PARTIAL PURIFICATION AND CHARACTERIZATION OF POLYPHENOL OXIDASE ENZYME FROM COMMON-MORNING GLORY (*Ipomoea purpurea*)

A THESIS SUBMITTED TO
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
KOCAELİ UNIVERSITY

BY

BEKTORE MANSUROV

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY

KOCAELİ 2021

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percent
=	:	Equals
±	:	Standard deviation
µl	:	Microliter
cm	:	Centimeter
g	:	Gram
L	:	Liter
M	:	Molar
ml	:	Milliliter
mM	:	Millimole
°C	:	Centigrade degrees
U	:	The enzyme unit
µmol/min	:	micromole/minute
U/g	:	Unit/gram
g/l	:	gram/liter
mg/ml	:	milligram/milliliter

Abbreviations

PPO	: Polyphenol Oxidase
IVCP-CE	: Crude extract prepared from *in vit*ro-cultured plant
LS	: *Linsmaier & Skoog*
LP-CE	: Crude extract prepared from leaves of local plant
TPP	: Three Phase Partitioning
HCl	: Hydrochloric acid
NaOH	: Sodium hydroxide
kDa	: Kilodalton
$(NH_4)_2SO_4$	: Ammonium sulfate
pH	: Power of Hydrogen
rpm	: Revolutions Per Minute
TEMED	: Tetrametiletilendiamin
t-butanol	: tert-Butyl alcohol, also called tert-butanol
SDS-PAGE	: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
PVPP	: Polyvinylpolypyrrolidone
H_2O_2	: Hydrogen peroxide
ADA	: 4-amino-N-N diethylaniline
*t*BC	: 4-tert-butyl catechol
BSA	: Bovine Serum Albumin
BCA	: Bicinchoninic acid
dH_2O	: Distilled water
APS	: Ammonium persulfate
I. purpurea	: *Ipomoea purpurea*
(v/v)	: Volume/Volume
(w/w)	: Weight/Weight
PDB	: Protein Data Bank

GÜNDÜZ SEFASI (*Ipomoea purpurea*) BİTKİSİNDEN POLİFENOL OKSİDAZ ENZİMİNİN KISMİ SAFLAŞTIRILMASI VE KARAKTERİZASYONU

ÖZET

Bu çalışmada, *in vivo* ve *in vitro* olarak yetiştirilen *Ipomoea purpurea* (*I. purpurea*) bitkilerinin polifenol oksidaz aktivitelerinin karşılaştırılması amaçlandı. Bu amaçla yerel bitki ile LS (*Linsmaier & Skoog*) ortamında *in vitro* büyütülen bitki yapraklarından ham ekstrakt örnekleri optimum koşullarda hazırlanarak polifenol oksidaz aktiviteleri açısından karşılaştırıldı. Buna göre, bitkisel ham ekstrakt örneklerinden *in vitro* olarak üretilen bitkiye ait yaprakların yerel bitki örneğine göre katekol substratına karşı daha yüksek polifenol oksidaz aktivitesine (yaklaşık 3 kat daha yüksek aktivite) sahip olduğu belirlendi. Daha sonra enzim, kültür bitkisinden Üçlü-Faz Ayırma (TPP) sistemi ile %57 aktivite geri kazanımı ile 10,5 kat saflaştırıldı. Enzim alt birimine ait moleküler ağırlığı SDS-PAGE ile yaklaşık 12.8 kDa olarak hesaplanırken enzim 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. Sonuç olarak *I. purpurea* bitkisinin bir polifenol oksidaz kaynağı olarak kullanılabileceği önerilmektedir.

Anahtar Kelimeler: Enzim Karakterizasyonu, Enzim Saflaştırması, *Ipomoea purpurea*, Polifenol Oksidaz, Üçlü-Faz Ayırma Sistemi.

PARTIAL PURIFICATION AND CHARACTERIZATION OF POLYPHENOL OXIDASE ENZYME FROM COMMON-MORNING GLORY (*Ipomoea purpurea*)

ABSTRACT

In this study, it was aimed to compare the polyphenol oxidase activity of *in vivo* and *in vitro* grown *Ipomoea purpurea* (*I. purpurea*) plants. Towards this aim, leaves from local plants and *in vitro* propagated plants using LS (*Linsmaier & Skoog*) medium were used to prepare crude extracts and their polyphenol oxidase activities were measured. Among the plant crude extracts, it was determined that the leaves of the *in vitro* plant possessed higher polyphenol oxidase activity (3-fold activity) than local plant when catechol used as substrate. Then, the enzyme was 10.5-fold purified with a 57% activity recovery using the Three Phase Partitioning (TPP) system from *in vitro* plants. Molecular weight of the enzyme subunit was determined approximately 12.8 kDa by SDS-PAGE, and 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°C-40°C and pH 7.0-9.0, respectively. In conclusion, it has been suggested that *I. purpurea* can be used as a source of polyphenol oxidase.

Keywords: Enzyme Characterization, Enzyme Purification, *Ipomoea purpurea*, Polyphenol Oxidase, Three-Phase Partitioning System.

INTRODUCTION

One of the most important problems faced by the world today, as a result of the consumption of excessive fossil resources by the industries, the balance of nature is disturbed, leading to global warming, water, air and soil pollution. For this reason, the economy based on fossil resources leaves its place to the economy based on biological resources in order to protect nature and meet the need for exhaustible energy and material. The mostly green leaves, seeds and fruits of plants constitute an inexhaustible source of raw materials for carbohydrates, proteins, enzymes, lipids, various phenolic substances and phytochemicals.

Phenolic substances compose the most important groups of natural antioxidants. These are polyphenolic components found in all parts of plants. Depending on their chemical structure, plant phenolic compounds can be separated into phenolic acids, flavonoids, tannins, stilbenes and lignans. It is known that they protect easily substances found in foods (fruits and vegetables) from oxidation.

Polyphenol oxidases, which play a role in the oxidation of phenolic substances, constitute a very large family of enzymes. Despite the uncertainty in the classification of PPOs, they can be divided into three groups according to the literature: laccases (or *p*-diphenol: oxygen-oxidoreductase, E.C. 1.10.3.2), catechol oxidases (or *o*-diphenol: oxygen-oxidoreductase, E.C. 1.10.3.1) and tyrosinases (or monophenol-monooxygenase, E.C. 1.14.18.1) (Kocabas *et al.*, 2011). On the other hand, peroxidases (E.C. 1.11.1.X), sometimes included in phenol oxidases, are enzymes that oxidize phenolic substances in the presence of hydrogen peroxide. Polyphenol oxidases have a wide range of uses in industry including food, chemical, pharmacy, wine, beer and fruit juice production (removal of phenolic substances), wastewater treatment, plastics, paper industry and melanin synthesis.

In this study, it was aimed to investigate the presence of polyphenol oxidase enzyme in extracts obtained from leaf parts of *Ipomoea purpurea*, known as a medicinal and ornamental plant, grown both *in vitro* and *in vivo*. At the same time, it was planned to analyze the biochemical properties of the enzyme by purifying it in a single step with the unconventional three-phase partitioning (TPP) method.

As a result, it has been shown for the first time that *I. purpurea* can form cheap and renewable biomass of agricultural origin for the production of polyphenol oxidase enzyme, which has an area of use in the industrial sector.

1. GENERAL INFORMATION

1.1. Global Warming

Global warming, as one of the world's most pressing issues, is a phenomenon characterized by a rise in temperature that is not limited to a single region but affects the entire globe (Held & Soden, 2006). The greenhouse effect caused by greenhouse gases such as carbon dioxide (CO_2), sulfur dioxide (SO_2), methane (CH_4), and nitrogen dioxide (NO_2) is the primary cause of global warming. Changes due to the global warming include increased sea levels resulting from melting of glaciers, severe storms and other extreme weather events. Climate change, referred as the increasing changes in the measures of climate over a long period, negatively affects the amount of precipitation and therefore leading to heat waves, droughts, floods, blizzards, thunderstorms, and hurricanes. These extreme weather events cause limitless damage to millions of lives across the world, from which we can clearly imagine the magnitude of the disaster we are facing. Recent analysis already shows that there has been an increase of 0.5 degree in temperature in 100 years. To mitigate global warming, we can reduce the source of greenhouse gases, preventing people from interfering with temperature rise (Sharma & Gahlawat, 2017).

Global warming has many serious effects. Until now, scientists cannot identify the specific cause of global warming due to the presence of many factors. However, we can identify a few hypotheses, which would affect climate change. One would be ice melting arising from thermal expansion. The glaciers are melting at a high rate, leading to significant sea level rise, which can be attributed to the thermal expansion of ocean water caused by global warming. When the water is heated, its density decreases, but the volume increases. This sea level rise will lead to millions of lives that will have to be homeless, particularly for those living in coastal regions (Haugan & Joos, 2004). Second would be on the human health. Because of increasing temperatures, many people will suffer from heat-related sickness and eventually death. Final would be on planting: an increase in sea

level will cause flooding of rivers and lakes, the destruction of plants, organisms in the soil, thereby reducing crop yields (Sorenson *et al.*, 1998).

Reforestation, that is, planting trees over a large area is believed as a good strategy to prevent global warming (Santos *et al.*, 2018). This method is an easy way of removing carbon dioxide from the atmosphere in the air (Dhillon & Wuehlisch, 2013).

1.2. Green Plants

The green plants are important not only for the human environment, but also for the sustainability of biological resources and environmental systems. After the transition from a fossil-fuel-based economy to a bio-based economy, green plants have gained a lot of attention. These renewable biomasses are used as raw materials in bio refineries, where they are recycled into value-added goods, biofuels, and bioenergy, preserving the environment while also meeting our growing energy and material demands.

Green bio refinery, which is one of the many bio refinery platforms, uses green plants such as grass and plant parts as raw materials. Green plant parts like tree leaves are defined as endless raw material reservoirs containing enzymes, lipids, proteins, carbohydrates and various phenolic compounds (Kamm *et al.*, 2006).

1.3. Phenolic Compounds (PCs)

The phenolic compounds contain a hydroxyl group and an aromatic ring in their structure. They are often found in most plant tissues, fruits, seeds, leaves, and edible parts like roots. PCs are the most common secondary metabolites and antioxidants of plants. They have unique flavor, taste and health specifications found in many vegetables and fruits. Polyphenols mainly contribute to plant defense system against radiation (ultraviolet), pathogen attack, plant color and environmental damage (de la Rosa *et al.*, 2019).

There are farther than 8,000 phenolic structures currently known. Depending on their chemical structure, plant phenolic compounds can be separated into phenolic acids (Figure 1.1), flavonoids, tannins, stilbenes and lignans (Dai & Mumper, 2010).

