyme activity multiplied by 100.

To determine the stability of *I. purpurea* PPO against the same pH range, the PPO sample was incubated in the buffers mentioned above for 30 and 60 min in a water bath. The enzyme activity was measured at the end of the incubation period using the standard experimental procedure. Percent residual activities were calculated by dividing the post-incubation enzyme activity by the pre-incubation activity multiplied by 100.

Effect of temperature on enzyme activity and stability

In order to detect the effect of temperature on *I. purpurea* PPO activity, enzyme activity was measured by setting a spectrophotometer temperature between 20°C and 70°C. The enzyme stability temperature was measured after keeping the PPO samples in a water bath at different temperatures between 20°C and 70°C (20, 30, 40, 50, 60 and 70°C) for both 30 and 60 min (Kavrayan and Aydemir, 2001; Gülçin et al., 2005; Kocabas et al., 2011). After the incubation, enzymes were first kept on ice, and then polyphenol oxidase activity measurements were made in a spectrophotometer device. Percent relative and residual activities were estimated as described above.

Statistical Analysis

All experiments were performed in triplicate. The data are represented as mean ± standard deviation (SD). The statistical differences between the means were compared using Duncan's multiple range test (DMRT) at $P \leq 0.05$ through the software "The Statistical Package for the Social Sciences" (SPSS, version 22, IBM Inc., Chicago, IL, USA).

RESULTS and DISCUSSION

Optimization of PPO Extraction Conditions

For PPO isolation from *I. purpurea*, the first step was optimizing extraction conditions, including PVPP concentration and pH of the extraction medium. PVPP is a compound capable of preventing hydrogen bonding between phenolics and PPO enzyme (Smith and Montgomery, 1985). For this reason, PVPP was used during extraction, and its concentration was optimized to improve PPO yield. PVPP was added to the extraction medium in a 5-75 mg PVPP ml^{-1} concentration range. As seen in Table 1, 25 mg PVPP ml^{-1} appeared to be a suitable concentration to remove phenolics for all samples tested. This was consistent

with previous reports where PVPP was used at a broad range of 10-60 mg ml^{-1} concentrations (Rocha and Morais, 2001; Kocabas et al., 2011; Pelalak et al., 2021). 25 mg PVPP ml^{-1} was chosen for further extraction optimization analysis.

Table 1 PVPP optimization results
Çizelge 1. PVPP optimizasyon bulguları

PVPP concentration (mg ml^{-1})	PPO activity (U ml^{-1})
5	1937±95 ^{d}
12.5	2310±108^{c}
25	3252±163^{a}
37.5	3007±136^{b}
50	1876±95^{d}
62.5	3028±151^{b}
75	1510±78^{e}

^{a,b,c} Values within a row with different superscripts differ significantly at P≤0.05

In order to optimize the pH of the environment, extraction media was prepared under conditions ranging from pH 4.0 to 9.0. Afterward, extracts were obtained, and enzyme activity was determined under standard conditions in the spectrophotometer. According to the results given in Table 2, the highest activity was observed in the extraction medium with pH 7.0. Thus, further screening experiments were performed at pH 7.0. In general, it is seen that the preferred pH during extraction of PPO from different plants falls in the range of 6.0-7.0. For example, pH 6.0 was used for artichoke (*Cynara scolymus* L.) (Aydemir, 2004), while pH 6.5 and 7.0 were chosen for cotton (*Gossypium hirsutum* L.) (Kouakou et al., 2009) and grape (*Vitis vinifera*) (Öztan, 2007), respectively.

Table 2 Extraction pH optimization results
Çizelge 2. Ekstraksiyon pH'sının optimizasyon bulguları

pH	PPO activity (U ml^{-1})
4.0	0^{g}
5.0	532±30^{f}
6.0	2201±102^{b}
6.5	3000±112^{c}
7.0	3328±164^{a}
8.0	1943±73^{d}
9.0	1250±61^{e}

^{a,b,c} Values within a row with different superscripts differ significantly at P≤0.05

Purification of PPO by TPP

The second step to obtain the PPO enzyme was the application of three-phase partitioning to the crude extract. Compared with other conventional purification methods, TPP is a simple purification technique with a short processing time and is economical (ammonium sulfate and t-butanol are readily available). This technique does not denature proteins during purification and generally works at room temperature (Rachana and Jose, 2014). Although TPP was chosen for many scientists to purify various important enzymes (Nadar and Rathod., 2017), studies on its use for PPO purification are restricted (Panadare and Rathod, 2018).