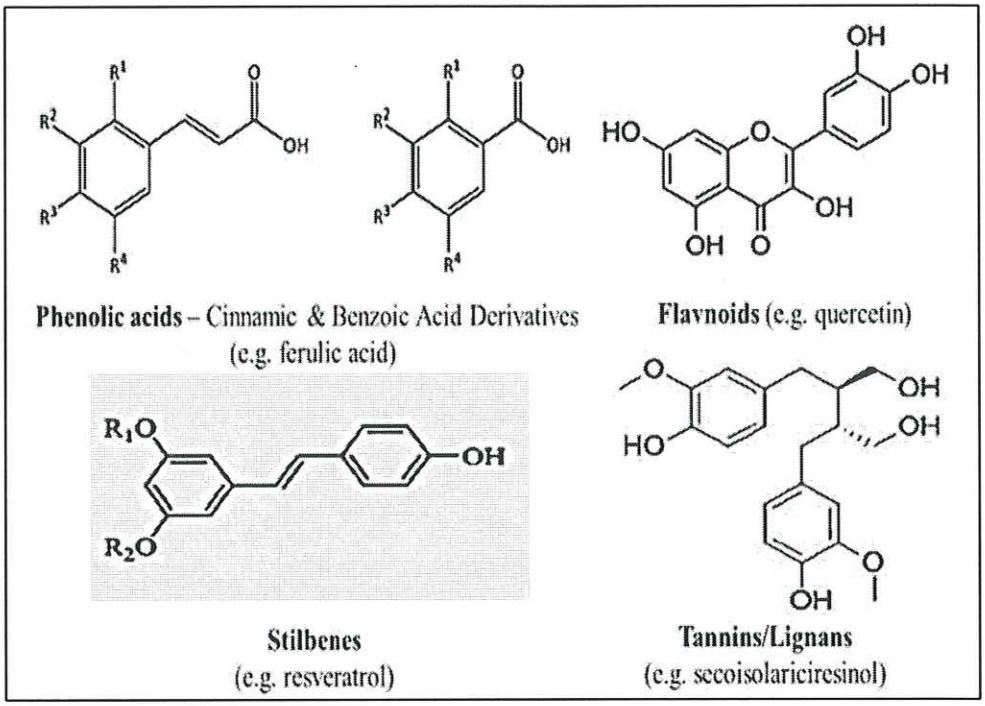

Figure 1.1. Structures of the different classes of Polyphenols (URL-1)

1.3.1. Phenolic acids

Phenolic acids are the most important secondary metabolites and bioactive groups in plant kingdom. Phenolic acid compounds and their functions are investigated in various agricultural, biological, chemical, and medical studies. They are mainly found in fruits, vegetables, herbs, spices and beverages. Phenolic acids consist of a group containing hydroxybenzoic and hydroxycinnamic acids (Figure 1.2). Hydroxybenzoic acids originated from benzoic acid share a common structure of C_6-C_1. They are found in soluble form (associated with sugars or organic acids) and bound with cell wall fractions as lignin. On the other hand, hydroxycinnamic acids are derived from cinnamic acid and usually presented as simple esters with glucose or hydroxy carboxylic acids. Phenolic acids can exist in food plants as glycosides or esters with other natural compounds like hydroxy fatty acids sterols, glucosides, and alcohols (Ghasemzadeh & Ghasemzadeh, 2011).

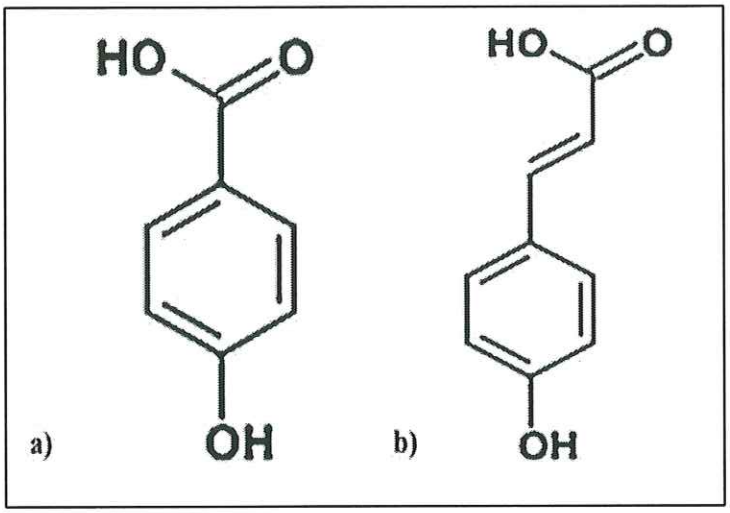

Figure 1.2. The main structures of the predominant phenolic acids in plants are: (a) Hydroxybenzoic acid and (b) Hydroxycinnamic acid (Russell & Duthie, 2011)

1.3.2. Flavonoids

Flavonoids are the richest PCs in food groups such as fruits and vegetables. More than 4000 different flavonoid varieties have been described to date. Interest in these compounds has increased especially after the discovery of their potential roles in preventing certain diseases such as heart disease and cancer. In addition to the detection and effects of these compounds on human health, studies on their chemistry, biochemistry, synthesis mechanisms and changes in flavonoids during bioavailability have also been introduced and discussed in recent years.

Flavonoids are the most bioactive compounds containing a phenyl benzopyran skeleton (two phenyl rings (A and B) combined with a heterocyclic pyran ring (ring C) (Figure 1.3). They can be separated into six groups or families based on the difference in the pyran ring: flavanones, flavan-3-ols, flavonols, flavones, isoflavones, and anthocyanidins (Çapanoğlu & Boyacıoğlu, 2009).

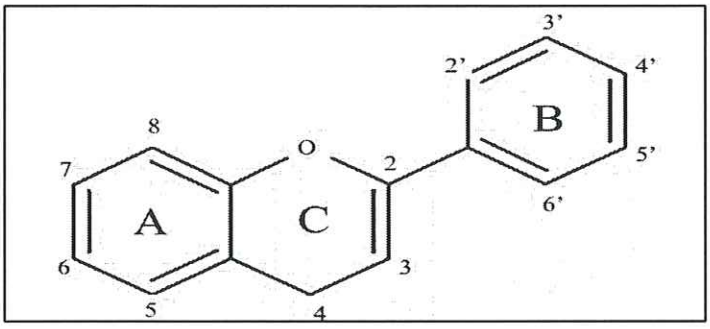

Figure 1.3. Phenyl benzopyrane skeleton, basic structure of flavonoids (de la Rosa et al., 2019)

1.3.3. Tannins

Tannins are another large group of polyphenols in the plant secondary metabolite family. They are classified into two classes: hydrolyzable tannins (HT) and nonhydrolyzable/condensed tannins (CT) (Figure 1.4). Hydrolysable tannins contain the polyesters of gallic acid (also called gallotannins) and hexahydroxydiphenic acid (also called ellagitannins), while condensed tannins a comprised of polymers of proanthocyanidins (favan-3-ol nuclei) and oligomers. Tannins are found in large quantities in many parts of the plant, namely bark, wood, leaves, fruits, roots and seeds. The biological role of many tannin plants is assumed to be related to defense mechanisms against microbic infection and other infectious agents (Sieniawska & Baj, 2017).

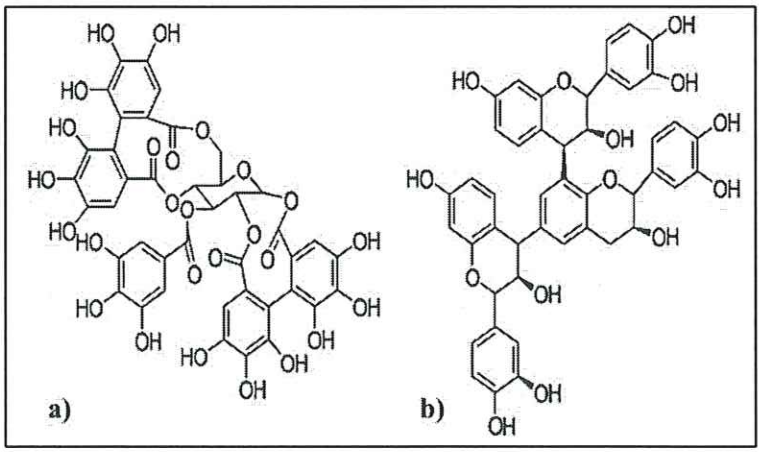

Figure 1.4. Structure of (a) hydrolyzable tannin, (b) condensed tannin (Raja et al., 2014)

1.3.4. Stilbenes and lignans

Stilbenes are natural small compounds (approximately 200-300 g/mol), found in a wide range of plant sources, aromatherapy products, and dietary supplements (Roupe *et al.*, 2008). Lignans are a large class of secondary metabolites in plants with a huge number of biological effects containing antitumor and antioxidant activities (Figure 1.5). Some plant lignans (sesamin *etc.*) have protective effects against hormone-related diseases such as breast cancer (Sato & Matsui, 2012). Stilbenes and lignans have been shown to protect against certain types of cancer and to exhibit antioxidant, antibacterial, antimicrobial, and cytotoxic properties (Tsopmo *et al.*, 2013).

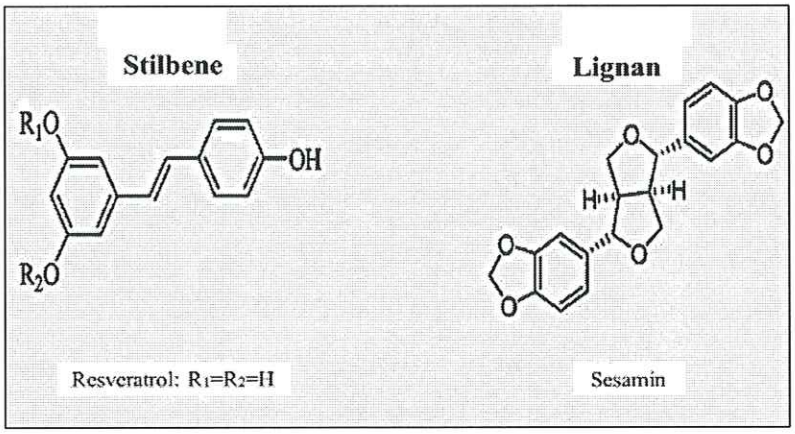

Figure 1.5. Structures of stilbene and lignan (Dai & Mumper, 2010)

1.4. Phenol Oxidative Enzymes

Nature is full of enzymes that catalyze the oxidation of phenolic compounds. The two main groups of these enzymes are peroxidases and polyphenol oxidases (Peter & Wollenberger, 1997).

1.4.1. Peroxidases

Peroxidases (E.C. 1.11.1.X) are oxidoreductases that use hydrogen peroxide or organic compounds as oxidizers. They are hemoproteins that contain protoporphyrin IX as the prosthetic group and are commonly found in microorganisms and plant sources. (Esposito & Durán, 2000).

1.4.2. Polyphenol oxidases (PPO)

Polyphenol oxidases (PPOs) (EC 1.14.18.1, EC 1.10.3.1, or EC 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 (or *p*-diphenol: oxygen-oxidoreductase, E.C. 1.10.3.2), catechol oxidase (or *o*-diphenol: oxygen-oxidoreductase, E.C. 1.10.3.1) and tyrosinase (or monophenol-monooxygenase, E.C. 1.14.18.1) (Kocabas *et al.*, 2011).

1.4.3. Laccase

Laccases (E.C. 1.10.3.2, *p*-benzenediol: oxygen oxidoreductase) are copper-containing enzymes that can oxidize both phenolic and non-phenolic lignin-related compounds, as well as a variety of organic and inorganic substrates such as mono-, di- and polyphenols, aminophenols, methoxyphenols, aromatic amines and ascorbate, and allow the formation of free radicals (Bourbonnais *et al.,* 1997, Li *et al.,* 1999). Laccase is made up of four copper atoms that are divided into three classes based on their electron paramagnetic resonance (EPR) properties: Type 1 is a blue copper zone, Type 2 is a natural copper zone, and Type 3 is a combined binuclear copper zone. (Figure 1.6) (Duran *et al.,* 2002).

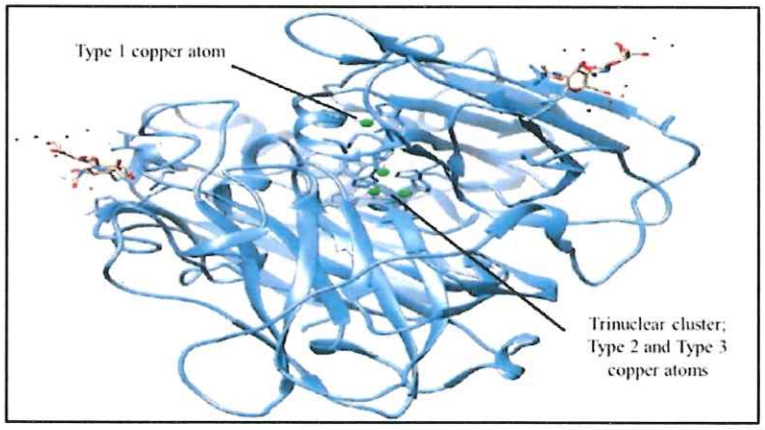

Figure 1.6. The construction of laccase from ***Trametes trogii*** (Charles Guest & Rashid, 2016)

1.4.4. Catechol oxidase (CO)

Catechol oxidase (CO) (also known as *o*-diphenol oxidoreductase; EC 1.10.3.1) is a type 3 copper enzyme. CO is commonly found in plant tissues, as well as in some insects (beetle) and crustaceans. It catalyzes the oxidation of varieties of *o*-diphenols to the corresponding *o*-quinones by molecular oxygen (Figure 1.7) (Messerschmidt, 2010, Koval *et al.,* 2006).

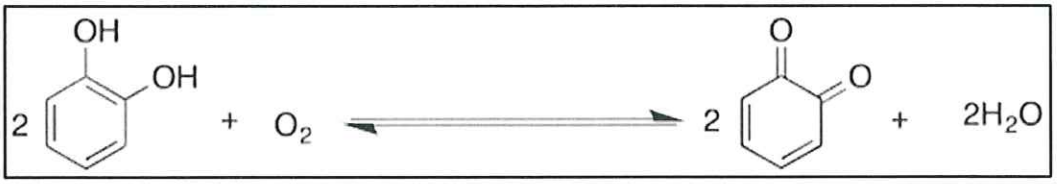

Figure 1.7. Reaction mechanism of catechol oxidase (URL-3)

1.4.5. Tyrosinase

Tyrosinase (EC 1.14.18.1, monophenol monooxygenase and E.C. 1.10.3.1) is a copper-containing metalloenzyme. It is widely distributed in nature, where it is found in every lifeform from bacteria to mammals. Tyrosinase is an important enzyme that appears in Mammalia with prolonged browning and is responsible for skin pigmentation deformities like blemishes and spots (Messerschmidt, 2010). The enzyme catalyzes two different oxygen dependent reactions that occur consequently: the *o*-hydroxylation of monophenols to *o*-diphenols (cresolase or monophenolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (catecholoxidase or diphenolase activity) (Figure 1.8). Due to its two-step catalytic feature, tyrosinase is considered as an attractive source for various biotechnological and industrial applications (Agarwal *et al.*, 2019).

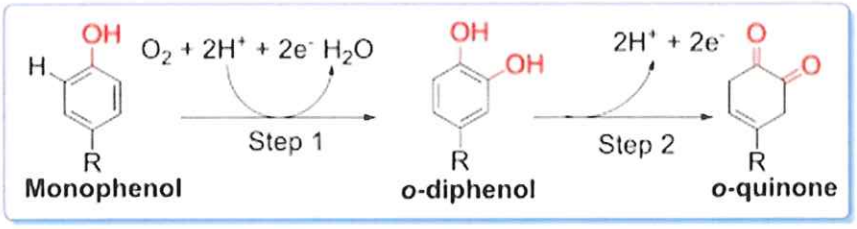

Figure 1.8. Tyrosinase structure (Nawaz et al., 2017)

1.5. Industrial Significance of PPOs

PPO activity is vital to determine the quality and shelf life of food products; 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 food industry (Figure 1.9).

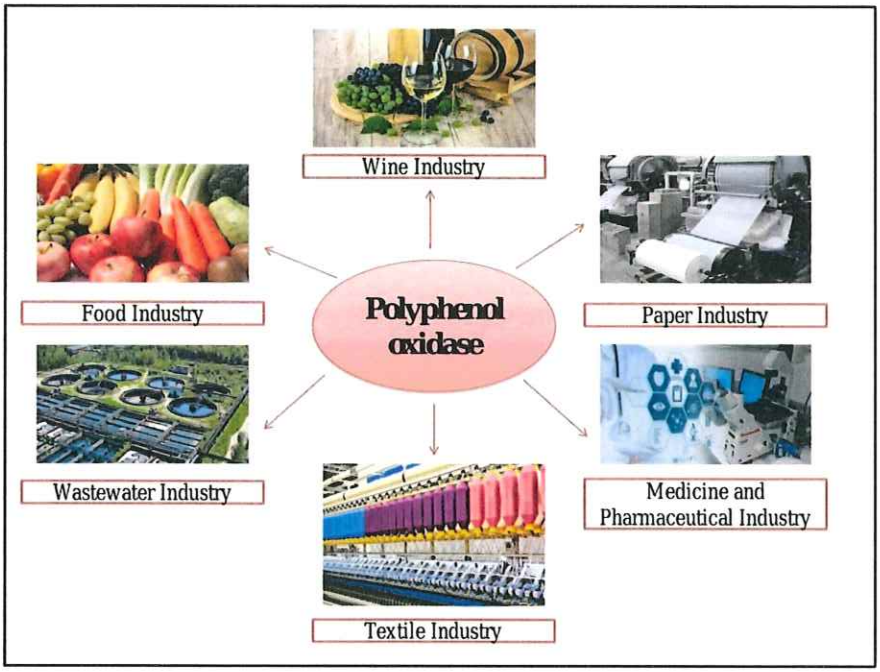

Figure 1.9. Industrial applications of PPO

In pharmaceutical industry, PPOs are frequently used in the treatment of Parkinson's disease (PD), phenylketonuria and leukemia as well as control of melanin synthesis. Under the action of PPO, L-tyrosine is converted into L-DOPA, which is used to supplement insufficient amounts of dopamine in Parkinson's disease (Asanuma *et al.*, 2003). Hor *et al.* (2020) supported the potential efficacy of polyphenols in providing neuroprotection against toxicity caused by oxidative stress in PD. According to a recent study, PPOs have also been found to prevent the growth of *Streptococcus sobrinus*, the

bacteria responsible for the formation of the oral cavity, from adhering to the tooth surface (Kamal-Alahmad *et al.*, 2015).

In food industry, they are generally used for color development, improvement of flavor in tea, cocoa and coffee production, and determination of food quality (Kamal-Alahmad *et al.*, 2015). Additionally, PPOs are applied for stabilization of beer and wine, fruit juice processing, sugar beetroot pectin gel transition (gelation) and flour products (paste) enhancing (Maki & Morohashi, 2006).

In environmental technology, the presence of toxic phenolic compounds and their derivatives in industrial wastewater resulting from coal recycling, petroleum refining, wood preservation, textile, paper, food, and chemical industries is a major problem. Especially, in developed countries, the removal of toxic compounds from wastewater becomes more stringent before it is discharged into the environment. Hence, it has been focused on the use of peroxidases and PPOs as an enzymatic approach to remove phenolics from industrial waste (Põllumaa *et al.*, 2001, Kamal-Alahmad *et al.*, 2015).

1.6. PPOs from Different Plant Sources

In countries where it is important to produce economically significant numbers of fruits and vegetables, green plants are used as a source of PPO. There are many studies on plant PPOs in the literature explored by the researchers especially with fruits and leaves.

Kocabas *et al.* (2011) screened various plants; mulberry, pear, cherry, quince, horse chestnut, apple, grape, and apricot in terms of their PPO activities. The authors compared their PPO activities with a common PPO source-mushroom (*Agaricus bisporus*). They suggested that Mulberry leaves (*Morus alba*) presented the highest activity (about 19 EU g^{-1} tissue). Apricot, pear, and cherry leaves also had high PPO activities (10, 15 and 12 EU g^{-1}, respectively). On the other hand, cherry, horse chestnut, apple, grape, and quince leaves presented low PO activity values.

Gul Guven *et al.* (2017) purified and characterized PPO from purslane (*Portulaca oleracea*). They measured the kinetic parameters for three substrates such as catechol

(Km: 4.4 mM, Vmax: 5503 U/min), L-Dopa (Km: 4.6 mM, Vmax: 1655 U/min) and 4-methylcatechol (Km: 6.75 mM, Vmax: 4504 U/min). The authors also calculated the optimum pH (7.0) and temperature (50°C) values using catechol as the substrate. They determined the molecular weight of purslane PPO as 163 kDa from denaturing SDS-PAGE and observed that ascorbic acid presented the most inhibitory effect (K_i value of 0.36 mM) among tested inhibitors including sodium azide and citric acid.

Alici & Arabaci, (2016) partially purified and characterized PPO by using three-phase partitioning (TPP) method from borage plant (*Trachystemon orientalis L.*). The molecular weight of the enzyme was 80 kDa and caffeic acid was the best substrate. The optimum pH and temperature values were varied depending on the substrate from 5.0 to 7.5 and from 5 to 30°C, respectively. Among the several PPO inhibitors tested, ascorbic acid was the most effective inhibitor with a K_i of 0.02 mM.

Bravo & Osorio, (2016) investigated the purification and characterization of PPO from the cape gooseberry (*Physalis peruviana*) through acetone precipitation and aqueous two-phase system (ATPS). The authors reported that the PPO enzyme was purified to 169-fold with 14% recovery. The enzyme was found to be more active against chlorogenic acid at optimum pH (5.0) and temperature (20°C) values than 4-methylcatechol and catechol. In inhibition tests, the strongest inhibitor was found to be ascorbic acid followed by L-cysteine and quercetin.

Siddiq & Dolan, (2017) extracted and characterized the PPO enzyme from blueberry (*Vaccinium corymbosum L.*). The enzyme presented optimum activity at pH values between 6.1 and 6.3. Temperature optima was observed as 35°C, although the enzyme was significantly active at a broad temperature range of 25-60°C. Blueberry PPO presented a wide range of activities against *di-* and *tri*-hydroxy phenolic substrates, with the maximum activity against catechol. Blueberry PPO indicated a Km of 15 mM and Vmax of 2.57 ΔA420 nm/min, when catechol used as substrate.

Lin *et al.* (2016) partially purified and characterized of PPO from Chinese parsley (*Coriandrum sativum*). The highest PPO activity was found at 35°C and pH 8.0 for 4-

methylcatechol, and at pH 7.0 and 40°C for catechol. L-cysteine has been found to be a potent inhibitor compared to citric acid. Two protein bands of approximately 46 kDa isoenzymes were determined using SDS-PAGE.

Aydin *et al.,* (2015) purified and partially characterized the PPO enzyme from Hemşin apple (*Malus communis L.*). The enzyme activity was optimum at pH 5.5 and 30°C when using catechol as the substrate. Some kinetic and thermal parameters were determined such as Vmax (333.3 EU/mL.min), Km (3.40 mM), k_{cat} (24.57 min^{-1}), ΔH (2.968 kcal/mol), Q_{10} (1.33) and V_0 (7.2x10^3 $mM^{-1}.min^{-1}$). In addition, the effects of some metal ions (i.e., Mg^{2+}, Pb^{2+}, Fe^{2+} etc.) were determined. Among them Cd^{2+} was the most potent inhibitor with a K_i value of 4.87 mM.

Yuzugullu Karakus *et al.,* (2021) purified and characterized PPO from the fennel seeds (*Foeniculum vulgare*) through TPP, which gave 20-fold purification with 120% activity recovery. The optimum activity was found at 30°C, while pH optimum varied from 5.0 to 7.0 depending on the substrate used. The molecular weight of the purified protein was estimated as 27.8 kDa from SDS-PAGE. The highest activity reported was against catechol followed by 4-tert-butylcatechol (4-tBC), 4-methyl catechol and pyrogallol.