For TPP systems, it is vital to optimize the system components like ammonium sulfate and t-butanol concentrations and the pH to extract a sufficient amount of saturated protein of interest from the complex mixture (CE extract) with the complex mixture minimal interaction with contaminating materials. In general, optimization trials are started with solutions prepared at 20% (w/v) saturation. The desired protein concentration in the middle or bottom phases is determined (Dennison and Lovrien, 1997). Therefore, the experiments were started with 20% (w/v) $(NH_4)_2SO_4$, since it was recommended in various reports (Niphadkar and Rathod, 2015, Panadare and Rathod, 2018). In the experiments, $(NH_4)_2SO_4$ was tested up to 70% (w/v) at pH 7.0 and room temperature on crude enzyme extract to t-butanol (1.0:1.0; v/v). Figure 1 reveals the effect of $(NH_4)_2SO_4$ concentration on PPO partition from the crude extract.

Ammonium sulfate saturations below 20% (w/v) usually lead to poor protein recovery, but higher concentrations cause protein to move from bottom to top (Bayraktar and Önal, 2013). Likewise, in this study, it was observed that the PPO enzyme accumulated in the bottom phase when the crude extract was saturated with 20%-40% (w/v) $(NH_4)_2SO_4$ (Figure 1). On the other hand, 50%, 60%, and 70% saturations resulted in PPO movement from the bottom to the middle, but the purification fold decreased. The highest fold purification of 8.2-fold along with 57% activity recovery of PPO in the bottom phase was obtained with 30% (w/v) $(NH_4)_2SO_4$.

Crude extract:t-butanol ratio is also an essential parameter in TPP systems and should be optimized. t-Butanol shows higher deactivation and lower interfacial precipitation through hydrophobic interactions with proteins than other organic solvents such as n-butanol, n-propanol, or isopropanol and does not denature proteins. Therefore, it is the most preferred organic solvent in TPP systems. Its lower concentrations might be insufficient to exert a synergistic effect with the salt used. At the same time, its higher amounts are expected to inhibit intermolecular interactions due to the increased viscosity of the TPP mixture (Dennison and Lovrien, 1997).

TPP systems were set up containing the crude extract saturated with 30% $(NH_4)_2SO_4$. t-Butanol was added into the solutions of crude extracts saturated with

$(NH_4)_2SO_4$ at ratios varying between 1.0:0.5 and 1.0:2.0 to determine the appropriate optimal volume added to the solution. As observed from Figure 2, the best PPO purification fold (8.2) and highest activity recovery (57%) were achieved from the bottom phase of the TPP system prepared by the addition of an equal volume of t-butanol to the crude extract saturated with 30% (w/v) $(NH_4)_2SO_4$. Also, in Figure 2, with ratios of crude extract to t-butanol of 1.0:0.5 and 1.0:2.0, the purification fold of the PPO enzyme at the bottom phase was decreased. On the other hand, with a 1.0:1.5 ratio, activity recovery was slightly increased.