1.7. *Ipomoea*

The genus *Ipomoea*, being the largest species of the *Convolvulaceae* family, is found in around 600-700 varieties all over the world in tropical and subtropical areas. The leaves of *Ipomoea* are usually heart-shaped, while the flowers are funnel-shaped (Austin, 1997; Meira *et al.*, 2012). Since the ancient times, the genus *Ipomoea* has been utilized constantly for different purposes, such as nutrition, medical, ritual and agriculture (Pereda-Miranda & Bah, 2005). The species of *Ipomoea* are also used as an ornamental plant for their flowers (Pereda-Miranda & Bah, 2005). *Ipomoea* is rich in phytochemicals including many medicinally important species including alkaloids, flavonoids, anthocyanins, glycolipids, lignans and phenolics compounds (Meira *et al.*, 2012). Most have been studied as active substances, but many are still being discovered (URL-2; Yeung, 1995; Srivastava, 2017).

Baucom *et al.*, (2011) investigated the importance of *Ipomoea* on agriculture and ecology in terms of ***Ipomoea's*** mating systems, growth form, flower color, molecular evolution, and parasitic interactions. The authors emphasized that *Ipomoea* species have potential for their use in different research areas including plant ecophysiology, comparative genomics, and evolutionary biology of transferable elements. Genomic resources for this group are under development, but it is promising that *Ipomoea* would be used as next model organism for ecological genomics.

1.7.1. *Ipomoea purpurea*

Ipomoea purpurea (L.) Roth (Common-morning glory, also known as Tall Morning-glory) is an annual/perennial ornamental plant. It is named as "Morning Glories" because it rises with the morning sun and blooms. *I. purpurea* is predominant in the American tropics, also commonly in agricultural fields and widely distributed in South Europe and in Turkey. Purpurea means "purple", which is the color of the flowers (Figure 1.10), in addition various mixes of plant colors are available (URL-2; Austin, 1997).

Figure 1.10. Common-morning glory (*Ipomoea purpurea*) (URL-2)

The advantages of *I. purpurea* include being a fast-growing plant and having ability of self-fertilization (around 30% of the flowers). For sowing, it is convenient to plant the seeds in the greenhouse in early spring or stick them in the ground in a sunny place after the hazardous effect of cold passed. The plant seeds are small sized (from 4 to 5.7 mm), looks like wrinkled brown peas, and the covering coat is particularly tough (Figure 1.11). Practically, since the plants are sensitive to cold, the protector of the seed coating delays

germination until the ambient conditions are warmed. There are some issues required attention. For example, before sowing, the seeds should be soaked or sepulchered, allowing it to germinate quickly (usually germinates in 1-3 weeks at 22°C). Mostly they bloom their flowers in autumn (Chace & Llewellyn, 2015).

Figure 1.11. *Ipomoea purpurea*'s seeds (URL-2)

I. purpurea is widely used for ornamental and medicinal purposes. In medicine, the stems, seeds, roots and flowers of *I. purpurea* have been utilized in many treatments such as laxative, hallucinogen, purgative, syphilis, infertility, rheumatism, fungal infection, liver protection, acne, urinary infection, diarrhea and constipation (Muhammad, 2019; Srivastava, 2017). Although it has a wide use, studies on *I. purpurea* are quite limited.

Acemi *et al.*, (2019) investigated the changes in photosynthetic pigment, protein and dry matter content of *I. purpurea* after chitosan treatment to determine its indirect effect on plant leaves. The authors observed that chitosan oligomers appeared very effective in stimulating the production of photosynthetic pigment when used in low concentrations. In addition to this, they suggested that the medium concentration of chitosan polymer in culture medium would positively affect total protein production and plant dry matter content in *I. purpurea*.

Atala *et al.*, (2014) performed three separate greenhouse experiments to test the twining induced in *I. purpurea* by applying different levels of artificial damage (0, 1, 5, 10 perforations to the leaves), volatile (ground leaves) and natural damage with snails. Plants

exposed to grounded leaves (volatiles) were reported to twin faster than control plants. Finally, twinning was started with more snail damage than undamaged plants.

Park *et al.*, (2007) showed that phytomelanins mainly accumulate in the outer epidermis and palisade layers of wild type *I. purpurea* seeds. Phytomelanins have been found to accumulate intensely when the outermost epidermal layer overlaps with the deposited layers of proanthocyanidine. Phytomelanin accumulation is proposed to occur in the outer epidermis and palisade layers, as they can pass from the phytomelanin precursor synthesized cells into the space where the phytomelanins polymerize.

1.8. Three-Phase Partitioning (TPP)

When we apply any purification method, we must pay attention that the method is environmentally safe and cost effective. Three-phase partitioning (TPP) is known as a rapidly developing, environmentally friendly and inexpensive biomolecule purification technology that combines salting out, isoelectric point precipitation, and solvent precipitation techniques (Figure 1.12). In TPP system, a protein sample is treated with an organic solvent (normally *t*-butanol) and a salt solution (mostly ammonium sulfate) for saturation. The sample is then mixed on a magnetic stirrer for 1 minute and centrifuged, and the resulting mixture consequences in a three-phase mixture of an upper organic phase, a lower aqueous phase, and a mid-phase protein (Dennison & Lovrien, 1997). Pigments, lipids and hydrophobic materials are concentrated in the upper phase, the protein is concentrated in the middle phase, saccharides and other polar components are concentrated in the lower phase (Liu *et al.*, 2019).

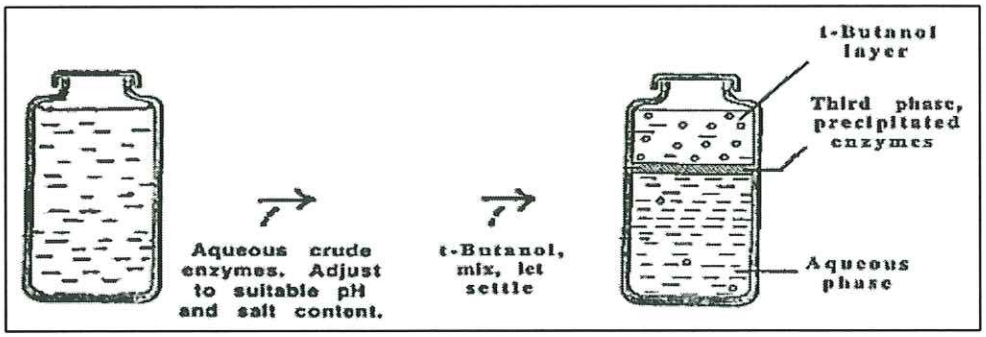

Figure 1.12. Schematic representation of TPP System (Dennison & Lovrien, 1997)

1.9. Applications of TPP

This non-chromatographic method can purify many proteins, especially enzymes, in a one-step protocol. The parameters for separation mechanisms like ammonium sulfate concentration, tert-butanol content and pH are optimized firsthand then the appropriate condition for precipitation such as protein, enzyme, vegetable oil, polysaccharide and other small molecule compounds are applied to the separation and purification of organics.

TPP has many advantages; it is a simple process with short processing time (i.e., time-efficient), ammonium sulfate and *t*-butanol are inexpensive chemicals, thus making TPP an economical protocol, TPP conditions do not denature proteins and convenient for working at room temperature. In most cases, enzymatic activity increases during TPP compared to crude extract and results in higher enzyme recovery (>100%). The desired protein particularly appears either in the middle or lower phases (Ketnawa *et al.*, 2017).

A small-scale separation of proteins from natural sources was accomplished through upstream and downstream protein purification systems. Several enzymes have been purified by TPP, containing lipase from *Rhizopus arrhizus* (Dobreva *et al.*, 2019), α-glucosidase from corn (Bayraktar *et al.*, 2018), protease from *Pangasianodon gigas* (Rawdkuen *et al.*, 2012), and laccase from *Trametes versicolor* (Wasak *et al.*, 2018). However, findings on plant PPO extraction utilizing three-phase partitioning are quite constrained, although it has been used for the purification of several main enzymes with TPP as mentioned above. There are only four reports on PPO extraction using TPP in the current literature. In these reports, the researchers used fennel seeds (*Foeniculum vulgare*) (Yuzugullu Karakus *et al.*, 2021), borage (*Trachystemon orientalis* L.) (Alici & Arabaci, 2016), potato peel (*Solanum tuberosum*) (Niphadkar & Rathod, 2015) and rosemary (*Rosmarinus officinalis* L.) (Yuzugullu Karakus *et al.*, 2020) as sources of PPO.

2. MATERIALS AND METHODS

2.1. 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 obtained from Sigma-Aldrich (St. Louis, MO, USA), Biolfe (Viale Monza, Milan, Italia), Duchefa Biochemie (RV Haarlem, Netherlands) and BioRad (California, USA).

2.2. Preparation of PPO Crude Extracts

Young leaves of *I. purpurea* were collected from its natural environment in Kocaeli (Turkey). The leaves were used in experiments within a few hours after collection or stored at -80°C until used for further experiments. To compare the effect of cultivation on PPO production, the seeds of *I. purpurea* were also grown in LS (*Linsmaier & Skoog*) medium in the Plant Tissue Culture laboratory of Biology department (Kocaeli University). Young leaves were collected immediately and used for further PPO extraction.

Plant leaves were thoroughly washed with dH_2O before use in the experiment. In the preparation of the crude extract, 30 grams of leaves were 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) in order to remove the phenolic substances in the environment, and the mixture was filtered through cheesecloth and centrifuged at 10000×*g* for 30 minutes at +4 degrees (Kocabas *et al.*, 2011). The resulting supernatant was collected and used as the crude enzyme extract for further purification.

2.3. PPO Purification

The PPO enzyme was purified using TPP system consisting of ammonium sulfate, t-butanol and crude enzyme extract. 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 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 magnetic stirrer for 1 minute and kept at room temperature for 60 minutes for phase separation. After standing, complete phase separation was attained by centrifugation at 4500*g* for 10 minutes (Alici & Arabaci, 2016; Duman & Kaya, 2014). The top phase, including t-butanol, where the protein was not expected to be present, was removed by careful pipetting. The precipitate containing the PPO enzyme (middle phase) was dissolved in 1 ml of 0.1 M sodium phosphate buffer solution (pH 7.0). Afterwards, activity and protein determination experiments were carried out using a spectrophotometer (Cary 60, Agilent) at 420 nm at room temperature on the middle and lower phase samples. The parameters (ammonium sulfate concentration, crude extract: *t*-butanol ratio, system pH) that play a role in the selective separation of biomolecules in three-phase partitioning systems were also optimized.

Crude extract activity (for *in vitro*-cultured plant: 4686 U, for local plant: 670 U) was acknowledged as 100%. A blank system was arranged to include $(NH_4)_2SO_4$, dH_2O, and tert-butanol (excluding the crude extract).

In the lower phase, the purification fold and enzyme activity recovery percentage were found to be higher than the middle phase activity. Therefore, the lower phase was chosen for next experiments.

2.4. Enzyme Activity Assay

PPO activity was measured by observing quinone production at 420 nm at room temperature on a spectrophotometer. To measure enzyme activity assay, the sample cuvette was prepared by adding 500 µl substrate (pyrocatechol) prepared at a

concentration of 100 mM, 1 ml sodium phosphate buffer solution (100 mM, pH 7.0) and 500 µL enzyme solution diluted in the appropriate amount. (Alici & Arabaci, 2016). The control (blank) cuvette was prepared in the same way but without the enzyme. All experiments were performed duplicate in enzyme activity.

2.5. Protein Determination

Protein concentration was performed according to the Bicinchoninic Acid (BCA). Bovine serum albumin (BSA) was used as the protein in drawing the standard graph (Figure APP-1) (Smith *et al.*, 1985).

2.6. Characterization of the PPO Enzyme

2.6.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was conducted to measure the purity of the TPP-purified PPO. The Bio-Rad Mini-Protean system was used in the analysis. Electrophoresis was performed with 5% (w/v) stacking gel and 15% (w/v) separating gel at a constant current of 20 mA in stacking and 40 mA in separating gels. Then bands were visualized by the Silver staining procedure (Laemmli, 1970; Blum *et al.*, 1987).

2.6.2 Activity staining

The activity staining procedure was carried out for the separation of laccase, CO and peroxidase activities from PPOs by polyacrylamide gel electrophoresis (Rescigno *et. al.*, 1997). The SDS-free polyacrylamide gel was designed to contain 10% (w/v) separation gel, and 4% (w/v) stacking gel and electrophoresis was carried out at 150 V for 45 minutes at 4°C (Kaptan, 2004). Thereafter, the separation gel was first washed with 20 ml of 0.1 M potassium phosphate buffer solution (pH 6.0) for 5 min. Then, the gel was treated with the following solutions: first it was exposed to 40 mM 4-amino-N-N diethylaniline (ADA) prepared in 10 mM HCl solution for laccase activity determination, then 10 mM H_2O_2 for peroxidase activity determination was applied and finally it was exposed to 40 mM 4-tert-butyl catechol (*t*BC) 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 *t*BC were analyzed (Rescigno *et al.*, 1997).

2.6.3. Effect of pH on enzyme activity

To determine the effect of different pH on *I. purpurea* PPO activity, it was measured using 100 mM catechol 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 (Gulcin *et al.,* 2005; Kavrayan & Aydemir, 2001; Kocabas *et al.,* 2011). The buffer solutions prepared are given in APP-A. pH analyses were performed by incubating the PPO sample in the above mentioned buffers for both 30 and 60 minutes in a water bath. The enzyme activity was measured at the end of the incubation period using the standard experimental procedure.

2.6.4. Effect of temperature on enzyme activity

In order to measure the effect of temperature on *I. purpurea* PPO enzyme activity, enzyme and substrate solutions were examined by setting a spectrophotometer temperature between 20°C and 70°C. The enzyme stability temperature was measured after keeping the PPO samples in a set water bath at different temperatures between 20°C and 60°C (20, 30, 40, 50, and 60°C) for about 1 hour. After the incubation, enzymes were first kept on ice and then phenol oxidase activity measurements were made in a spectrophotometer device.

3. RESULTS AND DISCUSSION

PPO enzymes have been widely used industrially in wastewater treatment, biosensor development, and the treatment of many diseases, including Parkinson's disease, vitiligo, cancer, phenylketonuria, and some *Streptococcus* infections. In addition, PPOs have been found useful for their applications in the synthesis of many pharmaceutical compounds and in the food industry to enhance the flavor of many hot and cold beverages (Gasmalla *et al.*, 2015, Jukanti, 2017). Due to the wide range of application areas of the PPO enzymes, its isolation and purification from different sources has gained great importance in recent years. The enzyme has been isolated and purified using common chromatography techniques from different plant (Mestmäcker *et al.*, 2018, Kumar *et al.*, 2014), bacterial (Sivapathasekaran *et al.*, 2009) and fungal (Singh *et al.,* 2020) sources and has been presented for application after characterization.

In this study, *I. purpurea* was chosen as PPO source due to their broad ornamental and medicinal uses and presence of their limited study in the literature. The enzyme was isolated, partially purified and characterized from *I. purpurea* as described in the "Materials and Methods" section. The protein content, activity, and specific activity values of the PPO preparation are given in the relevant sections and tables.

3.1. Optimization of PPO Extraction Conditions

For PPO isolation from *I. purpurea*, the first step was the optimization of extraction conditions. The leaves of *I. purpurea*, grown both naturally (local plant) and in LS medium (*in vitro*-cultured plant), were collected, and compared in terms of their PPO activities. Extraction conditions were investigated in terms of PVPP concentration and pH.

3.1.1. PVPP optimization

PVPP is a compound capable of preventing hydrogen bonding between phenolics and PPO enzyme (Smith & Montgomery, 1985). For this reason, PVPP was used during extraction and its concentration was optimized to improve PPO yield. PVPP was added to extraction medium in a concentration range of 5-75 mg PVPP/ml. As seen in Table 3.1, 25 mg PVPP/ml 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 concentrations (Kocabas *et al.*, 2011; Pelalak *et al.*, 2021; Rocha *et al.*, 2001). 25 mg PVPP/ml was chosen for further extraction optimization analysis.

Table 3.1. PVPP optimization results

PVPP concentration (mg/ml)	PPO activity of *in vitro*-cultured plant (U/ml)	PPO activity of local plant (U/ml)
5	1937±96	870±43
12.5	2310±115	985±49
25	**3252±160**	**1000±50**
37.5	3007±150	932±46
50	1876±94	812±40
62.5	3028±151	836±42
75	1510±75	803±40

3.1.2. pH optimization

To optimize the pH of the environment, extraction media was prepared under conditions ranging from pH 4.0 to 9.0. Afterwards, extracts were obtained, and enzyme activity was determined under standard conditions in the spectrophotometer. According to the results given in Table 3.2, the highest activity was observed in the extraction medium with pH 7.0. Thus, further screening experiments were performed at pH 7.0.

It was also observed that the samples of *I. purpurea*, which were grown in two different ways, reacted differently to pH changes in the extraction medium. Plant samples obtained from tissue culture appeared to be active in a wider range of pH than local plant samples.

In addition, explants exhibited higher PPO activities at pH values of 6.0-9.0. It is believed that this may provide an advantage in terms of the enzyme stability.

Table 3.2. pH optimization results

pH	PPO activity of *in vitro*-cultured plant (U/ml)	PPO activity of local plant (U/ml)
4	0	12±1
5	532±27	854±42
6	2201±110	690±34
7	**3328±165**	**1031±51**
8	1943±97	0
9	1250±62	0

3.2. Purification of PPO by Three-Phase Partitioning (TPP)

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

For TPP studies, two different samples of *I. purpurea* were used: 1) crude extract prepared from leaves of local plant (LP-CE), 2) crude extract prepared from *in vitro*-cultured plant (IVCP-CE). TPP conditions including $(NH_4)_2SO_4$ concentration, crude extract: *t*-butanol ratio and system pH were optimized for two samples (LP-CE and IVCP-CE) and purification results were compared.

PPO enzyme showed tendency to concentrate in different phase for LP-CE (middle phase) and IVCP-CE (bottom phase) samples. For this reason, only results from the phase (middle phase for LP-CE and bottom phase for IVCP-CE samples) where PPO activity detected are presented.

3.2.1. Determination of ammonium sulfate ($(NH_4)_2SO_4$) concentration

Ammonium sulfate concentration is very important in TPP systems. The concentration of ammonium sulfate should be optimized to extract a sufficient amount of saturated PPO enzyme from the complex mixture (crude extract) with minimal interaction with contaminating materials. Generally, optimization trials are started with solutions prepared at 20% (w/v) saturation and the concentration of the desired protein in the middle phase or in the bottom phase is determined (Dennison & Lovrien, 1997).

Here the experiments were started with 20% (w/v) $(NH_4)_2SO_4$, a concentration that is recommended in many reports (Niphadkar & Rathod, 2015, Senphan *et al.*, 2014, Panadare & Rathod, 2018) and experimented up to 70% (w/v) $(NH_4)_2SO_4$ at a constant 1:1 (v/v) ratio of crude enzyme extract to *t*-butanol at pH 7.0 and room temperature. Table 3.3 and Table 3.4 revealed the effect of $(NH_4)_2SO_4$ concentration on PPO partition from two different crude extracts, IVCP-CE and LP-CE, respectively.

Table 3.3. The effect of different $(NH_4)_2SO_4$ concentrations on PPO enzyme (obtained from IVCP-CE) distribution in TPP systems.

$(NH_4)_2SO_4$ concentration (%, w/v)*	Total activity (U)	Total amount of protein (mg)	SA (U/mg)	PF	AR (%)
20	3619±80	0.09	37700±80	8.0	55
30	**3766±100**	**0.09**	**38428±100**	**8.2**	**57**
40	3582±32	0.10	34114±32	7.3	54
50	1057±32	0.12	8385±32	1.8	16
60	10±1.6	0.14	72±2	0.02	0.15
70	3±0.16	0.20	11±3	0.002	0.05

*the specific activity of *I. purpurea* crude extract (IVCP-CE) is 4686 U/mg 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 1:1 crude extract: *t*-butanol. The precipitates in the lower phase formed were collected and analyzed. SA: Specific Activity; PF: Purification fold; AR: Activity recovery.

Generally, $(NH_4)_2SO_4$ saturations below 20% (w/v) leads to poor protein recovery, but higher concentrations cause protein to move from bottom phase to top phase (Bayraktar & Önal, 2013). Likewise, in this study, it was observed that the PPO enzyme accumulated in the lower phase when the crude extract prepared from IVCP-CE was saturated with

20%-40% (w/v) $(NH_4)_2SO_4$ (Table 3). On the other hand, 50%, 60% and 70% saturations resulted in PPO movement from bottom phase to middle phase (Figure 3.1). The highest fold purification of 8.2-fold along with 57% activity recovery of PPO in the lower phase was obtained with 30% (w/v) $(NH_4)_2SO_4$.

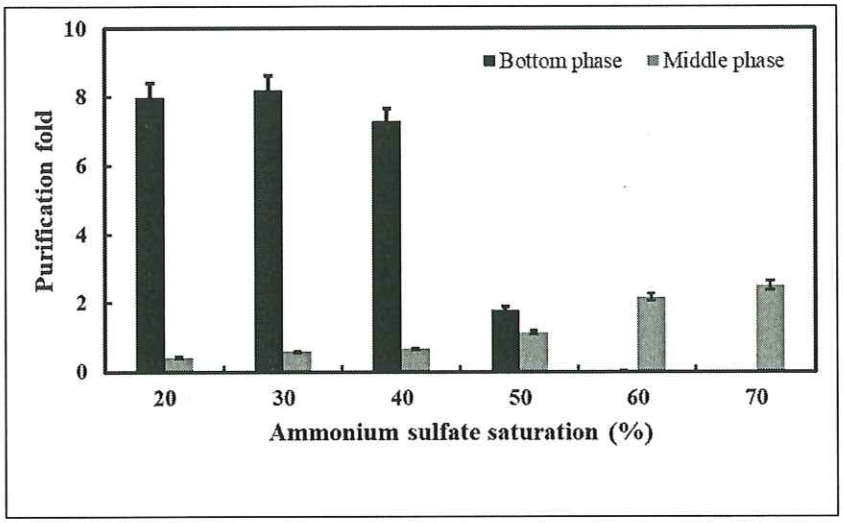

Figure 3.1. Effect of ammonium sulfate saturation on PPO partition into bottom and middle phases for TPP systems prepared using crude extract of IVCP-CE.

Table 3.4. The effect of different $(NH_4)_2SO_4$ concentrations on PPO enzyme (obtained from LP-CE) distribution in TPP systems.

$(NH_4)_2SO_4$ concentration (%, w/v)*	Total activity (U)	Total amount of protein (mg)	SA (U/mg)	PF	AR (%)
20	46±80	0.28	163±83	0.2	2
30	88±50	0.66	133±100	0.2	4
40	378±16	0.97	386±233	0.6	19
50	799±32	1.0	797±25	1.2	39
60	**1007±10**	**0.84**	**1188±26**	**1.8**	**50**
70	814±23	0.83	975±30	1.5	40

*the specific activity of *I. purpurea* (local plant) crude extract is 670 U/mg and the total protein amount is 3 mg. Equal volumes of *t*-butanol were added to the mixtures prepared with different $(NH_4)_2SO_4$ saturation at a ratio of 1:1 crude extract: *t*-butanol. The precipitates in the middle phase formed were collected and analyzed. SA: Specific Activity; PF: Purification fold; AR: Activity recovery

From crude extract of local plant, the PPO enzyme was purified to 1.8-fold with 50% activity recovery when the crude extract was saturated with 60% (w/v) $(NH_4)_2SO_4$ (Table

3.4). Contrary to the result obtained using IVCP-CE, it is seen that the PPO enzyme mainly concentrated into the middle phase rather than lower phase (Figure 3.2). Similar to the previous reports (Liu *et al.*, 2019), at high $(NH_4)_2SO_4$ concentrations, the enzyme was directed towards the middle phase, but the purification fold decreased.