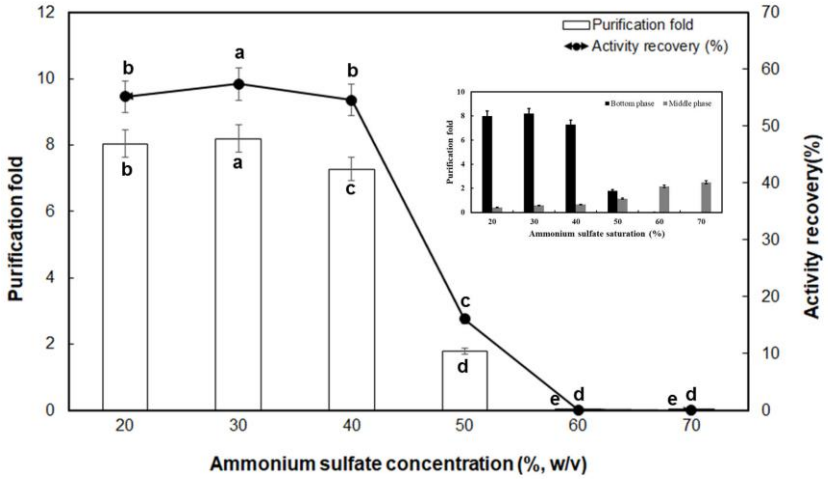

Figure 1. The effect of different $(NH_4)_2SO_4$ concentrations on PPO enzyme distribution in TPP systems. The specific activity of *I. purpurea* crude extract is 4686 U mg^{-1} and the total protein amount is 1.4 mg. Equal volumes of t-butanol were added to the mixtures prepared with different $(NH_4)_2SO_4$ saturation at a ratio of 1.0:1.0 crude extract: t-butanol. The bottom phase formed in all systems were collected and analyzed. Insets, Effect of ammonium sulfate saturation on PPO partition into the bottom and middle phases.

Şekil 1. Farklı $(NH_4)_2SO_4$ konsantrasyonlarının TPP sisteminde PPO enzim dağılımına etkisi. I. purpurea ham ekstraktının spesifik aktivitesi 4686 U mg^{-1}, toplam protein miktarı ise 1.4 mg olarak hesaplandı. Farklı *$(NH_4)_2SO_4$ doygunluğu ile hazırlanan karışımlara ham ekstrakt: t-butanol oranı 1.0:1.0 olacak şekilde eşit hacimlerde t-butanol ilave edildi. Tüm sistemlerde oluşan alt fazlar toplandı ve analiz edildi. Ekli küçük resim, Amonyum sülfat doygunluğunun PPO'nun alt ve orta fazlara dağılımına etkisi.*

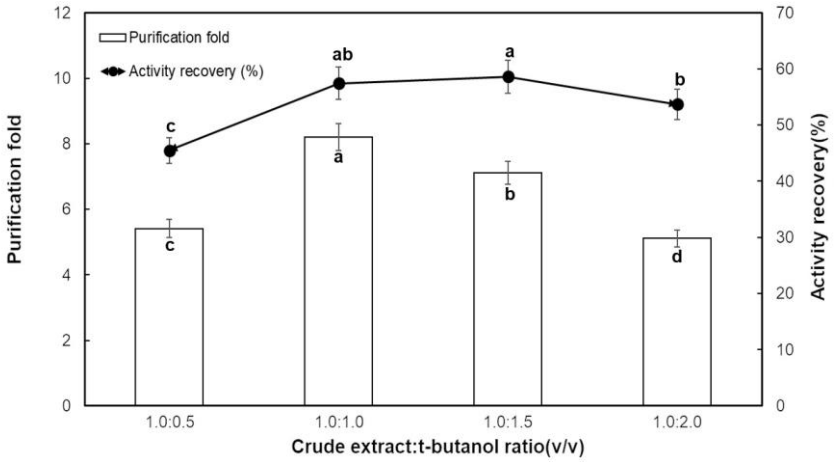

Figure 2. The effect of different crude extract: t-butanol ratios on PPO enzyme distribution in TPP systems. The specific activity of *I. purpurea* crude extract is 4686 U mg^{-1}. The total protein amount is 1.4 mg. t-Butanol was added to the mixtures prepared at 30% (w/v) $(NH_4)_2SO_4$ saturation to obtain different crude extract: t-butanol ratio. The bottom phase formed in all systems were collected and analyzed.

Şekil 2. Farklı ham ekstrakt:t-butanol otanlarının TPP sisteminde PPO enzim dağılımına etkisi. I. purpurea ham ekstraktının spesifik aktivitesi 4686 U mg^{-1}, toplam protein miktarı ise 1.4 mg olarak hesaplandı. %30 (w/v) $(NH_4)_2SO_4$ doygunluğunda hazırlanan karışımlara t-Butanol ilave edilerek farklı ham ekstrakt:t-butanol oranları elde edildi. Tüm sistemlerde oluşan alt faz toplandı ve analiz edildi.