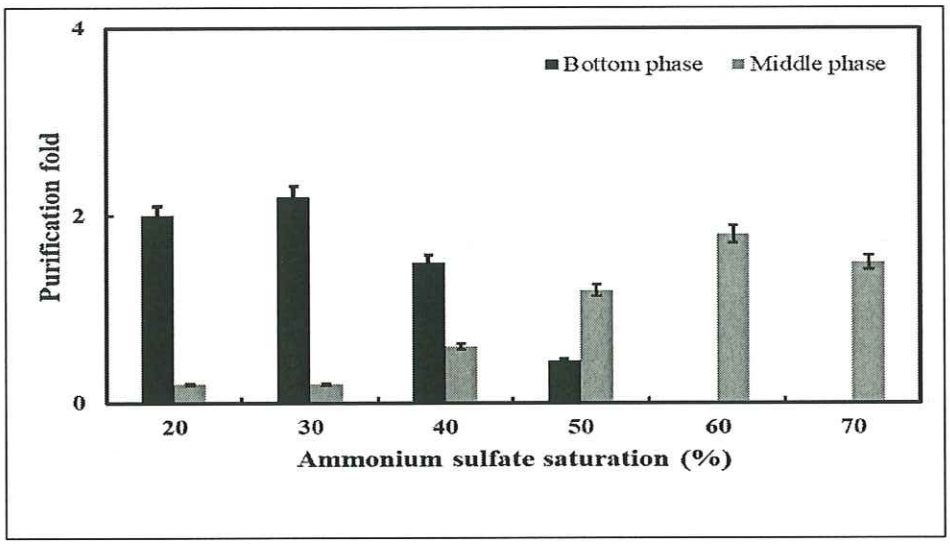

Figure 3.2. Effect of ammonium sulfate saturation on PPO partition into bottom and middle phases for TPP systems prepared by using crude extract of local plant

When the results obtained from TPP systems prepared using two different crude extracts (IVCP-CE and LP-CE) are compared, it is seen that the PPO partition is more efficient for TPP system prepared by using plant explants although activity recovery values were almost same. In addition, it might be advantageous to obtain the best partition result using lower $(NH_4)_2SO_4$ for the crude extract prepared from the IVCP-CE.

3.2.2. Determination of crude extract: *t*-butanol ratio

Crude extract: *t*-butanol ratio is also an important parameter in TPP systems and should be optimized. *t*-Butanol shows higher deactivation and lower interfacial precipitation through hydrophobic interactions with proteins compared to 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, while its higher amounts are

expected to inhibit intermolecular interactions as a result of increased viscosity of the TPP mixture (Dennison & Lovrien, 1997).

In terms of determining the appropriate optimal volume added to the solution, TPP systems were set up containing IVCP-CE saturated with 30% $(NH_4)_2SO_4$ and LP-CE saturated with 60% $(NH_4)_2SO_4$. *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. Table 3.5 and Table 3.6 revealed the effect of varying crude extract: *t*-butanol ratios on PPO partition from two different crude extracts, IVCP-CE and LP-CE, respectively.

Table 3.5. The effect of different crude extract: t-butanol ratios on PPO enzyme distribution in TPP systems prepared using crude extract of in vitro-cultured plants (IVCP-CE).

Crude extract: *t*-butanol ratio (v/v)*	Total activity (U)	Total amount of protein (mg)	SA (U/mg)	PF	AR (%)
1.0:0.5	2983±150	0.12	25372±380	5.4	45
1.0:1.0	**3766±100**	**0.09**	**38428±100**	**8.2**	**57**
1.0:1.5	3844±192	0.11	33322±500	7.1	59
1.0:2.0	3522±176	0.15	23959±359	5.1	53

*the specific activity of *I. purpurea* crude extract (IVCP-CE) is 4686 U/mg and 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 contain different crude extract: *t*-butanol ratio. SA: Specific Activity; PF: Purification fold; AR: Activity recovery.

As observed from Table 5, the best PPO purification fold (8.2) and highest activity recovery (57%) were achieved from the bottom phase of the TPP system prepared by addition of equal volume of *t*-butanol to the crude extract (IVCP-CE) saturated with 30% (w/v) $(NH_4)_2SO_4$. As is also presented in Table 5, with ratios of crude extract to t-butanol of 1.0:0.5 and 1.0:2.0 the purification fold of PPO enzyme at the bottom phase were decreased. On the other hand, with a doubled volume in t-butanol activity recovery was slightly increased.

Table 3.6. The effect of different crude extract: t-butanol ratios on PPO enzyme distribution in TPP systems prepared using crude extract of local plant (LP-CE).

Crude extract: *t*-butanol ratio (v/v)*	Total activity (U)	Total amount of protein (mg)	SA (U/mg)	PF	AR (%)
1.0:0.5	771±8	0.83	926±10	1.4	38

1.0:1.0	**1007±10**	**0.84**	**1188±26**	**1.8**	**50**
1.0:1.5	858±9	1.2	717±14	1.1	43
1.0:2.0	799±8	1.1	735±15	1.1	40

*the specific activity of *I. purpurea* crude extract (local plant) is 670 U/mg and the total protein amount is 3 mg. *t*-butanol was added to the mixtures prepared at 60% (w/v) $(NH_4)_2SO_4$ saturation to contain different crude extract: *t*-butanol ratio. SA: Specific Activity; PF: Purification fold; AR: Activity recovery.

For TPP systems prepared using crude extract of local plant, the highest fold purification of 1.8-fold along with 50% activity recovery of PPO in the middle phase was obtained with 60% (w/v) $(NH_4)_2SO_4$ (Table 3.6). Either increase or decrease in *t*-butanol volume resulted in a decrease in both purification fold and activity recovery values.

From the optimization of *t*-butanol volume experiments, it is clearly seen that the purification fold obtained by collecting the PPO in the middle phase in the TPP system prepared using local plant is significantly lower than the system using explants obtained by growing the seeds in plant tissue culture medium.

3.2.3. Determination of system pH

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).

To investigate the effect of the system pH on the separation of PPO enzyme in phases, the $(NH_4)_2SO_4$ concentration and crude extract: *t*-butanol ratio was kept constant in the systems; then the system pH was adjusted to the desired pH using 0.1 M sodium hydroxide (NaOH) and 0.1 M hydrochloric acid (HCl). Table 3.7 and Table 3.8 revealed the effect of varying system pH values on PPO partition from two different crude extracts, IVCP-CE-CE and LP-CE, respectively.

Table 3.7. The effect of the system pH on PPO enzyme (obtained from IVCP-CE) distribution in TPP systems.

System pH*	Total activity (U)	Total amount of protein (mg)	SA (U/mg)	PF	AR (%)
4	977±2	0.14	6523±36	1.4	14
5	3409±21	0.14	23640±67	5.1	49
6	3890±64	0.11	34737±43	7.6	55
7	3766±100	0.09	38428±100	8.2	57
7.5	**3976±18**	**0.08**	**48135±59**	**10.5**	**57**
8	3159±47	0.07	41796±24	9.16	45
9	2255±39	0.07	32220±52	7.06	32

*the specific activity of *I. purpurea* crude extract (IVCP-CE) is 670 U/mg and the total protein amount is 3 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. SA: Specific Activity; PF: Purification fold; AR: Activity recovery.

As shown in Table 3.7, the highest purification (10.5-fold) and the highest recovery (57%) were observed at pH 7.5. As is also presented in Table 7, at pH 7.0 and 7.5 values, activity recovery remained unchanged, but purification fold was increased when system pH was increased to 7.5. Reasonable 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 the occurrence of more hydrogen ions competing with the protein of interest for interaction with water molecules (Chew *et al.*,2019).

Table 3.8. The effect of the system pH on PPO enzyme (obtained from LP-CE) distribution in TPP systems.

System pH*	Total activity (U)	Total amount of protein (mg)	SA (U/mg)	PF	AR (%)
4	526±60	0.28	1867±39	1.0	23
5	1083±55	0.36	2972±71	1.6	48
6	1148±95	0.29	3928±50	2.1	50
7	1007±10	0.84	1188±26	1.8	50
7.5	**1358±25**	**0.32**	**4006±63**	**2.1**	**60**
8	961±72	0.31	3082±45	1.7	42
9	577±56	0.45	1279±20	0.7	25

*the specific activity of *I. purpurea* crude extract (local plant) 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 60% (w/v) $(NH_4)_2SO_4$ were adjusted to different pH. SA: Specific Activity; PF: Purification fold; AR: Activity recovery.

For TPP systems prepared using crude extract of local plant, the highest fold purification of 2.1-fold along with 60% activity recovery of PPO in the middle phase was obtained with 60% (w/v) $(NH_4)_2SO_4$ (Table 3.8). Purification fold values remained similar when the purification was performed between pH 5.0 and 8.0. On the other hand, at pH 4.0 and 9.0 both purification fold and activity values were remarkably reduced.

Overall results indicated that the best purification of the PPO enzyme was achieved from a TPP system consisting of 2 ml of crude enzyme (extracted from *I. purpurea* IVCP-CE) saturated with 30% (w/v) $(NH_4)_2SO_4$ and 2 ml of *t*-butanol at pH 7.5 (Table 3.9). The partitioned PPO enzyme was then concentrated and stored at -80°C to be used in electrophoretic and biochemical characterization studies.

Table 3.9. Summary of overall purification of PPO by three-phase partitioning.

Source	$(NH_4)_2SO_4$ concentration	Ratio of crude extract to t-butanol	pH	PF	AR
I. purpurea in vitro-cultured plant	30	1.0:1.0	7.5	10.5	57%
Local *I. purpurea* leaves	60	1.0:1.0	7.5	2.1	60%

PF: Purification fold; AR: Activity recovery.

A few PPO enzymes have been purified by TPP with different purification folds and yields (Table 3.10). Niphadkar & Rathod (2015) and Alici & 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 higher fold than potato and borage PPO enzymes. However, activity recovery value of the enzyme was the lowest among PPOs from other plants.

Table 3.10. Comparison of three phase partitioning systems for purification of PPO extracted from different sources.

Source	$(NH_4)_2SO_4$ concentration	Ratio of crude extract to *t*-butanol	pH	PF	AR
Solanum tuberosum[1]	40%	1.0:1.0	7.0	6.3	70%
Trachystemon orientalis[2]	15%	1.0:1.0	7.0	3.6	69%
Rosmarinus officinalis[3]	50%	1.0:1.0	6.5	14	230%
Foeniculum vulgare[4]	65%	1.0:0.5	6.0	20	120%
Ipomoea purpurea[5]	30%	1.0:1.0	7.5	10.5	57%

[1]Niphadkar & Rathod, 2015; [2]Alici & Arabaci, 2016; [3]Yuzugullu Karakus *et al.*, 2020; [4]Yuzugullu Karakus *et al.*, 2021; [5]Current study

3.3. Electrophoretic Analysis

3.3.1. SDS-PAGE electrophoresis

To check the purity and determine the molecular weight of the TPP-partitioned PPO, polyacrylamide gel electrophoresis was performed in the presence of anionic detergent (SDS). The molecular weight of the PPO enzyme was estimated as 12.8 kDa according to SDS-PAGE gel (Figure 3.3). The molecular weights of PPOs differ from one source to another. Examples of plant PPOs with varying molecular weights are given in Table 3.11. Among the plant PPO enzymes, *I. purpurea* PPO appeared to possess the lowest molecular weight.

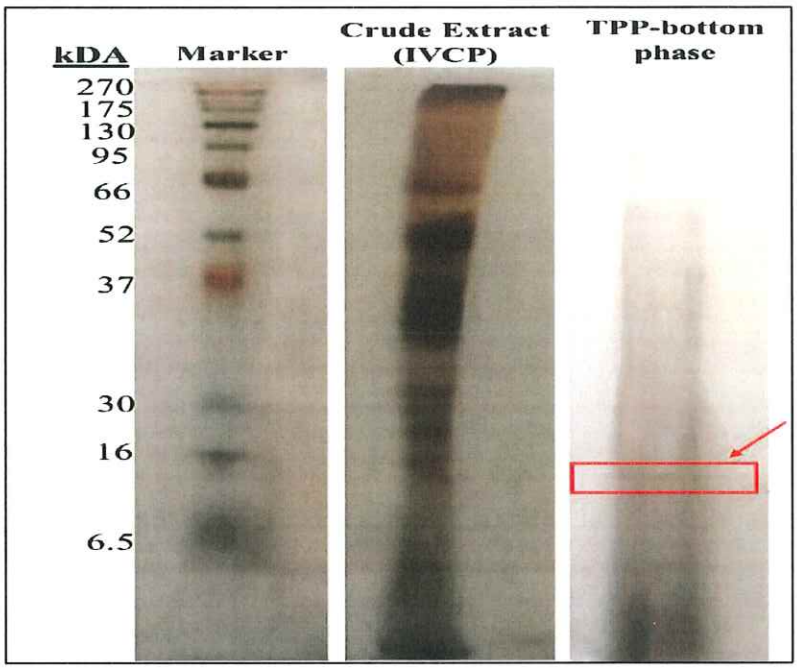

Figure 3.3. SDS-PAGE gel view of the polyphenol oxidase of *I. purpurea*. Molecular weight marker included 11 proteins with molecular weights varied from 6.5 to 270 kDa. 20 µg sample was loaded into each well. A band with a molecular weight of approximately 12.8 kDa corresponding to protein of interest was presented in rectangle.

Table 3.11. Comparison of plant PPOs in terms of molecular weight

Source	Molecular weight	Reference
Mamey	18	Palma-Orozco *et al.*, 2011
Silk flower	25	Yamamoto *et al.*, 2002
Common pawpaw	28	Fang *et al.*, 2007
Kalipatti sapota	24	Vishwasrao *et al.*, 2017
Sweet potato	38	Kanade *et al.*, 2007
Gooseberry	31	Bravo & Osorio, 2016
Borage	80.6	Alici & Arabaci, 2016
Chestnut kernel	32.5	Gong *et al.*, 2015
Rosemary	53	Yuzugullu Karakus *et al.*, 2020
Fennel	27.8	Yuzugullu Karakus *et al.*, 2021
Morning glory	12.8	Current study

3.3.2. Activity staining

Activity staining was performed in order to determine the type of the *I. purpurea* PPO. After SDS-free polyacrylamide gel electrophoresis, the gel was stained according to the method reported by Rescigno *et al.* (1997). The protocol was applied as described in Materials and Methods section.

As seen in Figure 3.4, 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) lead to amaranth pink band appearance corresponding to place where laccase activity was observed on ADA-stained gel. This indicated the presence of peroxidase activity. Lastly, the gel was exposed to *t*BC, resulting in dark blue color without any extra band. Dark blue color indicates that tBC was oxidized to corresponding quinones and catechol oxidase activity was detected at nearly same position with laccase and peroxidase bands. Considering all results obtained, it can be concluded that *I. purpurea* PPO exhibited laccase, peroxidase and catechol oxidase activities.

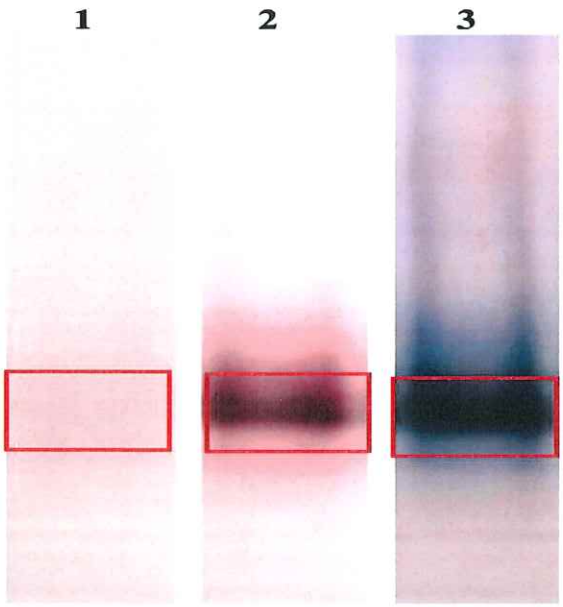

Figure 3.4. Gel view showing the activity staining results of I. purpurea PPO with ADA (1), H_2O_2 (2) and tBC (3).

3.4. Biochemical Characterization of Partially Purified *I. purpurea* PPO Enzyme

3.4.1. Effect of reaction temperature on PPO activity and stability

The reaction temperature is one of the key factors playing important role on oxygen solubility in reaction medium and affecting reaction kinetics (Panadare & Rathod, 2018). The effect of temperature on PPO activity was tested at temperatures between 20 and 70°C. Figure 3.5A exhibited the optimum temperature for *I. purpurea* PPO enzyme was 30°C when catechol used as substrate. Similarly, PPO enzymes from snake fruit, rosemary, fennel, mango, banana, and peppermint were optimum at 30°C using catechol (Table 3.12). 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 3.12). In this study, 93% and 87% of initial PPO activities were recovered at 50°C and 60°C, respectively, while at 70°C, the enzyme still maintained 59% of its activity (Figure 3.5A).

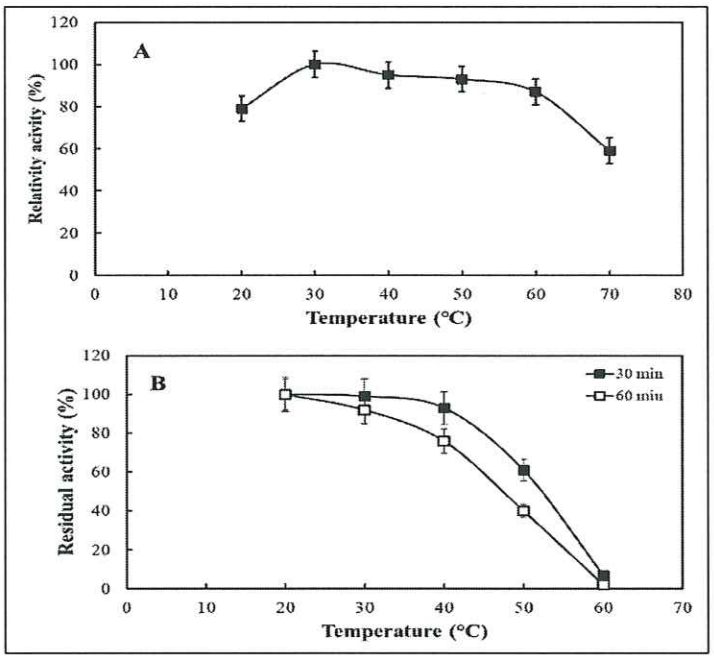

Figure 3.5. Effect of reaction temperature on *I. purpurea* PPO activity (A) and stability (B).

Table 3.12. Comparison of different plant PPOs in terms of pH and temperature optima

Source	Temperature	pH	Reference
Snake fruit	30°C	6.5	Zaini *et al.*, 2013

Chest nut kernel	40°C	7.0	Gong *et al.*, 2015
Honeydew peach	40°C	6.5-7.0	Liu *et al.*, 2015
Buriti	35°C	7.0	de Oliveira Carvalho & Orlanda, 2017
Blueberry	35°C	6.1-6.3	Siddiq & Dolan, 2017
Apricot	45°C	4.5	Derardja *et al.*, 2017
Rosemary	30°C	7.0	Yuzugullu Karakus *et al.*, 2020
Fennel	30°C	6.0	Yuzugullu Karakus *et al.*, 2021
Mango	30°C	7.0	Wang *et al.*, 2007
Banana	30°C	7.0	Ünal *et al.*,2007
Peppermint	30°C	7.0	Kavrayan & Aydemir, 2001
Morning glory	30°C	7.0	Current study

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 3.5B, from which it can be seen that the PPO enzyme is stable between 20 and 40°C and maintained 40% of its residual activity after incubation at 50°C for 60 min. On the other hand, enzyme was completely inhibited 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).

3.4.2. Effect of reaction pH on PPO activity and stability

The reaction pH is another important parameter that affects enzyme activity by altering the enzyme's net charge, thereby affecting enzyme's solubility, binding ability with different substrates/inhibitors and its folding. Enzymes mostly becomes inhibited at extreme pH values because of losing their ability to fold (Panadare & 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 3.6A. Accordingly, optimum pH was found 7.0 using catechol as substrate. Similarly, PPO enzymes from chest nut kernel, buriti, rosemary, mango, banana, and peppermint were optimum at pH 7.0 using catechol (Table 3.12). The PPO enzyme was observed to maintain more than 60% of its initial activity over the pH range 5.0-9.0 (Figure 3.6A).

The pH stability of the PPO was also investigated by preincubating the enzyme for 30 and 60 minutes 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 3.6B, the enzyme was the most stable at pH 7.0. An increase or decrease in pH above or below the stability range leads to a decrease in enzyme activity. From Figure 3.6B, 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 the acidic pH.

According to literature studies crude PPO extracted from peppermint (Kavrayan & Aydemir, 2001), pawpaw (Bello *et al.*, 2011), grape (Kaya & Bağci, 2021), artichoke (Aydemir *et al.*, 2003) and rosemary (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 with 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.

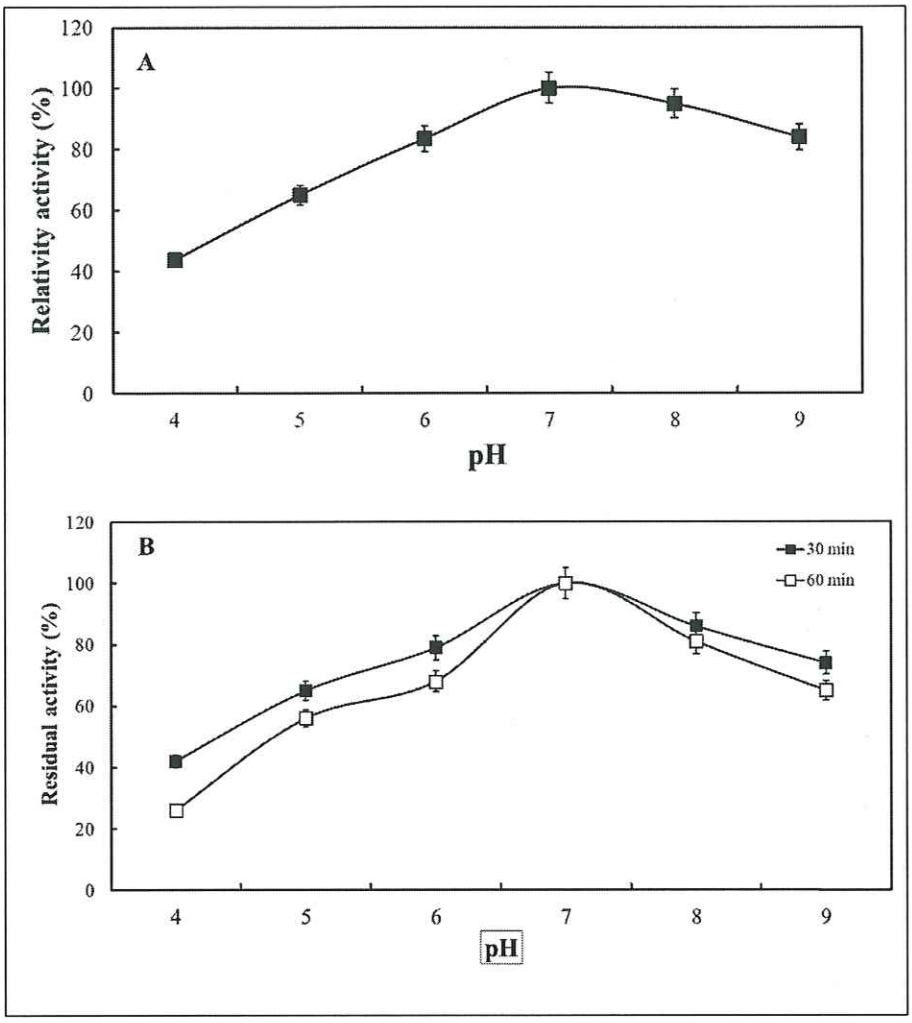

Figure 3.6. Effect of reaction pH on I. purpurea PPO activity (A) and stability (B)

4. CONCLUSIONS AND RECOMMENDATIONS

In this study, the leaves of *in vivo* and *in vitro* grown *I. purpurea* plant, which is used for ornamental and medicinal purposes, were screened for their PPO activities. Crude extract samples of *in vivo* (LP-CE) and *in vitro* (IVCP-CE) plants were obtained under optimized conditions where the extraction medium was prepared at pH 7.0 in the presence of 25 mg/ml PVPP. PPO activity was found 3-fold higher in IVCP-CE samples than that measured in LP-CE. This result has revealed that if a plant is exposed to different cultivation environment, its PPO content would also change.