The last important parameter affecting the protein enrichment and purification efficiency of TPP systems is the system pH. This effect is generally associated with changes in amino acid residues on the surface of proteins due to pH changes. In general, the cleavage of the target protein into the middle or aqueous phase in the TPP system is mainly based on the isoelectric point (Yan et al., 2018). The $(NH_4)_2SO_4$ concentration and crude extract: the t-butanol ratio was kept constant in the systems to investigate the effect of the system pH on the separation of PPO enzyme in phases. Then, the system pH was adjusted to the desired pH using 0.1 M sodium hydroxide (NaOH) and 0.1 M hydrochloric acid (HCl). Figure 3 indicates that the highest purification (10.5-fold) and the highest recovery (57%) were observed at pH 7.5. As shown in Figure 3, at pH 7.0 and 7.5 values, activity recovery remained unchanged, but the purification fold was increased when system pH was increased to 7.5. The moderate activity was also recovered when the system pH adjusted to pH 5.0, 6.0, or 8.0. Below pH 5.0, however, poor protein recovery and purification fold were observed. This can be explained by more hydrogen ions competing with the protein of interest for interaction with water molecules (Chew et al.,2019).

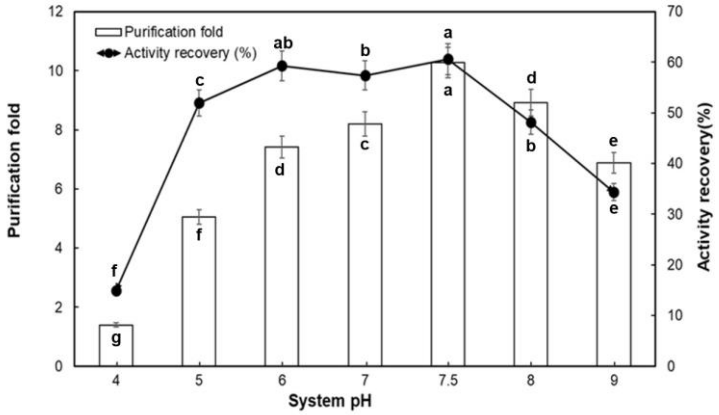

Figure 3. The effect of the system pH on PPO enzyme distribution in TPP systems. The specific activity of *I. purpurea* crude extract is 4686 U/mg, and the total protein amount is 1.4 mg. Prepared systems containing 1.0:1.0 crude extract t-butanol saturated with 30% (w/v) $(NH_4)_2SO_4$ were adjusted to different pH. The bottom phase formed in all systems were collected and analyzed.

Şekil 3. Sistem ph'sının TPP sisteminde PPO enzim dağılımına etkisi. I. purpurea ham ekstraktının spesifik aktivitesi 4686 U mg^{-1}, toplam protein miktarı ise 1.4 mg olarak hesaplandı. %30 (w/v) $(NH_4)_2SO_4$ doygunluğunda ve *1.0:1.0 oranında ham ekstrakt:t-butanol içerecek şekilde hazırlanan sistemler farklı pH'a ayarlandı. Tüm sistemlerde oluşan alt faz toplandı ve analiz edildi.*

In summary, the best purification of the PPO enzyme was achieved from a TPP system consisting of 2 ml of the crude enzyme (extracted from *I. purpurea* leaves) saturated with 30% (w/v) $(NH_4)_2SO_4$ and 2 ml of t-butanol at pH 7.5. A few PPO enzymes have been purified through TPP with different purification folds and yields. Niphadkar and Rathod (2015) and Alici and Arabaci (2016) have reported 70% and 69% activity recovery values of PPO corresponding to 6.3- and 3.6-fold purifications, respectively. Yuzugullu Karakus et al. (2020) and (2021) have reported 230% and 120% activity recoveries of PPO corresponding to 14- and 20-fold purifications, respectively. In this study, PPO was purified to a higher fold than potato and borage PPO enzymes. However, the activity recovery value of the enzyme was the lowest among PPOs from other plants.