Three-phase partitioning (TPP) system was carried out to purify the PPO enzyme from both LP-CE and IVCP-CE samples to test that TPP system would give different results depending on the PPO concentration that two samples have. TPP was particularly selected as a purification method due to its advantages like being time efficient and easily scaled up, supporting room temperature work, and allowing the recovery of chemicals over conventional chromatography methods. As expected, the TPP system gave better purification results with IVCP-CE samples rather than LP-CE although activity recovery values obtained from those samples were quite similar. Thus, by using TPP system, the PPO enzyme was purified 10.5-fold, with 57% recovery from *in vitro* cultured *I. purpurea* plant in a single step. Optimum conditions for TPP included addition of equal volume of *t*-butanol to the crude extract (IVCP-CE) saturated with 30% (w/v) $(NH_4)_2SO_4$ at pH 7.5.

The molecular weight of TPP-partitioned PPO was calculated by SDS-PAGE as 12.8 kDa. Activity staining results indicated that the enzyme exhibited laccase, peroxidase and catechol oxidase activities supporting that the PPO enzyme from *I. purpurea* is a highly functional enzyme. Biochemical characterization studies revealed that the effect of temperature and pH on enzyme activity resembles largely to other PPO enzymes obtained from different plant sources. On the other hand, its stability in alkaline pH environment gives the enzyme an advantage for industrial use over other PPO enzymes. In order to

increase the enzyme yield, it is suggested that the PPO enzyme activity can be increased by applying biotic/abiotic stress to the plant grown in IVCP environment. Additionally, it is highly recommended to perform a detailed study on enzyme characterization. Considering three different enzyme activities (laccase, catechol oxidase and peroxidase) observed from activity staining, it would be interesting to explore its substrate specificity. Kinetic parameters for different substrates can also be determined for a better comparison among plant PPOs.

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APPENDICES (APP)

APP-A

Preparation of Solutions

0.1 M Citrate buffer pH 4.0

Sodium citrate 9.838 g

Citric acid 12.782 g

Add dH_2O to a final concentration of 1 liter and adjust the pH to 4.0 with HCl and NaOH.

0.1 M Citrate buffer pH 5.0

Sodium citrate 16.63 g

Citric acid 8.127 g

Add dH_2O to a final concentration of 1 liter and adjust the pH to 5.0 with HCl and NaOH.

0.1 M Sodium Phosphate Buffer, pH 6.0

Monobasic 13.68 g

Dibasic 3.30 g

Add dH_2O to a final concentration of 1 liter and adjust the pH to 6.0 with HCl and NaOH.

0.1 M Sodium Phosphate Buffer, pH 7.0

Monobasic 6.08 g

Dibasic 16.34 g

Add dH_2O to a final concentration of 1 liter and adjust the pH to 7.0 with HCl and NaOH.

0.1 M Tris Buffer, pH 8.0

Tris 12.114 g

Add dH_2O to a final concentration of 1 liter and adjust the pH to 8.0 with HCl and NaOH.

0.1 M Glycine sodium hydroxide Buffer, pH 9.0

Glycine 0.66 g

Sodium hydroxide 0.046 g

Add dH_2O to a final concentration of 1 liter and adjust the pH to 9.0 with HCl and NaOH.

APP-B

SDS-PAGE Gel Electrophoresis

15% (v/v) Separating gel

30% Acrylamide – 2.5 ml

1.5 M Tris-HCl (pH 8.8) – 1.25 ml

ddH_2O – 1.18 ml

20% SDS – 0.025 ml

10% APS – 0.050 ml

TEMED – 0.005 ml

5% (v/v) Stacking gel

30% Acrylamide – 830 µl

1 M Tris-HCl (pH 6.8) – 630 µl

ddH_2O – 3.4 ml

20% SDS – 0.025 ml

10% APS – 0.050 ml

TEMED – 0.005 ml

10% (v/v) Ammonium Persulfate (APS)

APS – 100 mg

ddH_2O – 1 ml

The solution was made fresh before use.

4X Sample Dilution Buffer (SDB) or Gel loading dye

SDS – 0.8 gr

1 M Tris-HCl (pH 6.8) – 2.5 ml

Glycerol – 4 ml

β-mercaptoethanol – 2 ml

Bromophenol blue – 1 mg

ddH_2O (total) – 10 ml

SDB is stored at -80 degrees. The one to be used is stored at -20°C.

10x Electrode Running Buffer (ERB) (25 mM Tris, 192 mM Glycine)

15 g Tris, 72 g Glycine are dissolved in distilled water to a final concentration of 500 ml. It is stored at 4°C.

1x Electrode Running Buffer

During the processing of the gel, take 100 ml of the stock 10X ERB solution, dilute it in 900 ml of distilled water and add 1 g of SDS and mix. Afterwards, the gel is carried out by transferring it to the electrophoresis tank.

Stock Seperating Cool Buffer (1.5 M Tris-HCl Buffer, 100 ml (pH 8.8))

18.5 g Tris Base; 0.4 g SDS is dissolved in 80 ml distilled water. Adjust the pH to 8.7 with 10 M HCl. Passed through a sterile filter and stored at +4°C.

Stock Stacking Cool Buffer (1.0 M Tris-HCl Buffer, 100 ml (pH 6.8))

12 g Tris Base; 0.4 g SDS is dissolved in approximately 40 ml of distilled water. Its pH is adjusted to 6.8 with 10M HCl, the final volume is made up to 100 ml with dH_2O.

APP-C

Bovine serum albumin standard curve

Bovine serum albumin (BSA) was used as protein in drawing the standard graph. Standard protein solutions were prepared in concentrations of 0, 0.1, 0.125, 0.25, 0.5, 0.75, 1.0 mg/ml by diluting the stock BSA solution (2 mg/ml) with the same buffer in appropriate proportions. 0.1 ml different BSA concentrations and 2 ml of BCA working reagent are added to the 96-well plate. 15 minutes of incubation was performed in the 60°C drying-oven, and then the standard curve was created by measuring at 562 nm in the Elisa device (Figure APP-1).

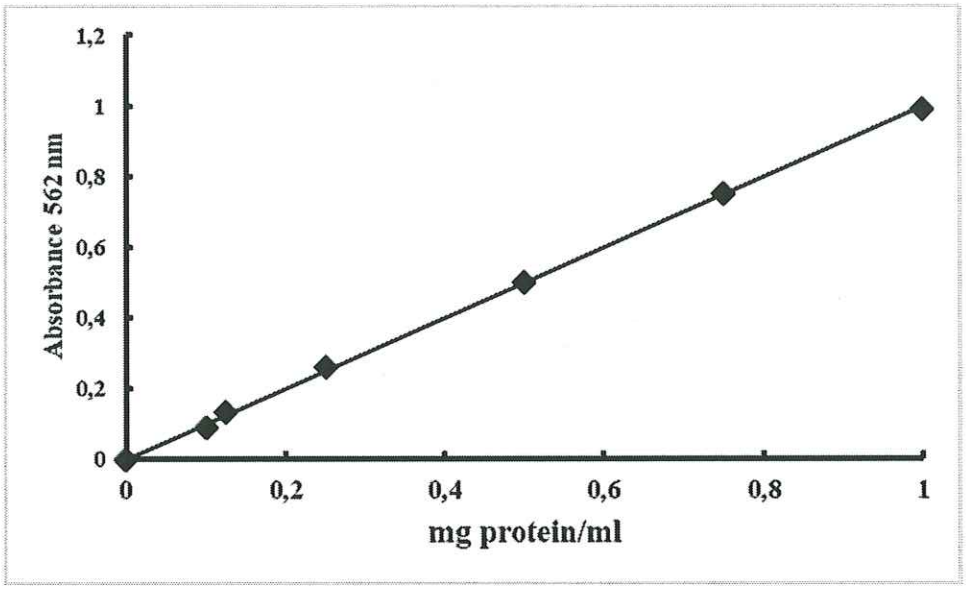

Figure APP-1. Standard BSA curve

APP-D

Silver Staining Protocol

Reagents

A. Fixation Solution

To prepare the fixer solution, fill the final volume with 100 ml of distilled water using 50 ml of ethanol, 12 ml of acetic acid and 50 µl of 37% formaldehyde. The solution can be used multiple times for subsequent experiments.

B. 50% Ethanol preparation

It is made by mixing 50 ml of pure ethanol with 50 ml of distilled water. Since it is used in the washing step, it should always be prepared fresh.

C. Pre-treatment Solution

Prepare by adding 0.02 g sodium thiosulfate ($Na_2S_2O_3.5H_2O$) to 100 ml distilled water.

D. Silver Nitrate Solution

0.2 g of silver nitrate is dissolved in 100 ml of distilled water and 75 µl of 37% formaldehyde is added just before use.

E. Developing Solution

6 g of sodium carbonate, 0.4 mg of sodium thiosulfate are dissolved in 100 ml of distilled water and 50 µl of 37% formaldehyde is added.

F. Terminating Solution

50 ml of methanol is mixed with 12 ml of acetic acid and the final volume is made up to 100 ml with distilled water.

Method

Silver staining method is given in Table APP-1.

Table APP-1. Silver staining procedure

	Step	Solution	Time of treatment
1.	Fixing	Fixer	Overnight
2.	Washing	50% Ethanol	3 x 20 min
3.	Pre-treatment	Pretreatment Solution	1 min
4.	Washing	Distilled water	3 x 20 sec
5.	Impregnate (4°C)	Silver Nitrate Solution	20 min
6.	Washing	Distilled water	3 x 20 sec
7.	Developing	Developing Solution	until the first bands come out.
8.	Washing	dH_2O	2 x 2 min
9.	Stop	Terminating Solution	The gel can be stored in 50% methanol solution after 10 minutes in the terminating solution.

PERSONAL PUBLICATIONS AND WORKS

Mansurov B., Yüzügüllü Karakuş Y., Purification of phenol oxidase enzyme from the Common-morning Glory (*Ipomoea purpurea*), *21. all-Slovak student scientific conference with international participation entitled "Chemistry and Technologies for Life"*, Bratislava, Slovakia, 6 November 2019.

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