Activity Staining of PPO

Activity staining was performed to detect the enzyme activity on a native gel. After SDS-free polyacrylamide gel electrophoresis (Kaptan, 2004), the gel was stained according to the method reported by Rescigno et al. (1997). The protocol was applied as described in the Materials and Methods section.

As seen in Figure 4, a light pink band was observed after staining with ADA (laccase substrate), supporting the presence of laccase activity. Further treatment of the gel with H_2O_2 (peroxidase substrate) leads to an amaranth pink band appearance corresponding to where laccase activity was observed on ADA-stained gel. This indicated the presence of peroxidase activity. Lastly, the gel was exposed to tBC, resulting in dark blue color without any extra band. The dark blue color indicates that tBC was oxidized to corresponding quinones, and catechol oxidase activity was detected at the same position as laccase and peroxidase bands. Considering all results obtained, it can be concluded that *I. purpurea* PPO exhibited laccase, peroxidase, and catechol oxidase activities.

Effect of Reaction pH on PPO Activity and Stability

The reaction pH is an important parameter that affects enzyme activity by altering the enzyme's net charge, thereby affecting the enzyme's solubility, binding ability with different substrates/inhibitors, and folding. Enzymes mostly become inhibited at extreme pH values because they lose their ability to fold (Panadare and Rathod, 2018). The effect of pH on PPO activity was investigated at a broad pH range of 4.0 to 9.0 and presented in Figure 5A. Accordingly, optimum pH was found at 7.0 using catechol as substrate. Similarly, using catechol, PPO enzymes from chest nut kernel, buriti, rosemary, mango, banana, and peppermint were optimum at pH 7.0 (Table 4). The PPO enzyme maintained more than 60% of its initial activity over the pH range 5.0-9.0 (Figure 5A).

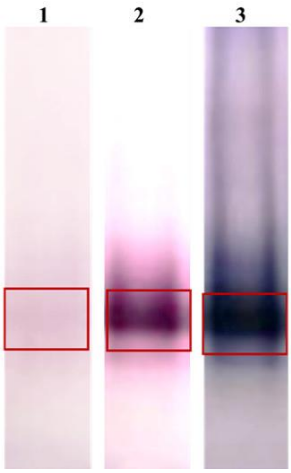

Figure 4. Gel view showing the activity staining results of *I. purpurea* PPO with ADA (1), H_2O_2 (2), and tBC (3).
Şekil 4. I. purpurea PPO'sunun ADA (1), H_2O_2 (2), ve tBC (3) ile aktivite boyama sonuçlarını gösteren jel

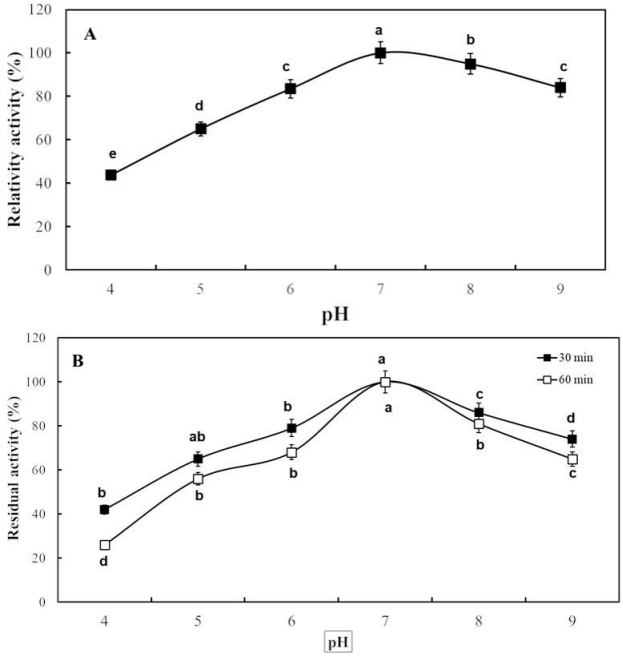

Figure 5. Effects of reaction pH on *I. purpurea* PPO activity (A) and stability (B). (A) Percent relative activities measured at various pH values (4.0-9.0) at room temperature in citrate, phosphate, Tris-HCl, and glycine-NaOH buffers. (B) Percent residual activities measured after pre-incubation of the PPO at pH values between 4.0 and 9.0. The values are presented as means ± SD.

Şekil 5. Reaksiyon pH'sının I. purpurea PPO aktivitesi (A) ve stabilitesi (B) üzerine etkisi. (A) Sitrat, fosfat, Tris-HCl ve glisin-NaOH tamponları kullanarak farklı pH değerlerinde oda sıcaklığında ölçülen yüzde relatif aktivite değerleri. (B) 4.0 ile 9.0 arasındaki pH değerlerinde hazırlanan tamponlarda PPO'nun ön inkübasyonundan sonra ölçülen bağıl aktivite yüzde değerleri. Değerler, ortalama ± SD olarak sunuldu.

The pH stability of the PPO was also investigated by preincubating the enzyme for 30 and 60 min at various pH values between 4.0 and 9.0 and measuring the residual activity at 30°C and pH 7.0 under standard experimental conditions. As is shown in Figure 5B, the enzyme was the most stable at pH 7.0. An increase or decrease in pH above or below the stability range leads to decreased enzyme activity. From Figure 5B, it is also seen that more than 65% of the residual activity was recovered at pH values of 6.0-9.0. However, only 26% and 56% of its residual activity was maintained at pH 4.0 and 5.0, respectively. This means the enzyme is more stable in neutral and alkaline pH rather than acidic pH.

Table 4 Comparison of different plant PPOs in terms of pH and temperature optima
Çizelge 4. pH ve sıcaklık optimumları açısından farklı bitki PPO'larının karşılaştırılması

Source	Temperature	pH	Reference
Snake fruit	30℃	6.5	Zaini et al., 2013
Chestnut kernel	40℃	7.0	Gong et al., 2015
Honeydew peach	40℃	6.5-7.0	Liu et al., 2015
Buriti palm	35℃	7.0	de Oliveira Carvalho and Orlanda, 2017
Blueberry	35℃	6.1-6.3	Siddiq and Dolan, 2017
Plums	25℃	6.0	Ioniță et al., 2017
Apricot	45℃	4.5	Derardja et al., 2017
Rosemary	30℃	7.0	Yuzugullu Karakus et al., 2020
Fennel	30℃	6.0	Yuzugullu Karakus et al., 2021
Mango	30℃	7.0	Wang et al., 2007
Banana	30℃	7.0	Ünal et al.,2007
Peppermint	30℃	7.0	Kavrayan and Aydemir, 2001
Morning glory	30℃	7.0	Current study

According to literature studies, crude PPO extracted from peppermint (Kavrayan and Aydemir, 2001), pawpaw (Bello et al., 2011), grape (Kaya and Bağci, 2021), artichoke (Aydemir et al., 2003), and rosemary (Yuzugullu Karakus et al., 2020) showed that the PPO enzyme is more stable in the pH ranges of 6.0-7.0, 6.0-8.0, 7.0, 6.0-8.0, 6.0-9.0, respectively. These observations are similar to the findings of I. purpurea plant, which shows that the enzyme is stable between pH 6.0 and 9.0. However, it was observed that PPO obtained from bush mango (*Irvingia gabonnensis*) had more activity in the pH stability range of 3.5-5.5 (Bello et al., 2011). This is presumably due to the existence of isoenzymes. Therefore, enzyme pH stability may vary depending on the material.

Effect of Reaction Temperature on PPO Activity and Stability

The reaction temperature is another critical factor that plays an essential role in oxygen solubility in reaction medium and affects reaction kinetics (Panadare and Rathod, 2018). The effect of temperature on PPO activity was tested at temperatures between 20 and 70°C. Figure 6A exhibited the optimum temperature for *I. purpurea* PPO enzyme was 30°C when catechol was used as substrate. Similarly, PPO enzymes from snake fruit, rosemary, fennel, mango, banana, and peppermint were optimum at 30°C using catechol (Table 4). On the other hand, the optimum temperature for some plant PPOs can change from 25°C to 65°C depending on the enzyme source (Table 4). In this study, 93% and 87% of initial PPO activities were recovered at 50°C and 60°C, respectively, while the enzyme maintained 59% of its activity at 70°C (Figure 6A).

The thermal stability of the TPP-partitioned PPO was tested by incubating the enzyme without substrate at different temperatures for 30 and 60 min. The results are given in Figure 6B, from which it can be seen that the PPO enzyme is stable between 20 and 40℃ and maintained 40% of its residual activity after incubation at 50°C for 60 min. On the other hand, the enzyme was inhibited entirely after incubation for 60 min at 60°C. Likewise, cotton PPO was reported to be inhibited after incubation for only 10 min at 60°C (Kouakou et al., 2009).

CONCLUSIONS

In this study, the leaves of in vitro grown *I. purpurea* plant, which is used for ornamental and medicinal purposes, were screened for their PPO activities. Crude extract samples were obtained under optimized conditions where the extraction medium was prepared at pH 7.0 in the presence of 25 mg ml^{-1} PVPP. Then, using the TPP system, the PPO enzyme was purified 10.5-fold, with 57% recovery in a single step. Optimum conditions for TPP included adding an equal volume of t-butanol to the crude extract saturated with 30% (w/v) $(NH_4)_2SO_4$ at pH 7.5. Activity staining results indicated that the enzyme exhibited laccase, peroxidase, and catechol oxidase activities, supporting that the PPO enzyme from I.

purpurea is highly functional. Biochemical characterization studies revealed that the effect of temperature and pH on enzyme activity resembles other PPO enzymes obtained from different plant sources. On the other hand, its stability in an alkaline pH environment gives the enzyme advantage for industrial use over other PPO enzymes.

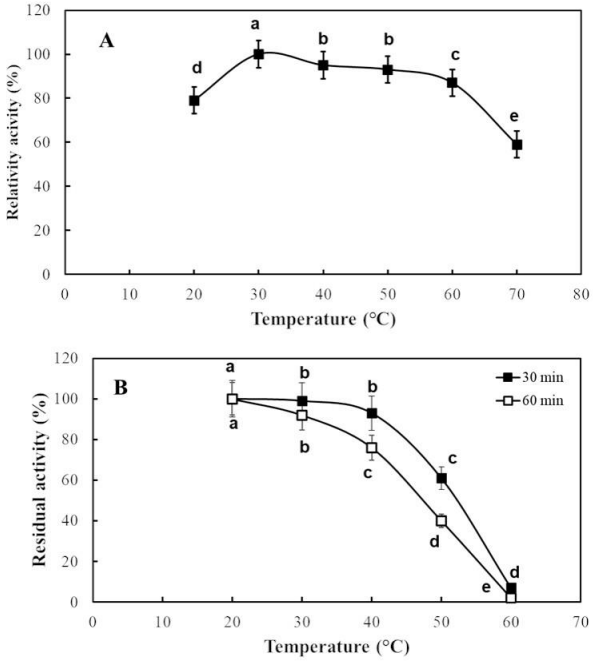

Figure 6. The changes in *I. purpurea* PPO activity (A) and stability (B) due to the reaction temperature. (A) A range of reaction temperatures between 20 and 70 ℃ at pH 7.0 were applied to test percentage relative activity changes. (B) Multiple temperatures ranging from 20 to 70˚C were applied on PPO to test percent residual activity changes. The values are presented as means ± SD.

Şekil 6. Reaksiyon sıcaklığına bağlı olarak I. purpurea PPO aktivitesi (A) ve stabilitesinde (B) gözlenen değişiklikler. Yüzde relatif aktivite değerindeki değişmleri test etmek için pH 7.0'da 20 ila 70 ℃ arasında bir dizi reaksiyon sıcaklığı uygulandı. (B) Yüzde bağıl aktivite değerindeki değişikliklerini test etmek için PPO'ya 20 ila 70°C arasında değişen çoklu sıcaklıklar uygulandı. Değerler, ortalama ± SD olarak sunuldu.

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Author's Contributions

BM prepared the crude extract from in vitro cultured *I. purpurea* leaves, purified the PPO enzyme, and performed biochemical characterization experiments. EK carried out statistical analysis. AA & YYK created the project. YYK guided the work and wrote the paper.

Statement of Conflict of Interest

Authors have declared no conflict of interest.

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